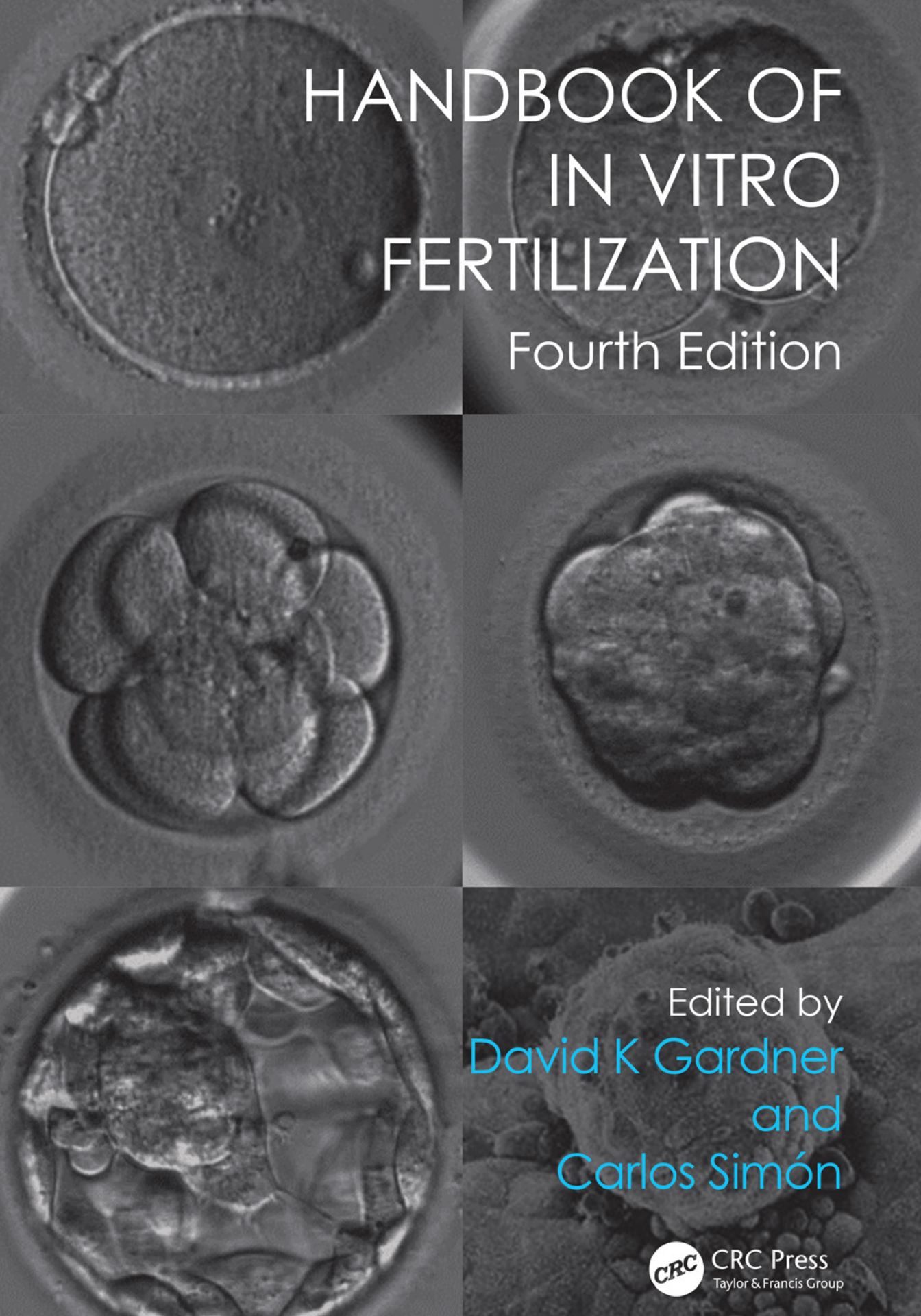

HANDBOOK OF IN VITRO FERTILIZATION

Fourth Edition

Edited by

David K Gardner

and

Carlos Simón

CRC Press
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Handbook of In Vitro Fertilization

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Preface

The past four decades have witnessed many remarkable advances in the field of assisted human conception. Following in the footsteps of Robert Edwards, Patrick Steptoe, and Jean Purdy, numerous scientists and physicians around the world have worked tirelessly to develop more effective and safer procedures to treat infertile couples. Along with improvements in the areas of ovarian stimulation, embryo culture and cryobiology, we have seen the introduction of assisted fertilization through intracytoplasmic sperm injection, and the development of techniques to remove and perform genetic analysis on polar bodies, blastomeres, and the trophectoderm. More recently, with the advent of time-lapse microscopy, we now have the capacity to analyze embryo morphology and the kinetics of development like never before.

While no single volume can adequately cover the enormity of the diverse field of reproductive medicine, it is the aim of this book to review the achievements of the biomedical community involved in assisted human conception, and to highlight ongoing and potential future treatments and procedures. There can be no doubt that both basic and clinical research has improved clinical outcomes worldwide, not only increasing pregnancy rates, but also reducing the time to pregnancy.

This book provides considerable background to many areas of human-assisted conception, and much practical information, which can be readily translated into clinical practice. To facilitate this, the chapters found within have been written by acknowledged pioneers and experts in each area. To all of them we are indebted for sharing their expertise. This book will be of enormous value to clinicians, embryologists, scientists, and all students of biomedical sciences.

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IVF: The First Four Decades

Mae Wu Healy, Micah J. Hill, and Alan DeCherney

Origins of Fertility and Reproductive Physiology

The concept of fertility has been linked throughout history to prosperity and womanhood. Dating back almost 40,000 years, cave paintings and sculptures depict females as well rounded figures showing importance on the ability to carry a child (1). Thus, the inability to procreate has caused much distress among many societies. Hippocrates, born in 460 BC, sought to logically explain different causes of infertility. He hypothesized infertility could be due to malposition of the cervix, softening of the endometrial cavity due to congenital origins, acquired from scarring after ulcers, obstruction of the opening of the uterus, excessive menstrual flow thus inability to fix the seed, or uterine prolapse (2).

Early understanding of reproductive physiology was limited. For instance, spermatozoa were thought to come initially from the bones (3). Another example is Aristotle's proposal that humans came from the egg, but sperm was what gave the egg its shape (4). In 1672, De Graaf described the physiology of the ovary and follicular function (5). Mistakenly, he described the follicle to be the egg (5). Further, Von Horne claimed infertility was due to a vicious humour inside the uterine cavity and recommended uterine irrigation to treat infertility (1).

In 1677, Antonii von Leeuwenhoek invented the microscope to study human semen, by which he discovered the spermatozoa a year later (1). He described them as "living animalcules in human semen…" believing the tails were operated by muscles, tendons, or joints (6,7). He also thought a spermatozoa contained an already formed embryo (1). The contributions of the egg and the sperm remained poorly understood for almost another 200 years until the work of Matthias Schleiden and Theodor Schwann. The pair hypothesized the egg and sperm were both individual cells with hereditary factors to contribute to each other (8). In 1875, Oskar Hertwig showed sea urchin fertilization could be achieved when one sperm cell penetrated the egg, thus illustrating the basics of genetic inheritance (9).

The Evolution of Artificial Insemination

Introduction

Almost a hundred years later, in 1786, John Hunter from London described the first artificial insemination in humans. Treating a male patient with severe hypospadias, Hunter advised him to collect his semen in a warmed syringe and inject it into his wife's vagina (10). Another pioneer, American J. Marion Sims, attempted 55 inseminations in the mid-1800s, reporting one pregnancy. At that time, he believed ovulation occurred during menstruation and focused much of his work on post-coital testing (10). In 1863, he published his work in the *Clinical Notes on Uterine Surgery*, which was regarded as both controversial and ahead of its time with its emphasis on treatment of infertility, including artificial insemination (10).

It was not until the 1890s that the idea of embryo transfer was described. Walter Heape, a professor and physician at the University of Cambridge in England, performed embryo transfer experiments between 1890 and 1897. The results were published in the *Proceedings of the Royal Society of London*,

illustrating the first case of embryo transplantation in rabbits. Heape described transferring two ova from an Angora doe rabbit that had been fertilized by a male Angora rabbit 32 hours prior. He placed the embryo into the fallopian tube of a Belgian Hare doe rabbit that had been mated with a Belgian Hale male rabbit three hours prior. The Belgian Hare doe delivered six babies, two with angora phenotypes and four with Belgian phenotypes (11,12).

Ahead of his time in 1932, Aldous Huxley wrote his science fiction novel *Brave New World* that conceptualized the technique of in vitro fertilization (IVF). Two years later, in an attempt to bridge fiction in vitro fertilization with reality, Pincus and Enzmann from the Laboratory of General Physiology at Harvard University, removed mammalian oocytes from the ovary and watched them undergo normal development in vitro (13). In 1948, Miriam Menken and John Rock retrieved eggs from over 800 women who were undergoing gynecology surgeries (14). They further exposed 138 of these oocytes to spermatozoa, reporting cleavage of the human embryos (14).

Donor Sperm, Rabbit Surrogacy, and Mice Embryo Transfers

In 1953, the first successful pregnancy from artificial insemination with frozen and thawed sperm was reported (10). With growing concern of transmitting HIV/AIDs with use of fresh sperm, the U.S. government began to require infectious disease screening, with a time period of quarantine prior to use (10). Thus, donor fresh sperm samples became exceedingly rare. Also, in an attempt to regulate the chances of unknowing marriage of biological siblings, the government restricted the number of times a single donor's sample could be used (10).

That same year, surrogacy became a reality. Min Chueh Chang, a scientist born in Tai Yuan, China, who studied in Cambridge, demonstrated this in rabbit experiments (15). First, Chang described growing rabbit embryos derived from oocytes fertilized by capacitated spermatozoa in a small Carrel flask (16). Six years later, he showed an egg from a female black rabbit could be fertilized in vitro by sperm from a black male rabbit. The embryo was then transferred to a white female rabbit, resulting in the birth of a black offspring (17). Chang also performed numerous studies on IVF in the hamster, mouse, and rat ova, all of which would serve as a foundation for Patrick Steptoe and Robert Edwards almost 20 years later (15).

While Chang continued his work on rabbits, in London developmental biologists Anne McLaren and Donald Michie were similarly working on this idea with mice. The pair developed and described mouse embryo transfer techniques of blastocysts to uterine-foster mothers (18). These findings were published in *Nature* in 1956 and released to the public under the headline "Brave New Mice" by the London *Daily Telegraph* (19).

Steptoe and Edwards

Many say the true start of IVF began with the work of Patrick Christopher Steptoe, a gynecologist, and Robert Geoffrey Edwards, a developmental geneticist. Together, their work pushed the limits of their time.

Patrick Steptoe was a member of the Royal College of Surgeons, licensed in 1939. During World War II, he volunteered for the Royal Navy Volunteer Reserves, serving as a Naval Surgeon until his ship was hit off Crete in 1941. He was captured and for 2 years remained a prisoner of war in Italy. Reports state he helped prisoners escape and thus landed himself in solitary confinement until he was released in 1943. After the war, Steptoe focused his work on sterilizations and gynecologic surgeries. Like many surgeons in the 1950s and 1960s, he sought alternatives to laparotomies. Learning of laparoscopy from Raoul Palmer in France and Hans Fragenheim in Germany, Steptoe began performing laparoscopic sterilization in England in the mid-1960s (20).

Concurrently, Robert Edwards, an expert in genetics, immunology, and embryology, worked with colleagues in Glasgow, Scotland, to produce the world's first embryonic stem cells from rabbit embryos. Intrigued by the idea of stem cells, Edwards turned to investigating human oocytes in vitro as a source of stem cells. Over the next several years, Edwards tried unsuccessfully to collaborate with clinicians in England to help him retrieve human eggs. Frustrated with these barriers, Edwards came to the United

States in 1965 and joined Georgeanna and Howard Jones at Johns Hopkins Hospital where they were performing ovarian wedge biopsies (21). One of his major accomplishments during his time in Baltimore, Maryland, was discovering that complete oocyte maturation in vitro took 37 hours (22). Thus, he concluded insemination should be carried out 35–40 hours after ovulation (22). By 1969, collaborating with his PhD student Barry Bavister, Edwards was able to fertilize human oocytes with spermatozoa (21).

In 1968, Edwards came across Steptoe's article in the *Lancet* on "Laparoscopy and Ovulation" (23). A chance meeting at a London conference initiated the famous partnership of Steptoe and Edwards (21). In 1970, Steptoe and Edwards performed their first laparoscopic oocyte retrieval (24), with the start of human embryo transfers a year later (21).

The First IVF Pregnancies

In 1973, across the world in Melbourne, Australia, Professors Carl Wood and John Leeton formed the Monash IVF research team. The group reported the first human IVF pregnancy (25). They described treating the patient with clomiphene citrate for 5 days with an ovulation trigger of human chorionic gonadotropin (HCG) 5000 International units (IU) on day 9 of her menstrual cycle. This was followed by laparotomy to remove the oocytes. About 74 hours after fertilization with her husband's sperm, an 8 cell zygote was transferred back to the patient's uterus (25). The pregnancy, unfortunately, resulted in an early miscarriage (25).

In 1976, Steptoe and Edwards reported the second IVF pregnancy, resulting in an ectopic pregnancy (26). The patient was stimulated with human menopausal gonadotropin (HMG) with an ovulation trigger of 5000 IU of HCG. The embryo transfer was performed between the morula and blastocyst stage, with a positive pregnancy test several weeks later. The patient then presented with pelvic and abdominal pain with a laparoscopy at 13 weeks gestation revealing a right tubal ectopic pregnancy. Pathology confirmed degenerate chorionic tissue (26).

During this time, U.K. colleagues, the media, and the Archbishop of Liverpool met the pair with criticism and accusations of malpractice. Despite this, Edwards and Steptoe persisted and finally achieved their first live birth with Louise Brown (27). The announcement of the successful birth of Louise in Oldham, England, on July 25, 1978, was met with some acceptance, but more skepticism and doubts (21). After 1978, the progress of Edwards and Steptoe stalled for 2 years due to the retirement of Steptoe from Britain's National Health Service (21). By 1980, their private laboratory in Bourn Hall near Cambridge was up and running and served as the center to one of the most well-known laboratories in the world today (21).

By 1978, Howard and Georgeanna Jones had retired from Johns Hopkins to start their own IVF program at the Eastern Virginia Medical School in Norfolk. During that year, out of 41 laparoscopic oocyte retrievals, only 13 patients had embryos that underwent cleavage, and no pregnancies were achieved. By 1981, after many failed cycles, Georgeanna Jones altered their protocol from a natural cycle to one with ovarian stimulation with HMG in hope of obtaining more oocytes. Following this change in protocol, they achieved America's first IVF pregnancy, the world's fourth IVF baby. This was their 13th attempted stimulated cycle. Elizabeth Jordan Carr was born in December 1981 at Norfolk General Hospital in Norfolk, Virginia.

Advancing the Field

Bourn Hall Meeting

During this time of rapid advancements in IVF, Edwards called together a gathering at Bourn Hall with the top physicians and scientists in the field (21). During this meeting, held in September of 1981, a consensus was formed, with five important concepts that have guided IVF protocols even to today. Agreed upon were stimulated cycles were better than natural cycles, to increase number of oocytes retrieved and allow for better prediction of ovulation timing. Secondly, ultrasounds should be used to monitor growth of follicles. Thirdly, progesterone supplementation should be used for luteal support. Two additional areas of weaknesses were identified, one of which was quality control in culture media and laboratory

processes. The other concern was the effect of gas on oocyte quality with laparoscopy (21). Over the next several decades, research and changes to the field were focused on improving each of these five concepts.

Pharmaceutical Advancements

The rising use of ovarian stimulation led to increased pharmaceutical development of stimulation medications. Starting in the 1950s, Gemzell described using human pituitary follicle-stimulating hormone (FSH), obtained at time of human autopsies, to make partially purified FSH preparations (28). In the early 1960s, Lunenfeld introduced HMG extracted from post-menopausal urine (29).

From here, the idea of making a more consistent preparation pushed pharmaceutical companies to isolate just the FSH from urine by treatment with polyclonal antibodies to remove the luteinizing hormone (LH), leading to urinary human FSH. In the 1990s, monoclonal antibodies specific to FSH were used to further refine the product, leading to highly purified urinary FSH. Keene and his colleagues described the first recombinant FSH produced from genetically engineered Chinese hamster ovaries (30). These described preparations are still being used today.

Furthermore, in 1981, Trounson and his colleagues described the use of clomiphene citrate with HMG in IVF treatment protocols (31). In Vienna, Feichtinger and Kemeter reported the first live births of twins using clomiphene citrate in August 1982 (32). Within the next 10 years, the first papers describing the use of letrozole, an aromatase inhibitor, for ovulation induction was published (33,34). Today, clomiphene citrate and letrozole are the most common oral agents used for controlled ovarian hyperstimulation (COH).

Technological and Laboratory Advancements

Around the world, groups worked on advancements in the laboratory that continued to improve the IVF process. In 1976, Menezo developed the B2 culture medium, known as the "French medium," that mimicked the follicular, tubal, and uterine environments of the sheep, rabbits, and humans (35). Simultaneously, the Melbourne group worked on improvements to the culture medias (36,37) in addition to developing Teflon-lined catheters to help improve embryo transfers (38). There were also new developments in laboratory assays. Frydman and Testart from the University Hospital in Clamart, France, created an assay to test for the initial rise of LH in plasma (39). This more accurately predicted the LH surge, thus helping to predict the ideal time for retrieval of the oocytes (39).

Better fertilization techniques continued to be investigated throughout the late 1980s. In 1987, Laws-King and colleagues described a micromanipulation method that involved taking a single spermatozoa and placing it under the zona pellucida (40). This helped overcome severe male factor infertility. Authors reported a high rate of fertilization with minimal damage to the oocyte (40). Shortly after this technique was described, the first birth using the subzonal sperm insemination (SUZI) method was described from the National University of Singapore (41).

In 1992, Gianpiero Palermo reported a significant breakthrough for male factor infertility. While attempting to perform subzonal injection of sperm for fertilization, Palermo noted if the sperm was instead injected through the zona pellucida and into the ooplasm of the metaphase-II oocyte, fertilization would also occur. His group published this as intracytoplasmic sperm injection (ICSI) in *The Lancet*, describing the first four pregnancies with this technique (42). Studies since have demonstrated better fertilization and pregnancy rates with ICSI over SUZI (43,44). Thus, ICSI has remained the standard of care, successfully treating cases of severe male factor infertility.

Additionally, other novel laboratory techniques continued to be investigated. Jacques Cohen, an embryologist who worked with Steptoe and Edwards in the 1980s at Bourn Hall, pioneered many of these new techniques. In 1990, Cohen, who was then at Emory University School of Medicine, introduced a new concept to assist in implantation. At that time, the rate of embryonic implantation, or babies born per embryo transferred, in the United States was <5% (45). Cohen's group hypothesized a substantial number of healthy IVF embryos failed implantation due to inability to hatch from the zona pellucida (46). Thus, they described piercing the zona pellucida with a microneedle until the needle tip was seen in the perivitelline space, otherwise known as assisted hatching (46). The result was a doubling in incidence of embryonic implantation from 11% to 23% (46). Since the technique was first described, there has been controversial debate of its contribution to increasing live birth rates. In 2012, the most recent Cochran

Review showed an increased chance of achieving a clinical pregnancy with assisted hatching; however, there is still insufficient evidence on live birth rate (47). Thus, since Cohen's group first introduced this technique, fertility clinics today still continue to use this for specific patient populations.

Ultrasound

In 1972, Kratochwil described ultrasonic tomography of the ovaries, overcoming poor visualization of the ovarian follicles during stimulation (48). This led to more accurate predictions of oocyte maturity and thus better timing of oocyte retrievals. Improvements in ultrasound technology also vastly improved both the number of oocytes retrieved during a cycle in addition to decreasing complications during the retrieval (49–52). Lenz and Lauritsen first described ultrasound guided trans-abdominal and trans-vesical aspirations under both local anesthesia or general anesthesia (49). In their group of 30 patients reported, oocytes were obtained in 57% of the cases. The only complication noted was transient hematuria (49). The technique was concluded to be atraumatic and inexpensive, thus was the recommended technique at that time over laparotomy and laparoscopic retrievals (49).

In 1983, Gleicher and his colleagues described the trans-abdominal ultrasound guided trans-vaginal needle oocyte aspiration. Their technique involved using a metal speculum with a tenaculum placed on the posterior lip of the cervix to manipulate the cervix upwards, exposing the posterior cul-de-sac. A 16 G spinal needle was passed through the cul-de-sac into the ovaries under ultrasound guidance. The benefits of this technique was a decrease in trauma to abdominal organs, notably the bladder, upon entry, in addition to not requiring general anesthesia (53). With this less invasive technique, their hope was to eventually make this an office procedure.

The introduction of vaginal ultrasound probes further changed many practices. In 1985, Wikland et al. reported the first trans-vaginal ultrasound-guided oocyte retrieval (TVOR) (54). Additionally, the vaginal probe needle introducer was introduced by Yale University to improve accuracy in follicular puncture and aspiration of oocytes. The benefits of TVOR were shortened operating time (55), no general anesthesia needed, less complications including pain, infection, and bleeding, in addition to being more cost effective (56). This described technique has thus remained the standard of care.

With improvements in oocyte retrievals under ultrasound guidance, investigators and physicians alike began looking into trans-abdominal ultrasound-guided embryo transfers. This was compared to transferring with the "clinical feel." This technique, described by Strickler et al. (57), allows for accurate positioning of the catheter tip in the uterine cavity, avoiding pressing the catheter tip in the fundus, allowing for better navigation through difficult utero-cervical junctions, and reassurance for both patient and physicians of proper placement of the embryo at the completion of an IVF cycle (58–60). This technique remains the recommended method to assist in embryo transfers today (61,62).

Cryopreservation

During the 1980s, there were increasing IVF cases of multiple gestations due to the high number of embryos being transferred back. The first triplets from IVF was reported in 1983 (63), followed by quadruplets in 1984 (64). The idea of cryopreserving embryos would allow patients to save additional embryos for the future, thus decreasing the number of embryos transferred back in the original fresh cycle. In 1983, the Monash group reported the first cryopreservation of a human embryo using the slow freezing method (65). More specifically, Trounson and Mohr described the techniques that would allow a four and eight cell embryo to survive the freeze, storage, and thaw process. Out of 15 patients described in their study, one became pregnant. The pregnancy resulted in a fetal loss at 24 weeks gestation after premature membrane rupture with chorioamnionitis (65). In 1985, Cohen's group reported the birth of the first term baby from a cryopreserved embryo (66).

The original slow rate freezing method was associated with low survival of the embryos once thawed and low pregnancy rates. This, with the high cost of cryopreservation, pushed others to search for better techniques. In the late 1980s, the ultra-rapid freezing protocol was described (67). This technique, known as vitrification, is based on the idea of applying a higher concentration of cryo-protectants and a rapid cooling speed to prevent the formation of intracellular ice crystals (68). This technique is faster to perform, offers better control of the process, and more importantly offers a high post-thaw survival rate

with improved pregnancy rates and live birth rates (69). Thus, since vitrification was described, embryologists have continued to view this as the gold standard. In addition, since further improvements on oocyte vitrification technique have been made, American Society for Reproductive Medicine (ASRM) in 2013, has declared egg freezing no longer experimental (70).

Since 2000, there have been many changes in the field of onco-fertility, a big part due to the advancements in cryopreservation. One strategy for fertility preservation is to take the patient, prior to their gonadotoxic therapy, through an IVF cycle, oocyte retrieval, and vitrify their oocytes or embryos (71). Another strategy is to remove a portion of ovarian tissue prior to therapy with a re-transplantation later on. Oktay and Karlikaya reported the first ovarian transplant after cryopreservation of the tissue (72). Four years later, Donnez and colleagues achieved the first live birth after orthotopic transplantation of the cryopreserved ovarian tissue (73). In the last decade, groups have been investigating fertility preservation among cancer patients both with oocyte cryopreservation and the use of in vitro maturation (74,75). In 2008, Porcu reported the first birth of healthy twins after oocyte cryopreservation and bilateral ovariectomy in a cancer patient survivor (76). Per ASRM in 2013, oncology patients should have discussed with them options of fertility preservation and future reproduction after gonadotoxic treatment prior to treatment (77).

Donor Oocytes

In 1983, the Monash team reported the first successful pregnancy in a woman using a donor embryo, resulting in a spontaneous abortion (66). Across the world, at the UCLA Medical Center, John E. Buster's group described the first successful pregnancy after oocyte donation, opening up possibilities to infertile women with diminished ovarian reserve or primary ovarian insufficiency (78,79). Since then, egg donation has become a viable option, with either vitrified eggs through a cryobank or fresh donor eggs, with good pregnancy and live birth rates.

Protocol Improvements

Ovarian stimulation protocols have improved throughout the years. During the first decade of natural and stimulated cycles, physicians were noting a high cancellation rate due to premature ovulation prior to oocyte retrievals. Thus, in 1982, Fleming and his colleagues described using gonadotropin-releasing hormone (GnRH) agonist to help eliminate the premature luteinization and to help better control ovarian stimulation (80). In 1991, Frydman and his colleagues described the use of a gonadotropin releasing hormone antagonist to help prevent both LH and progesterone rises during the stimulation (81). Both these techniques are integrated into common standard protocols today.

The benefit of a GnRH agonist was found to be more than solely suppressing premature luteinization. In the early 1980s, most fertility centers were using clomiphene citrate and gonadotropin with only two to four oocytes retrieved per cycle. In 1984, focusing on increasing the number of oocyte recruited and thus retrieved and fertilized, Porter and his colleagues reported the idea of inducing multifollicular development by pituitary desensitization (82). They were the first group to propose that GnRH agonist also increased the number of oocytes recovered. Per their study, they reported a total of 87 oocytes over 11 cycles with GnRH agonist as compared to 21 oocytes retrieved in 20 cycles without GnRH agonist (82).

Other groups continued to brainstorm ideas for improved follicular recruitment prior to ovarian stimulation. Gonen and colleagues in 1990 described using gonadotropin suppression with oral contraceptives before IVF (83). The idea was studied among 181 stimulation cycles compared to 113 stimulation cycles without pituitary suppression. Their data demonstrated oral contraceptive pills (OCPs) significantly helped recruit more follicles with more oocytes retrieved than the nontreatment group. In addition, it helped prevent spontaneous LH surges (83). Both Porter and Gonen's groups laid the foundation for pituitary suppression prior to a stimulated cycle.

In 1990, Gonen's group introduced the use of a gonadotropin-releasing hormone agonist for triggering follicular maturation (84). They compared using 0.5 mg of leuprolide acetate to 5000 IU of HCG in 18 cycles of IVF among 14 patients. One significant finding was LH and FSH serum levels remained elevated for only 34 hours after the GnRH agonist. Comparably, mean HCG levels stayed elevated for 6 days after administration. The mean number of oocytes retrieved and the number and quality of the

embryos between the two groups were not significantly different. Thus, authors concluded GnRH agonist is a good alternative to HCG trigger (84). Since that study, GnRH agonist was used relatively infrequently due to concern for lower pregnancy rates compared to HCG triggers. Over recent years, however, studies have shown improvement in both pregnancy rates and live birth rates with increased luteal support (85–87). In addition, the incidence of ovarian hyperstimulation as a GnRH agonist is significantly decreased compared to HCG triggers (88,89). Thus, the use of GnRH agonist trigger has increased substantially today and remains a good alterative to the HCG trigger (90).

Surrogacy

In 1985, the idea of human surrogacy became a reality. Utian et al., from Mount Sinai Medical Center in Cleveland, Ohio, described the first case in the *New England Journal of Medicine* (91). Their patient was a 37-year-old woman who had undergone bilateral salpingectomies for tubo-ovarian abscesses followed later by a cesarean hysterectomy for a uterine rupture from a prior pregnancy (91). The patient and husband went through an IVF protocol and created an embryo. This was transferred to the uterus of their 22-year-old friend, resulting in a successful pregnancy (91). Since then, surrogacy has remained a viable option to many with uterine factor infertility.

Male Infertility

Per ASRM, in 40% of infertile couples, the male partner is the sole cause or a contributing factor to the overall diagnosis. Thus, physicians over the past few decades have sought methods to overcome male factor infertility. In 1985, Temple-Smith and colleagues reported the first pregnancy from microsurgical epididymal sperm aspiration (MESA) from a man with secondary obstructive azospermia. His spermatozoa fertilized his wife's oocytes and the resultant embryo transferred back to the uterus (92). Over the next several years, more successful pregnancies were reported with intracytoplasmic sperm injection (ICSI) when sperm were aspirated from the epididymis or testes with MESA (93) or testicular sperm extraction (TESE) (94). This further broadened options for couples with obstructive azospermia or congenital bilateral absence of the vas deferens.

An important genetic finding in male infertility was the deletion of the azoospermia factor (AZF) gene located on the long arm of the Y chromosome (95). This Y-chromosome deletion causes severe oligospermia or azoospermia. Different categories, AZFa, AZFb, and AZFc deletions were further identified. Males with the AZFc deletion could undergo IVF with ICSI to reproduce. However, AZF deletions have the potential to be transmitted to sons, who likely will be infertile (95). Since the improvement in Y-DNA testing, it is a standard part of the male infertility workup for men with abnormal semen analysis.

Alternatives to Traditional IVF

In 1984, Asch and colleagues from the University of Texas described the first pregnancy with translaparoscopic gamete intra-fallopian transfer (GIFT) (96). The premise was to circumvent failed ovum pick up by the fimbriae or failed migration of the spermatozoa to the fallopian tube. The group treated a 35-year-old woman with HMG followed by laparoscopic oocyte retrieval notable for four eggs. These oocytes were then loaded into a catheter with the sperm preparation. The loaded content in the catheter was then injected 1.5 cm into the fimbriated end of the fallopian tube. The group's first attempt at GIFT resulted in a successful twin gestation pregnancy (96).

Following the introduction of GIFT in 1984, physicians across the world attempted to incorporate this technique into their practice. Devroey and his colleagues from Brussels, Belgium, focused their efforts on a 31-year-old female who failed six cycles of intrauterine insemination, IVF, and one unsuccessful cycle of GIFT (97). The group altered the idea of transferring gametes and worked on transferring instead a zygote. The new technique was coined zygote intrafallopian transfer (ZIFT). The couple had six oocytes that fertilized, three of which were replaced via laparoscopy within the fallopian tube, with the remainder of the three embryos frozen at the 4-cell stage. The patient had successful twin pregnancies (97). With improvements to IVF, GIFT and ZIFT have fallen out of favor for first line treatments among fertility clinics. However, they do still remain plausible options for couples that cannot undergo traditional IVF.

Screening for Genetic Diseases

As progress continued, the question emerged whether it was possible to screen embryos for inherited diseases. The idea was to allow parents the chance to avoid the decision of abortion during pregnancy if a genetic disorder was diagnosed. With the advent of polymerase chain reaction (PCR), it became possible to determine sex of the fetus by amplifying a repeated sequence unique only to the Y chromosome (98). Specifically, this would allow couples to avoid transmitting X-linked diseases. Thus, in 1989, Handyside and his colleagues described removing a single cell through the zona pellucida from a 6–10 cell cleavage stage embryo and determining the sex from the PCR (99). This important article in the *Lancet* laid the foundation for human pregenetic diagnosis and screening (99).

One year after their *Lancet* publication, Handyside reported in *Nature* two successful pregnancies after screening preimplantation embryos sexed by Y-specific DNA amplification (100). The couples had known X-linked adrenoleukodystrophy and X-linked mental retardation, and were brought through an IVF cycle, with biopsies performed at the 6–8 cell stage. From the 46 embryos biopsied, 50% of the embryos were male. Two female biopsied embryos were transferred per couple. Both women resulted in healthy female karyotype pregnancies (100).

Along similar lines, in 1990, Verlinsky and colleagues described the first polar body analysis of alpha-1-antitrypsin deficiency (101). The authors took a patient known to have this disorder, retrieved eight oocytes, and removed the first polar body by micromanipulation. Of these, six fertilized normally, showing removal of the first polar body did not affect subsequent fertilization or the ability to grow to blastocyst stage. Successful PCR analysis was performed, and two embryos from the oocytes containing the unaffected gene were transferred. However, there were no pregnancies in this report (101). Further, disadvantages include that it provides only genetic information about the maternal contribution with potential suboptimal diagnosis of aneuploidy.

In 1999, the current standard for pre-implantation genetic testing was described with trophectoderm biopsy (102–104). The first pregnancy following a blastocyst biopsy and preimplantation genetic diagnosis (PGD) was in 2002 by De Boer's group (103). Since then, successful prevention of diseases such as sickle cell anemia (104), retinoblastoma (105), beta-thalassemia (106), and BRCA 2 genes (107) have been described.

In Vitro Maturation

Attempts to harvest immature oocytes and mature them in vitro were initially described by Cha in 1991 (108). This technology was introduced as a potential advancement for donor oocyte programs. His group collected 270 immature oocytes from 23 ovaries (108). The oocytes underwent maturation in either mature follicular fluid or fetal cord serum. Cha reported transferring five embryos made from in vitro mature oocytes to a patient who delivered healthy triplet girls (108).

Uterine Transplantation

In 2015, Brannstrom and colleagues described the first successful uterine transplantation (109). A 35-year-old woman with Mayer-Rokitansky-Kuster-Hauser syndrome underwent a uterine transplant in 2013 at Sahlgrenska University Hospital in Gothenburg, Sweden. The patient started to have regular menstruation 43 days after transplantation. One year after transplantation, a single embryo was transferred back to the uterus, with successful implantation. A male baby was born at 31 weeks and 5 days (109).

Moral, Ethical, and Social Issues

Religion

The idea of IVF has been met from the very beginning with skepticism and doubt. As early as the 1940s, the Catholic Church outwardly objected to any form of artificial insemination. Two reasons were donor sperm was considered a form of adultery, and the process of providing a semen sample encouraged the

vice of masturbation. Others were concerned that this new technology may encourage eugenic government policies and involvement in family planning.

In 1982, the Vatican, emerging with strong disapproval of assisted reproduction, published the Dignitas Personae (110,111). In this document, they state "the doctor is at the service of persons and of human procreation. He does not have the authority to dispose of them or to decide their fate." Further, the document implies to aid infertile couples, adoption should be encouraged. They state the "sad reality, which often goes unmentioned, is truly deplorable: the various techniques of artificial reproduction, which would seem to be at service of life and which are frequently used with this intention, actually open the door to new threats against life," describing discarding of the embryos.

Additionally, the church holds it is ethically unacceptable to "dissociate procreation from the integrally personal context of the conjugal act." Moreover, when asked about preimplantation diagnosis, they state it is "shameful and utterly reprehensible, since it presumes to measure the value of a human life only within the parameters of 'normality' and physical well-being; thus opening the way to legitimizing infanticide and euthanasia as well." To the present day, the Vatican continues to hold these principles.

First Attempts at Legislation

In 1959, the first Congress of Infertility meeting was held in New York. Over the next several years, legislation was reviewed over 38 countries. Even from the beginning, clear consensus on legal positions did not exist among the different governments regarding artificial insemination. There was no standard on who was writing the laws, ranging from politicians to clinicians to scientists. "Reproductive tourism" expanded as patients sought care elsewhere if it was not offered in their own country. For example, patients in France would travel to Belgium for PGD or oocyte donor options. In other places, such as Nordic countries like Iceland and Finland, there simply was no legislation. Others had strict restrictions to certain parts of IVF. For instance, Denmark, Norway, and Sweden each prohibited gamete freezing and donation.

Specifically, in the United States, there were several big milestones that paved the way to the present legislation. The start of government involvement in reproductive rights was in 1973 with the *Roe versus Wade* case. The U.S. Supreme Court established the right for women to have an abortion in the first two trimesters. Per the Court, provisions included a "life of each human being begins at conception" and "unborn children have protectable interests in life, health, and well-being" (112).

Del-Zios Versus Van de Wiele Case

The first American attempt at IVF actually started several years before Drs. Jones' efforts. In September of 1973, at New York's Columbia-Presbyterian Hospital, Dr. William Sweeney was treating Doris Del-Zio, a 29-year-old with tubal infertility. After multiple failed attempts at other infertility treatments, Dr. Sweeny suggested IVF, which at that time had never been successful. Together with Dr. Landrum Shettles, Del-Zio was brought through ovarian stimulation and surgical aspiration of oocytes enough to fill two test tubes. The tubes were transferred to Dr. Shettles. Dr. Shettles then took Del-Zio's husband's sperm in another tube. The plan was to expose the oocytes to the sperm, allow them to grow for several days and then re-implant them back into Del-Zio's uterus. That same day, Dr. Shettles informed a colleague of his plans, who brought it up to her superior. By the next morning, the chairman of the OB/GYN department, Raymond Vande Wiele, learned of this plan. By 2 p.m. that day, Vande Wiele removed the tube from the incubator, exposing the cells to room temperature and stopping any cell division and thus America's first attempt at an in vitro baby. Vande Wiele's decision to stop this experiment was based on Shettles' violating federal regulation, which would endanger Columbia's grant funding for other research. In addition, Shettles was using equipment that was not sterile. Lastly, he stated if the test tube child was abnormal, it would be exposing the hospital to liability. Shettles resigned from the Columbia community a month later.

The Del-Zios couple, outraged by what had happened, filed a suit against Vande Wiele and Columbia-Presbyterian in 1974 for intentional infliction of emotional distress. They sought $1.5 million in damages. The trial finally took place in July of 1978, intensified by news of the pending arrival of England's world first test tube baby. Louise Brown was born on the seventh day of the Del-Zio trial. The trial continued until August 17th, with the conclusion that Columbia had engaged in behavior that was "utterly

intolerable in a civilized community"; however, the court only awarded Mrs Del-Zio $50,000 and Mr Del-Zio a mere $3.

Federal Control

As more controversy developed in the medical community and in the media, the government created the National Research Act in July 1974. This Act included protection of human subjects of biomedical and behavioral research. Ultimately, it prohibited any federal funding for human embryo research. This ban was in place until 1975 when reversed by Caspar Weinberger, secretary of Health, Education, and Welfare, following advice from the National Commission for the Protection of Human Subjects. The new regulations, however, then stated that IVF research with federal funding could only be used with the approval of a national Ethics Advisory Board. The caveat was the board did not exist until 1978.

Once formed, the Ethics Advisory Board unanimously recommended the federal government allowed federal funds for IVF. After which, Howard and Georgeanna Jones at Norfolk General Hospital in Norfolk, Virginia, filed for permission to open the first IVF clinic in the United States. Despite eight more months of opposition and debate, America's first IVF clinic was finally opened in March of 1980. After 16 months of attempts, the Jones' announced their first IVF pregnancy, with Elizabeth Carr born on December 28, 1981. Two years later, the first IVF clinic in New York City opened at Columbia University, with its codirector being Dr. Raymond Vande Wiele.

Baby M

As the discussion of surrogacy remained controversial, the Baby M Case came to light in 1986. Mrs Whitehead, a married women with children of her own, was asked to carry a child by Elizabeth Stern, who had multiple sclerosis and was advised not to carry a pregnancy. The pregnancy was achieved with Mrs Whitehead's oocytes fertilized with Mr William Stern's spermatozoa through artificial insemination. The Sterns offered Mrs Whitehead $10,000 to carry this baby. By the end of the pregnancy, the Whiteheads found themselves in a dilemma. How would they tell their existing children they sold their baby sister for money? The baby, who was born in March of 1986, was given two names at birth: Sarah by Mrs Whitehead and Melissa by the Sterns.

After the birth of baby M, the Whiteheads refused to give up custody, raising the issue of third party reproduction. The New Jersey Superior Court Judge Harvey R. Sorkow, after a year of deliberation, awarded custody of Baby M to the Sterns under "a best interest of the child analysis", with Whitehead given visitation rights. After this case, most European countries outlawed surrogacy. In 2004, Baby M, known as Melissa Stern, legally terminated Mrs Whitehead's parental rights and formalized the adoption process with the Sterns. This case set the precedence for many controversial cases that followed.

Asch Case

In 1994, Dr. Ricardo Asch, the pioneer of GIFT and at that time, the director of the UC-Irvine infertility clinic, was found to be using embryos from his patients without their consent. An estimated 100 patients may have had their eggs or embryos either transferred to other patients or sold to clinics for research. Furthermore, a number of infertility centers unknowingly used these stolen embryos from Dr. Asch's clinic. The theft of these embryos without the consent of the parents whom the gamete originated from has been described as a fundamental violation of human dignity. The importance of federally funded research is highlighted in this case, ensuring any research with federal funding proceeds forward with proper review.

More Legal Standardization

In 1992, the Federal Trade Commission interjected legally in a case of false advertising by an assisted reproductive technology (ART) clinic. Because of this, Congress enacted the Fertility Clinic Success Rate and Certification Act mandating all ART clinics to report their data yearly in a standardized manner to the Centers for Disease Control and Prevention (CDC). Later that year, the CDC collaborated

with the Society for Assisted Reproductive Technology (SART) to create the SART Registry. Initially, the registry was recommended to not be clinic specific, as they did not want clinics to be able to use this as an advertisement tool. However, votes were passed and the SART Registry was finalized as a clinic specific database of reported outcomes.

A year later in 1993, the National Institutes of Health Revitalization Act was passed by Senator Edward Kennedy of Massachusetts and Representative Henry Waxman of California. They overturned the Ethics Advisory Board approval requirement to use federal funding for human embryos. Instead, they allowed the National Institutes of Health to appoint a Human Embryo Research Panel to provide advice as to those areas acceptable for federal funding.

Then, in 1995, the Dickey-Wicker Amendment was passed. This restricted the use of federal funds for creating, destroying, or knowingly injuring human embryos. President Bill Clinton signed the bill, making it public law in January of 1996. The Bush administration further restricted federal funding to human cell lines as of August 2001. Most recently, President Obama, in his Executive Order in March 2009, lifted any restriction to federally funded stem cell research imposed by the president. The Dickey-Wicker amendment came out two days later as a part of the Omnibus Appropriations Act, 2009. This new amendment remains the only legal obstacle for federal funding of human embryo experimentation.

Present Day

Sir Robert Geoffrey Edwards was awarded the Nobel Prize in Physiology or Medicine in 2010 for the development of IVF. Today, there have been over 5 million births worldwide due to IVF, and growing. Approximately 1%–2% of all births in the United States are a result of IVF. Artificial reproductive technology has come a long way since Hippocrates and Aristotle, with great advancement made yearly. It is only logical to expect more improvements and breakthroughs in the future.

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REFERENCES

1. Morice P, Josset P, Chapron C, Dubuisson JB. History of infertility. *Hum Reprod Update*. 1995;1:497–504.
2. Hippocrates (trans. Littre, E.). *Oeuvres Completes*. Paris: Bailliere, 1839–1861.
3. Sauneron S. Le Germe Dans l'os. *BIFAO*. 1960;60:19–27.
4. Aristotle (trans. Louis, P.). *De La generation des animaux*. Paris: Les Belles Lettres, 1961.
5. De Graaf R. *De Mellerium Organis Generationi Inservientibus Tractatus Novus*, Lyon, 1672.
6. Bergeron A, Manjunath P. New insights towards understanding the mechanisms of sperm protection by egg yolk and milk. *Mol Reprod Dev*. 2006;73(10):1338–44.
7. Kremer J. The significance of Antoni van Leeuwenhoek for the early development of andrology. *Andrologia*. 1979;11:243–9.
8. Cobb M. Heredity before genetics: A history. *Nat Rev Genet*. 2006;7:953–8.
9. Hertwig O. Beiträge zur Kenntniss der Bildung, Befruchtung und Theilung des thierischen Eies. *Morphol. Jahrb*. 1876;1:347–434.
10. Ombelet W, Von Robays J. *History of Human Artifical Insemination*. Artificial insemination F, V&V in OBGYN, Monograph 2010, 1–5.
11. Heape W. Preliminary note on the transplantation and growth of mammalian ova within a uterine foster-mother. *Proc R Soc Lond B Biol Sci*. 1890;48:457–9.
12. Heape W. Further note on the transplantation and growth of mammalian ova within a uterine foster-mother. *Proc R Soc Lond B Biol Sci*. 1897;62:178–83.
13. Berelson B, Ford Foundation. *Population Council. Family Planning and Population Programs; A Review of World Developments*. Chicago: University of Chicago Press, 1966.

14. Menkin MF, Rock J. In vitro fertilization and cleavage of human ovarian eggs. *Am J Obstet Gynecol*. 1948;55:440–52.
15. Greep RO. Min Chueh Chang—October 10, 1908–June 5, 1991. *Biographical Memoirs National Academy of Sciences*. 1995;68:45–61.
16. Chang MC. Fertilization and normal development of follicular oocytes in the rabbit. *Science*. 1955;121:867–9.
17. Chang MC. Fertilization of rabbit ova in vitro. *Nature*. 1959;184(Suppl. 7):466–7.
18. Nayudu PL, Lopata A, Leung PC, Johnston WI. Current problems in human in vitro fertilization and embryo implantation. *J Exp Zool*. 1983;228:203–13.
19. Biggers JD. IVF and embryo transfer: Historical origin and development. *Reprodu Biomed Online*. 2012;25:118–27.
20. Steptoe PC. Gynecological endoscopy—Laparoscopy and culdoscopy. *J Obstet Gynaecol Br Commonw*. 1965;72:535–43.
21. Cohen J, Jones HW. In vitro fertilization: The first three decades. In: *In Vitro Fertilization: A Practical Approach*, Gardner DK (Ed.). New York NY: CRC Press, 2006, 1–15.
22. Edwards RG. Maturation in vitro of human ovarian oocytes. *Lancet*. 1965;2:926–9.
23. Steptoe PC. Laparoscopy and ovulation. *Lancet*. 1968;2:913.
24. Steptoe PC, Edwards RG. Laparoscopic recovery of preovulatory human oocytes after priming of ovaries with gonadotrophins. *Lancet*. 1970;1:683–9.
25. De Kretzer D, Dennis P, Hudson B, Leeton J, Lopata A, Outch K et al. Transfer of a human zygote. *Lancet*. 1973;2:728–9.
26. Steptoe PC, Edwards RG. Reimplantation of a human embryo with subsequent tubal pregnancy. *Lancet*. 1976;1:880–2.
27. Steptoe PC, Edwards RG. Birth after the reimplantation of a human embryo. *Lancet*. 1978;2:366.
28. Gemzell CA, Diczfalusy E, Tillinger G. Clinical effect of human pituitary follicle-stimulating hormone (FSH). *J Clin Endocrinol Metab*. 1958;18:1333–48.
29. Borth R, Lunenfeld B, de Watteville H. Activite gonadotrope d'un extrait d'urines de femmes en menopause. *Experimentia*. 1954;10:266.
30. Keene JL, Matzuk MM, Otani T, Fauser BC, Galway AB, Hsueh AJ et al. Expression of biologically active human follitropin in Chinese hamster ovary cells. *J Biol Chem*. 1989;264:4769–75.
31. Trounson AO, Leeton JF, Wood C, Webb J, Wood J. Pregnancies in humans by fertilization in vitro and embryo transfer in the controlled ovulatory cycle. *Science*. 1981;212:681–2.
32. Feichtinger W, Szalay S, Kemeter P, Beck A, Janisch H. Twin pregnancy after laparoscopic oocyte recovery, in-vitro fertilization and embryotransfer (author's transl). *Geburtshilfe Frauenheilkd*. 1982;42:197–9.
33. Mitwally MFM, Casper RF. Use of an aromatase inhibitor for induction of ovulation in patients with an inadequate response to clomiphene citrate. *Fertil Steril*. 2000;75:305–9.
34. Mitwally MFM, Casper RF. Aromatase inhibition: A novel method of ovulation induction in women with polycystic ovarian syndrome. *Reprod Technol*. 2000;10:244–7.
35. Menezo Y. Synthetic medium for gamete survival and maturation and for culture of fertilized eggs. *C R Acad Sci Hebd Seances Acad Sci D*. 1976;22:1967–70.
36. Mohr LR, Trounson AO. The use of fluorescein diacetate to assess embryo viability in the mouse. *J Reprod Fertil*. 1980;58:189–96.
37. Trounson AO, Willadsen SM, Rowson LE, Newcomb R. The storage of cow eggs at room temperature and at low temperatures. *J Reprod Fertil*. 1976;46:173–8.
38. Buttery B, Trounson A, McMaster R, Wood C. Evaluation of diagnostic ultrasound as a parameter of follicular development in an in vitro fertilization program. *Fertil Steril*. 1983;39:458–63.
39. Testart J, Frydman R, Feinstein MC, Thebault A, Roger M, Scholler R. Interpretation of plasma luteinizing hormone assay for the collection of mature oocytes from women: Definition of a luteinizing hormone surge-initiating rise. *Fertil Steril*. 1981;36:50–4.
40. Laws-King A, Trounson A, Sathananthan H, Kola I. Fertilization of human oocytes by microinjection of a single spermatozoon under the zona pellucida. *Fertil Steril*. 1987;48:637–42.
41. Ng SC, Bongso A, Ratnam SS, Sathananthan H, Chan CL, Wong PC et al. Pregnancy after transfer of sperm under zona. *Lancet*. 1988;2:790.
42. Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet*. 1992;340:17–8.

43. Levran D, Bider D, Yonesh M, Yemini Z, Seidman DS, Mashiach S et al. A randomized study of intracytoplasmic sperm injection (ICSI) versus subzonal insemination (SUZI) for the management of severe male-factor infertility. *J Assist Reprod Genet*. 1995;12:319–21.
44. van Rumste MM, Evers JL, Farquhar CM, Blake DA. Intra-cytoplasmic sperm injection versus partial zona dissection, subzonal insemination and conventional techniques for oocyte insemination during in vitro fertilisation. *Cochrane Database Syst Rev*. 2000:CD001301.
45. House of Representatives. *Consumer Protection Issues Involving in Vitro Fertilization Clinics. House Subcommittee on Regulation of Business Opportunities and Energy*. Washington DC, USA: US Goverment Printing Office, 1989.
46. Cohen J, Elsner C, Kort H, Malter H, Massey J, Mayer MP et al. Impairment of the hatching process following IVF in the human and improvement of implantation by assisting hatching using micromanipulation. *Hum Reprod*. 1990;5:7–13.
47. Carney SK, Das S, Blake D, Farquhar C, Seif MM, Nelson L. Assisted hatching on assisted conception (in vitro fertilisation (IVF) and intracytoplasmic sperm injection (ICSI). *Cochrane Database Syst Rev*. 2012;12:CD001894.
48. Kratochwil A, Urban G, Friedrich F. Ultrasonic tomography of the ovaries. *Ann Chir Gynaecol Fenn*. 1972;61:211–4.
49. Lenz S, Lauritsen JG. Ultrasonically guided percutaneous aspiration of human follicles under local anesthesia: A new method of collecting oocytes for in vitro fertilization. *Fertil Steril*. 1982;38:673–7.
50. Lenz S, Lauritsen JG, Kjellow M. Collection of human oocytes for in vitro fertilisation by ultrasonically guided follicular puncture. *Lancet*. 1981;1:1163–4.
51. Sterzik K, Jonatha W, Keckstein G, Rossmanith W, Traub E, Wolf A. Ultrasonically guided follicle aspiration for oocyte retrieval in an in vitro fertilization program: Further simplification. *Int J Gynaecol Obstet*. 1987;25:309–14.
52. Wikland M, Nilsson L, Hansson R, Hamberger L, Janson PO. Collection of human oocytes by the use of sonography. *Fertil Steril*. 1983;39:603–8.
53. Gleicher N, Friberg J, Fullan N, Giglia RV, Mayden K, Kesky T et al. EGG retrieval for in vitro fertilisation by sonographically controlled vaginal culdocentesis. *Lancet*. 1983;2:508–9.
54. Wikland M, Enk L, Hamberger L. Transvesical and transvaginal approaches for the aspiration of follicles by use of ultrasound. *Ann N Y Acad Sci*. 1985;442:182–94.
55. Deutinger J, Reinthaller A, Csaicsich P, Riss P, Fischl F, Bernaschek G et al. Follicular aspiration for in vitro fertilization: Sonographically guided transvaginal versus laparoscopic approach. *Eur J Obstet Gynecol Reprod Biol*. 1987;26:127–33.
56. Feldberg D, Goldman JA, Ashkenazi J, Shelef M, Dicker D, Samuel N. Transvaginal oocyte retrieval controlled by vaginal probe for in vitro fertilization: A comparative study. *J Utrasound Med*. 1988;7:339–43.
57. Strickler RC, Christianson C, Crane JP, Curato A, Knight AB, Yang V. Ultrasound guidance for human embryo transfer. *Fertil Steril*. 1985;43:54–61.
58. Buckett WM. A meta-analysis of ultrasound-guided versus clinical touch embryo transfer. *Fertil Steril*. 2003;80:1037–41.
59. Mirkin S, Jones EL, Mayer JF, Stadtmauer L, Gibbons WE, Oehninger S. Impact of transabdominal ultrasound guidance on performance and outcome of transcervical uterine embryo transfer. *J Assist Reprod Genet*. 2003;20:318–22.
60. Sallam HN, Sadek SS. Ultrasound-guided embryo transfer: A meta-analysis of randomized controlled trials. *Fertil Steril*. 2003;80:1042–6.
61. Matorras R, Urquijo E, Mendoza R, Corcostegui B, Exposito A, Rodriguez-Escudero FJ. Ultrasound-guided embryo transfer improves pregnancy rates and increases the frequency of easy transfers. *Hum Reprod*. 2002;17:1762–6.
62. Tang OS, Ng EH, So WW, Ho PC. Ultrasound-guided embryo transfer: A prospective randomized controlled trial. *Hum Reprod*. 2001;16:2310–5.
63. Kamel RM. Assisted reproductive technology after the birth of Louise Brown. *Journal Reprod Infertil* 2013;14:96–109.
64. Cohen J, Trounson A, Dawson K, Jones H, Hazekamp J, Nygren KG et al. The early days of IVF outside the UK. *Hum Reprod Update*. 2005;11:439–59.
65. Trounson A, Mohr L. Human pregnancy following cryopreservation, thawing and transfer of an eight-cell embryo. *Nature*. 1983;305:707–9.

66. Trounson A, Leeton J, Besanko M, Wood C, Conti A. Pregnancy established in an infertile patient after transfer of a donated embryo fertilised in vitro. *BMJ*. 1983;286:835–8.
67. Trounson A, Peura A, Kirby C. Ultrarapid freezing: A new low-cost and effective method of embryo cryopreservation. *Fertil Steril*. 1987;48:843–50.
68. Antinori M, Licata E, Dani G, Cerusico F, Versaci C, Antinori S. Cryotop vitrification of human oocytes results in high survival rate and healthy deliveries. *Reprod Biomed Online*. 2007;14:72–9.
69. AbdelHafez FF, Desai N, Abou-Setta AM, Falcone T, Goldfarb J. Slow freezing, vitrification and ultra-rapid freezing of human embryos: A systematic review and meta-analysis. *Reprod Biomed Online*. 2010;20:209–22.
70. Practice Committees of American Society for Reproductive M, Society for Assisted Reproductive T. Mature oocyte cryopreservation: A guideline. *Fertil Steril*. 2013;99:37–43.
71. Porcu E, Fabbri R, Damiano G, Giunchi S, Fratto R, Ciotti PM et al. Clinical experience and applications of oocyte cryopreservation. *Mol Cell Endocrinol*. 2000;169:33–7.
72. Oktay K, Karlikaya G. Ovarian function after transplantation of frozen, banked autologous ovarian tissue. *N Engl J Med*. 2000;342:1919.
73. Donnez J, Dolmans MM, Demylle D, Jadoul P, Pirard C, Squifflet J et al. Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. *Lancet*. 2004;364:1405–10.
74. Porcu E, Fabbri R, Damiano G, Fratto R, Giunchi S, Venturoli S. Oocyte cryopreservation in oncological patients. *Eur J Obstet Gynecol Reprod Biol*. 2004;113(Suppl. 1):S14–6.
75. Rao GD, Chian RC, Son WS, Gilbert L, Tan SL. Fertility preservation in women undergoing cancer treatment. *Lancet*. 2004;363:1829–30.
76. Porcu E, Venturoli S, Damiano G, Ciotti PM, Notarangelo L, Paradisi R et al. Healthy twins delivered after oocyte cryopreservation and bilateral ovariectomy for ovarian cancer. *Reprod Biomed Online*. 2008;17:265–7.
77. Ethics Committee of American Society for Reproductive M. Fertility preservation and reproduction in patients facing gonadotoxic therapies: A committee opinion. *Fertil Steril*. 2013;100:1224–31.
78. Buster JE, Bustillo M, Thorneycroft I, Simon JA, Boyers SP, Marshall JR et al. Non-surgical transfer of an in-vivo fertilised donated ovum to an infertility patient. *Lancet*. 1983;1:816–7.
79. Buster JE, Bustillo M, Thorneycroft IH, Simon JA, Boyers SP, Marshall JR et al. Non-surgical transfer of in vivo fertilised donated ova to five infertile women: Report of two pregnancies. *Lancet* 1983;2:223–4.
80. Fleming R, Adam AH, Barlow DH, Black WP, MacNaughton MC, Coutts JR. A new systematic treatment for infertile women with abnormal hormone profiles. *Br J Obstet Gynaecol*. 1982;89:80–3.
81. Frydman R, Cornel C, de Ziegler D, Taieb J, Spitz IM, Bouchard P. Prevention of premature luteinizing hormone and progesterone rise with a gonadotropin-releasing hormone antagonist, Nal-Glu, in controlled ovarian hyperstimulation. *Fertil Steril*. 1991;56:923–7.
82. Porter RR. Introduction to the complement system. *Philos Trans R Soc Lond B Biol Sci*. 1984;306:279–81.
83. Gonen Y, Jacobson W, Casper RF. Gonadotropin suppression with oral contraceptives before in vitro fertilization. *Fertil Steril*. 1990;53:282–7.
84. Gonen Y, Balakier H, Powell W, Casper RF. Use of gonadotropin-releasing hormone agonist to trigger follicular maturation for in vitro fertilization. *J Clin Endocrinol Metab*. 1990;71:918–22.
85. Humaidan P. Luteal phase rescue in high-risk OHSS patients by GnRHa triggering in combination with low-dose HCG: A pilot study. *Reprod Biomed Online*. 2009;18:630–4.
86. Humaidan P, Ejdrup Bredkjaer H, Westergaard LG, Yding Andersen C. 1,500 IU human chorionic gonadotropin administered at oocyte retrieval rescues the luteal phase when gonadotropin-releasing hormone agonist is used for ovulation induction: A prospective, randomized, controlled study. *Fertil Steril*. 2010;93:847–54.
87. Radesic B, Tremellen K. Oocyte maturation employing a GnRH agonist in combination with low-dose hCG luteal rescue minimizes the severity of ovarian hyperstimulation syndrome while maintaining excellent pregnancy rates. *Hum Reprod*. 2011;26:3437–42.
88. Fauser BC, de Jong D, Olivennes F, Wramsby H, Tay C, Itskovitz-Eldor J et al. Endocrine profiles after triggering of final oocyte maturation with GnRH agonist after cotreatment with the GnRH antagonist ganirelix during ovarian hyperstimulation for in vitro fertilization. *J Clin Endocrinol Metab*. 2002;87:709–15.
89. Kol S. Luteolysis induced by a gonadotropin-releasing hormone agonist is the key to prevention of ovarian hyperstimulation syndrome. *Fertil Steril*. 2004;81:1–5.

90. Kol S, Humaidan P, Alsbjerg B, Engmann L, Benadiva C, Garcia-Velasco JA et al. The updated Cochrane review 2014 on GnRH agonist trigger: Repeating the same errors. *Reprod Biomed Online*. 2015;30:563–5.
91. Utian WH, Sheean L, Goldfarb JM, Kiwi R. Successful pregnancy after in vitro fertilization and embryo transfer from an infertile woman to a surrogate. *N Engl J Med*. 1985;313:1351–2.
92. Temple-Smith PD, Southwick GJ, Yates CA, Trounson AO, de Kretser DM. Human pregnancy by in vitro fertilization (IVF) using sperm aspirated from the epididymis. *J In Vitro Fert Embryo Trans*. 1985;2:119–22.
93. Silber SJ, Nagy ZP, Liu J, Godoy H, Devroey P, Van Steirteghem AC. Conventional in-vitro fertilization versus intracytoplasmic sperm injection for patients requiring microsurgical sperm aspiration. *Hum Reprod*. 1994;9:1705–9.
94. Devroey P, Liu J, Nagy Z, Goossens A, Tournaye H, Camus M et al. Pregnancies after testicular sperm extraction and intracytoplasmic sperm injection in non-obstructive azoospermia. *Hum Reprod*. 1995;10:1457–60.
95. Reijo R, Alagappan RK, Patrizio P, Page DC. Severe oligozoospermia resulting from deletions of azoospermia factor gene on Y chromosome. *Lancet*. 1996;347:1290–3.
96. Asch RH, Ellsworth LR, Balmaceda JP, Wong PC. Pregnancy after translaparoscopic gamete intrafallopian transfer. *Lancet*. 1984;2:1034–5.
97. Devroey P, Braeckmans P, Smitz J, Van Waesberghe L, Wisanto A, Van Steirteghem A et al. Pregnancy after translaparoscopic zygote intrafallopian transfer in a patient with sperm antibodies. *Lancet*. 1986;1:1329.
98. Kogan SC, Doherty M, Gitschier J. An improved method for prenatal diagnosis of genetic diseases by analysis of amplified DNA sequences. Application to hemophilia A. *N Engl J Med*. 1987;317:985–90.
99. Handyside AH, Pattinson JK, Penketh RJ, Delhanty JD, Winston RM, Tuddenham EG. Biopsy of human preimplantation embryos and sexing by DNA amplification. *Lancet*. 1989;1:347–9.
100. Handyside AH, Kontogianni EH, Hardy K, Winston RM. Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. *Nature*. 1990;344:768–70.
101. Verlinsky Y, Ginsberg N, Lifchez A, Valle J, Moise J, Strom CM. Analysis of the first polar body: Preconception genetic diagnosis. *Hum Reprod*. 1990;5:826–9.
102. McArthur SJ, Leigh D, Marshall JT, de Boer KA, Jansen RP. Pregnancies and live births after trophectoderm biopsy and preimplantation genetic testing of human blastocysts. *Fertil Steril*. 2005;84:1628–36.
103. De Boer K, McArthur S, Murray C, Jansen R. First live birth following blastocyst biopsy and PGD analysis. *Reprod Biomed Online*. 2002;4:35.
104. Xu K, Shi ZM, Veeck LL, Hughes MR, Rosenwaks Z. First unaffected pregnancy using preimplantation genetic diagnosis for sickle cell anemia. *JAMA*. 1999;281:1701–6.
105. Xu K, Rosenwaks Z, Beaverson K, Cholst I, Veeck L, Abramson DH. Preimplantation genetic diagnosis for retinoblastoma: The first reported liveborn. *Am J Opthamol*. 2004;137:18–23.
106. Kokkali G, Vrettou C, Traeger-Synodinos J, Jones GM, Cram DS, Stavrou D et al. Birth of a healthy infant following trophectoderm biopsy from blastocysts for PGD of beta-thalassaemia major. *Hum Reprod*. 2005;20:1855–9.
107. Sagi M, Weinberg N, Eilat A, Aizenman E, Werner M, Girsh E et al. Preimplantation genetic diagnosis for BRCA1/2—A novel clinical experience. *Prenat Diagn*. 2009;29:508–13.
108. Cha KY, Koo JJ, Ko JJ, Choi DH, Han SY, Yoon TK. Pregnancy after in vitro fertilization of human follicular oocytes collected from nonstimulated cycles, their culture in vitro and their transfer in a donor oocyte program. *Fertil Steril*. 1991;55:109–13.
109. Brannstrom M, Johannesson L, Bokstrom H, Kvarnstrom N, Molne J, Dahm-Kahler P et al. Livebirth after uterus transplantation. *Lancet*. 2015;385:607–16.
110. Murphy TF. Dignity, marriage and embryo adoption: A look at Dignitas Personae. *Reprod Biomed Online*. 2011;23:860–8.
111. Pastor LM. Ethical analysis and commentary of Dignitas Personae document: From continuity toward the innovation. *Cuad Bioet*. 2011;22:25–46.
112. Missouri Revised Statute. Sections 1.205.1(2):2;1986.

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Evaluation and Preparation of the Infertile Couple for In Vitro Fertilization

David R. Meldrum

Thorough evaluation of the infertile couple before in vitro fertilization (IVF) is critical in achieving the best outcomes and avoiding complications. Most IVF programs organize the evaluation by using a checklist that the nurse coordinator and physician can assure is complete before proceeding with the cycle.

Evaluation of Oocyte Quality

Follicle-Stimulating Hormone (FSH)

A level exceeding 25 mIU/mL (about 12 mIU/mL using current assays) has been correlated with a low chance of pregnancy (1). More recent studies have shown that mild elevations in women fewer than 40 years of age predict a more modest reduction in the pregnancy rate, whereas an elevated level carries much more meaning in older women. Sometimes, particularly in older women, follicular maturation is very rapid and the FSH level can already be decreasing by day 3. Therefore, the level of estradiol (E2) should also be measured. The impact of an increased day 3 E2 level (over 70–80 pg/mL) in the presence of a normal FSH concentration is unclear. Using gonadotropin releasing hormone (GnRH) agonist and assisted hatching, an elevated E2 level correlated with increased cycle cancellation but not with a reduced pregnancy rate (2). FSH levels can be used to predict the optimal level of stimulation, because the ovarian response has been shown to vary inversely with the FSH level (1). For women with an FSH level greater than 10 mIU/mL, we generally choose a low responder protocol. FSH assays vary considerably in their normal ranges. When we switched to the Immulite system (Siemans Healthcare Global, Erlangen, Germany), a level of 12 mIU/mL corresponded to 25 mIU/mL with the assay used in the above report (1). In the absence of such direct comparison, one can use the College of American Pathologists survey booklet, which reports mean levels for all laboratories using each kit and standard sera (College of American Pathologists, Northfield, Illinois).

FSH levels also vary from cycle to cycle. A consistently elevated FSH predicts a poorer prognosis than a single elevated level with others being normal. The quality of ovarian stimulation is not improved when IVF is done in a cycle with a more normal FSH level (3). There is agreement that women with a single elevated FSH level have an increased cancellation rate, but studies conflict regarding the extent of reduction of pregnancy outcome (4,5). FSH levels are generally similar on days 2, 3, and 4 of the cycle, although with a prior elevated serum estradiol, day 2 may be best to reveal an elevated FSH level in someone with accelerated follicular development. Women with premenstrual spotting should be advised to count the first day of full flow as day 1.

Antral Follicle Count (AFC)

It is the antral follicles that respond to stimulation. With a high-quality trans-vaginal ultrasound scan, these can be accurately counted. AFC decreases with age in normal women (6). In women with 5–10 follicles per side, a normal response to stimulation is expected. With more than 10 follicles per side

(polycystic ovary [PCO]-like), a lower level of stimulation should be chosen than would otherwise be used based on weight and FSH level. A low AFC (fewer than 5 or 6 in total) predicts a lower prognosis (7,8) and should prompt use of a low responder protocol and a higher level of stimulation. AFC correlates positively with the number of oocytes retrieved and negatively with day 3 FSH and ampoules of gonadotrophins, with fewer than 10 total follicles predicting an increased chance of cancellation (9). By multivariate analysis, AFC was found to be a better predictor of ovarian response than FSH (10). As the outcome of IVF is very low in women above 40 years of age who develop fewer than three follicles with stimulation (11), a low AFC can be used together with other data (age, day 3 FSH, duration of infertility) to suggest egg donation as a better option.

Antimullerian Hormone (AMH)

AMH has been shown to be a better predictor of ovarian response and live birth than AFC (12), and can be measured during any phase of the menstrual cycle. As with AFC, AMH also provides some limited prediction for successful pregnancy when controlled for age (13). Very low or undetectable AMH levels can be associated in some women with a low but adequate ovarian response and pregnancies (14) and should not be used to exclude women from at least a trial of stimulation. The AMH level is increasingly being used to choose the appropriate level of stimulation; a level over 3.36 ng/mL was defined in one study as predicting OHSS with over 90% sensitivity (15).

Clomiphene Citrate Challenge Test (CCCT)

The CCCT has been used to identify patients with a low prognosis and low ovarian reserve who have a normal day 3 FSH level (16). This test has been supplanted by AFC and AMH for prediction of ovarian response and IVF success.

Identification of Patient Subgroups Requiring an Altered Approach to Stimulation

Polycystic Ovarian Syndrome (PCOS)

Women with PCOS produce more follicles with stimulation. More oocytes are retrieved, having a lower fertilization rate. The pregnancy rate is as good as other women having IVF. In a meta-analysis of 10 randomized trials, metformin increased implantation and reduced the miscarriage rate by 50% (17). The odds ratio for birth, 1.69, showed a strong trend but was not statistically significant. There was a dramatic reduction of ovarian hyperstimulation syndrome (OHSS) by over 70% (odds ratio 0.27, CI 0.16–0.46).

Metformin, which lowers circulating insulin levels and the ovarian production of androgens, has been found to reduce the follicular and estradiol response to stimulation and to increase the number of mature oocytes and embryo quality and the pregnancy rate in clomiphene-resistant women with PCOS (18). As insulin resistance is more common in women who are clomiphene-resistant, that clinical group and insulin resistance may be particularly strong indications for this adjunctive treatment. PCO women on metformin who are coasted had lower peak estradiol levels and fewer days of coasting (19). As insulin is one of the main factors that stimulate vascular endothelial growth factor production by luteinized granulosa cells, and metformin decreases ovarian response and circulating insulin levels, metformin is an important aid in reducing OHSS in these women. Even with GnRH agonist trigger and deferred embryo transfer, OHSS is not completely eliminated. Whether metformin will improve implantation of warmed embryos will be important to investigate.

Adjuncts to Stimulation Requiring Prior Treatment or Counseling

With increasing age, serum levels of the adrenal androgen, dehydroepiandrosterone (dhea) and circulating testosterone (T) decrease. T increases antral follicles and improves granulosa cell function (20). Half of the intrafollicular T results from conversion from dhea. Randomized studies have shown improved

stimulation with administration of oral dhea (21), T gel (22), and T patch (23), resulting in higher circulating T levels that are modest with dhea, moderate with T gel, and in the low male range with T patch. Consequently, it appears to require at least 2–3 months of oral dhea, a minimum of 3 weeks with T gel, and at least 5 days with the T patch, to increase the ovarian response to stimulation. Therefore, preparation for stimulation in such patients may need to be planned well in advance of the start of gonadotropins. Basal T levels also correlate with reduced ovarian response when controlled for age (24). Poor responders have been the predominant group where androgen treatment has been applied (21–23). Unfortunately, usual T assays are not accurate in that low range, so measuring serum T for this purpose is generally not practical.

Growth hormone (GH) and IGF-1 are reduced in poor responders (25) and in women failing to conceive with IVF (26). Because of reports of 3–4 fold increased pregnancy rates with GH treatment (27,28), this adjunct is being increasingly utilized in spite of being off label. It has not generally been started prior to stimulation, but because of the added cost, prior discussion with the couple is required, even though it is highly cost-effective (28). Unfortunately, due to its abuse by athletes and weight trainers, and the resulting jaundiced view by regulators, this adjunct has not been widely adopted as yet.

Evaluation of the Male Gamete

Semen Analysis with White Blood Cell Stain and Culture

A semen analysis is done before the cycle to assure that semen quality is not at a nadir for that individual due to recent factors such as stress or a febrile illness. Most IVF programs recommend intracytoplasmic sperm injection (ICSI) with any significant reduction of sperm quality. Pyospermia can reduce sperm function (29), although it does not alter fertilization, or embryo cleavage and quality (30). We attempt to clear pyospermia before proceeding to IVF; frequent ejaculation may augment the action of antibiotics. Semen culture is probably worthwhile as a routine, in order to prevent the very occasional contamination of the culture which will otherwise occur, resulting in loss of that cycle.

Strict Morphology

Cases of unexplained failure of fertilization have been found to be due to unrecognized subtle abnormalities of sperm structure. When strict morphology is decreased (<4% normal sperm according to the most recent World Health Organization standards), the chance of failed fertilization is increased. Insemination with a larger sperm number raises the fertilization rate, but embryo quality is compromised (31) and the percentages of implantation and ongoing pregnancy and delivery are reduced by 40%–50% (32). Intracytoplasmic sperm injection (ICSI) has been as successful as with other infertility factors. These findings suggest an embryotoxic effect of a high concentration of these very abnormal sperm which can be avoided by achieving fertilization with ICSI.

Antisperm Antibodies

Antisperm antibodies (ASAs) in the female can impair or prevent fertilization (33). Most programs no longer measure ASA routinely. For men with a history of testicular injury or surgery on the male collecting system, ICSI should be performed unless ASA are confirmed to be low (34).

Assessment of DNA Fragmentation

Fragmented DNA can be an unrecognized cause of infertility. This can now be determined clinically by flow cytometry (sperm chromatin structure assay [SCSA]) or other techniques such as single cell gel electrophoresis (COMET) and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labeling (TUNEL). Although there is a correlation of very abnormal sperm parameters with fragmented DNA (35), a high level of DNA fragmentation may occur with normal or mildly impaired sperm. If a prior failed IVF cycle was associated with poor progression of embryos from the cleavage to the blastocyst stage, measurement of sperm DNA fragmentation should be considered, because the male genome becomes activated at the cleavage stage. Recently there has also been a correlation with recurrent

spontaneous abortion (36). In one study, antioxidant therapy was shown to significantly reduce sperm DNA fragmentation from 22% to 9% (37). Frequent ejaculation should also be advised. The impact of a high SCSA can be lessened by density gradient centrifugation. A 450% improvement in nuclear integrity was achieved with a 45%–90% PureSperm (Nidacon, Gothenburg, Sweden) gradient (35). Recently, binding of sperm to hyaluronan has been used to choose sperm with low DNA fragmentation for ICSI (38). Retrieval of testicular sperm is also an option for men with continuing high DNA fragmentation. In the same individuals, the level of fragmentation in testicular sperm averaged 5%, compared to 24% in the ejaculate (39). As there is a correlation with motility and morphology, selecting the most active and morphologically normal sperm for ICSI will also choose the sperm most likely to have intact DNA. Some IVF laboratories have used high magnification (intracytoplasmic morphologically selected sperm injection, or IMSI) to better select the most normal appearing sperm, but that technique has not been widely adopted. Most practitioners reserve this test for couples with unexplained failed IVF.

Sexual Dysfunction

Rarely, anxiety can lead to total inability to provide a semen specimen on the day of retrieval. In our detailed instructions to patients, we state in bold print: "if you anticipate any problems providing a semen specimen on the day of retrieval, please tell us. We can arrange for you to freeze a specimen as a back-up." A supply of Viagra should be available for any male having difficulty collecting a specimen. The male should be reassured that if he is unable to provide a specimen, the oocytes can be cryopreserved with little or no impact on success of the cycle. However, if pre-implantation genetic diagnosis with deferred transfer is planned, testicular sperm extraction could be considered rather than using cryopreservation on two occasions, with its potential adverse effect and further delays for the couple.

Evaluation of Pelvic Factors Affecting Implantation

Chlamydia

A number of reports have found a negative relationship of chlamydia antibodies to successful pregnancy (40,41). In one study, a significantly higher miscarriage rate was noted (42). This may be due to chronic endometrial infection or permanent effects of prior infection. Unfortunately, the endometrium can be positive with negative cervical cultures (43). In fact, in one study of 28 infertile couples with negative cultures or DNA probe assays, 40% were found to have active chlamydia infection by polymerase chain reaction (PCR). Because of these findings, we have elected routinely to simultaneously treat both partners with a course of zythromycin. This may also eradicate unrecognized semen or pelvic infections which could compromise IVF outcome.

Trial Transfer

We have always done a rehearsal of the transfer with measurement and mapping of the endometrial canal. A controlled study has documented a significant increase in the pregnancy rate with this having been done and with a reduced incidence of difficult transfers (44). It is helpful to do this under ultrasound guidance, in order to define the optimal conditions for embryo transfer. Cervical dilation has been shown to reduce the incidence of difficult transfers (45), but should not be done at the time of oocyte retrieval unless deferred transfer is planned (46). Hysteroscopy has been used in very difficult cases to shave away ridges or cysts obstructing passage of the catheter (47).

Uterine and Tubal Abnormalities

Significant polyps or myomata are often easily visualized by pelvic ultrasound. However, most IVF practitioners perform a routine sonohysterogram or office hysteroscopy before IVF. Hysteroscopy has the advantage of visualizing small polyps or signs indicating chronic endometritis (endometrial hyperemia or edema, micropolyps) (48). A recent randomized study has shown a higher pregnancy rate following hysteroscopic excision of small (mean 16 mm) polyps (55% were under 1 cm), underlining the

importance of a thorough evaluation of the uterine cavity (49). Generally, a uterine septum should be incised before going on to IVF because of the higher risk of spontaneous abortion and premature labor.

Several studies have found approximately a 50% reduction in the rate of delivery and increased miscarriage in women with a hydrosalpinx compared with women with tubal disease without a hydrosalpinx (50). The success rate increases to normal after tubal repair or salpingectomy (51). Endometrial implantation markers are reduced in patients with a hydrosalpinx and revert to a normal pattern after salpingectomy (52). Occlusion of the proximal tube seems to be equally efficacious (53). For women having extensive pelvic adhesions, hysteroscopic occlusion can be utilized (54). Spontaneous pregnancy can occur when a unilateral hydrosalpinx is removed or successfully repaired (55). It has been suggested that only hydrosalpinges which are visible on trans-vaginal ultrasound should be removed (56). However, hydrosalpinges enlarge during stimulation (57) and visualization can be intermittent (in that study hydrosalpinx was defined during stimulation). Also, the above study had limited power and could have easily missed a significant impact on IVF success.

Endometriosis

Some studies have shown reduced rates of implantation with severe or extensive endometriosis, and unexplained failure of fertilization has been reported in some women with endometriomas. A meta-analysis showed an odds ratio for successful pregnancy with IVF of 0.56 in women with endometriosis with a significantly greater impact with higher stages of the disease (58). Even in the presence of mild endometriosis, defects of the secretory response of endometrial glandular cells and other endometrial abnormalities have been described (59). Two randomized studies have shown that a 3–6 month course of GnRH agonist leading directly into IVF was associated with an increased pregnancy rate in women with stage III and IV endometriosis (60,61). Cryopreservation of all embryos followed by deferred transfer avoids adding the extensive endometrial changes due to ovarian stimulation to the endometrial changes observed with endometriosis. The unchanged pregnancy rate with egg donation in recipients having endometriosis supports using this approach as a routine (62). The patient can also be placed on an oral contraceptive during the month between oocyte retrieval and the deferred embryo transfer to further normalize the endometrium.

Uterine Fibroids

Submucus fibroids markedly reduce the pregnancy rate with IVF (63,64). In the latter study, submucus myomas with less than 50% of their bulk in the uterine cavity were only associated with a trend toward an improved pregnancy rate following excision. However, the statistical power was low and miscarriages were not assessed. Studies have been conflicting regarding the role of intramural myomas, with some studies showing a significant reduction of outcome and others not showing an effect, possibly because size and position relative to the endometrial cavity are determining factors. Unfortunately, many studies define the distinction between a submucus and intramural myoma on the basis of procedures where fluid distends the uterine cavity under pressure, which may obscure significant distortion of the uterine cavity. For example, with one study showing no effect of intramural myomas, communication with the senior author indicated that women with distortion on ultrasound without fluid distention had myomectomy and were not included in their series. If significant distortion is observed without uterine distention, excision should be considered. Myomas enlarge substantially with pregnancy and are associated with increased miscarriage (65). Submucus myomas and intramural myomas over 4 cm should be excised before IVF (64,66).

Adenomyosis

Adenomyosis is associated with lower IVF success and increased miscarriage (67). For diagnosis, a high quality trans-vaginal ultrasound scan done by an experienced operator appears to be as effective as magnetic resonance imaging (MRI) (68). Signs of adenomyosis are scattered sono-dense punctate or linear densities, thickening of one or both uterine walls in the absence of myomata, and thickening of the junctional zone. Just as with endometriosis, deferred embryo transfer may avoid adding the endometrial changes of ovarian stimulation to the numerous alterations found with adenomyosis. Endometrial suppression is important prior to frozen embryo transfer (69).

Lifestyle Factors Important in Maximizing IVF Outcomes

Personal Habits

Smoking

Meta-analysis of studies on the effect of smoking by the female partner on IVF conception rate revealed an odds ratio of 0.54 (95% CL 0.34–0.75) (70). Secondhand smoke has been reported to have a similar impact (71). Smoking also increases the rate of spontaneous abortion. We very strongly recommend that all women stop smoking before having IVF. Smoking and secondhand smoke have a similar impact on IVF success in men (72). Fortunately the adverse effects of smoking go away within 3–6 months of smoking cessation. For either partner who is unable to quit, a marked increase of antioxidant intake may reverse some adverse effects, as smoking is a state of extreme oxidative stress.

Caffeine

A study of caffeine use found that daily intake of 2 mg or less (equivalent to one cup of decaffeinated coffee) was associated with the highest pregnancy rate with IVF (73). Another study showed no impact (74). Until further data are available, women should keep caffeine intake to a minimum.

Obesity

Obesity correlates negatively with implantation (75) and increases miscarriage (76) and preterm labor. Therefore, weight loss may improve IVF results and, most importantly, may reduce perinatal morbidity and mortality. Any level of exercise markedly increases IVF success for obese women (77), probably in part because obesity is a state of oxidative stress, which is countered by exercise.

Alcohol

Alcohol intake is associated with reduced IVF success and increased miscarriage for both the male and female partners (78).

Diet

For both partners, a "prudent" diet consisting of less red meat and saturated fat, more seafood, and more fruits and vegetables, often referred to as the "Mediterranean diet," was associated with better success with IVF (79,80). In a recent study, omega-3 fatty acids, at a dose of approximately 1800 mg and taken over a 6–7 month period, was associated with improvement of all sperm parameters, including sperm morphology (81). Omega-3 fatty acids are important constituents of sperm membranes and are decreased in men with asthenospermia (82). Omega-3s may also be helpful for the female partner in improving IVF outcome (83).

Supplements

Antioxidants and omega-3s can be augmented by diet or by supplements (see www.lifechoicesandfertility.com for a complete discussion and links to pertinent references). The website also has a more thorough discussion of lifestyle factors influencing fertility.

Mitochondrial function decreases with age. The oocyte mitochondria provide much of the energy for chromosomal segregation and cell division until the blastocyst stage, when new mitochondria again start to be produced. In the aged mouse, many of the abnormalities of oocyte mitochondrial function are reversed by treatment with Co-enzyme Q10, a mitochondrial nutrient and cofactor in energy production, even associated with improved chromosome segregation (84). There has only been a small trial in the human female, with a trend toward an increased pregnancy rate (85).

A study of actual behaviors of couples undergoing IVF (86) has shown that the above information should be again emphasized when proceeding to IVF is first being considered.

Psycho-Social Aspects

Stress, anxiety, and depression have been linked to lower IVF outcomes (87–89), and intervention improves the chance of success (90). Paying attention to these factors will also improve interactions between patients and staff, and will help couples' adjustment to the stresses of child rearing. Multiple pregnancies have been particularly shown to cause personal and marital stress. Early intervention may enhance the long-term well-being of these families.

An inventory of stressful life events during the prior 12 months revealed an adverse impact on IVF outcome (91). Couples should plan their IVF cycle for a time of lowest possible stress, and time should ideally be allowed to pass following significant stressful events before embarking on an IVF cycle.

In a study of vascular responses to stressful stimuli prior to IVF, a greater vascular response to stress was very strongly correlated with reduced IVF outcome (92). That finding supports a vascular mechanism, but also points out that there are individuals who have more exaggerated vascular constriction in response to stress. Since reduced blood flow to the mature follicle has been shown to be correlated with decreased embryo morphology and reduced pregnancy success (93), the major effect of stress may be on ovarian blood flow and oocyte quality. Testicular blood flow may also be affected, as stress in the male is associated with reduced semen quality (94). A simple question posed to an IVF couple as to whether either or both partners respond excessively to stress is likely to identify those individuals who will receive a greater benefit from psychological and behavioral interventions. The mind/body program appears to be the most effective approach for such individuals, by utilizing techniques that are intended to decrease the effects of stress on the individual.

An increasingly common intervention prior to and during IVF is acupuncture. A recent re-analysis of a large meta-analysis of randomized trials suggested a placebo effect; sufficient studies incorporating a true mock acupuncture control have not been carried out to document a true treatment effect. Because some studies found a negative impact, acupuncture should be used with caution (95).

General Health Screening

Most programs screen for HIV and hepatitis for safety of personnel. It would also be tragic to expend the amount of effort required to achieve an IVF pregnancy only to have the offspring at risk for a serious and potentially fatal disease. With hepatitis B, the female partner should be immunized. With HIV, anti-viral treatment, sperm separation, and ICSI are being used to avoid transmission of the virus to the female and offspring. PCR on the final frozen specimen can further assure that transmission is reduced to the lowest possible level. Remarkably, no instances of transmission have been reported with IVF/ICSI. Diagnosis of HIV in the female partner will allow treatment reduction of viral load with greater safety for the IVF laboratory and for the offspring.

Recent studies suggested a relationship of low vitamin D levels to reduced IVF outcome. However, in a large series of euploid embryo transfers, no relationship of levels to IVF outcomes was observed (96). This relatively large study was powered to detect an 18% difference in outcome. There was absolutely no trend toward any relationship of ongoing pregnancy to vitamin D levels. Nevertheless, restoring normal levels is logical.

Fetal levels of thyroid hormones and normal brain development during the first trimester of pregnancy are dependent on maternal thyroid levels. There has been considerable controversy whether thyroid stimulating hormone (TSH) levels over 2.5 mIU/mL call for initiation of thyroid replacement in infertile women. In one series of euploid transfers, TSH levels below the upper limit of normal did not predict outcomes, although only 80 women had TSH levels between 2.5 and 5 mIU/mL (97). In another study of recipients of oocyte donation, a marked decrease in pregnancy outcome was noted with "normal" levels above 2.5 mIU/mL, perhaps related to their older age and suggesting a uterine mechanism (98). TSH levels between 2.5 and 5 mIU/mL in 642 normal women during early pregnancy were compared to 3481 women with levels below 2.5 mIU/mL and were reported to be associated with increased spontaneous abortion (all were anti-peroxidase negative) (99). Thyroid replacement of euthyroid women with positive thyroid peroxidase antibodies beginning in the late first trimester was

associated with reduced miscarriage and premature labor (100). It appears that until further data are published, treatment of women prior to IVF with TSH levels ≥2.5 mIU/mL can be justified to reduce miscarriage to a minimum. If such women have positive anti-peroxidase antibodies, the decision to treat has very strong support.

Regular health screening such as pap smears or mammography can be easily forgotten during an extended course of fertility treatments. All appropriate health screening should be completed before embarking on pregnancy, to avoid a significant health issue being first recognized during pregnancy. For all egg donation recipients over age 45, we do a more extensive evaluation, including a stress electrocardiogram, chemistry panel, and chest X-ray.

REFERENCES

1. Muasher SJ, Oehninger S, Simonetti S, Matta J, Ellis LM, Liu HC et al. The value of basal and/or stimulated serum gonadotropin levels in prediction of stimulation response and in vitro fertilization outcome. *Fertil Steril*. 1988;50(2):298–307.
2. Frattarelli JL, Bergh PA, Drews MR, Sharara FI, Scott RT. Evaluation of basal estradiol levels in assisted reproductive technology cycles. *Fertil Steril*. 2000;74(3):518–24.
3. Scott RT, Jr., Hofmann GE, Oehninger S, Muasher SJ. Intercycle variability of day 3 follicle-stimulating hormone levels and its effect on stimulation quality in in vitro fertilization. *Fertil Steril*. 1990;54(2):297–302.
4. Martin JS, Nisker JA, Tummon IS, Daniel SA, Auckland JL, Feyles V. Future in vitro fertilization pregnancy potential of women with variably elevated day 3 follicle-stimulating hormone levels. *Fertil Steril*. 1996;65(6):1238–40.
5. Lass A, Gerrard A, Abusheikha N, Akagbosu F, Brinsden P. IVF performance of women who have fluctuating early follicular FSH levels. *J Assist Reprod Genet*. 2000;17(10):566–73.
6. Scheffer GJ, Broekmans FJ, Dorland M, Habbema JD, Looman CW, te Velde ER. Antral follicle counts by transvaginal ultrasonography are related to age in women with proven natural fertility. *Fertil Steril*. 1999;72(5):845–51.
7. Huang FJ, Chang SY, Tsai MY, Kung FT, Wu JF, Chang HW. Determination of the efficiency of controlled ovarian hyperstimulation in the gonadotropin-releasing hormone agonist-suppression cycle using the initial follicle count during gonadotropin stimulation. *J Assist Reprod Genet*. 2001;18(2): 91–6.
8. Nahum R, Shifren JL, Chang Y, Leykin L, Isaacson K, Toth TL. Antral follicle assessment as a tool for predicting outcome in IVF—Is it a better predictor than age and FSH? *J Assist Reprod Genet*. 2001;18(3):151–5.
9. Frattarelli JL, Lauria-Costab DF, Miller BT, Bergh PA, Scott RT. Basal antral follicle number and mean ovarian diameter predict cycle cancellation and ovarian responsiveness in assisted reproductive technology cycles. *Fertil Steril*. 2000;74(3):512–7.
10. Bancsi LF, Broekmans FJ, Eijkemans MJ, de Jong FH, Habbema JD, te Velde ER. Predictors of poor ovarian response in in vitro fertilization: A prospective study comparing basal markers of ovarian reserve. *Fertil Steril*. 2002;77(2):328–36.
11. Biljan MM, Buckett WM, Dean N, Phillips SJ, Tan SL. The outcome of IVF-embryo transfer treatment in patients who develop three follicles or less. *Hum Reprod*. 2000;15(10):2140–4.
12. Nelson SM, Fleming R, Gaudoin M, Choi B, Santo-Domingo K, Yao M. Antimullerian hormone levels and antral follicle count as prognostic indicators in a personalized prediction model of live birth. *Fertil Steril*. 2015;104(2):325–32.
13. Lliodromiti S, Kelsey TW, Wu, O, Anderson RA, Nelson, SM. The predictive accuracy of anti-Müllerian hormone for live birth after assisted conception: A systematic review and meta-analysis of the literature. *Hum Reprod Update*. 2014;20(4):560–70.
14. Reichman DE, Goldschlag D, Rosenwaks Z. Value of antimullerian hormone as a prognostic indicator of in vitro fertilization outcome. *Fertil Steril*. 2014;101(4):1012–8 e1.
15. Lee TH, Liu CH, Huang CC, Wu YL, Shih YT, Ho HN et al. Serum anti-Mullerian hormone and estradiol levels as predictors of ovarian hyperstimulation syndrome in assisted reproduction technology cycles. *Hum Reprod*. 2008;23(1):160–7.

16. Scott RT, Leonardi MR, Hofmann GE, Illions EH, Neal GS, Navot D. A prospective evaluation of clomiphene citrate challenge test screening of the general infertility population. *Obstet Gynecol*. 1993;82 (4 Pt 1):539–44.
17. Palomba S, Falbo A, La Sala GB. Effects of metformin in women with polycystic ovary syndrome treated with gonadotrophins for in vitro fertilisation and intracytoplasmic sperm injection cycles: A systematic review and meta-analysis of randomised controlled trials. *BJOG*. 2013;120(3):267–76.
18. Stadtmauer LA, Toma SK, Riehl RM, Talbert LM. Metformin treatment of patients with polycystic ovary syndrome undergoing in vitro fertilization improves outcomes and is associated with modulation of the insulin-like growth factors. *Fertil Steril*. 2001;75(3):505–9.
19. Stadtmauer LA, Toma SK, Riehl RM, Talbert LM. Impact of metformin therapy on ovarian stimulation and outcome in "coasted" patients with polycystic ovary syndrome undergoing in-vitro fertilization. *Reprod Biomed Online*. 2002;5(2):112–6.
20. Vendola KA, Zhou J, Adesanya OO, Weil SJ, Bondy CA. Androgens stimulate early stages of follicular growth in the primate ovary. *J Clin Invest*. 1998;101(12):2622–9.
21. Tartagni M, Cicinelli MV, Baldini D, Tartagni MV, Alrasheed H, DeSalvia MA et al. Dehydroepiandrosterone decreases the age-related decline of the in vitro fertilization outcome in women younger than 40 years old. *Reprod Biol Endocrinol*. 2015;13:18.
22. Kim CH, Howles CM, Lee HA. The effect of transdermal testosterone gel pretreatment on controlled ovarian stimulation and IVF outcome in low responders. *Fertil Steril*. 2011;95(2):679–83.
23. Fabregues F, Penarrubia J, Creus M, Manau D, Casals G, Carmona F et al. Transdermal testosterone may improve ovarian response to gonadotrophins in low-responder IVF patients: A randomized, clinical trial. *Hum Reprod*. 2009;24(2):349–59.
24. Barbieri RL, Sluss PM, Powers RD, McShane PM, Vitonis A, Ginsburg E et al. Association of body mass index, age, and cigarette smoking with serum testosterone levels in cycling women undergoing in vitro fertilization. *Fertil Steril*. 2005;83(2):302–8.
25. Bahceci M, Ulug U, Turan E, Akman MA. Comparisons of follicular levels of sex steroids, gonadotropins and insulin like growth factor-1 (IGF-1) and epidermal growth factor (EGF) in poor responder and normoresponder patients undergoing ovarian stimulation with GnRH antagonist. *Eur J Obstet Gynecol Reprod Biol*. 2007;130(1):93–8.
26. Mendoza C, Ruiz-Requena E, Ortega E, Cremades N, Martinez F, Bernabeu R et al. Follicular fluid markers of oocyte developmental potential. *Hum Reprod*. 2002;17(4):1017–22.
27. Duffy JM, Ahmad G, Mohiyiddeen L, Nardo LG, Watson A. Growth hormone for in vitro fertilization. *Cochrane Database Syst Rev*. 2010(1):CD000099.
28. Kolibianakis EM, Venetis CA, Diedrich K, Tarlatzis BC, Griesinger G. Addition of growth hormone to gonadotrophins in ovarian stimulation of poor responders treated by in-vitro fertilization: A systematic review and meta-analysis. *Hum Reprod Update*. 2009;15(6):613–22.
29. Wolff H, Politch JA, Martinez A, Haimovici F, Hill JA, Anderson DJ. Leukocytospermia is associated with poor semen quality. *Fertil Steril*. 1990;53(3):528–36.
30. Ricci G, Granzotto M, Luppi S, Giolo E, Martinelli M, Zito G et al. Effect of seminal leukocytes on in vitro fertilization and intracytoplasmic sperm injection outcomes. *Fertil Steril*. 2015;104(1):87–93.
31. Oehninger S, Kruger TF, Simon T, Jones D, Mayer J, Lanzendorf S et al. A comparative analysis of embryo implantation potential in patients with severe teratozoospermia undergoing in-vitro fertilization with a high insemination concentration or intracytoplasmic sperm injection. *Hum Reprod*. 1996;11(5):1086–9.
32. Grow DR, Oehninger S, Seltman HJ, Toner JP, Swanson RJ, Kruger TF et al. Sperm morphology as diagnosed by strict criteria: Probing the impact of teratozoospermia on fertilization rate and pregnancy outcome in a large in vitro fertilization population. *Fertil Steril*. 1994;62(3):559–67.
33. Mandelbaum SL, Diamond MP, DeCherney AH. Relationship of antisperm antibodies to oocyte fertilization in in vitro fertilization-embryo transfer. *Fertil Steril*. 1987;47(4):644–51.
34. Lombardo F, Gandini L, Dondero F, Lenzi A. Antisperm immunity in natural and assisted reproduction. *Hum Reprod Update*. 2001;7(5):450–6.
35. Tomlinson MJ, Moffatt O, Manicardi GC, Bizzaro D, Afnan M, Sakkas D. Interrelationships between seminal parameters and sperm nuclear DNA damage before and after density gradient centrifugation: Implications for assisted conception. *Hum Reprod*. 2001;16(10):2160–5.

36. Robinson L, Gallos ID, Conner SJ, Rajkhowa M, Miller D, Lewis S et al. The effect of sperm DNA fragmentation on miscarriage rates: A systematic review and meta-analysis. *Hum Reprod*. 2012;27(10):2908–17.
37. Greco E, Iacobelli M, Rienzi L, Ubaldi F, Ferrero S, Tesarik J. Reduction of the incidence of sperm DNA fragmentation by oral antioxidant treatment. *J Androl*. 2005;26(3):349–53.
38. Parmegiani L, Cognigni GE, Bernardi S, Troilo E, Ciampaglia W, Filicori M. "Physiologic ICSI": Hyaluronic acid (HA) favors selection of spermatozoa without DNA fragmentation and with normal nucleus, resulting in improvement of embryo quality. *Fertil Steril*. 2010;93(2):598–604.
39. Greco E, Scarselli F, Iacobelli M, Rienzi L, Ubaldi F, Ferrero S et al. Efficient treatment of infertility due to sperm DNA damage by ICSI with testicular spermatozoa. *Hum Reprod*. 2005;20(1):226–30.
40. Rowland GF, Forsey T, Moss TR, Steptoe PC, Hewitt J, Darougar S. Failure of in vitro fertilization and embryo replacement following infection with Chlamydia trachomatis. *J In Vitro Fert Embryo Transf*. 1985;2(3):151–5.
41. Lunenfeld E, Shapiro BS, Sarov B, Sarov I, Insler V, Decherney AH. The association between chlamydial-specific IgG and IgA antibodies and pregnancy outcome in an in vitro fertilization program. *J In Vitro Fert Embryo Transf*. 1989;6(4):222–7.
42. Licciardi F, Grifo JA, Rosenwaks Z, Witkin SS. Relation between antibodies to Chlamydia trachomatis and spontaneous abortion following in vitro fertilization. *J Assist Reprod Genet*. 1992;9(3):207–10.
43. Shepard MK, Jones RB. Recovery of Chlamydia trachomatis from endometrial and fallopian tube biopsies in women with infertility of tubal origin. *Fertil Steril*. 1989;52(2):232–8.
44. Mansour R, Aboulghar M, Serour G. Dummy embryo transfer: A technique that minimizes the problems of embryo transfer and improves the pregnancy rate in human in vitro fertilization. *Fertil Steril*. 1990;54(4):678–81.
45. Prapas N, Prapas Y, Panagiotidis Y, Prapa S, Vanderzwalmen P, Makedos G. Cervical dilatation has a positive impact on the outcome of IVF in randomly assigned cases having two previous difficult embryo transfers. *Hum Reprod*. 2004;19(8):1791–5.
46. Groutz A, Lessing JB, Wolf Y, Yovel I, Azem F, Amit A. Cervical dilatation during ovum pick-up in patients with cervical stenosis: Effect on pregnancy outcome in an in vitro fertilization-embryo transfer program. *Fertil Steril*. 1997;67(5):909–11.
47. Noyes N, Licciardi F, Grifo J, Krey L, Berkeley A. In vitro fertilization outcome relative to embryo transfer difficulty: A novel approach to the forbidding cervix. *Fertil Steril*. 1999;72(2):261–5.
48. Cicinelli E, Resta L, Nicoletti R, Tartagni M, Marinaccio M, Bulletti C et al. Detection of chronic endometritis at fluid hysteroscopy. *J Minim Invasive Gynecol*. 2005;12(6):514–8.
49. Perez-Medina T, Bajo-Arenas J, Salazar F, Redondo T, Sanfrutos L, Alvarez P et al. Endometrial polyps and their implication in the pregnancy rates of patients undergoing intrauterine insemination: A prospective, randomized study. *Hum Reprod*. 2005;20(6):1632–5.
50. Camus E, Poncelet C, Goffinet F, Wainer B, Merlet F, Nisand I et al. Pregnancy rates after in-vitro fertilization in cases of tubal infertility with and without hydrosalpinx: A meta-analysis of published comparative studies. *Hum Reprod*. 1999;14(5):1243–9.
51. Shelton KE, Butler L, Toner JP, Oehninger S, Muasher SJ. Salpingectomy improves the pregnancy rate in in-vitro fertilization patients with hydrosalpinx. *Hum Reprod*. 1996;11(3):523–5.
52. Daftary GS, Kayisli U, Seli E, Bukulmez O, Arici A, Taylor HS. Salpingectomy increases peri-implantation endometrial HOXA10 expression in women with hydrosalpinx. *Fertil Steril*. 2007;87(2):367–72.
53. Surrey ES, Schoolcraft WB. Laparoscopic management of hydrosalpinges before in vitro fertilization-embryo transfer: Salpingectomy versus proximal tubal occlusion. *Fertil Steril*. 2001;75(3):612–7.
54. Arora P, Arora RS, Cahill D. Essure((R)) for management of hydrosalpinx prior to in vitro fertilisation-a systematic review and pooled analysis. *BJOG*. 2014;121(5):527–36.
55. McComb PF, Taylor RC. Pregnancy outcome after unilateral salpingostomy with a contralateral patent oviduct. *Fertil Steril*. 2001;76(6):1278–9.
56. de Wit W, Gowrising CJ, Kuik DJ, Lens JW, Schats R. Only hydrosalpinges visible on ultrasound are associated with reduced implantation and pregnancy rates after in-vitro fertilization. *Hum Reprod*. 1998;13(6):1696–701.
57. Hill GA, Herbert CM, Fleischer AC, Webster BW, Maxson WS, Wentz AC. Enlargement of hydrosalpinges during ovarian stimulation protocols for in vitro fertilization and embryo replacement. *Fertil Steril*. 1986;45(6):883–5.

58. Barnhart K, Dunsmoor-Su R, Coutifaris C. Effect of endometriosis on in vitro fertilization. *Fertil Steril*. 2002;77(6):1148–55.
59. de Ziegler D, Borghese B, Chapron C. Endometriosis and infertility: Pathophysiology and management. *Lancet*. 2010;376(9742):730–8.
60. Surrey ES, Silverberg KM, Surrey MW, Schoolcraft WB. Effect of prolonged gonadotropin-releasing hormone agonist therapy on the outcome of in vitro fertilization-embryo transfer in patients with endometriosis. *Fertil Steril*. 2002;78(4):699–704.
61. Rickes D, Nickel I, Kropf S, Kleinstein J. Increased pregnancy rates after ultralong postoperative therapy with gonadotropin-releasing hormone analogs in patients with endometriosis. *Fertil Steril*. 2002;78(4):757–62.
62. Diaz I, Navarro J, Blasco L, Simon C, Pellicer A, Remohi J. Impact of stage III-IV endometriosis on recipients of sibling oocytes: Matched case-control study. *Fertil Steril*. 2000;74(1):31–4.
63. Eldar-Geva T, Meagher S, Healy DL, MacLachlan V, Breheny S, Wood C. Effect of intramural, subserosal, and submucosal uterine fibroids on the outcome of assisted reproductive technology treatment. *Fertil Steril*. 1998;70(4):687–91.
64. Shokeir T, El-Shafei M, Yousef H, Allam AF, Sadek E. Submucous myomas and their implications in the pregnancy rates of patients with otherwise unexplained primary infertility undergoing hysteroscopic myomectomy: A randomized matched control study. *Fertil Steril*. 2010;94(2):724–9.
65. Pritts EA, Parker WH, Olive DL. Fibroids and infertility: An updated systematic review of the evidence. *Fertil Steril*. 2009;91(4):1215–23.
66. Oliveira FG, Abdelmassih VG, Diamond MP, Dozortsev D, Melo NR, Abdelmassih R. Impact of subserosal and intramural uterine fibroids that do not distort the endometrial cavity on the outcome of in vitro fertilization-intracytoplasmic sperm injection. *Fertil Steril*. 2004;81(3):582–7.
67. Vercellini P, Consonni D, Dridi D, Bracco B, Frattaruolo MP, Somigliana E. Uterine adenomyosis and in vitro fertilization outcome: A systematic review and meta-analysis. *Hum Reprod*. 2014;29(5):964–77.
68. Valentin L. Imaging in gynecology. *Best Practice & Research Clin Obste Gyn*. 2006;20(6):881–906.
69. Niu Z, Chen Q, Sun Y, Feng Y. Long-term pituitary downregulation before frozen embryo transfer could improve pregnancy outcomes in women with adenomyosis. *GynEndocrin*. 2013;29(12):1026–30.
70. Hughes EG, Yeo J, Claman P, YoungLai EV, Sagle MA, Daya S et al. Cigarette smoking and the outcomes of in vitro fertilization: Measurement of effect size and levels of action. *Fertil Steril*. 1994;62(4):807–14.
71. Neal MS, Hughes EG, Holloway AC, Foster WG. Sidestream smoking is equally as damaging as mainstream smoking on IVF outcomes. *Hum Reprod*. 2005;20(9):2531–5.
72. Zitzmann M, Rolf C, Nordhoff V, Schrader G, Rickert-Fohring M, Gassner P et al. Male smokers have a decreased success rate for in vitro fertilization and intracytoplasmic sperm injection. *Fertil Steril*. 2003;79(Suppl. 3):1550–4.
73. Klonoff-Cohen H, Bleha J, Lam-Kruglick P. A prospective study of the effects of female and male caffeine consumption on the reproductive endpoints of IVF and gamete intra-Fallopian transfer. *Hum Reprod*. 2002;17(7):1746–54.
74. Choi JH, Ryan LM, Cramer DW, Hornstein MD, Missmer SA. Effects of caffeine consumption by women and men on the outcome of fertilization. *J Caffeine Res*. 2011;1(1):29–34.
75. Luke B, Brown MB, Stern JE, Missmer SA, Fujimoto VY, Leach R et al. Female obesity adversely affects assisted reproductive technology (ART) pregnancy and live birth rates. *Hum Reprod*. 2011;26(1):245–52.
76. Veleva Z, Tiitinen A, Vilska S, Hyden-Granskog C, Tomas C, Martikainen H et al. High and low BMI increase the risk of miscarriage after IVF/ICSI and FET. *Hum Reprod*. 2008;23(4):878–84.
77. Palomba S, Falbo A, Valli B, Morini D, Villani MT, Nicoli A et al. Physical activity before IVF and ICSI cycles in infertile obese women: An observational cohort study. *Reprod Biomed Online*. 2014;29(1):72–9.
78. Klonoff-Cohen H, Lam-Kruglick P, Gonzalez C. Effects of maternal and paternal alcohol consumption on the success rates of in vitro fertilization and gamete intrafallopian transfer. *Fertil Steril*. 2003;79(2):330–9.
79. Vujkovic M, de Vries JH, Lindemans J, Macklon NS, van der Spek PJ, Steegers EA et al. The preconception Mediterranean dietary pattern in couples undergoing in vitro fertilization/intracytoplasmic sperm injection treatment increases the chance of pregnancy. *Fertil Steril*. 2010;94(6):2096–101.
80. Twigt JM, Bolhuis ME, Steegers EA, Hammiche F, van Inzen WG, Laven JS et al. The preconception diet is associated with the chance of ongoing pregnancy in women undergoing IVF/ICSI treatment. *Hum Reprod*. 2012;27(8):2526–31.

81. Safarinejad MR. Effect of omega-3 polyunsaturated fatty acid supplementation on semen profile and enzymatic anti-oxidant capacity of seminal plasma in infertile men with idiopathic oligoasthenoteratospermia: A double-blind, placebo-controlled, randomised study. *Andrologia*. 2011;43(1):38–47.
82. Eslamian G, Amirjannati N, Rashidkhani B, Sadeghi MR, Baghestani AR, Hekmatdoost A. Dietary fatty acid intakes and asthenozoospermia: A case-control study. *Fertil Steril*. 2015;103(1):190–8.
83. Kim CH YJ, Ahn JW, Kang HJ, Lee JW, Kang BM. The effect of supplementation with omega-3-polyunsaturated fatty acids in intracytoplasmic sperm injection cycles for infertile patients with a history of unexplained total fertilization failure. *Fertil Steril*. 2010;94(4):S242.
84. Ben-Meir A, Burstein E, Borrego-Alvarez A, Chong J, Wong E, Yavorska T et al. Coenzyme Q10 restores oocyte mitochondrial function and fertility during reproductive aging. *Aging Cell*. 2015;14(5):887–95.
85. Bentov Y, Hannam T, Jurisicova A, Esfandiari N, Casper RF. Coenzyme Q10 supplementation and oocyte aneuploidy in women undergoing IVF-ICSI treatment. *Clin Med Insights Reprod Health*. 2014;8:31–6.
86. Domar AD, Conboy L, Denardo-Roney J, Rooney KL. Lifestyle behaviors in women undergoing in vitro fertilization: A prospective study. *Fertil Steril*. 2012;97(3):697–701 e1.
87. Sanders KA, Bruce NW. Psychosocial stress and treatment outcome following assisted reproductive technology. *Hum Reprod*. 1999;14(6):1656–62.
88. Klonoff-Cohen H, Chu E, Natarajan L, Sieber W. A prospective study of stress among women undergoing in vitro fertilization or gamete intrafallopian transfer. *Fertil Steril*. 2001;76(4):675–87.
89. Smeenk JM, Verhaak CM, Eugster A, van Minnen A, Zielhuis GA, Braat DD. The effect of anxiety and depression on the outcome of in-vitro fertilization. *Hum Rprod*. 2001;16(7):1420–3.
90. Domar AD, Rooney KL, Wiegand B, Orav EJ, Alper MM, Berger BM et al. Impact of a group mind/body intervention on pregnancy rates in IVF patients. *Fertil Steril*. 2011;95(7):2269–73.
91. Ebbesen SM, Zachariae R, Mehlsen MY, Thomsen D, Hojgaard A, Ottosen L et al. Stressful life events are associated with a poor in-vitro fertilization (IVF) outcome: A prospective study. *Hum Reprod*. 2009;24(9):2173–82.
92. Facchinetti F, Matteo ML, Artini GP, Volpe A, Genazzani AR. An increased vulnerability to stress is associated with a poor outcome of in vitro fertilization-embryo transfer treatment. *Fertil Steril*. 1997;67(2):309–14.
93. Lozano DH, Frydman N, Levaillant JM, Fay S, Frydman R, Fanchin R. The 3D vascular status of the follicle after HCG administration is qualitatively rather than quantitatively associated with its reproductive competence. *Hum Reprod*. 2007;22(4):1095–9.
94. Vellani E, Colasante A, Mamazza L, Minasi MG, Greco E, Bevilacqua A. Association of state and trait anxiety to semen quality of in vitro fertilization patients: A controlled study. *Fertil Steril*. 2013;99(6):1565–72.
95. Meldrum DR, Fisher AR, Butts SF, Su HI, Sammel MD. Acupuncture—Help, harm, or placebo? *Fertil Steril*. 2013;99(7):1821–4.
96. Franasiak JM, Molinaro TA, Dubell EK, Scott KL, Ruiz AR, Forman EJ et al. Vitamin D levels do not affect IVF outcomes following the transfer of euploid blastocysts. *Am J Obstet Gynecol*. 2015;212(3):315 e1–6.
97. Anderson KW, MD Franasiak, JM, Hong KH, Scott RT Jr. The impact of variations of TSH with the "low normal range" on euploid blastocyst implantation rate (IR). *Fertil Steril*. 2014;102(3):e31–2.
98. Sordia-Hernandez LH, Morales Martinez A, Gris JM, Herrero J, Hernandez Uzcanga C, Merino M et al. Normal "high" thyroid stimulating hormone (TSH) levels and pregnancy rates in patients undergoing IVF with donor eggs. *Clin Exp Obstet Gynecol*. 2014;41(5):517–20.
99. Negro R, Schwartz A, Gismondi R, Tinelli A, Mangieri T, Stagnaro-Green A. Increased pregnancy loss rate in thyroid antibody negative women with TSH levels between 2.5 and 5.0 in the first trimester of pregnancy. *J Clin Endo Metab*. 2010;95(9):E44–8.
100. Negro R, Formoso G, Mangieri T, Pezzarossa A, Dazzi D, Hassan H. Levothyroxine treatment in euthyroid pregnant women with autoimmune thyroid disease: Effects on obstetrical complications. *J Clin Endo Metab*. 2006;91(7):2587–91.

3

Carrier Screening

Stephanie Hallam and Gregory Porreca

Introduction

An important component of a healthy pregnancy and live birth is avoidance of prevalent and severe genetic disorders. Many of these can be readily screened by testing a sample from each member of the couple who will biologically contribute to an embryo. Carrier screening is the process of assessing the risk for a couple to have a child with an inherited genetic disorder due to pathogenic variants (referred to as mutations in the rest of this chapter) in one or more genes. Carrier screening may be used to determine a couple's risk for having a child with conditions like cystic fibrosis and sickle cell anemia. Knowing carrier status can lead to more informed decisions for couples making reproductive decisions. Additionally, knowing the mutations involved facilitates pre-implantation genetic diagnosis (PGD).

Modes of Inheritance

There are approximately 20,000 to 25,000 human genes spread among the 24 distinct human chromosomes (22 autosomes, plus the sex chromosomes, X and Y). Each individual typically has a total of 46 chromosomes, two each of the 22 autosomes (one of each from each parent) and either two X's (in females) or an X and a Y (in males). Chromosome disorders are generally those that can be detected by analysis of changes in the appearance of condensed chromosomes using staining techniques and a microscope (typically changes that impact more than 7 megabases of DNA). While some of these changes are inherited, most happen anew in the affected individual. Hence, testing of the parental chromosomes is rarely a useful means to determine the risk for their child to have a chromosome disorder.

Carrier screening generally focuses on smaller changes that look for mutations at the gene level. The primary modes of inheritance for gene-level disorders include autosomal recessive, autosomal dominant, X-linked recessive, and X-linked dominant. Other modes exist but are beyond the scope of this text. For purposes of carrier screening the focus is on the autosomal recessive and X-linked recessive disorders.

Autosomal recessive disorders manifest when a child inherits a mutation in the same gene from each parent. Note that the mutation itself does not need to be the same. In this scenario each biological parent is typically a carrier with one copy of the gene having a mutation and one copy that is normal. This carrier situation is usually asymptomatic such that the individual is unaware of their carrier status without genetic carrier screening. When both parents are carriers of a given gene disorder, the risk for an affected child is 1 in 4 or 25%; the risk to have a child that is a carrier, like themselves, is 1 in 2 or 50%; and the chance that the child will not have either mutation and be an unaffected, noncarrier is 1 in 4. See Figure 3.1 for an illustration of autosomal recessive inheritance.

The other category of inheritance to consider for carrier screening is X-linked recessive disorders. These are disorders that are due to genes that occur on the X chromosome. If the gene in question contains a mutation but occurs in a female with a second, normal copy of the gene, then the woman is a carrier but will be unaffected. If the X chromosome with the mutation is present in a male, then there is no functional copy of the gene and the disorder will manifest. An illustration of classic X-linked recessive inheritance where the mother is a carrier is provided in Figure 3.2. Note that there is a 1 in 2 or 50%

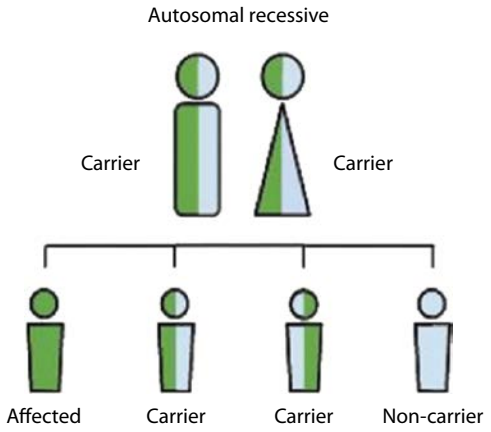

FIGURE 3.1 Autosomal recessive inheritance.

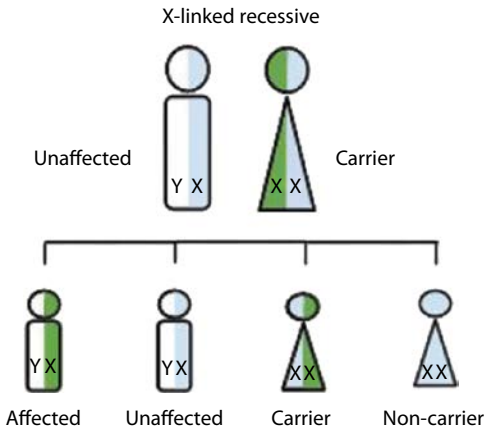

FIGURE 3.2 X-linked recessive inheritance.

chance that each child will inherit the X chromosome from the mother that has the mutation, but only the males are affected. If the couple is having a boy then the risk for an affected male is 1 in 2 or 50%, if the couple is having a baby, the risk for an affected child is 1 in 4 or 25%. If the condition associated with the X-linked mutation is not severe, such that males survive and reproduce, then an affected male will not pass the condition to any of his sons (they all inherit his Y chromosome) but all his daughters will be carriers (they all inherit his X with the mutated copy of the gene). Color blindness would be an example of this type of condition. Fragile X syndrome is also an example of X-linked inheritance; however, this disorder has additional complexity due to the type of mutation and the fact that it is not truly recessive; females that have one copy of a mutation may have some symptoms such as learning difficulties (when a full mutation is present) or premature ovarian insufficiency (when a permutation is present).

Types of DNA Mutations

There are a number of different types of changes that may occur in genes that are relevant to carrier screening. DNA, and hence genes, consist of four different units (bases): G, A, T, C; these are the letters of the genetic code. Many changes in the DNA replace one of these letters for another; others add and/or remove one or more of these letters. The type of change and where it occurs can determine whether

the change is pathogenic or benign. In a carrier screening situation there is the potential for confusion and unnecessary anxiety if the status of the DNA change detected is reported to the patient when the change is not well understood. Consequently, most carrier screening focuses only on known pathogenic DNA changes.

Carrier Rates, Detection Rates, and Residual Risk

Carrier screening typically has very high analytical sensitivity and specificity; i.e., if a mutation is included in the assay being performed, it can be readily detected, and mutations included in the assay are rarely reported as being present when they are in fact absent. The clinical, as opposed to analytical, sensitivity and specificity are related to the ability to accurately determine the true carrier status of an individual; so the clinical sensitivity is impacted by the detection rate, which is the percentage of mutations that could cause an individual to be a carrier that are included in the assay. For clinical specificity it is important to only report DNA changes that cause the disorder (those that are known to be pathogenic). Because there can be many different mutations that could cause an individual in a population to be a carrier of a disorder, and these may vary by ethnicity, a negative screening result reduces, but does not eliminate, the possibility to be a carrier of the disorder screened. For this reason results are provided as probabilities of still being a carrier after the screening test and this probability is called the residual risk. The residual risk is calculated using the carrier rate, which is the frequency of the disorder in the population to which the individual belongs, and the detection rate of the assay. These calculations are performed using Bayes' Theorem. A worked example for a theoretical disorder with a known carrier frequency of 1/30 and a test detection rate of 95% is shown in Table 3.1. A good introduction to risk calculation in genetic counseling is cited at the end of this chapter (1).

Commonly-Screened Disorders

Brief summaries of commonly screened disorders are provided below. For brevity, many details are omitted, but can be important in clinical practices. For those not already familiar, more complete information can be found in the clinical practice guidelines cited, as well as by consulting a board-certified genetics professional.

Cystic Fibrosis

Cystic fibrosis (CF) is caused by mutations in a transmembrane conductance regulator gene (CFTR) involved in transport of chloride ions. CF is a complex multisystem disease that affects the respiratory tract, exocrine pancreas, intestine, male genital tract, hepatobiliary system, and exocrine sweat glands. Pulmonary disease is the major cause of morbidity and mortality due to airway inflammation and endobronchial infection that progresses to end-stage lung disease. Pancreatic insufficiency with malabsorption also occurs in most individuals. Meconium ileus occurs in 15%–20% of newborns with CF. Because

TABLE 3.1

Example Residual Risk Calculation

Probability	Individual is a Carrier	Individual is NOT a Carrier	Sum
Prior	1/30	29/30	
Negative test result[a]	5%	1	
Joint calculation	$1/30 \times 5\%$	$29/30 \times 1$	
Joint values	0.00167	0.96667	0.96833
Residual risk calculation	= 0.00167/0.96833		
Residual risk value	1 in 581		

[a] Test has a 95% detection rate for the disorder.

95% of males with CF are infertile as a result of azoospermia caused by absent, atrophic, or fibrotic Wolffian duct structures, and because congenital absence of the vas deferens (CAVD) can also occur in men without other overt symptoms of classic CF, it is important to screen for CF mutations in the infertile population presenting with these complaints. The prevalence of CF in Northern Caucasians is approximately 1 in 2500–3200 live births. The prevalence is lower in Hispanics (1 in 13,500), Asian, Hawaiian and Pacific Islanders (1 in 31,000–100,000), and African Americans (1 in 15,100) (2). Life expectancy for those with CF continues to improve; in 2007 the average was 37.4 years (2). CF is inherited in an autosomal recessive manner; approximately 1 in 30 individuals in the United States is an asymptomatic carrier.

Disorders Prevalent in the Ashkenazi Jewish (AJ) Population

There are a number of disorders that are prevalent in the AJ population primarily as a result of founder mutations. However, other mutations do exist in other ethnic groups and, as a result of population admixture, some of the AJ mutations are present in individuals who do not identify as this ethnicity. AJ disorders that are frequently included in carrier screening panels include: Bloom syndrome, Canavan disease, DLD (dihyrolipoamide dehydrogenase deficiency), familial dysautonomia, familial hyperinsulinism, Fanconi anemia type C, Gaucher disease, glycogen storage disease type 1a, Joubert syndrome, maple syrup urine disease type 1A and 1B, mucolipidosis type IV, nemaline myopathy, Nieman-Pick type A, sickle cell anemia, Tay-Sachs disease, Usher syndrome type IF, and Usher syndrome type III. Most are severe, or have severe forms that result in significant morbidity and/or mortality. Additional disorders continue to be added to carrier screening panels. Table 3.2 shows the carrier frequency of these disorders in the AJ population. All are inherited in an autosomal recessive manner (familial hyperinsulinism also has additional modes of inheritance).

Fragile X Syndrome

Fragile X syndrome is caused by mutations in the *FMR1* gene. It results in moderate intellectual disability in males and mild intellectual disability in affected females. Males often have a characteristic appearance: large head, long face, prominent chin and forehead, protruding ears and large testes after puberty. Behavioral abnormalities, sometimes including autism spectrum disorder, are also common. Other disorders associated with premutations in the *FMR1* gene include primary ovarian insufficiency in females and fragile X associated tremor/ataxia syndrome (FXTAS) which may occur in older males and females, but is more common in males (3). Life expectancy overall for those with *FMR1* gene mutations is in the normal range.

The mutation that causes over 99% of *FMR1* related disease is a triplet repeat (CGG) of the DNA just upstream of the protein coding section of the gene. The disorders and risks for affected offspring relate to the number of copies of this CGG repeat. Normal size repeats are stably transmitted except in rare instances where the change is usually minor. Repeats greater than 45 typically expand only when transmitted by a female. Details are provided in Table 3.3.

Disease prevalence in males is approximately 1 in 5000, and is estimated to be approximately 1 in 10,000 females. The prevalence of female carriers has been reported to be anywhere from 1 in 77 to 1 in 382.

Hemoglobinopathies

The major hemogloginopathies are disorders that impact hemoglobin synthesis (4). Normal adult hemoglobin (hemoglobin A) consists of two alpha polypeptide chains (coded by the *HBA1* and *HBA2* genes), and two beta chains (coded by the *HBB* gene). Beta-thalassemia is caused by mutations in the *HBB* gene that reduce the amount of hemoglobin; alpha-thalassemia is caused by mutations in the *HBA1* and *HBA2* genes that reduce the amount of hemoglobin. Sickle cell disease is due to a specific mutation in the *HBB* gene. Other forms of hemoglobin exist as a result of the other genes involved in hemoglobin synthesis during development. Inappropriate persistence of these differentially expressed genes may occur if mutations exist in the gene cluster for the beta-like or alpha-like hemoglobin chain genes. Because of the overlapping prevalence of these disorders, the many forms of hemoglobin and the fact that hemoglobin

TABLE 3.2

Genetic Disorders Prevalent in the Ashkenazi Jewish Population

Disorder	Gene	Clinical Description	Life Expectancy	Carrier Frequency in AJ	Carrier Frequency Other Ethnicity
Bloom syndrome	BLM	Short stature, sun-sensitive facial lesions, immune deficiency, sterility, and increased cancer risk.	Less than 30 years	1 in 134	Unknown: expected to be very low
Canavan disease	*ASPA*	Severe neurodegenerative disorder with intellectual disability. Rapidly increasing head circumference, lack of head control, reduced visual responsiveness, abnormal muscle tone such as stiffness or floppiness. Inability to crawl, walk, sit, or talk.	Average lifespan is 3–10 years; survival into the 20s in rare case	1 in 55	Unknown: expected to be low
Dihydrolipoamide dehydrogenase deficiency		Variable age of onset and presentation from fatigue following even mild exertion, to episodes of hyperventilation, vomiting, and abdominal pain, which may be fatal. Developmental delay may also occur.	Variable: appropriate management can increase lifespan	1 in 107	Unknown: expected to be low
Familial dysautonomia	*IKBKAP*	Severe neurologic disorder affecting sensory and autonomic nervous system (swallowing, sweating, pain sensitivity). Increased risk for pulmonary infection and gastrointestinal complications.	50% die before end of fourth decade	1 in 31	Unknown: expected to be low
Familial hyperinsulinism	*ABCC8*	Overproduction of insulin causes hypoglycemia and seizures with associated brain damage and risk of death if not controlled. Poor muscle tone, poor feeding and breathing difficulties can also occur.	Variable, manage insulin/glucose levels. Can be normal in some.	1 in 68	Increased frequency seen in Finnish and Saudi Arabian populations. Other: expected to be low
Fanconi anemia (group C)	*FANCC*	Congenital abnormalities, bone problems (short stature, bone marrow failure), progressive pancytopenia, increased cancer risk, possible intellectual or learning disability.	20–30 years	1 in 100	U.S. Panethnic: 1 in 300 Increased frequency seen in Afrikaners
Gaucher disease	*GBA*	Variable from asymptomatic to severe hepatosplenomegaly, bone involvement, blood abnormalities (anemia, easy bruising, impaired clotting, etc). Neuropathic form is less severe.	Variable ranging from no outward symptoms to severe disability and death. Enzyme replacement therapy available and can reduce symptoms.	1 in 15	Panethnic: 1 in 100 to 1 in 200
Glycogen storage disease type 1a	*G6PC*	Enzyme defect causing severe hypoglycemia. Failure to thrive, enlarged liver and spleen, hypoglycemic episodes, gout, kidney problems, seizures, recurrent infections.	If treated with dietary management, normal life expectancy is possible. If untreated, early death likely.	1 in 64	Unknown: expected to be low

(*Continued*)

TABLE 3.2 (*Continued*)

Genetic Disorders Prevalent in the Ashkenazi Jewish Population

Disorder	Gene	Clinical Description	Life Expectancy	Carrier Frequency in AJ	Carrier Frequency Other Ethnicity
Joubert syndrome 2	*TMEM216*	Neurologic disorder with brain malformations. Decreased muscle tone, mild to moderate intellectual disability, and abnormal eye movements. Breathing difficulty, ataxia, failure to thrive, retinal degeneration, and renal dysfunction.	Can be shortened by kidney or liver failure; otherwise can be normal.	1 in 110	Unknown: expected to be low
Maple syrup urine disease type 1A/1B	*BCKDHA & BCKDHB*	Inability to breakdown amino acids: feeding problems, vomiting, and irritability. If untreated, maple syrup urine disease type 1A/1B may result in seizures, intellectual disability, coma, and death.	Death in infancy may occur; however, can be normal with early detection and controlled diet.	1 in 97	Mennonite: 1 in 7 Other: expected to be low
Mucolipidosis type IV	*MCOLN1*	Severe neurodegenerative disorder with visual impairment (corneal clouding, retinal degeneration) and intellectual disability. Symptoms also include limited or no ability to walk independently, limited speech, digestive problems, and anemia.	Typically survive to adulthood but lifespan may be shortened	1 in 89	Unknown: expected to be low
Nemalin myopathy		Variable severity and age of onset. Muscle weakness that is most pronounced in the face, neck, and limbs. Symptoms may include feeding and respiratory difficulties and delayed motor development. Cognition is generally normal, although verbal communication may be limited.	Variable: severe form, individuals may have a reduced lifespan due to breathing difficulties. In less severe forms lifespan may be normal.	1 in 168	Unknown: expected to be low
Nieman -Pick disease type A	*SMPD1*	Severe neurodegenerative disorder with failure to thrive and hepatosplenomegaly. A milder form (type B) is more variable and little or no neurologic involvement also exists.	Type A: 2–4 years Type B: Variable with possible survival to adulthood.	1 in 115	Unknown: expected to be low
Tay-Sachs disease	*HEXA*	Severe neurodegenerative disorder: progressive weakness, loss of motor skills, decreased attentiveness, increased startle response, seizures, blindness, spasticity, eventual incapacitation.	Average lifespan is <4 years	1 in 27	French Canadian: 1 in 73 Other: 1 in 300
Usher syndrome type 1F	*PCDH15*	Profound hearing loss at birth, progressive vision loss due to retinitis pigmentosa.	Normal	1 in 147	Unknown: expected to be low
Usher syndrome type 3	*CLRN1*	Post-lingual hearing loss that is moderate to severe, variable onset and severity of retinitis pigmentosa.	Normal	1 in 120	Increased frequency seen in Finnish population Other: expected to be low
Walker–Warburg syndrome	*FKTN*	Severe disease of the brain (seizures, developmental delay, mental retardation), muscles (weakness, feeding difficulty) and eyes (blindness).	Less than 3 years	1 in 150	Unknown: expected to be low

Source: SCOG guidelines and Jewish Genetic Disease Consortium; accessed August 25, 2015. http://www.jewishgeneticdiseases.org/jewish-genetic-diseases/

TABLE 3.3

Clinical Impact of Various Fragile X Repeat Sizes

Size Range	Mutation Type	Associated Disorders	Risk for Offspring
<45	None	None	None
45–54	Intermediate	None	Risk for expansion of repeat to premutation range upon transmission. Negligible risk for fragile X syndrome
55–199	Premutation (typically unmethylated)	Premature ovarian insufficiency; fragile X-associated tremor/ataxia syndrome	Risk for expansion of repeat upon transmission. Fragile X syndrome risk depends on size of the premutation
≥200	Full mutation (typically methylated)	Fragile X syndrome. All males; approximately 50% of females	Females have a 50% risk of passing on the full mutation with each child.

is a tetramer of alpha- and beta-globin polypeptides, hemoglobinopathies are a heterogeneous group of disorders with a wide range of clinical severity.

Generally speaking the hemoglobinopathies are characterized by microcytic anemia. They are most frequent in individuals of African, Mediterranean, Asian, Middle Eastern, Hispanic, and West Indian ancestry. Screening begins, for most ethnic groups that have an increased incidence of disease, with a complete blood count (CBC) to assess for a low mean corpuscular volume (MCV less than 80 fL).

Alpha-thalassemia

Alpha-thalassemia most frequently results from deletion of one or both of the alpha-globin genes; however, other types of gene mutations that disrupt protein amount exist. Because there are two genes (*HBA1* and *HBA2*), there are normally four copies of the alpha-globin gene in an individual (two from each parent). Deletion of one copy of the gene is clinically silent (α -/ α α); deletion of two copies typically results in mild, asymptomatic microcytic anemia. When two copies of the gene are deleted the arrangement may be both copies on one chromosome (- -/ α α); this is most frequent in Southeast Asians (carrier frequencies of approximately 1 in 20) (5); or one copy on each chromosome (α -/ α -), which is more common in those of African ancestry (carrier frequencies in some regions reaching 1 in 4) (5). If a child inherits one functional copy of the alpha-globin gene (α -/ - -), HbH disease with hemolytic anemia results. Absence of all four genes (--/--) results in Hb Bart's disease with hydrops fetalis, intrauterine death, and pre-eclampsia. In addition to the deletion mutations and those that impact alpha-globin chain amount, there are mutations in the alpha-globin genes that change the structure of the protein and may result in a similar clinical presentation.

Beta-thalassemia

Beta-thalassemias are categorized based on a description of the underlying mutation or by clinical manifestations. Individuals heterozygous (with one copy) of a beta-thalassemia mutation have beta-thalassemia minor, which is typically associated with asymptomatic mild anemia. Those who are homozygous (both copies of the gene are mutated) typically have beta-thalassemia major, or a milder form called beta-thalassemia intermedia may exist if some amount of HbA is produced. Beta-thalassemia major is characterized by severe anemia which causes extramedullary erythropoiesis, delayed sexual development, and poor growth. Death usually occurs by age 10 without periodic blood transfusion or bone marrow transplant. If production of fetal hemoglobin (HbF) persists, this can reduce the severity of symptoms. Carrier frequency for beta-thalassemia has been reported to be as high as 1 in 5 in Mediterranean populations (6).

Sickle Cell Disease

The mutation in the *HBB* gene that results in sickle cell disease alters the function of the beta-globin protein causing hemoglobin to polymerize when oxygen is low. This leads to formation of the characteristic

sickle shaped red blood cells that cause vaso-occlusion and chronic hemolytic anemia. Tissue ischemia, chronic pain, and organ damage result. Individuals with two copies of the sickle cell mutation do not make normal hemoglobin (HbA), instead they make sickle hemoglobin (HbS). Individuals with sickle cell anemia usually begin to show symptoms in early childhood; these include pain and swelling in the hands and feet, frequent infection, shortness of breath, fatigue, and delayed growth. Pulmonary hypertension can occur in about one third of adults and can lead to heart failure. Prevention or reduction of major symptoms is possible by maintaining good hydration, avoidance of climate extremes, and avoidance of activities that may cause inflammation. Hydroxyurea can reduce frequency and severity of vaso-occlusion events, can help to reduce the number of blood transfusions, and increase lifespan. Carrier frequency for sickle cell disease can be as high as 1 in 12 in individuals of African ancestry.

Because mutations in the *HBB* gene cause both beta-thalassemia and sickle cell anemia, co-inheritance of both is possible, with varying severity of disease from mild to severe.

Spinal Muscular Atrophy

Spinal muscular atrophy (SMA) is a severe neuromuscular disease caused by degeneration of the anterior horn cells in the spinal cord and brain stem nuclei resulting in proximal muscle weakness and paralysis. Poor weight gain, sleep difficulties, pneumonia, scoliosis, and joint contractures are common complications. Onset ranges from before birth to adolescence or young adulthood. Childhood SMA is divided into three categories determined by age of onset and clinical course. Children with type I disease usually have onset of symptoms by age 3 months and death from respiratory failure by age 2. Type II is milder, with the ability to sit but not walk unaided; survival is usually beyond 4 years of age. Type III is milder still, with onset in infancy or youth, and the ability to walk unaided.

The incidence of SMA is 1 in 10,000 in the United States (7). SMA is inherited in an autosomal recessive manner; approximately 1 in 50 individuals in the United States is an asymptomatic carrier.

Medical Society Recommendations Related to Carrier Screening

Many of the current professional medical society recommendations for carrier screening are based on testing specific subsets of populations for specific disorders based on ethnicity and disease prevalence. For example, testing for sickle cell and beta-thalassemia is recommended in those of African American descent but not for Northern European Caucasians, and many disorders are recommended for screening in the Ashkenazi Jewish population but generally not in other populations. Today, many nations are seeing increased mixing of ethnicity and, hence, determining who to test using ethnicity-based guidelines has become increasingly problematic. This was acknowledged by the American College of Obstetricians and Gynecologists (ACOG) when they updated their cystic fibrosis (CF) carrier screening recommendations in 2011 citing: "…it is becoming increasingly difficult to assign a single ethnicity to affected individuals. It is reasonable, therefore, to offer CF carrier screening to all patients" (8).

A summary of professional medical society recommendations is provided below. These guidelines have been generalized for purposes of this discussion; for more details of the specific recommendations in each case, please refer to the cited guideline. In addition, this discussion is somewhat U.S.-centric, and other guidelines may exist. Guidelines for some disorders do not exist from certain professional societies or countries due to one or more of the following: lack of evidence for a favorable cost benefit, deferral to other society recommendations, and lack of resource to review and prepare a guideline for the disorder.

Cystic fibrosis (CF) screening is recommended for women of reproductive age regardless of ethnicity by ACOG (8), the American College of Medical Genetics and Genomics (ACMG) (9), and the National Society of Genetic Counselors (NSGC) (10). The Society of Obstetricians and Gynecologists of Canada (SOGC) recommends CF testing only during pregnancy when there is a family history or clinical manifestation of CF (11). In Australasia, it is recommended that all couples who are pregnant or planning children are made aware of the availability of carrier testing (12). In the United Kingdom, systematic population screening is not recommended; however, this guideline is currently under review (13).

For disorders prevalent in the Ashkenazi Jewish (AJ) population:

- ACOG, ACMG, and SOGC recommend screening for Tay-Sachs disease, Canavan disease, and familial dysautonomia (14,15).
- ACMG additionally recommends screening for Niemann-Pick (type A), Bloom syndrome, Fanconi anemia group C, Gaucher disease, and Mucolipidosis IV. SOGC only recommends testing for these additional disorders and glycogen storage disease type 1a when there is a positive family history (16).

Tay-Sachs disease screening is also recommended in those of French Canadian and Cajun ancestry. Assessment of carrier status for Tay-Sachs disease typically includes biochemical testing to determine enzyme levels (hexosaminidase A). This test may be indicated for partners of known carriers, regardless of ethnicity. While this test may be the most sensitive for carrier detection, recent experience in our facility has shown that this assay can have poor specificity outside the target population.

Fragile X syndrome screening is recommended by ACOG, ACMG, and NSGC in individuals with a family history of intellectual disability suggestive of fragile X syndrome (17–19). ACOG additionally includes a family history of unexplained intellectual disability, developmental delay, autism, or primary ovarian insufficiency. SOGC guidelines are generally consistent with those of ACOG (20). Due to the inheritance pattern for this disorder, testing of females is most appropriate unless a male has symptoms of an FMR1-related disorder.

Hemoglobinopathy screening is recommended by ACOG and SCOG for the following ethnic groups (4,21):

- African ancestry: hemoglobin electrophoresis.
- Mediterranean ancestry: hemoglobin electrophoresis is recommended if anemia is present, the MCV is <80 fL, and iron studies are normal.
- Southeast Asian ancestry: hemoglobin electrophoresis is recommended if anemia is present, the MCV is <80 fL, and iron studies are normal. If hemoglobin electrophoresis is indicated but results are normal, molecular testing for alpha-thalassemia is recommended.

The SCOG and ACOG guidelines are generally consistent except that the SCOG includes additional ethnic groups: Middle East, Western Pacific Region, Caribbean, and South American.

Spinal muscular atrophy screening is recommended by ACMG regardless of ancestry or family history, while ACOG recommends it only when a family history is present (22,23).

Expanded Carrier Screening

As technologies improve and it becomes easier and less expensive to test for many different genetic disorders that may be individually rare, but collectively more frequent, there is a movement towards more expanded carrier screening panels with emerging guidelines. Because these guidelines cover a broad range of disorders, they focus on general considerations of how to select genes for inclusion, and the manner in which testing should be performed and how the associated care should be delivered. Recent guidelines related to expanded carrier screening are available from the ACMG (24) and subsequently a joint statement by the ACMG, ACOG, NSGC, Perinatal Quality Foundation, and the Society for Maternal–Fetal Medicine (25).

Overview of Technologies for Carrier Screening

A number of different technologies can be employed for genetic carrier screening. Because the nature of disease-causing variants differs from one disorder to the next, so too will the technologies that are best suited to test for a given disorder. Disease-causing variants can be single nucleotide variants (SNVs),

insertions and/or deletions of small stretches of DNA (indels), deletions or duplications of large stretches of DNA (copy number variations, CNVs), or variable length repeat sequences.

Technologies to Assess Variable Length Repeat Sequences

Some disorders, including Fragile X syndrome, are caused by abnormal numbers of trinucleotide repeats in the gene of interest. In order to test for carrier status in these cases, technologies that can both detect and accurately measure the length of a DNA sequence are employed. Historically both Southern blot analysis and the polymerase chain reaction (PCR) were used in combination to amplify and detect the full range of normal and mutant alleles (26). Gel electrophoresis was used to resolve the PCR reaction product length(s), which then allowed for accurate sizing of shorter alleles of the repetitive trinucleotide tract. The limitation of these early PCR approaches was an inability to amplify long trinucleotide repeats. Because the length of premutation-sized alleles is correlated with their potential to expand and become pathogenic in subsequent generations, inability to amplify a long trinucleotide repeat could result in a false negative test result. Southern blot analysis can detect the larger alleles and was therefore important to avoid these false negatives.

More recently, several methods have been developed that allow for detection of a long trinucleotide repeat even in cases where a full-length PCR product is unable to form. These rely on approaches including triplet repeat priming to generate stutter products and melt curve analysis to yield a signal indicating the presence of the long repeat. A full description of these methods is beyond the scope of this work, but see Lyon et al. (27) for a current review.

Technologies to Assess Single Nucleotide Variants and Insertions/Deletions

Disorders including cystic fibrosis and Tay-Sachs disease are caused primarily by SNVs and small indels in the relevant genes. Several classes of technologies have historically been used to assess carrier status for these disorders. The simplest of these are uniplex genotyping technologies that interrogate one variant per reaction (e.g., allele specific primer extension or allele-specific PCR). These technologies can employ multiplexing for the assay chemistry (e.g., multiplex PCR followed by allele specific primer extension) and/or for the detection method (e.g., fluorescent microspheres read by laser excitation and charge coupled device imaging). The inherent limitations on multiplexing in these methods impose a limit on the number of variants that can be simultaneously assessed.

More recently, several additional technologies have been developed and employed for SNV and indel detection in carrier screening. These are broadly referred to as "focused genotyping technology" and "next-generation DNA sequencing technology." Both technologies rely on multiplexed reaction chemistry and detection; however, each has specific strengths based on the technical differences between the methods.

Focused genotyping can be performed using a number of different platforms that employ microarray technology, enabling detection of hundreds of thousands of analytes on a single array. A variety of assay chemistries have been developed that interrogate specific SNVs and indels in a single multiplex reaction (28). Focused genotyping arrays can be designed to interrogate a wide array of variants that could be present in a sample, but this can only be accomplished if the specific variants of interest are known when the assay is being designed. As a result, carrier screening using focused genotyping is limited to the detection of previously-identified variants.

Next-generation DNA sequencing (NGS) is a class of new sequencing technologies based on highly multiplexed analysis that started as a research tool but is now being used for carrier screening as well as in other areas of clinical genetic testing. The technology differs from "first generation" DNA sequencing methods largely in the scale of analysis—the former analyze one to 100 DNA molecules per run, whereas the latter analyze hundreds of millions of DNA molecules or more per run (29). This technology has reduced the cost of human genome sequencing by over four orders of magnitude in 10 years since the first NGS platforms were reported (30–32).

Historically, for carrier screening, NGS sequencing technology was limited in its ability to fully interrogate regions of interest and to deliver exceedingly high analytical sensitivity (33). However, recently

technology has been developed to deliver near-complete region coverage coupled with analytical sensitivity exceeding 99% (34). In this context, NGS enables examination of the entire gene(s) of interest and therefore allows detection of almost any SNV or indel that is present. As a result, carrier screening tests that use NGS need not be limited to detection of previously-identified variants, and therefore can identify carriers that would be missed by focused genotyping technology (35,36).

Technologies to Assess Copy Number Variations

Disorders including spinal muscular atrophy (SMA) are caused primarily by the deletion of large stretches of DNA. In the case of SMA, a deletion of exon 7 of the SMN1 gene is present in the vast majority of carriers. Further complicating matters, the human genome contains a region of so-called paralogous sequence, known as the SMN2 pseudogene, that is almost identical in sequence to SMN1. As a result, technologies aiming to detect changes in the copy number of SMN1 exon 7 must be able to distinguish between it and SMN2 exon 7, even though their sequence is almost identical.

For this reason, technologies that can both provide an accurate measure of copy number variation and discriminate between very similar sequences are usually employed. The most common methods employ allele specific quantitative realtime PCR, which infers the amount of SMN1 exon 7 present from the number of PCR cycles required to meet a predefined amplification threshold (37), or multiplex ligation dependent probe amplification, which measures the amplification level of a probe pair that ligates together only in the presence of the region of interest (38).

Some SMA carriers are known as "2+0 carriers." These individuals have two copies of SMN1 exon 7 on one autosome, and zero copies of SMN1 exon 7 on the other autosome. As a result, even though a molecular analysis would show they have two copies of SMN1 exon 7, they can be at risk of having a child with SMA depending on the carrier status of their partner and which autosome is passed on to their child. Recently, variants have been discovered that are markers for 2+0 carrier status in individuals of certain ethnic backgrounds (39). In these patients, by interrogating these SNVs in conjunction with the copy number of SMN1 exon 7, a more accurate assessment of risk can be obtained.

Technologies that can both measure copy number and determine the genotypes of individual SNVs in the same assay are particularly well-suited to this combined analysis of exon 7 copy number and 2+0 marker status. Next-generation DNA sequencing is well suited for this purpose. To detect CNVs using NGS, reads derived from the region of interest can be counted and normalized to generate a "copy number call" for the region. Various normalization strategies have been devised for this purpose; a review of several methods has recently been published (40). To simultaneously interrogate a series of SNVs, the corresponding sequence can be captured and sequenced to generate genotype calls for the genomic positions harboring the SNVs.

REFERENCES

1. Young ID. *Introduction to Risk Calculation in Genetic Counseling*. 3rd ed. Oxford: Oxford University Press, 2007.
2. State of Lung Disease in Diverse Communities. American Lung Association. 2010. Accessed November 9, 2015. Available at: http://action.lung.org/site/DocServer/state-of-lung-disease-in-diverse-communities-2010.pdf.
3. Saul R. FMR1-Related Disorders. Genereviews. Last updated 2012. Accessed November 10, 2015. Available at: http://www.ncbi.nlm.nih.gov/books/NBK1384/.
4. Hemoglobinopathies in Pregnancy. ACOG Practice Bulletin No. 78. *Obstet Gynecol* 2007;109(1):229–37.
5. Origa R, Moi P, Galanello R, Cao, A. Alpha-thalassemia. Generreviews. Last updated 2013. Accessed August 24, 2015. Available at: http://www.ncbi.nlm.nih.gov/books/NBK1352/?report=printable.
6. Origa R. Beta-Thalassemia. Genereviews. Last updated 2015. Accessed November 10, 2015. Available at: http://www.ncbi.nlm.nih.gov/books/NBK1426/.
7. Prior T. Summary: Spinal Muscular Atrophy. Genereviews. Last updated 2013. Accessed November 10, 2015. Available at: http://www.ncbi.nlm.nih.gov/books/NBK1352/?report=printable.
8. Update on carrier screening for cystic fibrosis. Committee Opinion No. 486. American College of Obstetricians and Gynecologists. *Obstet Gynecol*. 2011;117:1028–31.

9. Watson MS, Cutting GR, Desnick RJ et al. Cystic fibrosis population carrier screening: 2004 Revision of American College of Medical Genetics Mutation Panel. *Genet Med*. 2004;6(5):387–91.
10. Langfelder-Schwind E, Karczeski B, Strecker MN et al. Molecular testing for cystic fibrosis carrier status practice guidelines: Recommendations of the National Society of Genetic Counselors. *J Genet Counsel*. 2013;23(1):5–15.
11. Cystic Fibrosis Carrier Testing in Pregnancy in Canada. SOGC Committee Opinion. *JOGC*. 2002;118:1–4.
12. Delatyck MB, Burke J, Christie L et al. Human Genetics Society of Australasia Position Statement: Population-Based Carrier Screening for Cystic Fibrosis. *Twin Res Hum Genet*. 2014; 24(10):578–83.
13. The UK NSC Recommendation on Cystic Fibrosis Screening in Pregnancy (currently under review). Cystic Fibrosis (pregnancy). Accessed November 10, 2015. Available at: http://legacy.screening.nhs.uk/cysticfibrosis-pregnancy.
14. Preconception and Prenatal Carrier Screening for Genetic Diseases in Individuals of Eastern European Jewish Descent. ACOG Committee Opinion No. 442. *Obstet Gynecol*. 2009;114(4):950–3.
15. Gross SJ, Pletcher BA, Monaghan KG. Carrier screening in individuals of Ashkenazi Jewish descent. *Genet Med*. 2008;10(1):54–6.
16. Carrier Screening for Genetic Disorders in Individuals of Ashkenazi Jewish Descent. SOGC Clinical Practice Guideline. *JOGC*. 2006;177:324–32.
17. Committee Opinion No. 469. Carrier Screening for Fragile X Syndrome. *Obstet Gynecol*. 2010;116(4):1008–10.
18. Sherman S, Pletcher BA, Driscoll DA. Fragile X Syndrome: Diagnostic and carrier testing. *Genet Med*. 2005;7(8):584–7.
19. Finucane B, Abrams L, Cronister A et al. Genetic counseling and testing for FMR1 gene mutations: Practice guidelines of the National Society of Genetic Counselors. *J Genet Couns*. 2012;21(6):752–60.
20. Fragile X Testing in Obstetrics and Gynaecology in Canada. Joint SOGC-CCMG Committee Opinion. *JOGC*. 2008;216:837–41.
21. Carrier Screening for Thalassemia and hemoglobinopathies in Canada. Joint SOGC-CCMG Clinical Practice Guideline. *JOGC*. 2008;218:950–9.
22. Prior TW. Carrier screening for spinal muscular atrophy. *Genet Med*. 2008;10(11):840–2.
23. Spinal Muscular Atrophy. ACOG Committee Opinion No. 432. *Obstet Gynecol*. 2009;113(5):1194–6.
24. Grody WW, Thompson BH, Gregg AR et al. ACMG position statement on prenatal/preconception expanded carrier screening. *Genet Med*, 2013;15:482–83.
25. Edwards JG, Feldman G, Goldberg J et al. Expanded carrier screening in reproductive medicine—Points to consider. *Obstet Gynecol*. 2015;125(3):653–62.
26. Pergolizzi RG, Erster SH, Goonewardena P, Brown WT. Detection of full Fragile X Mutation. *Lancet*. 1992;339(8788):271–2.
27. Lyons JI, Kerr GR, Mueller PW. Fragile X Syndrome. *J Mol Diagn*. 2015;17(5):463–71.
28. Ding C, Jin S. High-throughput methods for SNP genotyping. *Methods Mol Biol*. 2009;578:245–54.
29. Van Dijk EL, Auger H, Jaszczyszyn Y, Thermes C. Ten years of next-generation sequencing technology. *Trends Genet*. 2014;30(9):418–26.
30. Wetterstrand K. DNA Sequencing Costs. DNA Sequencing Costs. Accessed November 10, 2015. Available at: http://www.genome.gov/sequencingcosts/.
31. Margulies M, Egholm M, Altman WE et al. Genome sequencing in microfabricated high-density picolitre reactors. *Nature*. 2005;437(7057):376–80.
32. Shendure J, Porreca GJ, Reppas NB et al. Accurate multiplex polony sequencing of an evolved bacterial genome. *Science*. 2005;309(5741):1728–32.
33. Bell CJ, Dinwiddie DL, Miller NA et al. Carrier testing for severe childhood recessive diseases by next-generation sequencing. *Sci Transl Med*. 2011;3(65):65ra4.
34. Umbarger MA, Kennedy CJ, Saunders P et al. Next-generation carrier screening. *Genet Med*. 2014;16(2):132–40.
35. Neitzel D, Perreault-Micale C, Davie J, Mullen T. Pan-ethnic populations are enriched for rare and novel cystic fibrosis mutations. *Fertil Steril*. 2015;104 (3s): E206.
36. Perreault-Micale C, Davie J, Faulkner N. Next-generation DNS sequencing: Improving the accuracy of routine carrier screening. *Fertil Steril*. 2015;104 (3s): E210.

37. Anhuf D, Eggermann T, Rudnik-Schöneborn S, Zerres K. Determination of SMN1 and SMN2 Copy Number Using TaqMan Technology. *Hum Mutat*. 2003;22(1):74–8.
38. Scarciolla O, Stuppia L, De Angelis MV et al. Spinal muscular atrophy genotyping by gene dosage using multiple ligation-dependent probe amplification. *Neurogenetics*. 2006;7(4):269–76.
39. Luo M, Liu L, Peter I et al. An Ashkenazi Jewish SMN1 Haplotype specific to duplication alleles improves pan-ethnic carrier screening for spinal muscular atrophy. *Genet Med*. 2014;16:149–56.
40. Teo SM, Pawitan Y, Ku CS et al. Statistical challenges associated with detecting copy number variations with next-generation sequencing. *Bioinformatics*. 2012;28:2711–718.

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4

Testing Ovarian Reserve

Daniela Galliano, Nuria Pellicer, and Antonio Pellicer

Introduction

Ovarian reserve testing (ORT), or reproductive potential as a function of the number and quality of remaining oocytes, is increasingly important as a woman's age has become the main cause of infertility. Many middle-aged women seek the assistance of fertility specialists under the belief that assisted reproduction techniques (ART) can overcome the devastating effects of age (1). The reality, however, is quite different, and physicians need to counsel patients about their real chances of conception before undergoing ART. In this context, ORT has become a major issue.

The ovaries contain 6–7 million oocytes surrounded by a layer of granulosa cells which form the primordial follicle pool at weeks 15–18 of gestation (2–4). At birth, only 1–2 million primordial nongrowing follicles (PNGFs) remain (5). At menarche, 300,000–400,000 PNGFs are present in the ovaries (3,5,6), which are reduced during the reproductive years to fewer than 1000 at menopause (5,7). It has been calculated that only 12% of the initial ovarian reserve is preserved through the age of 30, declining to 3% by the age of 40. More recently, regression analyses of two relevant databases have tried to calculate the age of menopause based on the number of resting PNGFs in women (Figure 4.1) (8).

The biomarkers employed to evaluate ovarian reserve are ultrasonographic features which identify antral follicles or detect granulosa cell products of growing follicles in serum, thus reflecting the population of follicles with a certain degree of development and responsiveness to gonadotropins. This is not the true ovarian reserve, since there is another true ovarian reserve of PNGFs that are not identified by these markers. Thus, we should distinguish between the true ovarian reserve (number of PNGF) and the dynamic ovarian reserve (7,9).

Coinciding with the decrease in follicle number is a reduction in oocyte quality. This is believed to be due to an increase in meiotic nondisjunction, resulting in increased aneuploidy in the early embryo with age (10–13). Human aneuploidy is a key component of human reproductive aging. Underlying mechanisms may involve differences in quality between germ cells at the time they are formed, accumulated damage of oocytes over the course of a woman's life, or age-related changes in the quality of the surrounding granulosa cells that affect the oocyte (6,14).

Age is the best marker of quality. In fact, monthly fecundity gradually decreases from the mean age of 30 years onward (15,16), and natural fertility loss (represented by age at last child in conditions with unrestricted reproduction) already occurs by the mean age of 41 years, with a range between 23 and 51 years of age (17–19). But reproductive aging varies considerably among women, with some women remaining fertile until the fifth decade of life, while others lose fertility in their mid-thirties (7).

Due to the natural decline in fertility with age, women >35 years who have not conceived after 6 months of attempting pregnancy represent an indication for ORT (20). Moreover, a series of patients can be identified as having a higher risk for diminished ovarian reserve (DOR) based on their clinical history (Table 4.1) (20–41)

The ideal ORT markers should be highly reproducible, readily available, noninvasive, and cost effective. Moreover, they should be able to predict birth rate. Efforts have been made to discover the best way to test it, but neither single nor combined markers are currently satisfactory (42). Thus, the live birth end

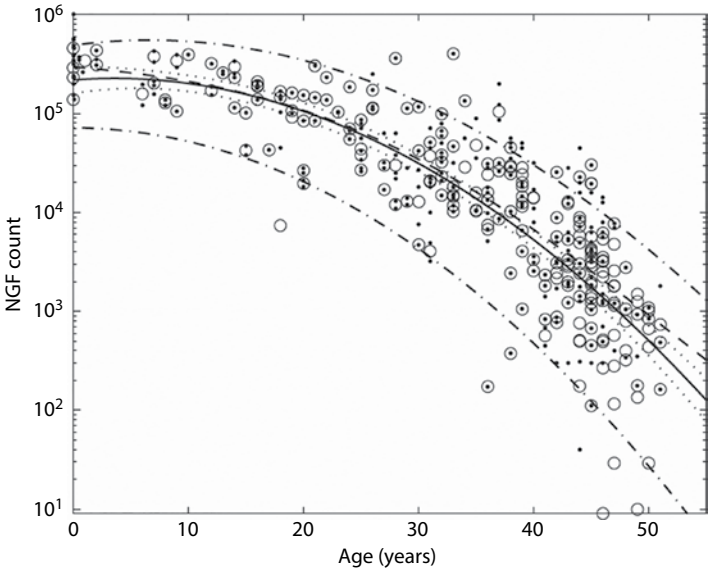

FIGURE 4.1 Logistic regression using two combined databases that represents the population of nongrowing primordial follicles (vertical axis) during a woman's life (horizontal axis). (Republished with permission of Depmann M et al. *J Clin Endocrinol Metab.* 2015;100:E845–51; permission conveyed through Copyright Clearance Center Inc.)

TABLE 4.1

Risk Factors for Diminished Ovarian Reserve (Other Than Age)

Genetic
- Familiar history of early menopause
- Chromosomal alterations (45,X0)
- Fragile X premutation carrier

Medical conditions
- Type I diabetes mellitus
- Autoimmune diseases

Conditions that can cause ovarian injury
- Endometriosis
- Pelvic infections

Iatrogenic
- Ovarian surgery
- Uterine artery embolization
- Oncologic treatment with gonadotoxic procedures

In-utero complications
- Exposure to excess androgens
- Small for gestational age infants
- Maternal nutritional restriction

Environmental
- Smoking
- Obesity
- Bisphenol A exposure
- Vitamin D deficiency
- Low weight gain in infancy

point has been substituted by other indirect measures of fertility potential, such as the number of follicles/oocytes obtained after ovarian stimulation, ART outcomes, or time to menopause (43).

Markers of Ovarian Reserve

Age

As mentioned above, age is the best marker of oocyte quality, indicated by the strong correlation between age and aneuploidy, as ascertained by increased trisomy 21 and miscarriage with age (44–46) and aneuploidiesin ART patients (13,47). Studies involving human oocytes have indeed confirmed age-related changes in spindle formation and chromosome alignment (29,31,37,40).

However, as illustrated in Figure 4.1, the confidence intervals are wide for any given age, which could result in under- or over-estimation of the number of PNGFs by 100-fold if age is employed as the sole ORT marker (9). The clinical implication of this is that age is only moderately predictive of ovarian response. Younger women will generally respond better than older women, given the age-related decline in PNGFs. However, for any given age, the large variation in PNGFs means that there can be a widely ranging ovarian response.

Basal Follicle Stimulating Hormone (FSH)

Granulosa cells of the developing cohort of follicles secrete estradiol (E2) and inhibin B, which help to maintain FSH within the normal range when the ovarian reserve is normal. When the follicular cohort is reduced, however, serum E2 and inhibin B levels decrease, resulting in an elevated early follicular phase FSH level. This higher FSH stimulates rapid ovarian follicular growth, which results in higher E2 as well as a shorter follicular phase. Elevated basal serum FSH levels were the first biochemical marker of ORT ever employed and they are still used. Values >10–20 IU/L are associated with DOR, but the test is not predictive of failure to conceive (48).

E2 and FSH should be measured together because high serum E2 levels could mask an elevated FSH. Furthermore, a single FSH determination has limited value because of inter-cycle variability (49). Moreover, assays for FSH have significant inter- and intra-cycle variability that limits their reliability (44,50,51). Thus, it is difficult to generalize FSH cutpoints (44).

FSH is commonly used as a marker of ovarian reserve, and high values have been associated with, but do not necessarily predict, both poor ovarian stimulation and the failure to conceive (44). Women having an abnormally elevated FSH value will also have DOR, but the variability in FSH levels requires repeat testing. Whereas consistently elevated FSH concentrations indicate a poor prognosis (52), a single elevated FSH value in women <40 years of age is not necessarily a bad sign (46).

A series of dynamic tests were developed for ORT based on the pituitary (FSH) and ovarian response (E2/inhibin B) to different hormonal challenges, but they are currently not used in clinical practice. This includes the clomiphene citrate challenge test (CCCT) (34,53), the gonadotropin-releasing hormone (GnRH) agonist stimulation test (54), and the exogenous FSH ovarian reserve test (55).

Inhibin B

Inhibin B is a dimeric polypeptide produced by granulosa cells predominantly during the follicular phase by the developing cohort of antral follicles. It acts as a negative feedback mechanism on FSH secretion, as well as influencing folliculogenesis (56–61). When the dynamic ovarian reserve decreases, serum inhibin B secretion also decreases and FSH increases, leading to a decrease in oocyte quality and fertility potential (53). However, inhibin B levels do not show a gradual decline with increasing female age and are considered a late marker of reduced follicle numbers, being considered a better indicator of ovarian activity than of ovarian reserve (53,62). Moreover, inhibin B exhibits high inter- and intra-cycle variability (63). In fact, studies relating inhibin B levels to IVF outcomes are contradictory (64–68).

Anti-Müllerian Hormone (AMH)

AMH is produced by the granulosa cells of small growing follicles (6–8 mm) (69) that have already been recruited, but not yet selected, for dominance. AMH is responsible for the regression of the female reproductive organs in male fetuses in utero, and it is a strong inhibitor of ovarian follicle development from primordial to primary follicle stage.

The development of AMH as a biomarker has been hampered by the existence of two different assay kits. Although the results obtained with these two kits are highly correlated, the standard curves are different, and there is no conversion factor (70). Thus, the threshold levels developed for one assay are not applicable to the other. Moreover, results can vary among different commercial laboratories using the same assay (37). However, newer assay kits based on a different technology are replacing the old assays and show good correlations with age and antral follicle count (AFC) in women of reproductive age (71). Moreover, comparison of these assays with the standard assay has confirmed good performance of both assays with a higher analytical sensitivity (72).

Serum AMH levels are reduced up to 20% in oral contraceptive (OC) users (73), but OC withdrawal restores AMH values because OCs do not reduce the ovarian reserve (74). However, the dynamic reserve of follicles stimulable by FSH is clearly reduced during OC intake, as shown by measurements of AMH.

AMH presents less intravariability across menstrual cycles, and although decreased serum AMH levels have been reported in the luteal phase, these fluctuations appear to be noncyclic and not significant (75–77). As a consequence, AMH can be tested at any time point of the cycle phase (78), as well as under ovarian suppression conditions like smoking, GnRH agonists, and pregnancy (79). However, this does not mean that AMH levels are static. The high inter-individual variability of AMH is probably secondary to the variability in the number of antral follicles among women of similar age (80), and also to ethnic variation, where African American and Hispanic patients have lower AMH levels than Caucasian women (81).

Perhaps the most interesting challenge today is to predict the reproductive lifespan of a woman and her time to menopause (82,83). Serum AMH levels are low during prepubertal development, rise during early puberty, peak around 20–25 years of age, and thereafter decrease progressively until undetectable levels around age 51, which is the age of menopause (82,83,84) (Figure 4.2). Serum levels within the

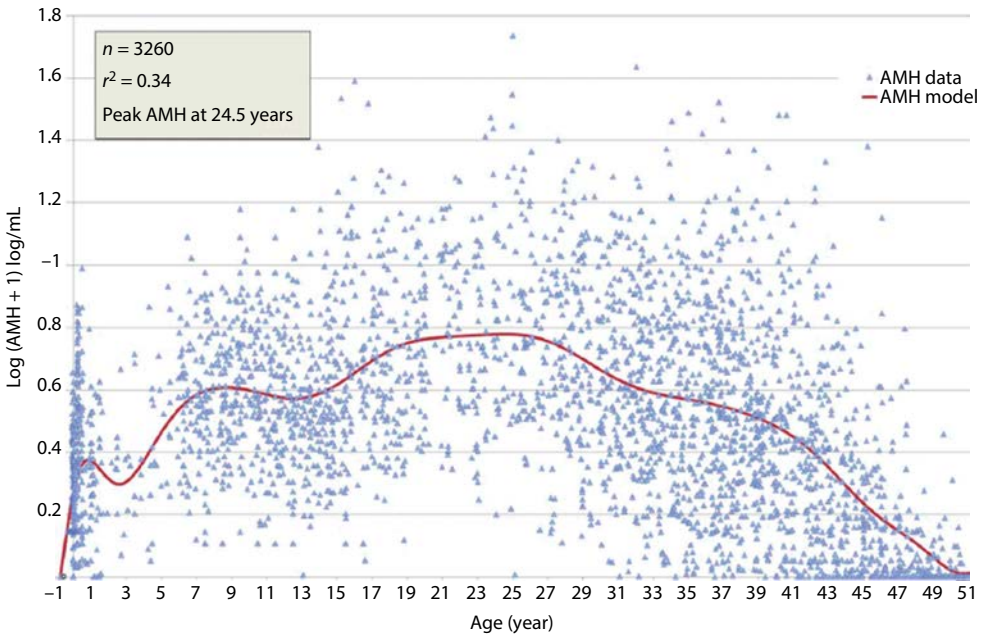

FIGURE 4.2 Serum AMH levels in 3200 women plotted in a validated mathematical model from different sources. (Reprinted from Kelsey TW et al. *PLoS One*. 2011;6(7):e22024. Doi: 10.1371/journal.pone.0022024. Epub 2011 Jul 15, with permission.)

range of 0.5–1.26 ng/mL of AMH suggest menopausal transition in 3–5 years (46). Given that AMH and age fit the pattern of histological oocyte decline better than any other biomarkers (85,86), AMH seems to be a promising marker of reproductive lifespan (87,88).

Likewise, in women receiving chemotherapy, AMH measured before and after treatment seems to be the best ORT to assess ovarian reserve and to encourage patients, including adolescents, to take fertility preservation measures before treatment (89–92).

In women with Turner syndrome, recent studies show that serum AMH values <4 pmol/L are indicative of imminent primary ovarian insufficiency (POI). The karyotype is also indicative of ovarian reserve, where women with mosaicisms 45,X0/46,XX have the best prognosis, normal pubertal development, and only 7% POI (93). Miscellaneous karyotypes are associated with 45% POI, while 45,X0 results in POI and absence of menarche (94). Fertility preservation might be an effective way to avoid POI.

AMH may also help to identify DOR or excessive ovarian response prior to ART (48,94–99), both of which are greatly associated with the likelihood of live birth (100). However, the role of AMH as a predictor of live birth in women undergoing ART is not entirely clear (101–103). In fact, it has been suggested that the association between AMH and increased cumulative live birth rate is attributed to oocytes and embryo quantity (104) rather than quality (105). Likewise, some researchers have reported a positive association between extreme (low and high) AMH levels and embryo quality (106–108), whereas others have not (92,109) Hence, patients should not be discouraged from undergoing ART based solely on low AMH serum levels.

AMH can predict an excessive ovarian response and risk of ovarian hyperstimulation syndrome (OHSS) with a sensitivity of 82% (105). Based on AMH levels and AFC, different algorithms have been introduced to select individualized ovarian stimulation regimens (110).

Antral Follicle Count (AFC)

AFC refers to the sum of small 2–10 mm antral follicles observed by 2D trans-vaginal ultrasound in the early follicular phase. It is widely employed because it has good inter-cycle and inter-observer reliability (63,111–114). The presence of 3–4 antral follicles in total in both ovaries is highly predictive of DOR, and AFC is generally considered to be the best predictor of oocyte yield (115–117). Also, AFC can predict excessive oocyte yield and risk of OHSS (83). Similar to AMH, AFC numbers are reduced by up to 18% in OC users (68), but OC withdrawal restores AFC (69).

While some do not believe that AFC reflects oocyte or embryo quality and live birth rates (83), others have described a live birth increase with increasing AFC after adjusting for age and oocyte yield, suggesting that AFC may indicate oocyte quality (118).

Combining Different ORT Markers

A recent study analyzed the added predictive value of all the markers described herein, including the age of the patient, serum FSH, AMH, and AFC (94) (Figure 4.3). The authors compared the different ORTs using a random intercept logistic regression model to predict DOR and obtained high accuracy for AMH (AUC 0.78) and for AFC (AUC 0.76), but only moderate accuracy for FSH (AUC 0.68). For prediction of pregnancy after IVF, all three ORTs showed only a very small or no predictive effect (94).

The multivariable analyses for DOR prediction showed that a model including age, AFC, and AMH had a significantly higher predictive accuracy than a model based on age alone (AUC 0.80 versus 0.61). Age was the strongest single predictor of pregnancy following IVF, with moderate accuracy (AUC 0.57). Multivariable analysis for predicting ongoing pregnancy indicated that no single or combined ORT significantly added predictive power to age (94) (Figure 4.3).

Genetic Predictors of Ovarian Reserve

Genetic polymorphism may alter tissue and cellular responsiveness to glycoproteins. In this context, polymorphisms in gonadotropins and their receptors have been related to altered response to ovarian

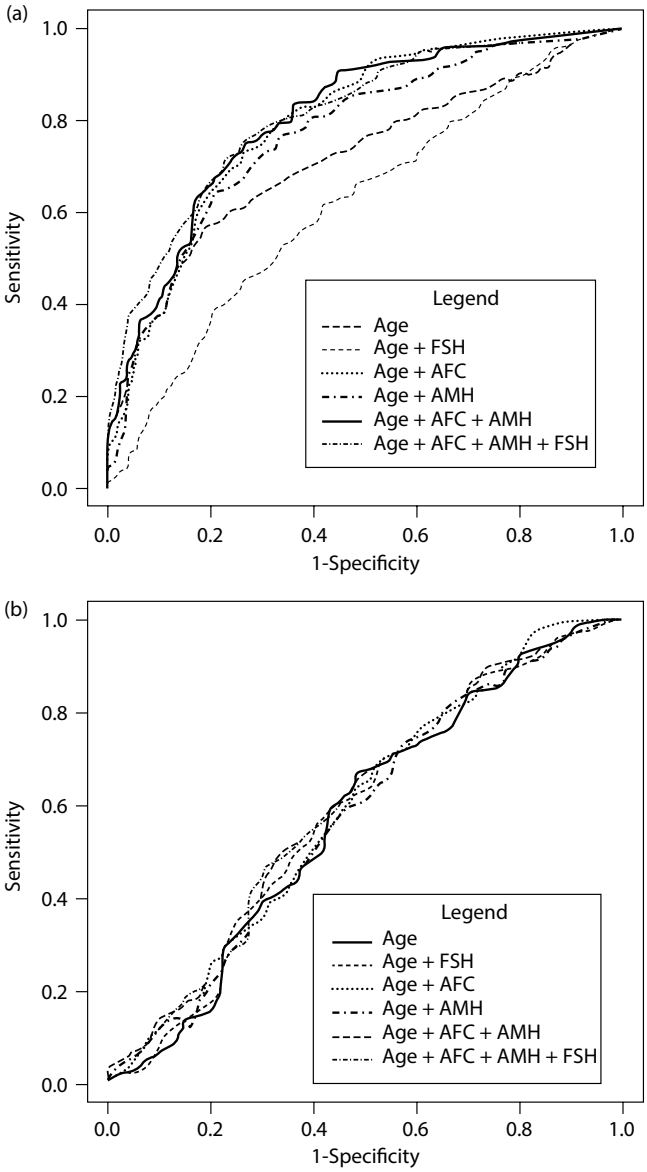

FIGURE 4.3 ROC curves of age, AFC, serum AMH, and serum FSH for predicting DOR and ongoing pregnancy from ORT. (A) Poor response prediction based on age and ORT. The ROC curves for Age + AMH, Age + AMH + AFC, and Age + AMH + AFC + FSH show a good discriminative capacity between normal and DOR patients. (B) Ongoing pregnancy prediction. The ROC curves indicate that the tests are not effective for predicting pregnancy. (Broer SL et al., *Hum Reprod Update*, 2013;19:26–36, by permission of Oxford University Press, on behalf of the European Society of Human Reproduction and Embryology)

stimulation, but are most likely not associated with modifications in ovarian reserve, AFC, or AMH levels. In fact, the common polymorphism Asn680Ser may influence the response of recruitable follicles to FSH, and perhaps some patients can be classified as DOR. The 680Ser genotype is a factor of major "resistance" to FSH stimulation, resulting in higher basal serum FSH levels and prolonged menstrual cycle duration. FSH sensitivity of 680Ser homozygote carriers can be overcome by higher FSH doses during COH protocols (119).

The number of CGG duplicates in the X-linked gene known as *FMR1* can be responsible for various clinical conditions. Premutation alleles range from 55 to 199 CGG repeats and can cause POI in 13% of female carriers (120). Up to 54 CGG repeats also increases the risk of POI (121). Moreover, it has been reported that women carrying a premutation allele have a higher likelihood of reaching menopause 5 years earlier (117). In addition, subfertile women who carry an allele with >35 CGG repeats have lower serum AMH levels compared with controls with normal alleles, suggesting a reduced ovarian reserve (122). These studies favor testing the premutation alleles in women with a family history of fragile X syndrome or POI, but not in the general population.

It has recently been reported that BRCA1 mutation carriers have lower serum AMH levels and DOR compared to other patients with cancer who preserve their fertility (123), but others have not found the same differences (124).

REFERENCES

1. Maheshwari A, Porter M, Shetty A, Bhattacharya S. Women's awareness and perceptions of delay in childbearing. *Fertil Steril*. 2008;90:1036–42.
2. Block E. Quantitative morphological investigations of the follicular system in women; variations at different ages. *Acta Anat (Basel)*. 1952;14:108–23.
3. Block E. A quantitative morphological investigation of the follicular system in newborn female infants. *Acta Anat (Basel)*. 1953;17:201–6.
4. Baker TG. A quantitative and cytological study of germ cells in human ovaries. *Proc R Soc Lond B Biol Sci*. 1963;158:417–33.
5. Markström E, Svensson ECh, Shao R, Svanberg B, Billig H. Survival factors regulating ovarian apoptosis—Dependence on follicle differentiation. *Reproduction*. 2002;123:23–30.
6. Velde ER, Pearson PL. The variability of female reproductive ageing. *Hum Reprod Update*. 2002; 8:141–54.
7. Monniaux D, Clement F, Dabiès-Tran R et al. The ovarian reserve of primordial follicles and the dynamic reserve of antral growing follicles: What is the link? *Biol Reprod*. 2014;90:1–11.
8. Depmann M, Faddy MJ, van der Schouw YT et al. The relationship between variation in size of the primordial follicle pool and age at natural menopause. *J Clin Endocrinol Metab*. 2015;100:E845–51.
9. Findlay JK, Hutt KJ, Hickey M, Richardson MA. What is ovarian reserve? *Fertil Steril*. 2015;103:629–30.
10. Battaglia DE, Goodwin P, Klein NA, Soules MR. Influence of maternal age on meiotic spindle assembly in oocytes from naturally cycling women. *Hum Reprod*. 1996;11:2217–22.
11. Kuliev A, Cieslak J, Verlinsky Y. Frequency and distribution of chromosome abnormalities in human oocytes. *Cytogenet Genome Res*. 2005;111:193–8.
12. Hunt PA, Hassold TJ. Human female meiosis: What makes a good egg go bad? *Trends Genet*. 2008;24:86–93.
13. Pellestor F, Anahory T, Hamamah S. Effect of maternal age on the frequency of cytogenetic abnormalities in human oocytes. *Cytogenet Genome Res*. 2005;111:206–12.
14. Warburton D. Biological aging and the etiology of aneuploidy. *Cytogenet Genome Res*. 2005;111:266–72.
15. Schwartz D, Mayaux MJ. Female fecundity as a function of age: Results of artificial insemination in 2193 nulliparous women with azoospermic husbands. Federation CECOS. *N Engl J Med*. 1982;306:404–6.
16. Noord-Zaadstra BM, Looman CW, Alsbach H et al. Delaying childbearing: Effect of age on fecundity and outcome of pregnancy. *BMJ*. 1991;302:1361–5.
17. Broekmans FJ, Faddy MJ, Scheffer G, te Velde ER. Antral follicle counts are related to age at natural fertility loss and age at menopause. *Menopause*. 2004;11:607–14.
18. Broekmans FJ, Soules MR, Fauser BC. Ovarian aging: Mechanisms and clinical consequences. *Endocrine Rev*. 2009;30:465–93.
19. O'Connor KA, Holman DJ, Wood JW. Declining fecundity and ovarian ageing in natural fertility populations. *Maturitas*. 1998;30:127–36.
20. Hardy R, Kuh D. Does early growth influence timing of the menopause? Evidence from a British birth cohort. *Hum Reprod*. 2002;17:2474–9.
21. Soto N, Iniguez G, Lopez P et al. Anti-Mullerian hormone and inhibin B levels as markers of premature ovarian aging and transition to menopause in type 1 diabetes mellitus. *Hum Reprod* 2009;24:2838–44.

22. Freeman EW, Gracia CR, Sammel MD et al. Association of anti-mullerian hormone levels with obesity in late reproductive-age women. *Fertil Steril*. 2007;87:101–6.
23. Su HI, Sammel MD, Freeman EW et al. Body size affects measures of ovarian reserve in late reproductive age women. *Menopause*. 2008;15:857–61.
24. Buyuk E, Seifer DB, Illions E et al. Elevated body mass index is associated with lower serum antimullerian hormone levels in infertile women with diminished ovarian reserve but not with normal ovarian reserve. *Fertil Steril*. 2011;95:2364–8.
25. Raffi F, Metwally M, Amer S. The impact of excision of ovarian endometrioma on ovarian reserve: A systematic review and meta-analysis. *J Clin Endocrinol Metab*. 2012;97:3146–54.
26. Berkane N, Moutafoff-Borie C. Impact of previous uterine artery embolization on fertility. *Curr Opin Obstet Gynecol*. 2010;22:242–7.
27. Dumesic DA, Patankar MS, Barnett DK et al. Early prenatal androgenization results in diminished ovarian reserve in adult female Rhesus monkeys. *Hum Reprod*. 2009;24:3188–95.
28. Richardson MC, Guo M, Fauser BCJM, Macklon NS. Environmental and developmental origins of ovarian reserve. *Hum Reprod Update*. 2014;20:353–69.
29. Merhi ZO, Seifer DB, Weedon J et al. Circulating vitamin D correlates with serum antimullerian hormone levels in late-reproductive-aged women: Women's Interagency HIV Study. *Fertil Steril*. 2012;98:228–34.
30. Dennis NA, Houghton LA, Jones GT et al. The level of serum anti-Mullerian hormone correlates with vitamin D status in men and women but not in boys. *J Clin Endocrinol Metab*. 2012;97:2450–5.
31. Ibañez L, Potau N, Enriquez G, de Zegher F. Reduced uterine and ovarian size in adolescent girls born small for gestational age. *Pediatr Res*. 2000;47:575–7.
32. Ibañez L, Valls C, Cols M et al. Hypersecretion of FSH in infant boys and girls born small for gestational age. *J Clin Endocrinol Metab*. 2002;87:1986–8.
33. Sir-Petermann T, Marquez L, Carcamo M et al. Effects of birth weight on anti-mullerian hormone serum concentrations in infant girls. *J Clin Endocrinol Metab*. 2010;95:903–10.
34. Matikainen TM, Moriyama T, Morita Y et al. Ligand activation of the aromatic hydrocarbon receptor transcription factor drives Bax-dependent apoptosis in developing fetal ovarian germ cells. *Endocrinology* 2002;143:615–20.
35. Waylen AL, Metwally M, Jones GL et al. Effects of cigarette smoking upon clinical outcomes of assisted reproduction: A meta-analysis. *Hum Reprod Update*. 2009;15:31–44.
36. Sharara FI, Beatse SN, Leonardi MR et al. Cigarette-smoking accelerates the development of diminished ovarian reserve as evidenced by the clomiphene citrate challenge test. *Fertil Steril*. 1994;62:257–62.
37. Fuentes A, Muñoz A, Barnhart K et al. Recent cigarette smoking and assisted reproductive technologies outcome. *Fertil Steril*. 2010;93:89–95.
38. Cooper GS, Baird DD, Hulka BS et al. Follicle-stimulating-hormone concentrations in relation to active and passive smoking. *Obstet Gynecol*. 1995;85:407–11.
39. Allard P, Colaiacovo MP. Bisphenol A impairs the double-strand break repair machinery in the germline and causes chromosome abnormalities. *Proc Nat Acad Sci USA*. 2010;107:20405–10.
40. Mok-Lin E, Ehrlich S, Williams PL, Petrozza J, Wright DL, Calafat AM, Ye X, Hauser R. Urinary bisphenol A concentrations and ovarian response among women undergoing IVF. *Int J Androl*. 2010;33:385–93.
41. Cresswell JL, Egger P, Fall CHD et al. Is the age of menopause determined in-utero? *Early Hum Dev*. 1997;49:143–8.
42. American College of Obstetricians and Gynecologists Ovarian reserve testing. Committee Opinion No. 618. *Obstet Gynecol*. 2015;125:268–73.
43. Freeman EW, Sammel MD, Lin H, Gracia CR. Anti-mullerian hormone as a predictor of time to menopause in late reproductive age women. *J Clin Endocrinol Metab*. 2012;97:1673–80.
44. Tabor A. Genetic amniocentesis. Indications and risks. *Dan Med Bull*. 1988;35:520–37.
45. Lamb NE, Yu K, Shaffer J et al. Association between maternal age and meiotic recombination for trisomy 21. *Am J Hum Genet*. 2005;76:91–9.
46. Volarcik K, Sheean L, Goldfarb J et al. The meiotic competence of in-vitro matured human oocytes is influenced by donor age: Evidence that folliculogenesis is compromised in the reproductively aged ovary. *Hum Reprod*. 1998;13:154–60.

47. Macklon NS, Geraedts JP, Fauser BC. Conception to ongoing pregnancy: The "black box" of early pregnancy loss. *Hum Reprod Update*. 2002;8:333–43.
48. Hendriks DJ, Mol BW, Bancsi LF et al. Antral follicle count in the prediction of poor ovarian response and pregnancy after in vitro fertilization: A metaanalysis and comparison with basal follicle-stimulating hormone level. *Fertil Steril*. 2005;83:291–301.
49. Kwee J, Schats R, McDonnell J et al. Intercycle variability of ovarian reserve tests: Results of a prospective randomized study. *Hum Reprod*. 2004;19:590–5.
50. Jayaprakasan K, Campbell B, Hopkisson J et al. Establishing the intercycle variability of three-dimensional ultrasonographic predictors of ovarian reserve. *Fertil Steril*. 2008;90:2126–32.
51. Esposito MA, Coutifaris C, Barnhart KT. A moderately elevated day 3 FSH concentration has limited predictive value, especially in younger women. *Hum Reprod*. 2002;17:118–23.
52. Roberts JE, Spandorfer S, Fasouliotis SJ et al. Taking a basal follicle-stimulating hormone history is essential before initiating in vitro fertilization. *Fertil Steril*. 2005;83:37–41.
53. Maheshwari A, Gibreel A, Bhattacharya S, Johnson NP. Dynamic tests of ovarian reserve: A systematic review of diagnostic accuracy. *Reprod Biomed Online*. 2009;18:717–34.
54. Broekmans FJ, Kwee J, Hendriks DJ et al. A systematic review of tests predicting ovarian reserve and IVF outcome. *Hum Reprod Update*. 2006;12:685–718.
55. Kwee J, Shats R, McDonnell J et al. The clomiphene citrate challenge test versus the exogenous follicle-stimulating hormone ovarian reserve test as a single test for identification of low responders and hyper-responders to in vitro fertilization. *Fertil Steril*. 2006;85:1714–22.
56. Groome NP, Illingworth PJ, O'Brien M et al. Detection of dimeric inhibin throughout the human menstrual cycle by two-site enzyme immunoassay. *Clin Endocrinol (Oxford).* 1994;40:717–23.
57. Groome NP, Illingworth PJ, O'Brien M et al. Measurement of dimeric inhibin B throughout the human menstrual cycle. *J Clin Endocrinol Metab.* 1996;81:1401–5.
58. Hall JE, Welt CK, Cramer DW. Inhibin A and inhibin B reflect ovarian function in assisted reproduction but are less useful at predicting outcome. *Hum Reprod*. 1999;14:409–15.
59. Findlay JK. An update on the roles of inhibin, activin, and follistatin as local regulators of folliculogenesis. *Biol Reprod*. 1993;48:15–23.
60. Findlay JK, Drummond AE, Britt KL et al. The roles of activins, inhibins and estrogen in early committed follicles. *Mol Cell Endocrinol*. 2000;163:81–7.
61. Hillier SG. Regulatory functions for inhibin and activin in human ovaries. *J Endocrinol*. 1991;131: 171–5.
62. Burger HG, Hale GE, Robertson DM, Dennerstein L. A review of hormonal changes during the menopausal transition: Focus on findings from the Melbourne Women's Midlife Health Project. *Hum Reprod Update*. 2007;13:559–65.
63. McIlveen M, Skull JD, Ledger WL. Evaluation of the utility of multiple endocrine and ultrasound measures of ovarian reserve in the prediction of cycle cancellation in a high-risk IVF population. *Hum Reprod*. 2007;22:778–85.
64. Bancsi LF, Broekmans FJ, Eijkemans MJ et al. Predictors of poor ovarian response in in vitro fertilization: A prospective study comparing basal markers of ovarian reserve. *Fertil Steril*. 2002;77:328–36.
65. Seifer DB, Lambert-Messerlian G, Hogan JW et al. Day 3 serum inhibin-B is predictive of assisted reproductive technologies outcome. *Fertil Steril*. 1997;67:110–4.
66. Scheffer GJ, Broekmans FJ, Dorland M et al. Antral follicle counts by transvaginal ultrasonography are related to age in women with proven natural fertility. *Fertil Steril.* 1999;72:845–51.
67. Scheffer GJ, Broekmans FJ, Looman CW et al. The number of antral follicles in normal women with proven fertility is the best reflection of reproductive age. *Hum Reprod*. 2003;18:700–6.
68. Rooij IA, Broekmans FJ, Scheffer GJ et al. Serum antimullerian hormone levels best reflect the reproductive decline with age in normal women with proven fertility: A longitudinal study. *Fertil Steril*. 2005;83:979–87.
69. Jeppesen JV, Anderson RA, Kelsey TW et al. Which follicles make the most anti-Mullerian hormone in humans? Evidence for an abrupt decline in AMH production at the time of follicle selection. *Mol Hum Reprod*. 2013;19:519–27.
70. La Marca A, Sighinolfi G, Radi D et al. Anti-mullerian hormone (AMH) as a predictive marker in assisted reproductive technology (ART). *Hum Reprod Update*. 2010;16:113–30.

71. Anderson RA, Anckaert E, Bosch E et al. Prospective study into the value of the automated Elecsys antimüllerian hormone assay for the assessment of the ovarian growing follicle pool. *Fertil Steril*. 2015;103:1074–80.
72. Van Helden J, Weiskirchen R. Performance of the two new fully automated anti-Müllerian hormone immunoassays compared with the clinical standard assay. *Hum Reprod*. 2015;30:1918–26.
73. Birch Petersen K, Hvidman HW, Forman JL et al. Ovarian reserve assessment in users of oral contraception seeking fertility advice on their reproductive lifespan. *Hum Reprod*. 2015;30:2364–75.
74. Berg MH, van Dulmen-den Broeder E, Overbeek A et al. Comparison of ovarian function markers in users of hormonal contraceptives during the hormone-free interval and subsequent natural early follicular phases. *Hum Reprod*. 2010;25:1520–7.
75. La Marca A, Stabile G, Artenisio AC, Volpe A. Serum anti-Mullerian hormone throughout the human menstrual cycle. *Hum Reprod*. 2006;21:3103–7.
76. Tsepelidis S, Devreker F, Demeestere I et al. Stable serum levels of anti-Mullerian hormone during the menstrual cycle: A prospective study in normo-ovulatory women. *Hum Reprod*. 2007;22:1837–40.
77. Van Disseldorp J, Lambalk CB, Kwee J et al. Comparison of inter-and intra-cycle variability of anti-Müllerian hormone and antral follicle counts. *Hum Reprod*. 2010;25:221–7.
78. La Marca A, Grisendi V, Griesinger G. How much does AMH really vary in normal women? *Int J Endocrinol*. 2013;2013:959487. Doi: 10.1155/2013/959487. Epub 2013 Nov 19.
79. Ledger WL. Clinical utility of measurement of anti-Mullerian hormone in reproductive endocrinology. *J Clin Endocrinol Metab*. 2010;95:5144–54.
80. Almog B, Shehata F, Suissa S et al. Age-related normograms of serum anti-Mullerian hormone levels in a population of infertile women: A multicenter study. *Fertil Steril*. 2011;95:2359–63.
81. Seifer DB, Golub ET, Lambert-Messerlian G et al. Variations in serum Mullerian inhibiting substance between white, black, and Hispanic women. *Fertil Steril*. 2009;92:1674–8.
82. Hagen CP, Aksglaede L, Sorensen K et al. Serum levels of anti-Mullerian hormone as a marker of ovarian function in 926 healthy females from birth to adulthood and in 172 Turner syndrome patients. *J Clin Endocrinol Metab*. 2010;95:5003–10.
83. Lie Fong S, Visser JA, Welt CK et al. Serum anti-Mullerian hormone levels in healthy females: A nomogram ranging from infancy to adulthood. *J Clin Endocrinol Metab*. 2012;97:4650–5.
84. Kelsey TW, Wright P, Nelson SM et al. A validated model of serum anti-Müllerian hormone from conception to menopause. *PLoS One*. 2011;6(7):e22024. Doi: 10.1371/journal.pone.0022024. Epub 2011 Jul 15.
85. Rosen MP, Johnstone E, McCulloch CE et al. A characterization of the relationship of ovarian reserve markers with age. *Fertil Steril*. 2012;97:238–43.
86. Hansen KR, Knowlton NS, Thver AC et al. A new model of reproductive aging: The decline in ovarian non-growing follicle number from birth to menopause. *Hum Reprod*. 2008;23:699–708.
87. Sowers MR, Eyvazzade AD, McConnell D et al. Anti-Müllerian hormone and inhibin B in the definition of ovarian aging and the menopause transition. *J Clin Endocrinol Metab*. 2008;93:3478–83.
88. Dolleman M, Verschuren WMM, Eijkemans MJC et al. Added value of anti-Müllerian hormone in prediction of menopause: Results from a large prospective cohort study. *Hum Reprod*. 2015;30:1974–81.
89. Beek RD, van den Heuvel-Eibrink M, Laven JS et al. anti-Mullerian hormone is a sensitive serum marker for gonadal function in women treated for Hodgkin's lymphoma during childhood. *J Clin Endocrinol Metab*. 2007;92:3869–74.
90. Anderson RA, Cameron DA. Pretreatment serum anti-Mullerian hormone predicts long-term ovarian function and bone mass after chemotherapy for early breast cancer. *J Clin Endocrinol Metab*. 2011;96:1336–43.
91. Dillon KE, Sammel MD, Prewitt M, et al. Pretreatment anti-Mullerian hormone levels determine rate of posttherapy ovarian reserve recovery: Acute changes in ovarian reserve during and after chemotherapy. *Fertil Steril*. 2013;99:477–83.
92. Krawczuk-Rybak M, Leszczynska E, Poznanska M et al. Anti-Mullerian hormone as a sensitive marker of ovarian function in young cancer survivors. *Int J Endocrinol*. 2013;2013:125080.
93. Lunding SA, Aksglaede L, Anderson RA et al. AMH as Predictor of Premature Ovarian Insufficiency: A Longitudinal Study of 120 Turner Syndrome Patients. *J Clin Endocrinol Metab*. 2015;100:E1030–8.
94. Anckaert E, Smitz J, Schiettecatte J et al. The value of anti-Mullerian hormone measurement in the long GnRH agonist protocol: Association with ovarian response and gonadotrophin-dose adjustments. *Hum Reprod*. 2012;27:1829–39.

95. Gnoth C, Schuring AN, Friol K et al. Relevance of anti-Mullerian hormone measurement in a routine IVF program. *Hum Reprod*. 2005;23:1359–65.
96. Broer SL, Mol BWJ, Hendriks D, Broekmans FJM. The role of anti-Mullerian hormone in prediction of outcome after IVF: Comparison with the antral follicle count. *Fertil Steril*. 2009;91:705–14.
97. Broer SL, Dolleman M, Opmeer BC et al. AMH and AFC as predictors of excessive response in controlled ovarian hyperstimulation: A meta-analysis. *Hum Reprod Update*. 2011;17:46–54.
98. Broer SL, van Disseldorp J, Broeze KA et al. Import study group. Added value of ovarian reserve testing on patient characteristics in the prediction of ovarian response and ongoing pregnancy: An individual patient data approach. *Hum Reprod Update*. 2013;19:26–36.
99. Styer AK, Gaskins AJ, Brady PC et al. Dynamic anti-Mullerian hormone levels during controlled ovarian hyperstimulation predict in vitro fertilization response and pregnancy outcomes. *Fertil Steril*. 2015. pii: S0015-0282(15)01673-8. Doi: 10.1016/j.fertnstert.2015.07.1161. [Epub ahead of print]
100. Sunkara SK, Rittenberg V, Raine-Fenning N et al. Association between the number of eggs and live birth in IVF treatment: An analysis of 400,135 treatment cycles. *Hum Reprod*. 2011;26:1768–74.
101. Fanchin R, Mendez Lozano DH, Frydman N et al. Anti-Müllerian hormone concentrations in the follicular fluid of the preovulatory follicle are predictive of the implantation potential of the ensuing embryo obtained by in vitro fertilization. *J Clin Endocrinol Metab*. 2007;92:1796–802.
102. Iliodromiti S, Kelsey TW, Wu O et al. The predictive accuracy of anti-Müllerian hormone for live birth after assisted conception: A systematic review and meta-analysis of the literature. *Hum Reprod Update*. 2014;20:560–70.
103. Tal R, Tal O, Seifer BJ, Seifer DB. Anti-Mullerian hormone as predictor of implantation and clinical pregnancy after assisted conception: A systematic review and meta-analysis. *Fertil Steril*. 2015;103:119–30.
104. Arce JC, La Marca A, Mirner Klein B et al. Anti-Mullerian hormone in gonadotropin releasing-hormone antagonist cycles: Prediction of ovarian response and cumulative treatment outcome in good-prognosis patients. *Fertil Steril*. 2013;99:1644–53.
105. Frattarelli JL, Lauria-Costab DF, Miller BT et al. Basal antral follicle number and mean ovarian diameter predict cycle cancellation and ovarian responsiveness in assisted reproductive technology cycles. *Fertil Steril*. 2000;74:512–7.
106. Brodin T, Hadziosmanovic N, Berglund L et al. Anti-Mullerian hormone levels are strongly associated with live-birth rates after assisted reproduction. *J Clin Endocrinol Metab*. 2013;98:1107–14.
107. Lin WQ, Yao LN, Zhang DX et al. The predictive value of anti-Mullerian hormone on embryo quality, blastocyst development, and pregnancy rate following in vitro fertilization-embryo transfer (IVF-ET). *J Assist Reprod Genet*. 2013;30:649–55.
108. Ebner T, Sommergruber M, Moser M et al. Basal level of anti-Mullerian hormone is associated with oocyte quality in stimulated cycles. *Hum Reprod*. 2006;21:2022–6.
109. Riggs R, Kimble T, Oehninger S et al. Anti-Mullerian hormone serum levels predict response to controlled ovarian hyperstimulation but not embryo quality or pregnancy outcome in oocyte donation. *Fertil Steril*. 2011;95:410–2.
110. La Marca A, Sunkara Sk. Individualization of controlled ovarian stimulation in IVF using ovarian reserve markers: From theory to practice. *Human Reprod Update*. 2014;20:124–40.
111. Bancsi LF, Broekmans FJ, Looman CW et al. Impact of repeated antral follicle counts on the prediction of poor ovarian response in women undergoing in vitro fertilization. *Fertil Steril*. 2004;81:35–41.
112. Frattarelli JL, Levi AJ, Miller BT, Segars JH. A prospective assessment of the predictive value of basal antral follicles in in vitro fertilization cycles. *Fertil Steril*. 2003;80:350–5.
113. Pache TD, Wladimiroff JW, de Jong FH et al. Growth patterns of nondominant ovarian follicles during the normal menstrual cycle. *Fertil Steril*. 1990;54:638–42.
114. Scheffer GJ, Broekmans FJ, Bancsi LF et al. Quantitative transvaginal two- and three-dimensional sonography of the ovaries: Reproducibility of antral follicle counts. *Ultrasound Obstet Gynecol*. 2002;20:270–5.
115. Layaprasakan K, Deh S, Batcha M et al. The cohort of antral follicles measuring 2–6 mm reflects the quantitative status of ovarian reserve as assessed by serum levels of anti-Mullerian hormone and response to controlled ovarian stimulation. *Fertil Steril*. 2010;94:1775–81.
116. Jayaprakasan K, Hilwah N, Kendall NR et al. Does 3D ultrasound offer any advantage in the pretreament assessment of ovarian reserve and prediction of outcome after assisted reproduction treatment? *Hum Reprod*. 2007;22:1932–41.

117. Hsu A, Arny M, Knee AB et al. Antral follicle count in clinical practice: Analyzing clinical relevance. *Fertil Steril*. 2011;95:474–9.
118. Holte J, Brodin T, Berglund L et al. Antral follicle counts are strongly associated with live-birth rates after assisted reprodction with superior treatment outcome in women with polycystic ovaries. *Fertil Steril*. 2011;96:594–9.
119. Behre HM, Greb RR, Mempel A et al. Significance of a common single nucleotide polymorphism in exon 10 of the follicle-stimulating hormone (FSH) receptor gene for the ovarian response to FSH: A pharmacogenetic approach to controlled ovarian hyperstimulation. *Pharmacogenet Genom*. 2005;15:451–6.
120. Sullivan AK, Marcus M, Epstein MP et al. Association of FMR1 repeat size with ovarian dysfunction. *Hum Reprod*. 2005;20:402–12.
121. Wittenberger MD, Hagerman RJ, Sherman SL et al. The FMR1 premutation and reproduction. *Fertil Steril*. 2007;87:456–65.
122. Gleichert N, Weghofer A, Oktay K, Barad DH. Correlation of triple repeats of the FMR1 (fragile X) gene to ovarian reserve: A new infertility test? *Acta Obstet Gynecol Scand*. 2009;88:1024–30.
123. Titus S, Li F, Stobezki R, Akula K et al. Impairment of BRCA1-related DNA double-strand break repair leads to ovarian aging in mice and humans. *Sci Transl Med*. 2013;5:172ra21.
124. Shapira M, Raanani H, Feldman B et al. BRCA mutation carriers show normal ovarian response in in vitro fertilization cycles. *Fertil Steril*. 2015. pii: S0015-0282(15)01674-X. DOI: 10.1016/j.fertnstert.2015.07.1162.

5

Basics of Ovarian Stimulation

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Relevant Ovarian Physiology

Ovarian stimulation in infertility treatment can be applied in several ways. Strategy and choice of drugs used depends on the indication and goal of the infertility treatment. To understand and apply ovarian stimulation it is necessary to understand the basic concepts of the (patho)physiology of ovarian function (i.e., follicle development).

Follicle Development and Selection

Follicular growth starts with the transition of primordial follicles into the antral follicle stage. This process, which takes several months, also known as initial recruitment, occurs continuously and randomly (1). The initial recruitment starts long before the onset of puberty and lasts until menopause. During initial recruitment some primordial follicles initiate growth, while others stay in the latent state for months or years. From the recruited primordial follicles the vast majority becomes atretic and will not reach the antral stage (see Figure 5.1). How this process of early follicle development and atresia is regulated, and which early stages of follicle development are affected by follicle-stimulating hormone (FSH), remains unclear. It is suggested that factors such as members of the TGF-β superfamily and factors regulating apoptosis play a significant role in this process (2,3).

The transition from the pre-antral to the antral stage and onwards, is clearly promoted by FSH. Cyclic recruitment, the process where antral follicles develop to advanced follicular stages, is initiated by FSH (4). Due to the perimenstrual rise of FSH levels, a cohort of antral follicles escapes apoptosis. Only the follicles of a more advanced stage of development during this rise of FSH will become gonadotropin-dependent and continue to grow. In these follicles FSH induces steroidogenesis by increasing granulosa cell aromatase activity (responsible for the conversion of androgens to estrogens), increasing the number of granulosa cells, and the generation of luteinizing hormone (LH) receptors, all collectively resulting in the increasing conversion of androgens into estradiol (E_2) secreted in the follicular compartment and diffused into the general circulation.

FSH Threshold and Window

The perimenstrual rise of FSH is the result of increased frequency of pulsatile gonadotropin-releasing hormone (GnRH) secretion induced by the feedback of declining levels of E_2, inhibin A, and progesterone in the late luteal phase when the corpus luteum vanishes. After this perimenstrual rise, FSH remains at the same level for several days. Subsequently, the FSH level declines due to the negative feedback of inhibin B and E_2 produced by the growing pool of recruited follicles (5,6). During this decline in FSH from the early-to-mid follicular phase onwards, only the most mature follicle is rescued from atresia and continues to grow. This follicle then becomes the dominant Graafian follicle from which the oocyte is released during ovulation. This dominant follicle grows faster than the others and produces higher amounts of E_2 and inhibins (7). Hence, in the human species, decremental follicular phase FSH concentrations are fundamental for single dominant follicle selection (see Figure 5.2) (4).

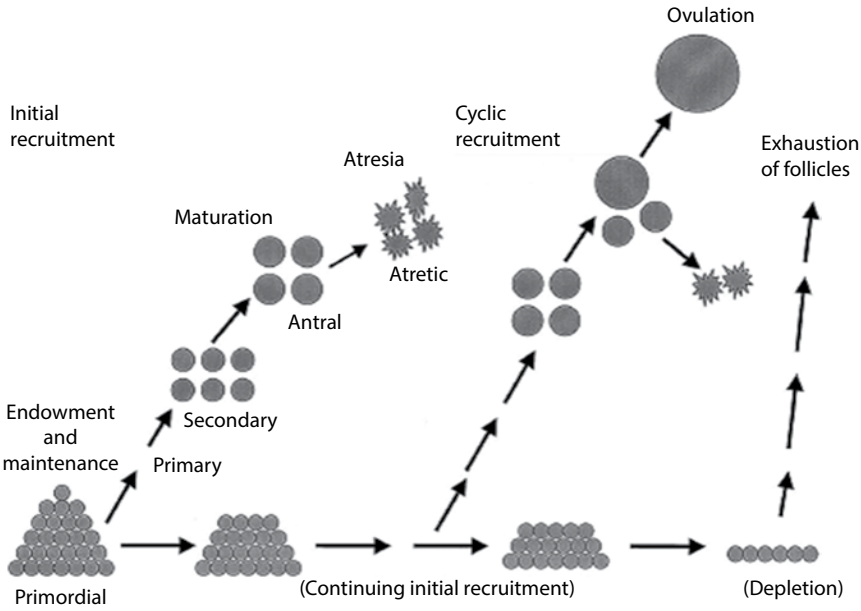


FIGURE 5.1 Life history of ovarian follicles: endowment and maintenance, initial recruitment, maturation, atresia or cyclic recruitment, ovulation, and exhaustion. A fixed number of primordial follicles are endowed during early life, and most of them are maintained in a resting state. Growth of some of these dormant follicles is initiated before and throughout reproductive life (initial recruitment). Follicles develop through primordial, primary, and secondary stages before acquiring an antral cavity. At the antral stage most follicles undergo atresia; however, under optimal gonadotropin stimulation that occurs after puberty, a few of them are rescued (cyclic recruitment) to reach the pre-ovulatory stage. Eventually, depletion of the pool of resting follicles leads to ovarian follicle exhaustion and senescence. (From McGee EA, Hsueh AJ. *Endocr Rev*. 2000;21:200–14, with permission.)

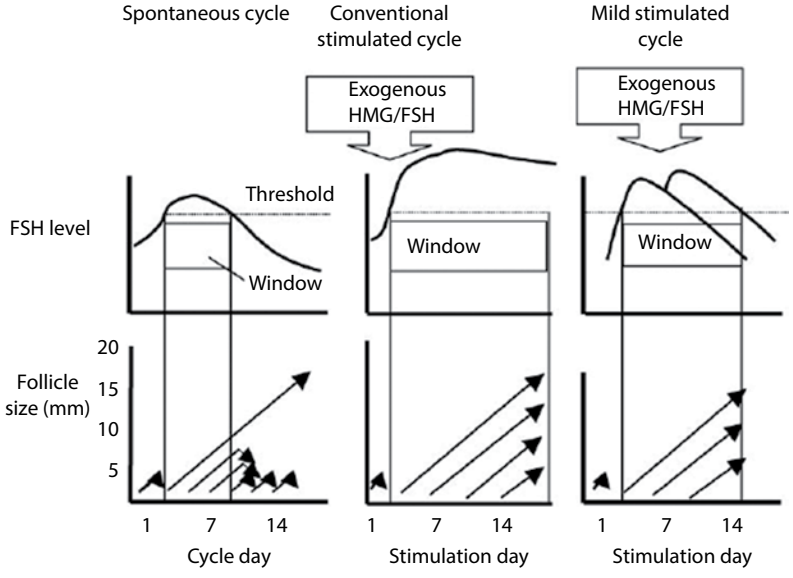


FIGURE 5.2 The FSH threshold and window concept for monofollicular selection (*left panel*), as conventionally applied to achieve multifollicular development (*middle panel*). Each arrow represents a developing follicle. The *right panel* represents the concept of extending the FSH window by administering exogenous FSH in the midfollicular phase to maintain FSH levels above the threshold, allowing multifollicular development to occur. (From Macklon NS et al. *Endocr Rev*. 2006;27:170–207, with permission.)

LH is also an important factor in single dominant follicle selection. The increased LH sensitivity and expression of LH receptors of the selected dominant follicle provides the dominant follicle the ability to respond to LH next to FSH. Maturation of the dominant follicle becomes less dependent on FSH and therefore survives the mid-follicular FSH decline. LH also initiates conversion of androstenedion to E_2 by the induction of the aromatase enzyme. This androgen secretion results in upregulation of E_2 levels pre-ovulatory, and finally in suppression of FSH levels and atresia of less mature antral follicles, due to the decline of FSH. Recently it has been suggested that cyclic fluctuations of anti-Mullerian hormone (AMH) produced by the granulosa cells of antral follicles also play a role in the selection of the dominant follicle. Probably the pre-ovulatory decline of intrafollicular AMH of growing antral follicles selected for dominance stimulates upregulation of E_2 levels pre-ovulatory (8).

Different Forms of Ovarian Stimulation

The principle of ovarian stimulation is to administer agents that increase FSH serum concentrations; either exceeding the FSH threshold at which follicle recruitment occurs (in case of ovulation induction) or extending the period or window in which the FSH threshold is exceeded. Exceeding physiological FSH levels and patterns will lead to multiple dominant follicle growth (9). The most common complication of ovarian stimulation is multiple pregnancies, with its associated increased complication rates during pregnancy along with compromised perinatal outcomes (10).

Ovulation Induction

Concept

The aim of ovulation induction is to induce mono-follicular growth and ovulation in anovulatory women. On the basis of this principle, ovulation induction is the preferred treatment in anovulatory women with childwish in order to restore normal fertility by inducing regular ovulatory cycles. This implies full restoration of the physiology of the normal menstrual cycle, including selection and growth of a single dominant follicle and ovulation. Ovulation induction in the treatment of ovulation disorders can be achieved in various ways depending on the underlying cause. The majority of women with ovulation disorders show FSH and E_2 levels between normal limits, also referred to as World Health Organization (WHO) class 2 anovulation. In 80%–90% of such cases polycystic ovary syndrome (PCOS) is diagnosed.

Hypogonadotropic hypoestrogenic anovulation showing low FSH and low E2 levels is seen in less than 10% of infertility patients and is caused by a central defect at the hypothalamic–pituitary level, known as WHO class 1 anovulation. WHO class 3 anovulation, which involves high FSH levels and low E2 levels, suggests a defect at ovarian level, usually caused by premature exhaustion of the follicle pool, ovarian insufficiency, or ovarian dysgenesis. In this category, ovulation induction is not preferred as infertility treatment (11).

Agents for Ovulation Induction

Clomiphene citrate. The most frequently used drug in ovulation induction is the anti-estrogen clomiphene citrate (CC). It is a nonsteroidal estrogen analogue with anti-estrogen effects on hypothalamic level. CC is only effective when the hypothalamic–pituitary–ovarian axis is intact, as is the case in WHO class 2 anovulation. Binding of CC to E2-receptors at hypothalamic level mimics hypo-estrogenic status, which induces an increase in GnRH pulse frequency resulting in higher FSH production on pituitary level (12). A 50% rise in FSH levels is seen if CC is used in the early- to mid-follicular phase (13).

The starting dose of CC is 50 mg per day during 5 consecutive days from cycle day 3 to cycle day 7. The limited duration is based on the principle that FSH levels decrease in the late follicular phase in order to induce monofollicular growth and ovulation. In some women persistent high levels of FSH are seen in the late follicular phase (14). Five to twelve days after the last day of CC administration the LH rise occurs.

In case of failure of follicle growth and ovulation, the daily dose of CC can be increased to 100 mg per day in the next cycle, with a maximum dose of 150 mg per day. In 60%–80% of cases, ovulation is seen with CC, with a cumulative pregnancy rate of approximately 70% within 6–12 months of treatment and a multiple pregnancy rate of less than 10% (15,16). As insulin resistance is an important factor in the pathophysiologic mechanism of polycystic ovary syndrome (PCOS), insulin-sensitizing drugs such as metformin alone or combined with CC are applied for ovulation induction. However, it has been shown that metformin alone or in combination with CC does not increase live birth rate and is associated with more gastrointestinal side effects (17).

This century, aromatase inhibitors were introduced for ovulation induction. These lower E2 levels by inhibiting the aromatization of androgens in estrogens. A recent systematic review showed that aromatase inhibitors were associated with higher live birth rate compared to CC, but the overall quality of the evidence was limited because of poor reporting of study methods and publication bias (18). Additional studies aiming on effectiveness and safety of aromatase inhibitors should be performed.

Gonadotropins. Gonadotropins are usually the second line treatment in women with WHO class 2 anovulation. In case of failure of ovulation or to conceive after CC, treatment with exogenous FSH is the next step. To achieve monofollicular growth with gonadotropins, specific treatment and monitoring protocols are necessary. The two most common protocols described in the literature and applied in clinical practice are the step-up and step-down protocols. Initial studies among the step-up protocol used a starting dose of 150 IU FSH per day (19). Due to a high complication rate, such as multiple pregnancy rates up to 36% and ovarian hyperstimulation syndrome of 14% per treatment cycle, this protocol has become obsolete.

Based on the concept that follicular development occurs above a certain FSH threshold, alternative step-up protocols were developed (20) (see Figure 5.3). The low-dose step-up protocol applies this concept by reaching the FSH threshold gradually and inducing monofollicular growth by a slight increase of the FSH level above this threshold. Based on consensus, the starting dose of FSH is 37.5–50 UI FSH per day, starting after spontaneous or progesterone induced bleeding. In the case of absent follicular response after 7–14 days, as monitored by ultrasound, the daily dose is increased by 50%, and additionally increased weekly by 37.5 IU in the case of persisting absence of follicular response, with a maximum of 225 IU per day. From a dominant follicle of at least 10 mm, the current daily dose of FSH

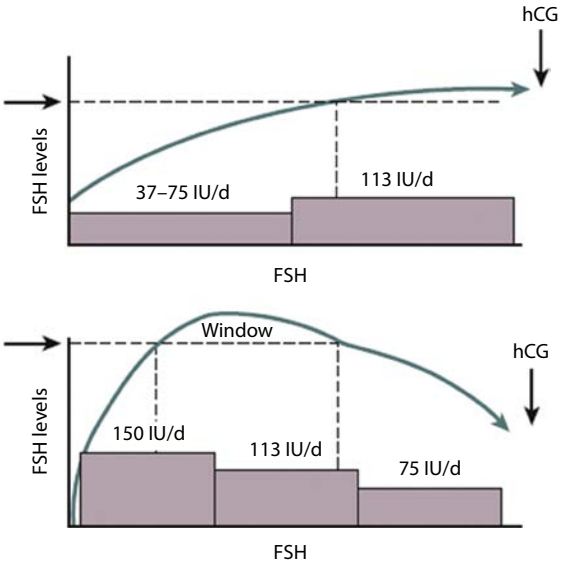


FIGURE 5.3 Schematic representation of serum follicle-stimulating hormone (FSH) levels and daily dose of exogenous FSH during low-dose step-up or step-down regimens for ovulation induction. (Reprinted from *Yen & Jaffe's Reproductive Endocrinology: Physiology, Pathophysiology, and Clinical Management*, 7th ed., Fauser BCJM, Medical approaches to ovarian stimulation for infertility, 712, Copyright 2014, with permission from Elsevier.)

TABLE 5.1

Clinical Results of Step-Up and Step-Down Administration of Recombinant Human FSH

	Step-Up Protocol (*n* = 85 Cycles)	**Step-Down Protocol (*n* = 72 Cycles)**	***p***
Duration of treatment (days)	15.2 ± 7	9.7 ± 3.1	<0.001
Total amount of rFSH (IU)	951 ± 586	967 ± 458	NS
Rate of monofollicular development (%)	68.2	32	<0.0001
Rate of bifollicular development (%)	15.3	23.6	NS
Rate of multifollicular (>3) development (1)	4.7	36	<0.0001
Estradiol plasma value at hCG (pg/mL)	454 ± 465	849 ± 1115	<0.05
hCG administration (%)	84.6	61.8	0.001
Rate of hyperstimulation (%)	2.25	11	0.001
No response (%)	11.8	8.33	NS
Progesterone >8 ng/mL (%) in luteal phase	70.3	61.7	0.02
Pregnancy/cycle (%)	18.7	15.8	NS

Source: Christin-Maitre S, Hugues JN. *Hum Reprod*. 2003;18:1626–31.

is continued until the follicle reaches the size of 18 mm and human chorion gonadotrophin (hCG) for ovulation triggering is given.

The low-dose step-down protocol uses the physiological pattern of ovulatory cycles, which starts with a high FSH level in the early follicular phase followed by a rapid decrease in the late follicular phase (21). The starting dose in the low-dose step-down protocol starts with a daily dose of FSH of 150 IU per. From a dominant follicle of at least 10 mm the daily dose of FSH is decreased to 112.5 IU per day, and 3 days later to 75 IU per day, until hCG is given for ovulation triggering.

A multicenter randomized trial showed that the step-up protocol resulted in a significantly higher rate of monofollicular cycles compared to the step-down protocol, 77% versus 35%, and lower ovarian hyperstimulation syndrome, 2% versus 11%. The pregnancy rate per started cycle was indifferent, 19% in the low-dose step-up versus 16% in the low-dose step-down protocol (see Table 5.1) (22). Therefore the low-dose step-up protocol remains the most applied schedule for ovulation induction with gonadotropins.

Pulsatile GnRH. Ovulation induction with gonadotropins is also applied in patients with hypogonadotropic hypogonadal anovulation. In these cases treatment with FSH should be combined with LH, as FSH alone does not result in pregnancy (23,24). A good alternative in WHO class 1 anovulation is pulsatile GnRH, provided that the pituitary function is intact. The management of GnRH is intravenous, with a pulse interval of 60–90 minutes using a dose of 2.5–10 µg per pulse. To prevent multifollicular growth, the dosage starts with the minimum and is increased until follicular growth and ovulation is induced, and continued throughout the luteal phase until menstruation or positive pregnancy test. With this regimen pregnancy rates of 83%–95% after six cycles have been reported, with a multiple pregnancy rate of 3%–8% (25,26).

Ovarian Stimulation for IUI

Concept

The aim of ovarian stimulation for intra uterine insemination (IUI) is to induce multiple follicle growth, multiple ovulation, and multiple fertilization in order to increase pregnancy chances in couples with unexplained subfertility with regular ovulatory cycles. The inherent risk associated with ovarian stimulation in normo-ovulatory women is multiple pregnancies. The concept of inducing multiple follicle growth is to increase the FSH level above the FSH threshold of monofollicular growth. Ovarian hyperstimulation for IUI can be achieved by clomiphene citrate or gonadotropins. Clomiphene citrate is normally given by daily doses of 50–100 mg from cycle day 3 to cycle day 7 and ovulation is triggered by

exogenous hCG. Ovarian response is monitored by ultrasound. Hyperstimulation by exogenous gonadotropin is normally started around cycle days 2–3, with daily doses of 50–75 IU in a fixed dose regimen. Monitoring of ovarian response is performed by ultrasound and/or E2 sampling. Ovulation triggering is also induced by exogenous hCG.

Agents for Ovarian Stimulation

A meta-analysis of seven trials involving 456 couples, comparing FSH with CC in ovarian hyperstimulation combined with IUI, showed that FSH resulted in higher pregnancy rates and no significant differences in the rate of ovarian hyperstimulation syndrome (OHSS), multiple pregnancy, miscarriage, or ectopic pregnancy (27). The most important complication of ovarian hyperstimulation in IUI is multiple pregnancy rate and OHSS. In case of ovarian stimulation by CC, a multiple pregnancy rate of 10% of pregnancies is seen and an OHSS rate of less than 1% (28). Multiple pregnancy rates in ovarian hyperstimulation with FSH and IUI occurs in 4%–8% in randomized trials, but uncontrolled studies also show rates up to 40% (29). It is obvious that the incidence of multiple pregnancy relates to dosing regimens of FSH and cancel criteria, therefore couples should be counseled extensively for this complication.

Co-Treatment GnRH Agonist and Antagonist

Premature LH surges during ovarian hyperstimulation for IUI occurs in 22%–43% of the cycles interfering with optimal timing of insemination, and seems to lead to lower pregnancy rates compared to cycles without premature LH surge (30). These LH surges can be prevented effectively by co-treatment with GnRH agonist or GnRH antagonist. However, GnRH agonist co-treatment does not improve pregnancy rates; in contrast, the pregnancy rate was significant higher in case of gonadotropins alone (OR 1.81, CI 95% 1.10–2.97). Furthermore, GnRH agonist co-treatment was significantly related to multiple pregnancy (OR 4.45, CI 95% 1.36–14.55) (27). Also, GnRH antagonist co-treatment seemed to be promising but the pooled OR for pregnancy rate did not reach statistical significance (OR 1.51, CI 95% 0.83–2.76). Furthermore, in contrast to GnRH agonist, the antagonist is not related to higher multiple pregnancy rates. For the present, there is no evidence that allows co-treatment of GnRH agonist or antagonist in IUI treatment with ovarian hyperstimulation with gonadotrophins.

Ovarian Stimulation for IVF

Concept

The concept of ovarian stimulation in vitro fertilization (IVF) is to induce growth of multiple dominant follicles in order to retrieve multiple mature and good quality oocytes for in vitro fertilization (9). This leads to more fertilized oocytes for embryo culture and final selection for transfer and cryopreservation. With this strategy embryo transfer can be performed in the majority of patients, and remaining embryos can be cryopreserved. This allows patients to gain subsequent pregnancy chances without the need for repeated ovarian stimulation and oocyte retrieval, which is time consuming and a burden to patients. As opposed to IUI in combination with ovarian stimulation, in IVF the multiple pregnancy rate can be controlled by reducing the number of embryos transferred.

Agents for Ovarian Stimulation

In the early years IVF was performed in the natural cycle. To increase the effectiveness of IVF, medication for ovarian stimulation has evolved from clomiphene citrate (CC), human menopausal gonadotropins (hMG), and purified urinary follicle stimulating hormone (uFSH) to human recombinant follicle stimulating hormone (rFSH) and, recently, long acting rFSH (31).

The first available gonadotropin was human menopausal gonadotropin (hMG), which was extracted from the urine of postmenopausal women. The initial preparations hMG were very impure and contained a lot of contaminating non active proteins. In the 1980s, improved purification techniques reduced

the amount of non active proteins and resulted in the development of purified urinary FSH (uFSH) preparations by using monoclonal antibodies. This reduced side effects such as local hypersensitivity reactions and increased the batch-to-batch consistency in bioactivity (32).

Development in DNA technology unraveled the structure of glycoprotein hormones. These hormones are heterodimers consisting of an identical α-subunit and a unique β-subunit that allows biological specificity for each individual hormone (33) (see Figure 5.4). Recombinant DNA techniques and transfection of human genes realized in vitro production of human recombinant FSH (rFSH) allowing the administration of FSH by protein weight instead of bioactivity (34). Additionally, to optimize IVF other interventions are introduced, such as GnRH analogue co-treatment for the prevention of spontaneous ovulation during ovarian stimulation and hCG administration for triggering the last maturation of oocytes in order to increase the number of mature oocytes at ovum pick up. Reports on IVF outcome in case of ovarian stimulation with CC with or without gonadotropins compared to IVF with conventional gonadotropins in a GnRH agonist protocol are conflicting (35). CC seems to be associated with higher cancellation rates and lower mean numbers of oocytes, but with lower OHSS rates (36).

For the clinical introduction of rFSH many randomized controlled trials are performed and published. The first meta analysis showed a slightly higher pregnancy rate per cycle and lower total gonadotropins required in favor of rFSH compared to uFSH (37). A more recent published meta analysis could not confirm these differences when comparing rFSH versus hMG or rFSH versus uFSH concerning all clinical outcomes, such as live birth rate and OHSS rate (38).

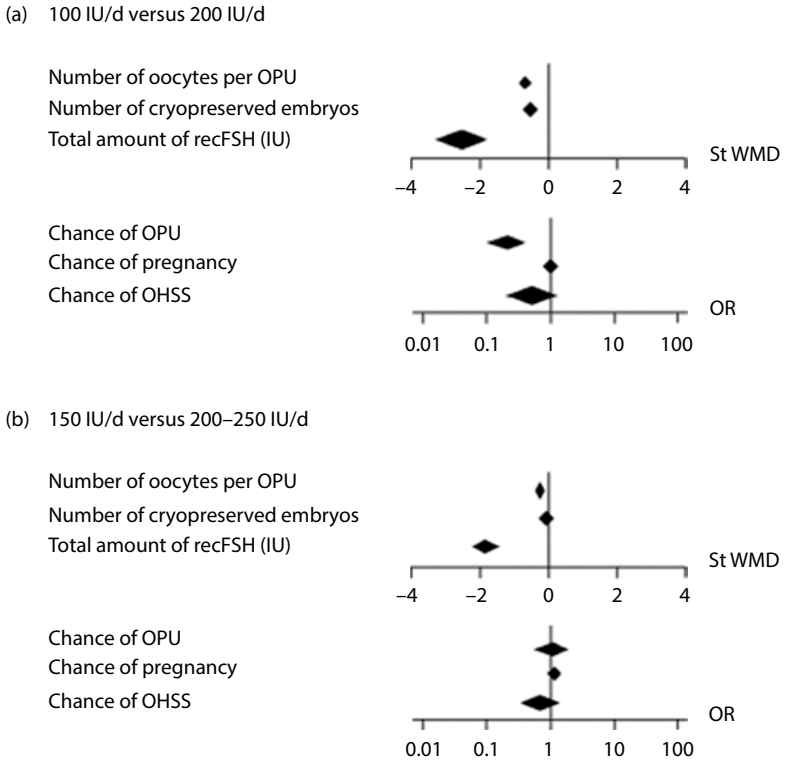


FIGURE 5.4 Clinical outcomes in relation to the daily dose of recombinant follicle-stimulating hormone for ovarian stimulation in in vitro fertilization in presumed normal responders younger than 39 years. The figures show Forest plot of mean difference of number of oocytes per oocyte pick-up, mean difference of number of cryopreserved embryos, mean difference of total amount of recFSH (IU), chance of ovum pick up (OPU), chance of clinical pregnancy, and chance of OHSS. The diamond represents the pooled weighted mean difference (WMD), and its width represents its 95% CI. Left side of the plot favors higher dose, right side of the plot favors lower dose. (From Sterrenburg M et al. *Hum Reprod Update*. 2001;17:184–96, with permission.)

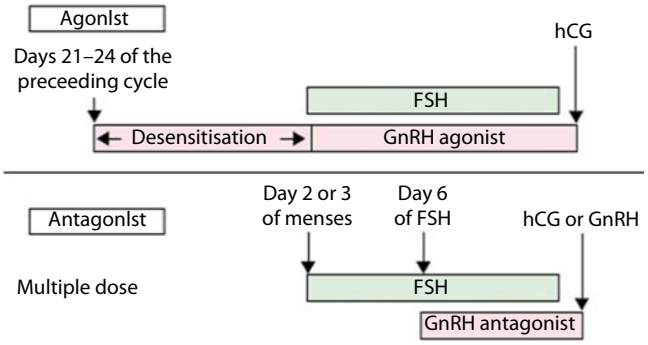


FIGURE 5.5 Schematic representration of most common GnRH agonist and antagonist protocol. (From Huirne JA, Lambalk CB. *Lancet*. 2001;24:1793–803.)

A decade ago the long-acting recombinant FSH agonist, named corifollitropin alfa or FSH-CTP, was introduced for IVF. A single dose of long-acting FSH is able to keep the circulating FSH level above the threshold necessary to support multi-follicular growth for seven days. In women with unexplained infertility a medium dose of 150–180 µg long-acting FSH seems to be equally effective compared to daily doses of rFSH in view of live birth rate (Risk Ratio [RR] 0.95, 95% confidence interval [CI] 0.84–1.07; 2363 participants, eight studies), but also in view of safety as OHSS rates are indifferent (RR 1.00, 95% CI 0.74–1.37; 3753 participants, nine studies) (39).

Several regimes have been applied with different starting days and doses of gonadotropins, with or without incremental doses. Due to lack of evidence of efficacy of these regimes, there is no consensus regarding starting day and starting dose. Starting doses vary between 100 and 300 IU per day. Just a few randomized trials are available for dose regimens. A systematic review, analyzing seven randomized trials, showed that although the higher gonadotropin doses resulted in 1 or 2 more oocytes, improved clinical outcomes such as pregnancy rates and embryo cryopreservation rates could not be found (see Figure 5.5) (40). This implies that the optimal daily gonadotropin dose is 150 IU in presumed normal responders younger than 39 years. Only one small trial studied ($n = 47$ patients) the effect of doubling the daily dose of gonadotropins in case of low response after 5 days of ovarian stimulation versus continuation of the same dose. This doubling did not affect the ovarian response, such as number of follicles, on ultrasound on the day of hCG administration or the number of oocytes retrieved (41). The absence of any effect is probably explained by the theory that follicular recruitment occurs only in the late luteal and early follicular phase of the menstrual cycle. However, a protocol starting exogenous FSH in the luteal phase of the preceding cycle did not result in higher oocyte yield in the case of poor response in previous IVF attempts (42).

Co-Treatment GnRH Agonist and Antagonist

In 1971 Schally and Guillemin isolated and discovered the structure of the decapeptide gonadotropin-releasing hormone (GnRH). The half-life of synthetic GnRH agonist is longer, with a more potent activity, than endogenous GnRH. The clinical use of GnRH agonists in IVF, such as buserelin, triptorelin, or leuprolin, was introduced in the early 1980s for the prevention of premature LH rise (43). Thereafter, down regulation of the pituitary by GnRH agonist before administration of gonadotropins has become the standard of care for IVF, also known as the long protocol. At least 30 years after the discovery of the GnRH agonists, GnRH antagonists such as ganirelix and cetrotide were introduced for clinical use and registered for IVF (44).

Suppression of the pituitary by GnRH agonist resulted in fewer cancelled cycles and improved IVF outcome (45). An accidental consequence of GnRH agonist treatment is the possibility of timing oocyte retrieval. The agonist effect of the agent initially induces stimulation of the pituitary and subsequently pituitary down-regulation. Various regimes of GnRH agonist are described in the literature, such as long protocols starting luteal or follicular, with continuation or stopping GnRH agonist at start of stimulation, or reduction of dose, and short protocols where the flare effect of GnRH agonist is used for ovarian

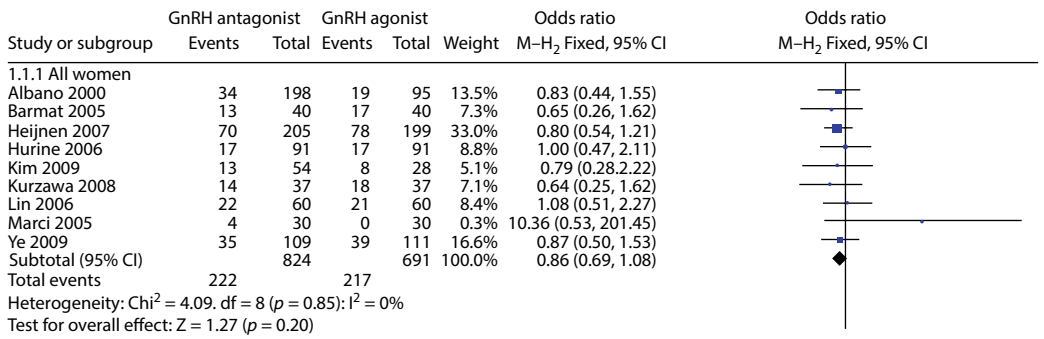


FIGURE 5.6 Forest plot of live birth rate per women randomized comparing GnRH antagonist versus GnRH agonist long protocol cotreatment for IVF. (From Al-Inany HG et al. *Cochrane Database Syst Rev.* 2011, Issue 5. Art. No.: CD001750. DOI: 10.1002/14651858.CD001750.pub3, with permission.)

stimulation. In the long protocol, GnRH agonist is usually started in the luteal phase of the preceding cycle, and stimulation with gonadotropins is started when the pituitary is down regulated and in the absence of follicular inactivity in the ovary (see Figure 5.5). In this regimen GnRH agonist is continued until ovulation triggering.

A meta-analysis showed a significant difference in clinical pregnancy rate (OR 1.50, 95% CI 1.16–1.93) and increased number of oocytes (Mean Difference [MD] 1.61, 95% CI 0.18–3.04) in favor of the long protocol compared to the short protocol (46). However, the long protocol required significantly more ampoules of FSH than the short protocol, with a mean difference of 12.9 ampoules per cycle (MD 12.90, 95% CI 3.29–22.51). Effectiveness in terms of live births is lacking, as live births are hardly reported.

The GnRH antagonist induces immediate gonadotropin suppression due to competitive occupancy of the GnRH receptor. In the GnRH antagonist protocol gonadotropins are usually started on cycle day 2 or 3 of the natural cycle (see Figure 5.5). The administration of GnRH antagonist can be restricted to the period of E2 rise when at risk of premature LH surge, which is the mid and late follicular phase, to a daily dose of 0.25 mg, i.e., from cycle day 6 onwards until ovulation triggering (47,48). Effectiveness in terms of live birth rates is comparable to the long GnRH agonist protocol (see Figure 5.6) and lower OHSS rates are seen in the antagonist protocol (49–51). In view of reduced treatment duration, less gonadotropins needed, with reduced OHSS rate and similar pregnancy rates, GnRH antagonist seems to be advantageous compared to the long GnRH agonist protocol. A drawback of the GnRH antagonist protocol is less flexibility in programming compared to the long GnRH protocol.

Additional Compounds and Approaches

Recently, recombinant LH and hCG have become available for oocyte maturation, and also induction of endogenous LH surge by a bolus of GnRH agonist. The induction of an endogenous LH surge seems to be more physiological compared to exogenous hCG, and possibly leading to improved endometrial receptivity. Oocyte maturation triggering by GnRH agonist can only be applied in the case of GnRH antagonist co-treatment. Studies on the effectiveness of the GnRH agonist bolus for oocyte triggering showed significant lower live birth rate (OR 0.70, 95% CI 0.54–0.91; 11 studies, 1198 women) and a higher early miscarriage rate (OR 1.74, 95% CI 1.10–2.75; 11 randomized controlled trials [RCTs], 1198 women), but lower OHSS rate in the GnRH agonist group compared to the hCG group (52). However, the lower live birth rate after GnRH agonist bolus is probably influenced by the luteal phase support applied. Regimens of luteal phase supplementation in these cases are not extensively studied yet, but optimizing the luteal phase will probably lead to improved results (53).

Since the clinical application of recombinant LH (rLH) and recombinant hCG (rhCG), several trials studied the effect of these agents for oocyte maturation for IVF. Urinary products tend to lack in purity, with batch-to-batch variation in activity leading to unpredictable results (54). A meta-analysis studied the safety and efficacy of rhCG and rLH versus uhCG and showed no evidence for differences in ongoing pregnancy rate, live birth rate, and OHSS (52).

Ovarian Response Prediction

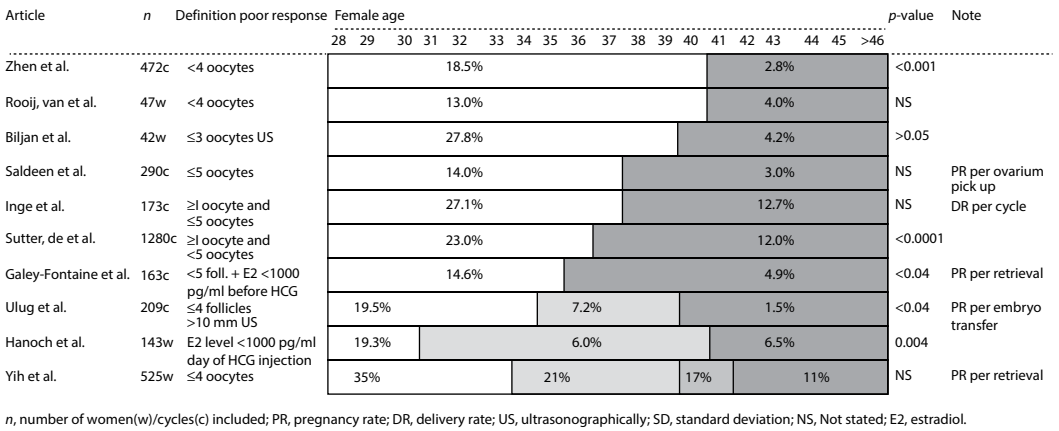
Initially, a large number of growing follicles and additionally a large number of oocytes were considered to be related to successful IVF. For this purpose, treatment protocols with high doses of exogenous gonadotropins with GnRH agonist co-treatment were developed. These protocols became more costly over the years due to the high usage of expensive drugs, intensive monitoring, side effects leading to drop out of treatment, and complications such as OHSS. Therefore, assessment of prognostic factors for ovarian response has become a more important issue in individual ovarian hyperstimulation for IVF.

IVF outcome is mainly determined by female age and ovarian response to ovarian hyperstimulation. Poor ovarian response is generally related to lower pregnancy chances modulated for female age compared to normal ovarian response (see Figure 5.7) (55). The mechanism behind poor response on ovarian hyperstimulation is most frequently the reduced number of FSH sensitive follicles, also known as diminished ovarian reserve.

Excessive ovarian response has long been accepted as the optimal outcome. However, increase in number of retrieved oocytes is not linearly related to increased pregnancy prospects. Reduced live birth rates are seen in cases of more than 15–20 oocytes retrieved (see Figure 5.8) (56,57). The impaired pregnancy outcome in excessive response is probably the consequence of less high quality oocytes and embryos with lower implantation potential, but also reduction of endometrial receptivity due to high E2 levels. Furthermore, excessive response is also related to higher risk for developing OHSS (58).

As ovarian response is an important determinant of IVF pregnancy chances and risks, prediction of ovarian response before starting IVF is becoming progressively important. Such approaches may optimize individual counseling and treatment. For this purpose, several ovarian reserve assessment tests have been studied extensively, such as the follicular phase FSH level, antral Mullerian hormone (AMH) level, and the antral follicle count (AFC).

The early follicular phase FSH level is the first marker that has been demonstrated to be an predicting factor of IVF outcome, but the accuracy in the prediction of poor ovarian response appeared to be moderate (59). In contrast to FSH, the accuracy of AMH and AFC were high in the prediction of poor response (see Table 5.2). A model including age and AFC or AMH had a significantly higher predictive accuracy for poor response than age alone. In the prediction of excessive response, AFC and AMH both add value to age alone (60). In predicting pregnancy after IVF, all three tests had very small or no predictive value. Age alone was the strongest predictor of pregnancy after IVF, and addition of any single test or a combination of tests did not lead to an increase in predictive accuracy.



n, number of women(w)/cycles(c) included; PR, pregnancy rate; DR, delivery rate; US, ultrasonographically; SD, standard deviation; NS, Not stated; E2, estradiol.

FIGURE 5.7 Female age category and pregnancy rate per cycle started. (From Oudendijk JF et al. *Hum Reprod Update*. 2012;18:1–11, with permission.)

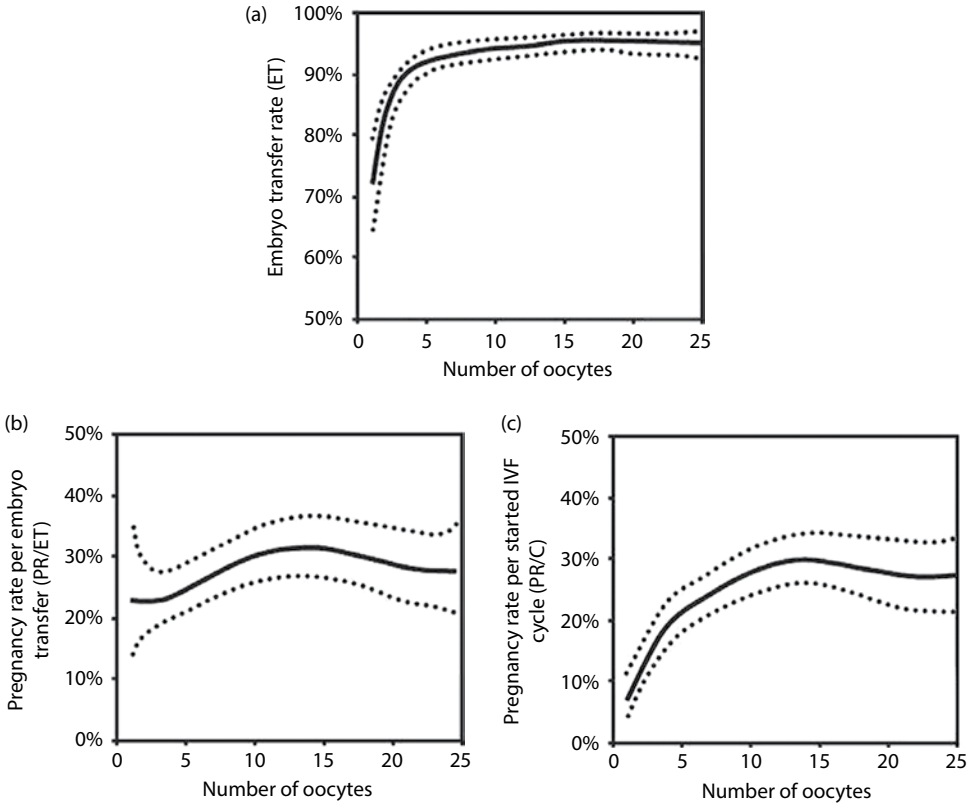


FIGURE 5.8 The number of retrieved oocytes (mean with 95% CI) in relation to embryo transfer rate (a); pregnancy rate per embryo transfer (b); pregnancy rate per started IVF cycle (c). The optimal number of obtained oocytes to conceive is 13. (From van der Gaast MH, et al. *Reprod Biomed Online*. 2006;13:476–80, with permission.)

Individualized Ovarian Stimulation Regimes

In the last decade new insights have resulted in milder ovarian hyperstimulation protocols. There is growing evidence that although a lower dose of gonadotropins leads to a lower number of oocytes, the implantation rate seems to be indifferent compared to conventional treatment (61,57). Studies on co-treatment with GnRH antagonist compared to agonist showed a slightly lower number of oocytes, but no difference in live birth rate and a lower incidence of OHSS in favor of the antagonist (50,51). These findings have resulted in a paradigm shift from quantity towards quality, aiming for a modest number of oocytes of high quality, resulting in good quality embryos with higher implantation potential, and optimal receptivity of the endometrium and reduction of OHSS.

Although ovarian response can be predicted quite accurately by the use of AMH or AFC, it remains a question whether adjustments of stimulation regimes will lead to the optimal ovarian response and, more importantly, to improved pregnancy rates. Studies on individualized regimens based on ovarian reserve testing showed contradictory results (62–67). There are only two studies available showing that an individualized starting dose based on a predicting algorithm leads to a reduction in the incidence of poor or excessive response and may lead to improved pregnancy rates (see Figures 5.9 and 5.10) (64,66). These findings, however, need to be confirmed by other studies. In view of expected hyper-responders, one pseudo randomized study reported that the use of a GnRH antagonist compared to GnRH agonist as co-treatment in predicted hyper-responders based on AMH levels, showed a reduction in the incidence of excessive response, the need for a complete cryopreservation, and cycle cancellation. Furthermore, an increase in clinical pregnancy rate per started cycle was seen in favor of GnRH antagonist (68).

TABLE 5.2

AUCs of Prediction Models of Age and ORTs for the Prediction of a Poor Response and Ongoing Pregnancy

	Three-test study group				Total study group			
	AUC	**95% CI**	***p*-value**	***n***	**AUC**	**95% CI**	***p*-value**	***n***
Poor response prediction								
Univariable models								
Age	0.61	0.54–0.68	NA	617	0.60	0.57–0.64	NA	4034
FSH	0.68	0.61–0.74	0.051	617	0.66	0.62–0.69	0.004	3652
AFC	0.76	0.70–0.82	<0.001	617	0.73	0.69–0.77	<0.001	2118
AMH	0.78	0.72–0.84	<0.001	617	0.81	0.77–0.84	<0.001	1274
Multivariable models								
Age and FSH	0.71	0.65–0.78	<0.001	617	0.69	0.66–0.72	<0.001	3652
Age and AFC	0.79	0.73–0.85	<0.001	617	0.76	0.72–0.80	<0.001	2118
Age and AMH	0.77	0.70–0.83	<0.001	617	0.80	0.76–0.84	<0.001	1274
Age and AMH and AFC	0.80	0.74–0.86	<0.001	617	0.80	0.74–0.86	<0.001	618
Age and AMH and AFC and FSH	0.81	0.75–0.86	<0.001	617	0.81	0.75–0.86	<0.001	617
Ongoing pregnancy prediction								
Univariable models								
Age	0.57	0.47–0.66	NA	420	0.56	0.54–0.59	NA	5207
FSH	0.53	0.43–0.62	0348	420	0.54	0.51–0.58	0.084	3521
AFC	0.50	0.40–0.59	0.100	420	0.52	0.48–0.57	0.612	1977
AMH	0.55	0.45–0.64	0.630	420	0.58	0.51–0.64	0.495	1008
Multivariable models								
Age and FSH	0.58	0.48–0.67	0.195	420	0.60	0.57–0.64	0.116	3521
Age and AFC	0.58	0.48–0.67	0.247	420	0.57	0.52–0.61	0.709	1977
Age and AMH	0.57	0.48–0.67	0.753	420	0.59	0.53–0.65	0.415	1008
Age and AMH and AFC	0.59	0.49–0.68	0.371	420	0.59	0.49–0.68	0.341	421
Age and AMH and AFC and FSH	0.58	0.49–0.68	0.414	420	0.58	0.49–0.68	0.414	420

Source: Broer SL et al. *Hum Reprod Update*. 2013;19:26–36, with permission.

Notes: AUC, area under the curve; ORT, ovarian reserve test; AMH, anti-Müllerian hormone; AFC, antral follicle count; FSH, follicle stimulating hormone. Poor response prediction. In the univariable analysis, it is shown that both AMH and AFC have a high accuracy, while FSH only has a moderate accuracy. In the multivariable models, the added value to the AUC of an ORT on female age is shown; the *p*-value indicates whether this added value is significant in comparison to age alone. All ORT show a significant rise in the AUC. Moreover, the added value of adding several ORTs to female age is shown. The model including age, AFC, and AMH reached the maximum predictive power. This level of accuracy, however, is also obtained when using a two factor model in the total study group Ongoing pregnancy. In the univariable analysis, it is shown that age is the strongest predictor compared with the single ORTs. The multivariable analysis shows that no single or combined ORT adds substantial predictive power to age alone. This is shown in the three test study group, as well as in the total study group.

Pretreatment of poor responders, such as transdermal testosterone or dehydroepiandrosterone (DHEA), and addition of aromatase inhibitors, rLH, or rhCG to ovarian stimulation were evaluated in a meta-analysis (69). Based on limited data, there seems to be an increase in clinical pregnancy rates and live birth in favor of transdermal testosterone, but because of the wide confidence intervals, further studies should confirm or reject these data. A beneficial effect of rLH, hCG, DHEA, or letrozole administration on pregnancy chances could not be found.

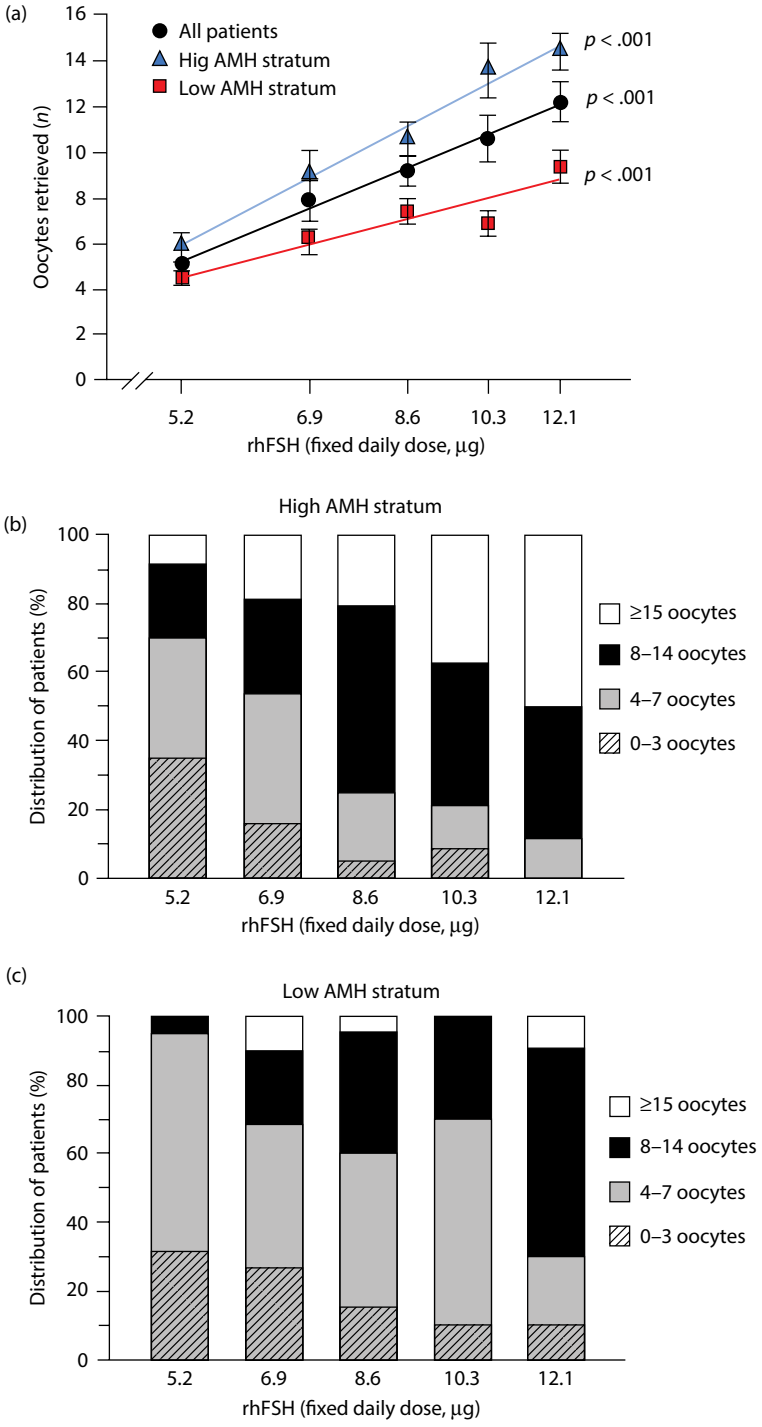


FIGURE 5.9 (a) Mean number of oocytes retrieved for patients in the five rhFSH dose groups, overall and by AMH stratum. The vertical bars represent standard errors. *p* values reflect the dose–response relationship. (b, c) Relative proportions of patients with different categories of number of oocytes retrieved by rhFSH dose group and AMH stratum (b: high AMH; c: low AMH). (From Arce JC et al. *Fertil Steril*. 2014;102:1633–40, with permission.)

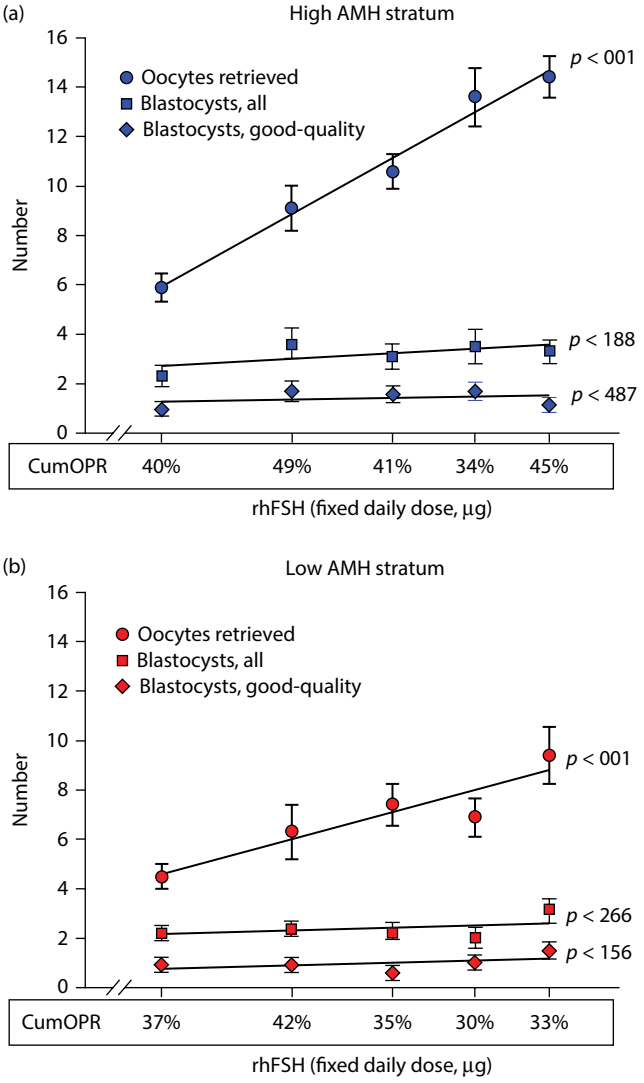


FIGURE 5.10 Mean rate of blastocysts to oocytes retrieved for patients in the five rhFSH dose groups, (a) overall and (b) by AMH stratum. The vertical bars represent standard errors. *p* values reflect the dose–response relationship. (From Arce JC et al. *Fertil Steril*. 2014;102:1633–40, with permission.)

Conclusions

Ovarian stimulation in any infertility treatment leads to higher pregnancy chances, but the side effects can lead to major complications such as multiple pregnancy and OHSS. The type and dosing of stimulation agents, stimulation regimes, and co-treatment with other agents influence the outcome in view of pregnancy rates, live birth rates, and complication rates. In the case of ovarian stimulation for IUI and IVF, but also in the case of ovulation induction, there is a need for more knowledge in the prediction of these outcomes. Additionally, development of individualized stimulation approaches might contribute to improvement of ovarian stimulation and treatment outcomes.

REFERENCES

1. McGee EA, Hsueh AJ. Initial and cyclic recruitment of ovarian follicles. *Endocr Rev.* 2000;21:200–14.
2. Adhikari D, Liu K. Molecular mechanisms underlying the activation of mammalian primordial follicles. *Endocr Rev.* 2009;30:438–64.
3. Edson MA, Nagaraja AK, Matzuk MM. The mammalian ovary from genesis to revelation. *Endocr Rev.* 2009;30:624–712.
4. Fauser BC, Van Heusden AM. Manipulation of human ovarian function: Physiological concepts and clinical consequences. *Endocr Rev.* 1997;18:71–106.
5. Baird DT. A model for follicular selection and ovulation: Lessons from superovulation. *J Steroid Biochem.* 1987;27:15–23.
6. Fluker MR, Marshall LA, Monroe SE, Jaffe RB. Variable ovarian response to gonadotropin-releasing hormone antagonist-induced gonadotropin deprivation during different phases of the menstrual cycle. *J Clin Endocrinol Metab.* 1991;72:912–9.
7. Zeleznik AJ, Benyo DF. Control of follicular development, corpus luteum function, and the recognition of pregnancy in higher primates. In: *The Physiology of Reproduction*, Knobil E, Neill J (Eds.). New York: Raven Press, 1994, 751–82.
8. Dewailly D, Andersen CY, Balen A, Broekmans F et al. The physiology and clinical utility of anti-Mullerian hormone in women. *Hum Reprod Update.* 2014;20:370–85.
9. Macklon NS, Stouffer RL, Giudice LC et al. The science behind 25 years of ovarian stimulation for in vitro fertilization. *Endocr Rev.* 2006;27:170–207.
10. Fauser BC, Devroey P, Macklon NS. Multiple birth resulting from ovarian stimulation for subfertility treatment. *Lancet.* 2005;365:1807–16.
11. De Vos M, Devroey P, Fauser BC. Primary ovarian insufficiency. *Lancet.* 2010;376:911–21.
12. Kerin JF, Liu JH, Phillipou G et al. Evidence for a hypothalamic site of action of clomiphene citrate in women. *J Clin Endocrinol Metab.* 1985;61:265–8.
13. Adashi EY: Clomiphene citrate: Mechanism(s) and site(s) of action—A hypothesis revisited. *Fertil Steril.* 1984;42:331–44.
14. Polson DW, Kiddy DS, Mason HD, Franks S. Induction of ovulation with clomiphene citrate in women with polycystic ovary syndrome: The difference between responders and nonresponders. *Fertil Steril.* 1989;51:30–4.
15. The Thessaloniki ESHRE/ASRM-sponsored PCOS consensus workshop group. Consensus on infertility treatment related to polycystic ovary syndrome. *Hum Reprod.* 2008;23(3):462–77.
16. Imani B, Eijkemans MJ, te Velde ER et al. Predictors of patients remaining anovulatory during clomiphene citrate induction of ovulation in normogonadotropic oligoamenorrheic infertility. *J Clin Endocrinol Metab.* 1998;83:2361–5.
17. Tang T, Lord JM, Norman RJ et al. Insulin-sensitising drugs (metformin, rosiglitazone, pioglitazone, D-chiro-inositol) for women with polycystic ovary syndrome, oligo amenorrhoea and subfertility. *Cochrane Dbf Syst Rev.* 2012, Issue 5. Art. No.: CD003053. DOI: 10.1002/14651858.CD003053.pub5.
18. Franik S, Kremer JAM, Nelen WLDM et al. Aromatase inhibitors for subfertile women with polycystic ovary syndrome. *Cochrane Db Syst Rev.* 2014, Issue 2. Art. No.: CD010287. DOI: 10.1002/14651858.CD010287.pub2.
19. Franks S, Mason HD, Polson DW et al. Mechanism and management of ovulatory failure in women with polycystic ovary syndrome. *Hum Reprod.* 1988;3:531–4.
20. Brown JB. Pituitary control of ovarian function: Concepts derived from gonadotrophin therapy. *Aust N Z J Obstet Gynaecol.* 1978;18:46–54.
21. van Santbrink EJ, Fauser BC. Urinary follicle-stimulating hormone for normogonadotropic clomiphene-resistant anovulatory infertility: Prospective, randomized comparison between low dose step-up and step-down dose regimens. *J Clin Endocrinol Metab.* 1997;82:3597–602.
22. Christin-Maitre S, Hugues JN. A comparative randomized multicentric study comparing the step-up versus step-down protocol in polycystic ovary syndrome. *Hum Reprod.* 2003;18:1626–31.
23. Schoot DC, Hop WC, Pache TD et al. Growth of the dominant follicle is similar to normal in patients with gonadotrophin-stimulated polycystic ovary syndrome exhibiting monofollicular development during a decremental dose regimen. *Acta Endocrinol.* 1993;129:126–9.

24. Burgues S. The effectiveness and safety of recombinant human LH to support follicular development induced by recombinant human FSH in WHO group I anovulation: Evidence from a multicentre study in Spain. *Hum Reprod*. 2001;16:2525–32.
25. Braat DD, Berkhout G, ten Brug CS, Schoemaker J. Multiple follicular development in women with normal menstrual cycles treated with pulsatile gonadotrophin-releasing hormone. *Hum Reprod*. 1991;6:1379–83.
26. Martin KA, Hall JE, Adams JM, Crowley WF Jr. Comparison of exogenous gonadotropins and pulsatile gonadotropin-releasing hormone for induction of ovulation in hypogonadotropic amenorrhea. *J Clin Endocrinol Metab.* 1993;77:125–9.
27. Cantineau AEP, Cohlen BJ. Ovarian stimulation protocols (anti-oestrogens, gonadotrophins with and without GnRH agonists/antagonists) for intrauterine insemination (IUI) in women with subfertility. *Coch Db Syst Rev.* 2007, Issue 2. Art. No.: CD005356. DOI: 10.1002/14651858.CD005356.pub2.
28. Veltman-Verhulst SM, Cohlen BJ, Hughes E et al. Intra-uterine insemination for unexplained subfertility. *Cochrane Db Syst Rev.* 2012, Issue 9. Art. No.: CD001838. DOI: 10.1002/14651858.CD001838.pub4.
29. The ESHRE Capri Workshop Group. Intrauterine Insemination. *Hum Reprod Update*. 2009;15:265–77.
30. Cantineau AE, Cohlen BJ, Dutch IUI study group. The prevalence and influence of luteinizing hormone surges in stimulated cycles combined with intrauterine insemination during a prospective cohort study. *Fertil Steril*. 2007;88:107–12.
31. Fauser BC, Mannaerts BM, Devroey P et al. Advances in recombinant DNA technology: Corifollitropin alfa, a hybrid molecule with sustained follicle-stimulating activity and reduced injection frequency. *Hum Reprod Update*. 2009;15:309–21.
32. Balen AH, Lumholtz IB. Consensus statement on the bio-safety of urinary-derived gonadotrophins with respect to Creutzfeldt-Jakob disease. *Hum Reprod*. 2005;20:2994–9.
33. Pierce JG, Parsons TF. Glycoprotein hormones: Structure and function. *Annu Rev Biochem*. 1981;60:465–95.
34. Fauser BC. Developments in human recombinant follicle stimulating hormone technology: Are we going in the right direction? *Hum Reprod*. 1998;13:36–46.
35. Kenny DT. In vitro fertilisation and gamete intrafallopian transfer: An integrative analysis of research, 1987–1992. *Br J Obstet Gynaecol*. 1995;102:317–25.
36. Gibreel A, Maheshwari A, Bhattacharya S. Clomiphene citrate in combination with gonadotropins for controlled ovarian stimulation in women undergoing in vitro fertilization. *Cochrane Db Syst Rev.* 2012;11:CD008528. Doi: 10.1002/14651858.CD008528.pub2.
37. Daya S. Updated meta-analysis of recombinant follicle-stimulating hormone (FSH) versus urinary FSH for ovarian stimulation in assisted reproduction. *Fertil Steril*. 2002;77:711–4.
38. Wely van M, Kwan L, Burt AL et al. Recombinant versus gonadotrophin for ovarian stimulation in assisted reproductive technology cycles. *Cochrane Db Syst Rev.* 2011;16(2):CD005354.
39. Pouwer AW, Farquhar C, Kremer JAM. Long-acting FSH versus daily FSH for women undergoing assisted reproduction. *Cochrane Db Syst Rev.* 2015, Issue 7. Art. No.: CD009577. DOI: 10.1002/14651858.CD009577.pub3.
40. Sterrenburg M, Veltman-Verhulst SM, Eijkemans MJ et al. Clinical outcomes in relation to the daily dose of recombinant follicle-stimulating hormone for ovarian stimulation in in vitro fertilization in presumed normal responders younger than 39 years: A meta-analysis. *Hum Reprod Update*. 2001;17:184–96.
41. van Hooff MH, Alberda AT, Huisman GJ et al. Doubling the human menopausal gonadotrophin dose in the course of an in-vitro fertilization treatment cycle in low responders: A randomized study. *Hum Reprod*. 1993;8:369–73.
42. Rombauts L, Suikkari AM, MacLachlan V et al. Recruitment of follicles by recombinant human follicle-stimulating hormone commencing in the luteal phase of the ovarian cycle. *Fertil Steril*. 1998;69:665–9.
43. Porter RN, Smith W, Craft IL et al. Induction of ovulation for in-vitro fertilisation using buserelin and gonadotropins. *Lancet*. 1984;2:1284–5.
44. Huirne JA, Lambalk CB. Gonadotropin-releasing-hormone-receptor antagonists. *Lancet*. 2001;24:1793–803.
45. Hughes EG, Fedorkow DM, Daya S et al. The routine use of gonadotropin-releasing hormone agonists prior to in vitro fertilization and gamete intrafallopian transfer: A meta-analysis of randomized controlled trials. *Fertil Steril*. 1992;58:888–96.

46. Maheshwari A, Gibreel A, Siristatidis CS et al. Gonadotrophin-releasing hormone agonist protocols for pituitary suppression in assisted reproduction. *Cochrane Db Syst Rev*. 2011, Issue 8. Art. No.: CD006919. DOI: 10.1002/14651858.CD006919.pub3.
47. Griesinger G, Felberbaum RE, Schultze-Mosgau A, Diedrich K. Gonadotropin-releasing hormone antagonists for assisted reproductive techniques: Are there clinical differences between agents? *Drugs*. 2004;64:563–75.
48. Hamdine O, Macklon NS, Eijkemans MJ et al. CETRO trial study group. Comparison of early versus late initiation of GnRH antagonist co-treatment for controlled ovarian stimulation in IVF: A randomized controlled trial. *Hum Reprod*. 2013;28:3227–35.
49. Kolibianakis EM, Collins J, Tarlatzis BC et al. Among patients treated for IVF with gonadotrophins and GnRH analogues, is the probability of live birth dependent on the type of analogue used? A systematic review and meta-analysis. *Hum Reprod Update*. 2006;12:651–71.
50. Al-Inany HG, Youssef MAFM, Aboulghar M et al. Gonadotrophin-releasing hormone antagonists for assisted reproductive technology. *Cochrane Db Syst Rev*. 2011, Issue 5. Art. No.: CD001750. DOI: 10.1002/14651858.CD001750.pub3.
51. Xiao JS, Su CM, Zeng XT. Comparisons of GnRH antagonist versus GnRH agonist protocol in supposed normal ovarian responders undergoing IVF: A systematic review and meta-analysis. *PLoS One*. 2014;9(9):e106854.
52. Youssef MAFM, Al-Inany HG, Aboulghar M, et al. Recombinant versus urinary human chorionic gonadotrophin for final oocyte maturation triggering in IVF and ICSI cycles. *Cochrane Db Syst Rev*. 2011, Issue 4. Art. No.: CD003719. DOI: 10.1002/14651858.CD003719.pub3.
53. Yding C, Andersen K, Vilbour A. Improving the luteal phase after ovarian stimulation: Reviewing new options. *Reprod BioMed Online*. 2014;28:552–9.
54. Zegers-Hochschild F, Fernandez E, Mackenna A et al. The empty follicle syndrome: A pharmaceutical industry syndrome. *Hum Reprod*. 1996;10:2262–5.
55. Oudendijk JF, Yarde F, Eijkemans MJ et al. The poor responder in IVF: Is the prognosis always poor? A systematic review. *Hum Reprod Update*. 2012;18:1–11.
56. Sunkara SK, Rittenberg V, Raine-Fenning N et al. Association between the number of eggs and live birth in IVF treatment: An analysis of 400 135 treatment cycles. *Hum Reprod*. 2011;26:1768–74.
57. van der Gaast MH, Eijkemans MJ, van der Net JB et al. Optimum number of oocytes for a successful first IVF treatment cycle. *Reprod Biomed Online*. 2006;13:476–80.
58. Delvigne A, Rozenberg S. Epidemiology and prevention of ovarian hyperstimulation syndrome (OHSS): A review. *Hum Reprod Update*. 2002;8:559–77.
59. Broer SL, van Disseldorp J, Broeze KA et al. Added value of ovarian reserve testing on patient characteristics in the prediction of ovarian response and ongoing pregnancy: An individual patient data approach. *Hum Reprod Update*. 2013;19:26–36.
60. Broer SL, Dólleman M, Opmeer BC et al. AMH and AFC as predictors of excessive response in controlled ovarian hyperstimulation: A meta-analysis. *Hum Reprod Update*. 2011;17:46–54.
61. Heijnen EM, Eijkemans MJ, de Klerk C et al. A mild treatment strategy for in-vitro fertilisation: A randomised non-inferiority trial. *Lancet* 2007;369:743–9.
62. Harrison RF, Jacob S, Spillane H et al. A prospective randomized clinical trial of differing starter doses of recombinant follicle-stimulating hormone (follitropin-b) for first time in vitro fertilization and intracytoplasmic sperm injection treatment cycles. *Fertil Steril*. 2001;75:23–31.
63. Klinkert ER, Broekmans FJ, Looman CW et al. Expected poor responders on the basis of an antral follicle count do not benefit from a higher starting dose of gonadotrophins in IVF treatment: A randomized controlled trial. *Hum Reprod*. 2005;20:611–5.
64. Popovic-Todorovic B, Loft A, Bredkjaeer HE et al. A prospective randomized clinical trial comparing an individual dose of recombinant FSH based on predictive factors versus a "standard" dose of 150 IU/day in "standard" patients undergoing IVF/ICSI treatment. *Hum Reprod*. 2003;18:2275–82.
65. Lekamge DN, Lane M, Gilchrist RB et al. Increased gonadotrophin stimulation does not improve IVF outcomes in patients with predicted poor ovarian reserve. *J Assist Reprod Genet*. 2008;25:515–21.
66. Arce JC, Andersen AN, Fernández-Sánchez M et al. Ovarian response to recombinant human follicle-stimulating hormone: A randomized, antimüllerian hormone-stratified, dose-response trial in women undergoing in vitro fertilization/intracytoplasmic sperm injection. *Fertil Steril*. 2014;102:1633–40.

67. Olivennes F, Trew G, Borini A et al. Randomized, controlled, open-label, non-inferiority study of the CONSORT algorithm for individualized dosing of follitropin alfa. *Reprod Biomed Online*. 2015;30:248–57.
68. Nelson SM, Yates RW, Lyall H et al. Anti-Müllerian hormone-based approach to controlled ovarian stimulation for assisted conception. *Hum Reprod*. 2009;24:867–75.
69. Bosdou JK, Venetis CA, Kolibianakis EM, Toulis KA, Goulis DG, Zepiridis L, Tarlatzis BC. The use of androgens or androgen-modulating agents in poor responders undergoing in vitro fertilization: A systematic review and meta-analysis. *Hum Reprod Update*. 2012;18:127–45.

6

Role of Antagonists in Human IVF: The Tool for a Safer and More Patient Friendly IVF

Evangelos Papanikolaou and Robert Najdecki

In the initial years of IVF the natural cycle IVF had been the protocol, resulting in an extreme delay in the achievement of the first birth. One of the main reasons was that, in the majority of cycles, a premature luteinizing hormone (LH) surge was noticed and thus, during the oocyte pick-up, no egg could be retrieved. The same was true even with the initiation of ovarian stimulation with urinary gonadotropins. The pregnancy results were still below 10%, as in many cycles only a few oocytes were retrieved or of poor quality due to premature LH surges. Some years later, a huge improvement in the IVF efficacy was observed by the introduction of the concept of hypophysis down regulation using gonadotropin releasing hormone (GnRH)-analogues (1). The main role of GnRH analogues was to achieve functional hypophysectomy and prevent premature LH surges. The above described down regulation was first achieved with the use of a GnRH-agonist, resulting in a significant decrease of incidence of such LH surges (2). In respect, an increase of successful oocyte pick-ups with many oocytes was observed, leading to a noticeable increase of pregnancies.

GnRH Analogues: The Way from Agonist to Antagonist

Although the rationale indicates the use of an antagonist to facilitate functional hypophysectomy, this was not possible in the initial years of research due to an extreme allergic reaction induced after the use of the then available GnRH-antagonist molecules.

Native GnRH is a decapeptide synthesized by a group of neurons that, in humans, is located in the arcuate nucleus of the medial basal hypothalamus and in the preoptic area of the ventral hypothalamus. The GnRH molecules are released to the anterior pituitary in a pulsatile pattern by a network of capillaries in which the blood flows from the hypothalamus to the pituitary. Then, GnRH binds to specific receptors in the pituitary cells, to regulate the synthesis and secretion of LH and follicle-stimulating hormone (FSH). Following the discovery of the amino acid sequence of GnRH, researchers manage to develop synthetic analogues, both agonistic and antagonistic. In the initial years, more than 1000 analogues of GnRH were synthesized, which were derived after alterations of the primary structure by the deletion and/or substitution of one or more amino acids by others.

Agonists were produced by the substitution of amino acids at position 6 of the native molecule or by the replacement of the C-terminal glycine-amide. These positions (6 and 10) of amino acid substitutions in synthetic GnRH agonists are important for the three-dimensional structure of the molecule. Moreover, they are the major sites of cleavage and enzymatic degradation by peptidases. Thus, modifications offering protection from these enzymes can prolong the half-life of these compounds. Initially, an agonistic effect was observed (flare-up), which was associated with an increase in the circulating levels of FSH and LH. However, after 1–3 weeks of continuous administration, down-regulation and desensitization of the pituitary induces a hypogonadotropic status (3).

Antagonists, conversely, do not induce such a flare-up effect, but an immediate, rapid, and reversible suppression of gonadotropin secretion, which is due to the competitive occupancy of GnRH receptors.

The third generation of GnRH antagonists has modifications in positions 1, 2, 3, 6, and 10 of the amino acid sequence and a structure offering metabolic stability and reduced allergic side effects.

The main advantage of the antagonist molecule is the short half-time life, which permits transient suppression of the axis and thus immediate recovery of the hypothalamic–hypophysis–ovarian axis within 24 hours. Therefore, compared to the use of the agonist analogues, with antagonist the female patient does not experience all the annoying menopausal symptoms after agonist down-regulation; second, we avoid the formation of ovarian cysts due to the flare-up effect; and third, the production of endogenous LH recovers earlier, allowing better quality follicles and oocytes and improved endometrial receptivity. On the other hand, local skin reactions at the injection site were common side effects with both analogues, but especially with the first generation antagonists due to increased histamine secretion. Nevertheless, only mild local reactions like redness and swelling might appear in less than 10% of the patients treated with the third generation antagonists (4). With both analogues, no significant changes have been observed in hematological profile.

Despite initial reservations in using the antagonist protocol, gradually, due to the more patient friendly profile and the reduced risk for OHSS, more and more fertility specialists around the globe have started using them.

Pregnancy Efficacy of the Antagonist Protocol

Although the antagonist protocol facilitates a more physiological mode down-regulation during ovarian stimulation, in the initial years after their launch in the market, reproductive physicians were reluctant to inscribe such protocol due to an uncertainty whether they were comparable regarding pregnancy rates with the gold-standard protocol used so far, the long-agonist protocol.

This attitude could be mainly attributed to the initial two meta-analyses published by the group of Al-Inany (5,6). In the second larger meta-analysis, including 27 randomized trials, Al-Inany and colleagues found that the clinical pregnancy rate was significantly lower in the antagonist group (6). At the same year a study by Kolibianakis et al. showed the opposite, that the chance of live birth is similar among the two analogues (7). These contradictory results added confusion to the medical community and the debate went on. Five years later, Al-Inany et al. reached the same conclusion as previously Kolibianakis had published, that there is no difference in the probability of live birth between the antagonist and the long agonist protocol (8). A new meta-analysis by Xiao et al. (9) in supposed normal ovarian responders also failed to find any difference among the two protocols (Figure 6.1 and Table 6.1). Finally, in 2016, Al-Inany, again with his team, analyzed 72 RCTs and reached the conclusion that the evidence suggested that if the chance of live birth following GnRH agonist is assumed to be 29%, the chance following GnRH antagonist would be between 25% and 33% (OR 1.02, 95% CI 0.85–1.23; 12 RCTs, $n = 2303$, $I2 = 27\%$, moderate quality evidence). On the other hand, controversy still exists, and some authors criticized the methodology of the published meta-analyses, stating that the delivery rates are higher with the long protocol (10).

Concluding, on the top of shorter stimulation period and less gonadotropins' dose, the overall pregnancy efficacy of the antagonist protocol seems to have improved after the first years (area under the learning curve) and ultimately to produce similar pregnancy outcomes as the gold standard long agonist protocol. Apart from this, the antagonist protocol has the significant advantage of more safety regarding the risk of ovarian hyperstimulation syndrome (OHSS), which renders antagonist the first choice for predicted high responder patients (13,14).

Antagonist Protocol Reduces Significantly the Risk of OHSS Syndrome

OHSS is the most common complication of IVF and although it is relatively rare (1%–6%) it can be fatal. There is strong evidence that when used during IVF treatment for patients with supposed normal responses, the GnRH antagonist protocol could significantly reduce the incidence of OHSS while yielding similar ongoing pregnancy and live birth rates, compared with those of the GnRH agonist standard

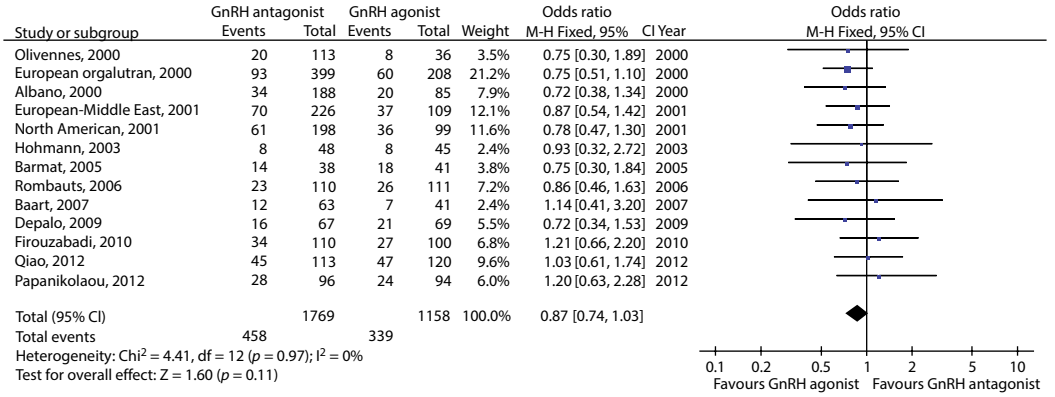


FIGURE 6.1 Ongoing pregnancy rate for the comparison of the GnRH-Agonist Long protocol versus the Antagonist protocol. (From Xiao J-s et al. *PLoS One*. 2014;9:9, with permission.)

TABLE 6.1

Meta-Analyses Published on the Comparison GnRH-Agonist versus Antagonist for Ovarian Stimulation in IVF

Authors (Ref)	RCT Trials No	Conclusion
Al-Inany et al. (5)	5	Clinical pregnancy rate was significantly lower with GnRH antagonist treatment than with the GnRH agonist long protocol
Al-Inany et al. (6)	27	Clinical pregnancy rate was significantly lower with GnRH antagonist treatment than with the GnRH agonist long protocol
Kolibianakis et al. (7)	22	Live birth rates were similar in the GnRH antagonist compared with the long GnRH agonist protocols
Al-Inany et al. (8)	45	Live birth rates were similar in the GnRH antagonist compared with the long GnRH agonist protocols
Bodri et al. (11)	8	In oocyte donors the ongoing pregnancy rates were similar in the GnRH antagonist compared with the long GnRH agonist protocols
Xiao et al. (9)	23	Ongoing pregnancy and live birth rates were similar in the GnRH antagonist compared with the long GnRH agonist protocols
Al-Inany et al. (12)	73	Live birth rates were similar, although non-conclusive evidence. The evidence suggested that if the chance of live birth following GnRH agonist is assumed to be 29%, the chance following GnRH antagonist would be between 25% and 33%

long protocol. The risk especially of OHSS cannot be neglected during ovarian stimulation, as with IVF relatively young women are likely to be treated and therefore any complication, including death, is unacceptable and devastating.

Predictive indicators for excess ovarian response and thus OHSS, is anti-mullerian hormone (AMH), antral follicle count, polycystic ovaries (PCO) history, or history of previous excess response (15). Since the long protocol produces one to two more follicles, a higher risk for hyperstimulation could be expected.

In the most recent meta-analysis, OHSS as an outcome measure was included in 20 studies (3693 cases) (9). The results showed (Figure 6.2) that the incidence of OHSS was lower in the GnRH antagonist group than in the GnRH agonist group, and this difference was statistically significant (OR: 0.59, 95%CI: 0.42, 0.82; $p = 0.002$).

However, apart from the inherited lower risk for OHSS, the antagonist protocol has the additional advantage of almost eliminating OHSS by offering the alternative of triggering ovulation with GnRH-agonist, which is impossible in the long protocol. Several studies have demonstrated that when hCG is replaced for agonist to trigger final oocyte maturation it leads to extremely low OHSS risk, since fewer

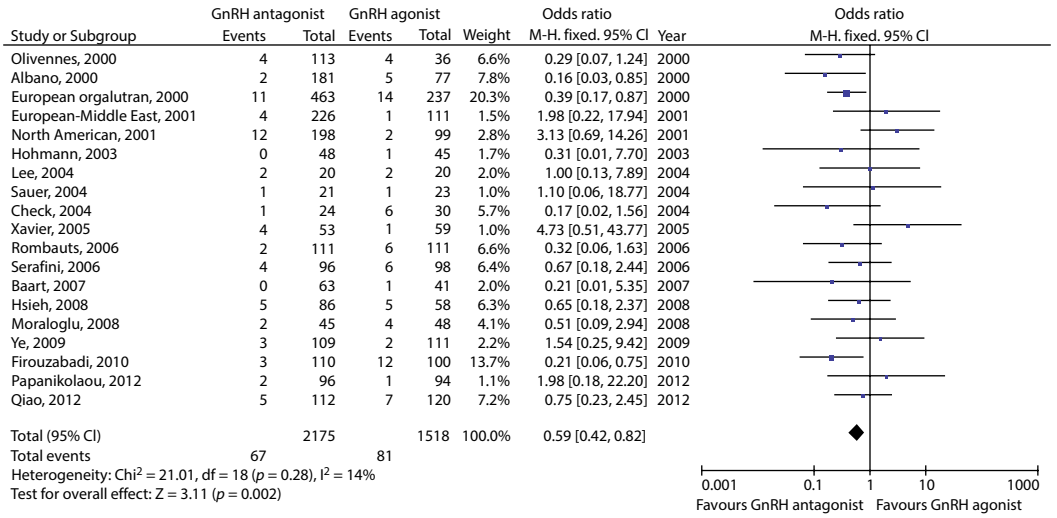


FIGURE 6.2 OHSS risk when comparing the GnRH-Agonist Long protocol versus the Antagonist protocol. (From Xiao J-s et al. *PLoS One*. 2014;9:9, with permission.)

corpora lutea are rescued and thus lower vascular endothelial growth factor (VEGF) is produced leading to reduced risk for OHSS (16). Therefore, nowadays, oocyte donors are stimulated only with the antagonist protocol and, similarly, PCO patients are treated preferentially within the antagonist protocol.

Cycle Programming with Antagonist

One of the advantages of using GnRH agonist down-regulation was that, after the period, within one to two weeks the physician could decide when a patient should start stimulation, offering in that way extreme comfort in scheduling the oocyte pick ups and avoiding weekends. With the antagonist protocol this flexibility was reduced and limited to plus/minus one day, either by delaying one day the simulation from cycle day-2 to day-3 or by delaying one day the ovulation triggering.

OCP (Oral Contraceptive Pill)

Therefore the use of the OCP was suggested for the preceding cycle, having an extra advantage of reducing the probability of identifying a follicular cyst at the beginning of the planned stimulation (17). Once the OCP is stopped, ovarian stimulation is allowed to start within 2–5 days irrespective of the resumption of period or not within this time lapse (18). However, the use of OCPs, apart of inducing discomfort like headaches and bloating in some patients, has a certain drawback, in that you cannot exactly predict the initiation of period, as it might range from 3 to 5 days (19).

A meta-analysis was performed by Griesinger et al. (20) with six RCTs (1370 individuals). All trials used combined OCPs with 30 μg of ethinyl E2 and 150 μg of gestogen (either desogestrel or levonorgestrel), and the duration of OCP pretreatment ranged from 14 to 28 days. The OCP-free interval between cessation of OCP treatment and initiation of stimulation was 2–3 days in two of the studies, whereas in the remaining four studies a 5-day interval was used (Table 6.2). Meta-analysis has shown that the probability of an ongoing pregnancy per randomized woman was found to be significantly lower in patients who received OCP pretreatment (RR 0.80, 95% CI: 0.66–0.97; $p = 0.02$; rate difference: –5%, 95% CI: –10% to –1%; $p = 0.02$; odds ratio 0.74, 95%CI: 0.58–0.96; $p = 0.02$; fixed effects model).

The reasons for this reduction in pregnancy rates when using OCP to program in an antagonist protocol might be several. The main drawback is the period free off OCP until the stimulation begins with the gonadotropins. If this interval was predefined—for example five days—many patients had not bled

TABLE 6.2

Comparison of Randomised Studies Comparing Pill versus No-Pill Antagonist Protocol for IVF

	OCP		NoOCP		
Study	**Events**	**Total**	**Events**	**Total**	**Risk Ratio**
Huirne, 2006	4	32	8	32	0.50 [0.17–1.50]
Rombauts, 2006	20	117	26	117	0.77 [0.46–1.30]
Kolibianakis, 2006	51	250	60	254	0.86 [0.62–1.20]
Cedrin, 2007	3	21	7	24	0.49 [0.14–1.66]
Kim, 2009	8	27	5	27	1.60 [0.60–4.27]
Tavnergen, 2009	55	223	71	219	0.76 [0.56–1.03]
Total	141	670	177	673	0.80 [0.66–0.97]

Source: Data from Griesinger G et al. *Fertil Steril*. 2010;94:2382–4.
Note: Relative risks for ongoing pregnancy with 95% CI per randomised patient.

until the initiation of the stimulation, meaning that their period (menstruation) could follow the start of stimulation and therefore the endometrium was unprepared until the luteal phase (21,22).

Another reason might be the progestagen component of the pill, as this might influence either the quality of the eggs or the endometrium receptivity later during the implantation period. For all these reasons, we need large randomized studies to explore different components and different OCP-free intervals before final conclusions could be drawn.

Luteal Estrogen Pretreatment

Another protocol to assist planning with the antagonist protocol is the initiation of estrogen in the late luteal phase of the preceding cycle. Blockeel et al. published a very interesting approach where, in the pretreatment group ($n = 44$), patients were administered oestradiol valerate at a daily dose of 4 mg (2-0-2) from day 25 of the preceding cycle onwards, during 6–10 consecutive days, depending on the day of the week (see Figure 6.3). The primary endpoint was the proportion of patients undergoing oocyte retrieval during a weekend day (i.e., Saturday or Sunday), which was significantly lower in the pretreatment group (1/37, 2.7%) compared with the control group (8/39, 20.5%; $p = 0.029$) (23). Eventually the clinical pregnancy rates per started cycle were similar in the pretreatment group (38.6%) compared with the control group (38.1%), which proves that the above strategy can ascribe flexibility regarding the starting day, even to the antagonist protocol, without compromising the pregnancy outcome.

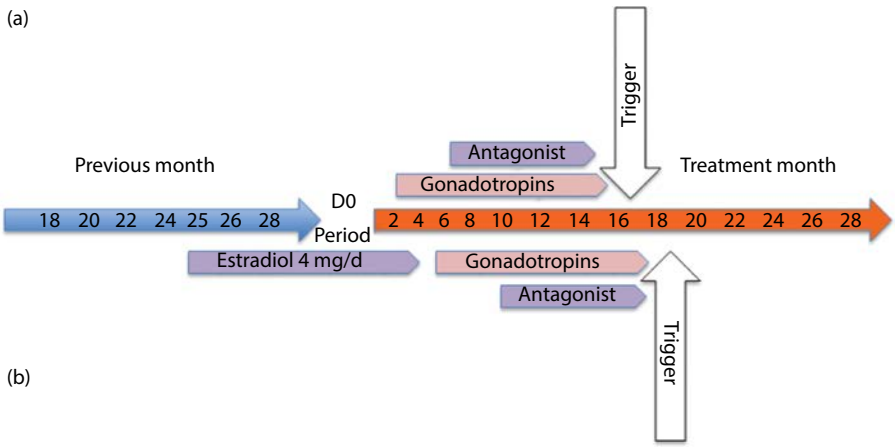


FIGURE 6.3 Estrogen pretreatment for cycle programming in antagonist protocol. (a) Classical antagonist protocol starting cycle Day 2. (b) Flexible, estrogen pretreatment antagonist protocol starting cycle Day 2 or 3 or 4 or 5.

What's New in the Future of Antagonists?

Degarelix is a new long acting injectable antagonist licensed already for the treatment of prostate cancer. Data is gradually becoming available in utilizing this new antagonist in women undergoing IVF with promising efficacy as well as tolerability for the patients (24).

Elagolix is the frontrunner among an emerging class of GnRH antagonists which, unlike their peptide predecessors, has a non-peptide structure, resulting in its oral bioavailability (25). Phase I and II clinical trials have demonstrated the safety of elagolix and its efficacy in partial and reversible suppression of ovarian estrogen production, resulting in improvements in endometriosis-related pain. Phase III clinical trials are currently underway, and elagolix may become a valuable addition to the armamentarium of pharmacological agents to treat endometriosis-related pain.

REFERENCES

1. Clayton RN, Catt KJ. Receptor-binding affinity of gonadotrophin-releasing hormone analogs: Analysis by radio-ligand receptor assay. *Endocrinology*. 1980;106:1154–9.
2. Smitz J, Ron-El R, Tarlatzis BC. The use of gonadotropin releasing hormone agonists for in vitro fertilization and other assisted procreation techniques: Experience from three centers. *Hum Reprod*. 1992;7(Suppl. 1):49–66.
3. Tarlatzis BC, Bili H. Safety of GnRH agonists and antagonists. *Expert Opin Drug Saf*. 2004;3(1):39–46.
4. Olivennes F, Diedrich K, Frydman R, Felberbaum RE, Howles CM. Cetrotide multiple dose international study group; cetrotide single dose international study group: Safety and efficacy of a 3 mg dose of the GnRH antagonist cetrorelix in preventing premature LH surges: Report of two large multicentre, multinational, Phase IIIb clinical experiences. *Reprod Biomed Online*. 2003;6:432–8.
5. Al-Inany HG, Aboulghar M. Gonadotrophin-releasing hormone antagonists for assisted conception. *Cochrane Db Syst Rev*. 2001;4:CD001750.
6. Al-Inany HG, Abou-Setta AM, Aboulghar M. Gonadotrophin-releasing hormone antagonists for assisted conception. *Cochrane Db Syst Rev*. 2006;19:CD001750.
7. Kolibianakis EM, Collins J, Tarlatzis BC et al. Among patients treated for IVF with gonadotrophins and GnRH analogues, is the probability of live birth dependent on the type of analogue used? A systematic review and meta-analysis. *Hum Reprod Update*. 2006;12:651–71.
8. Al-Inany HG, Youssef MA, Aboulghar M et al. Gonadotrophin-releasing hormone antagonists for assisted reproductive technology. *Cochrane Db Syst Rev*. 2011;11:CD0017503(1):39–46.
9. Xiao J-s, Su C-m, Zeng X-t. Comparisons of GnRH Antagonist versus GnRH Agonist Protocol in Supposed Normal Ovarian Respond Undergoing IVF: A Systematic Review and Meta-Analysis. *PLoS One*. 2014;9:9.
10. Orvieto R, Patrizio P. GnRH agonist versus GnRH antagonist in ovarian stimulation: An ongoing debate. *Reprod Biomed Online*. 2013;26: 4–8.
11. Bodri D, Sunkara SK, Coomarasamy A. Gonadotropin-releasing hormone agonists versus antagonists for controlled ovarian hyperstimulation in oocyte donors: A systematic review and meta-analysis. *Fertil Steril*. 2011;95(1):164–9.
12. Al-Inany HG, Youssef MA, Ayeleke RO et al. Gonadotrophin-releasing hormone antagonists for assisted reproductive technology. *Cochrane Db Syst Rev*. 2016;4:CD001750.
13. Humaidan P, Quartarolo J, Papanikolaou EG. Preventing ovarian hyper-stimulation syndrome: Guidance for the clinician. *Fertil Steril*. 2010;94(2):389–400.
14. Papanikolaou EG, Pados G, Grimbizis G et al. GnRH-agonist versus GnRH-antagonist IVF cycles: Is the reproductive outcome affected by the incidence of progesterone elevation on the day of HCG triggering? A randomized prospective study. *Hum Reprod*. 2012;27:1822–8.
15. Papanikolaou EG, Humaidan P, Polyzos NP et al. Identification of the high-risk patient for ovarian hyperstimulation syndrome. *Semin Reprod Med*. 2010;28(6):458–62.
16. Humaidan P, Papanikolaou EG, Tarlatzis BC. GnRH agonist to trigger final oocyte maturation: A time to reconsider. *Hum Reprod*. 2009;24(10):2389–94.

17. Meldrum D, Scott R, Levy MJ et al. A pilot study to assess oral contraceptive (OC) pre-treatment in women undergoing controlled ovarian hyperstimulation (COH) in ganirelix acetate cycles. *Fertil Steril*. 2002;78(Suppl. 1):S176.
18. Barma LI, Chantilis SJ, Hurst BS et al. A randomized prospective trial comparing gonadotropin-releasing hormone (GnRH) antagonist/recombinant follicle-stimulating hormone (rFSH) versus GnRH-agonist/rFSH in women pretreated with oral contraceptives before in vitro fertilization. *Fertil Steril*. 2005;83:321–30.
19. Huirne JA, van Loenen AC, Donnez J et al. Effect of an oral contraceptive pill on follicular development in IVF/ICSI patients receiving a GnRH antagonist: A randomized study. *Reprod Biomed Online*. 2006;13:235–45.
20. Griesinger G, Kolibianakis EM, Venetis C et al. Oral contraceptive pretreatment significantly reduces ongoing pregnancy likelihood in gonadotropin-releasing hormone antagonist cycles: An updated meta-analysis. *Fertil Steril*. 2010;94:2382–4.
21. Kim CH, Jeon GH, Cheon YP et al. Comparison of GnRH antagonist protocol with or without oral contraceptive pill pretreatment and GnRH agonist low-dose long protocol in low responders undergoing IVF/intracytoplasmic sperm injection. *Fertil Steril*. 2009;92:1758–60.
22. Tavmergen E, von Mauw E, Witjes H et al. Outcome of a trial to identify predictive factors for ovarian response in a GnRH antagonist protocol with or without oral contraceptive scheduling. Abstracts of the 25th Annual Meeting of ESHRE, Amsterdam, the Netherlands, 28 June–1 July, 2009. *Hum Reprod*. 2009;24 (i85 [O-211]).
23. Blockeel C, Engels S, De Vos M et al. Oestradiol valerate pretreatment in GnRH-antagonist cycles: A randomized controlled trial. *Reprod Biomed Online*. 2012;24(3):272–80.
24. Papanikolaou EG, Kyrou D, Kotanidis L et al. A single dose new long-acting GnRH-antagonist Degarelix effectively downregulates LH in stimulated IVF cycles triggered with agonist for oocyte maturation. A Proof of Concept Study. ESHRE July, 2016, Helsinki.
25. Ezzati M, Carr BR. Elagolix, a novel, orally bioavailable GnRH antagonist under investigation for the treatment of endometriosis-related pain. *Womens Hlth (Lond)*. 2015;11(1):19–28.

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Ultrasonography in IVF

Roger A. Pierson

Ultrasonographic imaging has become such an integral part of clinical care in the assisted reproductive technologies that it is difficult to imagine how IVF was done before we had the ability to visualize the ovaries and uterus easily. In the not-too-distant past, IVF was done using laparoscopic retrieval of oocytes following ovarian stimulation cycles monitored only by hormonal assay of systemic estradiol levels, embryos were transferred back into a uterus when we had no real idea about the physiologic status of the endometrium, and only a clinical touch was used to guide the placement of the embryo transfer catheter. Easily accessible and easy to use ultrasound imaging in the hands of the individuals performing the assisted reproductive technology (ART) procedures has delivered us from those uncertainties. At present, the quality and quantity of the information we receive from the images is an essential part of every procedure. Visualizing our work and its effects also has been a very important aspect of the incredible increases in ART success rates over the past decade. It is important to remember that the integrated understanding of anatomy, physiology, endocrinology, and pathology we have gained with imaging in the patients undergoing IVF are as important as the fantastic increase in knowledge and practice in the embryology laboratories. The confluence of, and synergy among, technologies used in ART care have greatly increased the probabilities of successful pregnancies.

The purpose of this chapter is to elucidate the primary uses of ultrasound imaging in IVF and to identify some areas where imaging has the potential to enhance our understanding in assisted reproduction. The essentials of ultrasonography in IVF are in monitoring the course of ovarian stimulation protocols, visually guided retrieval of oocytes, assessment of the endometrium, and visually guided embryo transfer. Each of these areas also provides a springboard for new research areas that may be incorporated into clinical care. Awareness of new frontiers is essential to progress in ART and in understanding the changes that will surely come. We rely so heavily on imaging in general gynecology, infertility workup, and early obstetrical care that it becomes challenging to narrow the focus to only IVF. However, with the general caveat that ultrasonography has forever changed our understanding of female reproduction, my goal is to provide a synopsis of imaging in IVF integrated into a framework within which the highest quality of care may be provided for patients who require ART to complete their families.

Ovarian Assessment

Monitoring the Course of Ovarian Stimulation

Ovarian stimulation protocols vary tremendously, and have evolved from fairly simplistic administration of exogenous hormones derived from urinary sources to sophisticated blends of GnRH analogs, recombinant follicle stimulating hormone (FSH) and luteinizing hormone (LH), and other compounds (1). The protocols are undergoing near constant refinement and are soon likely to be able to be tailored to the physiologies of individual patients. The common denominator in all ovarian stimulation protocols is that ultrasonography is used to monitor their effects on the ovaries of each patient. Although trans-abdominal ultrasonography has been used in the past, trans-vaginal ultrasonography (TVUS) is well-established as the optimal method for monitoring the course of follicular growth and development (2,3). TVUS allows a rapid, noninvasive, and highly visual approach to following the fates of individual follicles and cohorts of

follicles. When we combine our knowledge of natural ovarian physiology with concomitant assessment of circulating estradiol concentrations and oocyte development, the optimal timing for induction of the final stages of folliculogenesis and oogensis and oocyte retrieval may be determined (4–6).

All ovarian stimulation protocols have been designed to overwhelm the endogenous physiologic mechanism of selection for a single dominant follicle, obviate atresia in the cohort of other follicles recruited into the follicular wave, and foster and sustain the development of many follicles to an imminently preovulatory state, so that properly matured oocytes may be retrieved for IVF. Ultrasonography is essential in determining the numbers and fates of follicles stimulated and to tailor the ovarian response to the optimal number and quality of oocytes desired. In many cases, information such as the difference in ultrasonographically determined diameter between the largest follicle and first and other subordinate follicles may be used to assess the progress and quality of the ovarian stimulation. The follicular response of each woman to the stimulation protocol, the number of oocytes desired, and clinical assessment of the risk of ovarian hyperstimulation will dictate modifications to the stimulation protocol. It is important to note that the expected linear relationship between circulating estradiol concentrations and follicular diameter may not exist during ovarian stimulation (7). Similarly, we understand that all follicles do not contribute equally to the concentrations in the systemic circulation.

Human chorionic gonadotropin is usually administered to trigger the final phases of follicular maturation when the largest follicle first attains a predetermined diameter (e.g., 18–20 mm). The timing of administration of human chorionic gonadotropin (hCG) or recombinant LH administration is critical in initiating the final stages of oocyte maturation and establishing the time for oocyte retrieval which will yield the highest quality oocytes in the proper stage of development with the highest probability of fertilization. The relationships between follicle size and oocyte maturity remain not particularly well elucidated; however, oocyte maturity certainly plays a role in the ability of the resulting embryos to develop to the blastocyst stage (8–10). The timing of hCG administration varies among programs based upon individual clinician's feel for the stimulation cycle and laboratory logistics. Most commonly, 5000 or 10,000 IU hCG is administered. Oocyte retrieval is then typically scheduled for 30–34 hours thereafter. Many programs use only ultrasonographic monitoring to determine the course of ovarian stimulation, and it has been demonstrated that including estradiol monitoring during the stimulation protocol seldom changed the timing of hCG administration and did not affect pregnancy rates or the risks of ovarian hyperstimulation syndrome (11).

The morphological characteristics, individual physiologies, and most appropriate diameters of follicles which produce optimal oocytes prepared for fertilization remains the subject of intensive research (Figure 7.1). We know that mature oocytes yield the highest fertilization rates in standard IVF cycles; however, the widespread incorporation of intracytoplasmic sperm injection (ICSI) and the progress in understanding the potential of in vitro maturation (IVM) and/or fertilization of immature oocytes may require a reassessment of what constitutes an optimal oocyte. The role of ultrasonography in IVM–IVF protocols will very definitely revolve around the optimal timing of oocyte retrieval for optimal fertilization and cleavage rates (12–22).

Ovulation in natural cycles has been reported from follicles as small as 14 mm, and oocytes collected in IVF cycles from small follicles may indeed fertilize well; oocytes have been aspirated from follicles less than 10 mm in diameter and matured in vitro to increase the number of transferable embryos (23). Through research in animal models, we know that there appears to be a correlation between computer-assisted ultrasound image attributes of follicles and the ability of the oocyte to fertilize; however, similar studies in humans have apparently not yet been completed (24). The natural synergy among these observations is the start of an emerging area of research.

Examination of growth rates for individual follicles may be a useful measure with which to predict the number of follicles that may develop during ovarian stimulation protocols. This information is equally important when assessing the risks of ovarian hyperstimulation. In the past, follicular growth rates during induced cycles were observed to be faster than those of natural cycles (25). However, a mathematical equation developed to equate follicular growth rate to follicular age was used to conclude that the growth rates of individual follicles in spontaneous cycles were similar to those recruited by exogenous gonadotropins (26). Reduced growth rates of follicles were observed in cycles where a pregnancy was established, and it was concluded that growth rate was a more useful characteristic for prediction of ovulation

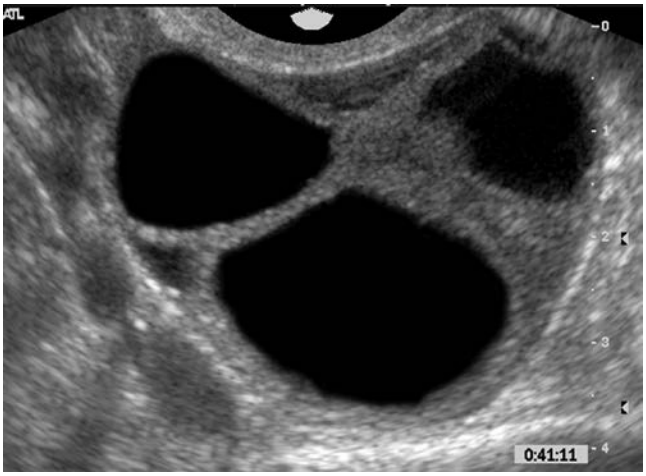

FIGURE 7.1 Image of an ovary at the end of an ovarian stimulation protocol approximately 24 hours prior to oocyte retrieval. Three dominant follicles are visible in the plane of section. The thick walls of the follicles are consistent with collection of mature oocytes with a high probability of fertilization.

than follicular diameter (27). Follow-up work does not appear to have been done. It will be logistically challenging to combine daily detailed ultrasound measurements of individually mapped follicles with per-follicle outcomes from the embryo laboratory and final pregnancy outcomes. However, the rationale that follicular growth rates may be more accurate in predicting the actual maturity of the ova is intriguing. Recent detailed studies on follicular growth have shown that follicles grow at approximately 1.5 mm per day, regardless of whether they developed during natural menstrual cycles, oral contraceptive cycles, or during ovarian stimulation (28). These data fit well with a mathematical model developed to predict the ovarian response to superstimulation protocols and based upon daily data on follicular growth rates (7). However, it is evident that new imaging-based studies tracking the development trajectories of individual follicles are required.

Color flow and power flow Doppler imaging also have been used to assess the state of relative maturity of dominant follicles (Figure 7.2) (29–34). Intrafollicular influences on oocyte development such as the developmental competence of the oocyte, oocyte metabolism, and mitochondrial function may be mediated, at least in part, by peri-follicular vascularity. It has been argued that blood flow to the follicle is

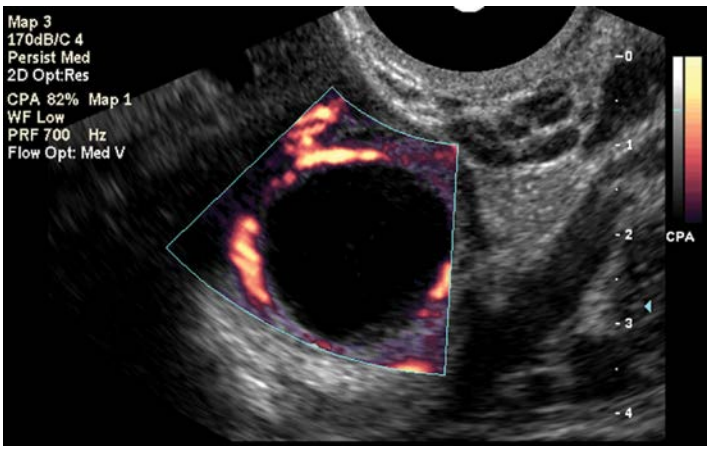

FIGURE 7.2 Power-flow Doppler image of a dominant pre-ovulatory follicle showing peri-follicular vascularization consistent with follicular maturity.

developmentally important for the generation of a normal follicle and oocyte (33,34). Preliminary studies using various indices of follicle vascularity support the notion that women who received embryos originating from oocytes nurtured in well-vascularized follicles had a higher pregnancy rate than women who received embryos derived from oocytes from more poorly vascularized follicles (29,30,35).

Assessment of Ovarian Follicular Reserve

Changes in demographic trends in the age at first pregnancy in our society have continued to yield more and more women seeking pregnancy when they are older and consequently less fertile. It is well documented that fertility declines progressively as age advances (36–39). In IVF, the main focus of attention is on assessment of what is termed the ovarian reserve. Ultrasonography is a significant part of the tests now being used to investigate follicular dynamics in aging women, as are detailed endocrine-based tests (40–43). A decrease in the ovarian reserve, or number of follicles capable of being stimulated, is a primary reason for declining fertility. Similarly, the ovarian response to exogenous gonadotropin stimulation also decreases, but the range of individual variation is extremely wide and it is well known that age is only a rough guesstimate of the ovarian reserve and hence the ovarian stimulation response (44–48).

There are several tests of "ovarian reserve" (reviewed in references (47,48)). In addition, there is evidence to suggest that ultrasonography may be used to estimate the number of antral follicles at specific times of the menstrual cycle and provide additional useful information of clinical relevance (49–58). Ultrasound assessments take place using antral follicle counts or measurement of ovarian volume. Early follicular phase antral follicle counts, typically done on Day 3 to Day 7 post-menstruation, may be used to predict the number of follicles likely to develop during ovarian stimulation with exogenous gonadotropins (51,58–61). Women having fewer than five follicles under 10 mm in diameter before ovarian stimulation begins have a relatively poor prognosis for success (58). Studies to determine the extent to which antral follicle counts correlate with endocrinologic measures of ovarian reserve (e.g., cycle day 3 follicle-stimulating hormone [FSH] and estradiol concentrations) remains to be widely confirmed (50). Ovarian volume assessments are based on the presumption that there is a significant correlation between the population of primordial follicles remaining in the ovary and the volume of the ovary, measured using either 2- or 3-dimensional ultrasonography (49,59,62,63). A clear relationship has been demonstrated between decreased ovarian volume and antral follicle counts and advancing age, combined with increased FSH (50,55,64). There remains a good deal of work yet to do in order to standardize the imaging based assessments; however, ultrasonography remains an important aspect of ovarian reserve estimation and relating this knowledge to the probability of a successful ovarian stimulation cycle (53,58,65).

Ovarian Hyperstimulation Syndrome

Ovarian hyperstimulation syndrome (OHSS) is an exaggerated ovarian response to ovarian stimulation that can develop into a life-threatening complication. In women with the disorder, ultrasonography demonstrates grossly enlarged ovaries containing numerous large follicular cysts with thin, highly echogenic borders, and dramatically increased local blood flow (66,67). The ovaries may enlarge to diameters in excess of 10 cm and echotexture interpreted as intrafollicular hemorrhage in some of the large cysts frequently may be observed. In severe cases, the ovaries may become too large to visualize in their entirety with TVUS, and trans-abdominal imaging must be used. Serial TVUS during ovarian stimulation cycles and careful tailoring of the dose of exogenous gonadotropins has helped to limit the risk of OHSS; however, prevention of OHSS is multi-factorial and requires ovarian stimulation protocols tailored to patient specifics and close monitoring of the ovarian response (66,68–71). Clinicians take an active role in the prevention of OHSS by aborting the treatment cycle, coasting strategies during stimulation, the use of low doses of ovulation triggering agents, and/or cryopreserving all embryos and single embryo replacement in subsequent cycles (69–72). When OHSS does occur, torsion of an enlarged ovary is a complication that must be kept in mind. When torsion is suspected, color flow Doppler imaging can help to establish an early and accurate diagnosis (73,74).

Computer Assisted Ultrasonographic Imaging of Follicular Development

The application of computer-assisted image analysis is demonstrating that ultrasound images have the potential to aid in the identification of healthy versus atretic follicles in natural and ovarian stimulation cycles (75–77). Physiologically dominant ovarian follicles are identifiable by ultrasonography at approximately Day 7 post menstruation in unstimulated cycles (77), and ovulatory and non-ovulatory follicles were identifiable in ovulation induction cycles (75). The image attributes of ultrasonographic images of normal pre-ovulatory follicles include thick, low-amplitude walls and a gradual transformation zone at the fluid–follicle interface. The walls of pre-ovulatory follicles are characterized by increased heterogeneity, increased wall breadth, and a more gradual transformation at the fluid–follicle wall interface. Atresia is characterized by thin walls, high numerical pixel value (bright) signals, and highly variable signals from the follicular fluid (78). Evaluation of the acoustic characteristics indicative of viability and atresia combined with the elucidation of the stage of oocyte development for optimal fertilization is an active area of research that has profound implications for development of safer and more effective ovarian stimulation protocols.

Ultrasound-Guided Oocyte Retrieval

The most visible use of imaging in IVF has been the tremendous advance facilitated by trans-vaginal retrieval of oocytes (79–91). Oocyte retrieval was a technology limiting step in the early days of IVF. Retrievals were done laparoscopically or using ultrasound guidance from trans-urethral, trans-vesicular, or trans-abdominal approaches (82,85,89,92–94). Trans-vaginal imaging and concerted efforts to develop effective, accurate tracking of the needles used for follicle aspiration was probably the single most important step in making IVF as safe and effective as it is today (79,87–89,95–99). Clinical complications appear to be rare and conservative treatment is typically followed in most cases (100).

Retrieval of oocytes in IVF cycles is now routinely performed under TVUS guidance (83). An aspirating needle is introduced through a guide attached to a trans-vaginal probe and is inserted into first one ovary, then the other, via the vaginal fornices. Almost all aspiration needles now in common use have a small band of highly reflective surface near the tip of the needle to facilitate visualization as the needle enters the ovary and once it is in the follicles (Figure 7.3). The path of the oocyte retrieval needle may be accurately visualized within biopsy guidelines imposed on the ultrasound screen as it is guided into each ovarian follicle. The highly reflective echoic tip of the needles makes identifying their path quite easy in most cases. The needle tip can be observed directly as it is maneuvered within the ovaries and into each follicle. The follicular fluid containing the oocyte–cumulus complex is then aspirated by application of gentle suction. The walls of the follicle collapse as the fluid is aspirated and the needle moved within the follicle to ensure that all of the follicular fluid is withdrawn (Figure 7.3).

There are two main types of aspiration needles used for oocyte retrieval: single and double lumen needles. Single lumen needles typically have a smaller diameter and tend to cause less discomfort (83). In many, if not most, IVF centers follicle aspirations are done using single lumen needles and no follicle flushing. The double lumen needles were developed for a technique involving constant infusion of oocyte collection media into the follicle at the same time as the follicular fluid is being removed. Flushing of the follicle can be well visualized with ultrasonography. The double lumen flushing technique is thought to increase the turbulence within the follicle, assist in dislodging the oocyte–cumulus complex from the follicle wall and increase the chances of oocyte collection. A single lumen needle flushing technique may also be used. In this technique, all of the follicular fluid is first aspirated from the follicle and the follicle is then refilled with collection medium and re-aspirated. A back and forth motion on the plunger of the infusion syringe may be used to increase the turbulence of flow. In a recent systematic review, no significant differences were observed in live birth rate, clinical pregnancy rates, and the number of oocytes retrieved between flushing and non-flushing techniques for oocyte retrieval. Follicular flushing also increased the procedural time required. The authors concluded that there is no advantage to use of routine follicular flushing during oocyte retrieval (101).

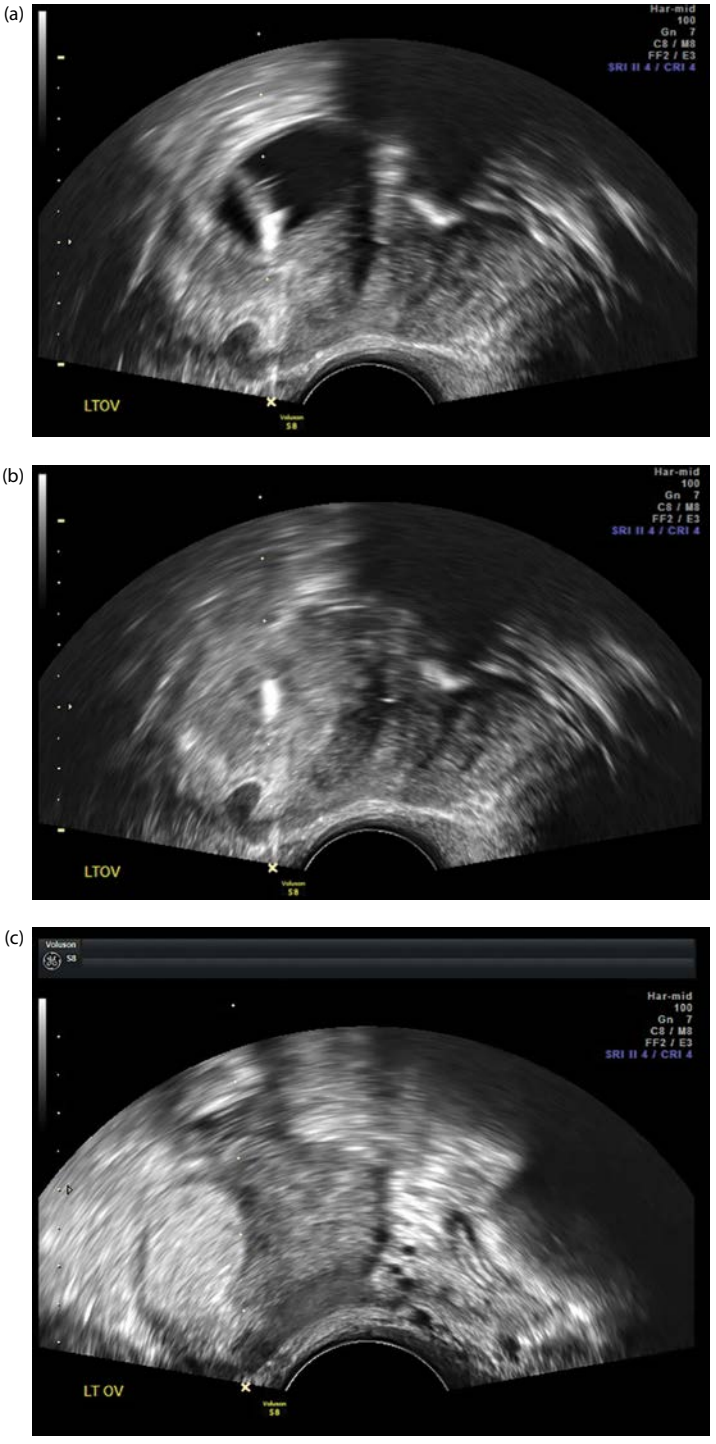

FIGURE 7.3 Ultrasound images taken during oocyte retrieval (a–c). (a) The highly echogenic band around the distal end of the needle and the tip of the follicle aspiration needle are clearly visualized in the superior most follicle. The needle is maneuvered within the ovary to aspirate all follicles. (b) Following complete evacuation of the follicular fluid, the echoic tip of the needle is visualized within the apposed walls of the collapsed follicle. (c) The hyperechoic area of the former follicle is visualized following follicle aspiration and removal of needle. (Images courtesy of Dr. Alex Hartman.)

Currently, new types of follicle aspiration needle sets are under development and smaller diameter needles are under evaluation (102–104). It has been determined that needle diameter does not affect oocyte yield; however, the time required for oocyte retrieval procedures was prolonged when smaller diameter needles were used. Similarly, equivalent oocyte retrieval numbers have been obtained using needles designed for aspiration of small follicles in an IVM program (103).

Unsuccessful oocyte retrieval following apparently normal ovarian stimulation reportedly occurs in 1%–7% of cycles—the so-called "empty follicle syndrome." The etiology appears to be multifactorial and may involve both technical and biological mechanisms (83,105). There is significant controversy over whether or not the syndrome actually exists and the nature of its prevalence and putative causative factors (106–111).

The complication rates of oocyte retrieval are reportedly extremely low, and almost all procedures are performed under conscious sedation on an outpatient basis (86,90,91,96,97,112–119).

Assessment of the Endometrium at Embryo Transfer

Endometrial Thickness and Pattern for Assessing Endometrial Receptivity

Ultrasonography has been used, with varying degrees of success, to correlate the probability of pregnancy in ovarian stimulation–ovulation induction cycles and IVF cycles (reviewed in references (120–122)). Most imaging studies attempt to predict the probability of implantation based on simple biophysical measurements of the endometrium. A thicker endometrium was observed on the day of oocyte retrieval in women who conceived during that cycle. In another study, no correlation was observed among endometrial pattern or thickness and estradiol levels, number of oocytes retrieved, or progesterone level on the day of embryo transfer; however, the authors appeared to appreciate the pattern of the endometrium on the day of hCG administration, but stated that pattern assessment was of no value (123). The endometrium on the day before embryo transfer was nearly 2 mm thicker in women who conceived (10.2 mm) than in those who did not (8.6 mm) (124). Only two pregnancies were reported when the endometrial thickness was less than 7.5 mm, and no pregnancies were observed when the endometrial thickness on the day of embryo transfer was less than 5 mm. However, no differences were observed in endometrial thickness among women who conceived compared to those that did not in a similar study (125). A more

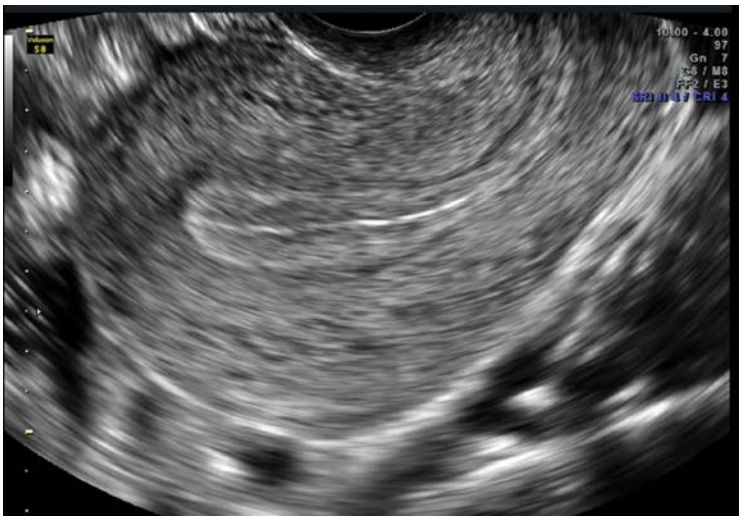

FIGURE 7.4 Midsagittal view of the uterus. The cervix is to the right of the image and the fundus is to the left. The endometrium is well demarcated, the junctional zone is visualized at the endometrial/myometrial interface, and there is differentiation between the stratum basalis and stratum functionalis of the endometrium. A pronounced, thick "triple-line" pattern associated with a higher probability of implantation following embryo transfer is demonstrated.

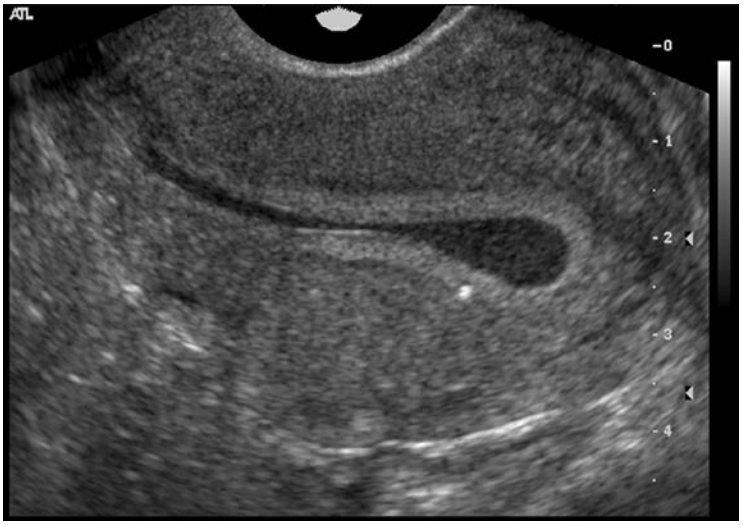

FIGURE 7.5 Midsagittal view of a uterus with a pronounced intra-luminal fluid collection. The cervix is visualized to the left of the image and the fundus to the right. Fluid collections on the day of embryo transfer are associated with a very low probability of pregnancy.

favorable outcome has been suggested when embryos were transferred when the endometrial thickness was greater than 9 mm and a "triple line" pattern was observed (126) (Figure 7.4) This observation was supported by a retrospective analysis in which the pregnancy rate was significantly higher in women who exhibited a triple-line pattern than in those with other endometrial patterns (127). These contradictory reports, and the apparent lack of correlation between ultrasonographic endpoints and histologic staging of the endometrium in women undergoing IVF can be interpreted to mean that ultrasonography using simple biophysical measures is not sensitive enough to be useful in predicting endometrial receptivity and the probability of implantation, with the exception of a strong negative correlation when the endometrium is thin (122). It is also possible that inconsistencies in the day on which measurements were done among the many studies, differences in measurement techniques, and the use of a wide variety of ovarian stimulation protocols which affect endometrial development differently have played a role in our seeming inability to interpret the data. Consensus is that implantation is more likely to occur as long as the endometrial thickness is greater than 6 mm.

Collections of fluid are sometimes found within the uterine lumen on the day of embryo transfer (128) (Figure 7.5). In a retrospective analysis of case records, approximately 5% of cycles were compromised by the presence of lumen fluid accumulation at some time during the IVF cycle procedures, and in 2% of the cases the fluid accumulations persisted until the day of embryo transfer. The pregnancy rate among women with fluid accumulations was markedly lower than those who did not exhibit intralumenal fluid. Interestingly, fluid accumulations were found in almost three times as many women with tubal factor infertility compared with other causes. Although luminal fluid collection does not appear to be a common problem in IVF cycles, it does appear to have a negative impact on implantation and pregnancy rates.

Spectral Doppler and Color Flow Doppler Ultrasonography

The history of Doppler ultrasonography of the uterine arteries in the literature is confusing because many reports failed to differentiate between spectral Doppler and color flow Doppler imaging. Early studies tend to be based upon spectral Doppler examinations, which are a means of evaluating the resistance to blood flow using calculations of the pulsatility index (PI), resistance index (RI), V_{max}, or the systolic to diastolic ratio (S/D ratio). Color flow Doppler and power flow Doppler imaging are means of turning motion, either toward or away from the transducer in the case of color flow Doppler, or motion

in any direction in the case of power flow Doppler, into a visually detectable color overlay on the two-dimensional ultrasound image (120,129).

Initially, attempts to determine if evaluation of blood flow in the uterine arteries could be useful were based on RI to look for differences in uterine receptivity. No differences were found between women who conceived and those who did not in a small series (130). When the PI of uterine arteries were examined and data were grouped into low, medium, and high categories, no differences were found between cycles where women either conceived or did not; however, no pregnancies were established in the women with high PI values (131). Elevated PI, as a measure of impedance to vascular flow in the uterine artery, was associated with a significantly lower pregnancy rate (132). Assessments of uterine artery RI have remained inconclusive, except that absent or low diastolic flow was associated with failure to conceive (133). Uterine artery vascular impedance measured by RI has not been found to be useful for predicting the probability of pregnancy. A subsequent study reported the PI and RI in the uterine arteries to be lower in conception cycles, and the authors suggested that a PI greater than 3.3 and an RI greater than 0.95 before embryo transfer were associated with a low probability of conception (134). A study of women undergoing cycles, in which embryos were produced using ICSI, reported no demonstrable differences in PI on the day of embryo transfer between conception and nonconception cycles (135). Some ultrasonographically detectable criteria were observed to be associated with negative pregnancy outcomes; however, no prognostic value has been observed in any measurement of vascular perfusion (reviewed in references (120,121,136).

Studies that evaluated endometrial perfusion on the day of hCG administration consistently reported that values for PI and V_{max} were not different, irrespective of whether or not conception was established (134,136–139). However, when only the color flow data were examined, absence of detectable subendometrial vascular flow, indicative of poor vascular penetration, was associated with failure of implantation (137). Power flow Doppler ultrasonography was subsequently used to examine women whose endometrial thickness was ≥10 mm. Intra-endometrial flow calculations of the maximal area that showed evidence of motion indicative of vascular flow of <5 mm^2 were associated with a lower pregnancy rate, and endometrial and subendometrial perfusion are reportedly impaired in women with unexplained infertility (138,140). In one report, spiral arterial flow and uterine artery flows were not different between pregnant and nonpregnant women; however, if spiral arterial flow could not be detected, no conceptions were observed (139). In an ancillary report, the presence of subendometrial flow was associated with pregnancy in an ovarian stimulation/IUI study (141). Subsequently, a high degree of endometrial perfusion visualized using three-dimensional (3D) ultrasonography was thought to indicate a more favorable endometrium (120,142).

Imaging-Based Uterine Scoring System

Imaging-based scoring systems to predict uterine sensitivity based on a uterine biophysical profile system have been proposed (120,143,144). The scoring systems were designed by assigning "points" for various criteria and then adding the cumulative columns. Comparisons of uterine scores in conception with non-conception cycles demonstrated no differences in any criteria measured, including endometrial thickness, endometrial pattern, PI, RI, color Doppler, or other vascular indices. However, in more recent work, a new endometrial scoring system based on two-dimensional (2D) imaging parameters and creation of a 3D mathematical surface for visual analysis has shown promising results in the ability to predict the probability of implantation from images taken on the day of oocyte retrieval (145) (Figure 7.6).

Three-Dimensional Imaging of the Endometrium

3D ultrasonography first became available in the late 1990s and 3D is now a part of almost all high-end imaging systems. There are several methods used to provide 3D information, and there are no studies comparing the same endpoints with different imaging systems (146). Descriptive study of endometrial development during the menstrual cycle has been done and provides an excellent reference for the normal changes in 3D volumes. An example of 3D imaging of the uterus is shown in Figure 7.7. The prospects for predicting the probability of implantation in IVF programs have now extended into 3D exploration of endometrial receptivity (120,147–150).

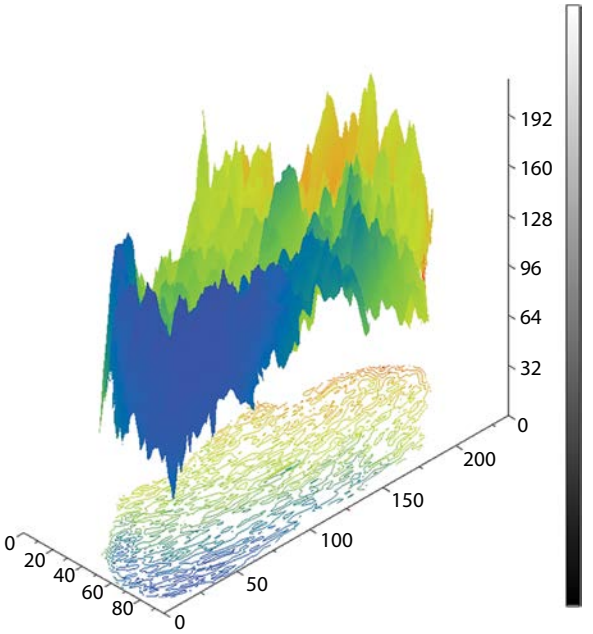

FIGURE 7.6 Three-dimensional mathematical surface model of the endometrium on the day of oocyte retrieval. The image may be manipulated in three-dimensional space within the computer and various aspects of the echoes representing the endometrium evaluated. A 3D colorometric visualization algorithm has been applied to enhance visualization when viewed with 3D glasses. (Image copyright Synergyne Imaging Technology Inc, 2015.)

The role of 3D volumes as predictors of endometrial receptivity and implantation has been contradictory. When endometrial volumes were compared among women who conceived and those who did not, pregnancy and implantation rates were significantly lower in women with volumes of less than 2 mL, and no pregnancies were established when endometrial volumes were less than 1 mL, a contemporary study found no relationship between 3D volume of the endometrium and conception (150,151). Nor was a correlation found among estradiol levels, endometrial thickness, or endometrial volume, leading the authors to conclude that there was no predictive value for conception in assessing endometrial volume. Endometrial thickness and endometrial volumes were not correlated with probability of pregnancy; however, 3D power flow Doppler indices used to measure endometrial perfusion may have some predictive value, although definitive studies do not appear to have been completed (139,142,152,153). Spiral artery blood flow measurements in 3D had positive predictive value when performed on the first day of ovarian stimulation, while women who became pregnant had lower RI and a higher 3D flow index than those that did not (139,142). Taken together, these observations provide rationale for further investigation of 3D ultrasonography in all of its iterations; however, it is clear a predictive index is beyond the limits of our current technology.

Motion Analysis

Motion analysis, or direct measurement of subendometrial contractions, is a method of evaluating the endometrium based on the observation that the uterus and endometrium are in constant motion (59,154–160). Objective assessment of these contractions has now been applied to assisted reproduction cycles (161–163). A computer is interfaced with an ultrasound instrument, digital frames are acquired, and the pixels comprising the endometrial image along a single line are isolated. A line is serially acquired from images taken 1 or 2 times per second over a 5–10 minute period, the pixel data from the line are concatenated, and the result is displayed as a graph of the velocity and amplitude of endometrial contractions (Figure 7.8).

Endometrial contractions may have a predictive effect on the probability of pregnancy in IVF cycles (158,164). Women with a higher frequency of uterine contractions were found to have lower pregnancy

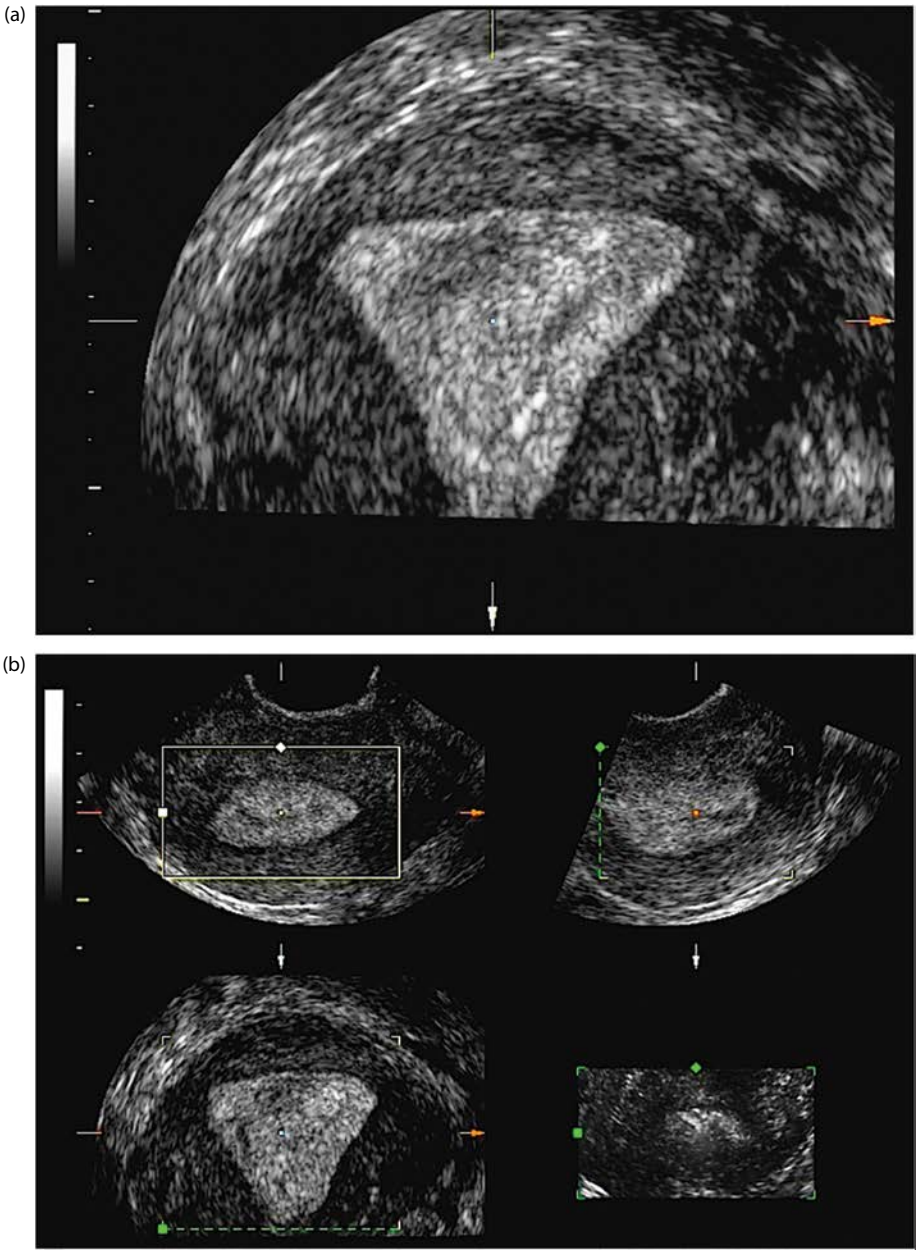

FIGURE 7.7 Three-dimensional ultrasonographic images of a uterus at approximately Day 5 post ovulation (a,b). The endometrium has homogenous echotexture and represents the secretory phase of the cycle. (a) Three dimensional coronal image. (b) Multi-planar views with the sagittal and transverse planes at the top. The coronal view seen in (a) is present at the lower left. The image at the lower right view is an automated rendering and is not clinically useful. (Images courtesy of Dr. Alex Hartman.)

rates (162). However, contradictory evidence has been reported (165). Exogenous progesterone has demonstrably reduced uterine contractility on the day of embryo transfer, and it has been hypothesized that progesterone supplementation before embryo transfer may improve endometrial receptivity by lowering the possibility that embryos might be expelled from the uterus by contractions (166–168). Uterine contractility at the time of blastocyst transfer was lower and reached a nadir seven days after hCG

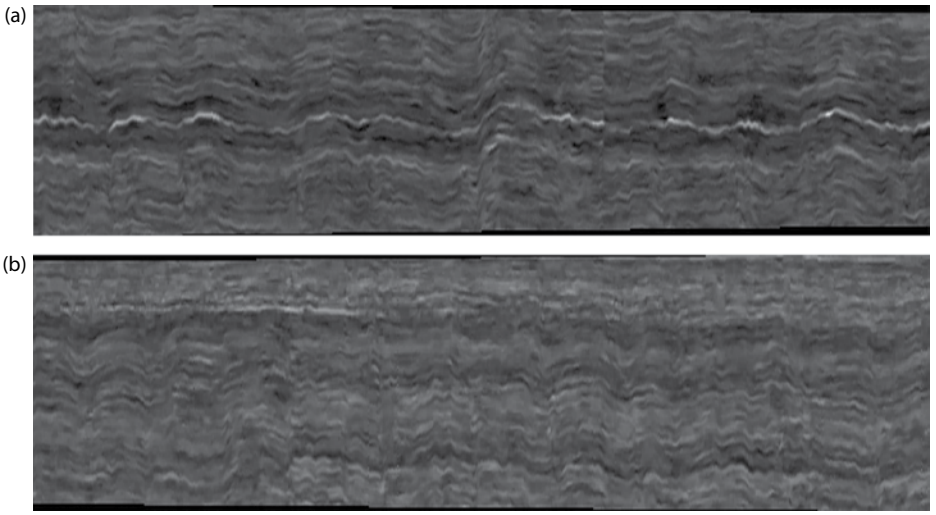

FIGURE 7.8 Ultrasound-based motion analysis images (a,b) of approximately 3 minutes duration showing individual women with normal (a: ~4 contractions per minute) and fast (b: ~7.5 contractions per minute) on the day of embryo transfer. The lumen is identified as the hyperechoic signal in the central aspect of the image and the endometrium is located above and below the lumenal echo. The endometrial/myometrial interface and sections of myometrial echoes are located above and below for reference. Contractile activity is visualized as the wave pattern as time proceeds from left to right aspects of the image. The amplitude and frequency of the contractions are easily visualized.

administration in IVF cycles. The low amplitude and frequency of contractions is hypothesized to facilitate blastocyst implantation (169). The effects of progesterone on uterine contractions have been demonstrated by the observation that higher progesterone concentrations correlated with lower amplitude and frequency uterine contractions (59,168). It was suggested that progesterone could be administered to reduce endometrial contractions and have a positive impact on pregnancy rates, although this hypothesis does not appear to have been critically tested (166). In addition, the effects of a selective oxytocin antagonist have been investigated for control of the frequency and power of endometrial contractions (170). The results of pharmaceutical intervention remain under investigation at this time.

Ultrasound-Guided Embryo Transfer

Ultrasonographic imaging is frequently used to guide the placement of the embryo transfer catheter in an effort to facilitate optimal embryo placement and enhance the probability of a successful pregnancy (171–180). Trans-abdominal ultrasound guidance is a more common means of directing the embryo transfer catheter, however, trans-vaginal scanning may also be used (177,181,182). It is also important to note that ultrasound guidance of embryo replacement does not prevent the establishment of an ectopic gestation (183). The recent advent of easy-to-use and relatively inexpensive 3D ultrasonography has facilitated a new wave of inquiry into the utility of 3D imaging to guide the embryo transfer catheter (179,184). Early impressions are that 3D imaging may be beneficial in identifying the site of optimal embryo placement with respect to anatomic variations in individual women (185).

Clinical and laboratory preparations for embryo transfer are the same, regardless of whether the transfer is to be ultrasound guided or not. Patients are placed in the lithotomy position and the cervix exposed using a bivalve speculum. Mucus and secretions are removed using culture media and the tip of the transfer catheter is introduced into the os cervix. The addition of trans-abdominal ultrasound imaging simply involves placement of the transducer, typically using a 3–4 MHz large aperture probe, on the lower abdomen and pelvis in the sagittal plane and imaging the full sagittal plane of the uterus and cervix through a full bladder window (176,186,187) (Figure 7.9). Most standard embryo transfer catheters

are easily visualized as a pair of highly echogenic lines within the cervix; however, transfer catheter systems are undergoing constant refinement, and new systems have been developed to increase the ease of imaging (188,189).

Once the catheter has been identified, the tip may be carefully guided through the uterine lumen using real-time imaging. Once the clinician attains the optimal place with the uterus, the embryos are gently expelled into the lumen (190). Opinion on exactly what optimal placement means is varied (62,184,185,191–196). The fluid droplet containing the embryos is visualized as a very small hypoechoic blip deposited at the tip of the transfer catheter. Specular reflection artifacts may sometimes be helpful in its identification (Figure 7.9).

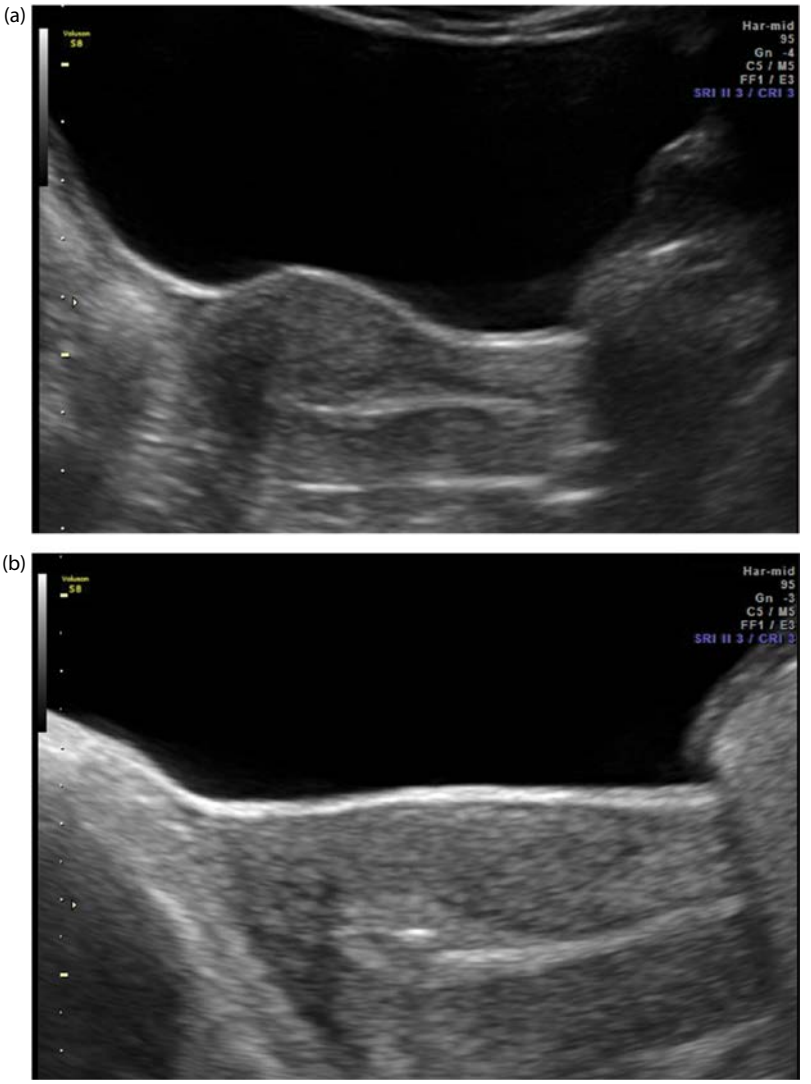

FIGURE 7.9 Midsagittal view of the uterus imaged with trans-abdominal ultrasonography during embryo transfer (a,b). (a) The bladder is fluid-filled, hypoechoic and fills the upper aspect of the image. The uterus is seen in midsagittal plane and the transfer catheter is visualized as the highly echogenic line in the middle of the uterus. (b) Enlargement showing the microdroplet of media containing the embryo visualized approximately 6 mm from the endometrial/myometrial interface at the fundal aspect of the uterus (hyperechoic focus). Specular echoes enhance the ability to visualize the microdroplet. The tip of the embryo transfer catheter is visualized to the right of the microdroplet as it is slowly removed from the lumen. The images document optimal replacement of the embryos. (Image courtesy of Dr. Alex Hartman.)

Trans-vaginal ultrasound guidance is done in a similar fashion, except that a probe designed for intracavitary use is introduced through the speculum and placed into contact with the anterior vaginal fornix (177,181). The transfer catheter is visualized and the tip guided to the optimal uterine location for embryo deposition. No differences in pregnancy rates were observed when trans-abdominal versus trans-vaginal ET catheter guidance was evaluated (182). The authors suggested that uterine position, parity, and the level of clinical comfort with the procedure should dictate the choice of imaging approach.

There is a measure of controversy regarding the usefulness of ultrasonographic guidance during embryo transfer versus non-visually guided clinical touch (174,179,180,186,189,191,193,195,197–202,424). Some clinicians prefer to rely on ultrasound guidance for mock transfers in cycles before IVF and embryo transfer and clinical touch in the actual procedure using physical measurements taken during the mock cycle. Other clinicians prefer to use ultrasound guidance for all procedures, and still others make a decision regarding its use based on whether or not the transfer is likely to be classified as easy or difficult (180,187,202). Two recent meta-analyses and a subsequent randomized controlled trial have been interpreted to mean that trans-abdominal ultrasound guidance versus clinical touch for embryo transfer significantly increased the pregnancy rate, although the rates of miscarriage, ectopic pregnancy, and multiple pregnancy were not affected (194–196,203). 3D and 4D ultrasonography have been posited as an optimal means of identifying a "maximal implantation point (MIP)" (185). It was reported that identification of the MIP was easily and reliably done using ultrasonographically-identifiable anatomic markers that could be individualized for each patient, and the ET catheter guided to it.

Concluding Remarks

Ultrasonography has provided us with direct visual access to all of the events involved in natural human reproduction, and has allowed us to elucidate our natural reproductive processes. When we contemplate the incredible progress made in our ability to understand assisted reproduction, it seems strange to envision ART care without ultrasound imaging. We take for granted the ability to see the effects of ovarian stimulation and optimize the protocol for individual patients, easy accessibility of the ovaries for oocyte retrieval, direct visualization of the endometrium at the time of embryo transfer, and embryo replacement under direct visual guidance.

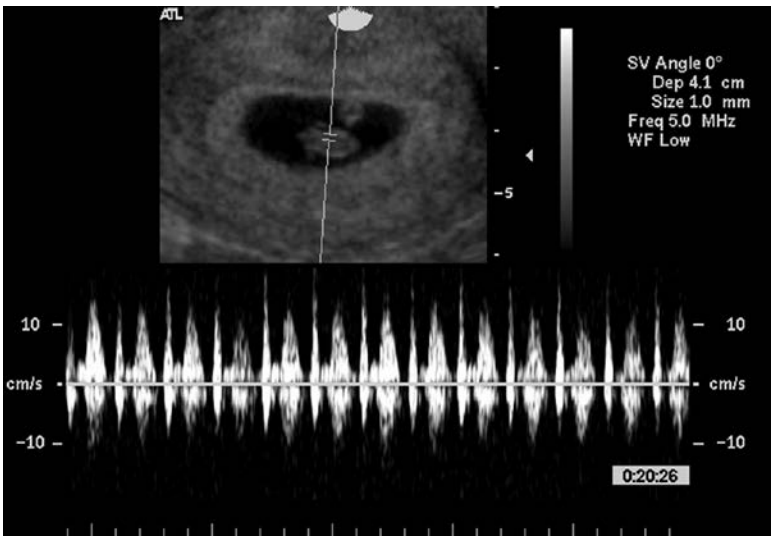

FIGURE 7.10 Gray-scale and spectral Doppler image of an embryo 5 weeks post transfer. The gestational sac, embryo and yolk sac are visualized in the top half of the image. The Doppler gate is placed directly over the embryonic heart and the resulting spectral Doppler trace is seen in the lower half of the image reflecting cardiac activity.

One of the most joyous occasions we have in our work is when we are able to confirm pregnancy in our patients. There is little that approaches the drama in the imaging suite when we can point out an embryo to our patients and listen to the heartbeat of their new life (Figure 7.10). The combined contributions to ART made by the dramatic advances made in the embryo laboratory, enhanced clinical knowledge, and advanced imaging techniques enrich not only our discipline and scientific knowledge, but also the lives of many couples who have struggled with infertility.

REFERENCES

1. Cohen J. A short review of ovarian stimulation in assisted reproductive techniques. *Reprod Biomed Online*. 2003;6(3):361–6.
2. Tarlatzis BC, Laufer N, Decherney AH. The use of ovarian ultrasonography in monitoring ovulation induction. *J In Vitro Fert Embryo Transf*. 1984;1(4):226–32.
3. Bonilla-Musoles F, Pardo G, Perez-Gil M, Serra V, Pellicer A. Abdominal ultrasonography versus transvaginal scanning: Accuracy in follicular development evaluation and prediction for oocyte retrieval in stimulated cycles. *J Clin Ultrasound*. 1989;17(7):469–73.
4. Wittmaack FM, Kreger DO, Blasco L, Tureck RW, Mastroianni L, Jr., Lessey BA. Effect of follicular size on oocyte retrieval, fertilization, cleavage, and embryo quality in in vitro fertilization cycles: A 6-year data collection. *Fertil Steril*. 1994;62(6):1205–10.
5. Nagai S, Yasumizu T, Kasai T, Hirata S, Mizuno K, Kato J. Effect of oocyte retrieval from a small leading follicle in fixed-schedule in vitro fertilization program. *J Obstet Gynaecol Res*. 1997;23(2):165–9.
6. Requena A, Neuspiller F, Cobo AC, Aragones M, Garcia-Velasco JA, Remohi J et al. Endocrinological and ultrasonographic variations after immature oocyte retrieval in a natural cycle. *Hum Reprod*. 2001;16(9):1833–7.
7. Sarty GE, Pierson RA. An application of Lacker's mathematical model for the prediction of ovarian response to superstimulation. *Math Biosci*. 2005;198(1):80–96.
8. Huang FJ, Huang HW, Lan KC, Kung FT, Lin YC, Chang HW et al. The maturity of human cumulus-free oocytes is positively related to blastocyst development and viability. *J Assist Reprod Genet*. 2002;19(12):555–60.
9. Kitai H, Inagaki N, Kimura H, Kuji N, Sueoka K, Suzuki S. A study on the correlation between maturation and quality of human oocytes. *Hum Cell*. 1997;10(4):263–70.
10. Salha O, Nugent D, Dada T, Kaufmann S, Levett S, Jenner L et al. The relationship between follicular fluid aspirate volume and oocyte maturity in in-vitro fertilization cycles. *Hum Reprod*. 1998;13(7):1901–6.
11. Lass A. Monitoring of in vitro fertilization-embryo transfer cycles by ultrasound versus by ultrasound and hormonal levels: A prospective, multicenter, randomized study. *Fertil Steril*. 2003;80(1):80–5.
12. Lin YC, Chang SY, Lan KC, Huang HW, Chang CY, Tsai MY et al. Human oocyte maturity in vivo determines the outcome of blastocyst development in vitro. *J Assist Reprod Genet*. 2003;20(12):506–12.
13. Russell JB. Immature oocyte retrieval combined with in-vitro oocyte maturation. *Hum Reprod*. 1998;13(Suppl. 3):63–70; discussion 1–5.
14. Russell JB. Immature oocyte retrieval with in-vitro oocyte maturation. *Curr Opin Obstet Gynecol*. 1999;11(3):289–96.
15. Thornton MH, Francis MM, Paulson RJ. Immature oocyte retrieval: Lessons from unstimulated IVF cycles. *Fertil Steril*. 1998;70(4):647–50.
16. Picton HM, Danfour MA, Harris SE, Chambers EL, Huntriss J. Growth and maturation of oocytes in vitro. *Reprod Suppl*. 2003;61:445–62.
17. Appeltant R, Somfai T, Kikuchi K, Maes D, Van Soom A. Influence of co-culture with denuded oocytes during in vitro maturation on fertilization and developmental competence of cumulus-enclosed porcine oocytes in a defined system. *Anim Sci J*. 2016;87(4):503–10.
18. Chian RC, Cao YX. In vitro maturation of immature human oocytes for clinical application. *Methods Mol Biol*. 2014;1154:271–88.
19. Moreno JM, Nunez MJ, Quinonero A, Martinez S, de la Orden M, Simon C et al. Follicular fluid and mural granulosa cells microRNA profiles vary in in vitro fertilization patients depending on their age and oocyte maturation stage. *Fertil Steril*. 2015;104(4):1037–46.e1.

20. Sanchez F, Romero S, De Vos M, Verheyen G, Smitz J. Human cumulus-enclosed germinal vesicle oocytes from early antral follicles reveal heterogeneous cellular and molecular features associated with in vitro maturation capacity. *Hum Reprod*. 2015;30(6):1396–409.
21. Sauerbrun-Cutler MT, Vega M, Keltz M, McGovern PG. In vitro maturation and its role in clinical assisted reproductive technology. *Obstet Gynecol Surv*. 2015;70(1):45–57.
22. Segers I, Mateizel I, Van Moer E, Smitz J, Tournaye H, Verheyen G et al. In vitro maturation (IVM) of oocytes recovered from ovariectomy specimens in the laboratory: A promising "ex vivo" method of oocyte cryopreservation resulting in the first report of an ongoing pregnancy in Europe. *J Assist Reprod Genet*. 2015;32(8):1221–31.
23. Triwitayakorn A, Suwajanakorn S, Pruksananonda K, Sereepapong W, Ahnonkitpanit V. Correlation between human follicular diameter and oocyte outcomes in an ICSI program. *J Assist Reprod Genet*. 2003;20(4):143–7.
24. Vassena R, Adams GP, Mapletoft RJ, Pierson RA, Singh J. Ultrasound image characteristics of ovarian follicles in relation to oocyte competence and follicular status in cattle. *Anim Reprod Sci*. 2003;76(1-2):25–41.
25. DeCherney AH, Laufer N. The monitoring of ovulation induction using ultrasound and estrogen. *Clin Obstet Gynecol*. 1984;27(4):993–1002.
26. Rossavik IK, Gibbons WE. Variability of ovarian follicular growth in natural menstrual cycles. *Fertil Steril*. 1985;44(2):195–9.
27. Zegers-Hochschild F, Gomez Lira C, Parada M, Altieri Lorenzini E. A comparative study of the follicular growth profile in conception and nonconception cycles. *Fertil Steril*. 1984;41(2):244–7.
28. Baerwald AR, Walker RA, Pierson RA. Growth rates of ovarian follicles during natural menstrual cycles, oral contraception cycles, and ovarian stimulation cycles. *Fertil Steril*. 2009;91(2):440–9.
29. Robson SJ, Barry M, Norman RJ. Power Doppler assessment of follicle vascularity at the time of oocyte retrieval in in vitro fertilization cycles. *Fertil Steril*. 2008;90(6):2179–82.
30. Borini A, Maccolini A, Tallarini A, Bonu MA, Sciajno R, Flamigni C. Perifollicular vascularity and its relationship with oocyte maturity and IVF outcome. *Ann NY Acad Sci*. 2001;943:64–7.
31. Palomba S, Russo T, Falbo A, Orio F, Jr., Manguso F, Nelaj E et al. Clinical use of the perifollicular vascularity assessment in IVF cycles: A pilot study. *Hum Reprod*. 2006;21(4):1055–61.
32. Palomba S, Russo T, Orio F, Jr., Falbo A, Tolino A, Zullo F. Perifollicular vascularity assessment for selecting the best oocytes for in vitro fertilization programs in older patients. *Fertil Steril*. 2008;90(4):1305–9.
33. Van Blerkom J. Epigenetic influences on oocyte developmental competence: Perifollicular vascularity and intrafollicular oxygen. *J Assist Reprod Genet*. 1998;15(5):226–34.
34. Van Blerkom J. Intrafollicular influences on human oocyte developmental competence: Perifollicular vascularity, oocyte metabolism and mitochondrial function. *Hum Reprod*. 2000;15(Suppl. 2): 173–88.
35. Borini A, Tallarini A, Maccolini A, Prato LD, Flamigni C. Perifollicular vascularity monitoring and scoring: A clinical tool for selecting the best oocyte. *Eur J Obstet Gynecol Reprod Biol*. 2004;115(Suppl. 1):S102–5.
36. American College of O, Gynecologists Committee on Gynecologic P, Practice C. Female age-related fertility decline. Committee Opinion No. 589. *Fertil Steril*. 2014;101(3):633–4.
37. Brezina PR, Kutteh WH, Bailey AP, Ding J, Ke RW, Klosky JL. Fertility preservation in the age of assisted reproductive technologies. *Obstet Gynecol Clin N Am*. 2015;42(1):39–54.
38. Laopaiboon M, Lumbiganon P, Intarut N, Mori R, Ganchimeg T, Vogel JP et al. Advanced maternal age and pregnancy outcomes: A multicountry assessment. *BJOG*. 2014;121(Suppl. 1):49–56.
39. Sauer MV. Reproduction at an advanced maternal age and maternal health. *Fertil Steril*. 2015;103(5):1136–43.
40. Bukulmez O, Arici A. Assessment of ovarian reserve. *Curr Opin Obstet Gynecol*. 2004;16(3):231–7.
41. Hansen KR, Hodnett GM, Knowlton N, Craig LB. Correlation of ovarian reserve tests with histologically determined primordial follicle number. *Fertil Steril*. 2011;95(1):170–5.
42. Wallace WH, Kelsey TW. Ovarian reserve and reproductive age may be determined from measurement of ovarian volume by transvaginal sonography. *Hum Reprod*. 2004;19(7):1612–7.
43. Wallace WH, Kelsey TW. Human ovarian reserve from conception to the menopause. *PLoS One*. 2010;5(1):e8772.

44. Brodin T, Hadziosmanovic N, Berglund L, Olovsson M, Holte J. Comparing four ovarian reserve markers—Associations with ovarian response and live births after assisted reproduction. *Acta Obstet Gynecol Scand*. 2015;95(10):1056–63.
45. Devine K, Mumford SL, Wu M, DeCherney AH, Hill MJ, Propst A. Diminished ovarian reserve in the United States assisted reproductive technology population: Diagnostic trends among 181,536 cycles from the Society for Assisted Reproductive Technology Clinic Outcomes Reporting System. *Fertil Steril*. 2015;104(3):612–19.e3.
46. Grive KJ, Freiman RN. The developmental origins of the mammalian ovarian reserve. *Development*. 2015;142(15):2554–63.
47. Parry JP, Moran T, Koch CA. Ovarian reserve testing. In: *Endotext*, De Groot LJ, Beck-Peccoz P, Chrousos G, Dungan K, Grossman A, Hershman JM et al. (Eds.). South Dartmouth (MA): MDText.com, Inc., 2000.
48. Practice Committee of the American Society for Reproductive M. Testing and interpreting measures of ovarian reserve: A committee opinion. *Fertil Steril*. 2015;103(3):e9–17.
49. Syrop CH, Dawson JD, Husman KJ, Sparks AE, Van Voorhis BJ. Ovarian volume may predict assisted reproductive outcomes better than follicle stimulating hormone concentration on day 3. *Hum Reprod*. 1999;14(7):1752–6.
50. Erdem A, Erdem M, Biberoglu K, Hayit O, Arslan M, Gursoy R. Age-related changes in ovarian volume, antral follicle counts and basal FSH in women with normal reproductive health. *J Reprod Med*. 2002;47(10):835–9.
51. Bancsi LF, Broekmans FJ, Eijkemans MJ, de Jong FH, Habbema JD, te Velde ER. Predictors of poor ovarian response in in vitro fertilization: A prospective study comparing basal markers of ovarian reserve. *Fertil Steril*. 2002;77(2):328–36.
52. Tufan E, Elter K, Durmusoglu F. Assessment of reproductive ageing patterns by hormonal and ultrasonographic ovarian reserve tests. *Hum Reprod*. 2004;19(11):2484–9.
53. Broekmans FJ, de Ziegler D, Howles CM, Gougeon A, Trew G, Olivennes F. The antral follicle count: Practical recommendations for better standardization. *Fertil Steril*. 2010;94(3):1044–51.
54. Broekmans FJ, Faddy M, te Velde ER. Ovarian reserve and reproductive age may be determined from measurement of ovarian volume by transvaginal sonography. *Hum Reprod*. 2005;20(4):1114–5; author reply 5–6.
55. Broekmans FJ, Faddy MJ, Scheffer G, te Velde ER. Antral follicle counts are related to age at natural fertility loss and age at menopause. *Menopause*. 2004;11(6 Pt 1):607–14.
56. Broekmans FJ, Scheffer GJ, Bancsi LF, Dorland M, Blankenstein MA, te Velde ER. Ovarian reserve tests in infertility practice and normal fertile women. *Maturitas*. 1998;30(2):205–14.
57. Hendriks DJ, Kwee J, Mol BW, te Velde ER, Broekmans FJ. Ultrasonography as a tool for the prediction of outcome in IVF patients: A comparative meta-analysis of ovarian volume and antral follicle count. *Fertil Steril*. 2007;87(4):764–75.
58. Hendriks DJ, Mol BW, Bancsi LF, Te Velde ER, Broekmans FJ. Antral follicle count in the prediction of poor ovarian response and pregnancy after in vitro fertilization: A meta-analysis and comparison with basal follicle-stimulating hormone level. *Fertil Steril*. 2005;83(2):291–301.
59. Lass A, Skull J, McVeigh E, Margara R, Winston RM. Measurement of ovarian volume by transvaginal sonography before ovulation induction with human menopausal gonadotrophin for in-vitro fertilization can predict poor response. *Hum Reprod*. 1997;12(2):294–7.
60. Durmusoglu F, Elter K, Yoruk P, Erenus M. Combining cycle day 7 follicle count with the basal antral follicle count improves the prediction of ovarian response. *Fertil Steril*. 2004;81(4):1073–8.
61. Vladimirov IK, Tacheva DM, Kalinov KB, Ivanova AV, Blagoeva VD. Prognostic value of some ovarian reserve tests in poor responders. *Arch Gynecol Obstet*. 2005;272(1):74–9.
62. Pope CS, Cook EK, Arny M, Novak A, Grow DR. Influence of embryo transfer depth on in vitro fertilization and embryo transfer outcomes. *Fertil Steril*. 2004;81(1):51–8.
63. Erdem M, Erdem A, Gursoy R, Biberoglu K. Comparison of basal and clomiphene citrate induced FSH and inhibin B, ovarian volume and antral follicle counts as ovarian reserve tests and predictors of poor ovarian response in IVF. *J Assist Reprod Genet*. 2004;21(2):37–45.
64. Bukman A, Heineman MJ. Ovarian reserve testing and the use of prognostic models in patients with subfertility. *Hum Reprod Update*. 2001;7(6):581–90.

65. Scheffer GJ, Broekmans FJ, Dorland M, Habbema JD, Looman CW, te Velde ER. Antral follicle counts by transvaginal ultrasonography are related to age in women with proven natural fertility. *Fertil Steril*. 1999;72(5):845–51.
66. Pierson RA, Chizen DR, Olatunbosun OA. Ultrasonographic assessment of ovulation induction. In: *Imaging in Infertility and Reproductive Endocrinology*, Jaffe R, Pierson RA, Abramowicz JA (Eds.). Philadephia: J.B. Lippincott, 1994, 155–66.
67. Battaglia C, Mancini F, Persico N, Zaccaria V, de Aloysio D. Ultrasound evaluation of PCO, PCOS and OHSS. *Reprod Biomed Online*. 2004;9(6):614–9.
68. Danninger B, Brunner M, Obruca A, Feichtinger W. Prediction of ovarian hyperstimulation syndrome by ultrasound volumetric assessment [corrected] of baseline ovarian volume prior to stimulation. *Hum Reprod*. 1996;11(8):1597–9.
69. Soave I, Marci R. Ovarian stimulation in patients in risk of OHSS. *Minerva Ginecol*. 2014;66(2):165–78.
70. Meldrum DR. Preventing severe OHSS has many different facets. *Fertil Steril*. 2012;97(3):536–8.
71. Papanikolaou EG, Humaidan P, Polyzos N, Kalantaridou S, Kol S, Benadiva C et al. New algorithm for OHSS prevention. *Reprod Biol Endocrinol*. 2011;9:147.
72. Orvieto R. Can we eliminate severe ovarian hyperstimulation syndrome? *Hum Reprod*. 2005; 20(2):320–2.
73. Ben-Ami M, Perlitz Y, Haddad S. The effectiveness of spectral and color Doppler in predicting ovarian torsion. A prospective study. *Eur J Obstet Gynecol Reprod Biol*. 2002;104(1):64–6.
74. Auslender R, Lavie O, Kaufman Y, Bardicef M, Lissak A, Abramovici H. Coiling of the ovarian vessels: A color Doppler sign for adnexal torsion without strangulation. *Ultrasound Obstet Gynecol*. 2002;20(1):96–7.
75. Pierson RA, Adams GP. Computer-assisted image analysis, diagnostic ultrasonography and ovulation induction: Strange bedfellows. *Theriogenology*. 1995;43:105–12.
76. Singh J, Pierson RA, Adams GP. Ultrasound image attributes of bovine ovarian follicles: Endocrine and functional correlates. *J Reprod Fertil*. 1988;112:345–69.
77. Singh J, Adams GP, Pierson RA. Promise of new imaging technologies for assessing ovarian function. *Anim Reprod Sci*. 2003;78(3–4):371–99.
78. Rezaei E, Baerwald AR, Pierson RA. Ultrasonographic image analysis of human dominant and 1st subordinate ovarian follicles. Proceedings of the 53rd Annual Conference of the Canadian Fertility and Andrology Society, Halifax, Nova Scotia: Canadian Fertility and Andrology Society, p. 48, 2007.
79. Russell JB, DeCherney AH, Hobbins JC. A new transvaginal probe and biopsy guide for oocyte retrieval. *Fertil Steril*. 1987;47(2):350–2.
80. Cohen J, Debache C, Pez JP, Junca AM, Cohen-Bacrie P. Transvaginal sonographically controlled ovarian puncture for oocyte retrieval for in vitro fertilization. *J In Vitro Fert Embryo Transf*. 1986;3(5):309–13.
81. Dellenbach P, Nisand I, Moreau L, Feger B, Plumere C, Gerlinger P. Transvaginal sonographically controlled follicle puncture for oocyte retrieval. *Fertil Steril*. 1985;44(5):656–62.
82. Hamberger L, Wikland M, Enk L, Nilsson L. Laparoscopy versus ultrasound guided puncture for oocyte retrieval. *Acta Eur Fertil*. 1986;17(3):195–8.
83. Feichtinger W. Current technology of oocyte retrieval. *Curr Opin Obstet Gynecol*. 1992;4(5):697–701.
84. Gembruch U, Diedrich K, Welker B, Wahode J, van der Ven H, Al-Hasani S et al. Transvaginal sonographically guided oocyte retrieval for in-vitro fertilization. *Hum Reprod*. 1988;3(Suppl. 2):59–63.
85. Feldberg D, Goldman JA, Ashkenazi J, Shelef M, Dicker D, Samuel N. Transvaginal oocyte retrieval controlled by vaginal probe for in vitro fertilization: A comparative study. *J Ultrasound Med*. 1988;7(6):339–43.
86. Schulman JD, Dorfmann AD, Jones SL, Pitt CC, Joyce B, Patton LA. Outpatient in vitro fertilization using transvaginal ultrasound-guided oocyte retrieval. *Obstet Gynecol*. 1987;69(4):665–8.
87. Wikland M, Enk L, Hammarberg K, Nilsson L. Use of a vaginal transducer for oocyte retrieval in an IVF/ET program. *J Clin Ultrasound*. 1987;15(4):245–51.
88. Wikland M, Enk L, Hammarberg K, Nilsson L, Hamberger L. Oocyte retrieval under the guidance of a vaginal transducer. *Ann N Y Acad Sci*. 1988;541:103–10.
89. Wiseman DA, Short WB, Pattinson HA, Taylor PJ, Nicholson SF, Elliott PD et al. Oocyte retrieval in an in vitro fertilization-embryo transfer program: Comparison of four methods. *Radiology*. 1989;173(1):99–102.

90. Healy MW, Hill MJ, Levens ED. Optimal oocyte retrieval and embryo transfer techniques: Where we are and how we got here. *Semin Reprod Med*. 2015;33(2):83–91.
91. Rose BI. Approaches to oocyte retrieval for advanced reproductive technology cycles planning to utilize in vitro maturation: A review of the many choices to be made. *J Assist Reprod Genet*. 2014;31(11): 1409–19.
92. Diedrich K, Van der Ven H, Al-Hasani S, Werner A, Krebs D. Oocyte retrieval for in vitro fertilization. *Ann Biol Clin (Paris)*. 1987;45(3):351–7.
93. Padilla SL, Butler WJ, Boldt JP, Reindollar RH, Tho SP, Holzman GB et al. Transurethral ultrasound-guided oocyte retrieval for in vitro fertilization. *Am J Obstet Gynecol*. 1987;157(3):622–6.
94. Parsons J, Riddle A, Booker M, Sharma V, Goswamy R, Wilson L et al. Oocyte retrieval for in-vitro fertilisation by ultrasonically guided needle aspiration via the urethra. *Lancet*. 1985;1(8437):1076–7.
95. Kemeter P, Feichtinger W. Trans-vaginal oocyte retrieval using a trans-vaginal sector scan probe combined with an automated puncture device. *Hum Reprod*. 1986;1(1):21–4.
96. Schulman JD, Dorfmann A, Jones S, Joyce B, Hanser J. Outpatient in vitro fertilization using transvaginal oocyte retrieval and local anesthesia. *N Engl J Med*. 1985;312(25):1639.
97. Sterzik K, Jonatha W, Keckstein G, Rossmanith W, Traub E, Wolf A. Ultrasonically guided follicle aspiration for oocyte retrieval in an in vitro fertilization program: Further simplification. *Int J Gynaecol Obstet*. 1987;25(4):309–14.
98. Yuzpe AA, Brown SE, Casper RF, Nisker J, Graves G, Shatford L. Transvaginal, ultrasound-guided oocyte retrieval for in vitro fertilization. *J Reprod Med*. 1989;34(12):937–42.
99. Ragni G, Lombroso GC, De Lauretis L, Scarduelli C, Wyssling H, Crosignani PG. Echoguided transvaginal oocyte retrieval: Effective and easy to learn technique. *Acta Eur Fertil*. 1991;22(2):89–90.
100. Sladkevicius P, Campbell S. Advances in ultrasound assessment in the establishment and development of pregnancy. *Br Med Bull*. 2000;56(3):691–703.
101. Roque M, Sampaio M, Geber S. Follicular flushing during oocyte retrieval: A systematic review and meta-analysis. *J Assist Reprod Genet*. 2012;29(11):1249–54.
102. Miller KA, Elkind-Hirsch K, Benson M, Bergh P, Drews M, Scott RT. A new follicle aspiration needle set is equally effective and as well tolerated as the standard needle when used in a prospective randomized trial in a large in vitro fertilization program. *Fertil Steril*. 2004;81(1):191–3.
103. Rose BI, Laky D. A comparison of the Cook single lumen immature ovum IVM needle to the Steiner-Tan pseudo double lumen flushing needle for oocyte retrieval for IVM. *J Assist Reprod Genet*. 2013;30(6):855–60.
104. Kushnir VA, Kim A, Gleicher N, Barad DH. A pilot trial of large versus small diameter needles for oocyte retrieval. *Reprod Biol Endocrinol*. 2013;11:22.
105. Bustillo M. Unsuccessful oocyte retrieval: Technical artefact or genuine "empty follicle syndrome"? *Reprod Biomed Online*. 2004;8(1):59–67.
106. Blazquez A, Guillen JJ, Colome C, Coll O, Vassena R, Vernaeve V. Empty follicle syndrome prevalence and management in oocyte donors. *Hum Reprod*. 2014;29(10):2221–7.
107. Kim JH, Jee BC. Empty follicle syndrome. *Clin Exp Reprod Med*. 2012;39(4):132–7.
108. Krishna D, Rajashekar L, Patil M. Empty follicle syndrome-still an enigma. *J Hum Reprod Sci*. 2008;1(2):86–9.
109. Mesen TB, Yu B, Richter KS, Widra E, DeCherney AH, Segars JH. The prevalence of genuine empty follicle syndrome. *Fertil Steril*. 2011;96(6):1375–7.
110. Stevenson TL, Lashen H. Empty follicle syndrome: The reality of a controversial syndrome, a systematic review. *Fertil Steril*. 2008;90(3):691–8.
111. van Heusden AM, van Santbrink EJ, Schipper I, de Jong D. The empty follicle syndrome is dead! *Fertil Steril*. 2008;89(3):746.
112. Barak Y, Lessing JB, Amit A, Kogosowski A, Yovel I, David MP et al. The development of an efficient ambulatory in vitro fertilization (IVF) and embryo transfer (ET) program using ultrasonically guided oocyte retrieval. *Acta Obstet Gynecol Scand*. 1988;67(7):585–8.
113. Tureck RW, Garcia CR, Blasco L, Mastroianni L, Jr. Perioperative complications arising after transvaginal oocyte retrieval. *Obstet Gynecol*. 1993;81(4):590–3.
114. Christiaens F, Janssenswillen C, Van Steirteghem AC, Devroey P, Verborgh C, Camu F. Comparison of assisted reproductive technology performance after oocyte retrieval under general anaesthesia (propofol) versus paracervical local anaesthetic block: A case-controlled study. *Hum Reprod*. 1998;13(9):2456–60.

115. El-Shawarby S, Margara R, Trew G, Lavery S. A review of complications following transvaginal oocyte retrieval for in-vitro fertilization. *Hum Fertil (Camb).* 2004;7(2):127–33.
116. Ng EH, Miao B, Ho PC. Anxiolytic premedication reduces preoperative anxiety and pain during oocyte retrieval. A randomized double-blinded placebo-controlled trial. *Hum Reprod*. 2002;17(5): 1233–8.
117. Tummon I, Newton C, Lee C, Martin J. Lidocaine vaginal gel versus lidocaine paracervical block for analgesia during oocyte retrieval. *Hum Reprod*. 2004;19(5):1116–20.
118. Kwan I, Bhattacharya S, Knox F, McNeil A. Pain relief for women undergoing oocyte retrieval for assisted reproduction. *Cochrane Database Syst Rev.* 2013;1:CD004829.
119. Siristatidis C, Chrelias C, Alexiou A, Kassanos D. Clinical complications after transvaginal oocyte retrieval: A retrospective analysis. *J Obstet Gynaecol*. 2013;33(1):64–6.
120. Bonilla-Musoles F, Raga F, Osborne NG, Castillo JC, Bonilla F, Jr. Endometrial receptivity: Evaluation with ultrasound. *Ultrasound Q*. 2013;29(1):3–20.
121. Turnbull LW, Lesny P, Killick SR. Assessment of uterine receptivity prior to embryo transfer: A review of currently available imaging modalities. *Hum Reprod Update*. 1995;1(5):505–14.
122. Heger A, Sator M, Pietrowski D. Endometrial receptivity and its predictive value for IVF/ICSI-outcome. *Geburtshilfe Frauenheilkd*. 2012;72(8):710–5.
123. Khalifa E, Brzyski RG, Oehninger S, Acosta AA, Muasher SJ. Sonographic appearance of the endometrium: The predictive value for the outcome of in-vitro fertilization in stimulated cycles. *Hum Reprod*. 1992;7(5):677–80.
124. Abdalla HI, Brooks AA, Johnson MR, Kirkland A, Thomas A, Studd JW. Endometrial thickness: A predictor of implantation in ovum recipients? *Hum Reprod*. 1994;9(2):363–5.
125. Coulam CB, Bustillo M, Soenksen DM, Britten S. Ultrasonographic predictors of implantation after assisted reproduction. *Fertil Steril*. 1994;62(5):1004–10.
126. Noyes N, Liu HC, Sultan K, Schattman G, Rosenwaks Z. Endometrial thickness appears to be a significant factor in embryo implantation in in-vitro fertilization. *Hum Reprod*. 1995;10(4):919–22.
127. Potlog-Nahari C, Catherino WH, McKeeby JL, Wesley R, Segars JH. A suboptimal endometrial pattern is associated with a reduced likelihood of pregnancy after a day 5 embryo transfer. *Fertil Steril*. 2005;83(1):235–7.
128. Chien LW, Au HK, Xiao J, Tzeng CR. Fluid accumulation within the uterine cavity reduces pregnancy rates in women undergoing IVF. *Hum Reprod*. 2002;17(2):351–6.
129. Zagzebski J. *Essentials of Ultasound Physics*. New York: C.V. Mosby, 1996, 220 p.
130. Sterzik K, Grab D, Sasse V, Hutter W, Rosenbusch B, Terinde R. Doppler sonographic findings and their correlation with implantation in an in vitro fertilization program. *Fertil Steril*. 1989;52(5):825–8.
131. Steer CV, Campbell S, Tan SL, Crayford T, Mills C, Mason BA et al. The use of transvaginal color flow imaging after in vitro fertilization to identify optimum uterine conditions before embryo transfer. *Fertil Steril*. 1992;57(2):372–6.
132. Serafini P, Batzofin J, Nelson J, Olive D. Sonographic uterine predictors of pregnancy in women undergoing ovulation induction for assisted reproductive treatments. *Fertil Steril*. 1994;62(4):815–22.
133. Tekay A, Martikainen H, Jouppila P. Blood flow changes in uterine and ovarian vasculature, and predictive value of transvaginal pulsed colour Doppler ultrasonography in an in-vitro fertilization programme. *Hum Reprod*. 1995;10(3):688–93.
134. Cacciatore B, Simberg N, Fusaro P, Tiitinen A. Transvaginal Doppler study of uterine artery blood flow in in vitro fertilization-embryo transfer cycles. *Fertil Steril*. 1996;66(1):130–4.
135. Aytoz A, Ubaldi F, Tournaye H, Nagy ZP, Van Steirteghem A, Devroey P. The predictive value of uterine artery blood flow measurements for uterine receptivity in an intracytoplasmic sperm injection program. *Fertil Steril*. 1997;68(5):935–7.
136. Friedler S, Schenker JG, Herman A, Lewin A. The role of ultrasonography in the evaluation of endometrial receptivity following assisted reproductive treatments: A critical review. *Hum Reprod Update*. 1996;2(4):323–35.
137. Zaidi J, Campbell S, Pittrof R, Tan SL. Endometrial thickness, morphology, vascular penetration and velocimetry in predicting implantation in an in vitro fertilization program. *Ultrasound Obstet Gynecol*. 1995;6(3):191–8.

138. Yang J, Wu M, Chen C, Jiang M, Ho H, Yang Y. Association of endometrial blood flow as determined by a modified colour Doppler technique with subsequent outcome of in-vitro fertilization. *Hum Reprod*. 1999;14(6):1606–10.
139. Schild RL, Knobloch C, Dorn C, Fimmers R, van der Ven H, Hansmann M. Endometrial receptivity in an in vitro fertilization program as assessed by spiral artery blood flow, endometrial thickness, endometrial volume, and uterine artery blood flow. *Fertil Steril*. 2001;75(2):361–6.
140. Raine-Fenning NJ, Campbell BK, Kendall NR, Clewes JS, Johnson IR. Endometrial and subendometrial perfusion are impaired in women with unexplained subfertility. *Hum Reprod*. 2004; 19(11):2605–14.
141. Riad ON, Hak AA. Assessment of endometrial receptivity using Doppler ultrasonography in infertile women undergoing intrauterine insemination. *Gynecol Endocrinol*. 2014;30(1):70–3.
142. Kupesic S, Bekavac I, Bjelos D, Kurjak A. Assessment of endometrial receptivity by transvaginal color Doppler and three-dimensional power Doppler ultrasonography in patients undergoing in vitro fertilization procedures. *J Ultrasound Med*. 2001;20(2):125–34.
143. Applebaum M. The uterine biophysical profile. *Ultrasound Obstet Gynecol*. 1995;5(1):67–8.
144. Salle B, Bied-Damon V, Benchaib M, Desperes S, Gaucherand P, Rudigoz RC. Preliminary report of an ultrasonography and colour Doppler uterine score to predict uterine receptivity in an in-vitro fertilization programme. *Hum Reprod*. 1998;13(6):1669–73.
145. Pierson RA, Mrázek M, Kuzcynski W, Klein BM, Arce J-C (Eds.). Endometrial quality at the end of controlled ovarian stimulation predicts on-going pregnancy rate after transfer of a single expanded or hatching/hatched blastocyst on day 5 in a fresh cycle. *Proceedings of the American Society for Reproductive Medicine ASRM* 2012, San Diego, CA. Fertility and Sterility 98: Supplement 3, ASRM Abstracts:S225.
146. Pierson RA. Three-dimensional ultrasonography of the embryo and fetus. In: *Fetal and Neonatal Ultrasonography*, Jaffe R, Bui TH (Eds.). New York: Parthenon Publishing Group, 1999, 317–26.
147. Alcazar JL. Three-dimensional ultrasound assessment of endometrial receptivity: A review. *Reprod Biol Endocrinol*. 2006;4:56.
148. Ledee N, Chaouat G, Serazin V, Lombroso R, Dubanchet S, Oger P et al. Endometrial vascularity by three-dimensional power Doppler ultrasound and cytokines: A complementary approach to assess uterine receptivity. *J Reprod Immunol*. 2008;77(1):57–62.
149. Ng EH, Chan CC, Tang OS, Yeung WS, Ho PC. Comparison of endometrial and subendometrial blood flow measured by three-dimensional power Doppler ultrasound between stimulated and natural cycles in the same patients. *Hum Reprod*. 2004;19(10):2385–90.
150. Raga F, Bonilla-Musoles F, Casan EM, Klein O, Bonilla F. Assessment of endometrial volume by three-dimensional ultrasound prior to embryo transfer: Clues to endometrial receptivity. *Hum Reprod*. 1999;14(11):2851–4.
151. Schild RL, Indefrei D, Eschweiler S, Van der Ven H, Fimmers R, Hansmann M. Three-dimensional endometrial volume calculation and pregnancy rate in an in-vitro fertilization programme. *Hum Reprod*. 1999;14(5):1255–8.
152. Schild RL, Holthaus S, d'Alquen J, Fimmers R, Dorn C, van Der Ven H et al. Quantitative assessment of subendometrial blood flow by three-dimensional-ultrasound is an important predictive factor of implantation in an in-vitro fertilization programme. *Hum Reprod*. 2000;15(1):89–94.
153. Wu HM, Chiang CH, Huang HY, Chao AS, Wang HS, Soong YK. Detection of the subendometrial vascularization flow index by three-dimensional ultrasound may be useful for predicting the pregnancy rate for patients undergoing in vitro fertilization-embryo transfer. *Fertil Steril*. 2003;79(3):507–11.
154. Martinez-Gaudio M, Yoshida T, Bengtsson LP. Propagated and nonpropagated myometrial contractions in normal menstrual cycles. *Am J Obstet Gynecol*. 1973;115(1):107–11.
155. de Vries K, Lyons EA, Ballard G, Levi CS, Lindsay DJ. Contractions of the inner third of the myometrium. *Am J Obstet Gynecol*. 1990;162(3):679–82.
156. Lyons EA, Taylor PJ, Zheng XH, Ballard G, Levi CS, Kredentser JV. Characterization of subendometrial myometrial contractions throughout the menstrual cycle in normal fertile women. *Fertil Steril*. 1991;55(4):771–4.

157. Ijland MM, Evers JL, Dunselman GA, van Katwijk C, Lo CR, Hoogland HJ. Endometrial wavelike movements during the menstrual cycle. *Fertil Steril*. 1996;65(4):746–9.
158. Fanchin R, Righini C, Ayoubi JM, Olivennes F, de Ziegler D, Frydman R. New look at endometrial echogenicity: Objective computer-assisted measurements predict endometrial receptivity in in vitro fertilization-embryo transfer. *Fertil Steril*. 2000;74(2):274–81.
159. van Gestel I, MM IJ, Hoogland HJ, Evers JL. Endometrial wave-like activity in the non-pregnant uterus. *Hum Reprod Update*. 2003;9(2):131–8.
160. Lesny P, Killick SR. The junctional zone of the uterus and its contractions. *BJOG*. 2004;111(11):1182–9.
161. Lesny P, Killick SR, Tetlow RL, Robinson J, Maguiness SD. Uterine junctional zone contractions during assisted reproduction cycles. *Hum Reprod Update*. 1998;4(4):440–5.
162. Fanchin R, Righini C, Ayoubi JM, Olivennes F, de Ziegler D, Frydman R. Uterine contractions at the time of embryo transfer: A hindrance to implantation?. *Contracept Fertil Sex*. 1998;26(7–8):498–505. (In French).
163. Fanchin R. Assessing uterine receptivity in 2001: Ultrasonographic glances at the new millennium. *Ann N Y Acad Sci*. 2001;943:185–202.
164. Fanchin R, Righini C, Olivennes F, Taylor S, de Ziegler D, Frydman R. Uterine contractions at the time of embryo transfer alter pregnancy rates after in-vitro fertilization. *Hum Reprod*. 1998;13(7):1968–74.
165. Vlaisavljevic V, Reljic M, Gavric-Lovrec V, Kovacic B. Subendometrial contractility is not predictive for in vitro fertilization (IVF) outcome. *Ultrasound Obstet Gynecol*. 2001;17(3):239–44.
166. Fanchin R, Righini C, de Ziegler D, Olivennes F, Ledee N, Frydman R. Effects of vaginal progesterone administration on uterine contractility at the time of embryo transfer. *Fertil Steril*. 2001;75(6):1136–40.
167. Baruffi R, Mauri AL, Petersen CG, Felipe V, Franco JG, Jr. Effects of vaginal progesterone administration starting on the day of oocyte retrieval on pregnancy rates. *J Assist Reprod Genet*. 2003;20(12):517–20.
168. Bulletti C, de Ziegler D. Uterine contractility and embryo implantation. *Curr Opin Obstet Gynecol*. 2005;17(3):265–76.
169. Fanchin R, Ayoubi JM, Righini C, Olivennes F, Schonauer LM, Frydman R. Uterine contractility decreases at the time of blastocyst transfers. *Hum Reprod*. 2001;16(6):1115–9.
170. Vishnova H, Pierson RA, Mrázek M, Garcia-Velasco JA, Blockeel C, Arce J-C (Eds.). Effects of barusiban, a selective oxytocin antagonist, on uterine contractility in the luteal phase after controlled ovarian stimulation. *Amercian Society for Reproductive Medicine ASRM* 2012, San Diego, CA. Fertility and Sterility, ASRM Abstracts:S183.
171. Strickler RC, Christianson C, Crane JP, Curato A, Knight AB, Yang V. Ultrasound guidance for human embryo transfer. *Fertil Steril*. 1985;43(1):54–61.
172. al-Shawaf T, Dave R, Harper J, Linehan D, Riley P, Craft I. Transfer of embryos into the uterus: How much do technical factors affect pregnancy rates? *J Assist Reprod Genet*. 1993;10(1):31–6.
173. al-Shawaf T, Yang D, al-Magid Y, Seaton A, Iketubosin F, Craft I. Ultrasonic monitoring during replacement of frozen/thawed embryos in natural and hormone replacement cycles. *Hum Reprod*. 1993;8(12):2068–74.
174. Kan AK, Abdalla HI, Gafar AH, Nappi L, Ogunyemi BO, Thomas A et al. Embryo transfer: Ultrasound-guided versus clinical touch. *Hum Reprod*. 1999;14(5):1259–61.
175. Coroleu B, Carreras O, Veiga A, Martell A, Martinez F, Belil I et al. Embryo transfer under ultrasound guidance improves pregnancy rates after in-vitro fertilization. *Hum Reprod*. 2000;15(3):616–20.
176. Rijnders RJ, Mol BW, Broekmans FJ. Use of ultrasound guidance during embryo transfer. *Hum Reprod*. 2000;15(11):2449.
177. Anderson RE, Nugent NL, Gregg AT, Nunn SL, Behr BR. Transvaginal ultrasound-guided embryo transfer improves outcome in patients with previous failed in vitro fertilization cycles. *Fertil Steril*. 2002;77(4):769–75.
178. Biervliet FP, Lesny P, Maguiness SD, Robinson J, Killick SR. Transmyometrial embryo transfer and junctional zone contractions. *Hum Reprod*. 2002;17(2):347–50.
179. Baba K, Ishihara O, Hayashi N, Saitoh M, Taya J, Kinoshita K. Three-dimensional ultrasound in embryo transfer. *Ultrasound Obstet Gynecol*. 2000;16(4):372–3.
180. de Camargo Martins AM, Baruffi RL, Mauri AL, Petersen C, Oliveira JB, Contart P et al. Ultrasound guidance is not necessary during easy embryo transfers. *J Assist Reprod Genet*. 2004;21(12):421–5.
181. Kojima K, Nomiyama M, Kumamoto T, Matsumoto Y, Iwasaka T. Transvaginal ultrasound-guided embryo transfer improves pregnancy and implantation rates after IVF. *Hum Reprod*. 2001;16(12):2578–82.

182. Porat N, Boehnlein LM, Schouweiler CM, Kang J, Lindheim SR. Interim analysis of a randomized clinical trial comparing abdominal versus transvaginal ultrasound-guided embryo transfer. *J Obstet Gynaecol Res*. 2010;36(2):384–92.
183. Sieck UV, Jaroudi KA, Hollanders JM, Hamilton CJ. Ultrasound guided embryo transfer does not prevent ectopic pregnancies after in-vitro fertilization. *Hum Reprod*. 1997;12(9):2081–2.
184. Letterie GS. Three-dimensional ultrasound-guided embryo transfer: A preliminary study. *Am J Obstet Gynecol*. 2005;192(6):1983–7; discussion 7-8.
185. Gergely RZ, DeUgarte CM, Danzer H, Surrey M, Hill D, DeCherney AH. Three dimensional/four dimensional ultrasound-guided embryo transfer using the maximal implantation potential point. *Fertil Steril*. 2005;84(2):500–3.
186. Tang OS, Ng EH, So WW, Ho PC. Ultrasound-guided embryo transfer: A prospective randomized controlled trial. *Hum Reprod*. 2001;16(11):2310–5.
187. Matorras R, Urquijo E, Mendoza R, Corcostegui B, Exposito A, Rodriguez-Escudero FJ. Ultrasound-guided embryo transfer improves pregnancy rates and increases the frequency of easy transfers. *Hum Reprod*. 2002;17(7):1762–6.
188. Letterie GS, Marshall L, Angle M. A new coaxial catheter system with an echodense tip for ultrasonographically guided embryo transfer. *Fertil Steril*. 1999;72(2):266–8.
189. Coroleu B, Barri PN, Carreras O, Belil I, Buxaderas R, Veiga A et al. Effect of using an echogenic catheter for ultrasound-guided embryo transfer in an IVF programme: A prospective, randomized, controlled study. *Hum Reprod*. 2006;21(7):1809–15.
190. Mansour RT, Aboulghar MA. Optimizing the embryo transfer technique. *Hum Reprod*. 2002;17(5):1149–53.
191. Hale L. Embryo transfer: How to ensure correct placement in utero. *Reprod Fertil Dev*. 2001;13(1):95–8.
192. Schoolcraft WB, Surrey ES, Gardner DK. Embryo transfer: Techniques and variables affecting success. *Fertil Steril*. 2001;76(5):863–70.
193. Coroleu B, Barri PN, Carreras O, Martinez F, Parriego M, Hereter L et al. The influence of the depth of embryo replacement into the uterine cavity on implantation rates after IVF: A controlled, ultrasound-guided study. *Hum Reprod*. 2002;17(2):341–6.
194. Buckett WM. A meta-analysis of ultrasound-guided versus clinical touch embryo transfer. *Fertil Steril*. 2003;80(4):1037–41.
195. Sallam HN. Should embryo transfer always be performed under ultrasound guidance? *Ultrasound Obstet Gynecol*. 2004;24(4):383–6.
196. Sallam HN, Sadek SS. Ultrasound-guided embryo transfer: A meta-analysis of randomized controlled trials. *Fertil Steril*. 2003;80(4):1042–6.
197. Wood EG, Batzer FR, Go KJ, Gutmann JN, Corson SL. Ultrasound-guided soft catheter embryo transfers will improve pregnancy rates in in-vitro fertilization. *Hum Reprod*. 2000;15(1):107–12.
198. Lindheim SR, Cohen MA, Sauer MV. Ultrasound guided embryo transfer significantly improves pregnancy rates in women undergoing oocyte donation. *Int J Gynaecol Obstet*. 1999;66(3):281–4.
199. Prapas Y, Prapas N, Hatziparasidou A, Vanderzwalmen P, Nijs M, Prapa S et al. Ultrasound-guided embryo transfer maximizes the IVF results on day 3 and day 4 embryo transfer but has no impact on day 5. *Hum Reprod*. 2001;16(9):1904–8.
200. Coroleu B, Barri PN, Carreras O, Martinez F, Veiga A, Balasch J. The usefulness of ultrasound guidance in frozen-thawed embryo transfer: A prospective randomized clinical trial. *Hum Reprod*. 2002;17(11):2885–90.
201. Garcia-Velasco JA, Isaza V, Martinez-Salazar J, Landazabal A, Requena A, Remohi J et al. Transabdominal ultrasound-guided embryo transfer does not increase pregnancy rates in oocyte recipients. *Fertil Steril*. 2002;78(3):534–9.
202. Shamonki MI, Schattman GL, Spandorfer SD, Chung PH, Rosenwaks Z. Ultrasound-guided trial transfer may be beneficial in preparation for an IVF cycle. *Hum Reprod*. 2005;20(10):2844–9.
203. Li R, Lu L, Hao G, Zhong K, Cai Z, Wang W. Abdominal ultrasound-guided embryo transfer improves clinical pregnancy rates after in vitro fertilization: Experiences from 330 clinical investigations. *J Assist Reprod Genet*. 2005;22(1):3–8.

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Oocyte Retrieval

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Introduction

The field of assisted reproductive technology (ART) has evolved greatly since its early beginnings in the 1940s. As ovarian stimulation methods developed to facilitate growth of multiple follicles, improved methods of retrieving follicular fluid and maximizing oocyte yields took precedence. As the field grew, techniques for oocyte retrieval were optimized, not only to increase efficiency, but also to improve the safety and consistency of the procedure. Prior to reviewing current methods used today, it is important to understand the history and evolution of the oocyte retrieval process.

History

Oocyte retrieval was initially performed through laparotomy by Miriam Menkin and John Rock in the 1940s as part of a series of experiments evaluating the potential for the fertilization of eggs through in vitro conditions (1). Although some progress was made during subsequent years, it was really the 1970s that saw the introduction of essential techniques involving aspiration of follicular fluid with needles connected to tubing and a collection device. Wedge resection of the ovary was also used to retrieve oocytes, with better rates of oocyte recovery as compared to aspiration. Although these approaches were suitable in research settings for patients who were already undergoing exploratory laparotomies for other indications, they had high intrinsic risks, including bleeding, damage to surrounding organs, infection, pain, and long recovery times.

The collaboration between Steptoe and Edwards was the necessary impetus for the advancement of the field of in vitro fertilization (IVF). Their collaboration was one of different areas of expertise but aligned passions. Edwards was a physiologist with an interest in pre-implantation genetic diagnosis. When he met Patrick Steptoe in 1968, he saw the direct utility of Steptoe's laparoscopic skills (2). A long-term partnership was borne, leading to the birth of the first IVF baby in 1978, and a worldwide discourse about reproduction. The early 1980s witnessed global interest in IVF, and the first baby from IVF in the United States was born at the end of 1981 through treatment at the Jones Institute (3).

As laparoscopic techniques developed and became more widely adopted in the 1970–1980s, studies emerged evaluating pre-ovulatory oocytes in stimulated vs unstimulated cycles, demonstrating reliable aspirations of enlarging follicles. The introduction of gamete intra-fallopian transfer (GIFT) in the mid-1980s helped to perfect oocyte aspirations laparoscopically. The technique allowed for a small needle port to be placed in the lower abdomen to introduce the aspiration needle. The needle would be placed into each follicle under laparoscopic guidance. Although less invasive and risky than laparotomy, laparoscopy still predisposed patients to significant surgical risks. Laparoscopy was also a costly procedure, requiring a full operating room set-up.

In the mid-1980s, ultrasound was introduced for oocyte retrievals. Before the advent of vaginal ultrasound, the trans-vesical approach was performed using abdominal ultrasound for guidance. The oocyte retrieval needle was placed in the end of a bladder catheter to protect the needle as it was introduced into the bladder. This technique was cumbersome, but was an important stepping stone to the use of

ultrasound guidance for retrievals. The development of vaginal ultrasound was a critical step in making oocyte retrievals easier for the patient.

Ultrasound Technology in Oocyte Retrieval

As mentioned above, the initial ultrasound-guided approach in infertility was trans-vesical. This route ameliorated difficulties associated with accessing the ovaries trans-abdominally through regional proximity. The use of ultrasound also permitted the evolution of oocyte retrieval as a same-day outpatient surgical procedure, and minimized the need for general anesthesia.

Due to the complications associated with trans-vesical procedures, trans-abdominal ultrasound became favored for several years. As initially postulated, trans-abdominal ultrasound guidance was limited due to the distance between the probe and ovaries. However, in rare patients, it facilitated access when the ovaries were transposed or enlarged above the pelvic brim. Trans-abdominal-guided oocyte retrieval continues to be used at our center for rare patients who have ovaries inaccessible by vaginal ultrasound.

The advent of modern oocyte retrieval was achieved with the development of the trans-vaginal probe. The vaginal route allows for the transducer to be close to the ovaries, allowing for better imaging of the follicles as the ovaries are typically enlarged and drape into the posterior cul-de-sac during stimulation.

The trans-vaginal approach to oocyte retrieval gained popularity through the 1980s due to its better and simpler access to the ovaries compared to trans-vesical aspiration. Large volume IVF clinics developed individualized techniques to standardize retrieval, thereby creating streamlined processes capable of accommodating high volumes of these procedures on a daily basis. Trans-vaginal probes initially suffered from poor resolution. Our initial experience at Boston IVF in 1986 with the trans-vaginal probe was challenging. The clinicians were essentially self-taught and the resolution of the machines did not allow for good imaging. However, we recognized the emergence of this technique as a new standard. The probes and the machines continued to improve in quality as more companies developed ultrasound machines for imaging. Diagnostic ultrasound also recognized the value of the vaginal route to better visualize not only the ovaries, but the uterus as well.

Modern Technique of Vaginal Ultrasound Retrieval

Vaginal Preparation

Strategies for preparatory cleansing of the vagina for oocyte retrieval vary from center to center. Although iodine based methods have traditionally been used in gynecologic surgery for pre-operative vaginal cleansing due to the powerful antimicrobial effects of iodine, povidone–iodine preparations have been shown to be toxic to eggs (4). Other methods of vaginal cleansing have been studied in gynecologic surgery, with acceptance of alternative preparations, including dilute chlorhexidine solutions and even milder solutions such as baby shampoo or saline irrigation, which have been found to confer a comparably reduced rate of post-operative infection to standard povidone–iodine preparation of the vagina with less risk of vaginal irritation (5,6).

A historic prospective randomized study demonstrated that disinfection of the vagina with betadine for trans-vaginal oocyte retrieval is related to a dramatically lower pregnancy rate per puncture (14.4% vs 26.4% for saline rinse), while rinsing with saline does not seem to increase the risk of infection (7). Another randomized trial of 721 patients found an increased risk of biochemical pregnancy in IVF patients who had a betadine scrub without a saline-mediated rinse prior to retrieval, as compared to those who had the iodine containing solution irrigated. The authors concluded that it is advisable to rinse the vagina with saline if an iodine scrub is used (8). A further study found that vaginal douching with aqueous povidone–iodine followed by normal saline irrigation immediately before oocyte retrieval is effective in preventing pelvic infection in patients with ovarian endometriomas without compromising the outcome of IVF treatment (9). These aggregate results suggest that, if povidone–iodine is used as a vaginal preparation, it should be combined with a thorough subsequent saline irrigation. However, a saline preparation is just as, if not more, effective at diluting vaginal bacteria and reducing infection risk.

At Boston IVF, we a gauze moistened with sterile buffered saline to clean the vaginal vault pre-procedure, with a final cleanse with culture media moistened gauze. Cleansing the vagina with saline appears to be the most accepted and standardized strategy, as evidenced by a recent worldwide survey of IVF practices including over 350 IVF centers in 71 countries. The survey showed that 72% of centers reported using saline alone, with just 15% reporting use of an antiseptic, and 4% use of an iodine containing solution (10).

Aspiration Technique

The technique of vaginal oocyte retrieval is fairly standard. The probe is draped with a sterile plastic protector and the needle guide is attached. The guide may be a permanent one that can be re-sterilized or it can be disposable. Most ultrasound machines display the needle guide track on the ultrasound machine and have variable controls for gain, depth, and frequency.

At our center, we use a standardized approach for oocyte retrieval, which is outlined in our internal standards in the International Organization for Standardization (ISO) guidelines that we use to guide us in all aspects of our clinical care. We have developed ISO standards for many aspects of IVF care in order to standardize how we perform procedures to allow for consistency throughout our organization.

The objective of oocyte retrieval is to minimize the number of punctures through the vaginal wall. At Boston IVF, we aim to reduce the number of puncture sites to one or two access punctures through the vagina per ovary. The follicles closest to the cortex are aspirated first. During the collapse of the follicle, the needle tip should be manipulated to stay in the center of the follicle as the wall collapses around it. Follicles are fully drained, and, once collapsed, the needle is oriented to the next follicle. Sometimes the needle tip must be withdrawn to the cortex to achieve an in line orientation for the subsequent follicle.

There are many manufacturers and options for retrieval needles. Typically the needle diameter is 16–17 gauge. The hub of the needle may be of various shapes to allow for comfort preferences. Studies evaluating needle diameter have shown that both pain and bleeding can be reduced when a smaller diameter needle is used. A randomized controlled trial demonstrated that the use of a 20 gauge needle (maximal diameter of 0.9 mm) could safely and effectively reduce patient perception of pain on a VAS (visual analog scale) as compared to the use of a 17 gauge needle (maximal diameter of 1.4 mm), without any differences between embryo development and pregnancy rates (11).

The test tubes attached to the tubing should rest in a warmer to minimize exposure to cooler temperatures. Most IVF centers use a suction machine to aspirate the fluid through the tubing and into the awaiting warmed tubes, with the assistance of a foot pedal. Ideal settings are about 145 mm of suction pressure. A study comparing 140 mm vs 120 mm pressure revealed increased pregnancy rates with 140 mmHg, further supporting the notion that this increased pressure does not damage oocytes (12). Moreover, a higher pressure option may exist on many machines which can be used when there is a clot or other debris in the needle, or when standard pressures are inadequate to drain the follicle of interest.

Pre-Operative Antibiotics

The effect of antibiotics for oocyte retrieval is not decisively known. However, there are many studies that have been conducted to assess the outcomes of preoperative antibiotics in IVF. In a retrospective study evaluating oocyte donors, 526 oocyte donors who received prophylactic antibiotics for oocyte retrieval were compared with a group of 625 who did not as part of an institutional change introducing routine preoperative antibiotic prophylaxis with cephalosporins (13). The incidence of infection after retrieval was reduced from 0.4% to 0% in the group receiving antibiotics (13). Notably, all donors had a vaginal preparation with povidone–iodine, unless they had a documented allergy.

An older study with an elegant design evaluated the impact of routine preoperative antibiotics on microbial growth at the catheter tip after embryo transfer. Antibiotic administration was associated with a reduction in positive microbiology cultures of embryo catheter tips in 78.4% of patients, and a highly significant decrease in implantation and pregnancy rates was noted in the women with positive microbial catheter-tip cultures (14). The most common microorganisms isolated from the vagina are Lactobacillus species, *Streptococcus viridans*, Enterococcus, and *Staphylococcus epidermis*, followed by *Ureaplasma urealyticum*, *Escherichia coli*, anaerobic gram-positive cocci, Prevotella, and *Mycoplasma hominis* (15).

Although the majority of these bacteria interfere with implantation and pregnancy rates, the finding of Lactobacillus on the catheter tip culture at the time of transfer has been correlated with increased pregnancy rates, highlighting the importance of some native flora in the establishment of pregnancy (15).

At our IVF center, we use preoperative antibiotics in the form of an IV administered cephalosporin prior to anesthesia induction. If the patient has a prior history of severe allergic response to penicillins or cephalosporins, doxycycline is typically used.

From the aforementioned worldwide survey, it appears that the rate of routine preoperative antibiotic use is moderate at 42%, with the caveat that a third of the remaining centers would administer preoperative antibiotics for those patients who have at least one risk factor for developing infection, thus increasing the actual rate of use (10).

Follicular Irrigation

The benefit of follicle irrigation or flushing has not been consistently demonstrated. Follicle flushing requires the added expense of a 2 channel retrieval needle, surplus culture media with which the flushing is performed, as well as more time in the operating room. Many studies which demonstrate a benefit do not thoroughly address whether the oocyte could merely have been present in the tubing prior to flushing. A small randomized trial in low responders evaluated whether follicular re-aspiration using a double lumen catheter improves oocyte recovery compared to a single aspiration. Although limited by a small number of patients in each arm (15), there were no significant differences in the number of oocytes retrieved or pregnancy rates, although there was a statistically and clinically significant increase in operating time when flushing was performed (16). This data is consistent with prior published randomized trials and reviews, with some demonstrating that follicular flushing can actually lead to decreased implantation and live birth rate in the poorest responders (17,18).

There may be a theoretical benefit to follicular flushing in IVM (in vitro maturation) cycles, where the immature oocyte is postulated to be more adherent and thus less likely to release from the follicle. However, this has not been conclusively studied. Regardless of preference for flushing follicles, a thorough flush of the tubing should be performed in order to ascertain the correct number of oocytes at the conclusion of the procedure. At our institution, we also flush the tubing when switching from one ovary to the other.

Which Follicles Are Suitable for Aspiration?

There is no specific consensus regarding at which follicular size it is optimal to trigger patients with human chorionic gonadotropin (hCG) or gonadotropin releasing hormone (GnRH) agonist. Most centers have a preferred number and range of follicular diameters, with a typical number of three follicles in the 17–18 mm range. Few studies have evaluated the correlation between follicular size and the resultant yield of the oocyte lot. An animal model using Egyptian Jennies which were stimulated for IVM demonstrated that medium and large follicles were more likely to yield oocytes than small follicles (62%, 60%, and 45.1% for large, medium, and small follicles, respectively), and these results were found to be statistically significant (19). A stringently designed clinical study correlating follicular diameter with oocyte recovery and maturity found that follicles 18–20 mm in size had the highest recovery rate at 80%, and that follicles greater than 15 mm in size were much more likely to yield a mature oocyte than smaller follicles (20). The authors concluded that follicles less than 11 mm at the time of retrieval had a yield and maturity rate that was so low that it was not worthwhile to retrieve them.

Retrieval for In Vitro Maturation Cycles

During IVM cycles, follicles are retrieved at much earlier stages of development, generally when the largest follicle is less than 10, 12, or 14 mm, depending on the program (21–23). Since the follicles are much smaller, modifications have been made to the aspiration needle to suit the task, resulting in the advent of a shorter, narrower needle for IVM procedures than for IVF. By design, these needles have less dead space, but generally require increased aspiration pressures**.** Although more punctures are typically required in IVM retrievals in order to maximize immature oocyte yields, comparable pain scores and infection rates are present between procedures (24). We do not perform IVM at our center.

Anesthesia for Oocyte Retrieval

At our center we have observed that the patient experience is improved with general anesthesia for oocyte retrieval. Additionally, we provide light sedation for patients undergoing minimal stimulation cycles in which only a few follicles are anticipated, but these patients may also choose IV sedation. Alternative options for anesthesia are reviewed on a case-by-case basis given a patient's past medical history, and all high-risk patients are offered a pre-operative anesthesia consultation to establish a plan for the day of retrieval.

When laparotomy and laparoscopy were the early standard method for performing oocyte retrievals, general anesthesia was most commonly used. Once trans-vesical and trans-abdominal approaches were introduced, an even broader range of anesthetic methods, including more minimal anesthetic methods such as light sedation, were popularized. A Cochrane review on pain relief for women undergoing oocyte retrieval identified 21 trials evaluating women undergoing oocyte retrieval. Five different categories of analgesia were examined, comparing conscious sedation, general anesthesia, acupuncture, paracervical block, and patient controlled anesthesia, some of which were compared directly to placebo (25). The results noted that many approaches were considered acceptable to women and were correlated with high degrees of satisfaction. The simultaneous use of more than one method resulted in better pain relief. Notably, a meta-analysis could not be performed due to significant heterogeneity in study designs (25).

Many studies have been conducted into the optimal choice of anesthetic agent for retrieval procedures, with most centers today using propofol. Propofol is a short-acting, intravenously administered hypnotic and amnestic agent commonly used for the induction and maintenance of anesthesia (26). It is a suitable agent for use in oocyte retrieval due to its safety profile and quick onset to action. Early studies evaluating this agent showed that propofol accumulates in follicular fluid, with increasing levels with longer case duration (27). This prompted further evaluation about the potential effects of propofol on oocytes and embryos (28). In a cohort of 130 women, for oocytes retrieved at the beginning, middle, and end of the procedure, no differences were found in egg maturity, fertilization, progression to cleavage stage, or cell count of the resultant embryos (29). In addition to its neutral impact on IVF outcomes, propofol is well tolerated. A prospective observational study evaluating 53 patients undergoing oocyte retrieval deemed that a propofol and minimal fentanyl-based regimen sufficiently maintained sedation according to the Bispectral Index and prevented recall (30).

Thiopental sodium can also be used for general anesthesia during oocyte retrieval. In a recent trial in which 180 patients were randomized to either thiopental anesthesia or propofol, similar fertilization rates and IVF outcomes were reported, with a slight but non-significant increase in pregnancy rates in the propofol group (31). A slight trend towards improved clinical outcome was also present in GIFT cycles in a more historic study (32). However, patients receiving thiopental were more likely to experience side effects such as nausea, emesis, and dizziness, as well as prolonged recovery (31). Thiopental may then be a reasonable anesthetic option for those patients who do not tolerate propofol.

Regional anesthesia is an option for oocyte retrieval, with paracervical block being the most common form. A randomized study evaluated the patient experience with paracervical block vs vaginal lidocaine gel administration. Although oocyte number and procedure duration were similar between groups, total pain experienced was greater with lidocaine vaginal gel compared with lidocaine paracervical block (33).

The effect of topical anesthetics on embryo progression was retrospectively studied with a comparison of the following anesthetic agents: EMLA cream (composed of 2.5% lidocaine and 2.5% prilocaine) one hour pre-operatively applied to vaginal fornices with a tampon placed to preserve the cream in the desired location, versus sedation with sevofluorane, propofol, or thiopental (34). Patient parameters were similar, however the number of oocytes retrieved in the EMLA cream group (average 4.5) was limited by the patient pain experience, with the highest number of oocytes retrieved in the sevofluorane group (average 7.2). Interestingly, fertilization rates were highest in the EMLA and sevofluorane groups, with similar overall rates of embryo development. This difference could be explained in the EMLA group by the oocytes likely being retrieved from the largest and most accessible follicles, leading to the improved per oocyte outcomes (34). Regardless, patients had a comparable level of anxiety between the groups, indicating that topical anesthetics are a reasonable strategy for oocyte retrieval in patients who have small numbers of follicles, or those who wish to avoid or have medical contraindications to general anesthesia.

For patients seeking a combination approach between Eastern and Western medicine, acupuncture can also be offered as an alternative or as a complement to traditional anesthetic methods, though its application may be logistically challenging. Several studies have evaluated acupuncture and electro-acupuncture in comparison to IV agents including opioids and IV anesthetic agents, with overall mixed results about the efficacy of pain relief. However, consistent findings reveal that pregnancy outcomes do not differ dramatically between groups (35–37). Thus, with highly motivated patients and a capacity to perform acupuncture or electro-acupuncture in the operating room, this may be a reasonable, though perhaps arduous, alternative to conventional anesthesia methods.

Complications

There are many theoretical complications involved in oocyte retrieval, but the true complication rate is quite low, especially with experienced surgeons performing the procedure. Complications range from mild bleeding which easily resolves to exceptionally rare complications such as bowel injury (0.001%), peritonitis (0.005%), and vertebral osteomyelitis as a result of hematogenous seeding from sepsis (38,39).

The most common minor complication in oocyte retrieval is vaginal bleeding. The incidence has been reported to range up to as high as 8.3%; however, more typical rates range from 1%–3%, with more significant blood loss of greater than 100 cc's occurring in only 0.8% of cases (24,38,40). A series of 7098 transvaginal retrievals over greater than 20 years reported a 0.06% risk of severe peritoneal bleeding requiring surgical intervention (41). It should be noted that many of the large studies documenting complication rates were published from IVF centers' early experiences, with the possibility that more clinical experience has decreased complication rates. A retrospective study of 4052 oocyte retrievals over a six year period found a 0.35% rate of requiring hospital admission after retrieval, with the majority of these admissions for bleeding (13). Although this population consisted of healthy donors, only six patients (0.15%) required re-operation, with one of the indicated surgeries for ovarian torsion and the remainder for bleeding, further reiterating the relative safety of oocyte retrieval (13).

Pain management can also complicate the post-operative care of patients undergoing oocyte retrieval. Consideration should be given to consultation with a specialist in patients who have a pre-existing dependence on pain medications. Hospitalization for pain management in the post-operative setting is rare—in one prospective trial of greater than 1000 patients over 1 years' duration, 0.7% required hospitalization (38), while the rate in a donor population was just 0.005% (13).

There is also a risk of pelvic infection with oocyte retrieval. Patients with endometriosis appear to be at higher risk for post-operative infection. Although a specific rate has not been published for this population, it appears to be higher than that of the general population, and resultant pelvic infection can be worse in this subset of patients (42). Although it is standard practice at our institution to administer pre-operative antibiotics, we especially recommend antibiotics in this subgroup of patients due to the burden of disease that can occur if these patients become infected. Moreover, it is advisable to avoid interrogating endometriosis lesions with the suction needle. If inadvertent perturbation of a lesion occurs, a prolonged outpatient antibiotic course can be considered.

Urologic complications are another real but very rare risk of oocyte retrieval. Case reports have demonstrated that ureteral injury can be recognized with clinical signs of delayed hematuria, with insidious symptoms of pelvic pain on the first post-operative day. Unfortunately, many of these symptoms can be masked by possible co-existing ovarian hyperstimulation (43,44). Patients with ureteral injury can require varying degrees of surgical intervention, and typically experience full recovery after short-term ureteral stent placement.

Another reported urologic complication is that of ureterovaginal fistula occurring seven days after oocyte retrieval. In the setting of this rare complication, the patient presented with right lower quadrant pain, fever, and eventually developed a clear vaginal discharge (45). Although quite rare, urologic complications should be considered as part of a thorough review of systems for a patient presenting with post-operative pain after oocyte retrieval.

Overall, the safety of oocyte retrieval is well documented. Complication rates can be minimized with the provision of pre-operative antibiotics, an established protocol for retrieval, good pain control with

suitable anesthetic selection, a well trained workforce, and good communication between team members caring for the patient.

Future Techniques

The technique of egg retrieval has evolved a long way over the last 40 years. It has become a very safe and effective process. The procedure will continue to improve, along with modification of needles and ultrasound technology. Patients are comfortable with current anesthesia methods for IVF, but further refinement will continue to improve the patient experience surrounding the egg retrieval process. It is also possible that with the development of 3D ultrasound and appropriate software, oocyte retrieval could become more automated.

REFERENCES

1. Rock J, Menkin MF. In vitro fertilization and cleavage of human ovarian eggs. *Science.* 1944;100(2588):105–7.
2. Johnson MH. Robert Edwards: The path to IVF. *Reprod Biomed Online.* 2011;23(2):245–62.
3. Jones HW. The use of controlled ovarian hyperstimulation (COH) in clinical in vitro fertilization: The role of Georgeanna Seegar Jones. *Fertil Steril.* 2008;90(5):e1–3.
4. Hershlag A. Betadine (povidone-iodine) is toxic to murine embryogenesis. *Fertil Steril.* 2003;79(5):1249–50.
5. Amstey MS, Jones AP. Preparation of the Vagina for Surgery. *JAMA.* 1981;245(8):839–841.
6. Lewis LA, Lathi RB, Crochet P, Nezhat C. Preoperative vaginal preparation with baby shampoo compared with povidone-iodine before gynecologic procedures. *J Minim Invas Gyn.* 2007;14:736–9.
7. Van Os HC, Roozenburg BJ. Vaginal disinfection with povidon iodine and the outcome of in-vitro fertilization. *Hum Reprod.* 1992;7(3):349–50.
8. Hannoun A, Awwad J, Zreik T, Ghaziri G, Abu-Musa A. Effect of betadine vaginal preparation during oocyte aspiration in in vitro fertilization cycles on pregnancy outcome. *Gynecol Obstet Invest.* 2008;66(4):274–8.
9. Tsai Y-C, Lin MYS, Chen S-H, Chung M-T, Loo T-C, Huang K-F, Lin L-Y. Vaginal disinfection with povidone iodine immediately before oocyte retrieval is effective in preventing pelvic abscess formation without compromising the outcome of IVF-ET. *J Assist Reprod Genet.* 2005;22(4):173–5.
10. Tobler KJ, Zhao Y, Weissman A, Majumdar A, Leong M, Shoham Z. Worldwide survey of IVF practices: Trigger, retrieval and embryo transfer techniques. *Arch Gynecol Obstet.* 2014;290(3):561–8.
11. Wikland M, Blad S, Bungum L, Hillensjö T, Karlström PO, Nilsson S. A randomized controlled study comparing pain experience between a newly designed needle with a thin tip and a standard needle for oocyte aspiration. *Hum Reprod.* 2011;26(6):1377–83.
12. Kumaran A, Narayan PK, Pai PJ, Ramachandran A, Mathews B, Adiga SK. Oocyte retrieval at 140-mmHg negative aspiration pressure: A promising alternative to flushing and aspiration in assisted reproduction in women with low ovarian reserve. *J Hum Reprod Sci.* 2015;8(2):98–102.
13. Bodri D, Guillén JJ, Polo A, Trullenque M, Esteve C, Coll O et al. Complications related to ovarian stimulation and oocyte retrieval in 4052 oocyte donor cycles. *Reprod Biomed Online.* 2008;17(2):237–43.
14. Egbase PE, Udo EE, Grudzinskas JG. Prophylactic antibiotics and endocervical microbial inoculation of the endometrium at embryo transfer. *Lancet.* 1999;354(9179):651–2.
15. Fujimoto VY, Agnew KJ, Eschenbach DA. Bacteria in the transfer catheter tip influence the live-birth rate after in vitro fertilization. *Fertil Steril* 2000;74(6):1118–1124.
16. Levens ED, Whitcomb BW, Payson MD, Larsen FW. Ovarian follicular flushing among low-responding patients undergoing assisted reproductive technology. *Fertil Steril.* 2009;91(4):1381–4.
17. Hill MJ, Levens ED. Is there a benefit in follicular flushing in assisted reproductive technology? *Curr Op Obstet Gynecol.* 2010;22(3):208–12.
18. Mok-lin E, Brauer AA, Schattman G, Zaninovic N, Rosenwaks Z, Spandorfer S. Follicular flushing and in vitro fertilization outcomes in the poorest responders: A randomized controlled trial. *Hum Reprod.* 2013;28(11):2990–5.

19. Abdoon ASS, Abdel-Rahman HA, Shawki SM, Kandil OM, Fathalla SI. Influence of follicle size, methods of retrieval on oocytes yield and morphology in Egyptian Jennies ovaries with special reference to maturation rate in vitro. *Vet Res Commun*. 2014;38(4):287–95.
20. Scott RT, Hofmann GE, Muasher SJ, Acosta AA, Kreiner DK, Rosenwaks Z. Correlation of follicular diameter with oocyte recovery and maturity at the time of trans-vaginal follicular aspiration. *J in Vitro Fertil Em*. 1989;6(2):73–5.
21. Asimakopoulos B, Kotanidis L, Nikolettos N. In vitro maturation and fertilization of vitrified immature human oocytes, subsequent vitrification of produced embryos, and embryo transfer after thawing. *Fertil Steril*. 2011;95(6):2123.e1–2123.e2.
22. Junk SM, Yeap D. Improved implantation and ongoing pregnancy rates after single-embryo transfer with an optimized protocol for in vitro oocyte maturation in women with polycystic ovaries and polycystic ovary syndrome. *Fertil Steril*. 2012;98(4):888–92.
23. Mikkelsen AL, Lindenberg S. Morphology of in-vitro matured oocytes: Impact on fertility potential and embryo quality. *Hum Reprod*. 2001;16(8):1714–8.
24. Seyhan A, Ata B, Son W-Y, Dahan MH, Tan SL. Comparison of complication rates and pain scores after transvaginal ultrasound-guided oocyte pickup procedures for in vitro maturation and in vitro fertilization cycles. *Fertil Steril*. 2014;101(3):705–9.
25. Kwan I, Bhattacharya S, Knox F, Mcneil A. Pain relief for women undergoing oocyte retrieval for assisted reproduction. *Cochrane Db Syst Rev*. 2005;3.
26. Chidambaran V, Costandi A, D'Mello A. Propofol: A review of its role in pediatric anesthesia and sedation. *CNS Drugs*. 2015;29(7):543–63.
27. Coetsier T, Dhont M, De Sutter P, Merchiers E, Versichelen L, Rosseel MT. Propofol anaesthesia for ultrasound guided oocyte retrieval: Accumulation of the anaesthetic agent in follicular fluid. *Hum Reprod*. 1992;7(10):1422–4.
28. Christiaens F, Janssenswillen C, Verborgh C, Moerman I, Devroey P, Van Steirteghem A et al. Propofol concentrations in follicular fluid during general anaesthesia for transvaginal oocyte retrieval. *Hum Reprod*. 1999;14(2):345–8.
29. Ben-Shlomo I, Moskovich R, Golan J, Eyali V, Tabak A, Shalev E. The effect of propofol anaesthesia on oocyte fertilization and early embryo quality. *Hum Reprod*. 2000;15(10):2197–9.
30. Circeo L, Grow D, Kashikar A, Gibson C. Prospective, observational study of the depth of anesthesia during oocyte retrieval using a total intravenous anesthetic technique and the Bispectral index monitor. *Fertil Steril*. 2011;6(3):635–637.
31. Goutziomitrou E, Venetis CA, Kolibianakis EM, Bosdou JK, Parlapani A, Grimbizis G et al. Propofol versus thiopental sodium as anaesthetic agents for oocyte retrieval: A randomized controlled trial. *Reprod Biomed Online*. 2015;31(6):752–9.
32. Pierce ET, Smalky M, Alper MM, Hunter JA, Amrhein RL, Pierce Jr EC. Comparison of pregnancy rates following gamete intrafallopian transfer (GIFT) under general anesthesia with thiopental sodium or propofol. *J Clin Anesth*. 1992;4(5):394–8.
33. Tummon I, Newton C, Lee C, Martin J. Lidocaine vaginal gel versus lidocaine paracervical block for analgesia during oocyte retrieval. *Hum Reprod*. 2004;19(5):1116–20.
34. Piroli A, Marci R, Marinangeli F, Paladini A, Di Emidio G, Artini PG et al. Comparison of different anaesthetic methodologies for sedation during in vitro fertilization procedures: Effects on patient physiology and oocyte competence. *Gynecol Endocrinol*. 2012;28(10):796–9.
35. Wang W, Zhang XH, Wang WH, Liu YL, Zhao LH, Xue SL et al. The time interval between hCG priming and oocyte retrieval in ART program: A meta-analysis. *J Assist Reprod Genet*. 2011;28(10):901–910.
36. Meng P, Wang L, Xu B, Sun H. Application of acupuncture compund anesthesia in transvaginal ultrasound-guided oocyte retrieval. *Zhongguo Zhen Jiu*. 2008;28(6):451–5.
37. Humaidan P, Stener-Victorin E. Pain relief during oocyte retrieval with a new short duration electro-acupuncture technique—An alternative to conventional analgesic methods. *Hum Reprod*. 2004;19(6):1367–72.
38. Ludwig AK, Glawatz M, Griesinger G, Diedrich K, Ludwig M. Perioperative and post-operative complications of transvaginal ultrasound-guided oocyte retrieval: Prospective study of >1000 oocyte retrievals. *Hum Reprod*. 2006;21(12):3235–40.
39. Almog B, Rimon E, Yovel I, Bar-am A, Amit A, Azem F. Vertebral osteomyelitis: A rare complication of transvaginal ultrasound- guided oocyte retrieval. *Fertil Steril*. 2000;73(6):1250–2.

40. Bennett SJ, Waterstone JJ, Cheng WC, Parsons J. Complications of transvaginal ultrasound-directed follicle aspiration: A review of 2670 consecutive procedures. *J Assist Reprod Gene.t* 1993;10(1):72–7.
41. Aragona C, Mohamed MA, Espinola MSB, Linari A, Pecorini F, Micara G et al. Clinical complications after transvaginal oocyte retrieval in 7,098 IVF cycles. *Fertil Steril.* 2011;95(1):293–4.
42. Romero B, Aibar L, Martínez Navarro L, Fontes J, Calderón M-A, Mozas J. Pelvic abscess after oocyte retrieval in women with endometriosis: A case series. *Iran J Reprod Med.* 2013;11(8):677–80.
43. Papler TB, Vrta E, Bokal I, Šalamun V, I DG, Smrkolj TD et al. Ureteral injury with delayed massive hematuria after transvaginal ultrasound-guided oocyte retrieval. *Case Rep Obstet Gynecol.* 2015;DOI 10.1155/2015/760805.
44. Grynberg M, Berwanger AL, Toledano M, Frydman R, Deffieux X, Fanchin R. Ureteral injury after transvaginal ultrasound-guided oocyte retrieval: A complication of in vitro fertilization-embryo transfer that may lurk undetected in women presenting with severe ovarian hyperstimulation syndrome. *Fertil Steril.* 2011;96(4):869–71.
45. Mongiu AK, Helfand BT, Kielb SJ. Ureterovaginal fistula formation after oocyte retrieval. *Urology.* 2009;73(2):444.e1–3.

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In Vitro Maturation for Clinical Use

Melanie L. Walls and Roger Hart

Introduction

In vitro maturation (IVM) is an alternative form of in vitro fertilization (IVF), whereby the patient receives little or no gonadotropin stimulation and collected oocytes complete their final stages of maturation in the laboratory. It was first described in an animal model (1) and later replicated in a human model, after oocytes collected from unstimulated follicles underwent spontaneous maturation (2). The first live birth was recorded in 1991, after oocyte maturation following collection by ovarian biopsy (3), and then in 1994 using trans-vaginal techniques (4). The primary purpose for IVM treatment is to avoid the adverse outcomes and additional costs associated with the administration of follicle stimulating hormone (FSH). However, IVM also has the potential to overcome other causes of infertility such as gamete donation, FSH resistance, avoiding the effects of an elevated estradiol, and for fertility preservation. It is estimated that more than 3000 children have been born worldwide from this method, and although some clinics routinely perform IVM, it is still considered a research technique. This chapter will explore the patient cohorts, treatment and laboratory protocols, success rates, and birth outcomes reported from around the world in clinics performing IVM, as well as addressing areas in need of further study and future trends for IVM.

Clinical Indications for In Vitro Maturation

Patient Cohort

For most clinics, the primary function for IVM treatment is to help women with polycystic ovaries (PCO) avoid ovarian hyperstimulation syndrome (OHSS). This is a severe medical condition where not only does the woman suffer from extreme discomfort due to the rapid development of ascites, but she runs the serious risk of a thrombotic event. However, IVM is now applied to a range of patients with fertility issues including fertility preservation, hormone sensitive cancers, FSH resistance, and cost conservative patients.

Polycystic Ovaries (PCO) and Polycystic Ovarian Syndrome (PCOS)

For women with PCO/PCOS, OHSS is a significant clinical consequence of gonadotropin stimulation, resulting in patient discomfort in the mild stages and significant morbidity in the major forms of the condition (5). IVM is currently the only treatment option which completely eliminates the risk of OHSS (6). In terms of success rates, the antral follicle count is a major determinant of IVM treatment (7), and patients with PCOS typically have a high antral follicle count and therefore respond better to the treatment. Women with PCOS typically have more oocytes collected from IVM cycles than patients without the condition, leading to a greater pregnancy rate (8,9).

The inclusion of women with regular menstrual cycles without PCO or PCOS in IVM treatment protocols is more controversial, and as the antral follicle count is the determining factor for IVM treatment, patients with fewer than five antral follicles should not be considered for this treatment (10). This taken

into account, IVM has proven to be a successful treatment option for non-PCOS patients who do have suitable antral follicle counts (11). The literature comparing patient cohorts is limited to those assessing stimulation protocols (10), with patient selection criteria emerging as a by-product for success, with further research needed in the area.

Fertility Preservation

Oncofertility is an emerging area of assisted reproductive technology (ART) whereby patients need to store their gametes and embryos prior to treatment for a range of cancers for which their future fertility is at risk (12). IVM is a viable oncofertility treatment option for patients with estrogen sensitive cancers, an additional diagnosis of PCOS, or as a methodology for maturing oocytes collected ex vivo at the time of oophorectomy or ovarian tissue cryopreservation.

Estrogen Sensitive Cancer/Avoidance of OHSS

Breast cancer is the most common malignancy in women seeking fertility preservation prior to chemo/radiotherapy. In patients where the cancer is estrogen sensitive, IVM provides a safer alternative to controlled ovarian hyperstimulation as it can be performed without using exogenous gonadotropins, alleviating the risk of increasing circulating estrogen levels, and may be their only treatment option (13,14). Additionally, IVM can be a suitable option for those patients whose malignancy is not estrogen sensitive, as it completely eliminates the risk of OHSS (6,15), therefore enabling the patient to proceed immediately into chemo/radiotherapy without delaying their lifesaving cancer treatments.

At the Time of Oophorectomy or Ovarian Tissue Cryopreservation

IVM has been successfully performed at the time of oophorectomy and laparoscopic ovarian wedge resection for ovarian tissue cryopreservation in patients with Hodgkin's lymphoma, breast cancer, and rectal cancer (16). Immature oocytes were aspirated from visible follicles in the laboratory prior to ovarian cortical tissue cryopreservation, with all patients achieving successful storage of at least one mature oocyte as well as ovarian tissue cryopreservation. This technique provides the patient with two options for future reproductive assistance.

In a recent case report (17), IVM was successfully performed with oocytes collected ex vivo, following laparoscopic oophorectomy (Figure 9.1). There were 22 GV stage oocytes collected from both ovaries,

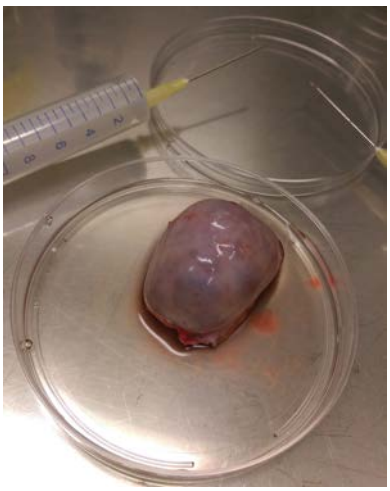

FIGURE 9.1 Ovary in the laboratory following laparoscopic oophorectomy and prior to ex vivo oocyte aspiration.

with 15 mature oocytes vitrified after 24 hours and four more vitrified after an additional 24 hours in maturation culture. It is unclear whether oocyte survival and subsequent fertilization and development will be successful; however, a large number of cryopreserved oocytes will provide the patient with the best chance of future fertility. A live birth after in vitro maturation of oocytes collected after oophorectomy has been reported (18), There is little literature available on oocyte survival rates following IVM; however, oocyte vitrification in standard IVF treatment significantly improves oocyte survival, fertilization, and embryo quality, as well as clinical and ongoing pregnancy rates, compared with slow freezing techniques (19), and hopefully this will translate to oocytes collected from IVM cycles. In situations where the patient is not currently in a relationship and under time limiting circumstances, oocyte cryopreservation, as opposed to embryo cryopreservation, is necessary and IVM is often the only treatment which is capable of achieving this outcome.

Conversion from IVF

IVM may be performed in order to avoid cycle cancellation or OHSS for patients who begin to recruit too many follicles. This method was shown to be successful in patients seeking fertility preservation (20), as these patients would otherwise have had their cycles cancelled, or led to a delay in their cancer treatment. Instead, they went on to have multiple mature oocytes and embryos stored for their future fertility. This protocol can be extended to other patients as a method of avoiding OHSS, providing the patient is appropriately counseled and consent given for the conversion of their treatment type.

Avoidance of OHSS

In addition to oncofertility related treatments, IVM can be used in cases to avoid OHSS. In rare circumstances, such as a patient who lives in a rural or remote area, a treatment such as IVM enables the patient to fly home directly after treatment, without increasing the risk of OHSS or a thrombotic event, as flying is not usually recommended after standard IVF treatment. This could also be useful in cases such as for a young egg donor with a good antral follicle count, where infertility is not an issue and a minimalist approach to stimulation might be preferable to avoid unnecessary side effects from ovarian stimulation. Whilst there is no documented evidence of IVM being employed in these cases, they are available in clinics that routinely perform IVM.

Prothrombotic Risk

It is well established that a serious cause of morbidity after IVF treatment is a thrombotic event, due to the elevated estradiol generated as part of an IVF cycle being an activator of the clotting cascade, and potentially exacerbated by the hemoconcentration associated with OHSS (21). IVM has the potential to reduce the risk of prothrombotic disorders by maintaining a low circulating estrogen level and eliminating the OHSS risk. There is yet to be any reported cases of IVM being used where the determining factor was a prothrombotic disorder. However, providing the antral follicle count is suitable to indicate IVM as a realistic treatment option, there is no evidence to suggest this methodology could not be successful.

IVM for FSH Resistant Ovaries

A pregnancy and live birth has been achieved in a patient with FSH resistant ovaries (22). Researchers performed their standard IVM protocol on a patient who was diagnosed with early onset ovarian failure and referred to the clinic for donor oocytes. A suitable antral follicle count meant IVM was a viable option with maturation, fertilization, and embryo development rates comparable to the clinics results in other IVM patients. The success of this case highlights the benefit of having IVM as a treatment option in ART clinics where the only other viable option to the patient is oocyte donation.

Clinical Protocols and Results

Priming Protocols

In theory, IVM requires no exogenous gonadotropin administration. Stimulation protocols and culture conditions have been the focus of most studies to date regarding IVM. As with conventional IVF, the in vivo preparation involving the patient is a fundamental part of the oocyte development prior to any treatments in vitro. The use of hormonal priming with exogenous gonadotropins, as opposed to natural cycle IVM, is still widely debated (23). The use of FSH and human chorionic gonadotropin (hCG) or human menopausal gonadotropin (hMG) to "prime" the follicles prior to oocyte collecting is a varied and sometimes contradictory practice.

In theory, FSH stimulation at the onset of a patient's menstrual cycle could increase the number of oocytes collected, make the collection process easier, and enhance maturation (23). The results, however, are conflicting and difficult to evaluate due to differences in priming and culture protocols. Initial investigations showed that FSH priming in ovulatory women without PCO did not improve oocyte numbers retrieved, their maturation rate, or the embryo cleavage rate or embryo development (11). However, a more recent study by the same individuals showed that FSH priming improved maturation potential and implantation rates in women diagnosed with PCOS (10). In more recent years, the use of FSH priming and collection from larger follicles has led to the highest reported success rates in IVM (9).

Follicular priming using human chorionic gonadotropin (hCG) is still a common practice; however, it is considered by many to not be true IVM, as it disrupts the oocyte cumulus cell interaction, induces initial stages of maturation in vivo, and is not necessary for a successful IVM program (9). Additionally, hCG priming can make results difficult to interpret and laboratory practices logistically challenging, as oocytes are at varying stages of development when they are collected. This in turn leads to differing injection times and embryo culture stages which, for a busy ART laboratory, is not an ideal practice. That being said, hCG priming has demonstrated effectiveness in advancing oocyte nuclear maturation in women with PCOS (24). It has also been found that increasing the timing of the hCG injection from 36 hours to 38 hours prior to oocyte retrieval has led to increasing oocyte maturation rates in women with PCOS (25).

A summary of the outcomes of hormonal priming and IVM success rates is shown in Table 9.1.

Oocyte Collection Procedure

Oocyte collection protocols for IVM vary widely between clinics; however, they are based around a standard trans-vaginal oocyte aspiration (TVOA) procedure with modifications to collect oocytes from much smaller follicles. The collection procedure for IVM is important in order to maximize oocyte yield, whilst maintaining oocyte integrity and minimizing developmental impacts for maturing oocytes. Figure 9.2 shows a typical IVM TVOA tube set up. Aspiration pressure has been reported in ranges from 7 kpa (52.5 mmHg) (28) to 200 mmHg (36), with some centers reporting lower aspiration of up to a third of that used for standard IVF collection (8). The use of a double or single lumen needle depends on whether the clinician performing the technique flushes the follicle with some reports of follicular flushing each follicle up to three times (15). Additionally, there are variations in solutions used to flush the follicles including Hartmann's (15) or Hepes (36) supplemented with heparin, and many clinics do not flush the follicles at all (37).

The exact impact and effects of flushing solutions and collection media on oocyte maturation and development are unknown. Simple flush medium is potentially problematic as it is not suited to support metabolic requirements of immature oocytes and the utilization of a specially formulated, amino acid enriched flushing media may be a better option. All of these slight modifications to IVM protocols may impact on the oocytes' competence, and it is paramount to oocyte integrity that it is supported from the moment it is removed from the follicle. Once removed, there is a cascade of events leading to a dramatic decrease in cyclic Adenosine Monophosphate (cAMP) activity within the oocyte. It is important that oocytes are transferred to media as quickly as possible to maintain developmental capacity and limit exposure to any contents of collection solutions that do not maximize an oocytes potential. Additionally,

TABLE 9.1

A Summary Table Reporting Clinical Outcomes of Human In Vitro Matured Oocytes according to Patient Selection and Hormonal Priming

Authors	Patient Cohort	Hormonal Priming	Mean # Oocytes per TVOA	% Mature	% Fertilised	% Cleaved	% Useable Blastocysts	Mean # Embryos Transferred	Implantation Rate (PR per ET)	# of Live Births
Cha et al. (26)	PCOS	Nil	13.6	62.2	ICSI/68	88	N/A	4.9	27.1	20
Cha et al. (27)	PCO/PCOS	Nil	15.5	–	–	–	N/A	5.0	21.9	24
Chian et al. (24)	PCOS	10,000 IU hCG vs nil	7.8	84.3	ICSI/90.7	94.9	N/A	2.8	38.5	3
			7.4	69.1	ICSI/83.9	95.7	N/A	2.5	27.3	9
Child et al. (28)	Normal vs. PCO vs. PCOS	10,000 IU hCG	5.1	78.4	ICSI/72.5	93.1	N/A	2.6	4.0	2
			10	76	ICSI/76.3	94.8	N/A	3.3	23.1	9
			11.3	77	ICSI/79.3	91.3	N/A	3.2	29.9	10
Da Silva-Buttkus et al. (29)	PCOS	10,000 IU hCG vs 150 IU FSH for 3 days	9.0	69.3	67.3	61.2		2.3	50	3
			8.9	55.1	57.5	51.9		1.4	0	0
Gougeon (30)	Previous OHSS	Nil vs 150 IU hMG for 2 days vs 10,000 IU hCG	18	74.8	ICSI/76	89.2	N/A	5.1	22.2	5
			16.6	70.7	ICSI/79.7	85.7	N/A	4.9	25.0	2
			17.6	73	ICSI/80.3	94.5	N/A	5.0	39.1	7
Gremeau et al. (31)	PCO/PCOS (included IVF controls)	10,000 IU hCG	15.8	65.01	62.9	N/A	N/A	1.9	12.9	16
Junk and Yeap (9)	PCO vs PCOS	100–150 IU FSH 3 days	8.8	68.9	73.9	N/A	41.1	1.0	44.4	8
			14.4	69.9	70.8	N/A	41.8	1.0	47.7	20
Le Du et al. (32)	PCOS	10,000 IU hCG	11.4	63.0	ICSI/70.1	96.3	N/A	2.5	22.5	6
Lin et al. (33)	PCOS only	10,000 IU hCG vs 10,000 IU hCG plus 75 IU FSH for 6 days	23.1	71.9	ICSI/69.5	88.1	N/A	3.8	36.4	–
			21.9	76.5	ICSI/75.8	89.4	N/A	3.8	31.4	21
Mikkelsen and Lindenberg (10)	PCOS only	150 IU FSH for 3 days vs nil	6.5	59	ICSI/70	56	N/A	1.8	33	3
			6.8	44	ICSI/69	64	N/A	1.7	0	0
Mikkelsen et al. (11)	Non-PCOS,	150 IU FSH for 3 days vs nil	4	85	ICSI/65	62	N/A	1.9	11.76	–
			3.7	76	ICSI/62	54	N/A	1.7	18.75	–

(*Continued*)

TABLE 9.1 (*Continued*)

A Summary Table Reporting Clinical Outcomes of Human In Vitro Matured Oocytes according to Patient Selection and Hormonal Priming

Authors	Patient Cohort	Hormonal Priming	Mean # Oocytes per TVOA	% Mature	% Fertilised	% Cleaved	% Useable Blastocysts	Mean # Embryos Transferred	Implantation Rate (PR per ET)	# of Live Births
Söderström-Anttila et al. (8)	PCO vs. PCOS	Nil	9.3	54.9	IVF/35	85.7	N/A	1.7	22.2	2
		Nil	14.3	58.2	ICSI/72.4	61.9	N/A	2.0	0	0
					IVF/43.8	82.5	N/A	1.7	52.9	6
					ICSI/78.4	70.9	N/A	1.8	22.2	1
Son et al. (34)	Previous risk of OHSS	10,000 IU hCG	5.98	11.6 (D0)	77.7	100	58.3	2.9	26.4	N/A
				52.5 (D1)	84.4	91.5	50.4			
				13.6 (D2)	81.6	72.2	11.3			
Son et al. (25)	PCOS	10,000 IU hCG 36 hrs before TVOA vs 10,000 IU hCG 38 hrs before TVOA	17.3	65.1	73.0	89.9	N/A	3.7	25.0	N/A
			19.1	72.5	73.4	92.4	N/A	3.6	40.9	N/A
Walls et al. (35)	PCOS	100–150 IU FSH for 3–5 days	18.75	IVF/79.2	IVF/59.6	N/A	41.2	1.0	42.9	6
				ICSI/71.8	ICSI/67.9	N/A	47.7			
Walls et al. (15)	PCO (included IVF controls)	100–150 IU FSH for 3–5 days	13.2	73	68	N/A	38	1.0	29.7 (Fresh) 35.5 (Frozen)	12 (Fresh) 21 (Frozen)
Yoon et al. (36)	Non-PCOS	Nil	9.0	74.3	72.6	89	N/A	3.6	17.6	6

Abbreviations: PR, Pregnancy Rate; ET, Embryos Transfer; IU, International Units; N/A, Information not available.

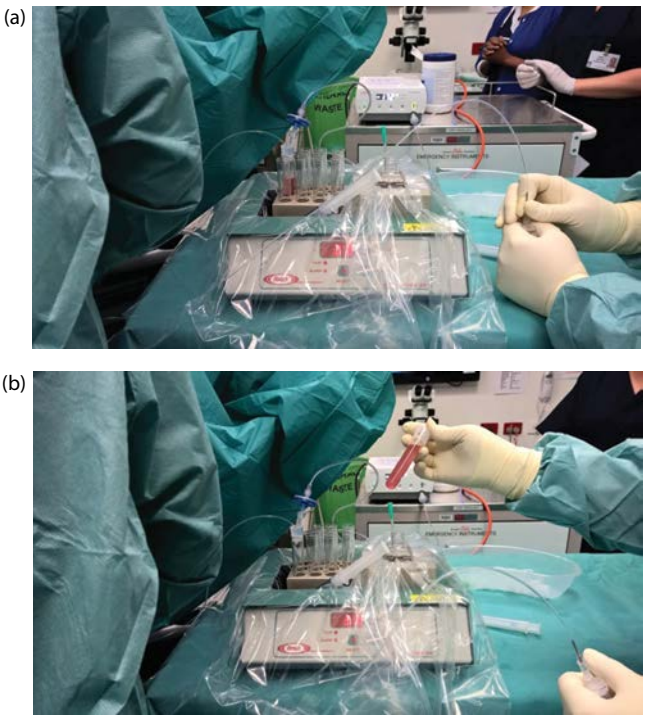

FIGURE 9.2 Demonstrates the tube set up and flushing procedure during an IVM, with 174 mmHg pressure and tube warmer at 37°C. Each follicle is flushed and the aspirated contents enter the test tube in the heat block (a). The test tube and contents are then passed to the Embryologist for oocyte identification (b).

most centers performing IVM will also use a mesh cell strainer (Figure 9.3) to filter follicular aspirates in order to identify the oocytes (8,37). This is not always the case, as some centers also report identifying oocytes by sight (9,15), and this may minimize manipulation and pipetting pressure. However, it may also contribute to the increase in length of the TVOA procedure, as without the expanded cumulus mass, oocytes are more difficult to identify (Figure 9.4).

Culture Conditions

After removal of the cumulus-oocyte complex from the follicle, there is a significant drop in the amount of gap junction communication between the cumulus cells and the oocyte, which needs to be managed in order to prevent spontaneous maturation and to promote synchrony between nuclear and cytoplasmic maturation (38). Various culture media have been formulated for the use in IVM. The two mostly widely used, commercially available IVM base media, Sage (Cooper Surgical, USA) and Medicult (Origio, Denmark) were found to have equal efficacy (39). Media specially formulated for blastocyst culture have been used successfully (9), but have also shown equal efficacy in terms of maturation, fertilization, and blastocyst development when compared with Sage media (40). In more recent years investigations have focused on factors which influence embryo development, and numerous additives have been tested with varying degrees of success, including an exogenous protein source, growth factors, steroids, energy sources, hormones, oocyte secreted factors, activators, and inhibitors.

Exogenous Gonadotropins in the Culture Media

Currently, hormonal additives are a universal component for all culture media in human IVM. Following the removal of immature oocytes from follicles there is a rapid and significant decrease in cAMP concentrations, which is thought to lead to asynchrony between oocyte nuclear and cytoplasmic maturation.

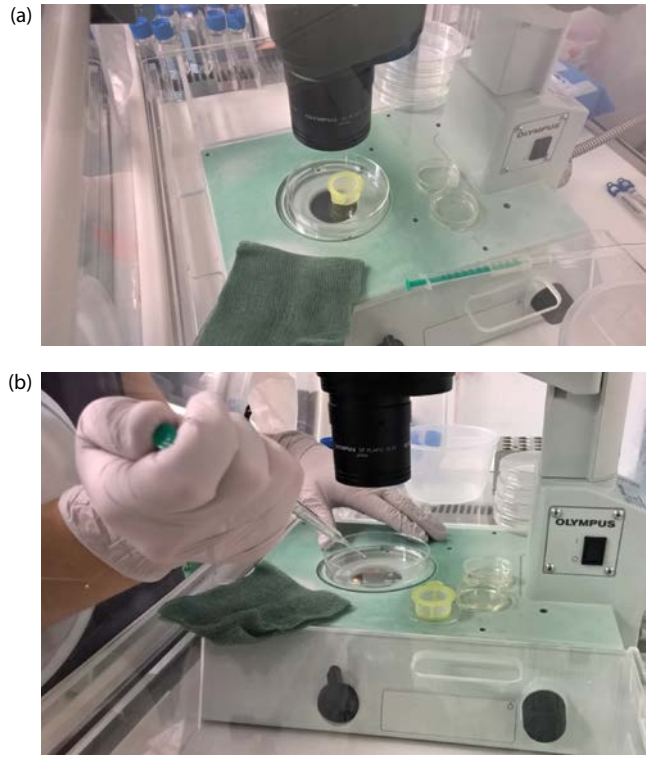

FIGURE 9.3 Typical IVM set up in theatre, including a cell strainer (a) used in oocyte collection procedures or glass pipette (b) to identify and collect by sight.

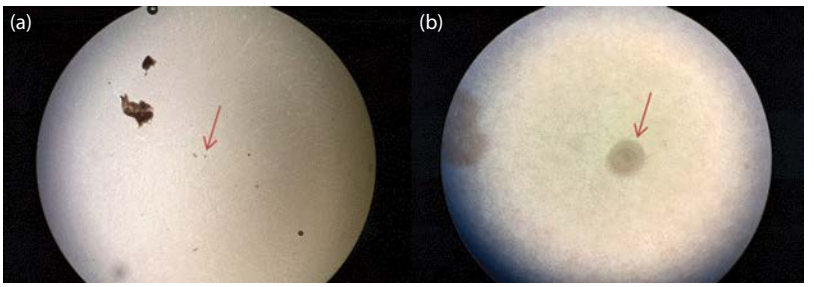

FIGURE 9.4 Oocyte located in petri dish under stereo microscope during IVM collection at low (a) and high (b) magnification on an SXZ12 stereo microscope (Olympus).

FSH is the most common method to encourage cumulus oocyte complex (COC) expansion and the subsequent rise in cAMP activity leading to increased oocyte maturation. Figure 9.5 demonstrates the coronal cells before and after maturation culture. The concentration of FSH added is relatively consistent, with 0.075 IU/mL being the dose most widely used in clinical trials (8,11,41). A much higher concentration of 0.75 IU/mL did not seem to have any beneficial impact on maturation rates (32).

In order to replicate the LH surge in vivo, recombinant LH and hCG are often added to the culture media. Oocytes can undergo spontaneous maturation without the exposure to LH or hCG; however, this may promote asynchrony between nuclear and cytoplasmic maturation, effecting developmental outcomes. The greatest implantation and live birth success rates in IVM protocols were achieved using a combination of FSH and hCG.

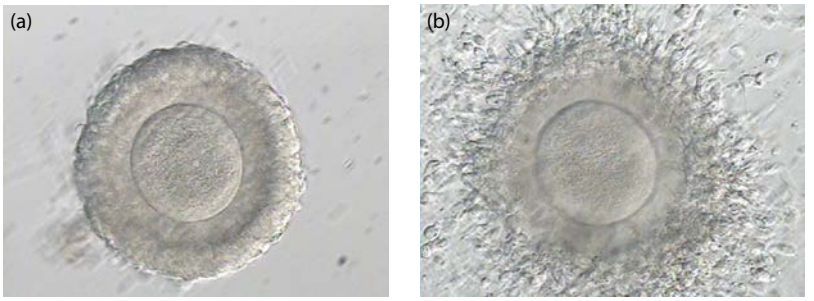

FIGURE 9.5 (a) Compact coronal cells surrounding GV stage oocyte at collection and (b) expanded coronal cells following 24 hours maturation culture.

Growth Factors in the Culture Medium

Growth factors in oocyte maturation culture media are less common and should be used with caution as the impacts on epigenetic variation are unknown. The addition of insulin like growth factor (IGF-I) to the culture media has been used by some who suggested it promotes granulosa cell division and therefore functions to encourage cumulus cell expansion and nuclear maturation (42). Similarly, epidermal growth factor (EGF) has been shown to aid in maturation rates (42) involving the GV breakdown and polar body extrusion (43). EGF has also been shown to play a role in enhancing fertilization when cultured with cumulus intact oocytes (44). More recently, EGF family members' recombinant human Areg and Ereg also increased maturation rates of oocytes in vitro (44). Growth hormone (GH) in an in vitro setting has been widely studied in animal models, but the research is still lacking in a human model.

Protein Source

A source of protein is essential in the IVM culture media. In humans a protein source may be derived from one of three main sources: human follicular fluid (HFF), inactivated autologous patient serum (human serum), or fetal bovine serum (FBS). HFF has been used in doses ranging from 30% (34) up to 70% (36). Patient serum is used at the concentration of either 10% (8,11) or 20% (28,33), with inactivated FBS also used in concentrations of 10% (45) or 20% (24,26). The use of human serum in culture media provides a number of nutrients and factors, not always found in synthetic serums, that are involved in the maturation process. However, there are a number of negative impacts for the developing embryos found in standard IVF embryo culture, as well as the risk of contributing unknown contaminants to the cultured embryos when using serum or HFF preparations. It is not clear whether the negative effects of serum in embryo culture translate to its use in oocyte maturation culture. In most circumstances the oocytes are only exposed to the maternal serum for the first 24–48 hours, depending on the protocol employed, and are then moved into commercial embryo culture media prior to insemination. Additionally, the culture environment for in vitro oocyte maturation is attempting to mimic the in vivo intra-follicular environment, and follicular fluid and serum have very similar elements.

Oxygen Tension in Culture

In conventional IVF, steps are now taken to mimic the environment in vivo by utilizing low oxygen tension, similar to that experience by the embryo in vivo (46). While many laboratories around the world continue to use atmospheric levels of oxygen, there is a general consensus in the ART field that low oxygen tension significantly improves clinical pregnancy and live birth rates (47). As a consequence, most centers also use low oxygen tension in their IVM culture. However, there is yet to be any research published comparing oxygen concentration during in vitro maturation on human oocytes.

Fertilization of IVM Oocytes

Due to extended maturation culture timing, it was originally assumed that oocytes obtained following IVM culture would require intracytoplasmic sperm injection (ICSI) due to hardening of the zona pellucida. The first study to compare insemination techniques following IVM treatment reported that although fertilization rates were significantly lower in the IVF inseminated group compared with the ICSI inseminated group, the embryo cleavage and implantation rates were higher for PCO and PCOS patients (8). A sibling oocyte study comparing fertilization techniques in IVM oocytes found no difference in fertilization, useable blastocyst development, total blastocyst development, or implantation rates between the two insemination techniques (35). It is important to remember that even though using sibling oocytes to compare techniques minimizes confounding factors affecting results, there is a potential flaw in the timing at which maturation/fertilization check is performed between ICSI and IVF. IVF oocytes are allowed an additional 24 hours prior to denudation and, therefore, additional time to undergo spontaneous maturation and potential for fertilization. Regardless of this, IVF appears to be an acceptable fertilization method for IVM oocytes, where sperm parameters are suitable.

IVM Embryo Culture

Until recently the most common culture period for human clinical IVM embryos are two to three days post insemination (8,26,28). However, blastocyst culture is also becoming more prevalent in IVM (9,15,40) and the determining factor for culture periods of IVM derived embryos should reflect the normal culture practices of the clinics performing the technique.

Recent advances in technology for embryo culture systems have also led to the inclusion of time lapse incubation systems being utilized for IVM. Analyses of embryo development using time lapse monitoring of embryo development found an increase in some abnormal phenotypic events in PCOS-IVM embryos. Furthermore, an increase in the rate of early arrest of IVM embryos was noted; however, IVM treatment did not alter morphokinetic development of embryos suitable for transfer of freezing (48).

Endometrial Priming

Endometrial priming is typically utilized through estrogen and progesterone supplementation, and has been shown to be beneficial when administration begins at the mid-follicular timing of the cycle (49). Recently, the highest implantation and live birth rates were achieved using a protocol of estrogen supplementation two days prior and progesterone supplementation on the day of TVOA (50) (Figure 9.6). However, when employing the same protocol, it was also shown that IVM embryos are significantly more likely to implant and result in a live birth following frozen embryo transfers (15). This demonstrates the potential asynchrony between the embryo and endometrium following IVM fresh transfers, and a freeze-all protocol may be beneficial to improve implantation and live birth rates.

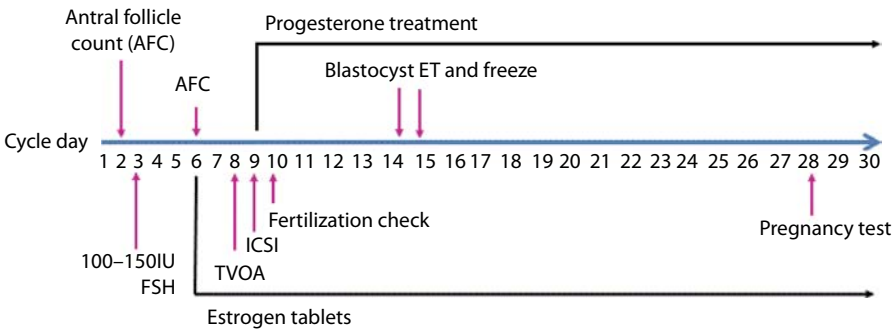

FIGURE 9.6 Protocol timeline for IVM hormonal priming and endometrial preparation.

Birth and Long-Term Outcomes of Children Following IVM Treatment

Children born from IVF have a greater risk of congenital abnormalities (51), imprinting disorders, and longer term health risks (52,53). Furthermore, women with PCOS are more likely to need fertility assistance and have significant health concerns (54), and their children are at a greater risk of medical complications in early life and childhood (55). Data available on the incidence of congenital birth defects following IVM treatment is limited, and incidence rates include 0% (9,56), 2.2% (57), and 7.9% (27). Several publications have now demonstrated no difference in congenital birth defects when compared to conventional IVF treatments, although sample sizes were small (15,58).

Table 9.2 demonstrates neonatal outcomes of IVM children reported in the literature. Other measurements of neonatal health including Apgar scores have been reported and are within normal ranges (56) or show no significant difference to controls in singleton live births (58,59,60). The incidence of adverse outcomes is often confounded by multiple births as many IVF centers worldwide still routinely transfer multiple embryos, even though evidence suggests this is not best practice (61). Regardless of multiple birth outcomes, the incidence of congenital birth defects, pre-term birth, and low birth weight, which are often associated with ART treatments, especially in PCOS patients, are low following IVM treatment.

TABLE 9.2

Reported Neonatal Outcomes Following IVM Treatment

Publication	Live Birth Twins	Live Birth Singletons	Congenital Birth Defects (%)	Mean Birthweight Grams (Twins)	Pre-Term Birth <37 Weeks (Twins)	Other Comments
Cha et al. (27)	4	24	2 (8.3)	3252 ± 516 (2361 ± 304)	1 2 (one set)	Additional case reported of omphalocele and death in utero following a twin pregnancy. Remaining infant resulted in a healthy live birth.
Mikkelsen (57)	2	46	1 (2.2)	3720	1 2 (one set)	One case of a stillbirth.
Söderström-Anttila et al. (56)	6	40	0 (0)	3550 ± 441 (2622 ± 194)	2 (4)	Reported minor developmental delay overexpression at 12 months with normal development at 2 year follow-up.
Fadini et al. (58)	43	153	10, 2 from twin births (6.5)	3269 ± 616 (2311 ± 577)	26 (15)	IVF controls included: Singleton IVM live births showed significantly higher birthweights and IVM twin live births reported significantly lower gestational age at birth.
Foix-L'Helias et al. (59)	4	34	Not reported	3119.5 ± 871	Not reported	Also reported on growth and development at birth, 12 and 24 months.
Walls et al. (15)	0	33	1 (3.0)	3364 ± 590 (N/A)	2 (N/A)	IVF controls included: No significant difference between IVF and IVM for any neonatal outcomes measured.

There is currently no long-term data on children born from IVM; however, the limited literature available on the follow-up to children born from this technique show favorable results. Children born from IVM treatment display normal rates of physical growth at all stages, as well as neurological and neuropsychological outcomes, compared to the general population, at 24 months (56).

In a cohort of French children born following IVM or standard ICSI treatment, at birth, female infants born following IVM displayed increased mean weight, height, head circumference, and BMI compared to their controls. At 2 years of age, mean weight and BMI remained significantly higher than controls, although the authors conceded that these findings may be related to underlying infertility and PCOS rather than the IVM procedure (59). Prior to this, a study had found no difference in height or weight between IVM conceived and control infants at 6 and 24 months of age. Furthermore, there was no difference in mental developmental index and psychomotor scores between the two groups according to the Bayley scale of infant development (60). So whilst the growth and development of children conceived through IVM technology appears reassuring to date, there is a clear need for future research into developmental outcomes beyond 24 months of age into adolescence and early adulthood.

The Risks of Aneuploidy and Epigenetic Variation

There is very little evidence of the effects of IVM on the risk of embryo chromosomal aneuploidy. Two case control studies utilizing fluorescence in-situ hybridization (FISH) to compare chromosomes 13, 15, 16, 18, 21, 22, X, and Y have found no difference in the incidence of chromosomal abnormality between IVF and IVM derived embryos (62,63). Only one study has reported on the use of array comparative genomic hybridization (aCGH), in which the phosphodiesterase inhibitor IBMX was added into the culture media, and rates of aneuploidy were found to be similar to the researchers previously published data from standard IVF treatments (64,65). The limited data available in regards to aneuploidy and IVM highlights need for more research in this area in both human and animal models.

Concerns have also been raised about the possible interference of IVM with epigenetic mechanisms and genomic imprinting. Research into the impacts of IVM on epigenetic variation in human oocytes is limited, and there is currently no information available on a genome-wide scale; however, for the selected genes LIT1, SNRPN, PEG3, and GTL2, there were no significant increases in imprinting mutations (66). Additionally, other research has shown no difference in epigenetic changes between samples from 11 IVM and 19 IVF control neonates (67). So while there is a clear need for further research, the limited data available so far is reassuring for IVM treatment.

Future Directions

In order for IVM to be considered a validated treatment option for ART clinics, there is a clear need for large scale randomized control trials. Additionally, there needs to be further research into the long-term outcomes of children born following this technique. Logistically, IVM is more laborious than standard IVM, with extra time needed for in vitro culture and a more standardized approach to protocols worldwide may lead to a more wide-scale adoption of the treatment. Research into additional culture media additives, including oocyte secreted factors GDF9 and BMP-15, as well as cAMP modulators (IBMX), has shown significant promise in animal trials (68), and initial testing of IBMX in human participants demonstrated its efficacy in terms of embryo chromosomal aneuploidy (69). Further large-scale trials into its effect on human IVM success rates may lead to the development of more successful, commercially available culture media which may help IVM move closer to the success rates achieved with standard IVF.

Conclusions

With success rates with IVM techniques improving, and research showing favorable outcomes in children conceived following this treatment, the routine use of IVM in clinical practice may not be unrealistic. The development of commercial culture media to further improve success rates, as well as standardizing

protocols, will aid in the functionality of laboratory practices. There remains a clear need for more robust research into the success of IVM treatment. Particularly, the lack of any randomized control trials and long-term data on the outcomes of children born from this technique. However, as many avenues of science move toward a more sustainable, minimally invasive approach, IVM fits neatly into this category as a financially beneficial and patient-friendly treatment option, providing a clinic can maintain success rates above a medically and ethically justified threshold, not dissimilar to standard IVF.

REFERENCES

1. Pincus G, Enzmann EV. The comparative behavior of mammalian eggs in vivo and in vitro. *J Exp Med*. 1935;62(5):665–75.
2. Edwards RG. Maturation in vitro of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. *Nature*. 1965;208(5008):349–51.
3. Cha KY, Koo JJ, Ko JJ, Choi DH, Han SY, Yoon TK. Pregnancy after in vitro fertilization of human follicular oocytes collected from nonstimulated cycles, their culture in vitro and their transfer in a donor oocyte program. *Fertil Steril*. 1991;55(1):109.
4. Trounson A, Wood C, Kausche A. In vitro maturation and the fertilization and developmental competence of oocytes recovered from untreated polycystic ovarian patients. *Fertil Steril*. 1994;62(2):353.
5. Saul T, Sonson JM. Ovarian hyperstimulation syndrome. *Am J Emergency Med*. 2009;27(2):250.e3–e4.
6. Lindenberg S. New approach in patients with polycystic ovaries, lessons for everyone. *Fertil Steril*. 2013;99(5):1170–2.
7. Tan SL, Child TJ, Gulekli B. In vitro maturation and fertilization of oocytes from unstimulated ovaries: Predicting the number of immature oocytes retrieved by early follicular phase ultrasonography. *Am J Obs Gyn*. 2002;186(4):684–9.
8. Söderström-Anttila V, Mäkinen S, Tuuri T, Suikkari A-M. Favourable pregnancy results with insemination of in vitro matured oocytes from unstimulated patients. *Hum Repro*. 2005;20(6):1534–40.
9. Junk SM, Yeap D. Improved implantation and ongoing pregnancy rates after single-embryo transfer with an optimized protocol for in vitro oocyte maturation in women with polycystic ovaries and polycystic ovary syndrome. *Fertil Steril*. 2012;98(4):888–92.
10. Mikkelsen A, Lindenberg S. Benefit of FSH priming of women with PCOS to the in vitro maturation procedure and the outcome: A randomized prospective study. *Reproduction*. 2001;122(4):587–92.
11. Mikkelsen AL, Smith SD, Lindenberg S. In-vitro maturation of human oocytes from regularly menstruating women may be successful without follicle stimulating hormone priming. *Hum Reprod*. 1999;14(7):1847–51.
12. Hart R. Preservation of fertility in adults and children diagnosed with cancer. *BMJ*. 2008;337:a2045.
13. Shalom-Paz E, Almog B, Shehata F, Huang J, Holzer H, Chian R-C et al. Fertility preservation for breast-cancer patients using IVM followed by oocyte or embryo vitrification. *Reprod Biomed Online*. 2010;21(4):566–71.
14. González C, Devesa M, Boada M, Coroleu B, Veiga A, Barri P. Combined strategy for fertility preservation in an oncologic patient: Vitrification of in vitro matured oocytes and ovarian tissue freezing. *JARG*. 2011;28(12):1147–9.
15. Walls ML, Hunter T, Ryan JP, Keelan JA, Nathan E, Hart RJ. In vitro maturation as an alternative to standard in vitro fertilization for patients diagnosed with polycystic ovaries: A comparative analysis of fresh, frozen and cumulative cycle outcomes. *Hum Reprod*. 2015;30(1):88–96.
16. Huang JYJ, Tulandi T, Holzer H, Tan SL, Chian R-C. Combining ovarian tissue cryobanking with retrieval of immature oocytes followed by in vitro maturation and vitrification: An additional strategy of fertility preservation. *Fertil Steril*. 2008;89(3):567–72.
17. Walls ML, Douglas K, Ryan JP, Tan J, Hart R. In-vitro maturation and cryopreservation of oocytes at the time of oophorectomy. *Gynecol Oncol Rep*. 2015;13:79–81.
18. Prasath EB, Chan ML, Wong WH, Lim CJ, Tharmalingam MD, Hendricks M et al. First pregnancy and live birth resulting from cryopreserved embryos obtained from in vitro matured oocytes after oophorectomy in an ovarian cancer patient. *Hum Reprod*. 2014;29(2):276–8.
19. Glujovsky D, Riestra B, Sueldo C, Fiszbajn G, Repping S, Nodar F et al. Vitrification versus slow freezing for women undergoing oocyte cryopreservation. *Cochrane Db Syst Rev*. 2014;9:Cd010047.

20. Walls ML, Ryan JP, Hart RJ. Conversion of IVF to IVM treatment for fertility preservation: Two case studies of successful oocyte and embryo storage prior to chemotherapy to avoid cycle cancellation. Poster session presented at: The 5th Congress of the Asia Pacific Initiative on Reproduction in Conjunction with the Fertility Society of Australia Annual Conference, April 4-6, Brisbane, Australia, 2014.
21. ASRM. Ovarian hyperstimulation syndrome. *Fertil Steril*. 2008;90(5):S188–S93.
22. Grynberg M, Peltoketo H, Christin-Maitre S, Poulain M, Bouchard P, Fanchin R. First birth achieved after in vitro maturation of oocytes from a woman endowed with multiple antral follicles unresponsive to follicle-stimulating hormone. *J Clin Endo Metab*. 2013;98(11):4493–8.
23. Suikkari A-M. In-vitro maturation: Its role in fertility treatment. *Curr Opin Obstet Gyn*. 2008;20(3):242–8. Doi: 10.1097/GCO.0b013e3282f88e33.
24. Chian RC, Buckett WM, Tulandi T, Tan SL. Prospective randomized study of human chorionic gonadotrophin priming before immature oocyte retrieval from unstimulated women with polycystic ovarian syndrome. *Hum Reprod*. 2000;15(1):165–70.
25. Son W-Y, Chung J-T, Chian R-C, Herrero B, Demirtas E, Elizur S et al. A 38 h interval between hCG priming and oocyte retrieval increases in vivo and in vitro oocyte maturation rate in programmed IVM cycles†. *Hum Reprod*. 2008;23(9):2010–6.
26. Cha KY, Han SY, Chung HM, Choi DH, Lim JM, Lee WS et al. Pregnancies and deliveries after in vitro maturation culture followed by in vitro fertilization and embryo transfer without stimulation in women with polycystic ovary syndrome. *Fertil Sertil*. 2000;73(5):978–83.
27. Cha KY, Chung HM, Lee DR, Kwon H, Chung MK, Park LS et al. Obstetric outcome of patients with polycystic ovary syndrome treated by in vitro maturation and in vitro fertilization–embryo transfer. *Fertil Steril*. 2005;83(5):1461–5.
28. Child TJ, Abdul-Jalil AK, Gulekli B, Lin Tan S. In vitro maturation and fertilization of oocytes from unstimulated normal ovaries, polycystic ovaries, and women with polycystic ovary syndrome. *Fertil Steril*. 2001;76(5):936–42.
29. Da Silva-Buttkus P, Marcelli G, Franks S, Stark J, Hardy K. Inferring biological mechanisms from spatial analysis: Prediction of a local inhibitor in the ovary. *Proc Nat Acad Sci*. 2009;106(2):456–461.
30. Gougeon A. Human ovarian follicular development: From activation of resting follicles to preovulatory maturation. *Annales Endocrinol* 2010;71(3):132–143.
31. Gremeau A-S, Andreadis N, Fatum M, Craig J, Turner K, McVeigh E, Child T. In vitro maturation or in vitro fertilization for women with polycystic ovaries? A case-control study of 194 treatment cycles. *Fertil Steril*. 2012;98(2):355–360.
32. Le Du A, Kadoch IJ, Bourcigaux N, Doumerc S, Bourrier M-C, Chevalier N et al. In vitro oocyte maturation for the treatment of infertility associated with polycystic ovarian syndrome: The French experience. *Hum Reprod*. 2005;20(2):420–4.
33. Lin YH, Hwang JL, Huang LW, Mu SC, Seow KM, Chung J et al. Combination of FSH priming and hCG priming for in-vitro maturation of human oocytes. *Hum Reprod*. 2003;18(8):1632–6.
34. Son W-Y, Lee S-Y, Lim J-H. Fertilization, cleavage and blastocyst development according to the maturation timing of oocytes in in vitro maturation cycles. *Hum Reprod*. 2005;20(11):3204–7.
35. Walls M, Junk S, Ryan J, Hart R. IVF versus ICSI for the fertilization of in-vitro matured human oocytes. *Reprod Biomed Online*. 2012;25(6):603–7.
36. Yoon H-G, Yoon S-H, Son W-Y, Lee S-W, Park S-P, Im K-S et al. Clinical assisted reproduction: Pregnancies resulting from in vitro matured oocytes collected from women with regular menstrual cycle. *JARG*. 2001;18(6):325–9.
37. Hreinsson J, Rosenlund B, Fridén B, Levkov L, Ek I, Suikkari AM et al. Recombinant LH is equally effective as recombinant hCG in promoting oocyte maturation in a clinical in-vitro maturation programme: A randomized study. *Hum Reprod*. 2003;18(10):2131–6.
38. Sasseville M, Gagnon MC, Guillemette C, Sullivan R, Gilchrist RB, Richard FJ. Regulation of gap junctions in porcine cumulus-oocyte complexes: Contributions of granulosa cell contact, gonadotropins, and lipid rafts. *Mol Endocrin*. 2009;23(5):700–10.
39. Pongsuthirak P, Vutyavanich T. Comparison of medicult and Sage media for in vitro maturation of immature oocytes obtained during cesarean deliveries. *JFIV Reprod Med Genet* 2015;3:136. doi: 10.4172/2375-4508.1000136.
40. Pongsuthirak P, Songveeratham S, Vutyavanich T. Comparison of blastocyst and Sage media for in vitro maturation of human immature oocytes. *Reprod Sci*. 2015;22(3):343–6.

41. Mikkelsen AL, Smith S, Lindenberg S. Impact of oestradiol and inhibin A concentrations on pregnancy rate in in-vitro oocyte maturation. *Hum Reprod*. 2000;15(8):1685–90.
42. Gomez E, Tarin J, Pellicer A. Oocyte maturation in humans: The role of gonadotropins and growth factors. *Fertil Steril*. 1993;60(1):40–6.
43. Das K, Stout L, Hensleigh H, Tagatz G, Phipps W, Leung B. Direct positive effect of epidermal growth factor on the cytoplasmic maturation of mouse and human oocytes. *Fertil Steril*. 1991;55(5):1000.
44. Goud PT. In-vitro maturation of human germinal vesicle stage oocytes: Role of cumulus cells and epidermal growth factor in the culture medium. *Hum Reprod*. 1998;13(6):1638–44.
45. Suikkari A-M, Tulppala M, Tuuri T, Hovatta O, Barnes F. Luteal phase start of low-dose FSH priming of follicles results in an efficient recovery, maturation and fertilization of immature human oocytes. *Hum Reprod*. 2000;15(4):747–51.
46. Johnson MH, Nasresfahani MH. Radical solutions and cultural problems: Could free oxygen radicals be responsible for the impaired development of preimplantation mammalian embryos in vitro? *Bioessays*. 1994;16(1):31–8.
47. Bontekoe S, Mantikou E, van Wely M, Seshadri S, Repping S, Mastenbroek S. Low oxygen concentrations for embryo culture in assisted reproductive technologies. *Cochrane Db Syst Rev*. 2012;7: Cd008950.
48. Walls ML, Ryan JP, Keelan JA, Hart R. In vitro maturation is associated with increased early embryo arrest without impairing morphokinetic development of useable embryos progressing to blastocysts. *Hum Reoprod*. 2015;30(8):1842–9.
49. Russell JB, Knezevich KM, Fabian KF, Dickson JA. Unstimulated immature oocyte retrieval: Early versus midfollicular endometrial priming. *Fertil Steril*. 1997;67(4):616–20.
50. Junk SM, Edirisinghe, WR, Matson PL, Yovich JL. The effect of in vitro maturation of mouse oocytes on zona pellucida hardness. Proceedings of the 13th Annual ESHRE Conference, 22–25 June, 1997, Edinborough, Scotland, 1997.
51. Davies MJ, Moore VM, Willson KJ, Van Essen P, Priest K, Scott H et al. Reproductive technologies and the risk of birth defects. *New Engl J Med*. 2012;366(19):1803–13.
52. Hart R, Norman RJ. The longer-term health outcomes for children born as a result of IVF treatment: Part I—General health outcomes. *Hum Reprod Update*. 2013;19(3):232–43.
53. Hart R, Norman RJ. The longer-term health outcomes for children born as a result of IVF treatment. Part II—Mental health and development outcomes. *Hum Reprod Update*. 2013;19(3):244–50.
54. Hart R, Doherty DA. The potential implications of a PCOS diagnosis on a woman's long-term health using data linkage. *J Clin Endo Metab*. 2015;100(3):911–9.
55. Doherty DA, Newnham JP, Bower C, Hart R. Implications of polycystic ovary syndrome for pregnancy and for the health of offspring. *Obs Gyn*. 2015;125(6):1397–406.
56. Söderström-Anttila V, Salokorpi T, Pihlaja M, Serenius-Sirve S, Suikkari A-M. Obstetric and perinatal outcome and preliminary results of development of children born after in vitro maturation of oocytes. *Hum Reprod*. 2006;21(6):1508–13.
57. Mikkelsen AL. Strategies in human in-vitro maturation and their clinical outcome. *Reprod Biomed Online*. 2005;10(5):593–9.
58. Fadini R, Mignini Renzini M, Guarnieri T, Dal Canto M, De Ponti E, Sutcliffe A et al. Comparison of the obstetric and perinatal outcomes of children conceived from in vitro or in vivo matured oocytes in in vitro maturation treatments with births from conventional ICSI cycles. *Hum Reprod*. 2012; 27(12):3601–8.
59. Foix-L'Helias L, Grynberg M, Ducot B, Frydman N, Kerbrat V, Bouyer J et al. Growth development of French children born after in vitro maturation. *PLoS One*. 2014;9(2):e89713.
60. Shu-Chi M, Jiann-Loung H, Yu-Hung L, Tseng-Chen S, Ming-I L, Tsu-Fuh Y. Growth and development of children conceived by in-vitro maturation of human oocytes. *Early Hum Dev*. 2006;82(10):677–82.
61. Pandian Z, Marjoribanks J, Ozturk O, Serour G, Bhattacharya S. Number of embryos for transfer following in vitro fertilisation or intra-cytoplasmic sperm injection. *Cochrane Db Syst Rev*. 2013;7:Cd003416.
62. Zhang XY, Ata B, Son W-Y, Buckett WM, Tan S-L, Ao A. Chromosome abnormality rates in human embryos obtained from in-vitro maturation and IVF treatment cycles. *Reprod Biomed Online*. 2010; 21(4):552–9.
63. Requena A, Bronet F, Guillén A, Agudo D, Bou C, García-Velasco JA. The impact of in-vitro maturation of oocytes on aneuploidy rate. *Reprod Biomed Online*. 2009;18(6):777–83.

64. Mertzanidou A, Wilton L, Cheng J, Spits C, Vanneste E, Moreau Y et al. Microarray analysis reveals abnormal chromosomal complements in over 70% of 14 normally developing human embryos. *Hum Reprod*. 2013;28(1):256–64.
65. Mertzanidou A, Spits C, Nguyen HT, Van de Velde H, Sermon K. Evolution of aneuploidy up to day 4 of human preimplantation development. *Hum Reprod*. 2013;28(6):1716–24.
66. Kuhtz J, Romero S, De Vos M, Smitz J, Haaf T, Anckaert E. Human in vitro oocyte maturation is not associated with increased imprinting error rates at LIT1, SNRPN, PEG3 and GTL2. *Hum Reprod*. 2014;29(9):1995–2005.
67. Pliushch G, Schneider E, Schneider T, El Hajj N, Rösner S, Strowitzki T et al. In vitro maturation of oocytes is not associated with altered deoxyribonucleic acid methylation patterns in children from in vitro fertilization or intracytoplasmic sperm injection. *Fertil Steril*. 2015;103(3):720–7.e1.
68. Rose RD, Gilchrist RB, Kelly JM, Thompson JG, Sutton-McDowall ML. Regulation of sheep oocyte maturation using cAMP modulators. *Theriogenology*. 2013;79(1):142–8.
69. Spits C, Guzman L, Mertzanidou A, Jacobs K, Ortega-Hrepich C, Gilchrist RB et al. Chromosome constitution of human embryos generated after in vitro maturation including 3-isobutyl-1-methylxanthine in the oocyte collection medium. *Hum Reprod*. 2015;30(3):653–63.

10

Sperm Preparation for IVF and ICSI

Christopher J. De Jonge

Introduction

Human spermatozoa are incapable of in vivo fertilization while bathed in the seminal plasma of the ejaculate. Once removed from seminal plasma, sperm must undergo maturational changes during which they acquire the ability to fertilize oocytes. This process, known as capacitation, was described more than 50 years ago by both Austin and Chang (1,2). Capacitation is prevented in ejaculated spermatozoa by at least one factor in seminal plasma (3). Additionally, prolonged or re-exposure to seminal plasma can inhibit the ability of spermatozoa to undergo the acrosome reaction in vitro (4) and diminish their capacity to fertilize (5).

In the female reproductive tract motile spermatozoa separate themselves from seminal plasma by actively migrating through the cervical mucus. This active migration selects for spermatozoa that are progressively motile, of more normal morphology, facilitates initiation of capacitation and, quite potentially, contributes to the identification of a cohort of spermatozoa that are candidates for fertilization (6). Due to the inhibitory effects of seminal plasma and the deleterious effects of other factors in the ejaculate on sperm function, it is critical that spermatozoa used for clinical procedures, such as in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI), be separated from the seminal plasma as quickly as possible after ejaculation and liquefaction.

While IVF started as a treatment for tubal infertility, the identification of a male population with poor semen quality compelled the development of a menu of sperm preparation techniques. These techniques generally fall into four categories: (1) simple dilution and washing; (2) sperm migration; (3) density gradient centrifugation; and (4) filtration or adherence. Regardless of the technique, the objective of sperm preparation for IVF is to isolate a population of motile, functionally competent spermatozoa that are free of inhibitory decapacitation factors. The technique should also reduce damage to the "healthy" spermatozoa from toxic factors like reactive oxygen species. Some of these techniques as well as their advantages and disadvantages are presented here.

Selection of technique based on specimen quality will not be presented because each laboratory should develop their own clinical protocol(s) based on their patient population, peer-reviewed scientific studies of high quality (limited bias, rigorous experimental and statistical design) and comparability, availability of resources, and safety considerations that include the unborn.

Sperm Collection

Ejaculated Specimens

The ejaculate should be collected by masturbation into a sterile glass or disposable plastic jar that has been screened for sperm toxicity using a bioassay. As soon as the seminal plasma has liquefied, the specimen is recommended to be analyzed according to WHO guidelines (7) and prepared for sperm isolation. If liquefaction is delayed or if the specimen is especially viscous several methods can be found in the WHO manual (7) to address these complicating issues. If, prior to processing, the initial specimen is determined to have insufficient numbers of viable sperm for IVF or ICSI then a second semen specimen

can often reliably be obtained (8). For men who are unable to ejaculate by masturbation, nontoxic condoms are commercially available; manufacturer's guidelines for their proper use should be strictly followed by patient and laboratory personnel. Ordinary contraceptive condoms must not be used (even those without spermicide) because of their sperm toxicity. Coitus interruptus is also not recommended because of the risk of incomplete recovery and potential iatrogenic contamination of the ejaculate.

Men who are unable to achieve erection, emission, or ejaculation because of neurological or psychogenic problems can frequently have an ejaculate collected by electro-ejaculation using direct vibratory stimulation of the glans penis or electrical stimulation of the prostate. Ejaculates from spinal cord injury patients will frequently have high sperm concentrations, decreased motility, and red blood cell contamination. Sperm may also be recovered from the urine of patients whose ejaculation is retrograde into the bladder. It is advisable that these patients be prescribed stomach-acid buffering medications to make the urine pH more hospitable for sperm. Care should be taken to avoid osmotic shock when processing sperm out of urine, as urine is hyperosmotic relative to commercially available media.

Surgically-Procured Specimens

The collection of epididymal and/or testicular spermatozoa requires an office or outpatient surgical procedure. Epididymal spermatozoa can be retrieved either by microsurgery or by percutaneous needle puncture. Since the typical indication for epididymal aspiration is obstructive azoospermia rather than testicular hypofunction, it is not uncommon for relatively large numbers of motile sperm to be obtained and most typically used, for ICSI. Any excess sperm may be frozen for future use. Depending on operator proficiency, epididymal aspirates can be obtained with minimal red blood cell and non-germ cell contamination, making the isolation and selection of motile sperm quite easy. If large numbers of epididymal spermatozoa are obtained, then density gradient centrifugation (see below) can sometimes be an effective method for sperm isolation in preparation for ICSI.

Testicular spermatozoa can be retrieved by open biopsy, with or without microdissection, or by percutaneous needle biopsy. The method for tissue procurement is often dependent upon the diagnosis of obstructed or non-obstructed azoospermia. Testicular specimens are invariably contaminated with large amounts of red blood cells and testicular tissue; additional steps are needed to isolate a clean preparation of spermatozoa. In order to free the seminiferous tubule-bound spermatozoa, it is necessary to use either enzymatic (collagenase) or mechanical methods. For the latter, testicular tissue in supportive culture medium is macerated using glass cover slips or slides until a fine slurry of dissociated tissue is produced, and the resulting suspension can then be processed by simple wash and dilution prior to ICSI. Testicular spermatozoa can also be obtained from a needle biopsy and processed similarly to an open biopsied specimen. Using this method only a small amount of tissue is usually retrieved, and with a resulting low sperm yield. Testicular tissue is often procured during a diagnostic biopsy many weeks or months prior to use for IVF and as such is frozen for future use in order to avoid further surgeries.

Sperm Preparation Methods

Simple Washing and Dilution

The sperm preparation method used for the first IVF cases involved dilution of the semen with culture medium (usually at two to ten times the volume) and separation of the spermatozoa, along with other cell types, from the liquid phase by centrifugation to form a pellet. After removal of the supernatant, the pellet is resuspended in another aliquot of culture medium. Repeat centrifugation, usually two or three times total, is often used to ensure removal of contaminating seminal plasma. The centrifugation is usually performed at 200–300*g* and is recommended to be performed at centrifugal forces less than 800*g* (9). Advantages of this method are that it is one of the simplest and the least expensive to perform. One disadvantage of this technique is nonviable and immotile spermatozoa as well as any leukocytes, squamous epithelial cells, or non-cellular debris that contaminated the original semen sample will still be present in the washed sample. Another disadvantage is the concern about potential damage caused by centrifugation.

Aitken and Clarkson (10) reported that techniques involving the repeated centrifugation of unselected populations of human spermatozoa generate cell suspensions with significantly reduced motility. Moreover, these detrimental effects of centrifugation were associated with a sudden burst of reactive oxygen species (ROS) produced by a discrete subpopulation of cells characterized by significantly diminished motility and fertilizing capacity. The ROS were found to impair the functional competence of normal spermatozoa in the same suspension, reflected by impaired capacity for sperm–oocyte fusion. It has also been shown that ROS can cause DNA damage in human spermatozoa when exposed for time periods consistent with clinical sperm preparation techniques for ICSI or IVF (11). Thus, sperm preparation techniques that involve a washing step in which semen is diluted with culture medium and centrifuged have mostly been abandoned for alternative selection techniques such as direct swim-up from semen or density gradient centrifugation.

Note in Brief: For sperm selection techniques that involve centrifugation it is advisable to use a "swing-out" bucket rotor rather than a "fixed" angle head rotor. In addition, locking safety tops are available for buckets to contain specimen accidents. Operators are encouraged to understand basic principles of centrifugation and, more specifically, density gradient centrifugation. Lastly, *g*-force, which stands for the acceleration due to gravity at the Earth's surface, is the standard unit for centrifugation and it is calculable for any centrifuge and rotor.

Sperm Migration

Motile spermatozoa separate themselves from seminal plasma in vivo by actively migrating through cervical mucus in the female reproductive tract. There are a variety of sperm preparation techniques that involve migration of spermatozoa, and the common element for all of these techniques requires the self-propelled, directional movement of spermatozoa, i.e., forward progressive motility. The recovery of motile sperm using sperm migration techniques, as with all sperm preparation techniques, is dependent upon the initial specimen quality. Poor quality specimens are recommended to be processed using alternative techniques, e.g., density gradient centrifugation (see below).

Swim-up from Washed Pellet

The swim-up of spermatozoa from a washed pellet technique was originally described by Mahadevan and Baker (12) and it is still a somewhat common method for patients with normozoospermia and female infertility (13). The procedure involves dilution and centrifugation (repeated 2–3 times) of a semen specimen to separate spermatozoa from seminal plasma. The pellet of spermatozoa formed after the final centrifugation can either be left intact or gently resuspended in the small residual volume of supernatant in the bottom of the centrifuge tube. To enhance the recovery of progressively motile sperm swimming up from an intact sperm pellet requires that the final centrifugation speed produce a loosely compacted pellet. Gently and slowly tilting the test tube and observing whether the pellet slightly tilts as well can verify this. Each laboratory should determine the centrifugation time and speed that will afford this attribute. If one chooses to further enhance the recovery of motile sperm, then the sperm pellet can be resuspended in a small volume. However, extreme care must be taken to ensure that no mixing occurs when overlaying the non-compacted pellet with culture medium. If mixing occurs, then the final supernatant, which contains sperm desired for IVF/ICSI, will likely be contaminated with immotile sperm, debris, and non-germ cells. This latter technical problem is less of an issue when the sperm pellet is left intact. Regardless of whether an intact or disrupted sperm pellet is used, culture medium is carefully layered over the pellet and the tube is incubated at 37°C for 45–60 minutes to allow the spermatozoa to swim up from the pellet.

As with all techniques involving the mixing of spermatozoa with medium, it is important to choose a culture medium that is buffered appropriately for the atmosphere in which the technique takes place. Therefore, if the incubator atmosphere is the same as the laboratory and the temperature is 37°C, then the medium should be buffered with HEPES or a similar buffer, and the caps of the swim-up tubes should be tightly closed. If the incubator atmosphere is 5%–6% CO_2 and the temperature is 37°C, then the medium is best buffered with sodium bicarbonate or a similar buffer, and the caps of the test tubes

should be loose. Adherence to the aforementioned will help to ensure culture pH that is compatible with essential sperm functional attributes.

To facilitate the release of motile spermatozoa from the sperm pellet, the test tube should be placed at a 45° angle to increase the surface area interface between the sperm pellet and the culture medium. Alternatively, aliquots of the resuspended pellet may be placed in four-well dishes before culture medium is layered over each aliquot. The use of four-well dishes will also increase the interface between the pellet and the culture medium (13). Evidence that sperm have successfully swum up into the overlaying culture medium is reflected by an increase in turbidity. If the culture medium appears clear, then more time may be needed to allow spermatozoa the opportunity to swim out of the pellet. After the incubation, the upper layer of culture medium containing spermatozoa is carefully aspirated without disrupting the interface and transferred to a clean test tube from which concentration, motility, and morphology can be assessed.

Advantages of the swim-up from washed pellet method include the recovery of a relatively high percentage of motile sperm and the absence of other cells and debris. Another advantage of this technique is that it consistently produces suspensions of spermatozoa with increased swimming velocity and more normal sperm morphology (14). The swim-up method also results in significant improvement of functional markers such as the rates of acrosome reaction, hypo-osmotic swelling, and nuclear maturity (15). A disadvantage of the swim-up from washed pellet is the low overall recovery of motile spermatozoa; motile spermatozoa trapped at the bottom of the pellet may never be able to reach the interface with the culture medium. Thus, the efficiency of the technique is based not only on the initial sperm motility in the ejaculate but also on the size, level of compaction, and exposed surface area of the final pellet. Another disadvantage is the previously discussed concern about potential damage caused by centrifugation of unselected populations of human spermatozoa.

Direct Swim-up from Semen

A sperm migration technique that avoids centrifugation of unselected sperm populations is the direct swim-up from semen, in which aliquots of liquefied semen are layered underneath culture medium in either a series of test tubes or four-well dishes. Similar to the swim-up from pellet method, the recovery of a highly motile sperm population by direct swim-up is increased by placing the tubes at a 45° angle to increase the surface area interface between the semen layer and the culture medium. Depending on the initial ejaculate volume, sperm concentration, and sperm motility, multiple test tubes or four-well dishes may be used to increase the recovery of motile spermatozoa. The interface can often be cleaner when the liquefied semen is layered under the culture medium with a syringe and needle rather than layering the culture medium over the semen (16).

The test tubes are incubated at 37°C for 45–60 minutes to allow the spermatozoa to swim up from the liquefied semen. Evidence that sperm have successfully swum up into the overlaying culture medium is reflected by an increase in turbidity. If the culture medium appears clear, then more time may be needed to allow spermatozoa the opportunity to swim out of the pellet. After incubation, the upper layer of culture medium in each tube is carefully aspirated and removed to a clean centrifuge tube. The suspension is then centrifuged at 300–500*g* for 4–10 minutes after which the supernatant is removed and the pellet resuspended in fresh culture medium to achieve the desired concentration of motile spermatozoa.

Advantages of the direct swim-up method include the recovery of a high percentage of motile sperm and the absence of contaminating dead or immotile spermatozoa, non-germ cells, and debris. In a comparison of four methods for sperm preparation, Ren (17) found that the direct swim-up method yielded the best sperm motility. Another advantage is the elimination of the centrifugation step prior to the swim-up, which reduces ROS production by white blood cells and dying spermatozoa. A disadvantage of the direct swim-up from semen is the low percentage recovery of total progressively motile spermatozoa.

Migration–Sedimentation

The migration–sedimentation method was developed by Tea and colleagues (18) and it combines the swim-up technique with a sedimentation step in special glass or plastic tubes containing an inner cone. Spermatozoa swim up directly from liquefied semen into the overlying culture medium and subsequently

settle gravitationally in the inner cone of the tube. Incubation is usually 60 minutes at 37°C, after which the medium in the cone is removed and centrifuged at 300*g* for 5–10 minutes. Sperm count and motility are then determined on the resuspended pellet.

The advantages of the migration–sedimentation method are similar to those of the direct swim-up technique: the migration–sedimentation method is a very gentle separation method and it yields a clean fraction of highly motile spermatozoa. In addition, ROS are reduced because of the lack of centrifugation prior to sperm migration. The disadvantages of the technique include a very low yield of motile spermatozoa and the requirement for special glass or plastic tubes.

A comparative study by Gabriel (19) demonstrated that specimens from fertile males processed using the migration–sedimentation method had the greatest increase in motility and the only increase in morphology versus specimens processed either by filtration (SpermPrep®) or swim-up from washed pellet. Specimens from subfertile males also showed significantly increased sperm motility and morphology when the migration–sedimentation method was used. Gabriel concluded that migration–sedimentation should be the method of choice unless the original sperm count is low.

Sanchez (20) modified the migration-sedimentation method to include an initial centrifugation of the neat semen at 400*g* for 10 minutes, with the resulting pellet diluted in 500 μL of seminal fluid before being placed under culture medium in special glass tubes and incubated for 2–3 hours at 37°C. After the incubation, the medium in the cone is removed and centrifuged at 300*g* for 5–10 minutes. Sperm count and motility are then determined on the resuspended pellet. The extra centrifugation step and the lengthened incubation allowed them to recover a sufficient number of motile spermatozoa even in cases with severe oligozoospermia and/or asthenozoospermia. Using this modified method, Sanchez demonstrated significantly better results in progressive motility, normal morphology, chromatin condensation, and reduction in the percentage of dead spermatozoa when compared with density gradient centrifugation. In spite of the findings of Sanchez, one must bear in mind the same cautions when subjecting unselected sperm populations to centrifugation. Commercial availability of the Tea-Jondet tubes is questionable.

Density Gradient Centrifugation

Density gradients may be either continuous or discontinuous, although discontinuous gradients have been used almost exclusively since the late 1980s (21). Discontinuous gradients are usually prepared with two or three layers. Colloidal silica with covalently bound silane molecules is probably the most common density gradient material currently used for clinical IVF and andrology. These products are made isosmotic by the inclusion of polysucrose, they have very low toxicity, are non-irritating, and are approved for human in vivo use. ALLGrad® (LifeGlobal Group, Guilford, Connecticut), PureSperm® (Nidacon International AB, Mölndal, Sweden), ISolate® (Irvine Scientific, Santa Ana, California), SpermGrad™ (Vitrolife AB, Göteborg, Sweden), and Sydney IVF Spermient™ (William A. Cook Australia Pty Ltd, Brisbane, Australia) are examples of silane-coated silica particle solutions that can be used for discontinuous gradients. A recent controlled trial comparing three commonly used commercial density gradient media showed that the products were largely comparable, with some differences being significant (22). Thus, it is important to stress that each laboratory should trial gradient media in their environment to determine which preparation provides optimal recoveries. Concerning is a recent report that has documented the induction of sperm DNA damage by one or more of the above-mentioned gradient media due to transition metals in the media and at concentrations promoting of oxidative DNA damage (23). As with any product, it is important to follow the manufacturer's recommendation for proper use and application.

The discontinuous density gradient method is most often made with two layers: a lower layer, higher density solution and an upper layer, lower density solution. The purpose of the two layers is to allow the greater density particles, i.e., spermatozoa, to pass through the upper layer and interface ahead of, and separately from, other particles. Use of a single layer gradient, such as 80%, does not allow for an initial separation of different density particles. The consequence can often be an interface that becomes overwhelmed by different density particles that block the ability of the greater density spermatozoa from passing through.

Most routinely for density gradient centrifugation, the ejaculate is placed on top of the density gradient medium and is centrifuged at 300–400*g* for 15–30 minutes. Since the density gradient medium is a

colloid rather than a solution, it has low viscosity and it does not retard the sedimentation of spermatozoa due to centrifugation (21). Highly motile spermatozoa move actively in the direction of the sedimentation gradient and can penetrate the boundary faster than poorly motile and/or immotile spermatozoa (12). Thus, the soft pellet at the bottom is enriched for highly motile spermatozoa. The pellet is washed with culture medium and centrifuged at 200*g* for 4–10 minutes. The wash and centrifugation is repeated to ensure removal of contaminating density gradient medium. (*Personal observation:* An indication that an additional wash to remove gradient media may be required is the appearance on wet mount of sperm stuck by their heads to the glass slide.) The final pellet is resuspended in culture medium so that concentration and motility can be determined.

Density gradient centrifugation usually results in a clean fraction of highly motile spermatozoa. Since the whole volume of the ejaculate is used in density gradient centrifugation (as it is in the swim-up techniques), it yields a significantly higher total number of motile spermatozoa and it can be used for patients with varying degrees of suboptimal semen parameters, e.g., oligozoospermia and asthenozoospermia. Other advantages of density gradient centrifugation include the elimination of leukocytes and the significant reduction of ROS (13). Additionally, Nicholson (24) demonstrated that centrifugation through one brand of silane-coated silica particles (PureSperm®) efficiently reduces bacterial contamination. Hammadeh (25) reported that another advantage of the density gradient method is the recovery of a higher percentage of morphologically normal spermatozoa than found in conventional swim-up or glass-wool filtration. The technique has also been shown to yield sperm populations with better DNA quality and chromatin packaging (26,27). Further, preliminary reports suggest that specimens known to be contaminated with sexually transmissible viruses can effectively be "cleaned up" using density gradient centrifugation, and the isolated spermatozoa can be used for therapy with low risk for horizontal disease transmission (28). One disadvantage of density gradient centrifugation is that the density gradient medium is a bit more expensive than either of the swim-up techniques.

Adherence: Filtration

These methods are based on the phenomenon that dead and moribund spermatozoa are extremely sticky and will attach to glass surfaces even in the presence of relatively high concentrations of protein (16).

Glass Wool Filtration

In this method, motile spermatozoa are separated from immotile spermatozoa by means of densely packed glass wool fibers. The principle of this technique involves both the self-propelled movement of the spermatozoa and the filtration effect of the glass wool. The method initially employed vertical Pasteur pipettes filled with glass wool fibers on to which the ejaculate was placed and allowed to filter by gravity (29). The method has evolved such that in a current variation (30), the filter is created by placing 30 mg of pre-cleaned glass wool microfibers in the barrel of a 3 mL disposable syringe and gently packing it down using the syringe plunger (minus its rubber tip). The syringe is suspended vertically in a 15 mL centrifuge tube and rinsed several times with culture medium to remove any loose wool fibers prior to filtration. Meanwhile, the ejaculate is washed with an equal volume of culture medium, pipetted into 15 mL centrifuge tubes (no more than 3 mL/tube), and centrifuged at 300*g* for 3 min. Each resulting pellet is resuspended in 1 mL of culture medium, and centrifuged again at 300*g* for 3 min. The pellet in one tube is resuspended with 300 µL of culture medium, and this single supernatant is sequentially added to resuspend the sperm pellet in any remaining tubes (the total volume should not exceed 400 µL). The washed sperm suspension is gently pipetted over the pre-wetted glass wool column and then allowed to filter by gravity into a clean 15 mL centrifuge tube. When the dripping stops, 100 µL of culture medium is added to the filter and allowed to drip through. The filter is removed and the filtrate can be assessed for sperm concentration and motility.

The success of this method is related to the kind of glass wool used—the chemical nature of the glass, the surface structure and charge of the glass wool, and the thickness of the glass wool fibers. Glass wool from Manville Fiber Glass Corporation (Denver, Colorado) or SpermFertil® columns from Mello (Holzhausen, Germany) have been tested extensively in clinical practice (13). Glass wool filtration and

two-layer, discontinuous density gradient centrifugation resulted in an average recovery of 50%–70% of the progressively motile and about 50% of the hypo-osmotic swelling (HOS)-positive spermatozoa (31). Additionally, glass wool filtration tended to be more successful than density gradient centrifugation when the ejaculates were asthenozoospermic or had an abnormal HOS test. After processing, the activity of the zona lysing enzyme acrosin increased approximately two- to three-fold, but no significant improvement in the percentage of normal sperm forms occurred. Glass wool filtration was also more effective in removing non-motile and HOS-negative spermatozoa than density gradient centrifugation when the percentage of these types of spermatozoa in the ejaculate is high.

This method can use the whole volume of the ejaculate and thus yield a significantly higher total number of motile spermatozoa, which means it can be used for patients with oligozoospermia and/or asthenozoospermia. It is also possible to prepare motile spermatozoa from patients with retrograde ejaculation (12). Another advantage of glass wool filtration is the elimination of up to 90% of the leukocytes present in the ejaculate (30). Since leukocytes are a major producer of ROS, elimination of a majority of leukocytes should significantly reduce ROS. Finally, glass wool filtration was also found to yield a significantly higher percentage of chromatin condensed spermatozoa than swim-up or density gradient centrifugation (32). Disadvantages of the glass wool filtration method include the added expense of the glass wool and a filtrate that is not as clean as it is with other sperm preparation methods since remnants of debris may still be present.

Sephadex Columns

Sperm separation using Sephadex beads is another filtration method (33). A kit based on this principle (SpermPrep®) currently has limited commercial availability (Fertility Technologies, Inc.). Basically, liquefied semen is diluted with culture medium and centrifuged at 400*g* for 6 minutes. The supernatant is discarded and the sperm pellet resuspended in culture medium to a concentration of 100 × 106 sperm/mL. One ml of the washed semen is placed in the filter column containing hydrated filtration beads and mixed gently. The bottom cap is removed from the filter column and fluid is allowed to filter for 15 minutes. The filtrate is centrifuged at 400*g* for 6 minutes and resuspended in 1 mL of culture medium before being assessed for concentration, motility, and morphology.

In a comparative study, the yield of spermatozoa post-processing was highest with SpermPrep® than with swim-up or migration–sedimentation in both fertile and sub-fertile men (17), and for that reason the authors recommended that specimens with a lower than normal sperm count but normal motility and morphology should be processed with SpermPrep®. Disadvantages of Sephadex bead filtration include the added expense of the kit and a filtrate that is not as clean as it is with other sperm preparation methods since remnants of debris may still be present. In addition, the pre-filtration centrifugation step might generate ROS.

Advanced Techniques

Several promising candidate techniques have emerged for application in the ART/ICSI laboratory setting, and they are (1) an electrophoretic method, commercially named SpermSep CS10 (NuSep Holdings, LTD, Homebush, NSW, Australia), (2) Magnetic-Activated Cell Sorting (MACS), (3) hyaluronic acid (HA) binding (PICSI® and SpermSlow™, Origio a/s, Denmark), and (4) Motile Sperm Organelle Morphology Examination (MSOME) or Intracytoplasmic Morphologically selected Sperm Injection (IMSI) are high magnification microscopic techniques for selecting individual sperm for ICSI based on morphology.

SpermSep, originally named Cell Sorter 10, was developed by Aitken and colleagues (34). The technique uses the ejaculated specimen, without any preparation, and isolates spermatozoa with minimal DNA damage based on size and overall greatest negative charge. In a prospective controlled trial the electrophoretic method produced sperm preparations that performed equally well as density gradient isolated sperm with no significantly different endpoints of recovery rates, fertilization, embryo cleavage, or embryo quality (35). Based on current information, the SpermSep electrophoretic method is not commercially available.

The MACs technique uses annexin V antibody-conjugated superparamagnetic beads that bind to spermatozoa whose plasma membranes have externalized phosphatidylserine (PS), which is a marker for signs of early apoptosis. Annexin V positive sperm (relective of apoptosis, i.e., programmed cell death) are bound by the magnetic field while those free of the magnetic beads pass through the column and have a far lower incidence of apoptotic markers (36). The MACs method is most frequently paired with density gradient centrifugation and/or swim-up to remove potential contaminants emanating from seminal plasma and magnetic beads. The final isolated sperm population has a lower incidence of DNA damage, markers of apoptosis, and caspase activation.

Mature spermatozoa have receptors for hyaluronic acid (HA) which stimulates activity of sperm hyaluronidase. The biological significance, in part, is that cumulus cells surrounding the mature oocyte are held together by hyaluronic acid, the substrate for hyaluronidase. Huszar and colleagues discovered that sperm bound to hyaluronic acid in vitro have markers of cellular maturity, minimal DNA fragmentation, normal shape, and low frequency of chromosomal aneuploidies (37). Two products utilizing HA as a component for selecting spermatozoa are commercially available (see above). Sperm isolated by either the PICSI dish or SpermSlow are used for ICSI (38).

Motile sperm organelle morphology examination (MSOME) (39) or intracytoplasmic morphologically selected sperm injection (IMSI) (40) are high magnification microscopic techniques for selecting individual sperm for ICSI based on morphology. MSOME/ICSI uses magnification of 6300× to evaluate six sperm organelles: acrosome, post-acrosomal lamina, neck, tail, mitochondria, and the nucleus. This technique requires significant operator time and advanced microscopy equipment.

Two recent evidence-based reviews offer insight regarding several of the aforementioned advanced techniques. A literature database review of ICSI alone versus IMSI yielded 294 records, of which only nine studies were parallel in design (41). Live birth was reported in only one of those studies and no significant difference was detected using IMSI vs ICSI. While a significant improvement in clinical pregnancy rate was found for IMSI, the quality of the evidence was determined to be very low quality.

The second literature database review yielded only two randomized controlled trials (RCTs) for evaluation (42). The first RCT compared HA plus ICSI versus ICSI only. Live birth was not reported in the trial. Further, no difference between the two techniques was detected, quite possibly because the quality of evidence was determined to be low quality due to poor reporting of methods and results. The second RCT compared the two HA techniques: SpermSlow and PICSI. Evidence was insufficient to detect a difference between the two techniques for rates of live birth, clinical pregnancy, or miscarriage. The authors concluded that the two RCTs collectively lacked evidence of adequate quality to determine if sperm selection using HA improves likelihood of live birth or pregnancy outcome after IVF/ICSI.

Post-Separation Treatment of Spermatozoa

Improvement of Motility and Sperm Function

Pentoxifylline

The use of methylxanthine derivatives like pentoxifylline for the stimulation of sperm functions, especially motility, is well known. Pentoxifylline is a non-specific inhibitor of phosphodiesterase that has stimulatory effects on sperm motility and motion characteristics such as sperm velocity and hyperactivation. The stimulatory effect is attributed to increased intracellular levels of 3',5'-cyclic adenosine monophosphate (cAMP) via inhibition of its breakdown by cAMP phosphodiesterase. Pentoxifylline is also reported to enhance the acrosome reaction presumably due to threshold levels of cAMP (43). The results of pentoxifylline treatment in assisted reproduction are equivocal. Depending on the time of stimulation relative to the capacitative state of the spermatozoa and the concentration of pentoxifylline in the medium, overstimulation can result in a premature acrosome reaction (13). Thus, pentoxifylline tends to be used on a limited basis in IVF programs, and some programs choose to use pentoxifylline only in the preparation of epididymal and testicular sperm for assisted in vitro fertilization.

Spermatozoa retrieved from the testis have not experienced the maturation-inducing influence(s) afforded during epididymal transport, and therefore, are in a different physiologic state than epididymal

or ejaculated spermatozoa. Treatment of immotile or very poorly motile fresh or cryopreserved testicular spermatozoa with pentoxifylline very frequently simulates some form of motion, whether it is twitching, non-progressive motility, or progressive motility. The goal in any ICSI procedure is to use spermatozoa that are viable, and motion is the best indicator ensuring both a functional (protective) plasma membrane and patent metabolic processes. The combination of these two attributes lends greater assurance that the DNA has not been made more vulnerable to the deleterious effects of ROS.

Platelet-Activating Factor (PAF)

PAF is a biologically active phospholipid thought to be a cellular mediator in reproduction that has been found in spermatozoa of many different species, including humans (44). PAF has been reported to have positive effects on motility, capactitation, acrosome reaction, and oocyte penetration (45,46), and these stimulatory actions on sperm function can be inhibited by PAF antagonists (47). Although the molecular mechanism of action has yet to be fully elucidated, the positive effect of PAF on sperm function has led to its use in assisted reproduction. Roudebush (48) reported that pregnancy rates in IUI cycles were significantly increased after the spermatozoa from normozoospermic males were prepared with a medium containing PAF.

Detection of Viability

Sperm motility is an important indicator of viability. In the absence of inherent or stimulated sperm motility, the assessment of viability becomes critical when performing ICSI. There is a simple vitality test based on the semi-permeability of the intact and physiologically functional plasma membrane which causes spermatozoa to swell under hypo-osmotic conditions, when an influx of water results in an expansion of cell volume (7). This vitality test is known as the hypo-osmotic swelling (HOS) test.

The HOS test can be used for specimens where the spermatozoa are all immotile. When setting up a dish for the ICSI procedure, a small (5 μL) drop of HOS solution is placed near the polyvinylpyrrolidone (PVP) drop, and two extra drops of culture medium are placed nearby. A small volume of sperm suspension is placed in one of the extra drops. When spermatozoa are located, they are picked up in the ICSI micropipette and placed in the HOS solution. Immediately or soon after contact with the hypo-osmotic medium, the tails of some spermatozoa will begin to coil or swell due to hypo-osmotic stress. Tail swelling or curling indicates the spermatozoon is viable and has a functional plasma membrane that is continuing to serve a protective function for the DNA. The spermatozoon is then picked up in the ICSI micropipette and placed in the other extra drop of medium in order to wash off excess hypo-osmotic medium from both the micropipette and the spermatozoon. The spermatozoon is then placed in the PVP drop in order to proceed with ICSI.

Summary

With the advent of IVF came a demand for clinically suitable and safe techniques to isolate spermatozoa from the ejaculate. As IVF matured to include ICSI, techniques for sperm isolation necessarily expanded in parallel. This chapter on sperm preparation techniques for IVF/ICSI serves as a descriptive overview of the most commonly used techniques, and some techniques whose utility has lapsed, and other techniques for which their promise is yet to be fulfilled.

The development of ICSI initially satisfied any pressing need to develop more sophisticated techniques than swim-up or density gradient centrifugation, because with ICSI the need for sperm is, essentially, reduced to unity—one sperm for one egg. Thus, for a time, attention was somewhat naively focused on simply the overall recovery of motile sperm which then often served, in part, as a compass to guide whether the IVF would proceed by insemination of oocytes or, instead, injection using ICSI. The consequence being that the "quality" of the product emanating from the andrology lab was only tepidly assessed.

Simultaneously, basic science began to reveal that the motile sperm being isolated for ART using some of the common techniques may not have "the right stuff." For example, whether occurring before

processing or as a result of the processing technique, consequences of oxidative stress and apoptotic processes became revealed in the form of DNA damage. Research also uncovered that sperm being ejaculated and subsequently isolated carried a legacy from their manufacturing that was not necessarily conspicuous but rather covert. For example, sperm that on the surface appeared "normal" displayed signs of plasma membrane and chromatin immaturity. The research laboratory findings impacted not only IVF but also ICSI, and those findings drove larger questions regarding potential impact of these "scarred" sperm on rates of fertilization, clinical pregnancy, and live birth.

There has, over the last decade or so, been a greater awareness and concern that the choice and application of a sperm preparation technique could be a major contributor in influencing whether a patient will become pregnant, regardless of the method (IVF vs ICSI) used for generating embryos. Even more so, emerging research findings shed light that some sperm being selected for therapeutic application could impact on the health and well-being of the offspring.

Today, basic and clinical laboratory initiatives align themselves with the mission of sperm isolation for therapeutic purposes with aspects of "quality" extending far beyond the traditional semen parameters of count, motility, and morphology. The sperm proteome, transcriptome, and epigenome are all new areas of focus and consideration in clinical methodology development for sperm selection. With that being said, whatever new technology or method arises, the sperm being selected for IVF/ICSI should not show evidence of having been "analyzed"—no "foot prints should be left in the sand"—because therein lays the problem of potential iatrogenic influences at fertilization, embryo development, or birth.

The ART field has come of age; it has matured from its infancy into a multi-billion dollar global industry. From the recombinant medications for ovarian stimulation to sophisticated algorithm-driven time-lapse imaging systems to monitor early embryo development, to high throughput screening assays to probe early embryonic genome, the exploitation of technologies has satisfied both clinical and consumer demand. Lagging behind, however, is a similar demonstration of technology exploitation for the clinical andrology laboratory. Some of the new horizon techniques hold promise to advance the field. However, before new technologies can be implemented in the clinical laboratory setting they must first be properly vetted, as evidenced by high quality supportive and comparable literature data generated from multiple diverse sources. The time is ripe for clinical andrology to come of age.

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REFERENCES

1. Austin CR. Observations on the penetration of sperm into the mammalian egg. *Aust J Scr Res.* 1951;4(series B):581.
2. Chang MC. Fertilizing capacity of spermatozoa deposited into the fallopian tubes. *Nature.* 1951;18:697.
3. Yanagimachi R. Mammalian fertilization. In: *The Physiology of Reproduction*, 2nd ed., Knobil E, Neill JD (Eds.). Raven Press, New York, 1994, 189.
4. Rogers BJ et al. Variability in the human-hamster in vitro assay for fertility evaluation. *Fertil Steril.* 1983;39:204.
5. Kanwar KC, Yanagimachi R, Lopata A. Effects of human seminal plasma on fertilizing capacity of human spermatozoa. *Fertil Steril.* 1979;31:321.
6. De Jonge CJ. Biological basis for human capacitation. *Hum Reprod Update.* 2005;11:205.
7. WHO. *WHO Laboratory Manual for the Examination and Processing of Human Semen*, 5th ed., World Health Organization, 2010.
8. Barash A, Lurie S, Weissman A, Insler V. Comparison of sperm parameters, in vitro fertilization results, and subsequent pregnancy rates using sequential ejaculates, collected two hours apart, from oligoasthenozoospermic men. *Fertil Steril.* 1995;64:1008.
9. Jeulin C, Serres C, Jouannet P. The effects of centrifugation, various synthetic media and temperature on the motility and vitality of human spermatozoa. *Reprod Nutr Dev.* 1982;22:81.

10. Aitken RJ, Clarkson JS. Significance of reactive oxygen species and antioxidants in defining the efficacy of sperm preparation techniques. *J Androl*. 1988;9:367.
11. Lopes S, Jurisicova A, Sun JG, Casper RF. Reactive oxygen species: Potential cause for DNA fragmentation in human spermatozoa. *Hum Reprod*. 1998;13:896.
12. Mahadevan M, Baker G. Assessment and preparation of semen for in vitro fertilization. In: *Clinical In Vitro Fertilization*, Wood C, Trounson A (Eds.). Springer-Verlag, Berlin, 1984, 83.
13. Henkel RR, Schill W-B. Sperm preparation for ART. *Reprod Biol Endocrinol*. 2003;1:108.
14. Oehninger S, Acosta R, Morshedi M, Philput C, Swanson RJ, Acosta AA. Relationship between morphology and motion characteristics of human spermatozoa in semen and in the swim-up sperm fractions. *J Androl*. 1990;11:446.
15. Erel CE, Senturk LM, Irez T, Ercan L, Elter K, Colgar U, Ertungealp E. Sperm-preparation techniques for men with normal and abnormal semen analysis. *J Reprod Med*. 2000;45:917.
16. Mortimer D, Mortimer ST. Methods of sperm preparation for assisted reproduction. *Ann Acad Med*. 1992;21:517.
17. Ren S-S, Sun GH, Ku CH, Chen DC, Wu GJ. Comparison of four methods for sperm preparation for IUI. *Arch Androl*. 2004;50:139.
18. Tea NT, Jondet M, Scholler R. A migration-gravity sedimentation method for collecting motile spermatozoa from human semen. In: *In Vitro Fertilization, Embryo Transfer and Early Pregnancy*, Harrison RF, Bonner J, Thompson W (Eds.). MTP Press Ltd., Lancaster, 1984, 117.
19. Gabriel LK, Vawda AI. Preparation of human sperm for assisted conception: A comparative study. *Arch Androl*. 1993;30:1.
20. Sanchez R, Stalf T, Khanaga O, Turley H, Gips H, Schill WB. Sperm selection methods for intracytoplasmic sperm injection (ICSI) in andrological patients. *J Assist Reprod Genet*. 1996;13:228.
21. Mortimer D. Sperm preparation methods. *J Androl*. 2000;21:357.
22. Malvezzi H, Sharma R, Agarwal A, Abuzenadah AM, Abu-Elmagd M. Sperm quality after density gradient centrifugation with three commercially available media: A controlled trial. *Reprod Biol Endocrinol*. 2014;12:121.
23. Aitken RJ, Finnie JM, Muscio L, Whiting S, Connaughton HS, Kuczera L, Rothkirch TB, De Iuliis GN. Potential importance of transition metals in the induction of DNA damage by sperm preparation media. *Hum Reprod*. 2014;29:2136.
24. Nicholson CM, Abramsson L, Holm SE, Bjurulf E. Bacterial contamination and sperm recovery after semen preparation by density gradient centrifugation using silane-coated silica particles at different g forces. *Hum Reprod*. 2000;15:662.
25. Hammadeh ME, Kühnen A, Amer AS, Rosenbaum P, Schmidt W. Comparison of sperm preparation methods: Effect on chromatin and morphology recovery rates and their consequences on the clinical outcome after in vitro fertilization embryo transfer. *Int J Androl*. 2001;24:360.
26. Tomlinson MJ, Moffatt O, Manicardi GC, Bizzaro D, Afnan M, Sakkas D. Interrelationships between seminal parameters and sperm nuclear DNA damage before and after density gradient centrifugation: Implications for assisted conception. *Hum Reprod*. 2001;16:2160.
27. Morrell JM, Moffatt O, Sakkas D, Manicardi GC, Bizzaro D, Tomlinson M, Nilsson H, Holmes PV. Reduced senescence and retained nuclear DNA integrity in human spermatozoa prepared by density gradient centrifugation. *J Assist Reprod Genet*. 2004;21:217.
28. Politch JA, Xu C, Tucker L, Anderson DJ. Separation of human immunodeficiency virus type 1 from motile sperm by the double tube gradient method versus other methods. *Fertil Steril*. 2004;81:440.
29. Paulson JD, Polakoski KL. A glass wool column procedure for removing extraneous material from the human ejaculate. *Fertil Steril*. 1977;28:178.
30. Jeyendran RS et al. Separation of viable spermatozoa by standardized glass wool column. In: *In Vitro Fertilisation*, Ratnam SS, Teoh E-S, Ng S-C (Eds.). Parthenon Publishing, Carnforth, England, 1987, 49.
31. Rhemrev J, Jeyendran RS, Vermeiden JP, Zaneveld LJ. Human sperm selection by glass wool filtration and two-layer, discontinuous Percoll gradient centrifugation. *Fertil Steril*. 1989;51:685.
32. Henkel R, Ichikawa T, Sánchez R, Miska W, Ohmori H, Schill WB. Differentiation of ejaculates in which reactive oxygen species are generated by spermatozoa or leukocytes. *Andrologia*. 1997;29:295.
33. Drobnis EZ, Zhong CQ, Overstreet JW. Separation of cryopreserved human semen using Sephadex columns, washing or Percoll gradients. *J Androl*. 1991;12:201.

34. Ainsworth C, Nixon B, Aitken R. Development of a novel electrophoretic system for the isolation of human spermatozoa. *Hum Reprod*. 2005;20:2261.
35. Fleming SD, Ilad RS, Griffin AM, Wu Y, Ong KJ, Smith HC, Aitken RJ. Prospective controlled trial of an electrophoretic method of sperm preparation for assisted reproduction: Comparison with density gradient centrifugation. *Hum Reprod*. 2008;23:2646.
36. Said T, Agarwal A, Grunewald S, Rasch M, Baumann T, Kriegel C, Li L, Glander HJ, Thomas AJ Jr, Paasch U. Selection of nonapoptotic spermatozoa as a new tool for enhancing assisted reproduction outcomes: An in vitro model. *Biol Reprod*. 2006;74:530.
37. Huszar G, Ozenci CC, Cayli S, Zavaczki Z, Hansch E, Vigue L. Hyaluronic acid binding by human sperm indicates cellular maturity, viability, and unreacted acrosomal status. *Fertil Steril*. 2003;79(Suppl 3):1616.
38. Nasr-Esfahani MH, Marziyeh T. Sperm selection for ICSI using the hyaluronic acid binding assay. *Methods Mol Biol*. 2013;927:263.
39. Bartoov B, Berkovitz A, Eltes F, Kogosowski A, Menezo Y, Barak Y. Real-time fine morphology of motile human sperm cells is associated with IVF-ICSI outcome. *J Androl*. 2002;23:1.
40. Bartoov B, Berkovitz A, Eltes F, Kogosovsky A, Yagoda A, Lederman H, Artzi S, Gross M, Barak Y. Pregnancy rates are higher with intracytoplasmic morphologically selected sperm injection than with conventional intracytoplasmic injection. *Fertil Steril*. 2003;80:1413.
41. Teixeira DM, Barbosa MA, Ferriani RA, Navarro PA, Raine-Fenning N, Nastri CO, Martins WP. Regular (ICSI) versus ultra-high magnification (IMSI) sperm selection for assisted reproduction. *Cochrane Db Syst Rev*. 2013;(7):CD010167.
42. McDowell S, Kroon B, Ford E, Hook Y, Glujovsky D, Yazdani A. Advanced sperm selection techniques for assisted reproduction. *Cochrane Db Syst Rev*. 2014;(10):CD010461.
43. Tesarik J, Mendoza C, Carrera A. Effects of phosphodiesterase inhibitors caffeine and pentoxifylline on spontaneous and stimulus-induced acrosome reaction in human sperm. *Fertil Steril*. 1992;58:1185.
44. Minhas BS, Kumar R, Ricker DD, Robertson JL, Dodson MG. The presence of platelet-activating factor-like activity in human spermatozoa. *Fertil Steril*. 1991;55:372.
45. Sengoku K, Tamate K, Takaoka Y, Ishikawa M. Effects of platelet-activating factor on human sperm function. *Hum Reprod*. 1993;8:1443.
46. Krausz C, Gervasi G, Forti G, Baldi E. Effect of platelet-activating factor on motility and acrosome reaction of human spermatozoa. *Hum Reprod*. 1994;9:471.
47. Cheminade C, Gautier V, Hichami A, Allaume P, Le Lannou D, Legrand AB. 1-O-Alkylgycerols improve boar sperm motility and fertility. *Biol Reprod*. 2002;66:421.
48. Roudebush WE, Toledo A, Mitchell-Leef D, Elsner C, Keenan D, Slayden S, Shapiro D, Massey J, Kort H. Short-term exposure of sperm to platelet-activating factor treatment will significantly improve intrauterine insemination pregnancy rates. *Hum Reprod*. 2003;18(Suppl 1):63.

11

Assisted Fertilization

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Introduction and History

Assisted fertilization (or in vitro fertilization) refers to a process when the oocyte is fertilized by a sperm "outside" of the body (i.e., in vitro) (1–3). The two techniques that are applied today are "conventional" IVF (2) and intracytoplasmic sperm injection (ICSI) (4). Robert Edwards, scientist (and first "Clinical Embryologist"), together with Patrick Steptoe, clinician, achieved the first successful live birth in 1978, which become a landmark in our field of assisted reproduction (2). Louise Brown, the first "test tube baby," was born following a natural IVF cycle. Professor Edwards has been awarded numerous honors, the most prestigious one, the Nobel Prize (in Physiology or Medicine), was given to him in 2010. This breakthrough was the result of decades of research to better understand reproductive physiology both in humans and in animals. Spallanzani's description in the late 1700s may be regarded as the first report on in vitro fertilization, when he demonstrated the fertilization of frog oocytes mixed with semen. Antoni van Leeuwenhoek (1632–1723) deserves credit of developing the microscope, amongst his many other discoveries, which has been an essential tool for observing gametes and the process of fertilization. Prevost and Dumas, in the early nineteenth century, were able to demonstrate that spermatozoa are produced by the testes and that motile sperms were required for fertilizing frog eggs. In 1826, Baer discovered the mammalian ovum. Edouard van Beneden was the first to observe fertilization in mammals in 1875. The beginning of the "modern era" may be regarded as starting when sperm capacitation was described simultaneously by Austin and Chang, in the middle of the twentieth century. Fertilization of rabbit ova was reported in 1959 by Chang (5). In 1965, Robert Edwards together with Georgeanna and Howard Jones (in the U.S.A,) attempted to fertilize human oocytes in vitro, followed by the report on pronuclear formation after insemination of human oocytes by Edwards in 1969 (6). A year later Edwards reported embryonic development to the 16 cell stage (7). Following the first successful IVF in humans in 1978 from the United Kingdom, soon other countries also reported pregnancies after IVF, including Australia, the United States, and France (8–10). Births after IVF have increased exponentially over the years; it is estimated that in 1990, a little more than a decade after the first IVF birth, about 95,000 babies were born. By 2000, it was thought that nearly 1 million babies were born, and in 2015, it had climbed to about five million.

Conventional IVF Insemination

The idea of attempting fertilization of the oocyte outside of the body ("in vitro" fertilization) was originally suggested for couples, where in vivo interaction between sperm and egg was not possible, such as cases with blocked (or missing) Fallopian tubes. The first, and many other initial IVF treatments, had this indication—which later was extended also for other indications, including a variety of both female and male factors (11,12).

Conventional insemination performed in the laboratory is mimicking in many aspects what happens in in vivo. Principally, the oocyte(s) obtained from a stimulated (or natural) cycle are placed in a petri dish (or in a tube) and mixed together with processed sperm obtained from the partner. Gametes are left in place in the petri dish for several hours (typically overnight), to allow the sperm to get through the outer

layers of the oocyte (cumulus and corona cells) and get into the egg. The morning after the insemination the cumulus and corona cells are removed and the oocyte(s) are examined for the presence of pronuclei, which if present, indicate fertilization. While this procedure appears to be simple and straightforward (today), there are several factors that can strongly impact outcomes, or influence efficiency of the procedure, which will be discussed in more detail.

The introduction of controlled ovarian hyperstimulation (COH) in the early 1980s is of significant importance (9,13,14), though not impacting the conventional insemination per se, but providing a higher number of available oocytes (than obtained from a natural cycle), which definitely makes the whole IVF process more efficient. Additionally, the introduction of ultrasound guided follicle aspiration around the same time provided a much more efficient and reliable way to retrieve the oocyte(s) (15).

Timing of Oocyte Collection

Appropriate timing of oocyte aspiration from the follicles is critical. Based on what we learned from the physiological events of the menstrual cycle and "programmed ovulation" (ovulation occurs about 40 hours after luteinizing hormone [LH] surge) (16–18); egg collection is typically performed between 34 and 38 hours after trigger in a stimulated cycle (using either human chorionic gonadotropin [hCG] or gonadotropin-releasing hormone [GnRH] agonist, depending on the suppression protocol) [19–24]. In some situations, when in-vitro oocyte maturation is aimed (for instance in polycystic ovary [PCO] syndrome cases), oocyte collection may be performed without hCG trigger (with "minimal stimulation" or no stimulation) (25–27).

Identification of Cumulus–Oocyte Complexes

Aspirated follicular fluid is examined in a petri dish using a dissecting microscope. Cumulus–oocyte complexes (CCOCs) are separated and cleaned from debris, blood, and blood clots (28). Trimming of cumulus is performed in most IVF programs, using needles (or other sharp tools). "Maturity" of cumulus–oocyte complexes used to be assessed meticulously in the early days of IVF, however, nowadays, in the era of ICSI, there is much less emphasis on it (29–31). Nuclear maturity of the oocyte is hard to judge at that time, as cumulus and corona cells obstruct the visualization of the polar body or germinal vesicle (or their absence). However, Veeck has developed a simple "maneuver" that flattens out the cells around the oocyte and thus one may be able to judge the egg maturity (29).

Timing and Process of Conventional insemination

Insemination is typically performed 4–5 hours post oocyte retrieval (40–41 h after ovulation trigger), corresponding to a physiologically correct timing. Timing of insemination may be altered if the morphological aspects of cumulus–oocyte complexes suggest that the eggs are mostly immature. If CCOCs are dense, smaller sized (indication for immaturity), then insemination may be delayed an additional 2–3 hours, which in turn may lead to higher fertilization rates (32). Insemination is performed typically in a fresh dish of culture medium in droplets under oil. Treated sperm is added to the insemination drop, having a final concentration between 100,000 and 250,000/mL (lower concentrations may be used if previous IVF yielded a high rate of 3PNs [three pronuclear oocytes]—conversely, higher concentrations may be used if previous IVF produced low fertilization) (33). Next, cumulus–oocyte complexes (CCOCs) are added to the fertilization drops. Typically, 2–6 CCOCs placed to a drop of roughly 100 μL (~25 μL of medium per CCOCs). Incubation of gametes takes place for approximately 15–17 hours (33). Interestingly, some studies have demonstrated that a much shorter incubation time may be sufficient to obtain a high fertilization rate with conventional insemination (34–36). For fertilization to occur, a single sperm must pass through the cumulus cells, interact with the zona pellucida, fuse with the oolemma, and decondense inside the ooplasm. This technique may be used for the non-male factor patients and male factor patients at the discretion of the embryology laboratory (33). In the past (prior ICSI), for patients with male-factor/decreased sperm parameters a high insemination concentration (HIC) procedure was recommended; however, when compared to ICSI, it did not provide similar outcomes (37–40).

Sperm Preparation for Conventional Insemination

For conventional insemination, typically fresh, ejaculated sperm is used (though cryopreserved ejaculated sperm can be used too). Before sperm collection, the male partner is instructed to abstain from ejaculation for approximately 3–5 days. Semen should be collected into a sterile cup, and one has to make sure that there is no contact with any material that may have a toxic effect on the sperm cells. Semen sample preparation is a critical step, as sperm has to be treated adequately to aid the process of capacitation—which is essential to obtain fertilization. Capacitation is a complex process, involving several major molecular changes and also resulting in hyperactivated motility (41). It is beyond the scope of this chapter to go into more details on sperm capacitation, but for a detailed review, please refer to the publication of Aitken (42). The specimen is maintained at room temperature after collection and during the preparation procedure. The sample should be allowed to liquefy at room temperature. If the sample has not liquefied after 20 minutes, 5 mg chymotrypsin may be added. Samples should be processed within one hour after obtaining, by the laboratory. Semen samples should also always be assessed for the basic sperm parameters before and after preparation, using the World Health Organization (WHO) as a reference guide for assessment (43). There are different techniques to prepare sperm for conventional insemination, the two most commonly used are (1) the swim-up method and (2) gradient density centrifugation (44–46). These two methods provide similar sperm yield (concentration and motility), however, gradient density centrifugation is more advantageous to remove debris from semen and to deselect grossly abnormal (morphological) sperm. Details of these two (and other) methods can be found in the following publications: (44–46), including how to best handle unusual issues (when semen contains blood, large number of white cells, or other debris, or has high viscosity, etc).

Evaluation of Fertilization after Conventional Insemination

During the co-incubation of gametes, hundreds of sperm cells penetrate into the cumulus mass and approach the zona pellucida. The acrosome reaction, which includes several parallel processes, might occur as early as when sperm pass through cumulus (47) but more likely it is induced by zona proteins, and especially by the glycoprotein ZP3 (48,49). Prior to the inclusion of the sperm in the oocyte, the spermatozoon has to penetrate the zona pellucida and fuse with the ooplasm. Only one sperm penetrating the oocyte induces the exocytosis of cortical granules content, which in turn alters the structure of the zona pellucida, making it impossible for other sperm to penetrate it (50). After the fusion of the gametes, a series of changes take place, starting with the activation of the egg (51). Calpain is activated within minutes of sperm entry, which inactivates mos protein (52). Mos protein is mostly destroyed within 15 to 30 minutes of sperm entry. Cyclins are also degraded and maturation promoting factor (MPF) inactivated within 10 minutes of sperm entry. The egg, activated by calcium and freed from the inhibitory action of mos protein, completes meiosis 2 and expels its second polar body, and the egg chromosomes and sperm nucleus form the female and male pronuclei. The mechanism of oocyte activation, involving the phospholipase C zeta released by the sperm (51) and the development of pronuclei, are truly fascinating processes, and more details can be found in these reviews: (51,53–55). At 18–20 hours post-insemination the corona and cumulus cells that remain attached to the oocyte are gently removed mechanically, using pipettes with 140–150 micrometer diameter. The greatest care is required to avoid any damage to the zona pellucida or to the egg. After the removal of the cumulus and corona cells, the oocytes/zygotes are washed and then transferred into a new and pre-equilibrated drop of cleavage medium, according to laboratory protocols. Oocyte is considered to be normally fertilized, when there are two pronuclei clearly visible, and there are also two polar bodies present in the perivitelline space. Normal fertilization rate varies widely after conventional insemination, depending on many factors, especially related to basic sperm parameters. It has been a common observation, when sperm parameters are adequate, and no other negative factors present, that a typical 60% to 80% fertilization may be achieved (56). However, in the decade after the introduction of IVF, it was observed that fertilization rates may be much lower when certain male-factor conditions are present, including lower concentration (oligozoospermia), lower motility (asthenozoospermia), or lower rate of normal morphology (teratozoospermia), or any of these three factors combined (57). There are other factors, beyond the basic sperm parameters, such as high

DNA fragmentation in sperm (that can be measured by different tests), where conventional insemination does not prove to be always very successful (58). Additionally, there have been different observations that demonstrated partial or complete fertilization failure of conventional insemination, even if sperm parameters were within the normal range (59,60).

Assisted Fertilization and Intracytoplasmic Sperm Injection (ICSI)

Development of the Micro-Injection Technique

Because of the inconsistent results with IVF (mild male-factor) and as well the very poor results of conventional IVF where severe male-factor was existing, a need was present for a new type of insemination technique, using microinjection. Probably the first microinjection, of spermatozoa into eggs of the starfish, was performed in the early 1900s by Kite, but results of this experiment were inconclusive (61). Nearly half a century later, Hiramoto reported microinjection of live sperm into unfertilized sea urchin eggs in an attempt to investigate oocyte activation (62). Uehara and Yanagimachi applied the technique of sperm injection to determine whether spermatozoa or sperm nuclei injected into the hamster egg cytoplasm can develop into male pronuclei (63,64). They also examined whether spermatozoa from various other species would develop into pronuclei when injected into hamster oocytes. It was also found that the nuclei of fresh, freeze-dried, and frozen-thawed human spermatozoa were able to develop into pronuclei in hamster oocytes, suggesting that the cytoplasmic factors controlling the transformation of sperm nuclei into male pronuclei are not species-specific (65,66). The injection of mouse sperm into rat oocytes resulted in a high proportion of activation and formation of both pronuclei. Capacitated and uncapacitated sperm reacted similarly when injected into oocytes, suggesting that sperm capacitation may not be necessary when spermatozoa are injected directly into cytoplasm (67). It was also concluded from the results of these studies that the interactions normally required for sperm to penetrate and fertilize oocytes are not biologically necessary and can be circumvented by direct injection of spermatozoa. This conclusion was further supported when single, uncapacitated rabbit sperm was injected into the cytoplasm of a series of superovulated rabbit oocytes, resulting in a relatively high survival rate (63%) and pronuclear formation rate (46%) (68). When intracytoplasmic injection of sperm was attempted in the mouse, a very high degeneration rate and an extremely low fertilization rate were obtained, which were most probably the result of certain technical parameters such as a relatively large injection pipette (69). A similar report was published by Ron-El et al. several years later, when intracytoplasmic injection of sperm was working satisfactorily in humans, confirming the technical difficulties deriving from the relatively small size of the oocyte as compared to the relatively large sperm (70). Most of the research on microfertilization described here was conducted by cytoplasmic sperm injection. With this method, a low fertilization rate and sometimes also a low developmental rate were obtained. For this reason, efforts have been made to establish alternative ways of micromanipulation that can be more efficient than intracytoplasmic injection. One of the first reports on micromanipulation of gametes in rodents aiming to establish a possible clinical use comes from Barg et al. who in 1986 injected immotile mouse sperm under the zona pellucida of mouse oocytes but did not observe fertilization (71). Most of the other reports, however, which described the insertion of sperm under the zona pellucida of oocytes (subzonal injection or SUZI) in rodents were successful in terms of fertilization (72,73). A majority of the reports also emphasized the importance of the acrosome reaction in the sperm used for microinjection in order to obtain fertilization (74–76). An alternative way of micromanipulation of mouse gametes was also reported. It involved a mechanical opening of the zona pellucida of the oocytes using a sharp microneedle (partial zona dissection or PZD) or a chemical opening using digestive enzymes or acidified solution (zona drilling or ZD) before exposing the oocyte to the sperm (77–80). The employment of PZD or ZD also offered an improvement in fertilization as compared to controls in the form of unmanipulated oocytes (77–79). Zona drilling of human oocytes using acid Tyrode's solution in order to alleviate male-factor infertility was first reported in 1988 by Gordon (81). In this publication, fertilization of oocytes was reported but pregnancy did not ensue in the ten couples who were involved in this treatment series. Although a few other investigators also tried to use the same approach (82–84), because of the very low success rate with this technique, its application did not gain wider use.

The first report on implantation of an embryo resulting from fertilization through opening the zona pellucida appeared in 1988 (85). Routine application of partial zona dissection (PZD) was attempted by some fertility centers to help couples with male-factor infertility or couples with previous failure in IVF, but results of this method were usually very poor in terms of normal fertilization and pregnancy rates, and it was always associated with a very high level of multiple pronuclear fertilization (83,84,86,87). When results of partial zona dissection were compared to those from standard insemination, no clear advantage was found in terms of fertilization rate (88), which explains why this type of micromanipulation method did not gain wider acceptance in infertility treatment. The first report of the insertion of spermatozoa under the zona pellucida (SUZI) of human oocytes to result in a relatively high rate of fertilization without damage to the oocyte was published in 1987 (89). This technique was introduced into several IVF laboratories with a relatively consistent result. A total fertilization rate of between 10% and 30% was usually obtained, which was related to the type of infertility problem or to certain technical aspects, such as the number of sperm microinjected. The abnormal fertilization rate tended to rise faster than the normal fertilization rate when increasing numbers of sperm were microinjected (82,90–97). Despite the relatively low fertilization, subzonal injection of spermatozoa seemed to offer a higher fertilization rate than conventional insemination, especially in cases of very severe male infertility (98). When results of subzonal sperm injection were compared to the results of partial zona dissection, then most authors found that SUZI provided higher normal fertilization and thus a better chance of pregnancy (99–101).

There were only very few early reports describing the intracytoplasmic injection of spermatozoa (ICSI) into the oocyte as a preclinical evaluation of this method for potential use in male-factor infertility (102,103). In both of these publications, normal (2-PN) fertilization was reported, but pregnancy did not ensue after the transfer of the derived embryos (102,103). The first pregnancies, followed by live births, reported after the use of ICSI appeared in 1992 (4) by Palermo, who developed this breakthrough technique at the Dutch speaking Brussels Free University (AZ-VUB). However, in the beginning (in the first 300 microinjection cycles performed at the AZ-VUB) SUZI was more frequently used than ICSI (104). But soon enough, because of the consistently better results with ICSI, intracytoplasmic injection was used more and more regularly in the second series of 300 microinjection cycles (105). Finally, SUZI was completely abandoned following a comparative trial on sibling oocytes between SUZI and ICSI, and ICSI became the sole method of assisted fertilization for the alleviation of male infertility at the Brussels Free University (106). Subsequently, other infertility centers also compared the results of SUZI with ICSI and without any exception the outcome of ICSI was superior to the results of SUZI, which further explains its rapid spread all over the world (107–109).

Timing of Oocyte Collection and Preparation for ICSI

Timing of oocyte collection and identification of CCOCs for ICSI is performed the same way as described earlier for conventional insemination. However, preparation of oocytes for ICSI is markedly different than preparation for conventional insemination. Two to three hours after egg retrieval (38–39 hours after ovulation trigger), the cells of the cumulus and corona radiata are removed by incubation for about 30–60 seconds in HEPES-buffered medium containing hyaluronidase. Initially, up to 80 IU hyaluronidase/mL concentration was used (using bovine derived product), but today most labs use much lower concentrations (about 10 IU/mL, and recombinant product) (110–112). The removal of the cumulus and corona cells is enhanced by aspiration of the complexes in and out of hand-drawn glass or plastic pipettes with different diameter openings—250–300, 200 and 150 µm. The oocytes are subsequently rinsed several times then observed under the inverted microscope at 200× magnification for nuclear maturity and for other morphological characteristics (113). Just prior to the ICSI procedure the oocytes are observed again to see whether more oocytes have extruded the first polar body. ICSI is carried out typically on all morphologically intact oocytes that have extruded the first polar body.

Sperm Preparation from Ejaculate from Epididymis and from Testis for ICSI

The preparation of sperm from ejaculate is performed the same way for ICSI as it is performed for conventional insemination (as described earlier). In cases with extreme low concentration (alone or in combination with other sperm impairments—oligo-astheno-teratozoospermia), a high speed "concentration"

centrifugation is performed (1800 g) after (or instead) of gradient centrifugation (44,114–116). The prepared sperm suspension can be kept in the 37°C incubator (5% O_2, 5% CO_2, 90% N_2) or alternatively in a pre-gassed and well-closed tube at room temperature until the time for intracytoplasmic injection of the oocytes (115).

Epididymal sperm: the epididymal fluid is immediately diluted in medium (usually HEPES buffered) and a tiny portion examined for number, motility, and quality of progression. If there is no motility or poor motility, another aspiration is made (usually more proximally at the epididymis) (117). Motile spermatozoa are more likely to be obtained at the most proximal portion of the caput epididymis or even the vasa efferentia (118). The sperm fractions with the highest concentration and motility are pooled and treated in the same way as ejaculated semen. Whenever possible, a part of the freshly recovered epididymal sperm is frozen for later use to avoid the micro-surgical epididymal sperm aspiration procedure in subsequent cycles. If frozen epididymal sperm is being used it is put on a two-layer Percoll (95%–47.5%), and centrifuged for 20 min at 300 g. The 95% fraction is washed with "sperm preparation" medium for 5 min at 1800 g and the pellet used for injection after one additional centrifugation in a 1.5 mL Eppendorf tube (5 min, 1800 g) (119).

Testicular sperm: fresh human testicular tissue is obtained usually by excisional biopsy or by fine needle aspiration and put into a Falcon petri dish (or conical tube) containing HEPES-buffered medium (120,121). The testicular tissue is teased apart (after biopsy) with microscopic glass slides (or needles, or any other sharp and sterilized tools) on the stage of a stereo microscope at 40× magnification. The petri dish is then checked for the presence of spermatozoa under an inverted microscope at 200× or 400× magnification. If no sperm cells are observed in the teased testicular tissue, another biopsy specimen is excised. The contents of the petri dish are transferred into a 10 mL Falcon tube and incubated until the moment of injection. Just prior to injection, the remaining tissue is removed from the tube and the fluid centrifuged for 5 min at 300 g. If motile spermatozoa are observed during the first examination, the supernatant is centrifuged again for 5 min at 1800 g (119). If there is very little or no sperm found in the biopsied testicular tissue, then further processing of the biopsy sample may be performed. This additional step can be mechanical, further tearing up the testicular tissue with tools (122), or erythrocyte lysing buffer can be used (removing red blood cells from the testicular tissue preparation can aid enormously to visualize/find sperm) (123). Additionally, elastase and/or collagenase "digestion" can also be performed on the testicular tissue (which can take a few hours, so the biopsy procedure may be planned the day before the ICSI procedure, in cases where a very low sperm count is anticipated, such as non-obstructive azoospermia), which is a very effective way to recover sperm cells from testicular sperm extraction (TESE) samples (specially, if combined with "vortexing" the sample) (124,125).

Timing and Process of ICSI Insemination

ICSI is usually performed 3–5 hours after oocyte retrieval (39–41 hours after ovulation trigger), as it is estimated this will provide the most optimal outcomes (126). Timing of ICSI, relative to the enzymatic exposure of CCOCs and to the mechanical denudation, does not seem to correlate significantly with outcomes (127). The ICSI procedure is carried out on the heated stage of an inverted microscope at 400× or 200× magnification using a Hoffman Modulation Contrast System (if a plastic ICSI dish is used) (128). The microscope is typically equipped with two coarse positioning manipulators and with two three-dimensional hydraulic remote-control micro manipulators (or with similar tools). A single spermatozoon is selected from the sperm droplet of the ICSI dish (usually the central droplet) and is aspirated into the tip of the injection pipette after rendering it immotile by breaking the tail by pushing it with the injection pipette against the bottom of the dish. Presence of Ca++ in the medium that is used for microinjection was found to be important by Gearon to obtain activation of oocytes at a high rate (129). Appropriate immobilization of sperm by squeezing the tail was emphasized by Palermo and others (130–132). Squeezing the tail of the sperm is also believed to destabilize/interrupt the membrane of the sperm, which could possibly lead to acrosome reaction and consequently contribute to adequate oocyte activation (and improved fertilization) (133). For ICSI, the petri dish is moved in order to visualize an oocyte in one of the droplets surrounding the sperm suspension. The oocyte is immobilized by the holding pipette. The polar body is held at 6 o'clock (or 12 o'clock) and the micro pipette is pushed through the zona pellucida and the oolemma into the ooplasm at 3 o'clock (making sure that the plasma membrane is broken). The site of

sperm deposition was demonstrated to be of importance to obtain optimal results by Nagy and colleagues (134). A single spermatozoon is injected into the ooplasm with the smallest amount of medium possible.

It was described first by Nagy and colleagues that the breakage of the oolemma can differ depending on technical and oocyte parameters (134). Oolemma reactions were categorized in five groups, based on how easy or difficult it was to break the membrane of the oocyte and which kind of injection technique was used in order to be able to deliver the sperm inside the cytoplasm, and the outcomes analyzed showed significant correlations (134). Later, others described similar findings (135). As a final step, oocytes are washed in culture medium after injection and transferred back into a culture dish and back to the incubator (128).

Sperm Selection Methods for ICSI

Traditionally, sperm for ICSI is selected by morphological evaluation of the sperm in the ICSI dish (after semen was processed as described earlier) using 200× or 400× magnification (4,115,136). The typical fertilization rate ranges from 50% to 80% (of the injected MII oocytes), depending on many factors, including sperm source/parameters and egg quality (115,119). However, in the last one or two decades, there have been several investigations that aimed to improve results of ICSI by applying different methods of sperm selection. Among the different techniques studied were annexin V magnetic activated cell separation (MACS) (137,138) and glass wool filtration (139); electrophoresis (surface charge based selection) (140); and hyaluronic acid binding (sperm "maturity" assessed by membrane characteristics) (141–145). One of the most extensively studied methods is intracytoplasmic morphologically selected sperm injection (IMSI), which is based on motile sperm organelle morphology examination (MSOME) (146). Initial studies have shown improvement in ICSI outcomes when MSOME was applied (147–149), however, more recent studies could not demonstrate the benefit of IMSI over traditional ICSI (150–152). Many of these methods help to select sperms with lower DNA fragmentation (decreased stress/ROS exposure) (58), and improved maturity that gives improved ICSI outcomes (not only at the level of fertilization, but potentially also improved embryo viability). Currently, none of these experimental sperm selection methods are used widely in the daily routine in IVF laboratories, but we can expect that future developments may result in techniques that will provide a meaningful improvement. For details on the different sperm selection techniques, please refer to the following reviews: (153,154).

Fertilization after ICSI and Potential Use and Improvements of the Technique

Evaluation of fertilization after ICSI is very similar to what we do after conventional insemination (see earlier); however, there are some differences. Timing of two-pronuclear evaluation (2-PN) may be different (optimal time is 16–18 hours after ICSI and 18–20 hours after conventional IVF), as the time course of oocyte activation and pronuclear development usually happens sooner after ICSI than after conventional IVF (also the disappearance of pronuclei after ICSI occur sooner), as was reported initially in 1994 by Nagy and colleagues, using the traditional evaluation at frequent time intervals (155,156), and later also by using the time-lapse approach (157). Another aspect, where observed outcomes may be different for conventional IVF versus ICSI is the occasional degeneration of oocytes. After conventional IVF, oocyte degeneration is virtually nonexistent, but after ICSI some oocytes may degenerate as an undesired side effect of the injection procedure. Many parameters can contribute to oocyte degeneration (including injection needle shape, sharpness, etc., as well as operator experience) but it was observed also that certain oolemma characteristics are more frequently associated with oocyte degeneration after ICSI (134). To overcome "hard" zona pellucida (and more fragile oolemma), Nagy introduced the laser-assisted ICSI procedure, where, using laser beam (the same as used for assisted hatching, but at a much lower power setting) a tiny channel is drilled in the zona first, then the injection needle is introduced through that hole (158). Laser-assisted ICSI provides a virtually atraumatic injection, and thus prevents oocyte degeneration—an outcome that was confirmed later by other studies as well (159–161).

One of the novel uses of ICSI, suggested originally by Nagy and colleagues, was to re-inseminate oocytes that failed to fertilize after conventional insemination, a procedure called "rescue ICSI" (162). It was observed that IVF failed fertilized oocytes had very high fertilization rates after ICSI re-insemination, although at the same time an exponentially increasing abnormal fertilization (3PN or >3PN

fertilization) was also observed (correlating with oocyte "aging"), also increasing chromosomal abnormalities detected in the derived embryos (of the normally fertilized eggs); and the embryos, if transferred, had very low implantation potential (162,163). To overcome oocyte aging related anomalies with ICSI re-insemination, it was suggested to shorten the time of conventional insemination (to 4–6 hours, instead of overnight), and perform ICSI re-insemination on oocytes that had no sign of activation (no 2nd polar body extrusion) (164–167). Although "rescue ICSI" with short IVF insemination was demonstrated to be valuable, because of the logistics of this approach (the time of procedure to perform rescue ICSI would be typically in the evening, after a morning oocyte retrieval), and because of the ever-extending use of ICSI, this method has not become a routine procedure.

Outcomes with ICSI Insemination

Early reports about the results of ICSI (104,106,168) lead to the conclusion that intracytoplasmic sperm injection can be used successfully to treat couples who have failed IVF or who have too few spermatozoa for conventional methods of in vitro insemination (168). Consequently, ICSI has been fast adopted all over the world, and the results of it are highly satisfactory, according to reports (109,169–178). At the same time, the proportion of cases with ICSI treatment increased rapidly compared to other types of infertility treatments (www.sart.org).

At the time of ICSI introduction, it was not known what sperm parameters, if any, can impact outcomes (or what extent/threshold of sperm impairment may be associated negatively with ICSI). The first study that examined the influence of different sperm factors on the outcome of ICSI was published in early 1995 (115). When correlation(s) between the three basic sperm parameters (total sperm count, motility, and morphology) and the fertilization, embryo development, and pregnancy rates were studied, it was observed that there was no important influence either from the type or from the extent of sperm impairment on the outcome of ICSI (115).

This conclusion was also soon confirmed by other groups (179,180). ICSI in patients with extreme oligospermia is associated with high fertilization rates and offers the chance of pregnancy to these infertile couples who otherwise have no chance for conception (181). Nagy and colleagues have shown that actually only one condition had a strongly negative influence on the outcome of ICSI, where an immotile (presumably dead) spermatozoon is injected into the oocyte (115).

Total immotility (obtained from ejaculate or from epididymis) as a result of necrozoospermia is not necessarily a constant situation, because many of those patients who had not a single motile sperm at the time of the first ICSI cycle presented motile sperm on the occasion of the next trial (or even on the same day, if a second ejaculate was asked) and many of these cases resulted in pregnancy in subsequent cycles (182). To overcome the problem of having only immotile sperm in the ejaculate, a testicle biopsy can be considered at the time of the oocyte retrieval because testicular sperm can provide better results in this situation (183,184). Another possible solution to overcome the negative impact of injecting immotile ejaculated sperm is to perform a hypo-osmotic swelling test (on ejaculated sperm), and reactive/living sperm can be used that also provide better results with ICSI (185,186). It was also found that total globozoospermia (or round-headed spermatozoa, which is a rare type of teratozoospermia characterized by multiple structural abnormalities, including the lack of acrosome), also resulted in very low fertilization (or complete fertilization failure in some cases), nevertheless pregnancies were possible occasionally (187–190). Because in globozoospermia the acrosome is missing, the sperm does not carry or deliver the oocyte activating factor, phospholipase C-zeta, which in turn results in fertilization failure. To overcome this problem (and other cases with low or failed ICSI fertilization), some investigators have recommended applying artificial oocyte activation after ICSI, in selected cases, using calcium ionophores (or other approaches) (191–193). Otherwise, as Svalander has also suggested, the outcome of ICSI is unrelated to "strict criteria" sperm morphology (194,195) or to chromatin condensation, as it was judged using aniline blue staining (196). The fact that initial pregnancy loss was similar in all different level of morphological abnormalities indicates that morphological abnormality of spermatozoa possibly does not necessarily reflect a genetic abnormality of the male gametes (197,198), but rather the inability of the sperm to penetrate the egg. As was reported by others, even pregnancy and birth can be achieved with a morphologically highly defective sperm—for instance, with tail stump syndrome—after ICSI (199). In the last two decades, since ICSI was

introduced, there have been numerous studies that have been published looking at the correlation between different sperm parameters (and their extent) and outcomes of ICSI, and none of them have shown radically different findings than the initial studies. Due to the limits of this chapter, it is not possible to discuss them, but for recent reviews on this topic, please refer to these publications: (200–203).

Another relatively frequent finding is the presence of anti-sperm antibodies (ASAbs) in semen, which frequently results in low or no fertilization when conventional IVF is performed (204). In contrast, the outcome of ICSI in cases of ASAbs has demonstrated high normal fertilization embryo development and pregnancy rates, and it is independent of the level of ASAbs bound to the spermatozoa, or of the type of immunoglobulin, or of the localization of ASAbs on the spermatozoa (205,206).

In some cases of male-factor infertility there is no sperm present in the ejaculate (azoospermia). Obstructive azoospermia is when the vasa deferentia are blocked (for instance after vasectomy) or missing (for instance in cystic fibrosis) (207), and non-obstructive azoospermia (secretory azoospermia) is when vasa deferentia are patent but the testes are not producing sperm (for instance in Sertoli-cell-only [SCO] syndrome), or when spermatogenesis is blocked (maturation arrest) (208). In these cases, sperm may be obtained surgically either from the epididymis (obstructive cases only), or from the testes (both obstructive and non-obstructive cases) (209–211). One of the first studies that looked at ICSI outcomes with surgically retrieved sperm (fresh or frozen epididymal and testicular sperm) and compared them to outcomes with fresh sperm ICSI was published in 1995 by Nagy and colleagues (119). It was concluded that, with appropriately treated epididymal and testicular sperm, it was possible to achieve high normal fertilization, embryo cleavage, and pregnancy rates via ICSI, although the fertilization rates were somewhat lower with surgically retrieved sperm than with freshly ejaculated sperm. Many other groups have observed the same outcome, thus confirming the initial experience of the Brussels group, that ICSI provides adequate outcomes when using fresh epididymal sperm (212–216) and also when using percutanously retrieved sperm (217–219), frozen-thawed epididymal sperm (220–222), and freshly retrieved testicular spermatozoa in obstructive cases (223–227) and also in non-obstructive cases (228,229). Cryopreservation of testicular and epididymal spermatozoa has become a frequent procedure, with mostly adequate outcomes—a simple procedure that may help to avoid repetition of surgical interventions to retrieve spermatozoa for successive ICSI cycles (230–233). One of the important observations from these initial studies was that, unlike sperm from fresh ejaculate, testicular sperm (freshly retrieved) does not need to be motile to achieve high fertilization with ICSI (234). The likely explanation is that freshly retrieved testicular sperm are "alive" (independently whether displaying motility or not), unlike ejaculated sperm, which may be "dead" when motility is not present. For this reason, cryopreservation of testicular sperm may pose a practical problem, as the cryopreservation process itself can damage/kill sperm, and therefore thawed testicular sperm that is immotile may not perform as well as a fresh sperm. Additional to this observation, there have been many other observations made (and published) in the last two decades, analyzing various aspects of ICSI outcomes using surgically retrieved sperms, adding some more details to the studies cited earlier (208,235–238).

The ICSI technique has significantly contributed to the success of in vitro fertilization, as in almost all cases it provides a secure way of fertilization, and thus gives a chance for the infertile couple to be pregnant. However, even ICSI fails occasionally, which is usually not expected. When analyzing a total of 2732 ICSI cycles, total fertilization failure occurred only in 76 cases (2.8%), and it was mostly associated with an extremely low number of oocytes obtained, or immotile or round-headed sperm (thus fertilization failure may not be because of the technique itself) (239). Importantly, in the same study it was also observed that over 80% of couples who returned after the failed ICSI fertilization achieved fertilization in their subsequent cycle—thus an initial unexpected ICSI failure is not necessarily associated with poor future outcomes (239).

Obstetrical and Perinatal Outcomes after ICSI

Since the introduction of ICSI there have been some major concerns regarding risks that are intrinsic to the technique. One of the points is that ICSI is an "invasive" procedure and can cause damage to the oocyte. This damage can be immediate, resulting in the degeneration of the oocyte after only a few minutes of injection. However, damage to the oocyte can be less obvious or not visible by the usual light microscopic evaluation. Some early publications have suggested that disruption of the meiotic spindle

can occur as a result of microinjection (240). This might cause de novo chromosome aberrations, as was published by Jean (241). Another worry originated from the fact that not only the sperm is injected into the cytoplasm of the oocyte but also other components (polyvinylpyrrolidone and serum proteins). Yet another concern was about the elimination of sperm selection by the cumulus-corona cells and by the zona pellucida when ICSI is performed, so that successful pregnancy can be achieved despite the presence of severely impaired spermatozoa in a population at high risk for chromosomal aberrations (242). Therefore cytogenetic screening is suggested for males with severe male subfertility who opt for ICSI to exclude a higher risk for spontaneous abortion and fetal chromosomal abnormalities. Attention was also drawn to the fact that a range of indications for ICSI includes andrological problems to a high degree, which themselves could be the result of chromosomal anomalies or hereditary disorders. One example is vas deferens aplasia in one form of cystic fibrosis (CF) mutation. On the other hand, it is likely that some forms of severe male factor infertility may be genetically transmitted through the azoospermia factor (AZF) or deleted in azoospermia (DAZ) (243,244), even though in many cases the responsible genetic marker(s) are not yet identified (245); and although ICSI offspring have been shown to be completely normal, it is possible that the sons of these infertile couples will also require ICSI when they grow up and wish to have a family (118,246). The use of preimplantation genetic testing in some male factor cases can help to prevent passing over responsible genetic factors for male infertility, such as in cases of CF and Klinefelter syndrome (247,248), but this option seems limited at present.

Obstetrical outcomes after ICSI do not reveal anything unexpected, and are comparable to obstetrical outcomes following conventional IVF, and for both groups the major risk factors are associated with multiple pregnancy (249–253). On the other hand, the very first report, in 1995, on fetal prenatal karyotypes after ICSI, a letter to *The Lancet*, raised some worries about the high incidence of sex chromosomal abnormalities (five of 15 karyotypes were reported as abnormal) and questioned the safety of the ICSI method (254). However, all five abnormalities were related to the sex chromosomes (two patients with 47,XXY; two patients with 45,X, and one patient with mozaic abnormal). In contrast, Liebaers and colleagues reported a much lower rate of abnormal fetal karyotypes on a much larger patient population (255); six chromosomal anomalies were observed in 585 fetal karyotypes (1%). There are several other reports in the literature on the incidence of fetal chromosomal anomalies, and practically all observed a slightly (but significantly) higher rate of abnormalities (256–259). Currently, it is viewed that this higher rate of prenatal chromosome anomalies after ICSI is likely related to the patient population requiring ICSI, who have a higher frequency of nullisomy or disomy for the sex chromosomes in spermatozoa from men with severe oligo-astheno-teratozoospermia (259,260).

Initial report(s) from the Brussels' University on children born after ICSI showed that the incidence of major malformations was not different from the incidence observed in IVF or in the general population (261–263). Bonduelle and colleagues have reported a 2.6% rate of major congenital malformations (23 out of 877 children born) (263), and others had similar observations (264). However, some other investigators did observe a statistically significant increase in the incidence of congenital anomalies, where some increase was attributed to the higher frequency of hypospadias and cryptorchidism (265–269). Yet, more recent studies with a large data set, as well as meta-analyses, have not found a significant increase in the incidence of birth defects of ICSI children (270–274), suggesting that the increased risk, if any, is more likely associated with the patient population and not the technique itself. When analyzing the development and health of children at early ages born after the ICSI procedure, most investigators agreed that some differences in the health of children conceived may exist, but this is likely the result of the age (and other health/medical conditions) of the woman (and man) receiving the ICSI treatment (275–277).

Conventional IVF or ICSI?

Before ICSI was developed, there was very little option other than conventional IVF, even for cases of extreme oligo-astheno-teratozoospermia. Some investigators tried to use conventional IVF even for sperm retrieved from the epididymis, with very limited success (278). Since the introduction of ICSI it has become widely applied all over the world for the alleviation of male factor infertility, as conventional IVF (and alternative micromanipulation techniques) do not provide satisfactory results (279–281).

However, initially the indication for ICSI was extremely strict at the Brussels University, where the procedure was developed in 1992; only patients who had less than 500,000 total motile sperm in the ejaculate, or who had two (or more) failed conventional IVF treatment cycles were eligible (106). One of the reasons for that caution was because ICSI was a novel procedure, introduced in humans without prior animal experiments (as ICSI did not work in animal models at that time), and therefore it was not known if it carried any short or long-term risks.

There have been numerous studies that have compared outcomes of conventional IVF with ICSI for different indications (and different sperm parameters/conditions), and most of them have concluded that ICSI is superior when male factor conditions (even when mild) exist (282–288). In addition to male factor infertility, for other indications, such as endometriosis and "unexplained infertility," ICSI also performed better compared to conventional IVF, with normal sperm parameters (289,290). Some studies, excluding male factor infertility, did not find improved outcomes with ICSI, but did observe a lower rate of unexpected total fertilization failure compared to conventional IVF (291–294). Not surprisingly, there have been a number of opinion articles commenting on when to perform conventional IVF and when to perform ICSI, ranging from "conservative" views (favoring more conventional IVF) to more "progressive" views (suggesting ICSI in all cases) (295–299). The arguments in favor of conventional IVF are (1) a more "natural" process; (2) less invasive; (3) less complex (simpler "logistics"); and (4) less costly. Additionally, as was recently demonstrated, it provides significantly higher fertilization rates for cases with low oocyte maturity (300). The arguments in favor of ICSI are fewer, but perhaps some are stronger: (1) it provides information on oocyte maturity and quality/morphology (as for ICSI cumulus and corona cells have to be removed); and (2) it provides a more standard/secure way of fertilization (as partial or total fertilization failure with conventional insemination can happen not only when sperm parameters are impaired, but occasionally also when all sperm parameters are "normal"). As a practical matter, it appears that more and more IVF treatment cycles are performed using ICSI insemination (with or without "adequate" indications); for instance, in the United States, the national average was 67% ICSI cycles in 2013, which is up 12% from 2003, when the figure was 55% (the proportion of patients with male factor has not changed during this 11-year period). Although it is evident that ICSI insemination is not associated with higher pregnancy rates than conventional IVF, it does seem to provide a more consistent fertilization outcome, avoiding unexpected fertilization failure—a likely reason for its ever increasing use.

A possible solution to resolve (at least partly) the dilemma of conventional IVF or ICSI is to perform both procedures on every (or almost every) patient. If the patient (couple) had prior natural or IUI or conventional IVF conception (this latter with good fertilization) and sperm is adequate, then one may consider performing 100% conventional insemination (as there is evidence of prior natural fertilization). However, if the patient has never conceived before (no evidence of sperm fertilizing the oocyte), one should consider performing a split IVF/ICSI insemination, if sperm parameters are adequate, and there are a reasonable number of oocytes available. Obviously, if sperm parameters are impaired (or there is history of low prior IVF fertilization, or any other indication), then one should consider performing 100% ICSI insemination. One of the additional benefits of routine use of split IVF/ICSI insemination is not only to optimize fertilization, but also at the same time to optimize the availability of the best embryo cohort, as, frequently, either conventional insemination or ICSI insemination will result in a better embryo development (within the same cohort of oocytes).

Additionally, ICSI is the ultimate choice of insemination for some specific indications. For instance, ICSI is advised for patients who are planning to perform preimplantation genetic testing (for single gene defects) (301). ICSI is also the choice for insemination for in vitro matured (IVM) oocytes (302), as was demonstrated by Nagy and colleagues achieving the first pregnancy by this approach (303). Additionally, ICSI is also the standard procedure to inseminate oocytes previously cryopreserved for fertility preservation or for donation purposes (304,305).

Summary

Since the first success of in-vitro fertilization, marked by the birth of Louise Brown in 1978, IVF has been the ultimate choice of method to alleviate infertility due to various indications. It was found,

however, that IVF does not always provide satisfactory results in respect of normal fertilization, and consequently lowers pregnancy rates, especially when male factor is present. Many efforts have been made to find ways that increases the chance of gamete fusion in cases where in vitro insemination failed previously, including improved IVF procedures. Other approaches, using micromanipulations of gametes, tried to eliminate some of the biological barriers involved in the path of gamete fusion by creating a hole on the zona pellucida (PZD) or by inserting some spermatozoa under the zona pellucida into the perivitelline space (SUZI). None of these approaches helped to improve results in a constant way in poor IVF fertilization cases. A more direct method of gamete micromanipulation was described in 1988 by the Norfolk IVF center, where a single sperm was brought into the cytoplasm of an oocyte after passing the limits of the zona pellucida and oolemma, and fertilization, but not ongoing pregnancy, was obtained. The successful introduction of this method (called intracytoplasmic sperm injection—ICSI) into daily laboratory practice and the first pregnancy was achieved in 1991 at the Dutch-speaking Brussels Free University (Vrije Universiteit Brussel). With further improvements, this method ensured a consistently much higher fertilization rate than any other alternative approaches, especially in male factor infertility. Among other significant findings, it was demonstrated that there is no important influence either from the type or from the extent of sperm impairment on the outcome of ICSI. Even in the most extreme cases of male factor infertility, where virtual azoospermia or total astheno- or total teratozoospermia was diagnosed in the initial semen sample (from the ejaculate), high fertilization and pregnancy rates were obtained by ICSI. Only one condition had a strongly negative influence on the result of ICSI, where immotile (presumably dead) spermatozoa were injected into the oocyte. Furthermore, it was also established that ICSI can provide high normal fertilization, cleavage, and pregnancy rates when fresh or frozen–thawed epididymal and testicular spermatozoa are used, though fertilization rates may be lower than with ejaculated sperm. For all these reasons, today ICSI is the preferred mode of oocyte insemination for the treatment of most or all indications for IVF. ICSI is able to overcome almost all types of male factor related infertility; however, ICSI has not changed much the oocyte quality-related outcomes (associated with the reproductive age of the women), which is considered the holy grail of assisted reproduction treatment.

REFERENCES

1. Nargund G, Fauser BC, Macklon NS, Ombelet W, Nygren K, Frydman R. The ISMAAR proposal on terminology for ovarian stimulation for IVF. *Hum Reprod*. 2007;22(11):2801–4.
2. Steptoe PC, Edwards RG. Birth after the reimplantation of a human embryo. *Lancet*. 1978;2(8085):366.
3. Edwards R, Patrizio P, Edgar D, Field C, Brinton L. Defining IVF terminology. *Reprod Biomed Online*. 2007;14(5):553–4.
4. Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet*. 1992;340(8810):17–8.
5. Chang MC. Fertilization of rabbit ova in vitro. *Nature*. 1959;184(Suppl 7):466–7.
6. Edwards RG, Bavister BD, Steptoe PC. Early stages of fertilization in vitro of human oocytes matured in vitro. *Nature*. 1969;221(5181):632–5.
7. Edwards RG, Steptoe PC, Purdy JM. Fertilization and cleavage in vitro of preovulator human oocytes. *Nature*. 1970;227(5265):1307–9.
8. Lopata A, Johnston IW, Hoult IJ, Speirs AI. Pregnancy following intrauterine implantation of an embryo obtained by in vitro fertilization of a preovulatory egg. *Fertil Steril*. 1980;33(2):117–20.
9. Trounson AO, Leeton JF, Wood C, Webb J, Wood J. Pregnancies in humans by fertilization in vitro and embryo transfer in the controlled ovulatory cycle. *Science*. 1981;212(4495):681–2.
10. Cohen J, Trounson A, Dawson K, Jones H, Hazekamp J, Nygren KG et al. The early days of IVF outside the UK. *Hum Reprod Update*. 2005;11(5):439–59.
11. Thatcher SS, DeCherney AH. A critical assessment of the indications for in-vitro fertilization and embryo transfer. *Hum Reprod*. 1989;4(8 Suppl):11–6.
12. Hull MG. Indications for assisted conception. *Br Med Bull*. 1990;46(3):580–95.
13. Fleming R, Adam AH, Barlow DH, Black WP, MacNaughton MC, Coutts JR. A new systematic treatment for infertile women with abnormal hormone profiles. *Br J Obstet Gynaecol*. 1982;89(1):80–3.

14. Rogers PA, Milne BJ, Trounson AO. A model to show human uterine receptivity and embryo viability following ovarian stimulation for in vitro fertilization. *J In Vitro Fert Embryo Transf.* 1986;3(2):93–8.
15. Lenz S, Lauritsen JG. Ultrasonically guided percutaneous aspiration of human follicles under local anesthesia: A new method of collecting oocytes for in vitro fertilization. *Fertil Steril*. 1982;38(6):673–7.
16. Zanartu J, Rosner JM, Guiloff E, Ibarra-Polo AA, Croxatto HD, Croxatto HB et al. Attempts to programme ovulation with exogenous oestrogens and LH-RH analogue. *Br Med J.* 1975 7;2(5970):527–9.
17. Mauvais-Jarvis P. Hypophyseal gonadotropins and control of ovulation. II. Hypothalamic control. Physiology of ovulation. *Presse Med*. 1967;75(6):283–7.
18. Testart J, Frydman R. Minimum time lapse between luteinizing hormone surge or human chorionic gonadotropin administration and follicular rupture. *Fertil Steril.* 1982;37(1):50–3.
19. Humaidan P, Bredkjaer HE, Bungum L, Bungum M, Grondahl ML, Westergaard L et al. GnRH agonist (buserelin) or hCG for ovulation induction in GnRH antagonist IVF/ICSI cycles: A prospective randomized study. *Hum Reprod*. 2005;20(5):1213–20.
20. The International Recombinant Human Chorionic Gonadotropin Study Group. Induction of ovulation in World Health Organization group II anovulatory women undergoing follicular stimulation with recombinant human follicle-stimulating hormone: A comparison of recombinant human chorionic gonadotropin (rhCG) and urinary hCG. *Fertil Steril*. 2001;75(6):1111–8.
21. The European Recombinant Human Chorionic Gonadotrophin Study Group. Induction of final follicular maturation and early luteinization in women undergoing ovulation induction for assisted reproduction treatment–recombinant HCG versus urinary HCG. The European Recombinant Human Chorionic Gonadotrophin Study Group. *Hum Reprod*. 2000;15(7):1446–51.
22. Corsan GH, Kemmann E. Luteal hCG in ovulation induction. *Fertil Steril*. 1989;51(3):549–50.
23. Sas M, Kincses L. Ovulation induction stimulated with HCG in functional sterility patients treated with clomiphene. *Ther Hung*. 1978;26(1):12–5.
24. Laufer N, DeCherney AH, Tarlatzis BC, Zuckerman AL, Polan ML, Dlugi AM et al. Delaying human chorionic gonadotropin administration in human menopausal gonadotropin-induced cycles decreases successful in vitro fertilization of human oocytes. *Fertil Steril*. 1984;42(2):198–203.
25. Chian RC, Buckett WM, Abdul Jalil AK, Son WY, Sylvestre C, Rao D et al. Natural-cycle in vitro fertilization combined with in vitro maturation of immature oocytes is a potential approach in infertility treatment. *Fertil Steril*. 2004;82(6):1675–8.
26. Tan SL, Child TJ. In-vitro maturation of oocytes from unstimulated polycystic ovaries. *Reprod Biomed Online*. 2002;4(Suppl 1):18–23.
27. Child TJ, Abdul-Jalil AK, Gulekli B, Tan SL. In vitro maturation and fertilization of oocytes from unstimulated normal ovaries, polycystic ovaries, and women with polycystic ovary syndrome. *Fertil Steril*. 2001;76(5):936–42.
28. Daya S, Kohut J, Gunby J, Younglai E. Influence of blood clots in the cumulus complex on oocyte fertilization and cleavage. *Hum Reprod*. 1990;5(6):744–6.
29. Veeck LL. Oocyte assessment and biological performance. *Ann NY Acad Sci*. 1988;541:259–74.
30. Veeck LL. The morphological assessment of human oocytes and early concepti. In: *Handbook of the Laboratory Diagnosis and Treatment of Infertility*, Keel BA, Webster BW (Eds.). Boca Raton: CRC Press, 1990, 353.
31. Coticchio G, Sereni E, Serrao L, Mazzone S, Iadarola I, Borini A. What criteria for the definition of oocyte quality? *Ann N Y Acad Sci*. 2004;1034:132–44.
32. Trounson AO, Mohr LR, Wood C, Leeton JF. Effect of delayed insemination on in-vitro fertilization, culture and transfer of human embryos. *J Reprod Fertil*. 1982;64(2):285–94.
33. Trounson A, Gardner DK. *Handbook of in vitro Fertilization*. Boca Raton: CRC Press, 1993.
34. Gianaroli L, Cristina Magli M, Ferraretti AP, Fiorentino A, Tosti E, Panzella S et al. Reducing the time of sperm-oocyte interaction in human in-vitro fertilization improves the implantation rate. *Hum Reprod*. 1996;11(1):166–71.
35. Dai SJ, Qiao YH, Jin HX, Xin ZM, Su YC, Sun YP et al. Effect of coincubation time of sperm-oocytes on fertilization, embryonic development, and subsequent pregnancy outcome. *Syst Biol Reprod Med*. 2012;58(6):348–53.
36. Kattera S, Chen C. Short coincubation of gametes in in vitro fertilization improves implantation and pregnancy rates: A prospective, randomized, controlled study. *Fertil Steril*. 2003;80(4):1017–21.

37. Kastrop PM, Weima SM, Van Kooij RJ, Te Velde ER. Comparison between intracytoplasmic sperm injection and in-vitro fertilization (IVF) with high insemination concentration after total fertilization failure in a previous IVF attempt. *Hum Reprod*. 1999;14(1):65–9.
38. Oehninger S, Kruger TF, Simon T, Jones D, Mayer J, Lanzendorf S et al. A comparative analysis of embryo implantation potential in patients with severe teratozoospermia undergoing in-vitro fertilization with a high insemination concentration or intracytoplasmic sperm injection. *Hum Reprod*. 1996;11(5):1086–9.
39. Hall J, Fishel S, Green S, Fleming S, Hunter A, Stoddart N et al. Intracytoplasmic sperm injection versus high insemination concentration in-vitro fertilization in cases of very severe teratozoospermia. *Hum Reprod*. 1995;10(3):493–6.
40. Fishel S, Lisi F, Rinaldi L, Lisi R, Timson J, Green S et al. Intracytoplasmic sperm injection (ICSI) versus high insemination concentration (HIC) for human conception in vitro. *Reprod Fertil Dev*. 1995;7(2):169–74; discussion 74–5.
41. Bomsel-Helmreich O, Testart J. Effects of seminal plasma from intact and vasectomized males on capacitation in the rabbit. *J Reprod Fertil*. 1972;31(3):516–7.
42. Aitken RJ, Nixon B. Sperm capacitation: A distant landscape glimpsed but unexplored. *Mol Hum Reprod*. 2013;19(12):785–93.
43. World Health Organization. Laboratory manual of the WHO for the examination of human semen and sperm-cervical mucus interaction. *Ann Ist Super Sanita*. 2001;37(1):I–XII, 1–123.
44. Henkel RR, Schill WB. Sperm preparation for ART. *Reprod Biol Endocrinol*. 2003;1:108.
45. Ricci G, Perticarari S, Boscolo R, Montico M, Guaschino S, Presani G. Semen preparation methods and sperm apoptosis: Swim-up versus gradient-density centrifugation technique. *Fertil Steril*. 2009;91(2):632–8.
46. Mortimer D. Sperm preparation methods. *J Androl*. 2000;21(3):357–66.
47. Cross NL, Morales P, Overstreet JW, Hanson FW. Induction of acrosome reactions by the human zona pellucida. *Biol Reprod*. 1988;38(1):235–44.
48. Wassarman PM. Regulation of mammalian fertilization by zona pellucida glycoproteins. *J Reprod Fertil Suppl*. 1990;42:79–87.
49. Wassarman PM. Zona pellucida glycoproteins. *Annu Rev Biochem*. 1988;57:415–42.
50. Tsaadon A, Eliyahu E, Shtraizent N, Shalgi R. When a sperm meets an egg: Block to polyspermy. *Mol Cell Endocrinol*. 2006;252(1–2):107–14.
51. Raz T, Shalgi R. Early events in mammalian egg activation. *Hum Reprod*. 1998;13(Suppl 4):133–45.
52. Watanabe N, Vande Woude GF, Ikawa Y, Sagata N. Specific proteolysis of the c-mos proto-oncogene product by calpain on fertilization of Xenopus eggs. *Nature*. 1989;342(6249):505–11.
53. Swann K, Lai FA. The sperm phospholipase C-zeta and Ca2+ signalling at fertilization in mammals. *Biochem Soc Trans*. 2016;44(1):267–72.
54. Swann K, Windsor S, Campbell K, Elgmati K, Nomikos M, Zernicka-Goetz M et al. Phospholipase C-zeta-induced Ca2+ oscillations cause coincident cytoplasmic movements in human oocytes that failed to fertilize after intracytoplasmic sperm injection. *Fertil Steril*. 2012;97(3):742–7.
55. Li L, Lu X, Dean J. The maternal to zygotic transition in mammals. *Mol Aspects Med*. 2013;34(5):919–38.
56. Wolf DP, Byrd W, Dandekar P, Quigley MM. Sperm concentration and the fertilization of human eggs in vitro. *Biol Reprod*. 1984;31(4):837–48.
57. Hall J, Fishel S. In vitro fertilization for male infertility: When and how? *Baillieres Clin Obstet Gynaecol*. 1997;11(4):711–24.
58. Evenson DP, Wixon R. Clinical aspects of sperm DNA fragmentation detection and male infertility. *Theriogenology*. 2006;65(5):979–91.
59. Audibert F, Hedon B, Arnal F, Humeau C, Badoc E, Virenque V et al. Results of IVF attempts in patients with unexplained infertility. *Hum Reprod*. 1989;4(7):766–71.
60. Oehninger S, Acosta AA, Kruger T, Veeck LL, Flood J, Jones HW, Jr. Failure of fertilization in in vitro fertilization: The "occult" male factor. *J In Vitro Fert Embryo Transf*. 1988;5(4):181–7.
61. Lillie FR. Studies of fertilisation VI. The mechanism of fertilisation in Arbacia. *J Exp Zool*. 1914;16(4):523–90.
62. Hiramoto Y. Microinjection of the live spermatozoa into sea urchin eggs. *Exp Cell Res*. 1962;27:416–26.
63. Uehara T, Yanagimachi R. Behavior of nuclei of testicular, caput and cauda epididymal spermatozoa injected into hamster eggs. *Biol Reprod*. 1977;16(3):315–21.

64. Uehara T, Yanagimachi R. Microsurgical injection of spermatozoa into hamster eggs with subsequent transformation of sperm nuclei into male pronuclei. *Biol Reprod.* 1976;15(4):467–70.
65. Naish SJ, Perreault SD, Zirkin BR. DNA synthesis following microinjection of heterologous sperm and somatic cell nuclei into hamster oocytes. *Gamete Res.* 1987;18(2):109–20.
66. Naish SJ, Perreault SD, Foehner AL, Zirkin BR. DNA synthesis in the fertilizing hamster sperm nucleus: Sperm template availability and egg cytoplasmic control. *Biol Reprod.* 1987;36(1):245–53.
67. Thadani VM. A study of hetero-specific sperm-egg interactions in the rat, mouse, and deer mouse using in vitro fertilization and sperm injection. *J Exp Zool.* 1980;212(3):435–53.
68. Keefer CL. Fertilization by sperm injection in the rabbit. *Gamete Res.* 1989;22(1):59–69.
69. Markert CL. Fertilization of mammalian eggs by sperm injection. *J Exp Zool.* 1983;228(2):195–201.
70. Ron-El R, Liu J, Nagy Z, Joris H, Van den Abbeel E, Van Steirteghem A. Intracytoplasmic sperm injection in the mouse. *Hum Reprod.* 1995;10(11):2831–4.
71. Barg PE, Wahrman MZ, Talansky BE, Gordon JW. Capacitated, acrosome reacted but immotile sperm, when microinjected under the mouse zona pellucida, will not fertilize the oocyte. *J Exp Zool.* 1986;237(3):365–74.
72. Lassalle B, Courtot AM, Testart J. In vitro fertilization of hamster and human oocytes by microinjection of human sperm. *Gamete Res.* 1987;16(1):69–78.
73. Mann JR. Full term development of mouse eggs fertilized by a spermatozoon microinjected under the zona pellucida. *Biol Reprod.* 1988;38(5):1077–83.
74. Yamada K, Stevenson AF, Mettler L. Fertilization through spermatozoal microinjection: Significance of acrosome reaction. *Hum Reprod.* 1988;3(5):657–61.
75. Lacham O, Trounson A, Holden C, Mann J, Sathananthan H. Fertilization and development of mouse eggs injected under the zona pellucida with single spermatozoa treated to induce the acrosome reaction. *Gamete Res.* 1989;23(2):233–43.
76. Palermo G, Van Steirteghem A. Enhancement of acrosome reaction and subzonal insemination of a single spermatozoon in mouse eggs. *Mol Reprod Dev.* 1991;30(4):339–45.
77. Wazzan WC, Gwatkin RB, Thomas AJ, Jr. Zona drilling enhances fertilization by mouse caput epididymal sperm. *Mol Reprod Dev.* 1990;27(4):332–6.
78. Barlow P, Van Der Zwalmen P, Schoysman R, Leroy F. Improvement of fertilizing performance by normal and abnormal mouse semen after zona opening of mature oocytes. *Mol Reprod Dev.* 1991;30(4): 385–9.
79. Mordel N, Ohad S, Zentner B, Schenker JG, Gordon J, Laufer N. Enhancing in vitro fertilization of mouse oocytes by partial zona pellucida digestion. *J Assist Reprod Genet.* 1992;9(2):128–32.
80. Talansky BE, Gordon JW. Cleavage characteristics of mouse embryos inseminated and cultured after zona pellucida drilling. *Gamete Res.* 1988;21(3):277–87.
81. Gordon JW, Grunfeld L, Garrisi GJ, Talansky BE, Richards C, Laufer N. Fertilization of human oocytes by sperm from infertile males after zona pellucida drilling. *Fertil Steril.* 1988;50(1):68–73.
82. Ng SC, Bongso A, Chang SI, Sathananthan H, Ratnam S. Transfer of human sperm into the perivitelline space of human oocytes after zona-drilling or zona-puncture. *Fertil Steril.* 1989;52(1):73–8.
83. Cohen J, Malter H, Wright G, Kort H, Massey J, Mitchell D. Partial zona dissection of human oocytes when failure of zona pellucida penetration is anticipated. *Hum Reprod.* 1989;4(4):435–42.
84. Malter HE, Cohen J. Partial zona dissection of the human oocyte: A nontraumatic method using micromanipulation to assist zona pellucida penetration. *Fertil Steril.* 1989;51(1):139–48.
85. Cohen J, Malter H, Fehilly C, Wright G, Elsner C, Kort H et al. Implantation of embryos after partial opening of oocyte zona pellucida to facilitate sperm penetration. *Lancet.* 1988;2(8603):162.
86. Garrisi GJ, Talansky BE, Grunfeld L, Sapira V, Navot D, Gordon JW. Clinical evaluation of three approaches to micromanipulation-assisted fertilization. *Fertil Steril.* 1990;54(4):671–7.
87. Tucker MJ, Bishop FM, Cohen J, Wiker SR, Wright G. Routine application of partial zona dissection for male factor infertility. *Hum Reprod.* 1991;6(5):676–81.
88. Calderon G, Veiga A, Penella J, Barri PN. Two years of assisted fertilization by partial zona dissection in male factor infertility patients. *Fertil Steril.* 1993;60(1):105–9.
89. Laws-King A, Trounson A, Sathananthan H, Kola I. Fertilization of human oocytes by microinjection of a single spermatozoon under the zona pellucida. *Fertil Steril.* 1987;48(4):637–42.
90. Fishel S, Jackson P, Antinori S, Johnson J, Grossi S, Versaci C. Subzonal insemination for the alleviation of infertility. *Fertil Steril.* 1990;54(5):828–35.

91. Fishel S, Antinori S, Jackson P, Johnson J, Lisi F, Chiariello F et al. Twin birth after subzonal insemination. *Lancet*. 1990;335(8691):722–3.
92. Alikani M, Adler A, Reing AM, Malter HE, Cohen J. Subzonal sperm insertion and the frequency of gamete fusion. *J Assist Reprod Genet*. 1992;9(2):97–101.
93. Sakkas D, Lacham O, Gianaroli L, Trounson A. Subzonal sperm microinjection in cases of severe male factor infertility and repeated in vitro fertilization failure. *Fertil Steril*. 1992;57(6):1279–88.
94. Palermo G, Joris H, Devroey P, Van Steirteghem AC. Induction of acrosome reaction in human spermatozoa used for subzonal insemination. *Hum Reprod*. 1992;7(2):248–54.
95. Imoedemhe DA, Sigue AB. Subzonal multiple sperm injection in the treatment of previous failed human in vitro fertilization. *Fertil Steril*. 1993;59(1):172–6.
96. Terriou P, Giorgetti C, Hans E, Spach JL, Salzmann J, Carlon N et al. Subzonal sperm insemination and total or extreme asthenozoospermia: An effective technique for an uncommon cause of male infertility. *Fertil Steril*. 1993;60(6):1057–61.
97. Catt J, Krzyminska U, Tilia L, Csehi E, Ryan J, Pike I et al. Subzonal insertion of multiple sperm is a treatment for male factor infertility. *Fertil Steril*. 1994;61(1):118–24.
98. Svalander P, Wikland M, Jakobsson AH, Forsberg AS. Subzonal insemination (SUZI) or in vitro fertilization (IVF) in microdroplets for the treatment of male-factor infertility. *J Assist Reprod Genet*. 1994;11(3):149–55.
99. Cohen J, Talansky BE, Malter H, Alikani M, Adler A, Reing A et al. Microsurgical fertilization and teratozoospermia. *Hum Reprod*. 1991;6(1):118–23.
100. Sakkas D, Gianaroli L, Diotallevi L, Wagner I, Ferraretti AP, Campana A. IVF treatment of moderate male factor infertility: A comparison of mini-Percoll, partial zona dissection and sub-zonal sperm insertion techniques. *Hum Reprod*. 1993;8(4):587–91.
101. Payne D, Warnes GM, Flaherty SP, Matthews CD. Local experience with zona drilling, zona cutting and sperm microinjection. *Reprod Fertil Dev*. 1994;6(1):45–50.
102. Lanzendorf SE, Maloney MK, Veeck LL, Slusser J, Hodgen GD, Rosenwaks Z. A preclinical evaluation of pronuclear formation by microinjection of human spermatozoa into human oocytes. *Fertil Steril*. 1988;49(5):835–42.
103. Ng SC, Bongso A, Ratnam SS. Microinjection of human oocytes: A technique for severe oligoasthenoteratozoospermia. *Fertil Steril*. 1991;56(6):1117–23.
104. Palermo G, Joris H, Derde MP, Camus M, Devroey P, Van Steirteghem A. Sperm characteristics and outcome of human assisted fertilization by subzonal insemination and intracytoplasmic sperm injection. *Fertil Steril*. 1993;59(4):826–35.
105. Van Steirteghem AC, Liu J, Joris H, Nagy Z, Janssenswillen C, Tournaye H et al. Higher success rate by intracytoplasmic sperm injection than by subzonal insemination. Report of a second series of 300 consecutive treatment cycles. *Hum Reprod*. 1993;8(7):1055–60.
106. Van Steirteghem AC, Nagy Z, Joris H, Liu J, Staessen C, Smitz J et al. High fertilization and implantation rates after intracytoplasmic sperm injection. *Hum Reprod*. 1993;8(7):1061–6.
107. Fishel S, Timson J, Lisi F, Jacobson M, Rinaldi L, Gobetz L. Micro-assisted fertilization in patients who have failed subzonal insemination. *Hum Reprod*. 1994;9(3):501–5.
108. Tarin JJ. Subzonal insemination, partial zona dissection or intracytoplasmic sperm injection? An easy decision. *Hum Reprod*. 1995;10(1):165–70.
109. Catt J, Ryan J, Pike I, O'Neill C. Fertilization rates using intracytoplasmic sperm injection are greater than subzonal insemination but are dependent on prior treatment of sperm. *Fertil Steril*. 1995;64(4):764–9.
110. De Vos A, Van Landuyt L, Van Ranst H, Vandermonde A, D'Haese V, Sterckx J et al. Randomized sibling-oocyte study using recombinant human hyaluronidase versus bovine-derived Sigma hyaluronidase in ICSI patients. *Hum Reprod*. 2008;23(8):1815–9.
111. Van de Velde H, Nagy ZP, Joris H, De Vos A, Van Steirteghem AC. Effects of different hyaluronidase concentrations and mechanical procedures for cumulus cell removal on the outcome of intracytoplasmic sperm injection. *Hum Reprod*. 1997;12(10):2246–50.
112. Taylor TH, Elliott T, Colturato LF, Straub RJ, Mitchell-Leef D, Nagy ZP. Comparison of bovine- and recombinant human-derived hyaluronidase with regard to fertilization rates and embryo morphology in a sibling oocyte model: A prospective, blinded, randomized study. *Fertil Steril*. 2006;85(5):1544–6.
113. Ebner T, Moser M, Tews G. Is oocyte morphology prognostic of embryo developmental potential after ICSI? *Reprod Biomed Online*. 2006;12(4):507–12.

114. Bourne H, Richings N, Liu DY, Clarke GN, Harari O, Baker HW. Sperm preparation for intracytoplasmic injection: Methods and relationship to fertilization results. *Reprod Fertil Dev.* 1995;7(2):177–83.
115. Nagy ZP, Liu J, Joris H, Verheyen G, Tournaye H, Camus M et al. The result of intracytoplasmic sperm injection is not related to any of the three basic sperm parameters. *Hum Reprod.* 1995;10(5):1123–9.
116. De Vos A, Nagy ZP, Van de Velde H, Joris H, Bocken G, Van Steirteghem A. Percoll gradient centrifugation can be omitted in sperm preparation for intracytoplasmic sperm injection. *Hum Reprod.* 1997;12(9):1980–4.
117. Silber SJ, Nagy ZP, Liu J, Godoy H, Devroey P, Van Steirteghem AC. Conventional in-vitro fertilization versus intracytoplasmic sperm injection for patients requiring microsurgical sperm aspiration. *Hum Reprod.* 1994;9(9):1705–9.
118. Silber SJ, Nagy Z, Liu J, Tournaye H, Lissens W, Ferec C et al. The use of epididymal and testicular spermatozoa for intracytoplasmic sperm injection: The genetic implications for male infertility. *Hum Reprod.* 1995;10(8):2031–43.
119. Nagy Z, Liu J, Cecile J, Silber S, Devroey P, Van Steirteghem A. Using ejaculated, fresh, and frozen-thawed epididymal and testicular spermatozoa gives rise to comparable results after intracytoplasmic sperm injection. *Fertil Steril.* 1995;63(4):808–15.
120. Silber SJ, Van Steirteghem AC, Liu J, Nagy Z, Tournaye H, Devroey P. High fertilization and pregnancy rate after intracytoplasmic sperm injection with spermatozoa obtained from testicle biopsy. *Hum Reprod.* 1995;10(1):148–52.
121. Gorgy A, Podsiadly BT, Bates S, Craft IL. Testicular sperm aspiration (TESA): The appropriate technique. *Hum Reprod.* 1998;13(4):1111–3.
122. Verheyen G, De Croo I, Tournaye H, Pletincx I, Devroey P, van Steirteghem AC. Comparison of four mechanical methods to retrieve spermatozoa from testicular tissue. *Hum Reprod.* 1995;10(11):2956–9.
123. Nagy ZP, Verheyen G, Tournaye H, Devroey P, Van Steirteghem AC. An improved treatment procedure for testicular biopsy specimens offers more efficient sperm recovery: Case series. *Fertil Steril.* 1997;68(2):376–9.
124. Crabbe E, Verheyen G, Silber S, Tournaye H, Van de Velde H, Goossens A et al. Enzymatic digestion of testicular tissue may rescue the intracytoplasmic sperm injection cycle in some patients with non-obstructive azoospermia. *Hum Reprod.* 1998;13(10):2791–6.
125. Crabbe E, Verheyen G, Tournaye H, Van Steirteghem A. The use of enzymatic procedures to recover testicular germ cells. *Hum Reprod.* 1997;12(8):1682–7.
126. Dozortsev D, Nagy P, Abdelmassih S, Oliveira F, Brasil A, Abdelmassih V et al. The optimal time for intracytoplasmic sperm injection in the human is from 37 to 41 hours after administration of human chorionic gonadotropin. *Fertil Steril.* 2004;82(6):1492–6.
127. Van de Velde H, De Vos A, Joris H, Nagy ZP, Van Steirteghem AC. Effect of timing of oocyte denudation and micro-injection on survival, fertilization and embryo quality after intracytoplasmic sperm injection. *Hum Reprod.* 1998;13(11):3160–4.
128. Joris H, Nagy Z, Van de Velde H, De Vos A, Van Steirteghem A. Intracytoplasmic sperm injection: Laboratory set-up and injection procedure. *Hum Reprod.* 1998;13(Suppl 1):76–86.
129. Gearon CM, Taylor AS, Forman RG. Factors affecting activation and fertilization of human oocytes following intracytoplasmic injection. *Hum Reprod.* 1995;10(4):896–902.
130. Palermo GD, Schlegel PN, Colombero LT, Zaninovic N, Moy F, Rosenwaks Z. Aggressive sperm immobilization prior to intracytoplasmic sperm injection with immature spermatozoa improves fertilization and pregnancy rates. *Hum Reprod.* 1996;11(5):1023–9.
131. Vanderzwalmen P, Bertin G, Lejeune B, Nijs M, Vandamme B, Schoysman R. Two essential steps for a successful intracytoplasmic sperm injection: Injection of immobilized spermatozoa after rupture of the oolema. *Hum Reprod.* 1996;11(3):540–7.
132. Chen SU, Ho HN, Chen HF, Huang SC, Lee TY, Yang YS. Intracytoplasmic sperm injection (ICSI) for severe semen abnormalities: Dissecting the tail of spermatozoa at the tip. *Hum Reprod.* 1996;11(12):2640–4.
133. Sathananthan AH, Szell A, Ng SC, Kausche A, Lacham-Kaplan O, Trounson A. Is the acrosome reaction a prerequisite for sperm incorporation after intra-cytoplasmic sperm injection (ICSI)? *Reprod Fertil Dev.* 1997;9(7):703–9.
134. Nagy ZP, Liu J, Joris H, Bocken G, Desmet B, Van Ranst H et al. The influence of the site of sperm deposition and mode of oolemma breakage at intracytoplasmic sperm injection on fertilization and embryo development rates. *Hum Reprod.* 1995;10(12):3171–7.

135. Palermo GD, Alikani M, Bertoli M, Colombero LT, Moy F, Cohen J et al. Oolemma characteristics in relation to survival and fertilization patterns of oocytes treated by intracytoplasmic sperm injection. *Hum Reprod*. 1996;11(1):172–6.
136. Van Steirteghem A, Nagy P, Joris H, Verheyen G, Smitz J, Camus M et al. The development of intracytoplasmic sperm injection. *Hum Reprod*. 1996;11(Suppl 1):59–72; discussion 81–5.
137. Herrero MB, Delbes G, Chung JT, Son WY, Holzer H, Buckett W et al. Case report: The use of annexin V coupled with magnetic activated cell sorting in cryopreserved spermatozoa from a male cancer survivor: Healthy twin newborns after two previous ICSI failures. *J Assist Reprod Genet*. 2013;30(11):1415–9.
138. Grunewald S, Paasch U. Sperm selection for ICSI using annexin V. *Methods Mol Biol*. 2013;927:257–62.
139. Van den Bergh M, Revelard P, Bertrand E, Biramane J, Vanin AS, Englert Y. Glass wool column filtration, an advantageous way of preparing semen samples for intracytoplasmic sperm injection: An autocontrolled randomized study. *Hum Reprod*. 1997;12(3):509–13.
140. Simon L, Murphy K, Aston KI, Emery BR, Hotaling JM, Carrell DT. Optimization of microelectrophoresis to select highly negatively charged sperm. *J Assist Reprod Genet*. 2016;33(6):679–88.
141. Huszar G, Jakab A, Sakkas D, Ozenci CC, Cayli S, Delpiano E et al. Fertility testing and ICSI sperm selection by hyaluronic acid binding: Clinical and genetic aspects. *Reprod Biomed Online*. 2007;14(5):650–63.
142. Huszar G, Ozenci CC, Cayli S, Zavaczki Z, Hansch E, Vigue L. Hyaluronic acid binding by human sperm indicates cellular maturity, viability, and unreacted acrosomal status. *Fertil Steril*. 2003;79(Suppl 3):1616–24.
143. Huszar G, Ozkavukcu S, Jakab A, Celik-Ozenci C, Sati GL, Cayli S. Hyaluronic acid binding ability of human sperm reflects cellular maturity and fertilizing potential: Selection of sperm for intracytoplasmic sperm injection. *Curr Opin Obstet Gynecol*. 2006;18(3):260–7.
144. Worrilow KC, Eid S, Woodhouse D, Perloe M, Smith S, Witmyer J et al. Use of hyaluronan in the selection of sperm for intracytoplasmic sperm injection (ICSI): Significant improvement in clinical outcomes–Multicenter, double-blinded and randomized controlled trial. *Hum Reprod*. 2013;28(2):306–14.
145. Parmegiani L, Cognigni GE, Ciampaglia W, Pocognoli P, Marchi F, Filicori M. Efficiency of hyaluronic acid (HA) sperm selection. *J Assist Reprod Genet*. 2010;27(1):13–6.
146. Berkovitz A, Eltes F, Lederman H, Peer S, Ellenbogen A, Feldberg B et al. How to improve IVF-ICSI outcome by sperm selection. *Reprod Biomed Online*. 2006;12(5):634–8.
147. Bartoov B, Berkovitz A, Eltes F, Kogosovsky A, Yagoda A, Lederman H et al. Pregnancy rates are higher with intracytoplasmic morphologically selected sperm injection than with conventional intracytoplasmic injection. *Fertil Steril*. 2003;80(6):1413–9.
148. Vanderzwalmen P, Hiemer A, Rubner P, Bach M, Neyer A, Stecher A et al. Blastocyst development after sperm selection at high magnification is associated with size and number of nuclear vacuoles. *Reprod Biomed Online*. 2008;17(5):617–27.
149. Wilding M, Coppola G, di Matteo L, Palagiano A, Fusco E, Dale B. Intracytoplasmic injection of morphologically selected spermatozoa (IMSI) improves outcome after assisted reproduction by deselecting physiologically poor quality spermatozoa. *J Assist Reprod Genet*. 2011;28(3):253–62.
150. Gatimel N, Parinaud J, Leandri RD. Intracytoplasmic morphologically selected sperm injection (IMSI) does not improve outcome in patients with two successive IVF-ICSI failures. *J Assist Reprod Genet*. 2016;33(3):349–55.
151. Teixeira DM, Barbosa MA, Ferriani RA, Navarro PA, Raine-Fenning N, Nastri CO et al. Regular (ICSI) versus ultra-high magnification (IMSI) sperm selection for assisted reproduction. *Cochrane Db Syst Rev*. 2013;7:CD010167.
152. De Vos A, Polyzos NP, Verheyen G, Tournaye H. Intracytoplasmic morphologically selected sperm injection (IMSI): A critical and evidence-based review. *Basic Clin Androl*. 2013;23:10.
153. Said TM, Land JA. Effects of advanced selection methods on sperm quality and ART outcome: A systematic review. *Hum Reprod Update*. 2011;17(6):719–33.
154. Rappa KL, Rodriguez HF, Hakkarainen GC, Anchan RM, Mutter GL, Asghar W. Sperm processing for advanced reproductive technologies: Where are we today? *Biotechnol Adv*. 2016;34(5):578–87.
155. Nagy ZP, Liu J, Joris H, Devroey P, Van Steirteghem A. Time-course of oocyte activation, pronucleus formation and cleavage in human oocytes fertilized by intracytoplasmic sperm injection. *Hum Reprod*. 1994;9(9):1743–8.

156. Nagy ZP, Janssenswillen C, Janssens R, De Vos A, Staessen C, Van de Velde H et al. Timing of oocyte activation, pronucleus formation and cleavage in humans after intracytoplasmic sperm injection (ICSI) with testicular spermatozoa and after ICSI or in-vitro fertilization on sibling oocytes with ejaculated spermatozoa. *Hum Reprod*. 1998;13(6):1606–12.
157. Payne D, Flaherty SP, Barry MF, Matthews CD. Preliminary observations on polar body extrusion and pronuclear formation in human oocytes using time-lapse video cinematography. *Hum Reprod*. 1997;12(3):532–41.
158. Nagy ZP, Oliveira SA, Abdelmassih V, Abdelmassih R. Novel use of laser to assist ICSI for patients with fragile oocytes: A case report. *Reprod Biomed Online*. 2002;4(1):27–31.
159. Demirol A, Benkhalifa M, Sari T, Gurgan T. Use of laser-assisted intracytoplasmic sperm injection (ICSI) in patients with a history of poor ICSI outcome and limited metaphase II oocytes. *Fertil Steril*. 2006;86(1):256–8.
160. Rienzi L, Ubaldi F, Martinez F, Minasi MG, Iacobelli M, Ferrero S et al. Clinical application of laser-assisted ICSI: A pilot study. *Eur J Obstet Gynecol Reprod Biol*. 2004;115(Suppl 1):S77–9.
161. Moser M, Ebner T, Sommergruber M, Gaisswinkler U, Jesacher K, Puchner M et al. Laser-assisted zona pellucida thinning prior to routine ICSI. *Hum Reprod*. 2004;19(3):573–8.
162. Nagy ZP, Joris H, Liu J, Staessen C, Devroey P, Van Steirteghem AC. Intracytoplasmic single sperm injection of 1-day-old unfertilized human oocytes. *Hum Reprod*. 1993;8(12):2180–4.
163. Nagy ZP, Staessen C, Liu J, Joris H, Devroey P, Van Steirteghem AC. Prospective, auto-controlled study on reinsemination of failed-fertilized oocytes by intracytoplasmic sperm injection. *Fertil Steril*. 1995;64(6):1130–5.
164. Nagy ZP, Rienzi LF, Ubaldi FM, Greco E, Massey JB, Kort HI. Effect of reduced oocyte aging on the outcome of rescue intracytoplasmic sperm injection. *Fertil Steril*. 2006;85(4):901–6.
165. Chen L, Xu Z, Zhang N, Wang B, Chen H, Wang S et al. Neonatal outcome of early rescue ICSI and ICSI with ejaculated sperm. *J Assist Reprod Genet*. 2014;31(7):823–8.
166. Jin H, Shu Y, Dai S, Peng Z, Shi S, Sun Y. The value of second polar body detection 4 hours after insemination and early rescue ICSI in preventing complete fertilisation failure in patients with borderline semen. *Reprod Fertil Dev*. 2014;26(2):346–50.
167. Coticchio G. Polarization microscopy and rescue ICSI. *Reprod Biomed Online*. 2013;26(3):222–3; discussion 4–5.
168. Palermo GD, Cohen J, Alikani M, Adler A, Rosenwaks Z. Development and implementation of intracytoplasmic sperm injection (ICSI). *Reprod Fertil Dev*. 1995;7(2):211–7; discussion 7–8.
169. Payne D, Flaherty SP, Jeffrey R, Warnes GM, Matthews CD. Successful treatment of severe male factor infertility in 100 consecutive cycles using intracytoplasmic sperm injection. *Hum Reprod*. 1994;9(11):2051–7.
170. Tucker MJ, Wright G, Morton PC, Mayer MP, Ingargiola PE, Jones AE. Practical evolution and application of direct intracytoplasmic sperm injection for male factor and idiopathic fertilization failure infertilities. *Fertil Steril*. 1995;63(4):820–7.
171. Harari O, Bourne H, McDonald M, Richings N, Speirs AL, Johnston WI et al. Intracytoplasmic sperm injection: A major advance in the management of severe male subfertility. *Fertil Steril*. 1995;64(2):360–8.
172. McLachlan RI, Fuscaldo G, Rho H, Poulos C, Dalrymple J, Jackson P et al. Clinical results from intracytoplasmic sperm injection at Monash IVF. *Reprod Fertil Dev*. 1995;7(2):247–53.
173. Hamberger L, Sjogren A, Lundin K, Soderlund B, Nilsson L, Bergh C et al. Microfertilization techniques–the Swedish experience. *Reprod Fertil Dev*. 1995;7(2):263–7; discussion 8.
174. Palermo GD, Cohen J, Rosenwaks Z. Intracytoplasmic sperm injection: A powerful tool to overcome fertilization failure. *Fertil Steril*. 1996;65(5):899–908.
175. Sobek A, Hrbkova K, Priesnitz J. The first pregnancy in the Czech Republic achieved by oocyte fertilization using ICSI–intracytoplasmic sperm injection. *Ceska Gynekol*. 1996;61(1):3–6.
176. Michelmann HW. The intracytoplasmatic spermatozoa injection–The way out of the "male fertility crisis"? *Dtsch Tierarztl Wochenschr*. 1996;103(10):384–6.
177. Lunglmayr G, Obruca A. Assisted reproductive technology in the management of azoospermic men–The Austrian experience. *Andrologia*. 1996;28(Suppl 1):83–6.
178. Oehninger S. Intracytoplasmic sperm injection: Results from Norfolk, USA. *Hum Reprod*. 1996;11(Suppl 1):73–5; discussion 81–5.

179. Aboulghar MA, Mansour RT, Serour GI, Amin YM. The role of intracytoplasmic sperm injection (ICSI) in the treatment of patients with borderline semen. *Hum Reprod*. 1995;10(11):2829–30.
180. Michelmann HW. Minimal criteria of sperm quality for insemination and IVF therapy. *Int J Androl*. 1995;18(Suppl 2):81–7.
181. Gil-Salom M, Minguez Y, Rubio C, Ruiz A, Remohi J, Pellicer A. Intracytoplasmic sperm injection: A treatment for extreme oligospermia. *J Urol*. 1996;156(3):1001–4.
182. Vandervorst M, Tournaye H, Camus M, Nagy ZP, Van Steirteghem A, Devroey P. Patients with absolutely immotile spermatozoa and intracytoplasmic sperm injection. *Hum Reprod*. 1997;12(11):2429–33.
183. Tournaye H, Liu J, Nagy Z, Verheyen G, Van Steirteghem A, Devroey P. The use of testicular sperm for intracytoplasmic sperm injection in patients with necrozoospermia. *Fertil Steril*. 1996;66(2):331–4.
184. Kahraman S, Tasdemir M, Tasdemir I, Vicdan K, Ozgur S, Polat G et al. Pregnancies achieved with testicular and ejaculated spermatozoa in combination with intracytoplasmic sperm injection in men with totally or initially immotile spermatozoa in the ejaculate. *Hum Reprod*. 1996;11(6):1343–6.
185. Casper RF, Meriano JS, Jarvi KA, Cowan L, Lucato ML. The hypo-osmotic swelling test for selection of viable sperm for intracytoplasmic sperm injection in men with complete asthenozoospermia. *Fertil Steril*. 1996;65(5):972–6.
186. Verheyen G, Joris H, Crits K, Nagy Z, Tournaye H, Van Steirteghem A. Comparison of different hypo-osmotic swelling solutions to select viable immotile spermatozoa for potential use in intracytoplasmic sperm injection. *Hum Reprod Update*. 1997;3(3):195–203.
187. Liu J, Nagy Z, Joris H, Tournaye H, Devroey P, Van Steirteghem A. Successful fertilization and establishment of pregnancies after intracytoplasmic sperm injection in patients with globozoospermia. *Hum Reprod*. 1995;10(3):626–9.
188. Lundin K, Sjogren A, Nilsson L, Hamberger L. Fertilization and pregnancy after intracytoplasmic microinjection of acrosomeless spermatozoa. *Fertil Steril*. 1994;62(6):1266–7.
189. Bourne H, Richings N, Harari O, Watkins W, Speirs AL, Johnston WI et al. The use of intracytoplasmic sperm injection for the treatment of severe and extreme male infertility. *Reprod Fertil Dev*. 1995;7(2):237–45.
190. Trokoudes KM, Danos N, Kalogirou L, Vlachou R, Lysiotis T, Georghiades N et al. Pregnancy with spermatozoa from a globozoospermic man after intracytoplasmic sperm injection treatment. *Hum Reprod*. 1995;10(4):880–2.
191. Tesarik J, Sousa M. More than 90% fertilization rates after intracytoplasmic sperm injection and artificial induction of oocyte activation with calcium ionophore. *Fertil Steril*. 1995;63(2):343–9.
192. Montag M, Koster M, van der Ven K, Bohlen U, van der Ven H. The benefit of artificial oocyte activation is dependent on the fertilization rate in a previous treatment cycle. *Reprod Biomed Online*. 2012;24(5):521–6.
193. Ebner T, Montag M. Artificial oocyte activation: Evidence for clinical readiness. *Reprod Biomed Online*. 2016;32(3):271–3.
194. Ombelet W, Menkveld R, Kruger TF, Steeno O. Sperm morphology assessment: Historical review in relation to fertility. *Hum Reprod Update*. 1995;1(6):543–57.
195. Svalander P, Jakobsson AH, Forsberg AS, Bengtsson AC, Wikland M. The outcome of intracytoplasmic sperm injection is unrelated to "strict criteria" sperm morphology. *Hum Reprod*. 1996;11(5):1019–22.
196. Hammadeh ME, al-Hasani S, Stieber M, Rosenbaum P, Kupker D, Diedrich K et al. The effect of chromatin condensation (aniline blue staining) and morphology (strict criteria) of human spermatozoa on fertilization, cleavage and pregnancy rates in an intracytoplasmic sperm injection programme. *Hum Reprod*. 1996;11(11):2468–71.
197. Martin RH, Rademaker A. The relationship between sperm chromosomal abnormalities and sperm morphology in humans. *Mutat Res*. 1988;207(3–4):159–64.
198. Rosenbusch B, Strehler E, Sterzik K. Cytogenetics of human spermatozoa: Correlations with sperm morphology and age of fertile men. *Fertil Steril*. 1992;58(5):1071–2.
199. Stalf T, Sanchez R, Kohn FM, Schalles U, Kleinstein J, Hinz V et al. Pregnancy and birth after intracytoplasmic sperm injection with spermatozoa from a patient with tail stump syndrome. *Hum Reprod*. 1995;10(8):2112–4.
200. Hotaling JM, Smith JF, Rosen M, Muller CH, Walsh TJ. The relationship between isolated teratozoospermia and clinical pregnancy after in vitro fertilization with or without intracytoplasmic sperm injection: A systematic review and meta-analysis. *Fertil Steril*. 2011;95(3):1141–5.

201. Tournaye H. Male factor infertility and ART. *Asian J Androl*. 2012;14(1):103–8.
202. Devroey P, Van Steirteghem A. A review of ten years experience of ICSI. *Hum Reprod Update*. 2004;10(1):19–28.
203. Ortega C, Verheyen G, Raick D, Camus M, Devroey P, Tournaye H. Absolute asthenozoospermia and ICSI: What are the options? *Hum Reprod Update*. 2011;17(5):684–92.
204. de Almeida M, Gazagne I, Jeulin C, Herry M, Belaisch-Allart J, Frydman R et al. In-vitro processing of sperm with autoantibodies and in-vitro fertilization results. *Hum Reprod*. 1989;4(1):49–53.
205. Nagy ZP, Verheyen G, Liu J, Joris H, Janssenswillen C, Wisanto A et al. Results of 55 intracytoplasmic sperm injection cycles in the treatment of male-immunological infertility. *Hum Reprod*. 1995;10(7):1775–80.
206. Lahteenmaki A, Reima I, Hovatta O. Treatment of severe male immunological infertility by intracytoplasmic sperm injection. *Hum Reprod*. 1995;10(11):2824–8.
207. Lewis-Jones DI, Gazvani MR, Mountford R. Cystic fibrosis in infertility: Screening before assisted reproduction: Opinion. *Hum Reprod*. 2000;15(11):2415–7.
208. Wosnitzer M, Goldstein M, Hardy MP. Review of Azoospermia. *Spermatogenesis*. 2014;4:e28218.
209. Silber SJ. Microsurgical TESE and the distribution of spermatogenesis in non-obstructive azoospermia. *Hum Reprod*. 2000;15(11):2278–84.
210. van Wely M, Barbey N, Meissner A, Repping S, Silber SJ. Live birth rates after MESA or TESE in men with obstructive azoospermia: Is there a difference? *Hum Reprod*. 2015;30(4):761–6.
211. Makhseed M, Al Salem MH, Ahmed MA. Percutaneous testicular sperm aspiration and intracytoplasmic sperm injection in obstructive and non-obstructive azoospermia: An easy alternative to TESE and MESA. *Urol Int*. 2002;68(2):86–90.
212. Schlegel PN, Palermo GD, Alikani M, Adler A, Reing AM, Cohen J et al. Micropuncture retrieval of epididymal sperm with in vitro fertilization: Importance of in vitro micromanipulation techniques. *Urology*. 1995;46(2):238–41.
213. Son IP, Hong JY, Lee YS, Park YS, Jun JH, Lee HJ et al. Efficacy of microsurgical epididymal sperm aspiration (MESA) and intracytoplasmic sperm injection (ICSI) in obstructive azoospermia. *J Assist Reprod Genet*. 1996;13(1):69–72.
214. Mansour RT, Aboulghar MA, Serour GI, Fahmi I, Ramzy AM, Amin Y. Intracytoplasmic sperm injection using microsurgically retrieved epididymal and testicular sperm. *Fertil Steril*. 1996;65(3):566–72.
215. Madgar I, Seidman DS, Levran D, Yonish M, Augarten A, Yemini Z et al. Micromanipulation improves in-vitro fertilization results after epididymal or testicular sperm aspiration in patients with congenital absence of the vas deferens. *Hum Reprod*. 1996;11(10):2151–4.
216. Watkins W, Nieto F, Bourne H, Wutthiphan B, Speirs A, Baker HW. Testicular and epididymal sperm in a microinjection program: Methods of retrieval and results. *Fertil Steril*. 1997;67(3):527–35.
217. Shrivastav P, Nadkarni P, Wensvoort S, Craft I. Percutaneous epididymal sperm aspiration for obstructive azoospermia. *Hum Reprod*. 1994;9(11):2058–61.
218. Meniru GI, Tsirigotis M, Zhu J, Craft I. Successful percutaneous epididymal sperm aspiration (PESA) after more than 20 years of acquired obstructive azoospermia. *J Assist Reprod Genet*. 1996;13(5):449–50.
219. Craft I, Tsirigotis M, Bennett V, Taranissi M, Khalifa Y, Hogewind G et al. Percutaneous epididymal sperm aspiration and intracytoplasmic sperm injection in the management of infertility due to obstructive azoospermia. *Fertil Steril*. 1995;63(5):1038–42.
220. Patrizio P, Ord T, Balmaceda JP, Asch RH. Successful fertilization, pregnancy, and birth using epididymal sperm frozen 24 hours after conventional oocyte insemination. *Fertil Steril*. 1995;64(4):863–5.
221. Oates RD, Lobel SM, Harris DH, Pang S, Burgess CM, Carson RS. Efficacy of intracytoplasmic sperm injection using intentionally cryopreserved epididymal spermatozoa. *Hum Reprod*. 1996;11(1):133–8.
222. Holden CA, Fuscaldo GF, Jackson P, Cato A, Southwick GJ, Hauser R et al. Frozen-thawed epididymal spermatozoa for intracytoplasmic sperm injection. *Fertil Steril*. 1997;67(1):81–7.
223. Bourne H, Watkins W, Speirs A, Baker HW. Pregnancies after intracytoplasmic injection of sperm collected by fine needle biopsy of the testis. *Fertil Steril*. 1995;64(2):433–6.
224. Abuzeid MI, Chan YM, Sasy MA, Basata S, Beer M. Fertilization and pregnancy achieved by intracytoplasmic injection of sperm retrieved from testicular biopsies. *Fertil Steril*. 1995;64(3):644–6.
225. Hovatta O, Moilanen J, von Smitten K, Reima I. Testicular needle biopsy, open biopsy, epididymal aspiration and intracytoplasmic sperm injection in obstructive azoospermia. *Hum Reprod*. 1995;10(10):2595–9.

226. Gil-Salom M, Minguez Y, Rubio C, Remohi J, Pellicer A. Intracytoplasmic testicular sperm injection: An effective treatment for otherwise intractable obstructive azoospermia. *J Urol*. 1995;154(6):2074–7.
227. Rosenlund B, Sjoblom P, Dimitrakopoulos A, Hillensjo T. Epididymal and testicular sperm for intracytoplasmic sperm injection in the treatment of obstructive azoospermia. *Acta Obstet Gynecol Scand*. 1997;76(2):135–9.
228. Kahraman S, Ozgur S, Alatas C, Aksoy S, Balaban B, Evrenkaya T et al. High implantation and pregnancy rates with testicular sperm extraction and intracytoplasmic sperm injection in obstructive and non-obstructive azoospermia. *Hum Reprod*. 1996;11(3):673–6.
229. Schlegel PN, Palermo GD, Goldstein M, Menendez S, Zaninovic N, Veeck LL et al. Testicular sperm extraction with intracytoplasmic sperm injection for nonobstructive azoospermia. *Urology*. 1997;49(3):435–40.
230. Gil-Salom M, Romero J, Minguez Y, Rubio C, De los Santos MJ, Remohi J et al. Pregnancies after intracytoplasmic sperm injection with cryopreserved testicular spermatozoa. *Hum Reprod*. 1996;11(6):1309–13.
231. Romero J, Remohi J, Minguez Y, Rubio C, Pellicer A, Gil-Salom M. Fertilization after intracytoplasmic sperm injection with cryopreserved testicular spermatozoa. *Fertil Steril*. 1996;65(4):877–9.
232. Podsiadly BT, Woolcott RJ, Stanger JD, Stevenson K. Pregnancy resulting from intracytoplasmic injection of cryopreserved spermatozoa recovered from testicular biopsy. *Hum Reprod*. 1996;11(6):1306–8.
233. Hovatta O, Foudila T, Siegberg R, Johansson K, von Smitten K, Reima I. Pregnancy resulting from intracytoplasmic injection of spermatozoa from a frozen-thawed testicular biopsy specimen. *Hum Reprod*. 1996;11(11):2472–3.
234. Nagy ZP, Joris H, Verheyen G, Tournaye H, Devroey P, Van Steirteghem AC. Correlation between motility of testicular spermatozoa, testicular histology and the outcome of intracytoplasmic sperm injection. *Hum Reprod*. 1998;13(4):890–5.
235. Dohle GR, Elzanaty S, van Casteren NJ. Testicular biopsy: Clinical practice and interpretation. *Asian J Androl*. 2012;14(1):88–93.
236. Vloeberghs V, Verheyen G, Tournaye H. Intracytoplasmic spermatid injection and in vitro maturation: Fact or fiction? *Clinics (Sao Paulo)*. 2013;68(Suppl 1):151–6.
237. Popal W, Nagy ZP. Laboratory processing and intracytoplasmic sperm injection using epididymal and testicular spermatozoa: What can be done to improve outcomes? *Clinics (Sao Paulo)*. 2013;68(Suppl 1):125–30.
238. Silber SJ. Sperm retrieval for azoospermia and intracytoplasmic sperm injection success rates–A personal overview. *Hum Fertil (Camb)*. 2010;13(4):247–56.
239. Liu J, Nagy Z, Joris H, Tournaye H, Smitz J, Camus M et al. Analysis of 76 total fertilization failure cycles out of 2732 intracytoplasmic sperm injection cycles. *Hum Reprod*. 1995;10(10):2630–6.
240. Flaherty SP, Payne D, Swann NJ, Mattews CD. Aetiology of failed and abnormal fertilization after intracytoplasmic sperm injection. *Hum Reprod*. 1995;10(10):2623–9.
241. Jean M, Rival JM, Mensier A, Mirallie S, Lopes P, Barriere P. Prenatal diagnosis of ring chromosome 14 after intracytoplasmic sperm injection. *Fertil Steril*. 1997;67(1):164–5.
242. Baschat AA, Kupker W, al Hasani S, Diedrich K, Schwinger E. Results of cytogenetic analysis in men with severe subfertility prior to intracytoplasmic sperm injection. *Hum Reprod*. 1996;11(2):330–3.
243. Tiepolo L, Zuffardi O. Localization of factors controlling spermatogenesis in the nonfluorescent portion of the human Y chromosome long arm. *Hum Genet*. 1976 28;34(2):119–24.
244. Reijo R, Lee TY, Salo P, Alagappan R, Brown LG, Rosenberg M et al. Diverse spermatogenic defects in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein gene. *Nat Genet*. 1995;10(4):383–93.
245. Shan Z, Hirschmann P, Seebacher T, Edelmann A, Jauch A, Morell J et al. A SPGY copy homologous to the mouse gene Dazla and the Drosophila gene boule is autosomal and expressed only in the human male gonad. *Hum Mol Genet*. 1996;5(12):2005–11.
246. Page DC, Silber S, Brown LG. Men with infertility caused by AZFc deletion can produce sons by intracytoplasmic sperm injection, but are likely to transmit the deletion and infertility. *Hum Reprod*. 1999;14(7):1722–6.
247. Liu J, Lissens W, Silber SJ, Devroey P, Liebaers I, Van Steirteghem A. Birth after preimplantation diagnosis of the cystic fibrosis delta F508 mutation by polymerase chain reaction in human embryos resulting from intracytoplasmic sperm injection with epididymal sperm. *JAMA*. 1994;272(23):1858–60.

248. Staessen C, Coonen E, Van Assche E, Tournaye H, Joris H, Devroey P et al. Preimplantation diagnosis for X and Y normality in embryos from three Klinefelter patients. *Hum Reprod*. 1996;11(8):1650–3.
249. De Sutter P. Single embryo transfer (set) not only leads to a reduction in twinning rates after IVF/ICSI, but also improves obstetrical and perinatal outcome of singletons. *Verh K Acad Geneeskd Belg*. 2006;68(5–6):319–27.
250. De Neubourg D, Gerris J, Mangelschots K, Van Royen E, Vercruyssen M, Steylemans A et al. The obstetrical and neonatal outcome of babies born after single-embryo transfer in IVF/ICSI compares favourably to spontaneously conceived babies. *Hum Reprod*. 2006;21(4):1041–6.
251. Wisanto A, Magnus M, Bonduelle M, Liu J, Camus M, Tournaye H et al. Obstetric outcome of 424 pregnancies after intracytoplasmic sperm injection. *Hum Reprod*. 1995;10(10):2713–8.
252. Wisanto A, Bonduelle M, Camus M, Tournaye H, Magnus M, Liebaers I et al. Obstetric outcome of 904 pregnancies after intracytoplasmic sperm injection. *Hum Reprod*. 1996;11(Suppl 4):121–9; discussion 30.
253. Wennerholm UB, Bergh C, Hamberger L, Nilsson L, Reismer E, Wennergren M et al. Obstetric and perinatal outcome of pregnancies following intracytoplasmic sperm injection. *Hum Reprod*. 1996; 11(5):1113–9.
254. In't Veld P, Brandenburg H, Verhoeff A, Dhont M, Los F. Sex chromosomal abnormalities and intracytoplasmic sperm injection. *Lancet*. 1995;346(8977):773.
255. Liebaers I, Bonduelle M, Van Assche E, Devroey P, Van Steirteghem A. Sex chromosome abnormalities after intracytoplasmic sperm injection. *Lancet*. 1995;346(8982):1095–0.
256. Jozwiak EA, Ulug U, Mesut A, Erden HF, Bahceci M. Prenatal karyotypes of fetuses conceived by intracytoplasmic sperm injection. *Fertil Steril*. 2004;82(3):628–33.
257. Gjerris AC, Loft A, Pinborg A, Christiansen M, Tabor A. Prenatal testing among women pregnant after assisted reproductive techniques in Denmark 1995–2000: A national cohort study. *Hum Reprod*. 2008;23(7):1545–52.
258. Woldringh GH, Besselink DE, Tillema AH, Hendriks JC, Kremer JA. Karyotyping, congenital anomalies and follow-up of children after intracytoplasmic sperm injection with non-ejaculated sperm: A systematic review. *Hum Reprod Update*. 2010;16(1):12–9.
259. Bonduelle M, Van Assche E, Joris H, Keymolen K, Devroey P, Van Steirteghem A et al. Prenatal testing in ICSI pregnancies: Incidence of chromosomal anomalies in 1586 karyotypes and relation to sperm parameters. *Hum Reprod*. 2002;17(10):2600–14.
260. Moosani N, Pattinson HA, Carter MD, Cox DM, Rademaker AW, Martin RH. Chromosomal analysis of sperm from men with idiopathic infertility using sperm karyotyping and fluorescence in situ hybridization. *Fertil Steril*. 1995;64(4):811–7.
261. Bonduelle M, Desmyttere S, Buysse A, Van Assche E, Schietecatte J, Devroey P et al. Prospective follow-up study of 55 children born after subzonal insemination and intracytoplasmic sperm injection. *Hum Reprod*. 1994;9(9):1765–9.
262. Bonduelle M, Legein J, Derde MP, Buysse A, Schietecatte J, Wisanto A et al. Comparative follow-up study of 130 children born after intracytoplasmic sperm injection and 130 children born after in-vitro fertilization. *Hum Reprod*. 1995;10(12):3327–31.
263. Bonduelle M, Wilikens A, Buysse A, Van Assche E, Wisanto A, Devroey P et al. Prospective follow-up study of 877 children born after intracytoplasmic sperm injection (ICSI), with ejaculated epididymal and testicular spermatozoa and after replacement of cryopreserved embryos obtained after ICSI. *Hum Reprod*. 1996;11(Suppl 4):131–55; discussion 56–9.
264. Palermo GD, Colombero LT, Schattman GL, Davis OK, Rosenwaks Z. Evolution of pregnancies and initial follow-up of newborns delivered after intracytoplasmic sperm injection. *JAMA*. 1996;276(23):1893–7.
265. Hansen M, Kurinczuk JJ, Bower C, Webb S. The risk of major birth defects after intracytoplasmic sperm injection and in vitro fertilization. *N Engl J Med*. 2002;346(10):725–30.
266. Olson CK, Keppler-Noreuil KM, Romitti PA, Budelier WT, Ryan G, Sparks AE et al. In vitro fertilization is associated with an increase in major birth defects. *Fertil Steril*. 2005;84(5):1308–15.
267. Massaro PA, MacLellan DL, Anderson PA, Romao RL. Does intracytoplasmic sperm injection pose an increased risk of genitourinary congenital malformations in offspring compared to in vitro fertilization? A systematic review and meta-analysis. *J Urol*. 2015(5 Suppl);193:1837–42.
268. Bang JK, Lyu SW, Choi J, Lee DR, Yoon TK, Song SH. Does infertility treatment increase male reproductive tract disorder? *Urology*. 2013;81(3):644–8.

269. Kurinczuk JJ, Hansen M, Bower C. The risk of birth defects in children born after assisted reproductive technologies. *Curr Opin Obstet Gynecol*. 2004;16(3):201–9.
270. Yan J, Huang G, Sun Y, Zhao X, Chen S, Zou S et al. Birth defects after assisted reproductive technologies in China: Analysis of 15,405 offspring in seven centers (2004 to 2008). *Fertil Steril*. 2011;95(1):458–60.
271. Tsai YR, Huang FJ, Lin PY, Kung FT, Lin YJ, Lan KC. Clinical outcomes and development of children born to couples with obstructive and nonobstructive azoospermia undergoing testicular sperm extraction-intracytoplasmic sperm injection: A comparative study. *Taiwan J Obstet Gynecol*. 2015;54(2):155–9.
272. Fedder J, Loft A, Parner ET, Rasmussen S, Pinborg A. Neonatal outcome and congenital malformations in children born after ICSI with testicular or epididymal sperm: A controlled national cohort study. *Hum Reprod*. 2013;28(1):230–40.
273. Lie RT, Lyngstadaas A, Orstavik KH, Bakketeig LS, Jacobsen G, Tanbo T. Birth defects in children conceived by ICSI compared with children conceived by other IVF-methods; a meta-analysis. *Int J Epidemiol*. 2005;34(3):696–701.
274. Bonduelle M, Camus M, De Vos A, Staessen C, Tournaye H, Van Assche E et al. Seven years of intracytoplasmic sperm injection and follow-up of 1987 subsequent children. *Hum Reprod*. 1999;14(Suppl 1):243–64.
275. Belva F, Henriet S, Liebaers I, Van Steirteghem A, Celestin-Westreich S, Bonduelle M. Medical outcome of 8-year-old singleton ICSI children (born >or = 32 weeks' gestation) and a spontaneously conceived comparison group. *Hum Reprod*. 2007;22(2):506–15.
276. Punamaki RL, Tiitinen A, Lindblom J, Unkila-Kallio L, Flykt M, Vanska M et al. Mental health and developmental outcomes for children born after ART: A comparative prospective study on child gender and treatment type. *Hum Reprod*. 2016;31(1):100–7.
277. Fauser BC, Devroey P, Diedrich K, Balaban B, Bonduelle M, Delemarre-van de Waal HA et al. Health outcomes of children born after IVF/ICSI: A review of current expert opinion and literature. *Reprod Biomed Online*. 2014;28(2):162–82.
278. Silber SJ, Ord T, Balmaceda J, Patrizio P, Asch RH. Congenital absence of the vas deferens. The fertilizing capacity of human epididymal sperm. *N Engl J Med*. 1990;323(26):1788–92.
279. Ben-Shlomo I, Bider D, Dor J, Levran D, Mashiach S, Ben-Rafael Z. Failure to fertilize in vitro in couples with male factor infertility: What next? *Fertil Steril*. 1992;58(1):187–9.
280. Coates TE, Check JH, Choe J, Nowroozi K, Lurie D, Callan C. An evaluation of couples with failure of fertilization in vitro. *Hum Reprod*. 1992;7(7):978–81.
281. Tournaye H, Devroey P, Camus M, Staessen C, Bollen N, Smitz J et al. Comparison of in-vitro fertilization in male and tubal infertility: A 3 year survey. *Hum Reprod*. 1992;7(2):218–22.
282. Verheyen G, Tournaye H, Staessen C, De Vos A, Vandervorst M, Van Steirteghem A. Controlled comparison of conventional in-vitro fertilization and intracytoplasmic sperm injection in patients with asthenozoospermia. *Hum Reprod*. 1999;14(9):2313–9.
283. Tournaye H, Verheyen G, Albano C, Camus M, Van Landuyt L, Devroey P et al. Intracytoplasmic sperm injection versus in vitro fertilization: A randomized controlled trial and a meta-analysis of the literature. *Fertil Steril*. 2002;78(5):1030–7.
284. Pinheiro RC, Lambert J, Benard F, Mauffette F, Miron P. Effectiveness of in vitro fertilization with intracytoplasmic sperm injection for severe male infertility. *CMAJ*. 1999;161(11):1397–401.
285. Okada H, Dobashi M, Yamazaki T, Hara I, Fujisawa M, Arakawa S et al. Conventional versus microdissection testicular sperm extraction for nonobstructive azoospermia. *J Urol*. 2002;168(3):1063–7.
286. Kihaile PE, Misumi J, Hirotsuru K, Kumasako Y, Kisanga RE, Utsunomiya T. Comparison of sibling oocyte outcomes after intracytoplasmic sperm injection and in vitro fertilization in severe teratozoospermic patients in the first cycle. *Int J Androl*. 2003;26(1):57–62.
287. Xie BG, Huang YH, Zhu WJ, Jin S. Comparison of the outcome of conventional in vitro fertilization and intracytoplasmic sperm injection in moderate male infertility from ejaculate. *Urol Int*. 2015;94(1):111–6.
288. Shuai HL, Ye Q, Huang YH, Xie BG. Comparison of conventional in vitro fertilisation and intracytoplasmic sperm injection outcomes in patients with moderate oligoasthenozoospermia. *Andrologia*. 2015;47(5):499–504.
289. Komsky-Elbaz A, Raziel A, Friedler S, Strassburger D, Kasterstein E, Komarovsky D et al. Conventional IVF versus ICSI in sibling oocytes from couples with endometriosis and normozoospermic semen. *J Assist Reprod Genet*. 2013;30(2):251–7.

290. Johnson LN, Sasson IE, Sammel MD, Dokras A. Does intracytoplasmic sperm injection improve the fertilization rate and decrease the total fertilization failure rate in couples with well-defined unexplained infertility? A systematic review and meta-analysis. *Fertil Steril*. 2013;100(3):704–11.
291. Eftekhar M, Mohammadian F, Yousefnejad F, Molaei B, Aflatoonian A. Comparison of conventional IVF versus ICSI in non-male factor, normoresponder patients. *Iran J Reprod Med*. 2012;10(2):131–6.
292. Hwang JL, Seow KM, Lin YH, Hsieh BC, Huang LW, Chen HJ et al. IVF versus ICSI in sibling oocytes from patients with polycystic ovarian syndrome: A randomized controlled trial. *Hum Reprod*. 2005;20(5):1261–5.
293. van Rumste MM, Evers JL, Farquhar CM. ICSI versus conventional techniques for oocyte insemination during IVF in patients with non-male factor subfertility: A Cochrane review. *Hum Reprod*. 2004;19(2):223–7.
294. Staessen C, Camus M, Clasen K, De Vos A, Van Steirteghem A. Conventional in-vitro fertilization versus intracytoplasmic sperm injection in sibling oocytes from couples with tubal infertility and normozoospermic semen. *Hum Reprod*. 1999;14(10):2474–9.
295. Tucker M, Graham J, Han T, Stillman R, Levy M. Conventional insemination versus intracytoplasmic sperm injection. *Lancet*. 2001;358(9293):1645–6.
296. McDonough PG. Standard IVF and ICSI–How much do the conclusions differ? *Fertil Steril*. 2002;78(3):653.
297. Aboulghar M, Mansour R, Serour G. Standard IVF and ICSI–How much do the conclusions differ? *Fertil Steril*. 2002;78(3):652–3; author reply 3.
298. Orief Y, Dafopoulos K, Al-Hassani S. Should ICSI be used in non-male factor infertility? *Reprod Biomed Online*. 2004;9(3):348–56.
299. Palermo GD, Neri QV, Rosenwaks Z. To ICSI or Not to ICSI. *Semin Reprod Med*. 2015;33(2):92–102.
300. Taylor TH, Wright G, Jones-Colon S, Mitchell-Leef D, Kort HI, Nagy ZP. Comparison of ICSI and conventional IVF in patients with increased oocyte immaturity. *Reprod Biomed Online*. 2008;17(1):46–52.
301. Basille C, Frydman R, El Aly A, Hesters L, Fanchin R, Tachdjian G et al. Preimplantation genetic diagnosis: State of the art. *Eur J Obstet Gynecol Reprod Biol*. 2009;145(1):9–13.
302. Chian RC, Lim JH, Tan SL. State of the art in in-vitro oocyte maturation. *Curr Opin Obstet Gynecol*. 2004;16(3):211–9.
303. Nagy ZP, Cecile J, Liu J, Loccufier A, Devroey P, Van Steirteghem A. Pregnancy and birth after intracytoplasmic sperm injection of in vitro matured germinal-vesicle stage oocytes: Case report. *Fertil Steril*. 1996;65(5):1047–50.
304. Nagy ZP, Chang CC, Shapiro DB, Bernal DP, Elsner CW, Mitchell-Leef D et al. Clinical evaluation of the efficiency of an oocyte donation program using egg cryo-banking. *Fertil Steril*. 2009;92(2):520–6.
305. Garcia-Velasco JA, Domingo J, Cobo A, Martinez M, Carmona L, Pellicer A. Five years' experience using oocyte vitrification to preserve fertility for medical and nonmedical indications. *Fertil Steril*. 2013;99(7):1994–9.

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12

Analysis of Fertilization

Basak Balaban

Introduction

Fertilization is a complex process involving successive molecular events. In general it can be defined as the process when the haploid spermatozoa fuses with the fully grown and mature oocyte at the metaphase II stage of development to form a distinctly new entity, the embryo. Sperm–oocyte interaction in vivo begins with the transport of spermatozoa through the female reproductive tract, many acrosome-intact spermatozoa reaching the mature oocyte within an hour. Only a selected group of spermatozoa are capable of successfully penetrating oocytes and they need to undergo certain modifications in the female genital tract, namely capacitation, hyperactivation, and acrosome reaction (1). The last step, the acrosome reaction, begins at the time of spermatozoa binding to the zona pellucida of the oocyte, which may be induced in vivo by cumulus cells and follicular fluid (2). Fusion then occurs between sperm plasma membrane in the region of the equatorial segment and the oolemma. The spermatozoa becomes immotile at this point as it is then incorporated into the oocyte, and all of the spermatozoon, including the sperm tail, is fused into the ooplasm. Fertilization is completed with the sequential steps: activation of the oocyte with membrane fusion and release of the sperm activating factor (PLC-zeta); release of intracellular calcium stores within the oocyte to induce the exocytosis of the cortical granules to initiate the block to polyspermy and prevent further sperm entry; completion of metaphase II leading to the formation of the second polar body and the appearance of pronuclei followed by nuclear syngamy whereby the genomes of both gametes are merged to form a new embryonic genome, resulting in the zygote (3).

Even though the analysis of normal and abnormal fertilization is inaccessible in natural conception cycles, the capability of observation of particular events by in vitro fertilization from the time of fusion until the formation of the zygote at syngamy provides valuable information that relates to healthy ongoing pregnancy and offspring. As abnormal fertilization in in vitro applications can give rise to nonviable embryos, the noninvasive assessments of zygote and embryos is therefore clinically relevant in the routine practice of assisted reproduction techniques.

Here the specific features of fertilization in assisted reproduction—conventional in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI), the effect of spermatozoa and metaphase II oocyte on fertilization, embryo development, clinical and neonatal outcome, the noninvasive assessment of zygote viability and quality by pronuclear morphology and its predictive value on embryo quality, further development, chromosomal status and implantation potential—will be considered. The dynamics of pronuclear formation and thus the limited predictive value of morphological assessments will also be discussed by studies of dynamic observations of zygote and embryo development.

Fertilization Process by Assisted Reproduction

Conventional In Vitro Fertilization (IVF)

As the natural barriers of spermatozoon to penetrate through the cervical mucus and follow the genital tract to reach the oocyte are bypassed by in vitro fertilization, less concentration of motile spermatozoa

is needed for fertilization. Despite this necessity the semen samples are still treated with special preparation techniques, to select the most progressive spermatozoa, and to eliminate the spermatozoa with functional disorders (see Chapter 10).

Intra-Cytoplasmic Sperm Injection (ICSI)

A single spermatozoon, typically selected by morphological appearance, is micro-injected into the cytoplasm of the mature oocyte at metaphase II stage for the ICSI procedure, thus the essential steps for spermatozoa to bypass for successful fertilization such as progressive movement, integrity of sperm functions for zona pellucida and oolemma penetration, and fusion with oolemma remains less important. As the barriers of natural selection of healthy, viable, and chromosomally normal spermatozoa are bypassed by the direct injection of spermatozoa within the oocyte, the selection of the single spermatozoon plays a critical role for ICSI cycles (4) (Chapters 10 and 11).

Contribution of Oocyte Characteristics on Fertilization and Embryo Development In Vitro

Assessment of human metaphase II mature oocytes in IVF is mainly performed by the examination of the morphological characteristics. Despite the fact that the morphology of oocytes is difficult to evaluate in IVF cases as the cumulus corona cells surrounding the oocyte eliminate precise examination, ICSI procedures allow the assessment of various morphological variations of metaphase II oocytes that could correlate with fertilization capacity, embryo quality, and development, as well as the clinical and neonatal outcome. Optimal oocyte morphology is defined as an oocyte with spherical structure enclosed by a uniform zona pellucida, with a uniform translucent cytoplasm free of inclusions and a size-appropriate polar body (5,6). However, Metaphase II-stage (MII) oocytes retrieved from patients after ovarian stimulation are known to show significant morphological variations that may affect the developmental competence and implantation potential of the derived embryo. Morphological abnormalities of the oocyte can be observed under two different subgroups: extracytoplasmic abnormalities and cytoplasmic abnormalities (5–8).

Cytoplasmic abnormalities of MII oocytes include different types and degrees of cytoplasmic granulations (slightly diffused or excessive whole/centrally located granulation) and the appearance of refractile bodies, smooth endoplasmic reticulum clusters (sERCs), or vacuolization in the ooplasm (Figures 12.1a,b and 12.2a,b).

Despite the fact that the various degrees of diffused granulation in the cytoplasm does not seem to affect the fertilization capacity, embryo development, or pregnancy outcome, condensed granulation that is centrally located within the cytoplasm with a clear border might have a detrimental effect on the fertilization rate, embryo quality, and, more importantly, chromosomal normality. Decreased pregnancy rates with such embryos, as well as increased abortion rates, are reported (Figure 12.3a,b).

Fluid filled large vacuoles within the cytoplasm have a significant detrimental effect on the fertilization capacity of the oocyte, with a cut-off value suggested >14 μms in diameter. Large vacuoles are assumed to negatively affect a large proportion of the cytoskeleton (e.g., microtubules) which cannot function as it is supposed to. In addition to the detrimental effect on fertilization rates, large vacuoles can also negatively affect the embryo quality and blastocyst formation, as well as the euploidy rates of the embryos obtained (Figure 12.4a,b).

The presence of translucent vacuole-like structures, referred to as smooth endoplasmic reticulum clusters(sERC), is known to negatively affect the neonatal safety of the offspring, and thus their utilization for fertilization in vitro continues to be debated (Figures 12.5a,b and 12.6a,b).

A variety of extracytoplasmic anomalies exist which in part negatively influence fertilization: consistency and thickness of the zona, polar body (Pb)1 decay, and debris within the perivitelline space (PVS). Characteristics of the zona pellucida and the PVS are most probably associated with the health of the

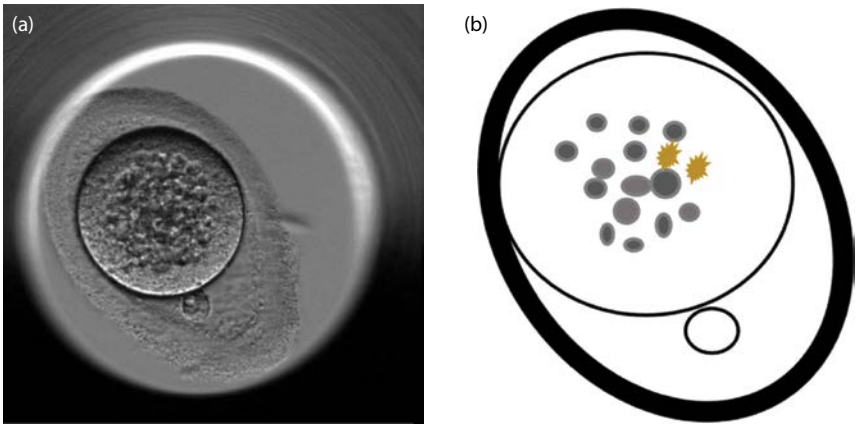


FIGURE 12.1 (a,b) Centrally located granulation and refractile bodies.

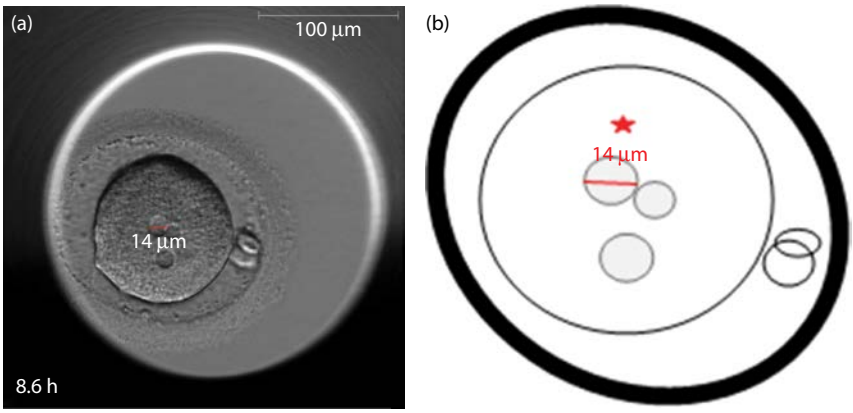


FIGURE 12.2 (a,b) Multiple vacuoles of various sizes, *vacuole with 14 µm diameter, large perivitelline space.

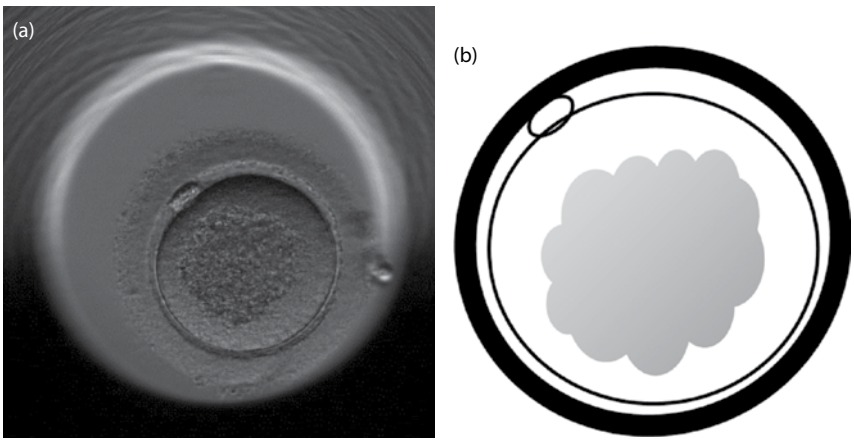


FIGURE 12.3 (a,b) Centrally located condensed granulation.

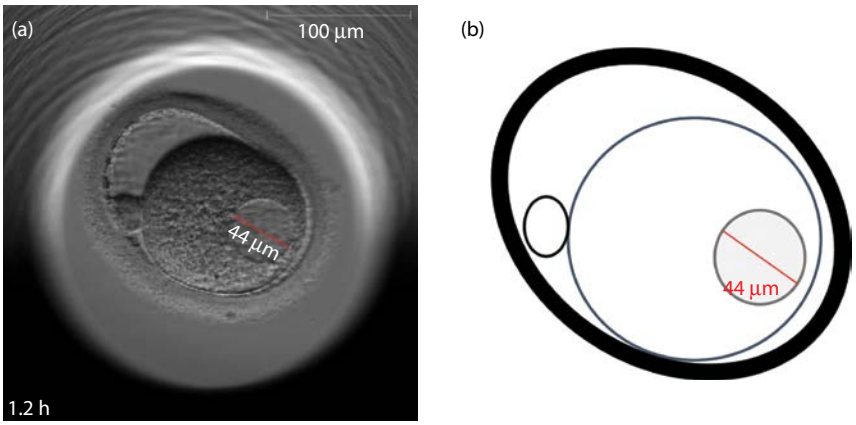


FIGURE 12.4 (a,b) Large vacuole with 44 µm diameter, large perivitelline space.

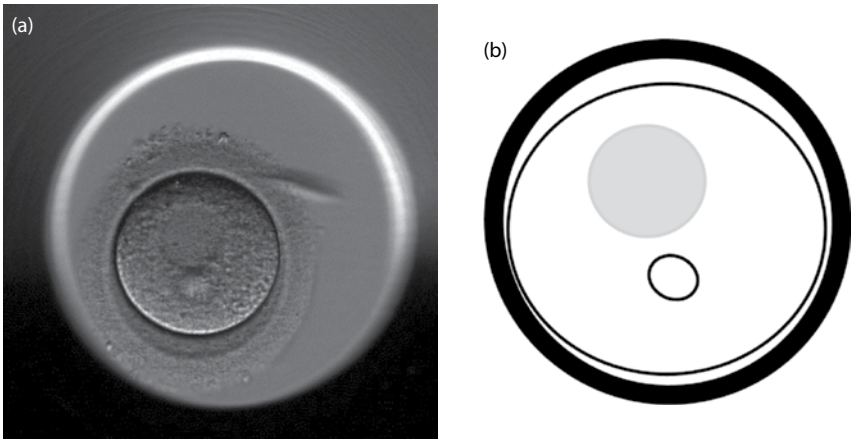


FIGURE 12.5 (a,b) Translucent vacuole type structures—smooth endoplasmic reticulum clusters.

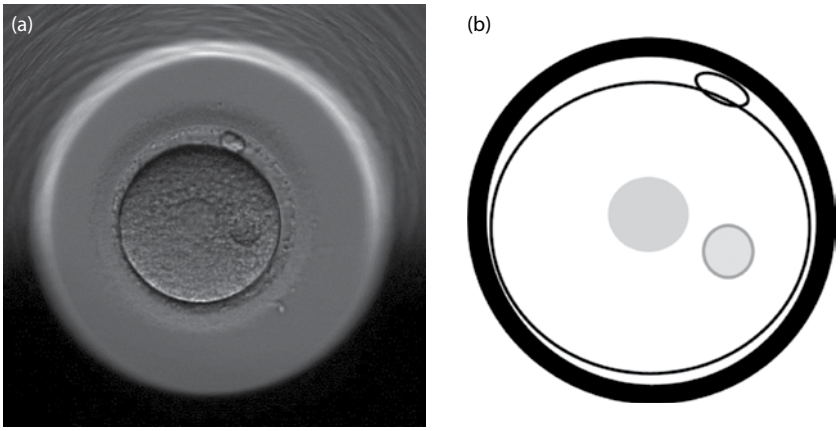


FIGURE 12.6 (a,b) Smooth endoplasmic reticulum clusters.

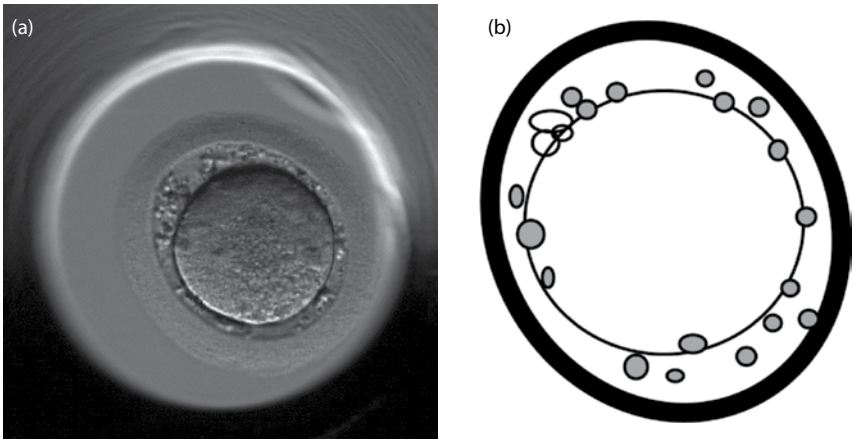


FIGURE 12.7 (a,b) Debris in perivitelline space, fragmented first polar body.

developing follicle, e.g., its vascularization and oxygen content. Any disturbance during growth might severely alter oocyte morphology, resulting in a pool of gametes with different prognoses (9) (Figure 12.7a,b).

Contribution of the Spermatozoon Characteristics on Fertilization and Embryo Development In Vitro

The effect of the spermatozoa, paternal genome, on fertilization rates, embryo quality, and especially the clinical outcome, is less evident. All the natural barriers that healthy spermatozoa with high fertilization capacity have to successfully overcome are bypassed and ignored during the ICSI procedure, for which a single spermatozoon is manually selected and inserted in the cytoplasm of the mature metaphase II oocyte. As the ICSI procedure is mainly applied for male factor infertility cases where poorer-quality spermatozoa is found, determination of defects that may adversely impact not only the fertilization, embryo viability, and implantation potential, but also the fetal outcome, is of critical importance. Studies have shown that the risk of neonatal and obstetric outcomes, as well as congenital malformation rates, for children born after the ICSI procedure for male factor infertility with ejaculated spermatozoa is not statistically different when compared with IVF treatments (10,11). For severe male factor infertility when epididymal or testicular spermatozoa are used, the overall neonatal health in terms of birth parameters, major anomalies, and chromosomal aberrations of children born still seems reassuring in comparison to the outcome of children born after the use of ejaculated sperm (12). Other publications have also shown that ICSI with epididymal or testicular spermatozoon does not lead to more stillbirths or congenital malformations compared with ICSI using ejaculated spermatozoon, and that the procedure is equally as safe as even conventional IVF treatment (13,14). Despite this promising evidence, the search for the ideal normal spermatozoa that would guarantee fertilization, good quality embryo, and viable blastocyst development, and hence successful clinical outcome, with the delivery of the healthy offspring, is still being investigated by novel technologies, applicable in addition to classical morphological evaluation or as a sole method of selection, with the aim of eliminating the utilization of spermatozoon with genetic integrity problems and DNA damage (4).

Fertilization Abnormalities by IVF

Abnormal fertilization by conventional IVF is mainly associated with abnormal polyspermy, due to dispermic oocyte penetration resulting in a tripronucleate (3PN) oocyte. Reasons for the appearance

of 3PN include uncontrolled multiple sperm penetrations, fertilization by a diploid sperm or oocyte, inhibition of second polar body extrusion, and formation of two female pronuclei (or possibly one diploid), together with a single sperm pronucleus (monospermic digyny). The identification and elimination of polysypermic oocytes are critical, as it is known that significantly higher spontaneous abortions are expected from the transfer of embryos derived from triploid zygotes (15). In addition to the dispermy and digyny, the origin of tripronucleate oocytes may also be connected with their chromosomal constitution. New micromanipulation techniques, such as microsurgical removal of a single pronucleus, may provide an opportunity to repair the abnormalities of tripronuclear zygotes, especially for patients with high rate of hyperploidy (16). Escriba et al. (17) demonstrated heteroparental blastocyst production from microsurgically corrected tripronucleated human embryos from conventional IVF, which represents a hope for future studies using micromanipulation for hyperploidic embryos.

Another frequently seen abnormal fertilization type in human oocytes after IVF procedure is the visualization of a single pronucleus at the time of assessment of fertilization, instead of two distinct pronuclei as a sign of both female and male gametes. Despite the fact that earlier studies had shown high diploidy rates for embryos derived from single pronucleus after standard IVF, with 50% of the blastomeres having Y chromosome and healthy deliveries reported after the transfer of embryos derived with mononucleated zygotes (assuming that the second pronucleus can be observed at a later reassessment)—or the fusion of the two pronuclei might appear at static observations by a certain time period (18,19)—recent molecular cytogenetic studies of human single pronucleate oocytes suggest a significantly lower rate of diploid embryos; hence, it is not recommended to use such zygotes in assisted reproduction purposes (20).

Fertilization Abnormalities by ICSI

As only one spermatozoon is injected into a mature oocyte in the ICSI procedure, the formation of the third pronucleus appears as a result of decondensation of the unextruded polar body (21). The failure of the second polar body extrusion might be connected to damage to the metaphase plate region during ICSI, to reorientation of the first meiotic spindle or damage to the oocyte cytoskeleton during sperm injection, to misorientation of the second meiotic spindle, to high estradiol (E2) levels on the day of hCG administration, to the origin of spermatozoa, to utilization of testicular spermatozoon, or to spermatozoon from severe oligoasthenoteratozoospermic patients (15). Typically it is recommended to eliminate embryos derived from tripronuclear zygotes formed after ICSI, as high rates of spontaneous abortions and mosaic embryos are reported (22).

A report in which ten relevant studies for the removal of the third pronucleus were reviewed found the transfer of corrected embryos only once, which resulted in live birth. This study indicated that obstacles associated with the procedure, such as the identification of the supernumerary pronucleus, the presence of two centrosomes in dispermic oocytes, and cytogenetically abnormal patterns after intracytoplasmic sperm injection, are still to be clarified, possibly offering patients with exclusively abnormally or few normally fertilized oocytes the opportunity to benefit from epronucleation to guarantee embryo transfer, or to increase the number of embryos that could be transferred (23).

The percentage of haploid embryos obtained from mononucleated zygotes after ICSI are reported to be higher, and varying percentages from 10% to 30% of embryos have been shown to contain Y chromosomes. It has been suggested that monopronucleated ICSI zygotes may usually be parthenogenetically activated and not fertilized (18). Mateo et al., in contrast, reported that even though the majority of monopronuclear zygotes after ICSI result from fertilization, as all embryos obtained were chromosomally abnormal, a significantly low percentage of embryos developed to good quality blastocysts, suggesting the deselection of embryos derived from monopronuclear zygotes (24). The study by Azavedo et al. also revealed that after the analysis of chromosome status of ICSI embryos derived from monopronucleated zygotes, the diploidy rate is low, and thus such embryos should not be used in assisted reproduction treatments (20).

Examination of Pronuclear Morphology and Predictive Value for Clinical Outcome

One of the most-cited and frequently used scoring schemes to assess the quality and viability of embryos to be transferred, by looking to the pronuclear morphology of normally fertilized zygotes with two distinct pronucleus clarified in the oocyte cytoplasm, was published by Tesarik and Greco (25). The morphological parameters evaluated included number of nucleolar precursor bodies (NPB) and their distribution in each pronucleus, defined as polarized or non-polarized. The distribution of NPB is considered polarized when all NPB present in a pronucleus were present in the pronuclear hemisphere whose pole was the point of contact with the other pronucleus, and it is considered non-polarized when at least one NPB is found in the opposite hemisphere. The relative size of both pronuclei (equal or unequal) and their position with regard to one another (in apposition versus at distance) were also noted. Five patterns based on these parameters were defined in the pronuclear grading scheme of Tesarik and Greco. The developmental fate analysis of zygotes with different patterns of pronuclear stage morphology revealed that these patterns can significantly predict the percentage of arrested embryos, embryos with multinucleated blastomeres (MNB), and good quality embryos. Pattern 0, which are zygotes giving rise to embryos transferred in 100% implantation cycles, are defined as the ideal zygotes with the number of NPB not showing big differences between both pronuclei, never differing by more than three, NPB being always polarized when they are fewer than seven and never polarized when they are more than seven in a pronucleus, the number of NPB in a pronucleus never fewer than three, and the distribution of NPB being either polarized or non-polarized in both pronulei, but never polarized in one pronucleus and non-polarized in the other. Pattern 1 represented zygotes which had a big difference (>3) in the number of NPB; Pattern 2 small number (<7) of NPB without polarization in at least one of the pronucleus; Pattern 3 large number (>7) of NPB with polarization in at least one pronucleus; Pattern 4 very small number (<3) of NPB in at least one pronucleus; and Pattern 5 represents zygotes with polarized distribution of NPB in one pronucleus and non-polarized in the other. The proportion of arrested embryos developing from Pattern 0 zygotes was markedly reduced compared to any other pattern, and developmental arrest was mostly seen with embryos developing from Patterns 1 and 3. Patterns 1 and 5 developed multinucleated blastomeres almost twice as frequently as Pattern 0. The development into good-morphology embryos was significantly more frequent for Pattern 0 zygotes as compared to Pattern 1 and 2. When two or more pronuclear abnormalities were seen in a single zygote, the incidence of developmental arrest and multinucleated blastomeres were increased. In a follow-up study they examined the clinical validation of their grading scheme and found in 380 fresh embryo transfer cycles that the clinical pregnancy and implantation rates were significantly higher for the group of patients who received embryos originated from Pattern 0 zygotes, and that the transfer of only one Pattern 0 embryo was sufficient for the optimal chance of pregnancy, whereas transfer of two Pattern 0 embryos mostly resulted in twin pregnancy (13). In correlation with the results of the study by Tesarik et al. (26), Balaban et al. (27) demonstrated that embryos showing an ideal pronuclear Pattern 0 cleaved earlier and faster and resulted in better quality cleavage stage embryos and blastocysts. The incidence of blastocyst formation was very high; 72% in zygotes showing Pattern 0, compared with 12.7% in zygotes with double abnormalities assessed by the Tesarik and Greco grading scheme. In addition to better embryo quality and blastocyst formation, higher implantation and pregnancy rates were obtained when at least one blastocyst derived from Pattern 0 zygote was included in the set of embryos transferred. Balaban and Tesarik et al. (28) also showed that both embryo cleavage characteristics (such as cleavage rate, embryo quality, and blastocyst formation) and chromosome constitution of the embryo were related with pronuclear morphology. Embryos developing from zygotes with the normal PN Pattern 0 cleaved faster and formed embryos with better morphology as compared with zygotes with abnormal pronuclear patterns. Aneoploidy rate of embryos derived from zygotes with the normal pronuclear pattern was significantly lower when compared with embryos derived from single or double pronuclear abnormality. Chromosomally normal embryos with the normal Pattern 0 progressed to the blastocyst stage at a significantly higher rate than chromosomally normal embryos with single or double pronuclear abnormality. The same relationship applied to chromosomally abnormal embryos (Figure 12.8).

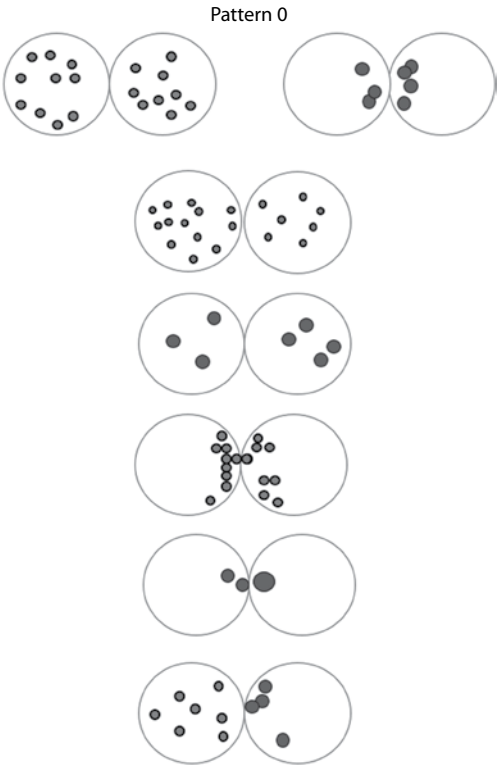


FIGURE 12.8 Schematic presentation of pronuclear morphology corresponding to individual patterns. Pattern 0 corresponds to normal zygotes whereas all the others are asymmetrical scores. (Adapted from Tesarik J, Greco E. *Hum Reprod*. 1999;14:1318–23.)

An alternative grading scheme that has also been widely cited and utilized is the one published by Scott and Smith (29) which is based on empirical observations correlated with pregnancy. The basis of this grading scheme is a combination of pronuclear size, nucleoli number and distribution, and cytoplasmic appearance, which prospectively showed an increase on the incidence of implantation when utilized for day one embryo transfers and to select embryos for cryopreservation. As a continuum of this study, Scott et al. (30) published a revised pronuclear grading scheme with the aim of better predictivity and easy and fast use for laboratory staff. The new grading scheme examined the cleavage stage and blastocyst stage embryo characteristics as well as their implantation potential in correlation with zygote morphology. The revised grading system was named the Z scoring scheme, where the zygotes were scored from Z1 to Z4, Z1 and Z2 representing the ideal zygotes that would give rise to implanting embryos. Similar to the previous scheme, the grading took into account the nuclear size and alignment and nucleoli number and distribution. The nucleoli needed to be aligned, or be beginning to align, at the pronuclear junction, between three and seven per nucleus, with no more than one nucleolus difference between the nuclei, and equal in size. Although the size of the nucleoli was not measured, zygotes with either very small pinpoint nucleoli or very large ones were designated as Z3. The study showed that embryos derived from Z1 and Z2 scored zygotes have a higher rate of blastocyst formation. The pregnancy and implantation of embryos derived from Z1 and Z2 scored zygotes were also significantly higher (Figure 12.9).

Consistent with the earlier hypothesis (31) indicating that nucleolar patterns are closely linked to embryo viability, thereby reinforcing the relevance of polarity in human oocytes and embryos, it appears that pronuclear scoring can successfully be incorporated into the practice of embryo evaluation for prediction of implantation potential and embryo normality of the individual embryo (32). A systematic review of 40 studies examining the effectiveness of zygote morphology evaluation in fresh IVF and ICSI cycles revealed that the majority of the evidence-based clinical trials supported the utilization of

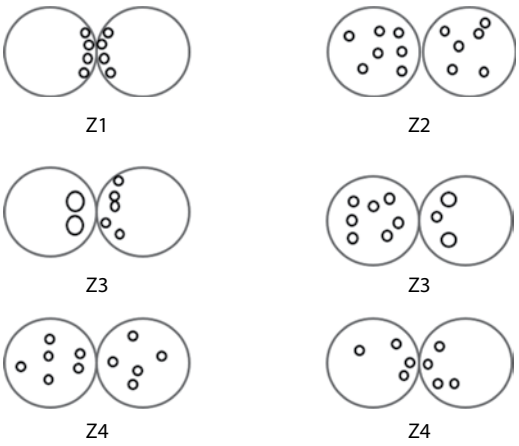


FIGURE 12.9 Diagrammatic representation of Z scoring. Z1 and Z2 represents normal zygotes whereas all the other arrangements are considered asymmetrical. (Data from Scott L et al. *Hum Reprod*. 2000;15:2394–403.)

the grading schemes by Tesarik and Greco or the Scott Z scoring system, with minor modifications (33). This review also demonstrated that zygote morphology correlated significantly with embryo quality and cleavage, blastocyst stage, and embryonic chromosome status, in a high proportion of the studies which assessed the specific outcome.

Timing of assessment of pronuclear scoring is of major importance as the pronuclear formation is a dynamic event that will dissappear by the beginning of the cleavage events of the embryo. The systematic review showed that various timings, differing from 14th to 18th hour post-insemination, were being used in clinical trials for IVF and/or ICSI cases. Taking into consideration the dynamic development of a human embryo, the heterogeneous results reported might be correlated to these timing differences of examination of pronuclear sizes, as well as polarization dynamics of the NPBs. In the time course leading to the initiation of pronuclear formation, zygotes arising from IVF are observed to be approximately an hour behind those arising from ICSI, and thus the widely-used and recommended timing based on earlier studies for 16th–18th hour of assessment may not be appropriate, not only for the observation of visualization of normal fertilization with two distinct pronuclei and two polar bodies, but also for the examination of pronuclear morphology. The Istanbul consensus workshop proceedings, published by Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, suggested that the fertilization as well as pronuclear morphological characteristics of a zygote should be assessed at the 17th hour, with minus or plus an hour maximum flexibility (5). A simplified consensus grading scheme for pronuclear scoring was also suggested in the consensus paper. According to this grading system, three categories were defined, by which Category 1 represent symmetrical zygotes that are equivalent to Z1 and Z2 as described by Scott and Smith, Category 2 represent non-symmetrical zygotes with all other arrangements of pronuclear appearances, and Category 3 represent abnormal zygotes with 0 or 1 NPB within the pronuclei (Figures 12.10 and 12.11a,b).

Consensus scoring system for pronuclei

Category	Rating	Description
1	Symmetrical	Equivalent to Z1 and Z2
2	Non-symmetrical	Other arrangements, including peripherally sited pronuclei
3	Abnormal	Pronuclei with 0 or 1 NPB

FIGURE 12.10 Alpha and ESHRE Special Interest Group of Embryology Consensus Grading Scheme for pronuclear evaluation modified by Scott L et al. (Data from Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology. *Hum Reprod*. 2011;26:1270–83; Scott L et al. *Hum Reprod*. 2000;15:2394–403.)

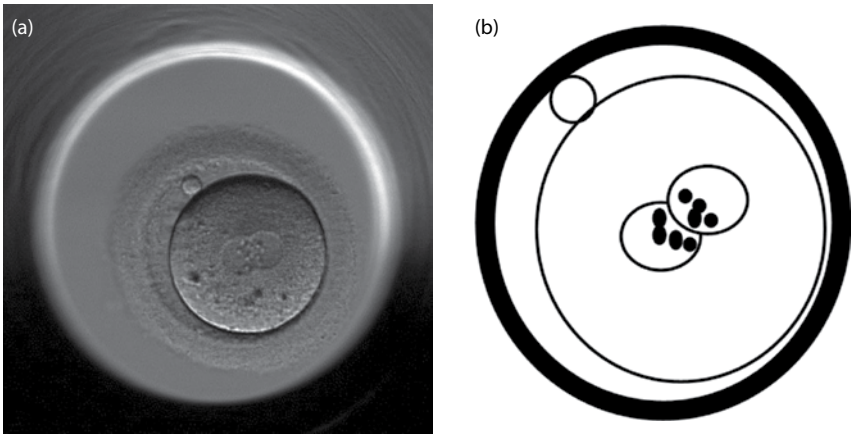


FIGURE 12.11 (a,b) Category 1 pronuclear score – according to Alpha and ESHRE Special Interest Group of Embryology Consensus Grading Scheme (Data from Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology. *Hum Reprod*. 2011;26:1270–83.)

Dynamics of Fertilization, Pronuclear Development, and Morphology

One of the major drawbacks of morphological evaluations and grading schemes of embryos, starting from the zygote level to the blastocyst stage, is that the assessment is performed at static developmental time points by microscopy. As the human embryo is a highly dynamic and complex entity, the static evaluation of such a cell will always have limitations for viability and implantation competency assessments, by missing some of the important cleavage events that could be visualized by continuous monitoring. Time-lapse incubation systems have allowed the predictive value and objectivity of morphological grading schemes to be revisited, and opened a debate for the necessity of a revised scoring system, emphasizing the need for a new look at embryological parameters.

The study by Montag et al. (34) analyzed pronuclear morphology based on time-lapse imaging for the distribution of NPB at 14th–15th, 16th–18th, and 19th–20th hours, and found out that at 14th–15th hours to 16th–18th hours similar pronucleus scorings were present in 75% zygotes, whereas 25% showed change in the pattern, which resulted in another pronuclear score. The change in the pronuclear score from 16th–18th hours to 19th–21st hours was 33.8%, whereas 66.2% maintained similar pronuclear scores. The documented changes were mostly from an asymmetric distribution of NPB towards a symmetric or perfectly aligned distribution. The change from a symmetric to an asymmetric pattern was less common. These dynamic changes reported in the study can perhaps explain the contradictory situation in the literature where static observations for zygote morphology were reported. The prospective study by Azzarello et al. (35) was performed on 159 embryos, all of which were transferred. The pronuclear morphology of the 46 embryos which resulted in live birth was compared with that of 113 embryos which resulted in no live birth. Pronuclear morphological assessment was performed on day of embryo transfer, using six different scoring systems at different times. The study showed that no embryo with pronuclear breakdown earlier than 2 hours 45 minutes resulted in live birth, all six pronuclear assessment models showed no significant distribution of scores between the live birth and no live birth groups at the 16th and 18th hour post-fertilization and 40 minutes before pronuclear breakdown. The outcomes of assessments changed significantly over time, and the time of pronuclear breakdown was found to be the optimal stage to evaluate pronuclear morphology. In agreement with published works, this study also demonstrated that pronuclear morphology changes over time, showing that the single static observation is lacking in comparison to dynamic continuous observation. Even though the examination of the pronuclear morphology did not improve the embryo selection, the timing of pronuclear breakdown was suggested to be a more objective criterion for prediction of live birth. The study by Aguilar et al. (36) compared the timings of early fertilization events by time-lapse imaging, namely the second polar body extrusion, first and second pronuclei

appearance, pronuclear syngamy (known as abuttal), pronuclear movements within the cytoplasm, pronuclear morphology, pronuclear symmetry, pronuclear fading and the length of S-phase (defined as the time from pronuclear appearance to pronuclear fading) in implanted versus nonimplanted embryos in a two year cohort study. The timings at which second polar body extrusion (3.3–10.6 hours), pronuclear fading (22–25.9 hours) and length of S-phase (5.7–13.8 hours) occurred were linked successfully to embryo implantation, and all the other parameters defined were unrelated with embryo implantation competency.

Consequently, studies on the continuous observation of early fertilization events offer a new opportunity to revisit the changes in fertilization phenomenon and show the necessity of a closer examination of the dynamics of pronuclear morphological changes.

Conclusion

A successful fertilization process is a complex and highly dynamic biological phenomenon that includes several events which are closely correlated with the developmental capability and viability of the resulting embryo. The events, in summary, are: the exclusion of the second polar body; the appearance and fading of the pronuclei; and pronuclear syngamy, which all happen before or after the conventional first embryo observation performed at 16–22 h post insemination. Continuous observation of embryo development by time-lapse technology will not only provide valuable information for cleavage or blastocyst stage characteristics of the embryo, but also may detect some critical abnormalities that start at the very early stage of the fertilization process. Despite the fact that the majority of clinical time-lapse studies identify information on the cleaved embryo, more future trials will guide us through the details of the human first cell cycle, which may introduce alternative objective parameters for the selection of embryos with higher implantation probabilities that will lead us to a successful clinical outcome.

REFERENCES

1. Mortimer ST. A critical review of the physiological importance and analysis of sperm movement in mammals. *Hum Reprod Update*. 1997;3:403–40.
2. Tesarik J. Comparison of acrosome reaction-inducing activities of human cumulus oophorus, follicular fluid, and ionophore A23187 in human sperm population of proven fertilizing ability in vitro. *J Reprod Fertil*. 1985;74:383.
3. Kupker W, Diedrich K, Edwards RG. Principles of mammalian fertilization. *Hum Reprod*. 1998;13(Suppl 1):20–32.
4. Sakkas D. Novel technologies for selecting the best sperm for in vitro fertilization and intracytoplasmic sperm injection. *Review. Fertil Steril*. 2013;99(4):1023–9.
5. Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology. The Istanbul consensus worskhop on embryo assessment: Proceedings of an expert meeting. *Hum Reprod*. 2011;26:1270–83.
6. Rienzi L, Balaban B, Ebner T, Mandelbaum J. The oocyte. *Hum Reprod*. 2012;27:2–21.
7. Balaban B, Urman B. Effect of oocyte morphology on embryo development and implantation. *Reprod Biomed Online*. 2006;12:608–15.
8. Ebner T. Is oocyte morphology prognostic of embryo development potential after ICSI? *Reprod Biomed Online*. 2016;12:507–12.
9. Balaban B, Ebner T. Morphological selection of gametes and embryos: Oocyte. In: *A Practical Guide to Selectiong Gametes & Embryos*, Montag M (ed.), Boca Raton, London, New York: CRC Press, Taylor & Francis Group, 2014, 81–96.
10. Bonduelle M, Liebaers I, Deketelaere V, Derde MP, Camus M, Devroey P, Van Steirteghem A. Neonatal data on a cohort of 2889 infants born after ICSI (1991–1999) and of 2995 infants born after IVF (1983–1999). *Hum Reprod*. 2002;17:671–94.
11. Hourvitz A, Pri-Paz S, Dor J, Seidman DS. Neonatal and obstetric outcome of pregnancies conceived by ICSI or IVF. *Reprod Biomed Online*. 2005;11:469–75.
12. Belva F, De Schrijver F, Tournaye H, Liabaers I, Devroey P, Haentjens P, Bonduelle M. Neonatal outcome of 724 children born after ICSI using non-ejaculated sperm. *Hum Reprod*. 2011;26:1752–8.

13. Fedder J, Loft A, Parner ET, Rasmussen S, Pinborg A. Neonatal outcome and congenital malformations in children born after ICSI with testicular or epididymal sperm: A controlled national cohort study. *Hum Reprod*. 2013;28:230–40.
14. Guo YH, Dong RN, Su YC, Li J, Zhang YJ, Sun YP. Follow-up of children born after intracytoplasmic sperm injection with epididymal and testicular spermatozoa. *Chin Med J*. 2013;126:2129–33.
15. Feng H, Hershlag A. Fertilization abnormalities following human in vitro fertilization and Intracytoplasmic sperm injection. *Microscopy Res Tech*. 2003;61:358–61.
16. Ivakhnenko V, Gieslak J, Verlinsky Y. A microsurgical technique for anucleation of multipronuclear human zygotes. *Hum Reprod*. 2000;15:911–6.
17. Escriba MJ, Martin J, Rubio C, Valbuena D, Remohi J, Pellicer A, Simon C. Heteroparental blastocyst production from microsurgically corrected tripronucleated human embryos. *Fertil Steril*. 2006;86:1601–7.
18. Palermo GD, Munne S, Cohen J, Rozenwaks Z. Genetics of abnormal human fertilization. *Hum Reprod*. 1995;10(Suppl):120–7.
19. Staessen C, Janssenswillen C, Devroey P, VanSteirteghem A. Cytogenetic and morphological observation of single pronucleated human oocytes after in-vitro fertilization. *Hum Reprod*. 1993;8:221–3.
20. Azevedo AR, Pinho MJ, Silva J, Sa R, Thorsteinsdottir S, Barros A, Sousa M. Molecular cytogenetics of human single pronucleated zygotes. *Reprod Sci*. 2014;21:1472–82.
21. Andrea R, Sachs BA, Ginsburg ES. Factors associated with the formation of triploid zygotes after intracytoplasmic sperm injection. *Fertil Steril*. 2000;73:1109–14.
22. Ma S, Kalousek DK, Yuen BH, Moon YS. The chromosome pattern of embryos derived from tripronuclear zygotes studied by cytogenetic analysis and fluorescense in situ hybridization. *Fertil Steril*. 1995;63:1246–50.
23. Rosenbusch BE. Selective microsurgical removal of a pronucleus from tripronuclear human oocytes to restore diploidy: Disgarded but valuable? *Fertil Steril*. 2009;92:897–903.
24. Mateo S, Parriego M, Boada M, Vidal F, Coroleu B, Veiga A. In vitro development and chromosome constitution of embryos derived from monopronucleated zygotes after intracytoplasmic sperm injection. *Fertil Steril*. 2013;99:897–902.
25. Tesarik J, Greco E. The probability of abnormal preimplantation development can be predicted by a single static observation on pronuclear stage morphology. *Hum Reprod*. 1999;14:1318–23.
26. Tesarik J, Junca AM, Hazout A, Aubriot FX, Nathan C, Bacrie PC, Hassan MD. Embryos with high implantation potential after intracytoplasmic sperm injection can be recognized by a simple, non-invasive examination of pronuclear morphology. *Hum Reprod*. 2000;15:1396–9.
27. Balaban B, Urman B, Isiklar A, Alatas C, Aksoy S, Mercan R, Mumcu A, Nuhoglu A. The effect of pronuclear morphology on embryo quality parameters and blastocyst transfer outcome. *Hum Reprod*. 2001;16:2357–61.
28. Balaban B, Yakin K, Urman B, Isiklar A, Tesarik J. Pronuclear morphology predicts embryo development and chromosome constitution. *Reprod Biomed Online*. 2004;8:695–700.
29. Scott LA, Smith S. The successful use of pronuclear embryo transfers the day after oocytes retrieval. *Hum Reprod*. 1998;13:1003–13.
30. Scott L, Alvero R, Leondires M, Miller B. The morphology of human pronuclear embryos is positively related to blastocyst development and implantation. *Hum Reprod*. 2000;15:2394–403.
31. Edwards R, Beard H. Oocyte polarity and cell determination in early mammalian embryos. *Mol Hum Reprod*. 1997;3:863–906.
32. Balaban B, Urman B. Embryo culture as a diagnostic tool. *Review. Reprod Biomed. Online* 2003;7:671–82.
33. Nicoli A, Palomba S, Capodanno F, Fini M, Falbo A, La Sala GB. Pronuclear morphology evaluation for fresh in vitro fertilization(IVF) and intracytoplasmic sperm injection (ICSI) cycles: A systematic review. *J Ovarian Res*. 2013;6:64.
34. Montag M, Liebenthron J, Koster M. Which morphological scoring system is relevant in human embryo development? *Placenta*. 2011;32(Suppl 3):252–6.
35. Azzarello A, Hoest T, Mikkelsen AL. The impact of pronuclei morphology and dynamicity in live birth outcome after time-lapse culture. *Hum Reprod*. 2012;27:2649–57.
36. Aguilar J, Motato Y, Escriba MJ, Ojeda M, Munoz E, Meseguer M. The human first cell cycle: Impact on implantation. *Reprod Biomed Online*. 2014;28:475–84.

13

Human Embryo Development and Assessment of Viability

David K. Gardner and Markus Montag

Introduction

The exquisite structures that comprise the stages of the pre-implantation period represent one of the most dynamic periods of human development. During the first five days of life, the single-celled fertilized oocyte undergoes remarkable changes, not only in its appearance, but also in its underlying genetic control and physiology. The pronucleate oocyte, like the MII oocyte itself, has a low metabolic rate, consuming limited oxygen, and possesses little capacity to utilize glucose. This low oxidative state, and hence reduction in the generation of reactive oxygen species, represents a means to minimize damage to intracellular structure and function, and to maintain the integrity of the chromosomal DNA (a particularly useful strategy if you are an oocyte and need to reside in the ovary for up to 40 years). For the first two days of development the embryo is predominantly under the control of maternally-derived stable mRNAs and proteins synthesized during oocyte maturation. During this time there is limited biosynthesis, indeed the entire pre-implantation period is not associated with net growth per se, rather it is focused on early differentiation. After the 8-cell stage the embryo undergoes one of the most significant, and yet often most overlooked, developmental processes, the formation of the first transporting epithelium of the conceptus at compaction. The energetically expensive process of blastocoel formation ensues, together with spatial cell allocation and an exponential increase in mitoses. Concomitantly, there is a surge in the requirements for both energy and for biosynthetic precursors, both of which are met by an increase in glucose and oxygen consumption (although there is more to this story, as shall be discussed in detail below). The resultant blastocyst, which forms just four days after fertilization, is metabolically as distinct from the pronucleate oocyte as any two tissues could be, and represents one of the most active, and metabolically unique types of cell in the body.

In order for a pronucleate oocyte to develop into a competent blastocyst, which will then give rise to a healthy baby, the molecular and biochemical transitions eluded to above need to be seamless. Deviations away from the set plan, inducing altered patterns of either metabolism or gene expression, are associated with loss of viability. The ability of the pronucleate oocyte to develop successfully therefore depends upon many factors, some of which are beyond the control of the IVF clinic, including patient etiology, genetics, diet, smoking, and lifestyle. All of these factors can have a significant impact on IVF outcome, and furthermore these factors can be compounding. However, there are certainly factors that we can take control of from a treatment perspective, including patient stimulation, quality control/assurance/management systems, and appropriate embryo culture system, all of which have been covered extensively elsewhere in this book.

Providing that the clinical and laboratory systems have been established appropriately to ensure the successful development of embryos, how do we assess the developmental competence of the fertilized oocytes created through in-vitro fertilization (IVF)/intracytoplasmic sperm injection (ICSI)? Given the documented negative outcomes associated with the establishment of a multiple pregnancy, the goal of human assisted reproductive technology (ART) has become increasingly focused on the delivery of a single healthy baby following the transfer of a single embryo. Consequently, there is an urgent need to be able to quantitate the viability of a given embryo within a cohort. In order to make more informed decisions regarding the fate of each embryo, here we present background on human embryo development and physiology. In particular, the

relative merits of using embryo morphology, morphokinetics, and metabolism for selection/de-selection are presented. Through a clearer understanding of such parameters we can make appropriate decisions regarding the assessment of embryo viability and hence the ranking of embryos for transfer and cryopreservation.

Quantification of Embryo Viability: Morphology

The developmental profile of a viable human embryo adheres to a specific time frame. Should an embryo develop too slowly, or indeed too quickly, then questions are raised regarding its developmental potential, and even its karyotype. Prior to the development of time-lapse microscopy, analysis of embryo morphology was limited to discrete time-points on each day. Key time points from such an approach have been detailed (Table 13.1). Although such stochastic assessments cannot capture all events during development, if embryos are examined at specific times then key morphological features can be quantitated. The significance of each parameter during the pre-implantation period to pregnancy outcome has been thoroughly reviewed by Gardner and Balaban (1), and the key features associated with either high or low viability are described in Figures 13.1 and 13.2.

TABLE 13.1

Key Morphological Features of the Human Embryo during Pre-Implantation Development

The Fertilized Embryo Is Examined for	Score
18–19 h post insemination/ICSI	
1. Equal size and symmetry of PN	10
2. Alignment between the PN and polar bodies	5
3. Lack of heterogeneity and granularity in cytoplasm	5
4. Presence of PN with both polarized or both not-polarized NPB	10
5. A difference of less than 3 in the number of NPB in the PN	10
6. Polar bodies are not displaced from each other	10
25–26 h post insemination/ICSI	
1. Embryos that have already cleaved to form a 2-cell embryo with even blastomeres and no fragments	15
2. Zygotes that have progressed to nuclear membrane breakdown	5
42–44 h post insemination/ICSI	
1. Number of blastomeres should be greater or equal to 4	10
2. Fragmentation of less than 20%	10
3. No multinucleated blastomeres	5
66–68 h post insemination/ICSI	
1. Number of blastomeres should be greater or equal to 8	10
2. Fragmentation of less than 20%	10
3. No multinucleated blastomeres	5
94–96 h post insemination/ICSI	
1. Compaction	10
2. Signs of blastocoel formation	15
106–108 h post insemination/ICSI	
1. Full blastocoel cavity	10
2. Inner cell mass with tightly packed numerous cells	10
3. Trophectoderm with many cells forming epithelium	15

Source: Modified from Gardner DK, Sakkas D. *Placenta*. 2003;24(Suppl B):S5–12.

Note: A multiple step scoring system that encompasses several criteria would allow the allocation of a score to those embryos that attain a defined hurdle at each step of assessment. The scoring would therefore be based on positive points at each step. Embryos that do not show the required pattern of development at the specific time would not score. The maximum score for a perfect embryo over 5 days would be 180.

Ideal features shared by pronucleate oocytes with high viability:

(i) Number of nucleolar precursor bodies (NPB) in both pronuclei never differed by more than 3
(ii) NPB are always polarized or not-polarized in both pronuclei but never polarized in one pronucleus and not in the other
(iii) Angle β from the axis of the pronuclei and the furthest polar body is less than 50°

Ideal features shared by 2-cell embryos with high viability:

(i) Mononucleated blastomeres
(ii) Equal cell size
(iii) <20% fragmentation

Ideal features shared by 4-cell embryos with high viability:

(i) Mononucleated blastomeres
(ii) Equal cell size
(iii) <20% fragmentation

Ideal features shared by day 3 embryos with high viability:

(i) Mononucleated blastomeres
(ii) Equal cell size
(iii) <20% fragmentation
(iv) At least 4 blastomeres

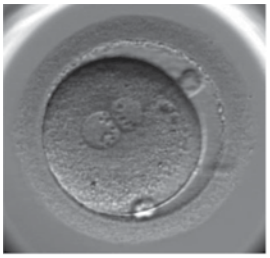
Ideal features shared by morulae with high viability:

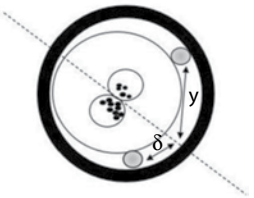
(i) Visibly compacted cells denoted by the slight reduction in overall size of the embryo and increase in space between the embryo and zona pellucida
(ii) Lack of fragments

Ideal features shared by blastocysts with high viability:

(i) Expanded blastocoel cavity by day 5
(ii) Well formed ICM clearly composed of many cells
(iii) Cohesive epithelium made up from many cells in the TE
(iv) Signs of the zona pellucida thinning

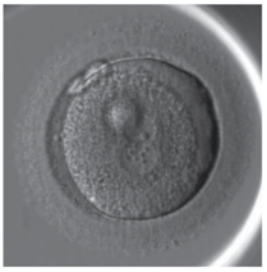
FIGURE 13.1 Key morphological features of embryos with high viability. ICM: inner cell mass, TE: trophectoderm. (From Gardner DK, Balaban B. *Mol Hum Reprod*. 2016;22(10):704–18, with permission.)

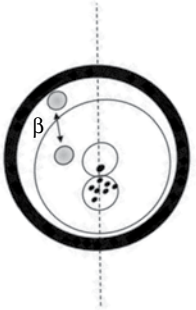




Suboptimal features shared by pronucleate oocytes with medium/low viability:

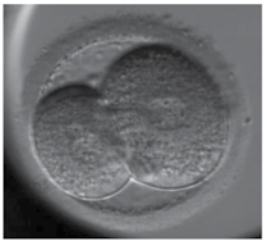
(i) Number of nucleolar precursor bodies (NPB) differ by more than 3
(ii) Angle β from the axis of the pronuclei and the furthest polar body is more than 50˚

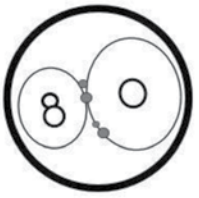




Suboptimal features shared by pronucleate oocytes with medium/low viability:

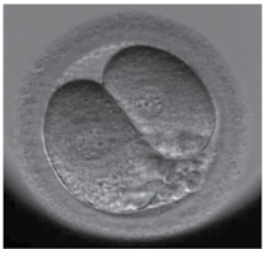
(i) Number of nucleolar precursor bodies (NPB) differ by more than 3
(ii) Distribution of NPB being polarized in one pronuclei, whereas it's non-polarized in the other





Suboptimal features shared by 2-cell embryos with medium/low viability:

(i) Multinucleated blastomeres
(ii) Unequal cell size

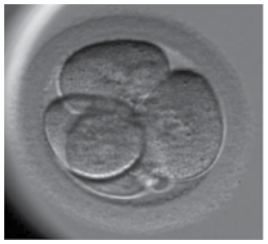


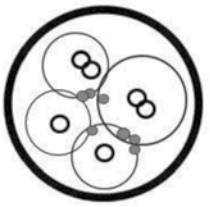
Suboptimal features shared by 2-cell embryos with medium/low viability:

(i) Unequal cell size
(ii) 20% fragmentation

FIGURE 13.2 Key morphological features of embryos with medium to low viability. Although it is possible for embryos with medium to low viability to share some of the features of those embryos with high viability (such as polarized nuclei at the pronucleate oocyte stage, mononucleate blastomere with <20% fragmentation during the cleavage stages, and even an expanded blastocoel cavity on day 5 of development), other aspects of their morphology do not align with embryos of higher viability. For example, in the pronucleate oocyte, although both pronuclei can be aligned, developmental capacity is deemed reduced due to other characteristics being altered, such as differences in the number of nucleolar precursor bodies differing by more than 3, or the angle ß from the axis of the pronuclei and the furthest polar body being greater than 50°. Similarly, in the cleavage stages, although the blastomeres may have <20% fragmentation, they may exhibit other characterisitics associated with reduced viability, such as multinucleation and/or unequal size. Finally, in the blastocyst, although the blastocoel is expanded, the ICM and TE may be poorly developed, reflecting compromised implantation potential.

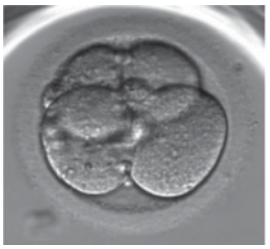
(Continued)





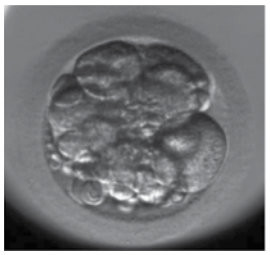
Suboptimal features shared by 4-cell embryos with medium/low viability:

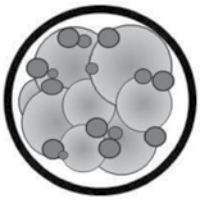
(i) Multinucleated blastomeres
(ii) Unequal cell size



Suboptimal features shared by 4-cell embryos with medium/low viability:

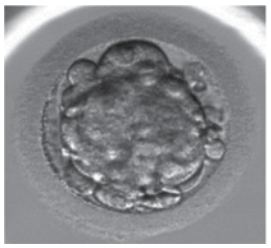
(i) Unequal cell size





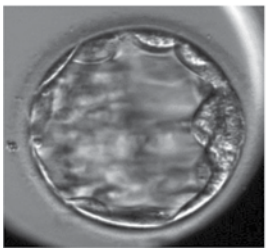
Suboptimal features shared by day 3 embryos with medium/low viability:

(i) Unequal cell size
(ii) 35% fragmentation



Suboptimal features shared by morulae with medium/low viability:

(i) Non-participation of all cells in compaction
(ii) 20% fragmentation



Suboptimal features shared by blastocysts with medium/low viability:

(i) Loosely formed ICM composed of few cells
(ii) Loosely formed epithelium made up from few cells in the TE

FIGURE 13.2 (Continued) For the embryos shown, this information could be retrieved by analysis of a time-lapse sequence. However, without time-lapse imaging, it is not possible to perform a reliable evaluation of the nuclear state of the 2-cell or 4-cell stage, and hence occurrence of multinucleation may remain undetected in the majority of embryos. (From Gardner DK, Balaban B. *Mol Hum Reprod*. 2016;22(10):704–18, with permission.)

With the advent of more physiological culture systems, capable of maintaining the human embryo to the blastocyst stage (2), there has been a growing trend to move to day 5/6 transfers in order to increase implantation and live birth rates (3,4). Assessment of morphology at the blastocyst stage has the advantage of being able to assess the development of the two cell types within the embryo at this stage; the inner cell mass (ICM) and trophectoderm (TE). Gardner and Schoolcraft developed an alphanumeric system to establish which parameters were associated with implantation and pregnancy (5), thereby facilitating the routine transfer of a single blastocyst (6). This grading scheme is shown in Figure 13.3. Blastocysts exhibiting an AA phenotype were initially associated with the highest implantation potential (6,7)

Follow up studies reported that the ICM grade was more important than TE grade in predicting pregnancy outcome (8–10). However, Ahlstrom and colleagues subsequently stated that although blastocyst expansion and ICM grade were significant predictors, they were not predictors of live birth when analyzed by stepwise logistic regression (11). Rather, it appeared that the TE was the most important determinant of successful transfer outcome, and that a 4BA blastocyst had a statistically greater chance of forming a live newborn compared to a 4AB blastocyst. Further, a 3BA blastocyst was more likely to go to term than a 4AB. These findings have been repeated in the study of Hill and colleagues, who found that live birth rates were 57%, 40%, and 25% for the TE grades of A, B, and C, respectively (12). An analysis of over 3000 cycles by Thompson et al. further revealed that TE grade and degree of expansion were the parameters most strongly associated with pregnancy and live birth rate, observations also reported by Ebner and colleagues (13,14).

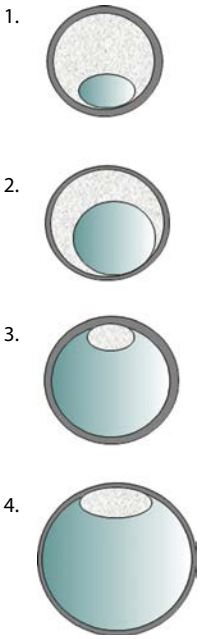


FIGURE 13.3 Scoring system for human blastocysts. Initially blastocysts are given a numerical score from 1 to 6 based upon their degree of expansion and hatching status: (1) Early blastocyst: the blastocoel being less than half the volume of the embryo; (2) Blastocyst: the blastocoel being greater than or equal to half of the volume of the embryo; (3) Full blastocyst: the blastocoel completely fills the embryo; (4) Expanded blastocyst: the blastocoel volume is now larger than that of the early embryo and the zona is thinning; (5) Hatching blastocyst: the trophectoderm has started to herniate through the zona; (6) Hatched blastocyst: the blastocyst has completely escaped from the zona. The initial phase of the assessment can be performed on a dissection microscope. The second step in scoring the blastocysts should be performed on an inverted microscope. For blastocysts graded as 3 to 6 (i.e. full blastocysts onwards) the development of the inner cell mass (ICM) and trophectoderm can then be assessed: ICM Grading—(A) Tightly packed, many cells; (B) Loosely grouped, several cells; (C) Very few cells. Trophectoderm Grading—(A) Many cells forming a tightly knit epithelium; (B) Few cells; (C) Very few cells forming a loose epithelium. (From Gardner DK, Schoolcraft WB. In vitro culture of human blastocyst In: *Towards Reproductive Certainty: Fertility and Genetics Beyond 1999*, Jansen R, Mortimer D (Eds). Carnforth, UK: Parthenon Publishing, 1999, 378–88, with permission.)

What is the physiology underlying the significance of the TE as the most important parameter, given that one could consider the ICM grade to have the greatest impact on transfer outcome as it contains progenitors of the fetus itself? The answer to this intriguing situation may be explained by the significance of the TE during the initiation and progression of implantation, and that a healthy TE ensures appropriate implantation is achieved. A higher number of TE cells will ensure greater signaling and interaction with the endometrium. The TE produces human chorionic gonadotrophin (hCG) as one of the earliest signals to the mother (15). However, it has recently been proposed that lactate production by the blastocyst, derived predominantly from the TE, has several physiological functions in regulating the implantation process (16), which gives further credence to the importance of a good TE grade in predicting implantation potential. So is the ICM grade of relevance? Van den Abbeel et al. have reported that all three parameters of the blastocyst (degree of expansion, ICM, and TE quality) were significantly associated with pregnancy and live birth rates (17). Of greater interest they also reported that transfer of blastocysts with an "A" grade ICM reduced the incidence of pregnancy loss (17). Furthermore, it has since been reported that ICM grade is positively associated with birth weight (18). Importantly, from a physiological perspective, it must be appreciated that the TE and ICM do not exist in isolation, but rather co-exist as a functional unit. While it is the TE which creates a unique environment for the ICM by the synthesis of blastocoel fluid (characterized by a higher lactate environment than the surrounding culture medium) (19), it is the ICM itself that regulates the proliferation and activity of the TE in the mouse blastocyst (20–22). Consequently, it would appear prudent to keep grading both the ICM and TE and to use both parameters in decisions regarding the fate of an embryo.

With the successful introduction of commercially available time-lapse systems, it is now feasible to analyze the entire pre-implantation period as a continuum. This has not only facilitated the precise determination of each cleavage event, but has revealed hitherto uncharacterized events such as direct cleavage and reverse cleavage. Furthermore, it has shed new light on the incidence and dynamics of multinucleation. Consequently, we have entered a new era of quantitative algorithms to model the kinetics of human embryo development with either development in vitro or outcome following transfer.

Quantification of Embryo Viability: Morphokinetics

Morphokinetic Analysis of Human Embryo Development and the Creation of Algorithms for Embryo De-Selection

From the beginnings of clinical IVF, assessment of human embryo morphology was considered the best means to characterize embryo viability. Morphological assessment is based on defined criteria for a given day of development, and covers both cellular stage and quality. In order to enable an objective classification scheme, Scientists in Reproductive Medicine (ALPHA) and European Society for Human Reproduction and Embryology (ESHRE) have published a consensus paper that assures aligned nomenclature and defines the time of observation (23,24).

With the introduction of time-lapse systems, embryos can be imaged every few minutes without the need to remove them from the incubation chamber. In contrast, standard embryo assessment requires the removal of the culture dish from the incubator at specific time-points. Inevitably this disturbs continuous culture and at the same time exposes embryos to light both during handling and while viewing at the microscope stage (25,26). These drawbacks are clearly avoided using the continuous observation possibilities of time-lapse imaging, which allows linking every morphological event to the specific time when it occurred. This is now referred to as morphokinetics. As a result, the scheme described in this paper can be considered an algorithm that helps to differentiate embryos according to their viability.

Morphokinetic Variables by Time-Lapse Imaging

Morphokinetic analysis of human embryos enables every aspect of embryo development, starting as early as extrusion of the second polar body and continuing up to the time of hatching at the blastocyst stage, to be assigned a precise time (27). Of note, time-lapse imaging was able to detect some morphokinetic events, which are clearly linked to embryo viability, but were previously unknown due to the

limitations imposed by standard embryo assessment at fixed time points. One such event is reverse cleavage, and refers to embryos that undergo either complete or partial division of one blastomere into two daughter cells that subsequently fuse back to continue as one blastomere. One study has reported 0% implantation rate for embryos that were transferred after reverse cleavage (28), which makes reverse cleavage a highly accurate morphokinetic de-selection criteria for non-viable embryos. Another example is direct cleavage, which characterizes embryos in which one blastomere immediately divides into more than two daughter cells. Direct cleavage can occur at any of the early cell stages and is linked to impaired embryo development, reduced implantation rate (29), and increased aneuploidy rates (30).

The possibility to assess morphokinetic variables at high accuracy and in an objective way has stimulated the search for algorithms that use morphokinetics to predict outcome measures such as development to blastocyst (31), potential risk for aneuploidy (32), and the chance for implantation (33). Consideration of the potential of using morphokinetic algorithms for viability assessment is split into a discussion on selection of the most viable embryos versus de-selection of the least viable embryos. Both strategies have been proposed in current attempts to establish morphokinetic algorithms and have to be discussed in line with the influence of external factors on certain morphokinetic variables.

Published Algorithms for Blastocyst Prediction

One of the first published algorithms developed for blastocyst prediction was based on data from less than 100 frozen pronuclear stage oocytes that were thawed and cultured to the blastocyst stage (31). This algorithm used early time intervals, namely the duration of the 2-cell and 3-cell stage to generate two scores (Low and High) (34). It was later refined by splitting the range of the same variables into a three score model (35). In both studies a correlation between blastocyst prediction algorithms and implantation outcome was reported. However, when tested on a multicentric dataset, these results could not be confirmed, and the authors concluded that blastocyst prediction is not necessarily applicable in predicting implantation (36).

Subsequent publications explored the possibility of using time-lapse data only for blastocyst prediction. Milewski and colleagues used the time of division to the 2-cell stage (t2) and 5-cell stage (t5), as well as the duration of the 2-cell stage, to formulate a logistic regression model (37). Although the model succeeded in predicting blastocyst formation as early as on day 2 to 3, it was not tested for use in predicting implantation. Cetinkaya and colleagues also combined relative timings, and compared these to absolute time parameters up to the 8-cell stage (38). They reported a formula that was able to cover the cleavage synchronicity from the 2-cell to the 8-cell stage $[((t3 - t2) + (t5 - t4))/(t8 - t2)]$ and enabled an estimate for blastocyst formation on day 3 with a high sensitivity and specificity. The latter two algorithms may well be suited for deciding on day 3 if it is worthwhile to continue the culture of embryos to day 5 and obtain blastocyst stage embryos for transfer; however, their performance regarding implantation prediction still needs to be investigated and validated.

A recent retrospective study reported morphokinetic parameters for blastocyst formation and blastocyst implantation (39). The authors showed that for blastocyst prediction the time of formation of the morula stage (tM; 81.28–96.0 hours after ICSI) and the interval t8–t5 with a range of <8.78 hours were the most predictive parameters. However, the same parameters with identical time ranges were less predictive for implantation prediction, and the interval t8–t5 required a major adaption (<5.67 hours) in order to give a reasonable implantation prediction, but only when combined with another variable—the time of the expanding blastocyst (tEB).

Published Algorithms for Implantation Prediction

Most of the published implantation prediction algorithms combine morphokinetic timing variables and morphological events. The hierarchical algorithm proposed by Meseguer and colleagues (33) is based on symmetry at the 2-cell stage, an optimal range for the time of the division to the 5-cell stage (t5), the duration of the 3-cell stage, and the speed of development from the 1-cell to the 3-cell stage. The ranges

for the different variables were obtained by quartile analysis in a retrospective study of 247 embryos with known implantation outcome. The algorithm was then applied in a randomized controlled trial (RCT) and compared to a control group that was cultured in a standard incubator using traditional embryo assessment at fixed time-points (40). Although this study showed an increase in the overall pregnancy rate and a decrease in the early pregnancy loss rate, it was criticized due to the study design; particularly it has been interpreted that the incubation conditions were the main cause for the beneficial effects reported, rather than a benefit due to the applied algorithm.

The Meseguer algorithm was developed further on 754 embryos by integrating multinucleation at the 4-cell stage as another exclusion criteria and by adapting the ranges for certain variables (41), prior to being tested in a retrospective multicentric study on 865 cycles. The study results confirmed the applicability in daily clinical use, the limitations being the retrospective nature of the evaluation. The blastocyst prediction algorithm by Milewski et al. (37) was developed into a day 3 implantation prediction algorithm based on 410 transferred embryos and integrating time points for t2, t3, t4, t5, as well as duration of the 2-cell and 3-cell stage, fragmentation, and maternal age (42). When tested on 112 transferred embryos of which 40 implanted, it predicted implantation with a sensitivity of 72.5% and a specificity of 65.3%.

Recently, a day 3 selection algorithm was published, which showed a high sensitivity and specificity when applied to IVF and ICSI embryos and when using different culture media (43). This algorithm used conventional morphology as a primary exclusion criterion, excluded embryos that showed direct or reverse cleavage, favored embryos that reached at least the 8-cell stage at 68 hours, stayed in the 3-cell stage for a limited time period, and developed from the time of pronuclear (PN) fading (tPNf) to t5 within a given range. Due to the highly selective nature of this algorithm, only 12.5% of all embryos fell into the best category in the retrospective analysis. This algorithm was validated on 66 embryos, out of which 14 (21.2%) were in the prime category and 50% of these implanted. Still, a validation in an independent data set with a substantially larger sample size and preferentially in a prospective multicentric setting has yet to be presented.

Goodman and colleagues combined various morphokinetic and morphological parameters in a grading scheme (44). The starting parameter was an acceptable morphology on the day of transfer. Next, embryos received negative points for a short duration of the 2-cell stage, presence of multinucleation, and presence of irregular division. Positive points were allocated for a favorable range of t5 (45.8–57.0 hours), a short duration of the 3-cell stage (<0.1 hours), a range for the duration of t8–t5 (1.4–7.0 hours) and the start of the formation of the blastocoel cavity before 100 hours post insemination. These parameters were derived from a pre-study performed by the same group, where the retrospective analysis showed a 50% clinical pregnancy rate for patients that received the transfer of embryos that ranked top. When the authors performed a RCT, they cultured the control group in the same integrated time-lapse system as the test group. However, the control group was only assessed at the standard time points, and additional information derived from time-lapse like direct cleavage was not taken into account. In the study group the grading scheme and thus full time-lapse information was applied to select the best embryo for transfer. The study saw an increase in the clinical pregnancy rate from 62.9% to 68.1%, which was not significant. However, the sample size justification in the study was calculated on the base of a 50% clinical pregnancy rate of the pre-study and an expected absolute increase of 10% to reach a 60% clinical pregnancy rate after the intervention. With a power of 80% at an alpha of 0.05 the number of patients for randomization was set at 232. Interestingly, this study was not properly powered from the beginning, which was overlooked by the reviewers of the respective journal. Using a publicly available sample size calculator (http://clincalc.com/Stats/SampleSize.aspx) clearly shows that this study would have required a sample size of 774 patients. Using only 232 patients would only give a power between 30% and 35%, i.e., in two out of three studies one would *not* see any significant difference, and draw the wrong conclusion, even if the actual improvement was the expected 10% absolute (i.e., 20% relative). The encouraging improvement in the clinical pregnancy rate of absolute 5% does show the benefit of adding morphokinetics to morphological assessments in a time-lapse system. However, to prove this increase to be significant would require a sample size of 1311 patients in each arm for a study to obtain a power of 80% to detect a significant difference at an alpha level of 5%.

Limitations of Specific Algorithms

The majority of algorithms for implantation prediction were developed on monocentric and thus clinic-specific data. Some of these algorithms were later tested in other clinics and eventually failed to deliver comparably good implantation results due to a required shift in the morphokinetic variables in other laboratory settings (45–47). The major reasons for these differences are the laboratory conditions (36,48,49). For example, embryos cultured at low oxygen proceed in early development much faster compared to those cultured at ambient air (49,50). Therefore, an algorithm that has been developed under ambient air conditions may fail when applied in a laboratory with reduced oxygen, as observed by Freour and colleagues (47).

The obvious limitation of most of the published algorithms is the rather low numbers of embryos that were used, ranging from 132 to 754. The outlined shortcomings of specifically developed algorithms provoked the question if it would ever be possible to find a universal morphokinetic algorithm, which will work across different clinics and independent of conditions such as oxygen and/or insemination technique? Constructing a universal algorithm requires a large dataset of embryos. If the endpoint is blastocyst prediction, this task is rather easy, as data from every embryo that was cultured can be used. Although morphokinetic parameters do correlate with blastocyst formation (51), the limitations of blastocyst prediction algorithms in regard to implantation prediction have already been discussed (36). A far better strategy is to use data from transferred embryos, and where the outcome for each embryo is known; whether it implanted, as verified by ultrasound scan, or if it did not implant. Such data are called Known Implantation Data (KID). Evidently, the most powerful algorithm would be based on data from live-born children.

Development of a Universal Algorithm for Day 3 Embryo Transfer

The development of a universal algorithm for implantation prediction of human embryos has recently been published (52). This algorithm was developed on KID from 3275 embryos transferred on day 3. The data were derived from 24 clinics and covered a variety of culture conditions, including high and low oxygen, single step and sequential culture media, variable carbon dioxide levels, and different insemination techniques like IVF and ICSI. The KIDScore D3 algorithm is based on morphokinetic variables that are easy to annotate (tPNf, t2, t3, t4, t5, t8; number of cells at 66 hours post insemination). It was constructed as a decision tree model that ranks embryos and assigns a score from 0 to 5, which reflects the relative implantation potential. The algorithm is shown in Figure 13.4 and as it is an integrated part of the software of a commercial time-lapse system it can be applied on day 3 to every embryo. This algorithm focuses on de-selection rather than selection criteria and uses relative formulae for some of the more condition-sensitive time-values. All embryos that are not showing 2 PN are scored 0, embryos that reach t3 too fast are scored 1, embryos that develop too slow are scored 2, embryos that show an irregular division are scored 3, embryos that show a regular division but do not reach the desired stage on day 3 are scored 4, and finally embryos that fulfill all parameters are scored 5. The difference in relative implantation between the score 1 and score 5 was seven-fold, with a high specificity and sensitivity. As the algorithm is focused on morphokinetic variables, it enables differentiation between the implantation potential of embryos that show similar morphological quality (Figure 13.5). The algorithm gave equally good results when validated at various clinic- or condition-specific settings, thereby fulfilling the criteria required to be classified as a universal time-lapse algorithm for implantation prediction. Interestingly, when this algorithm was tested on blastocyst prediction, it outperformed some of those algorithms that were specifically designed for blastocyst prediction (52).

In contrast to de-selection algorithms, like KIDScore D3, algorithms that are based on a selection principle tend to be more clinic-specific. Although selection algorithms may identify the embryo(s) with the highest potential, they may reject embryos with a medium to low implantation-competency. De-selection algorithms on the other hand provide a ranking of the entire cohort of embryos from a given patient. This is an important feature if it comes to practicability in daily routine use. Patients who present with suboptimal embryos benefit from a morphokinetic de-selection algorithm as it does not reject any embryos and instead ranks the less optimal embryos despite their lower viability.

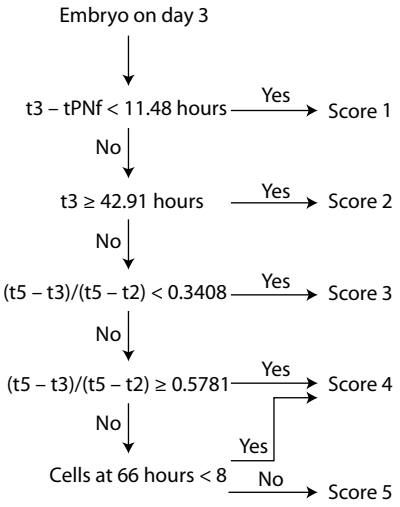


FIGURE 13.4 KIDScore D3 uses a low number of variables for scoring embryos based on a decision tree model. The model scheme shown in the figure does not need to be calculated manually for each embryo. As soon as the embryo is annotated in the software, the algorithm can be applied on the annotation data and the score will be automatically calculated and displayed. The different scores reflect the relative implantation potential, which is lowest for score 1 and highest for score 5. The two decision pathways that give the score 4 category are similar in the underlying implantation potential. The KIDScore algorithm is described in detail elsewhere (see Petersen et al. (52)).

Consequently, morphokinetic assessment of embryo viability based on universal de-selection algorithms takes the move to elective single embryo transfer one step further. As the criteria used for viability assessment are based on objective time-lapse parameters, this approach will promote standardization in embryo evaluation among embryologists. The major advantage of universal algorithms is their immediate applicability from day one of using time-lapse equipment without the need to adapt or harvest

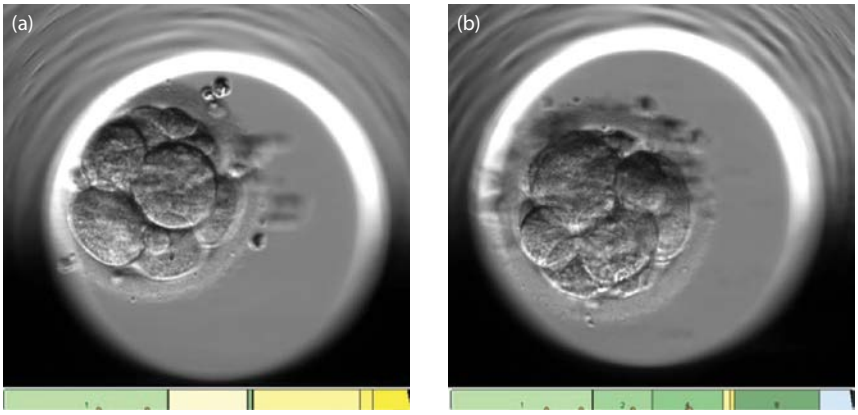


FIGURE 13.5 In traditional embryo assessment, embryos are graded at fixed time-points by the stage of development and morphology. In Figure (a) and (b) two embryos with similar morphology and cell number are shown on day 3. By standard assessment parameters, these two embryos can hardly be distinguished in regard to differences of their implantation potential. The division chart below each embryo gives an overview of the developmental progression of both embryos, which is different in the way that embryo (a) shows an irregular development compared to (b). Based on time-lapse information, the KIDScore D3 algorithm automatically assigns a score to these embryos, where the embryo in (a) scores 1 and the embryo in (b) scores 5. Based on data from a large number of embryos, the relative implantation potential of these embryos differs five-fold. (Figure courtesy of Vitrolife A/S Denmark.)

clinic-specific data. This does not exclude the later development of a specific algorithm in a given clinic, provided that enough data from embryos with known implantation data are available, and that a professional statistical approach is undertaken.

Viability assessment by morphokinetic ranking is often criticized with the argument that a freeze-all approach followed by subsequent transfer of one embryo after the other in a replacement cycle will at the end give the same result (53). The proponents of this approach very often overlook the fact that IVF patients present with a high dropout rate, and that in many societies time to pregnancy becomes an important aspect of IVF therapy due to advanced maternal age. The major advantage of a time-lapse based approach is the reduction in time to pregnancy, as recently shown in a meta-analysis of six randomized controlled trials where patients in the time-lapse group showed a higher ongoing pregnancy rate and a reduced early pregnancy loss rate (54).

Quantification of Embryo Viability: Metabolism

The transformation of the fertilized oocyte into the blastocyst is not only characterized by major morphological events, but also by dramatic changes in its physiology, reflected in changes in the relative activity of the metabolic pathways which provide not only energy, but also the biosynthetic intermediates required to support proliferation.

Given that the oocyte has remained dormant for so long before being ovulated, it is not surprising that the fertilized oocyte has limited requirement for energy (in the form of adenosine triphosphate [ATP]), and hence it is characterized by a high level of ATP (55) (of note, cells which are actively utilizing ATP are typically characterized by low levels of ATP, and yet measurement of high ATP levels in cells is often reported as being positively associated with metabolic activity and consequently promoted as a marker of oocyte health; it is not). This high level of ATP is responsible for the down-regulation of glycolytic activity, and hence low glucose use, by its allosteric regulation of one of the rate-limiting enzymes in this pathway, phosphofructokinase (PFK) (56). Upon activation of the majority of the embryonic genome around the 8-cell/morula stage (57,58), and the ensuing exponential increase in embryo cell number, the embryo's demand for energy increases rapidly as blastomeres proliferate, which requires the biosynthesis of both nucleic acids and membranes. To reiterate, the high levels of ATP characteristic of the oocyte, fertilized oocyte, and early cleavage stages are a direct result of their relative quiescence, i.e., they have limited demand for energy and hence ATP is not utilized. Given their resultant inability to use glucose for energy production, early developmental stages are consequently dependent upon the utilization of pyruvate, lactate, and aspartate, which undergo limited oxidation through both the tricarboxylic acid (TCA) cycle (pyruvate and lactate) (59,60) and the malate–aspartate shuttle (aspartate) (61).

As development proceeds and consequently ATP consumption increases, the inhibition on PFK is removed, and glucose is able to be utilized to a greater extent through glycolysis, reflected by a significant increase in its utilization after compaction (62). By the blastocyst stage, glucose is the preferred and predominant nutrient consumed. Intriguingly, the blastocyst does not oxidize all of the glucose, but rather converts around 50% to lactate, even in the presence of oxygen, a phenomenon referred to as aerobic glycolysis. This rather unique pattern of metabolism was first observed in cancer cells by Otto Warburg (63), and hence is also known as the "Warburg Effect." For many years it was held that blastocysts are like cancers in their metabolic profile and regulation of function (64,65). However, with hindsight it is actually more appropriate to consider that cancers are more like blastocysts, and that cancers utilize all the specialized metabolic processes that a blastocyst employs to facilitate invasion of the endometrium and to promote angiogenesis (16). Detailed analysis of the regulation of the metabolic function of the cleavage stage embryo and blastocyst have recently been published (66,67), and a comprehensive overview is provided in Figures 13.6 and 13.7. Why it is important to understand the complexities of embryo metabolism, is that should any aspect of metabolic regulation be compromised, then there are downstream consequences for the viability of the embryo (68–71). Hence, understanding metabolic function has been of immense value in developing culture media, which support appropriate metabolic functions and thereby reduce metabolic stress (65,72–74).

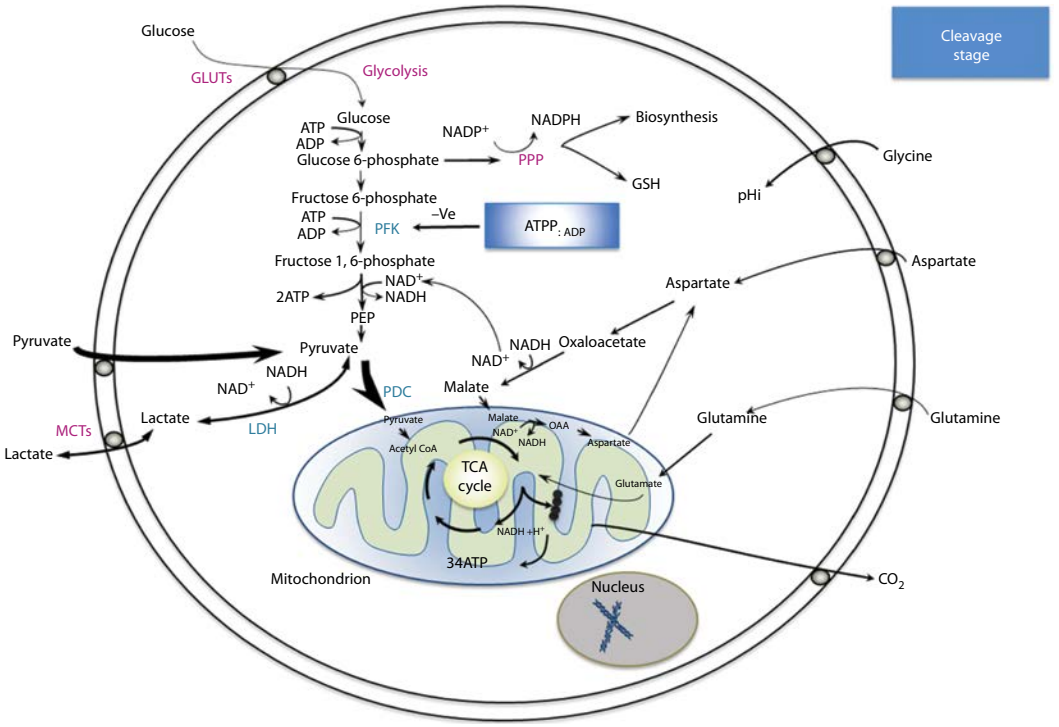


FIGURE 13.6 Metabolism of the pronucleate oocyte and cleavage stage embryo. Prior to compaction the embryo has a metabolism based around low levels of oxidation of pyruvate, lactate, and specific amino acids. The ovulated oocyte is surrounded by, and is directly connected to, cumulus cells which actively produce pyruvate and lactate from glucose (118,119). This creates a high concentration of pyruvate and lactate and a low concentration of glucose around the fertilized oocyte. Upon dispersal of the cumulus cells, the human zygote and cleavage stage embryo still find themselves in a relatively high concentration of pyruvate (0.32 mM) and lactate (10.5 mM), and low levels of glucose (0.5 mM) within the ampulla (119). The early embryo is characterized by a high ATP:ADP level (55), which in turn allosterically inhibits PFK, thereby limiting the flux of glucose through the glycolytic pathway prior to compaction. Significantly, the relative abundance of nutrients affects the metabolism of the embryo. For example, the ratio of pyruvate:lactate in the surrounding environment directly affects the ratio of NADH:NAD^+ in the embryo, which in turn controls the redox state of the cells and hence the flux of nutrients through specific energy generating pathways (120). Lactate dehydrogenase comprises ~5% of the total protein of the mouse oocyte (121). The oocyte, pronucleate oocyte and all stages of development to the blastocyst exhibit LDH isoform I (122), which then changes to predominantly isoform V at the late blastocyst stage upon outgrowth (123). Isoform I favors the formation of pyruvate, whereas Isoform V favors lactate formation. This switch in isoforms is consistent with the changes in patterns of energy metabolism as the embryo develops, but does not explain the significant production of lactate by the blastocyst (see Figure 13.2). Amino acids fill several niches in embryo physiology, such as the use of glycine as buffer of intracellular pH (pHi). Several amino acids are also utilized as energy sources by the early embryo such as glutamine and aspartate. Aspartate can be utilized through the malate–aspartate shuttle (61), the significance of which during embryo development we are only beginning to understand. The thickness of the lines represents the relative flux of metabolites through that pathway. GLUTs, glucose transporters; GSH, reduced glutathione; LDH, lactate dehydrogenase; OAA, oxaloacetate; PDC, pyruvate dehydrogenase complex; pHi, intracellular pH; PFK, phosphofructokinase; PK, pyruvate kinase; PPP, pentose phosphate pathway. (Adapted from Gardner DK, Wale PL. *Fertil Steril*. 2013;99(4):1062–72.)

Metabolic Function: Carbohydrates

Given the significance of the appropriate and stage-specific utilization of metabolites, it is a logical progression that the quantification of nutrient uptake, ideally combined with a measure of its utilization, will be of value in assessing embryonic developmental potential, both in culture and post transfer (viability). Proof of concept data was first reported in 1980 by Renard et al., who observed a positive relationship between glucose uptake by day 10 cow blastocysts and subsequent pregnancy after transfer (75). This

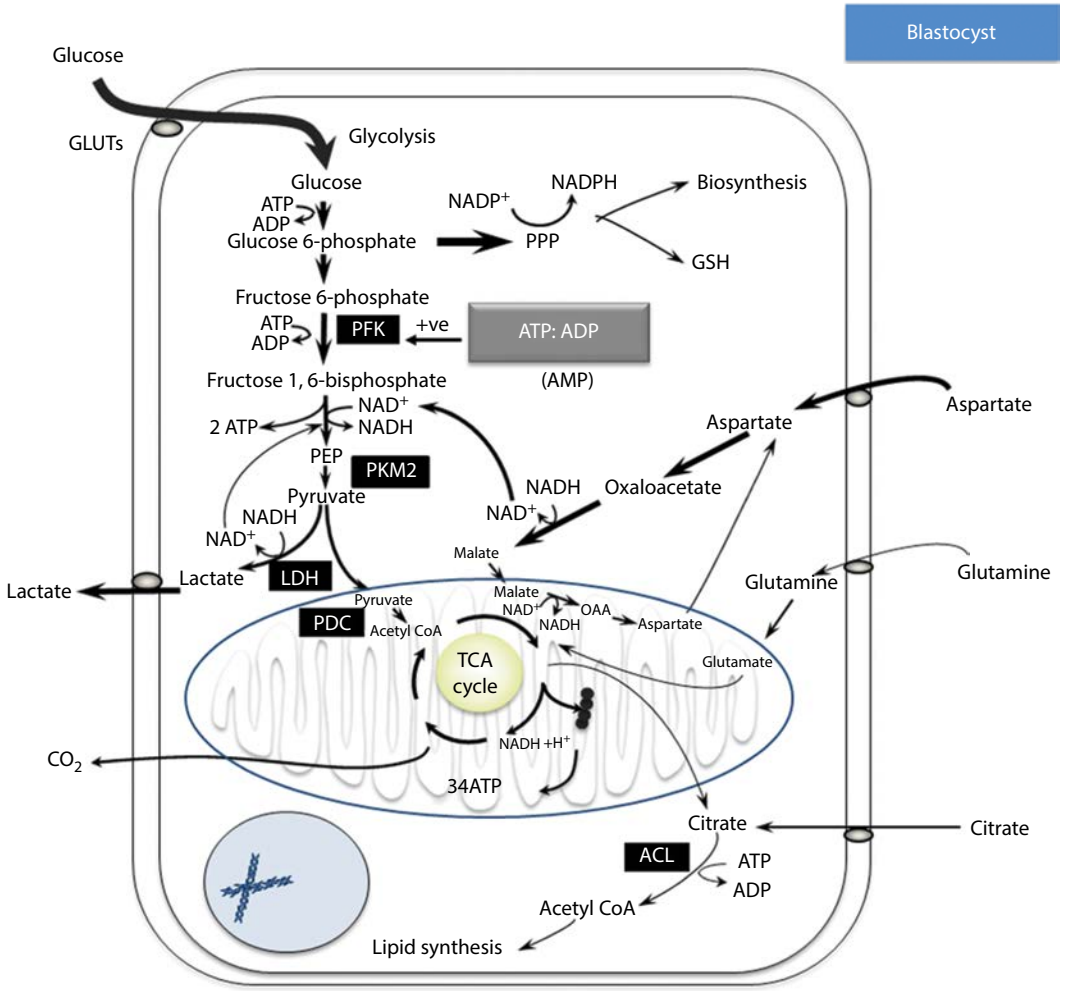


FIGURE 13.7 Metabolism of the blastocyst. After compaction the embryo exhibits greatly increased oxygen consumption (124–126) and an increased capacity to use glucose as an energy source. The increase in oxygen consumption plausibly reflects the considerable energy required for the formation and maintenance of the blastocoel, while the increase in glucose utilization reflects an increased demand for biosynthetic precursors (64). Consequently, there is a reduction in the ATP:ADP ratio (55), and a concomitant increase in AMP, which will have a positive allosteric effect on PFK, thereby facilitating a higher flux of glucose through glycolysis. Rather than oxidize the glucose consumed, the blastocyst exhibits high levels of aerobic glycolysis (72). Although this may appear energetically unfavorable, it does ensure that the biosynthetic arm of the pentose phosphate pathway has maximum substrate availability at all times. Activity of the pentose pathway will ensure reducing equivalents are available for biosynthesis and ensure production of glutathione (reduced), a key intracellular antioxidant. In order for high levels of glycolysis to proceed the blastomeres need to regenerate cytosolic NAD^+. This can be achieved through the generation of lactate from pyruvate. A second means of generating cytosolic NAD^+ is through the activity of the malate-aspartate shuttle. Although it is evident that blastocysts do use aerobic glycolysis, it is proposed that the significant increase in oxygen utilization at this stage of development could be largely attributed to the activity of the malate-aspartate shuttle and the resulting demand for oxygen to convert intramitochondiral NADH to ATP. Indeed, many tumors that exhibit aerobic glycolysis also have high levels of the malate–aspartate shuttle (105). Furthermore, inhibition of this shuttle has dire consequences for subsequent fetal development (71,127). It has been proposed that the considerable amount of lactate produced by the blastocyst could facilitate a number of key processes involved in the implantation process by creating a microenvironment around the embryo. Specifically, several of the pathways employed by cancers for invasion and proliferation depend upon high levels of lactate production, and consequently mirror those developed by the blastocyst to promote implantation. Lactate has been shown to have a profound effect on the mechanisms involved in extracellular matrix breakdown, can induce angiogenesis and indeed modulate the local immune response in order to prevent rejection (16). A further key regulatory enzyme in glycolysis is pyruvate kinase (PK). (*Continued*)

FIGURE 13.7 (Continued) In proliferating cells and in cancer cells, a specific isoform is present, PKM2 (128,129). This particular isoform of PK has been shown to promote aerobic glycolysis and anabolic metabolism (130), and recently has been identified in the mammalian blastocyst (131). Further work is warranted on establishing the regulation of PKM2 in the embryo, with specific reference to its control by exogenous factors and signaling pathways involved. The pyruvate dehydrogenase complex catalyses the irreversible conversion of pyruvate to acetyl Co-A, and consequently functionally linking glycolysis to the activity of the oxidative TCA cycle. The activity of this enzyme complex is tightly regulated through 3 main mechanisms: (1) phosphorylation/dephosphorylation, (2) the redox state (NAD^+:NADH, ATP:ADP and the acetylCoA:CoA ratios), and (3) through transcriptional regulation. However, relatively little is known about the regulation of this complex in mammalian embryos during the pre-implantation period. Citrate, formed from either mitochondrial metabolism, or provided in the culture medium, could serve as a precursor in lipid synthesis, required for membrane generation associated with proliferation. As the embryo develops it exhibits a growing number of receptors for specific growth factors (132,133). Given that growth factor signaling can reorganize metabolic fluxes independently of traditional allosteric means, it will be important to determine how exogenous factors affect key metabolic processes within the embryo. PFK, phosphofructokinase; PK, pyruvate kinase; LDH, lactate dehydrogenase, PDC, pyruvate dehydrogenase complex; ACL, acetyl-citrate lyase; OAA, oxaloacetate; PPP, pentose phosphate pathway; OAA, oxaloacetate; GSH, reduced glutathione. (Adapted from Gardner DK, Wale PL. *Fertil Steril*. 2013;99(4):1062–72.)

relationship between blastocyst glucose uptake and the establishment of viable pregnancies was later confirmed in the mouse (76). A landmark study followed, in which mouse blastocyst glucose uptake was quantitated together with the appearance of lactate in the culture medium, i.e., the metabolic fate of the nutrient was determined indirectly (69). For blastocysts developed in vivo, the amount of glucose converted to lactate is approximately 50%, and hence this pattern of activity can be considered as the "metabolic norm" (72). It was subsequently determined that those mouse blastocysts which exhibited the highest rate of glucose uptake, together with the conversion of around half of it to lactate, were the most viable within a cohort. In contrast, if a blastocyst lost its ability to regulate how much lactate is produced, i.e., exhibited the production of increasing amount of lactate, then their resultant viability following embryo transfer to the uterus was significantly compromised (69). This resulted in the creation of the "Rate and Fate" hypothesis in which it was determined that not only is the *rate* at which a nutrient is consumed related to viability, but also the subsequent metabolic *fate* of the nutrient. In other words, should an embryo utilize an inappropriate metabolic pathway at any given time, then resultant viability is compromised.

Early attempts to quantitate human embryo metabolism focused predominantly on the cleavage stages, given that it was not feasible to culture the human embryo to the blastocyst stage until the late 1990s. Consequently, the relationship between pyruvate uptake on days 2 and 3 and resultant development and viability was initially investigated (62,77–79). Although it was evident that differences in pyruvate uptake existed between those embryos which developed in vitro and those that arrested, the culture conditions used in these early studies were relatively simple, and limited data was obtained with regards to subsequent pregnancy outcome. Of note, Hardy et al. (62) reported that pyruvate uptake of embryos which arrested was lower than those which developed normally. Our own analysis of pyruvate uptake by the same embryos on days 2 and 3 of culture, during the initial clinical evaluation of the first of the physiologically based media we formulated, revealed a significant relationship between the days, inferring that those embryos that are metabolically most active on day 2 are those which are most active on day 3 (80). With the development of more physiological media in the 1990s (81,82), it became possible to analyze the metabolism of the later stage embryo prior to transfer. Early studies revealed that blastocysts with the same alphanumeric score, e.g., 3AA, from the same patient exhibited a wide range of glucose uptakes, indicating that morphological grade was not directly related to the physiological status of the embryo (83). Given the significance of glucose uptake by blastocysts in animal models, the uptake of glucose by individual human embryos on days 4 and 5, followed by single embryo transfer, allowed for the relationship between nutrient uptake and subsequent pregnancy to be established. Consistent with the animal data, glucose uptake by those embryos which were considered viable by the delivery of a healthy baby exhibited a significantly higher glucose uptake on both day 4 and day 5 of development, thereby establishing in the human that embryo viability post compaction is positively associated with a high glucose uptake (84) (Figure 13.8). Furthermore, it was revealed that sex differences existed, with female embryos consuming significantly more glucose than males on day 4 of development. This phenomenon

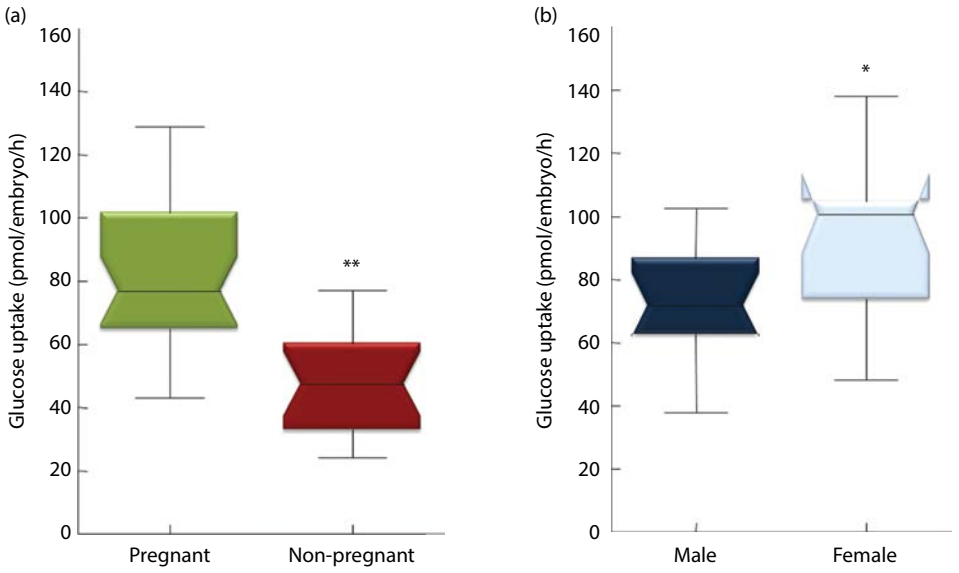


FIGURE 13.8 Relationship between glucose consumption on day 4 of development and human embryo viability and embryo sex. (a) Glucose uptake on day 4 of embryonic development and pregnancy outcome (positive fetal heart beat). Notches represent the confidence interval of the median, and the depth of the box represents the interquartile range (50% of the data), whiskers represent the 5 and 95% quartiles. The line across the box is the median glucose consumption. **significantly different from pregnant ($p < 0.01$). (b) Glucose uptake by male and female embryos on day 4 of development. *significantly different from male embryos ($p < 0.05$). (Adapted from Gardner DK et al. *Hum Reprod.* 2011;26(8):1981–6.)

was previously reported in the mouse and attributed to the window in development in which both X chromosomes are active in female embryos (85,86). The result of having two active X chromosomes, even transiently, is an altered transcriptome (87) and proteome (88). Of note, several key enzymes involved in metabolic function are X-linked. Such data now await validation on a much larger set of patients.

The main reason why such analyses have not been widely adopted clinically is simply due to the lack of methods sensitive enough to accurately quantitate nutrient uptake and release by single embryos. However, it is envisaged that the introduction of solid state and/or of microfluidic devices will facilitate the routine quantification of single embryo metabolism in the near future (89–91).

Metabolic Function: Amino Acids

Studies on mammalian embryo culture revealed that amino acids are key regulators of pre-implantation development (92–95). Amino acids have been shown to have several key roles in regulating the physiology of the pre-implantation embryo (2,96,97). Amongst their roles is the ability to act as energy substrates (98) and also to regulate carbohydrate metabolism (65,73). Consequently, their uptake and utilization warrants analysis, as this has the potential to reflect viability.

Pioneering work in this area was undertaken by the Leese laboratory and their collaborators in the United Kingdom. Houghton et al. (99) measured amino acid turnover by individual donated human embryos and observed different patterns of amino acid utilization between embryos that went on to form a blastocyst and those embryos that failed to develop. Those embryos that did develop consumed more leucine from the culture medium, and the profiles of alanine, arginine, glutamine, methionine, and asparagine flux were related to blastocyst formation (but not necessarily blastocyst quality). In a follow up study, Brison et al. (100) observed changes in the levels of amino acids in the spent medium of human zygotes cultured for 24 h to the 2-cell stage. Asparagine, glycine, and leucine were all significantly associated with clinical pregnancy and live birth. Subsequently it was determined that there appear to be gender differences with regards to amino acid utilization (101,102), consistent with data on glucose

consumption and with differences in the proteomes of male and female embryos (86,88). Similar to the data on carbohydrate use, these exciting data await validation in a larger patient population.

Of note, there has been a resurgence of interest into the roles of amino acids in embryo development, particularly their role in regulating the malate–aspartate shuttle (61,71), their role as signaling molecules (103), and as markers of blastocyst viability (104). It is evident that there are exciting times ahead with regards to amino acids and early embryonic development, supporting their role as a potential embryo biomarker.

Metabolic Function: Oxygen

An overall reflection of metabolic state can be attained through the measurement of oxygen consumption, and hence respiration rate. This may at first appear confusing, given that the blastocyst stage embryo utilizes aerobic glycolysis, but one has to consider that ~50% of the glucose consumed is oxidized, and so measuring oxygen consumption makes great sense (particularly if it can be quantitated together with nutrient uptakes, and thereby assist in determining the rate and fate of said nutrients). Furthermore, the activity of the malate–aspartate shuttle can contribute to oxygen consumption (105). Excitingly, recent data on oxygen consumption by cleavage stage human embryos indicates that viability is associated with an increased oxygen consumption rate, reflecting an increase in respiration rate (106). Of note, the concentration of oxygen itself has a profound impact on the metabolism of both carbohydrates and amino acids, with atmospheric levels (~21%) significantly altering metabolic function in a stage-specific fashion (70,107).

An interesting observation from the initial analysis of amino acid utilization by human embryos was that those embryos that developed to blastocyst exhibited a lower turnover of amino acids than "non-viable" embryos (99), leading to the hypothesis that a "quiet metabolism" is optimal (108). In essence it was proposed that embryos with a low metabolic activity reflected a less stressed physiology, and consequently those embryos classified as viable would be those that had low nutrient uptake and turnover (109). However, a growing number of recent studies have generated data which do not provide support for this hypothesis (69,84,110); rather they indicate that viability is associated with increased metabolic activity. So how can this apparent paradox be resolved? An examination of the studies upon which the "quiet hypothesis" was built reveals that a common factor among them is the use of 20% oxygen, either for embryo culture and analysis, or during the actual analysis of metabolism. Given the documented negative impact of 20% oxygen on gene expression, embryonic proteome, and metabolism, described above, the significance of the "quiet hypothesis" for embryos cultured under physiological oxygen conditions must be carefully reviewed and studies performed to determine what is the optimal nutrient utilization profile under physiological conditions.

Is There a Role for Metabolomics in Embryo Selection?

The approaches described above employed target metabolomics approaches, i.e., the nutrients being measured were identified ahead of time and the assays were both specific and of high sensitivity for specific carbohydrates and amino acids. An alternative approach to assessing the overall metabolic state of the embryo is to use a metabolic profiling platform such as near infrared (NIR) spectroscopy, which creates a spectrum of metabolites from which algorithms can be generated associated with implantation potential (111). Although a series of preliminary studies showed some benefits of metabolomics approaches, they were largely based on retrospective studies and performed in a single research laboratory as distinct from a real clinical setting (112,113). However, subsequent randomized clinical trials, which compared morphological assessment to NIR to rank embryos within a cohort that had good morphology, failed to show compelling benefits for embryos selected for either transfer or cryopreservation (114,115).

Quantification of Embryo Viability: A Combined Approach

When Morphokinetics and Metabolism Combine

Recently there have been studies in which both morphokinetics and analysis of embryo physiology have been combined in an attempt to further increase our understanding of pre-implantation embryo

development and our ability to identify the most viable embryos (104,116). In both studies, one on metabolism and the other on proteomics, markers were identified at the blastocyst stage that could be used in conjunction with morphokinetics parameters determined during the cleavage stages. In the case of metabolism, this was an increase in the uptake of glucose and aspartate, combined with a lower glycolytic rate at the blastocyst stage, whereas in the proteomics analysis it was the presence of Interleukin-6 (IL-6) in the culture medium. Further work is required in this area to establish the relative weightings of such parameters for their inclusion in future selection algorithms.

Conclusions

Embryo morphology, morphokinetics, and metabolism all appear linked to the developmental potential of the human embryo. Furthermore, these three parameters have some degree of independence, and hence it would appear prudent to build future algorithms around both embryo quality parameters, such as blastomere symmetry, multinucleation, and degree of fragmentation, together with targeted metabolic data. The more we know about the physiology of each developing embryo, the more likely it is that we shall be able to select as a matter of routine the most viable embryos within a cohort.

As we move to single embryo transfer, the technologies outlined in this chapter to assess human embryo viability are needed in human IVF now more than ever. Such technologies will not only facilitate the ranking of embryos in terms of their viability, but will greatly reduce the time to pregnancy, whether the transfers are fresh or following cryopreservation. The widespread introduction of pre-implantation genetic screening, made more accurate and affordable through the introduction of new molecular approaches such as next generation sequencing, will ensure the identification of euploid embryos, and hence will complement, and not replace, the quantification of the pregnancy potential of each embryo. Future platforms for embryo culture and analysis could well be one and the same, in which a microfluidic device, or a dish with solid state sensing capacity, resides within a time-lapse incubator. From such a system we will be able to acquire morphological data, morphokinetic data, and real-time metabolic measurements, all of which, when combined, will provide further insights into embryo development, and will increase the ability to identify the most viable embryos within a cohort.

REFERENCES

1. Gardner DK, Balaban B. Assessment of human embryo development using morphological criteria in an era of time-lapse, algorithms and OMICS: Is looking good still important? *Mol Hum Reprod*. 2016;22(10):704–18.
2. Gardner DK, Lane M. Culture and selection of viable blastocysts: A feasible proposition for human IVF? *Hum Reprod Update*. 1997;3(4):367–82.
3. Papanikolaou EG, Camus M, Kolibianakis EM, Van Landuyt L, Van Steirteghem A, Devroey P. In vitro fertilization with single blastocyst-stage versus single cleavage-stage embryos. *N Engl J Med*. 2006;354:1139–46.
4. Papanikolaou EG, Kolibianakis EM, Tournaye H, Venetis CA, Fatemi H, Tarlatzis B et al. Live birth rates after transfer of equal number of blastocysts or cleavage-stage embryos in IVF. A systematic review and meta-analysis. *Hum Reprod*. 2008;23(1):91–9.
5. Gardner DK, Schoolcraft WB. In vitro culture of human blastocyst In: *Towards Reproductive Certainty: Fertility and Genetics Beyond 1999*, Jansen R, Mortimer D (Eds.). Carnforth, UK: Parthenon Publishing, 1999, 378–88.
6. Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB. Blastocyst score affects implantation and pregnancy outcome: Towards a single blastocyst transfer. *Fertil Steril*. 2000;73(6):1155–8.
7. Gardner DK, Stevens, J., Sheehan, C.B., Schoolcraft, W.B. Analysis of blastocyst morphology. In: *Human Preimplantation Embryo Selection*, Elder KC, J (Ed.) London: Informa Healthcare, 2007, 79–87.
8. Richter KS, Harris DC, Daneshmand ST, Shapiro BS. Quantitative grading of a human blastocyst: Optimal inner cell mass size and shape. *Fertil Steril*. 2001;76(6):1157–67.

9. Shapiro BS, Richter KS, Harris DC, Daneshmand ST. A comparison of day 5 and day 6 blastocyst transfers. *Fertil Steril*. 2001;75(6):1126–30.
10. Kovacic B, Vlaisavljevic V, Reljic M, Cizek-Sajko M. Developmental capacity of different morphological types of day 5 human morulae and blastocysts. *Reprod Biomed Online*. 2004;8(6):687–94.
11. Ahlstrom A, Westin C, Reismer E, Wikland M, Hardarson T. Trophectoderm morphology: An important parameter for predicting live birth after single blastocyst transfer. *Hum Reprod*. 2011;26(12):3289–96.
12. Hill MJ, Richter KS, Heitmann RJ, Graham JR, Tucker MJ, DeCherney AH et al. Trophectoderm grade predicts outcomes of single-blastocyst transfers. *Fertil Steril*. 2013;99(5):1283–9 e1.
13. Thompson SM, Onwubalili N, Brown K, Jindal SK, McGovern PG. Blastocyst expansion score and trophectoderm morphology strongly predict successful clinical pregnancy and live birth following elective single embryo blastocyst transfer (eSET): A national study. *J Assist Reprod Genet*. 2013;30(12):1577–81.
14. Ebner T, Tritscher K, Mayer RB, Oppelt P, Duba HC, Maurer M et al. Quantitative and qualitative trophectoderm grading allows for prediction of live birth and gender. *J Assist Reprod Genet*. 2016;33(1):49–57.
15. Herrler A, von Rango U, Beier HM. Embryo-maternal signalling: How the embryo starts talking to its mother to accomplish implantation. *Reprod Biomed Online*. 2003;6(2):244–56.
16. Gardner DK. Lactate production by the mammalian blastocyst: Manipulating the microenvironment for uterine implantation and invasion? *Bioessays*. 2015;37(4):364–71.
17. Van den Abbeel E, Balaban B, Ziebe S, Lundin K, Cuesta MJ, Klein BM et al. Association between blastocyst morphology and outcome of single-blastocyst transfer. *Reprod Biomed Online*. 2013;27(4):353–61.
18. Licciardi F, McCaffrey C, Oh C, Schmidt-Sarosi C, McCulloh DH. Birth weight is associated with inner cell mass grade of blastocysts. *Fertil Steril*. 2015;103(2):382–7 e2.
19. Brison DR, Hewitson LC, Leese HJ. Glucose, pyruvate, and lactate concentrations in the blastocoel cavity of rat and mouse embryos. *Mol Reprod Dev*. 1993;35(3):227–32.
20. Ansell JD, Snow MH. The development of trophoblast in vitro from blastocysts containing varying amounts of inner cell mass. *J Embryol Exp Morphol*. 1975;33(1):177–85.
21. Snow MH, Aitken J, Ansell JD. Role of the inner cell mass in controlling implantation in the mouse. *J Reprod Fertil*. 1976;48(2):403–4.
22. Gardner RL, Johnson MH. An investigation of inner cell mass and trophoblast tissues following their isolation from the mouse blastocyst. *J Embryol Exp Morphol*. 1972;28(2):279–312.
23. The Istanbul consensus workshop on embryo assessment: Proceedings of an expert meeting. *Hum Reprod*. 2011;26(6):1270–83.
24. Istanbul consensus workshop on embryo assessment: Proceedings of an expert meeting. *Reprod BioMed Online*. 2011;22(6):632–46.
25. Zhang JQ, Li XL, Peng Y, Guo X, Heng BC, Tong GQ. Reduction in exposure of human embryos outside the incubator enhances embryo quality and blastulation rate. *Reprod Biomed Online*. 2010;20(4):510–5.
26. Wale PL, Gardner DK. The effects of chemical and physical factors on mammalian embryo culture and their importance for the practice of assisted human reproduction. *Hum Reprod Update*. 2016;22(1):2–22.
27. Montag M, Kajhoj TQ, Agerholm IE. Description of time-lapse systems: EmbryoScope. In: *Time-lapse Microscopy in In-Vitro Fertilization*, Mesegeur M (Ed.). Cambridge: Cambridge University Press, 2016, 11–30.
28. Liu Y, Chapple V, Roberts P, Matson P. Prevalence, consequence, and significance of reverse cleavage by human embryos viewed with the use of the Embryoscope time-lapse video system. *Fertil Steril*. 2014;102(5):1295–300 e2.
29. Rubio I, Kuhlmann R, Agerholm I, Kirk J, Herrero J, Escriba MJ et al. Limited implantation success of direct-cleaved human zygotes: A time-lapse study. *Fertil Steril*. 2012;98(6):1458–63.
30. Zaninovic N, Zhan Q, Rosenwaks Z. Time-lapse implementation in a clinical setting: Outcome results. In: *Time-Lapse Microscopy in In-Vitro Fertilization*, Mesgeur M (Ed.). Cambridge: Cambridge University Press, 2016, 144–64.
31. Wong CC, Loewke KE, Bossert NL, Behr B, De Jonge CJ, Baer TM et al. Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. *Nat Biotechnol*. 2010;28(10):1115–21.
32. Campbell A, Fishel S, Bowman N, Duffy S, Sedler M, Hickman CF. Modelling a risk classification of aneuploidy in human embryos using non-invasive morphokinetics. *Reprod Biomed Online*. 2013;26(5):477–85.

33. Meseguer M, Herrero J, Tejera A, Hilligsoe KM, Ramsing NB, Remohi J. The use of morphokinetics as a predictor of embryo implantation. *Hum Reprod*. 2011;26(10):2658–71.
34. Conaghan J, Chen AA, Willman SP, Ivani K, Chenette PE, Boostanfar R et al. Improving embryo selection using a computer-automated time-lapse image analysis test plus day 3 morphology: Results from a prospective multicenter trial. *Fertil Steril*. 2013;100(2):412–9 e5.
35. VerMilyea MD, Tan L, Anthony JT, Conaghan J, Ivani K, Gvakharia M et al. Computer-automated time-lapse analysis results correlate with embryo implantation and clinical pregnancy: A blinded, multicentre study. *Reprod Biomed Online*. 2014;29(6):729–36.
36. Kirkegaard K, Campbell A, Agerholm I, Bentin-Ley U, Gabrielsen A, Kirk J et al. Limitations of a time-lapse blastocyst prediction model: A large multicentre outcome analysis. *Reprod Biomed Online*. 2014;29(2):156–8.
37. Milewski R, Kuc P, Kuczynska A, Stankiewicz B, Lukaszuk K, Kuczynski W. A predictive model for blastocyst formation based on morphokinetic parameters in time-lapse monitoring of embryo development. *J Assist Reprod Genet*. 2015;32(4):571–9.
38. Cetinkaya M, Pirkevi C, Yelke H, Colakoglu YK, Atayurt Z, Kahraman S. Relative kinetic expressions defining cleavage synchronicity are better predictors of blastocyst formation and quality than absolute time points. *J Assist Reprod Genet*. 2015;32(1):27–35.
39. Motato Y, de los Santos MJ, Escriba MJ, Ruiz BA, Remohi J, Meseguer M. Morphokinetic analysis and embryonic prediction for blastocyst formation through an integrated time-lapse system. *Fertil Steril*. 2016;105(2):376–84 e9.
40. Rubio I, Galan A, Larreategui Z, Ayerdi F, Bellver J, Herrero J et al. Clinical validation of embryo culture and selection by morphokinetic analysis: A randomized, controlled trial of the EmbryoScope. *Fertil Steril*. 2014;102(5):1287–94 e5.
41. Basile N, Vime P, Florensa M, Aparicio Ruiz B, Garcia Velasco JA, Remohi J et al. The use of morphokinetics as a predictor of implantation: A multicentric study to define and validate an algorithm for embryo selection. *Hum Reprod*. 2015;30(2):276–83.
42. Milewski R, Milewska AJ, Kuczynska A, Stankiewicz B, Kuczynski W. Do morphokinetic data sets inform pregnancy potential? *J Assist Reprod Genet*. 2016;33(3):357–65.
43. Liu Y, Chapple V, Feenan K, Roberts P, Matson P. Time-lapse deselection model for human day 3 in vitro fertilization embryos: The combination of qualitative and quantitative measures of embryo growth. *Fertil Steril*. 2016;105(3):656–62 e1.
44. Goodman LR, Goldberg J, Falcone T, Austin C, Desai N. Does the addition of time-lapse morphokinetics in the selection of embryos for transfer improve pregnancy rates? A randomized controlled trial. *Fertil Steril*. 2016;105(2):275–85 e10.
45. Best L, Campbell A, Duffy S, Montgomery S, Fishel S. Testing a published selection algorithm on independent time-lapse data. *Hum Reprod*. 2013;28(Suppl 1):87–90.
46. Yalcinkaya E, Ergin EG, Caliskan E, Oztel Z, Ozay A, Ozornek H. Reproducibility of a time-lapse embryo selection model based on morphokinetic data in a sequential culture media setting. *J Turkish German Gynecol Assoc*. 2014;15(3):156–60.
47. Freour T, Le Fleuter N, Lammers J, Splingart C, Reignier A, Barriere P. External validation of a time-lapse prediction model. *Fertil Steril*. 2015;103(4):917–22.
48. Dal Canto M, Coticchio G, Mignini Renzini M, De Ponti E, Novara PV, Brambillasca F et al. Cleavage kinetics analysis of human embryos predicts development to blastocyst and implantation. *Reprod Biomed Online*. 2012;25(5):474–80.
49. Kirkegaard K, Hindkjaer JJ, Ingerslev HJ. Effect of oxygen concentration on human embryo development evaluated by time-lapse monitoring. *Fertil Steril*. 2013;99(3):738–44 e4.
50. Wale PL, Gardner DK. Time-lapse analysis of mouse embryo development in oxygen gradients. *Reprod Biomed Online*. 2010;21(3):402–10.
51. Cruz M, Garrido N, Herrero J, Perez-Cano I, Munoz M, Meseguer M. Timing of cell division in human cleavage-stage embryos is linked with blastocyst formation and quality. *Reprod Biomed Online*. 2012;25(4):371–81.
52. Petersen B, Boel M, Montag M, Gardner DK. Development of a generally applicable morphokinetic algorithm capable of predicting the implantation potential of embryos transferred on Day 3. *Hum Reprod*. 2016; 31(10):2231–44.

53. Armstrong S, Vail A, Mastenbroek S, Jordan V, Farquhar C. Time-lapse in the IVF-lab: How should we assess potential benefit? *Hum Reprod*. 2015;30(1):3–8.
54. Montag M, Pribenszky C. A meta-analysis of the benefit of using time-lapse imaging in assisted reproduction to improve pregnancy rate and reduce early pregnancy loss. *Hum Reprod*. 2016;31(Suppl 1):i96–i97.
55. Leese HJ, Biggers JD, Mroz EA, Lechene C. Nucleotides in a single mammalian ovum or preimplantation embryo. *Anal Biochem*. 1984;140(2):443–8.
56. Barbehenn EK, Wales RG, Lowry OH. Measurement of metabolites in single preimplantation embryos; a new means to study metabolic control in early embryos. *J Embryol Exp Morphol*. 1978;43:29–46.
57. Hamatani T, Ko M, Yamada M, Kuji N, Mizusawa Y, Shoji M et al. Global gene expression profiling of preimplantation embryos. *Hum Cell*. 2006;19(3):98–117.
58. Xue Z, Huang K, Cai C, Cai L, Jiang CY, Feng Y et al. Genetic programs in human and mouse early embryos revealed by single-cell RNA sequencing. *Nature*. 2013;500(7464):593–7.
59. Biggers JD, Whittingham DG, Donahue RP. The pattern of energy metabolism in the mouse oocyte and zygote. *Proc Nat Acad Sci USA*. 1967;58(2):560–7.
60. Wales RG, Whittingham. The metabolism of specifically labelled lactate and pyruvate by two-cell mouse embryos. *J Reprod Fertil*. 1973;33(2):207–22.
61. Lane M, Gardner DK. Mitochondrial malate-aspartate shuttle regulates mouse embryo nutrient consumption. *J Biol Chem*. 2005;280(18):18361–7.
62. Hardy K, Hooper MA, Handyside AH, Rutherford AJ, Winston RM, Leese HJ. Non-invasive measurement of glucose and pyruvate uptake by individual human oocytes and preimplantation embryos. *Hum Reprod*. 1989;4(2):188–91.
63. Warburg O. On the origin of cancer cells. *Science*. 1956;123(3191):309–14.
64. Gardner DK. Embryo development and culture techniques. In: *Animal Breeding: Technology for the 21st Century*, Clark J (Ed.). London: Harwood Academic, 1998, 13–46.
65. Gardner DK. Changes in requirements and utilization of nutrients during mammalian preimplantation embryo development and their significance in embryo culture. *Theriogenology*. 1998;49(1): 83–102.
66. Gardner DK, Wale PL. Analysis of metabolism to select viable human embryos for transfer. *Fertil Steril*. 2013;99(4):1062–72.
67. Gardner DK, Harvey AJ. Blastocyst metabolism. *Reprod Fertil Dev*. 2015;27(4):638–54.
68. Menke TM, McLaren A. Mouse blastocysts grown in vivo and in vitro: Carbon dioxide production and trophoblast outgrowth. *J Reprod Fertil*. 1970;23(1):117–27.
69. Lane M, Gardner DK. Selection of viable mouse blastocysts prior to transfer using a metabolic criterion. *Hum Reprod*. 1996;11(9):1975–8.
70. Wale PL, Gardner DK. Oxygen regulates amino acid turnover and carbohydrate uptake during the preimplantation period of mouse embryo development. *Biol Reprod*. 2012;87(1):24.
71. Mitchell M, Cashman KS, Gardner DK, Thompson JG, Lane M. Disruption of mitochondrial malate-aspartate shuttle activity in mouse blastocysts impairs viability and fetal growth. *Biol Reprod*. 2009;80(2):295–301.
72. Gardner DK, Leese HJ. Concentrations of nutrients in mouse oviduct fluid and their effects on embryo development and metabolism in vitro. *J Reprod Fertil*. 1990;88(1):361–8.
73. Gardner DK, Sakkas D. Mouse embryo cleavage, metabolism and viability: Role of medium composition. *Hum Reprod*. 1993;8(2):288–95.
74. Lane M, Gardner DK. Amino acids and vitamins prevent culture-induced metabolic perturbations and associated loss of viability of mouse blastocysts. *Hum Reprod*. 1998;13(4):991–7.
75. Renard JP, Philippon A, Menezo Y. In-vitro uptake of glucose by bovine blastocysts. *J Reprod Fertil*. 1980;58(1):161–4.
76. Gardner DK, Leese HJ. Assessment of embryo viability prior to transfer by the noninvasive measurement of glucose uptake. *J Exp Zool*. 1987;242(1):103–5.
77. Conaghan J, Hardy K, Handyside AH, Winston RM, Leese HJ. Selection criteria for human embryo transfer: A comparison of pyruvate uptake and morphology. *J Assist Reprod Genet*. 1993;10(1):21–30.
78. Gott AL, Hardy K, Winston RM, Leese HJ. Non-invasive measurement of pyruvate and glucose uptake and lactate production by single human preimplantation embryos. *Hum Reprod*. 1990;5(1):104–8.

79. Leese HJ, Hooper MA, Edwards RG, Ashwood-Smith MJ. Uptake of pyruvate by early human embryos determined by a non-invasive technique. *Hum Reprod*. 1986;1(3):181–2.
80. Gardner DK, Spitzer A, Osborn JC. Development of a complex serum-free medium and its effects on the development and metabolism of the preimplantation human embryo. *Proc Am Fertil Soc*. 1991:164.
81. Gardner DK. Mammalian embryo culture in the absence of serum or somatic cell support. *Cell Biol Int*. 1994;18(12):1163–79.
82. Barnes FL, Crombie A, Gardner DK, Kausche A, Lacham-Kaplan O, Suikkari AM et al. Blastocyst development and birth after in-vitro maturation of human primary oocytes, intracytoplasmic sperm injection and assisted hatching. *Hum Reprod*. 1995;10(12):3243–7.
83. Gardner DK, Lane M, Stevens J, Schoolcraft WB. Noninvasive assessment of human embryo nutrient consumption as a measure of developmental potential. *Fertil Steril*. 2001;76(6):1175–80.
84. Gardner DK, Wale PL, Collins R, Lane M. Glucose consumption of single post-compaction human embryos is predictive of embryo sex and live birth outcome. *Hum Reprod*. 2011;26(8):1981–6.
85. Gutierrez-Adan A, Perez-Crespo M, Fernandez-Gonzalez R, Ramirez M, Moreira P, Pintado B et al. Developmental Consequences of Sexual Dimorphism During Pre-implantation Embryonic Development. *Reprod Domest Anim*. 2006;41(Suppl 2):54–62.
86. Gardner DK, Larman MG, Thouas GA. Sex-related physiology of the preimplantation embryo. *Mol Hum Reprod*. 2010;16(8):539–47.
87. Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P, Gutierrez-Adan A. Sex determines the expression level of one third of the actively expressed genes in bovine blastocysts. *Proc Nat Acad Sci USA*. 2010;107(8):3394–9.
88. Epstein CJ, Smith S, Travis B, Tucker G. Both X chromosomes function before visible X-chromosome inactivation in female mouse embryos. *Nature*. 1978;274(5670):500–3.
89. Heo YS, Cabrera LM, Bormann CL, Smith GD, Takayama S. Real time culture and analysis of embryo metabolism using a microfluidic device with deformation based actuation. *Lab Chip*. 2012;12(12):2240–6.
90. Urbanski JP, Johnson MT, Craig DD, Potter DL, Gardner DK, Thorsen T. Noninvasive metabolic profiling using microfluidics for analysis of single preimplantation embryos. *Anal Chem*. 2008;80(17):6500–7.
91. Gardner DK, Meseguer M, Rubio C, Treff NR. Diagnosis of human preimplantation embryo viability. *Hum Reprod Update*. 2015;21(6):727–47.
92. Bavister BD, Arlotto T. Influence of single amino acids on the development of hamster one-cell embryos in vitro. *Mol Reprod Dev*. 1990;25(1):45–51.
93. Gardner DK, Lane M. Amino acids and ammonium regulate mouse embryo development in culture. *Biol Reprod*. 1993;48(2):377–85.
94. Mehta TS, Kiessling AA. Development potential of mouse embryos conceived in vitro and cultured in ethylenediaminetetraacetic acid with or without amino acids or serum. *Biol Reprod*. 1990;43(4):600–6.
95. Spindle AI, Pedersen RA. Hatching, attachment, and outgrowth of mouse blastocysts in vitro: Fixed nitrogen requirements. *J Exp Zool*. 1973;186(3):305–18.
96. Bavister BD, McKiernan SH. Regulation of hamster embryo development in vitro by amino acids. In: *Preimplantation Embryo Development*, Bavister BD (Ed.). New York: Springer-Verlag, 1993, 57–72.
97. Gardner DK. Dissection of culture media for embryos: The most important and less important components and characteristics. *Reprod Fertil Dev*. 2008;20(1):9–18.
98. Rieger D, Loskutoff NM, Betteridge KJ. Developmentally related changes in the uptake and metabolism of glucose, glutamine and pyruvate by cattle embryos produced in vitro. *Reprod Fertil Dev*. 1992;4(5):547–57.
99. Houghton FD, Hawkhead JA, Humpherson PG, Hogg JE, Balen AH, Rutherford AJ et al. Non-invasive amino acid turnover predicts human embryo developmental capacity. *Hum Reprod*. 2002;17(4):999–1005.
100. Brison DR, Houghton FD, Falconer D, Roberts SA, Hawkhead J, Humpherson PG et al. Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. *Hum Reprod*. 2004;19(10):2319–24.
101. Picton HM, Elder K, Houghton FD, Hawkhead JA, Rutherford AJ, Hogg JE et al. Association between amino acid turnover and chromosome aneuploidy during human preimplantation embryo development in vitro. *Mol Hum Reprod*. 2010;16(8):557–69.
102. Sturmey RG, Bermejo-Alvarez P, Gutierrez-Adan A, Rizos D, Leese HJ, Lonergan P. Amino acid metabolism of bovine blastocysts: A biomarker of sex and viability. *Mol Reprod Dev*. 2010;77(3):285–96.

103. Tan BS, Rathjen PD, Harvey AJ, Gardner DK, Rathjen J. Regulation of amino acid transporters in pluripotent cell populations in the embryo and in culture; novel roles for sodium-coupled neutral amino acid transporters. *Mech Dev*. 2016;141:32–9.
104. Lee YS, Thouas GA, Gardner DK. Developmental kinetics of cleavage stage mouse embryos are related to their subsequent carbohydrate and amino acid utilization at the blastocyst stage. *Hum Reprod*. 2015;30(3):543–52.
105. Greenhouse WV, Lehninger AL. Magnitude of malate-aspartate reduced nicotinamide adenine dinucleotide shuttle activity in intact respiring tumor cells. *Cancer Res*. 1977;37(11):4173–81.
106. Tejera A, Herrero J, Viloria T, Romero JL, Gamiz P, Meseguer M. Time-dependent O(2) consumption patterns determined optimal time ranges for selecting viable human embryos. *Fertil Steril*. 2012;98(4):849–57 e3.
107. Wale PL, Gardner DK. Oxygen affects the ability of mouse blastocysts to regulate ammonium. *Biol Reprod*. 2013;89(3):75.
108. Baumann CG, Morris DG, Sreenan JM, Leese HJ. The quiet embryo hypothesis: Molecular characteristics favoring viability. *Mol Reprod Dev*. 2007;74(10):1345–53.
109. Leese HJ, Baumann CG, Brison DR, McEvoy TG, Sturmey RG. Metabolism of the viable mammalian embryo: Quietness revisited. *Mol Hum Reprod*. 2008;14(12):667–72.
110. Meseguer MT, A, Herrero, J, de los Santos M, Viloria T, Remohi J. Oxygen consumptio increases during cytokinesis in implanting human embryos; a time-lapse and microsensro combined analysis. *Hum Reprod*. 2012;27(Supp 2):ii22–4.
111. Botros L, Sakkas D, Seli E. Metabolomics and its application for non-invasive embryo assessment in IVF. *Mol Hum Reprod*. 2008;14(12):679–90.
112. Vergouw CG, Botros LL, Roos P, Lens JW, Schats R, Hompes PG et al. Metabolomic profiling by near-infrared spectroscopy as a tool to assess embryo viability: A novel, non-invasive method for embryo selection. *Hum Reprod*. 2008;23(7):1499–504.
113. Ahlstrom A, Wikland M, Rogberg L, Barnett JS, Tucker M, Hardarson T. Cross-validation and predictive value of near-infrared spectroscopy algorithms for day-5 blastocyst transfer. *Reprod Biomed Online*. 2011;22(5):477–84.
114. Hardarson T, Ahlstrom A, Rogberg L, Botros L, Hillensjo T, Westlander G et al. Non-invasive metabolomic profiling of Day 2 and 5 embryo culture medium: A prospective randomized trial. *Hum Reprod*. 2012;27(1):89–96.
115. Vergouw CG, Heymans MW, Hardarson T, Sfontouris IA, Economou KA, Ahlstrom A et al. No evidence that embryo selection by near-infrared spectroscopy in addition to morphology is able to improve live birth rates: Results from an individual patient data meta-analysis. *Hum Reprod*. 2014; 29(3):455–61.
116. Dominguez F, Meseguer M, Aparicio-Ruiz B, Piqueras P, Quinonero A, Simon C. New strategy for diagnosing embryo implantation potential by combining proteomics and time-lapse technologies. *Fertil Steril*. 2015;104(4):908–14.
117. Gardner DK, Sakkas D. Assessment of embryo viability: The ability to select a single embryo for transfer–a review. *Placenta*. 2003;24 Suppl B:S5–12.
118. Leese HJ, Barton AM. Production of pyruvate by isolated mouse cumulus cells. *J Exp Zool*. 1985;234(2):231–6.
119. Gardner DK, Lane M, Calderon I, Leeton J. Environment of the preimplantation human embryo in vivo: Metabolite analysis of oviduct and uterine fluids and metabolism of cumulus cells. *Fertil Steril*. 1996;65(2):349–53.
120. Lane M, Gardner DK. Lactate regulates pyruvate uptake and metabolism in the preimplantation mouse embryo. *Biol Reprod*. 2000;62(1):16–22.
121. Brinster RL. Lactate dehydrogenase activity in the preimplanted mouse embryo. *Biochim Biophys Acta*. 1965;110(2):439–41.
122. Auerbach S, Brinster RL. Lactate dehydrogenase isozymes in the early mouse embryo. *Exp Cell Res*. 1967;46(1):89–92.
123. Gibbons J, Hewitt E, Gardner DK. Effects of oxygen tension on the establishment and lactate dehydrogenase activity of murine embryonic stem cells. *Cloning Stem Cells*. 2006;8(2):117–22.
124. Mills RM, Brinster RL. Oxygen consumption of preimplanation mouse embryos. *Expl Cell Res*. 1967;47:337–44.

125. Thompson JG, Partridge RJ, Houghton FD, Cox CI, Leese HJ. Oxygen uptake and carbohydrate metabolism by in vitro derived bovine embryos. *J Reprod Fertil*. 1996;106(2):299–306.
126. Trimarchi JR, Liu L, Porterfield DM, Smith PJ, Keefe DL. Oxidative phosphorylation-dependent and -independent oxygen consumption by individual preimplantation mouse embryos. *Biol Reprod*. 2000;62(6):1866–74.
127. Wakefield SL, Lane M, Mitchell M. Impaired mitochondrial function in the preimplantation embryo perturbs fetal and placental development in the mouse. *Biol Reprod*. 2011;84(3):572–80.
128. Mazurek S. Pyruvate kinase type M2: A key regulator of the metabolic budget system in tumor cells. *The Int J Biochem Cell Biol*. 2011;43(7):969–80.
129. Vander Heiden MG, Lunt SY, Dayton TL, Fiske BP, Israelsen WJ, Mattaini KR et al. Metabolic pathway alterations that support cell proliferation. *Cold Spring Harb Sym*. 2011;76:325–34.
130. Christofk HR, Vander Heiden MG, Harris MH, Ramanathan A, Gerszten RE, Wei R et al. The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. *Nature*. 2008;452(7184):230–3.
131. Redel BK, Brown AN, Spate LD, Whitworth KM, Green JA, Prather RS. Glycolysis in preimplantation development is partially controlled by the Warburg Effect. *Mol Reprod Dev*. 2012;79(4):262–71.
132. Hardy K, Spanos S. Growth factor expression and function in the human and mouse preimplantation embryo. *J Endocrinol*. 2002;172(2):221–36.
133. Thouas GA, Dominguez F, Green MP, Vilella F, Simon C, Gardner DK. Soluble ligands and their receptors in human embryo development and implantation. *Endocr Rev*. 2015;36(1):92–130.

14

Embryo Culture Systems

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Introduction

The initial success of clinical IVF was compromised by suboptimal culture conditions, resulting in impaired embryo development, and all too frequently complete developmental arrest around the 8-cell stage (1–4). Consequently, it became the paradigm to transfer human embryos to the uterus asynchronously on days 1, 2, or 3. Indeed, it was advocated that if the laboratory conditions were not optimized then embryos should be transferred as soon as possible back to the uterus to avoid suboptimal conditions (5). Fortunately, research over the past 25 years has resulted in the development of more physiological and effective culture systems capable of maintaining both development and viability of the pre-implantation embryo, culminating in the routine culture of human blastocysts (6–8). Improvements in embryo culture media formulations, combined with increases in efficiency and safety of the overall culture system, have led directly to a significant increase in embryo implantation rates (for both cleavage and blastocyst transfers), and a decline in pregnancy loss, thereby facilitating the routine introduction of single embryo transfer. Furthermore, more suitable culture conditions produce embryos more able to survive cryopreservation (9). Consequently, improvements in embryo culture technology have significantly contributed to the increase in the overall success rates of human assisted conception per cycle.

In this chapter the types of embryo culture media and their individual components are considered, followed by a discussion of media in the context of the embryo culture system. To do this we have included information and references, some of which date back over 50 years, but feel that it is important to have an appreciation of where we have come from if we are truly to be able to decide where we are going. It is, therefore, the aim of this chapter to provide the reader with the necessary information to make an informed decision on the type of culture system best suited for their clinical requirements.

Types of Media for Embryo Culture

Culture media employed for clinical IVF have varied greatly in their composition, which has led to a great deal of confusion concerning the formulation of embryo culture media and the role of individual components in embryo development. An understanding of the role of culture media and their components was initially hampered by the routine inclusion of serum in human embryo culture media. Serum has both the ability to mask potential embryo toxins and suppress the beneficial effects of other medium components. In light of this there was considerable research into the development of serum-free embryo culture media. Such studies have been invaluable in our understanding of the embryo's requirements during the pre-implantation period.

Media used to culture the human pre-implantation embryo historically have fallen into one of five categories.

Simple Salt Solutions with Carbohydrates

These media were originally formulated to support the development of zygotes from certain inbred strains of mice and their F1 hybrids (10,11). Examples of this type of media used in clinical IVF are M16

TABLE 14.1

Composition (mM) of Simple Salt Solution with Added Energy Substrates Used in Embryo Culture

Component	Whitten (19)	Brinster (85)	Whitten and Biggers (10)	M16 1971 (12)	Earle's[a] 1971 (13)	HTF[a] 1985 (15)	CZB 1989 (17)	MTF 1990 (24)	KSOM[b] 1993 (41)	P1[a] 1998 (16)
NaCl	118.46	119.23	68.49	94.66	116.30	101.60	81.62	114.19	95.00	101.6
KCl	4.74	4.78	4.78	4.78	5.36	4.69	4.83	4.78	2.50	4.69
KH_2PO_4	1.18	1.19	1.19	1.19	–	0.37	1.18	1.19	0.35	–
NaH_2PO_4	–	–	–	–	1.02	–	–	–	–	–
$CaCl_2 \cdot 2H_2O$	–	1.71	–	1.71	1.80	2.04	1.70	1.71	1.71	2.04
$MgSO_4 \cdot 7H_2O$	1.18	1.19	1.19	1.19	0.81	0.20	1.18	1.19	0.20	0.20
$NaHCO_3$	24.88	25.00	25.07	25.00	26.18	25.00	25.12	25.00	25.00	25.00
Ca lactate	2.54	–	1.71	–	–	–	–	–	–	–
Na lactate (D/L)	–	25.00	21.58	23.28	–	21.40	31.30	4.79	10.00@	21.4
Na Pyruvate	–	0.25	0.33	0.33	0.10	0.33	0.27	0.37	0.20	0.33
Glucose	5.55	–	5.56	5.56	5.55	2.78	–	3.40	0.20	–
BSA (mg/mL)	1.00	1.00	4.00	4.00	[b]	5.00	5.00	4.00	1.00	[c]

Note: CZB contains 110 μM EDTA, 1.0 mM glutamine and 5.5 mM glucose after 48 h of culture from the zygote stage. KSOM contains 10 μM EDTA and 1.0 mM glutamine. P1 contains 50 μM taurine and 0.5 mM citrate.

[a] Used in clinical IVF.

[b] Modifications to this medium have included the addition of specific groups of amino acids resulting in significant improvements to mouse zygote development in culture.

[c] Medium supplemented with synthetic serum substitute.

(12), T6 (12), and Earle's (13), and were employed in the initial phase of human IVF (14). Derivatives of such types of media include Human Tubal Fluid medium (HTF) (15) and, later on, medium P1 (16). Renewed interest in embryo culture in the 1980s culminated in the development of new simple media such as CZB (17) and SOM (18). Such "simple" media typically lack amino acids (with the exception of CZB and SOM, both of which contain glutamine as the sole amino acid), are supplemented with either whole serum or serum albumin, and are recommended for the cleavage stage embryo only, i.e., pronucleate oocyte to the 8-cell stage (Table 14.1). They do not readily support human blastocyst development given the omission of amino acids.

Complex Tissue Culture Media

These media are commercially available and are designed to support the growth of somatic cells in culture, e.g., Hams' F-10 (20) and αMEM. Such media are far more complex, containing amino acids, vitamins, nucleic acid precursors, and transitional metals, and are typically supplemented with 5%–20% serum. Importantly, such media were not formulated with the specific needs of the human embryo in mind, and they contain components which are now known to be detrimental to the developing embryo. Although tissue culture media were used in the early days of human IVF (21–23), they are no longer considered suitable for clinical ART.

Sequential Media

The approach taken in our laboratories has been to not only learn from the environment to which embryos are exposed in vivo (24,25), but also to study the physiology and metabolism of the embryo in culture in order to determine what causes intracellular stress (in particular metabolic stress) to the embryo (26–34). By being able to identify and monitor such stress we have been able to develop stage specific culture media that substantially reduce culture-induced trauma. Of note, these media were designed specifically for the culture of the human embryo, being based on the nutrients available to the human embryo in vivo (25) (Table 14.2). The development and characterization of such sequential media has been published in detail elsewhere (7,37–39).

TABLE 14.2

Composition of the Original Sequential Media G1/G2

	G1	G2
Component	**Concentration (mM)**	
NaCl	90.08	90.08
KCl	5.5	5.5
Na_2HPO_4	0.25	0.25
$MgSO_4 \cdot 7H_2O$	1.0	1.0
$CaCl_2 \cdot 2H_2O$	1.8	1.8
$NaHCO_3$	25.0	25.0
Sodium pyruvate	0.32	0.10
Sodium lactate (L)	10.5	5.87
Glucose	0.5	3.15
Alanine	0.1	0.1
Aspartic acid	0.1	0.1
Asparagine	0.1	0.1
Arginine	–	0.6
Cystine	–	0.1
Glutamate	0.1	0.1
Alanyl glutamine	1.0	0.5
Glycine	0.1	0.1
Histidine	–	0.2
Isoleucine	–	0.4
Leucine	–	0.4
Lysine	–	0.4
Methionine	–	0.1
Phenylalanine	–	0.2
Proline	0.1	0.1
Serine	0.1	0.1
Taurine	0.1	–
Threonine	–	0.4
Tryptophan	–	0.05
Tyrosine	–	0.2
Valine	–	0.4
EDTA	0.01	0.00
HSA	5 mg/mL	5 mg/mL
Hyaluronan	0.125 mg/mL	0.125 mg/mL

Note: Formulations available from References 35 and 36.

Simplex Optimized Media

Simplex media were developed using a computer program to generate successive media formulations based on the response of mouse embryos in culture to form blastocysts (40,41). Once a specific medium was formulated, tested, and blastocyst formation as the sole parameter analyzed, the program generated new formulations for use in the next series of cultures. This procedure was performed several times to generate several media that supported high rates of blastocyst development of embryos derived from the oocytes of outbred mice (CF1) crossed with the sperm of an F1 hybrid male, and were termed SOM (18) and later KSOM (which contains around 10 times more potassium than SOM) (42). Such media were subsequently modified by another laboratory to include amino acids (KSOMAA) (43). However, this last phase of medium development was based on independent studies on the mouse embryo (44) and did not involve the simplex procedure. This single medium formulation, KSOMAA, has been used to produce human blastocysts in culture (45). In such types of media, the embryo therefore has to adapt to its surrounding at it develops and

differentiates. Technically, therefore, the Simplex media developed for mouse embryos can be considered as examples of simple salt solutions with added carbohydrates and later on, amino acids (Table 14.1).

Time-Lapse Media

The advent of time-lapse microscopy has created a demand for uninterrupted culture (although this approach is not physiological, as shall be stressed later). Such media have, therefore, been specifically designed to cope with the extended build-up of ammonium from amino acid breakdown and metabolism (46), and the levels of nutrients by necessity balanced between the different requirements of the cleavage and blastocyst stages.

Composition of Embryo Culture Media

The composition of embryo culture systems can be broken down into the following components:

- Water
- Ions
- Carbohydrates
- Amino acids
- Vitamins
- Nucleic acid precursors
- Chelators
- Antioxidants
- Antibiotics
- Proteins
- Macromolecules
- Hormones, cytokines, and growth factors
- Buffer system

Each component of embryo culture media is considered in detail below. A common theme that emerges is the interaction of specific medium components with each other, often making their study in isolation rather difficult to interpret.

Water

Water is the major component of any medium, making up the majority of the contents. The source and purity of water used for media preparation is therefore a major factor in assuring the quality of media. The ability of embryos to develop in culture is positively correlated to water quality. Whittingham (12) demonstrated that the development of 2-cell mouse embryos to the blastocyst in culture was enhanced when the media was prepared using triple distilled water as opposed to double or single distilled water. However, the process of distillation has inherent problems, due to the possible leaching of ions and pyrogens from the glassware. A more reliable water purification system is ultrafiltration, which produces pyrogen-free water, with a resistance greater than 18 meg. Depending upon the local water source, however, it may be necessary to distil or pre-filter the original supply before processing. An alternative to in-house water preparation is commercially available high quality water, which should come endotoxin tested and have endotoxin levels less than 0.1 IU/mL.

Ions

The ionic basis of culture media used for clinical IVF varies markedly (Table 14.1). Surprisingly, even today relatively little is known about the role of ions during human pre-implantation embryo development.

The ionic composition of oviduct fluid from the human and mouse has been sampled by micropuncture and analyzed using an electron probe (47,48). Mammalian oviduct fluid is characterized by high potassium and chloride concentrations, and a high overall osmolality. Interestingly, high osmolality balanced salt solutions with added carbohydrates as energy sources do not support high levels of embryo development in vitro (in the absence of amino acids) (49,50).

Optimization of the ionic component of media has been compounded by the ability of embryos from certain strains of mice to develop apparently normally in culture to the blastocyst stage in a wide range of ionic concentrations. However, the suitability of using in vitro development to the blastocyst stage as the sole criterion for assessing the suitability (or otherwise) of a culture medium is highly questionable (51,52). The only true test of a medium's suitability is to transfer embryos to recipient females and quantify fetal development. Unfortunately, however, there is relatively little information available regarding embryo viability in animal models, and so almost all data has come from in vitro studies. Wales (53) used the development of 2-cell mouse embryos to the blastocyst in order to determine the range of ion concentrations capable of supporting development in vitro. Embryos formed blastocysts in medium with a potassium concentration ranging between 0.4 and 48 mM, a magnesium concentration between 0 and 9.6 mM, a calcium concentration between 0.1 and 10.2 mM, and a phosphate concentration between 0 and 7.2 mM, with a narrower range of optima for all ions. Studies on the hamster have also shown that the first cleavage and development of 2-cell embryos to the blastocyst occur in a wide range of sodium, magnesium, calcium, and potassium concentrations (54,55). Unfortunately it is difficult to interpret the effects of individual ions on embryo development and viability, as there are many subtle interactions which exist between ions, carbohydrates, and amino acids (see below).

High potassium levels in culture media have been reported to have a beneficial effect on sperm capacitation (56) and embryo development in vitro (42,53,57). However, there is conflicting data on the positive effects of potassium on embryo development (51,58,59).

High concentrations of NaCl (125 mM) in the culture media are detrimental to mouse embryo development to the blastocyst in vitro (18,50). Reducing the sodium chloride concentration to 85 mM in the medium increases the rates of both mRNA (43) and protein (60) synthesis of cleavage stage mouse embryos in vitro.

Studies on the effect of magnesium and calcium in the medium for the development of 2-cell mouse embryos in culture determined that magnesium was not essential for development to the blastocyst stage; however, calcium in medium is essential for embryos to undergo compaction in vitro (53,61). Early hamster embryos up to 6 hours following fertilization have a reduced ability to regulate intracellular calcium levels. This is exacerbated by low magnesium:calcium ratios in the medium (62,63). This reduced ability of embryos to regulate ionic homeostasis is directly related to a loss in viability (63), and increased calcium mobilization is reported to alter levels of gene expression (64). Interestingly, the appearance of the appropriate transporter systems in the hamster embryo correlates with the dispersion of the cumulus cells, i.e., prior to this time the cumulus cells may have a protective action. Therefore, the premature removal of cumulus cells in an intracytoplasmic sperm injection (ICSI) procedure may render the oocyte susceptible to ionic stress. The ionic composition of the culture medium is an important consideration, as external ion concentrations can have a profound effect on intracellular ion levels and therefore the regulation of normal cellular processes.

There has been much discussion regarding the rationale for the inclusion of phosphate in embryo culture medium. In a simple culture medium containing glucose, such as HTF or Earle's balanced salts, the presence of phosphate results in retarded human embryo development (65). Interestingly, phosphate is only inhibitory (with the exception of the hamster 2-cell embryo) in the presence of glucose, the mechanism of which is discussed in detail below. However, when phosphate is present in more physiologically defined media, i.e., the presence of specific amino acids, it does not have an inhibitory effect.

Further to their specific functions, the ions in any medium make the largest single contribution to osmotic pressure. The optimal osmolality for the development of human embryos in culture has not been determined. However, mouse (66) and hamster (54) embryos will develop in a wide range of osmolalities (200–350 mOsmols). Although conventional embryo culture media has an osmolality of between 275 and 295 mOsmols, enhanced development of mouse embryos appears to occur at reduced osmolalities (10,18). Again, however, it is important to note that such studies were performed using simple

embryo culture media, i.e., balanced salt solutions, in the absence of amino acids. It is now evident that the inclusion of osmolytes, such as betaine, or specific amino acids, such as glycine, in the culture medium can reduce any osmotic stress (32,33,49,50,67,68), thereby allowing apparently normal embryo development to occur over a wider range of osmotic pressures and ion concentrations.

Carbohydrates

Carbohydrates are present within the luminal fluids of the female reproductive tract. Their levels vary both between the oviduct and uterus and within the cycle (25,69). Therefore, the developing embryo is exposed to gradients of carbohydrates as it develops. Furthermore, the cumulus cells surrounding the oocyte and early embryo readily produce both pyruvate and lactate from glucose (24,25,70). Together with amino acids, carbohydrates represent the main energy substrates for the embryo. Embryo culture media contain pyruvate, lactate and glucose, all of which have been shown to interact with each other (71) and whose effects on embryonic metabolism are stage specific (31).

Analysis of carbohydrate uptakes in vitro revealed that the human embryo has an initial preference for pyruvate (72–74) whilst glucose uptake increases with development (72–75). Such studies on nutrient uptakes reflect the findings of earlier culture experiments which found that the mouse oocyte and zygote appeared to have an absolute requirement for pyruvate as an energy source (76). The omission of pyruvate from the medium for the development of the human embryo results in 84% of embryos arresting development at or prior to the 8-cell stage. Pyruvate as the sole energy substrate is also able to support the development of human zygotes to the blastocyst stage (77). It has therefore become dogma over the years that the first cleavage division of the mouse embryo is dependent upon the presence of pyruvate in the culture medium (76). However, it has now been shown that, in the presence of aspartate and lactate, there exists sufficient activity of the malate–aspartate shuttle in the embryo's mitochondria to overcome this dependence on pyruvate. Indeed, viable mouse fetuses can be obtained from zygotes cultured in the complete absence of pyruvate, reflecting the interaction of carbohydrates and amino acids (78).

Interestingly, in the mouse embryo, lactate can be utilized as an energy source from the 2-cell stage and acts synergistically with pyruvate (79). There have been conflicting studies on the optimal concentration of lactate in the culture medium to support mouse embryo development to the blastocyst stage. Cross and Brinster (79) reported that a lactate concentration of 30 mM is optimal to support zygote development to the blastocyst, whilst other studies have reported 10 mM to be optimal (71,80). A subsequent study showed that mouse zygotes cultured to the 8-cell stage in the presence of high lactate concentration (20 mM) were more viable than embryos cultured in a low lactate (4.79 mM) concentration (51). However, when the culture period was extended to the morula stage prior to transfer, the reverse was true, with viability increased by culture in lower lactate. Significantly, the regulation of metabolism of these carboxylic acids changes with development, further highlighting the physiological differences between the zygote and blastocyst stages (31,81). Of physiological significance, the relative ratio of pyruvate to lactate affects the intracellular ratio of NAD^+ to NADH, the former being a key regulator of not only glycolytic enzyme activity, but also a key regulator of ADP ribosylation and the activity of sirtuins, both of which are implicated in the regulation of gene expression (82,83).

An important point to note is that in almost all of the early embryo culture media formulations lactate was present as a 50:50 mixture of both the D- and L-isomer in sodium lactate syrup. As only the L-isomer is biologically active, the effective lactate concentration in embryo culture media is half of that given in the formulation. Lactate is a weak acid which readily enters the embryo, and at concentrations of 5 mM or greater induces a significant drop in intracellular pH (84). Therefore, it is recommended that sodium lactate salt is used in culture medium preparation in order to avoid the presence of excess lactate present as the D-isomer, which although not biologically active, can still induce a fall in pHi and therefore affect cellular physiology.

Glucose as the sole substrate cannot support mouse embryo development prior to the late 4-/early 8-cell stage (85,86). This inability to utilize glucose as an energy source during the first three cell cycles has been attributed to a blockade in glycolysis (87–89), induced by the high ATP levels of the oocyte and early cleavage stages (90). Studies on the mouse (17), hamster (91), sheep (92), cattle (93–95), and human (65,77) have all demonstrated that glucose in the presence of phosphate is responsible for the retardation

or developmental arrest of cleavage stage embryos in culture. However, this inhibition of glucose can be alleviated by the inclusion of amino acids (7,96–98), EDTA (27,99), and vitamins (28), highlighting the interactions which exist between medium components and the potential hazards of using simple salt solutions for embryo culture. In light of the potential toxicity of glucose in such media as HTF, it has been advocated to remove it from embryo culture media (16,65,100). Such a course of action may work for the culture of the cleavage stage embryo, but the removal of glucose from medium used for blastocyst culture results in a significant reduction in subsequent fetal development, highlighting its intrinsic role in the development of a viable embryo (82,101–103). Indeed, the removal of glucose from a culture medium should be considered as alleviating a culture-induced artefact, by the introduction of a second artefact, i.e., the removal of glucose from the culture medium when it is present in both oviduct and uterine fluids (25), and when the oocyte and embryo have a specific carrier for this hexose (104–107). Of note, expression of the lactate transporters MCT1 and MCT4 at the blastocyst stage requires the presence of glucose during the cleavage stages, hence the presence of glucose before compaction is actually programming the resultant metabolic behavior of the blastocyst (108,109). Further reasons for the inclusion of glucose in embryo culture medium are that not only is it required for energy production, but post compaction it is also essential for biosynthesis. The metabolism of glucose through the pentose phosphate pathway not only generates NADPH, required for lipid/membrane biosynthesis, but also generates ribose moieties for nucleic acid and triacylglycerol biosynthesis (27,103). Furthermore, at the time of implantation the environment around the blastocyst is relatively anoxic (110,111). This means that glycolysis may well be the only means of generating energy before angiogenesis in the endometrium is complete (82,112,113). A source of this glucose for glycolysis could be the embryo's own glycogen stores. Should the embryo have prematurely used such glucose stores during development, because there was no glucose present in the culture medium, then these embryos will have a reduced ability to implant. Indeed, mouse blastocysts in culture which exhibit excessive lactate production from their endogenous energy reserves have a significantly reduced developmental potential after transfer (114).

In conclusion, the pre-implantation embryo undergoes a switch in carbohydrate utilization during development. Initially pyruvate/lactate are the preferred nutrients, with glucose utilization increasing significantly post-compaction. Such changes in utilization mirror the availability of carbohydrates within the female tract. Pyruvate and lactate are at their highest concentration within the human fallopian tube, whilst glucose is at its lowest level. In contrast, within the uterus, pyruvate and lactate concentration is at its lowest and glucose at its highest (25), the presence of glucose ensuring the viability of the developing blastocyst. The complexities of embryo metabolism, and its relationship to viability and epigenetic state, have recently been reviewed (81–83,103).

Amino Acids

For the first two decades of human IVF, almost all of the embryo culture media lacked amino acids. In contrast to the early simple media, both oviduct and uterine fluids contain significant levels of amino acids (24,115–119). Oocytes and embryos possess specific transport systems for amino acids (120) and maintain an endogenous pool of amino acids (121). Indeed, amino acids are readily taken up and metabolized by the embryo (122,123). It is therefore no surprise that amino acids can be considered amongst the most important regulators of mammalian embryo development (8).

Oviduct and uterine fluids are characterized by high concentrations of the amino acids alanine, aspartate, glutamate, glycine, serine, and taurine (116–119). With the exception of taurine the amino acids at high concentrations in oviduct fluid bear a striking homology to those amino acids present in Eagle's non-essential group, i.e., those not required to support somatic cells in culture (124). Analysis of human and mouse oviduct fluids have also demonstrated that there are significant levels of glutamine present. Studies on the embryos of several mammalian species, such as mouse (44,101,125,126), hamster (6,67,127,128), sheep (92,98,129), and cow (37,93), have all demonstrated that the inclusion of specific amino acids in the culture medium enhances embryo development to the blastocyst stage. In the mouse embryo it has been determined that inclusion of Eagle's non-essential amino acids and glutamine in the medium significantly increases the rate of zygote development to the blastocyst in culture and, indeed,

can alleviate the 2-cell block (101). Non-essential amino acids and glutamine stimulate cleavage rates (44,130,131), blastocyst formation, and hatching of cultured mouse embryos (44,52,131). Significantly, the inclusion of amino acids in the culture medium is associated with the production a mouse blastocysts in vitro at the same time as they would form in vivo (132). It has been demonstrated that even a transient exposure (less than 5 minutes) of mouse zygotes to medium lacking amino acids impairs subsequent developmental potential (Figure 14.1) (101). During this 5 minute period in a simple medium lacking amino acids the embryo loses its entire endogenous pool, which takes several hours of active transport to replenish after returning the embryo to medium with amino acids. This has implications for the collection of oocytes and, more importantly, the manipulation of denuded oocytes during ICSI, where plausibly the inclusion of amino acids in the holding medium will decrease or prevent intracellular stress.

After compaction, non-essential amino acids and glutamine stimulate cleavage of the trophectoderm and increase blastocoel formation and hatching (52). In contrast, Eagle's essential amino acids, i.e., those required for somatic cell function, which are at lower concentrations in the oviduct, reduce the cell number of blastocysts from cultured zygotes (44,52). This inhibition of early cleavage development by essential amino acids has also been documented in the cow (133). Interestingly, however, after the 8-cell stage, essential amino acids stimulate cleavage rates and increase development of the inner cell mass in the blastocyst (52). Studies on the development of single amino acids on hamster embryos by Bavister and co-workers (67,128,134) found that asparagine, aspartate, glycine, histidine, serine, and taurine stimulated hamster zygote development to the blastocyst in culture, whilst cysteine, isoleucine, leucine, phenylalanine, threonine, and valine were inhibitory. All the inhibitory amino acids are present in Eagle's essential amino acids, whilst the stimulatory amino acids to hamster embryo development other than histidine are found in Eagle's non-essential amino acids. Evidently, the term essential and non-essential have little meaning with regards to embryology; rather, they have served as convenient groupings in the initial analysis of amino acids (44). Other terms such as "Cleavage amino acids" and "ICM amino acids" may be functionally more relevant (135). As research continues, other amino acids may be added to such groupings.

The beneficial effects of amino acids and glutamine on early embryo development have been proposed to come from their use not solely as energy substrates, but also as intracellular osmolytes (49,68) and

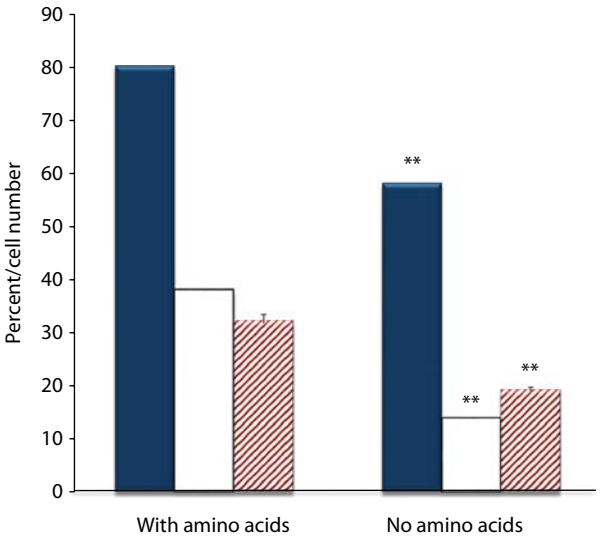

FIGURE 14.1 Effect of collection of CF1 mouse zygotes in medium without amino acids on subsequent development. Zygotes were collected in medium either (left) containing non-essential amino acids and glutamine or (right) in the same medium without the amino acids. Embryos were in the collection medium for less than 5 minutes. Solid bars represent morula/blastocyst development. Open bars represent blastocyst development. Shaded bars represent blastocyst cell number. **Significantly reduced compared to collection with amino acids ($p < 0.01$). (Data from Gardner DK, Lane M. *Hum Reprod*. 1996;11(12):2703–12).

regulators of intracellular pH (26,136). The use of amino acids as intracellular regulators is common amongst unicellular organisms, and the use of such amino acids such as glycine, alanine, serine, proline, and taurine by the pre-compacted embryo may well stem from the simplistic organization of individual cells within the embryo. Prior to compaction each cell is in direct contact with the external medium, whilst post-compaction the embryo has a transporting epithelium and can therefore actively regulate its internal environment. In support of this hypothesis is the observation that the beneficial effects of betaine and glycine in media containing a high sodium concentration is restricted to stages prior to compaction (32,60). Furthermore, it has been demonstrated that the formation of a transporting epithelium at compaction marks the ability of the embryo to regulate against an acid load. When the embryo is subsequently de-compacted experimentally, it loses its ability to regulate pHi (26).

Most importantly, amino acids have been reported to increase viability of cultured embryos from several species after transfer to recipients (37,51,98,137,138), as well as increasing embryo development in culture. In the mouse, culture with those amino acids present at high levels in the oviduct (i.e., those in the non-essential group) to the 6–8 cell stage prior to transfer significantly increased implantation rates and fetal development after transfer (131). In contrast, embryos cultured to the morula or blastocyst stage prior to transfer had greatest fetal development rates after culture with all 20 amino acids, confirming that the pre-implantation embryo undergoes a switch in amino acids requirements as development proceeds from the zygote to the blastocyst stage (somewhat similar to that observed for carbohydrates).

The addition of glutamine to a simple culture medium significantly increased development of human blastocysts in culture and increased subsequent pregnancy rates (139). Based on the available data, sequential media were formulated to support the human blastocyst in culture, G1 (pre-compaction) (37) and G2 (post-compaction) (140), to include the amino acids that stimulate cleavage stages for development prior to compaction, and include all of the amino acids that stimulate blastocyst formation and inner cell mass development for development post-compaction (37,140).

It was discussed earlier that the malate–aspartate shuttle is active in the embryo, and that through the addition of sufficient aspartate and lactate to the culture medium, pyruvate is no longer an obligatory nutrient for the early embryo (78). Further research determined that should the activity of this shuttle be inhibited in the cleavage stage, then subsequent blastocyst development and metabolism are compromised, culminating in impaired implantation and decreased fetal:placental weight ratio (141), thereby placing greater significance on the role of specific amino acids. Although the precise requirements for amino acids in human embryo culture have yet to be fully determined, it is evident that their inclusion is critical for optimal embryo development in culture and healthy fetal development.

Ammonium

Although amino acids in culture media regulate embryo development, their presence in culture media represents a potential problem as they spontaneously breakdown at 37°C, and are also metabolized by the embryo, both of which produce ammonium. Consequently, there is a build-up of embryo-toxic ammonium ions in the medium, a phenomenon which does not occur within the dynamic environment of the tract, as levels of ammonium are close to zero in oviduct fluid (117,142,143). Ammonium ions in the medium can not only alter embryo differentiation, metabolism, and gene expression in vitro (144,145), but can also significantly reduce implantation and fetal development rates after transfer (131). Of all amino acids, glutamine is the most labile. The substitution of this amino acid with the more stable alanyl-glutamine or glycyl-glycine significantly reduces the generation of ammonium in the culture medium (132). Significantly, clinical effects have been reported, showing that increasing concentrations of ammonium in the culture medium have a negative impact on human blastocyst development (146). More recent work has revealed that even a low concentration of <100 μM of ammonium can affect the metabolism of the cleavage stage human embryo, and further exposure to ammonium leads to slower cleavage divisions, culminating in abnormal blastocyst gene expression (Figure 14.2) (145). Furthermore, it appears that these perturbations in cellular function induced by ammonium are further compromised by its interaction with atmospheric oxygen (147).

Of note, it has been a matter of contention as to the level of concern one should place on ammonium toxicity in culture medium (148,149), even though data are readily available on the appearance

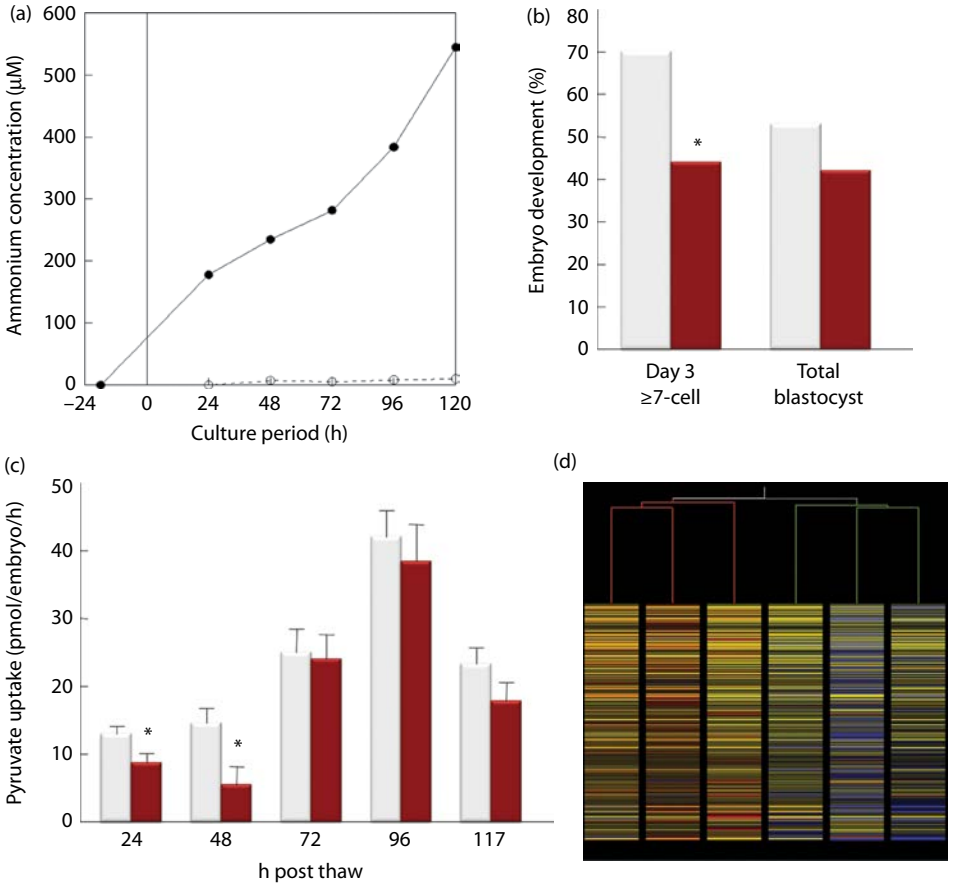

FIGURE 14.2 (a) Production of ammonium into the culture medium (lacking embryos) by the spontaneous breakdown of amino acids in culture media. Solid circles, KSOM^{AA}; open circles, G1/G2. The media were placed in the incubator at 4 pm the day before culture for equilibration purposes. The line at time zero represents when embryos would be placed into culture (although these measurements were taken in the absence of embryos). Medium KSOM^{AA} contains 1 mM glutamine and therefore releases significant levels of ammonium into the culture medium. Media G1/G2 do not contain glutamine, but rather the stable dipeptide form, alanyl-glutamine, and therefore these media do not release significant levels of ammonium. At a concentration of just 75 μmol/L, ammonium can induce a 24 h developmental delay in mouse fetal development by day 15 and induces the neural tube defect exencephaly in 20% of all fetuses (131). (b) Ammonium significantly reduces the development of the cleavage stage human embryo. Pronucleate oocytes were exposed to an increasing ammonium gradient consisting of 75, 150, 225, and 300 μM ammonium on successive days of culture. Control media (open bars), presence of ammonium (solid red bars); significantly different from no ammonium, $p < 0.05$. (c) Ammonium significantly compromises human embryo metabolism. Pyruvate uptake was significantly reduced by ammonium at 24 and 48 h of culture. Control media (open bars), presence of ammonium (solid red bars); significantly different from no ammonium, $p < 0.05$. (d) Ammonium significantly impairs human blastocyst gene expression. Heat map representation with hierarchical clustering of altered genes in human blastocysts following ammonium exposure and separation of control (green lines) and three ammonium (red lines) samples into distinct branches. Gene expression is related to colour, with red representing the highest levels of gene up-regulation and blue down-regulation. (Adapted from Gardner DK et al. *Reproduction*. 2013;146(1):49–61).

of ammonium in the culture medium over time (44,142,146,150). Of relevance to this discussion is the reported impact of culture media composition on the live birth rates and subsequent development of the children conceived (151,152). In these studies the effects of two commercial media were analyzed in a day 2 transfer program, and differences in embryo growth kinetics, subsequent birth weight (which persisted through the first 2 years of life) were documented. One of the two media employed during this analysis contained free glutamine, and hence embryos in this group (which exhibited the growth delay)

were likely exposed to a level of ammonium known to adversely affect human embryo development, gene expression and physiology. As such, the early exposure of embryos to ammonium may explain the effects of the two different media on subsequent birth rate and development.

The most effective means of alleviating the detrimental effects of ammonium on embryo development in vitro is the renewal of the culture medium after 48 h of culture. However, if this is not feasible, due to the wish to leave the embryos undisturbed during time-lapse, then it is essential to ensure that the lowest effective concentrations of amino acids are used (46).

Vitamins

Although vitamins are present in complex media formulations, their roles in supporting embryo development remain relatively unknown. Whilst both human (153,154) and mouse zygotes (10) will form blastocysts in culture in the absence of vitamins, the rabbit blastocyst requires vitamins for blastocoel expansion (155). However, Kane (155) found that B12, one of the vitamins present in tissue culture medium Ham's F-10, caused a decrease in blastocyst expansion. Furthermore, a distinction should be made between the type of blastocyst formed by the rabbit compared to that of the human and mouse. The rabbit blastocyst undergoes prolific expansion whilst the volume of the human and mouse blastocyst are only slightly larger than that of the oocyte. In the mouse, culture of zygotes to the blastocyst stage has been shown to be inhibited by the water-soluble vitamins present in both Ham's F-10 medium and MEM (156). Specifically, nicotinamide inhibited blastocyst cell number in vitro and reduced viability after transfer in the absence of amino acids (156). Interestingly, however, the vitamins present in MEM had no detrimental effect on mouse zygote development to the blastocyst stage when amino acids were also present (51). These data again highlight the interactions that exist between various medium components. Importantly, vitamins and amino acids act in synergy to prevent perturbations in metabolism and loss of viability induced by suboptimal culture conditions (27,28). As B-group vitamins are an integral part of carbohydrate and amino acid metabolism, certain vitamins may therefore have an important role to play in human embryo development and the regulation of metabolic function.

Nucleic Acid Precursors

The development of human and mouse zygotes to blastocysts in culture does not require the presence of nucleic acid precursors in the medium, although mouse embryos can incorporate exogenous radio-labeled nucleosides into their RNA and DNA (1). The ability of embryos to grow in the absence of nucleosides indicates that de novo pathways of nucleic acid synthesis are active at this stage. Loutradis et al. (157) observed that hypoxanthine, present in Ham's F-10, induced a block in mouse embryo development at the 2-cell stage in vitro. Hypoxanthine is thought to inhibit the purine salvage pathway (158). Subsequent studies have revealed that both adenosine and inosine are also detrimental to the development of mouse embryos after the first cleavage division (159). Without further research into the role of nucleotides in embryo development, their omission from embryo culture media formulations would currently seem advisable.

Chelators

The addition of chelators of heavy metal ions to culture media has been reported to enhance the development of pre-implantation embryos in vitro. Addition of ethylenediaminetetraacetic acid (EDTA) to the culture media increases the development of mouse zygotes beyond the 2-cell stage and increased development to the blastocyst stage (17,41,101,160,161,162). However, the stimulatory effect of EDTA was only evident at concentrations between 10 and 150 μM, whilst a concentration of 200 μM inhibited development to the blastocyst (162). Initial reports on the human embryo showed that the inclusion of EDTA to medium HTF without glucose and phosphate significantly increased the development of zygotes to the blastocyst stage in vitro (65). In light of these studies many new media formulated for the development of mammalian embryos in culture such as CZB (17), KSOM (18,42), G1 (37), and mHTF (65) contain EDTA.

Of clinical importance, the beneficial effect of EDTA on embryo development in vitro has been isolated to the cleavage stage embryo (99,101,163). The presence of EDTA at 100 µM in the medium after compaction significantly reduces fetal development after transfer (7,101). It is, therefore, apparent that whilst EDTA stimulates development prior to compaction, the presence of EDTA for blastocyst development compromises the subsequent developmental competence of the embryos. Furthermore, in the cow, the presence of 100 µM EDTA in the culture medium for development post-compaction specifically retarded development of the inner cell mass (ICM) (164). Interestingly, the ICM is dependent upon glycolysis for its energy production (165). Therefore, the detrimental effect of EDTA on ICM development may be explained by altered ICM energy production resulting in reduced fetal development, as ICM development is directly related with fetal development after blastocyst transfer (52). As analysis of the glycolytic enzyme 3-phophoglycerate kinase in 2-cell, 8-cell, and blastocyst stage embryos revealed that enzyme activity was significantly reduced by 10 µM EDTA as well as by 100 µM EDTA, it would be best to err on the side of caution and not expose blastocysts to EDTA (99). A recent report by Otsuki and colleagues describes an increased incidence of monozygotic twins derived from blastocysts, cultured in the presence of EDTA, which exhibited loose inner cell mass development. They attribute this phenomenon to the presence of the EDTA during blastocyst formation (166). Clearly, such observations warrant further investigation.

Another chelator of free metal ions, transferrin, has also been demonstrated to increase development of mouse zygotes through the 2-cell block to the blastocyst stage (167,168). It is proposed that transferrin increases embryo development by the chelation of ferric ion, thus preventing the formation of free oxygen radicals in the culture medium, which cause oxidative stress to the embryo. However, it has been shown that in a medium containing EDTA and non-essential amino acids and glutamine, the inclusion of transferrin did not increase mouse embryo development to the blastocyst stage (101).

Antioxidants

Oxygen required by cells for oxidative metabolism is also the source of oxidative stress. It has therefore been proposed that one of the causes of impaired embryo development in culture and subsequent loss of viability is oxidative stress (169). Of note, the fluids within the female reproductive tract include an elegant antioxidant system to provide protection and repair against such oxidative stress (169). There have been several studies examining the effects of known antioxidants on pre-implantation embryo development over the past two decades, although data to date for individual antioxidants remain rather contradictory. Supplementation of medium with superoxide dismutase (SOD), which dismutases superoxide radicals, increased the development of mouse zygotes beyond the 2-cell block to the blastocyst stage (170,171). However, several studies have reported that SOD had no effect on either mouse (172), rabbit (173), or bovine (174) embryo development in vitro.

Similarly, Legge and Sellens (175) reported that addition of glutathione to the medium stimulated development of mouse zygotes in culture, whereas Nasr-Esfahani and Johnson (176) reported that the addition of glutathione to the medium did not increase embryo development in culture. However, glutathione is only protective as an antioxidant when in its reduced form (GSH), and GSH is not particularly stable in solution, and hence reports that glutathione has no effect may be confounded by its breakdown in an aqueous solution. Glutathione is present in the fluid of the reproductive tract and therefore may feasibly play a role in embryo development (177). Moreover, the beneficial effects of the addition of cysteamine to the medium for bovine (178–181) and pig (182) oocyte development have been attributed to an increase in intracellular glutathione levels (183). Therefore, it is highly probable that the maintenance of a high intracellular pool of glutathione sustained through the addition of its precursors and/or other antioxidants, such as lipoate (which can convert glutathione to its reduced form upon oxidation) (184), may be more important for high rates of development of the oocyte and early embryo rather than the addition of GSH to culture media. The addition of the water soluble antioxidant ascorbate has been shown to be highly beneficial when added to media used in the slow freezing of embryos (185). Presumably, the presence of such an antioxidant is beneficial in reducing the impact of reactive oxygen intermediates generated during the freezing process. However,

ascorbate (like GSH) is highly labile once in solution, and for its beneficial effects to be conferred it must be added to the medium immediately prior to use.

Interestingly it has been shown that pyruvate, present in all embryo culture media, is a powerful antioxidant (186), and readily decreases intracellular hydrogen peroxide levels within the embryo (187,188). As pyruvate is present in all media for embryo development, embryo culture media are supplemented with an antioxidant by default. Similarly, the amino acid taurine, present in such media as G1 (37,140) and P1 (16), may also serve as an antioxidant (189).

The conflicting reports as to the benefits of the addition of antioxidants to culture media may in part be explained by their use in isolation and not as part of a more complete antioxidant system. For example, when SOD is present to dismutate superoxide radicals to hydrogen peroxide, then catalase and/or glutathione may be required to remove the peroxide formed. The presence of more than one antioxidant may therefore facilitate the cycling of antioxidant back into the reduced forms. Recent work has revealed that the proposed approach of employing groups of, rather than individual, antioxidants can indeed have a profound effect on the development and viability of mouse embryo culture at 20% oxygen. Troung and colleagues examined the individual and combined effects of acetyl-L-carnitine, acetyl-L-cysteine, and α-lipoic acid, and confirmed that while each antioxidant conferred a significant beneficial effect, there was a synergy when the three antioxidants were present as a group (Figure 14.3) (190). Furthermore, the presence of this group of antioxidants also conferred benefit to embryos cultured at 5% oxygen, although the absolute levels of increase in development was not as large compared to embryos exposed to atmospheric oxygen. Of note, when embryos were cultured individually, compared to those in groups, atmospheric oxygen had a greater effect on embryo development, confirming the findings of Kelley and Gardner (191), whereby one stress, e.g., atmospheric oxygen, predisposes the embryo to greater

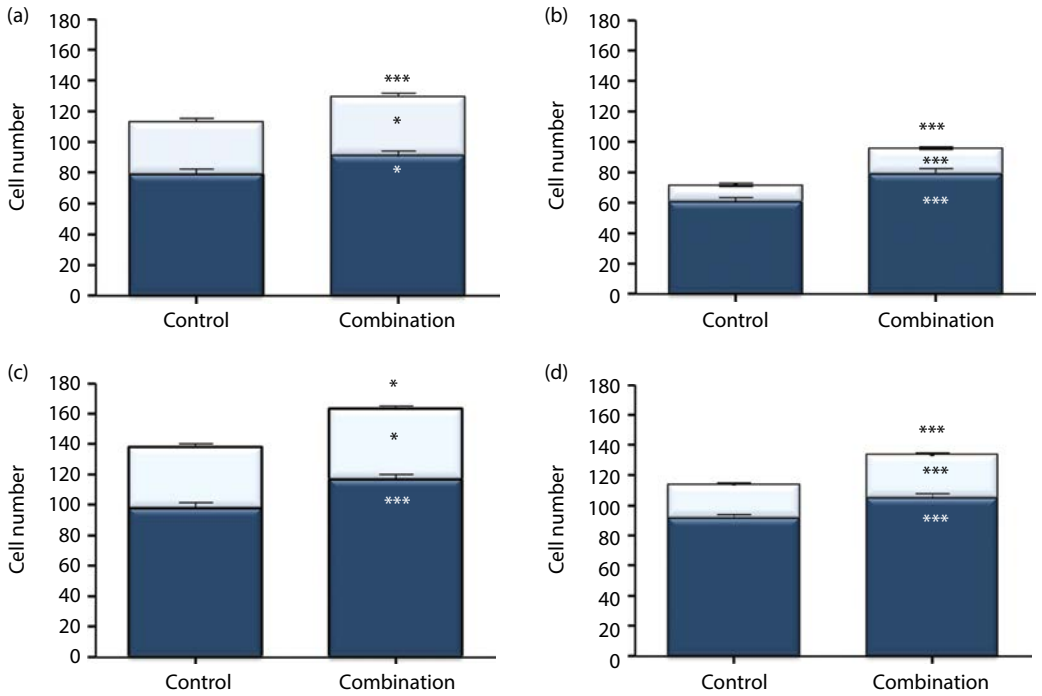

FIGURE 14.3 Effect of antioxidants in combination (acetyl-L-carnitine, acetyl-L-cysteine, and α-lipoic acid) on blastocyst cell numbers and allocation in embryos cultured in groups and individually at 5% and 20% oxygen. Light and dark bar portions represent the average ICM and TE cells, respectively. Control, embryos cultured without antioxidants; Combination, embryos cultured with 10 μM acetyl-L-carnitine, 10 μM acetyl-L-cysteine and 5 μM α-lipoic acid. (a) Embryos cultured in groups in 20% oxygen. (b) Embryos cultured individually in 20% oxygen. (c) Embryos cultured in groups in 5% oxygen. (d) Embryos cultured individually in 5% oxygen. *$p < 0.05$; ***$p < 0.001$. (Adapted from Truong TT, Soh YM, Gardner DK. *Hum Reprod*. 2016;31(7):1445–54, with permission).

susceptibility to a second stress, e.g., individual culture. These data are in line with the double stress hypothesis presented by Wale and Gardner (147,192). Of potential significance to clinical IVF was the finding that the benefits of the above group of antioxidants were also manifest post-transfer, with higher fetal size and weights reported for embryos cultured in the presence of antioxidants (190). Such exciting data now require clinical validation through prospective randomized trials.

Antibiotics

Traditionally, antibiotics such as penicillin, streptomycin, or gentamycin have been routinely included in embryo culture medium. However, a study reported improved cleavage rates of human embryos in medium free of antibiotics, questioning the practice of routinely adding antibiotics to the culture medium (193). However, it is important to note that the washing of embryos in medium supplemented with antibiotics can remove any bacterial contamination (194), and this may be an important consideration for a clinical setting. In contrast, there can be no debate that the inclusion of antibiotics in medium for the preparation of sperm is a necessity.

Protein

Serum

Historically, the most commonly used protein source in human IVF and embryo culture was patient's serum, added to the culture medium at a concentration of 5%–20%. In some programs fetal cord serum has been used in preference (23). The use of serum in embryo culture medium has several inherent drawbacks: the expense and time required for its collection and processing (and screening of the fetal cord serum) and the risk of infection to the laboratory staff and embryos. Furthermore, proteins in serum have macromolecules attached, such as hormones, vitamins, and fatty acids, as well as chelated metal ions and pyrogens (195,196). As the concentration of such macromolecules and other serum components varies between patients, and even within the menstrual cycle, it makes any comparison between batches of medium which contain serum almost impossible. Furthermore, serum from several groups of patients, such as those with endometriosis, polycystic ovarian syndrome, or unexplained infertility, appears to be embryo-toxic (197–201). There are several reasons for the elimination of serum from mammalian embryo culture systems. From a physiological perspective, the mammalian embryo is never exposed to serum in vivo (202). The fluids of the female reproductive tract are not simple serum transudates (203), but rather specialized environments for the development of the embryo (25). Serum can best be considered a pathological fluid formed by the action of platelets. More disturbing, however, is the evidence that serum is detrimental to the developing mammalian pre-implantation embryo in culture. Studies on the embryos of mice, sheep, and cattle have demonstrated that serum in the culture medium induces morphological, metabolic, epigenetic, and ultrastructural changes in blastocysts cultured from the zygote stage. The trophectoderm of such blastocysts develops a vesicular appearance due to the sequestering of lipid in the blastomeres (37,98,129,204,205).

Furthermore, when ruminant pronucleate oocytes were cultured to the blastocyst stage in the presence of serum they possessed mitochondria with abnormal folding of the cristae (129,204). Such blastocysts exhibit elevated levels of lactate production, plausibly associated with mitochondrial damage and impaired oxidative capacity (98). Finally, the inclusion of serum in the culture medium is associated with the birth of abnormally large lambs after the transfer of blastocysts to recipient ewes (129,206). Such data is of great concern and the mechanism(s) by which serum imparts such detrimental effects is the focus of much research. The overexpression of certain growth factor genes in this phenomenon is a plausible mechanism (34,207,208). For example, fetal overgrowth in the sheep following embryo culture in the presence of serum has been associated with a decreased expression of M6P/IGF-IIR through loss of methylation (208). M6P/IGF-IIR has a role in fetal organogenesis. Interestingly, this locus, although imprinted in mice, sheep, and cows, is not imprinted in the human (209). Subsequently, this specific absence of imprinting may mean that the human embryo is less susceptible to this particular epigenetic

disturbance. This may therefore explain why Menezo and colleagues (210) did not report any adverse effects in children following blastocyst co-culture in the presence of serum, but that studies on mice, sheep, and cattle have all revealed long-term effects on embryos cultured in the presence of serum.

So why has serum been included in human embryo culture media? Undoubtedly the main reason for the inclusion of serum in media used in human IVF is the limited ability of simple salt solutions and tissue culture media to support embryo development in the absence of serum. In a suboptimal medium serum can act as a chelator and a buffer to minimize pH fluctuations when the medium is outside of a CO_2 environment. It may serve to supplement simplistic media with known regulators of embryo development, such as amino acids, whilst, when added to more complex tissue culture media such as Ham's F-10, it may help by binding the embryo toxic transitional metals present. However, with the development of more physiological embryo culture media and the inclusion of appropriate chelators, the requirement for serum in embryo culture has been eliminated (7,37,140,202,211). Serum should now reside only in the annals of embryo culture, and certainly not in the media.

Human Serum Albumin

Protein can be added to culture media in the form of serum albumin. The addition of a macromolecule such as serum albumin prevents gametes and embryos from becoming "sticky," whereby their surface charges make them stick to both glass and plastic. One of the roles of macromolecules, therefore, is to facilitate gamete and embryo manipulation. Furthermore, albumin can negate the effects of toxins (212). Human serum albumin (HSA) has been used successfully in the culture of human embryos (202,213–215). Several commercially available serum products have been used to great success in replacing serum in human embryo culture systems. These range from therapeutic albumin solutions (213,215,216) to globulin enriched albumin solutions such as Plasmanate (217), Plasmatein (218), and Synthetic Serum Substitute (SSS) (219–221). Of the latter products, SSS appears to be the most effective, containing 84% HSA and 16% α- and β-globulins, with less than 1% γ-globulin. It has been proposed that the glycoprotein components of serum (α- and β-globulins) have a role in supporting embryo development in culture. Glycoproteins, which possess numerous hydroxyl groups, may confer benefit to the embryo by altering the solvent properties of the medium, making it more akin to the tubal environment (218,222). However, there have been no prospective randomized trials using such supplements.

Although serum albumin is a relatively pure fraction, it is still contaminated with fatty acids and other small molecules (223), transition metals (224,225), as well as many components which are poorly characterized. Albumin has been shown to contain an embryotrophic factor, which stimulates cleavage and growth in rabbit morulae and blastocysts. This has been determined to be citrate (226). Not only are there significant differences between sources of serum albumin (227,228), but also between batches from the same source (227,229). Furthermore, some HSA preparations contain the preservative sodium caprylate, which binds to the hydrophobic domains of the proteins and, therefore, cannot be removed by dialysis. The effects of such a preservative on embryo development have yet to be determined. Further, the processing of albumin can also lead to the introduction of known chemicals such as octanoic acid, which has been shown to be detrimental to embryo development (230).

As well as the concerns raised above relating to the purity and efficacy of serum albumin, there are now concerns that serum albumin is the source of detectable levels of di(2-ethylhexyl)phthalate and mono(2-ethylhexyl)phthalate as well as polybrominated diphenyl ethers in human embryo culture media (231,232). Such data infer that the use of serum albumin in human IVF warrants renewed consideration, and further clinical studies on alternatives such as recombinant albumin (which eliminate the problems inherent with using blood derived products including lot-to-lot variability) should be considered.

Recombinant Albumin

Recombinant human albumin has been shown to be an effective replacement for serum derived albumin in animal IVF and embryo culture (9,233), and its clinical efficacy has been validated in a prospective randomized trial and found to be equally as effective as human serum albumin in supporting IVF, embryo development in vitro, and subsequent pregnancies (234). Furthermore, the inclusion of recombinant human albumin appears to confer increased cryotolerance to bovine embryos cultured in its

presence (9), and supports equivalent cryo survival in the human (235). Given the growing concerns surrounding the safety of human IVF, it would appear that evaluation of recombinant albumin, to minimize risk and to increase consistency of function, much like the move from urinary to recombinant gonadotropins, is overdue. When the use of recombinant albumin was first considered, the cost of the recombinant protein was almost one hundred times that of serum-derived albumin. However, over the past two decades, the costs for such recombinant materials has decreased considerably, making the inclusion of recombinant albumin in human IVF and embryo culture media a more feasible proposition. Hence, it is time to re-evaluate its use clinically, and prospective randomized trials are required to establish its role in human IVF.

Glycosaminoglycans

Hyaluronan, a glycosaminoglycan, is present in the female reproductive tract, and its concentration increases at the time of implantation in the mouse (236). Hyaluronan is a high molecular mass glycosaminoglycan that can be readily obtained endotoxin- and prion-free from a yeast fermentation procedure. Hyaluronan can not only substitute for albumin when added to the culture medium, but can also act in synergy with serum albumin to further increase mouse blastocyst development in culture (237). The addition of hyaluronan to the culture medium has also been shown to increase blastocyst development in porcine embryos (238) and increase the cryotolerance of embryos (239–241). Perhaps of greatest significance, however, is the finding that the addition of hyaluronan to embryo culture medium significantly increases mouse blastocyst implantation and fetal development after transfer (237). Interestingly, this increase in post-transfer outcome could be attributed to the presence of hyaluronan in the medium used for transfer (Figure 14.4). These data have since been repeated clinically, and in the largest prospective trial to date, which enrolled 1282 cycles of IVF, it was determined that the use of hyaluronan-enriched medium was associated with significant increases in clinical pregnancy rates and implantation rates, both for day 3 and day 5 embryo transfers. The beneficial effect was most evident in women who were >35 years of age, in women who had only poor-quality embryos available for transfer, and in women who had previous implantation failures (241). A recent Cochrane report confirmed improved pregnancy and take home baby rates when hyaluronan is included in the transfer medium (242).

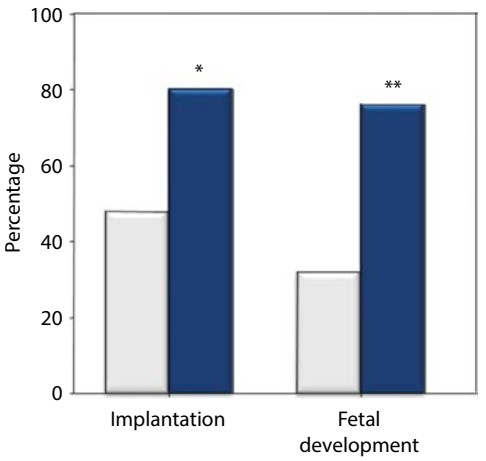

FIGURE 14.4 Effect of hyaluronan in the transfer medium on subsequent viability of cultured mouse blastocysts. Mouse zygotes were cultured to the blastocyst stage in sequential media DM2/DM3 without protein or macromolecule supplement. Blastocysts were placed in either medium DM3 or DM3 containing hyaluronan and transferred to pseudopregnant recipients and subsequent viability was assessed. Open bars represent medium DM3 without macromolecule. Solid bars represent medium DM3 supplemented with hyaluronan. *Significantly different to medium DM3 ($p < 0.05$). **Significantly different to medium DM3 ($p < 0.01$). (Data from Gardner DK, Rodriegez-Martinez H, Lane M. *Hum Reprod*. 1999;14(10):2575–80).

Hormones, Cytokines, and Growth Factors

Although the mouse blastocyst is capable of metabolizing exogenous steroid hormones (243), there is limited data on the direct action of hormones on the early embryo (244–247). Certainly, estradiol appears to have a direct negative impact on mouse embryo development (248,249). However, prolactin at a concentration of 300 ng/mL has been shown to improve the rate of blastocyst formation from cultured 2-cell mouse embryos (250). Available evidence indicates that the effects of maternal hormones on the developing embryo are mediated through the cells of the reproductive tract (251), and therefore their direct inclusion in embryo culture media does not appear to be justified.

The role of cytokines and growth factors in the development of the pre-implantation mammalian embryo has been the subject of intensive research over many years (reviewed by Thouas et al. (252)). In the human, early studies by Martin et al. demonstrated that adding heparin-binding epidermal growth factor to the culture medium significantly increased both blastocyst development and subsequent hatching (253). Similarly, the addition of IGF I stimulated human blastocyst formation and increased development of the inner cell mass (254). A recent proteomic analysis of human uterine fluids has confirmed the presence of an extensive list of cyotkines and growth factors (255). Furthermore, the levels of specific factors such as vascular endothelial growth factor (VEGF) have been shown to change with both stages of the cycle and fertility status. This is of great interest given that VEGF has been shown to affect pre-implantation and post-implantation development (255,256). In spite of such data, cytokines and growth factors remain conspicuously absent from clinical embryo culture media. An exception to this is a study on the effects of granulocyte-macrophage colony-stimulating factor (GM-CSF) (257). However, it was reported that GM-CSF only had a beneficial clinical effect when the levels of HSA were reduced in the medium (257), an observation previously reported in the mouse model (258). Therefore, further research on the effects of such factors at the physiological, genomic, and proteomic levels is required in order to lead to a better understanding of their role in human IVF media (252,259,260). Further, it is envisaged that such factors should not be included in embryo culture media in isolation, but rather be present as a functional group of factors (259). Clearly the long-term follow up of any children born after in vitro exposure to such factors is important.

Buffer System

Most embryo media utilize a bicarbonate/CO_2 buffer system to maintain a physiological pH of between 7.2 and 7.4 in the medium. The inclusion of sodium bicarbonate in the medium requires the use of a CO_2 incubator to maintain a 5%–6% CO_2 atmosphere. An advantage of the bicarbonate/CO_2 system is that it is the physiological buffering system in fluid surrounding mammalian cells. A major drawback, however, is the rapid pH increase that occurs when medium is exposed to air, resulting in impaired embryo development or even cellular necrosis if prolonged. A practical solution to this problem is the use of an oil overlay to reduce gas exchange when culture dishes are taken out of the incubator, e.g., for embryo scoring. This approach only delays and does not prevent changes in pH, and is not feasible for oocyte collection or prolonged embryo manipulation. A possible alternative, therefore, is the use of phosphate-buffered medium, which does not require a CO_2 environment to maintain its pH in air. Unfortunately, phosphate buffered media appear to be detrimental to embryo development in vitro (261–263). The detrimental effect of phosphate on embryo development is exacerbated in a simple medium, such as phosphate buffered saline (PBS), i.e., the reduced rates of development associated with phosphate would be at its greatest in a medium such as PBS. Furthermore, during cryopreservation procedures, PBS has limited buffering capacity, therefore rendering the embryos liable to pH stress. Therefore, the use of PBS for embryo collection, manipulation, or cryopreservation should be avoided. An alternative to a phosphate buffer system is N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES) (264). HEPES has been used successfully as a buffer in media for human oocyte collection and embryo handling. In such media it is usual to replace 20 mM bicarbonate with HEPES, leaving 5 mM bicarbonate (265). The rationale for this is that embryos require bicarbonate for development (76,263). At lower

concentrations of HEPES (<20 mM), the bicarbonate competes with the HEPES, which subsequently loses its buffering capacity as the bicarbonate dissociates and consequently increases the pH (266). More recently another zwitterionic buffers 4-morpholinepropanesulfonic acid (MOPS) (264) has been used successfully for embryo handling and manipulation. Both HEPES and MOPS have been used to great effect in human IVF.

Oxygen

The concentration of oxygen in the lumen of the rabbit oviduct is reported to be 2%–6% (267–269), while the oxygen concentration in the oviduct of hamster and rhesus monkey is similar at 8% (269). Of note, the oxygen concentration in the uterus is significantly lower than in the oviduct ranging from 5% in the hamster and rabbit to 1.5% in the rhesus monkey (269,270).

Numerous studies on different mammalian species have demonstrated that culture at a reduced oxygen concentration results in enhanced development in vitro. In the mouse, culture at oxygen concentrations as low as 1% were sufficient to support embryo development to the blastocyst stage (271). It has been established that a reduced oxygen concentration between 5% and 8% enhances development to the blastocyst stage in the mouse (101,171,272), the rabbit (273), and in domestic animal species such as sheep (274), goat (227), and cow (274), compared to 20% oxygen. Similarly, it has been observed that human blastocysts cultured in a low oxygen environment (5%) have significantly more cells than those cultured in a high oxygen environment (20%) (132). Even a transient exposure for 1 h to 20% oxygen reduced mouse embryo development in vitro (271). It has subsequently been determined that equilibrating culture dishes at 20% oxygen for 5 h prior to culture in 7% oxygen decreases mouse zygote development to the blastocyst stage and resultant blastocyst cell numbers. This inhibition can be attributed to the fact that it takes more than 5 h for the oxygen concentration to fall to embryo-safe levels (101). The negative impact of atmospheric oxygen on the pronucleate oocyte can be observed as early as the first cleavage division, which is delayed compared to embryos cultured in 5% oxygen (275). Two time-lapse analysis studies, one on the mouse, and a follow-up study on the human, have both revealed the sensitivity of the cleavage stage embryo to atmospheric oxygen (275,276). Furthermore, the detrimental effects of atmospheric oxygen are not restricted to embryo development either before or after compaction, but rather exposure to atmospheric oxygen during any phase of pre-implantation development has a significant detrimental effect. The damaging effect cannot be reversed by subsequently reducing the oxygen concentration down to physiological levels (Figure 14.5).

Animal models have revealed the extent of cellular damage done by embryo culture in 20% oxygen; resultant blastocysts exhibit an altered gene expression and perturbed proteome compared to embryos developed in vivo (34,277,278). In contrast, culture in 5% oxygen had significantly less effect on both embryonic gene expression and proteome. Similarly, 20% oxygen has been shown to adversely affect embryonic metabolism (33). More recent data has revealed that not only does 20% oxygen compromise the ability of the embryo throughout the pre-implantation period to utilize both carbohydrates and amino acids (279), but also that atmospheric oxygen impairs the ability of the embryo to regulate against ammonium stress (147). Therefore, not only does oxygen induce its own trauma on the embryo, but also increases the embryo's susceptibility to other stressors present in the culture system or laboratory, as described above for individual embryo culture (190–192). Furthermore, atmospheric oxygen has recently been linked to changes to the embryonic epigenome (reviewed by Wale and Gardner (192) and Gardner (280)).

In support of the utilization of physiological levels of oxygen in human embryo culture, clinical data including a randomized controlled trial, demonstrated that more physiological conditions increased both implantation and live birth rates (281–284). However, in spite of the animal and clinical data describing the detrimental effects of atmospheric oxygen, it has been reported in a recent online survey, in which 265 clinics from 54 different countries participated, that <25% of IVF human embryo culture is performed exclusively under physiological (~5%) oxygen (285). Although this survey represents only a small fraction of the world's IVF clinics, what is notable from an extensive literature review of the past 10 years is a clear geographic difference with regard to the use of 5% oxygen. The authors of this chapter

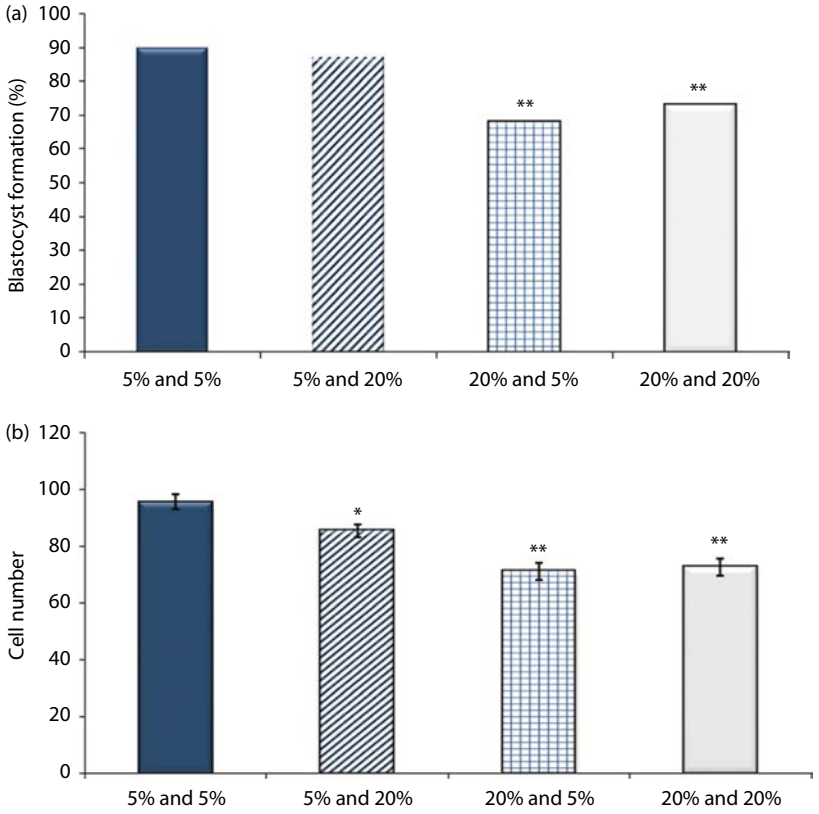

FIGURE 14.5 Atmospheric oxygen is damaging to the mammalian embryo before and after compaction. Pronucleate mouse oocytes were cultured for 48 h in medium G1 in the presence of either 5% or atmospheric (~20%) oxygen. After 48 h the resultant embryos were transferred to medium G2 and either exposed to the same oxygen concentration or the alternate oxygen concentration, making a total of four treatment groups. (a) Blastocyst development was significantly impaired if embryos were exposed to 20% oxygen during the first 48 h of culture. (b) If embryos were exposed to 5% oxygen for the first 48 h, blastocyst cell number was significantly reduced if they saw 20% for the second 48 h of culture, compared to the group exposed to 5% throughout culture. Should embryos be exposed to 20% oxygen for the first 48 h of culture, there was a significant decrease in blastocyst cell number, which could not be rescued by exposing embryos to 5% for the second 48 h of culture. $*p < 0.05$; $**p < 0.01$. (Data from Wale PL, Gardner DK. *Reprod Biomed Online.* 2010;21(3):402–10).

presented a case for the clinical introduction of physiological oxygen in human IVF over 25 years ago (286). In the intervening two decades the rationale for the discontinuation of atmospheric oxygen has become compelling, and the justification for employing atmospheric oxygen in a clinical IVF laboratory is non-existent. One could now go as far as to say that the use of atmospheric oxygen for human IVF is unethical.

Carbon Dioxide

Carbon dioxide is not only required to maintain the pH of bicarbonate buffered medium, but is readily incorporated into protein and nucleic acids by the mouse embryo at all stages prior to implantation (287). Culture systems for the pre-implantation mammalian embryo routinely employ a carbon dioxide concentration of 5% coupled with a bicarbonate concentration of around 25 mM. However, analysis of the uterine environment in the guinea pig on day 4 of pregnancy determined the carbon dioxide concentration to be 10% (288). Studies on the hamster 8-cell embryo showed that development to the blastocyst

stage is increased by a carbon dioxide concentration of 10% (55,289). Similarly, rabbit zygote development to the hatching blastocyst stage is increased in a carbon dioxide concentration of 10% compared to 5% (290,291). It has been proposed that the beneficial effects of a high carbon dioxide concentration is due to a decrease in the pH of the medium as the beneficial effects of increased carbon dioxide could be replicated with a weak acid (289). The pH of media containing 25 mM bicarbonate that is equilibrated in a CO_2 environment can be calculated using the Henderson–Hasselbach equation. Interestingly, at 5% CO_2, the pH of medium containing 25 mM bicarbonate is 7.45, while a CO_2 concentration of 6% is required to maintain the external pH of the medium at around 7.4. The optimal concentration of carbon dioxide for human embryo development has yet to be determined; however, the CO_2 concentration is related to the pH of the medium, which also needs to be considered. Typically, Fyrite has been employed to quantitate CO_2 within the incubation chamber used. However, the accuracy of such a procedure is rather low. Fortunately, there are now available new hand-held infra red (IR) CO_2 analysis meters with very high accuracy (to 0.1%).

pH

The pH of fluid collected from the reproductive tract of the rhesus monkey was reported to alter in parallel with changes in the bicarbonate concentration increasing from 7.1–7.3 during the follicular phase of the estrus cycle to 7.5–8.0 at the time of ovulation and during the luteal phase (292). Dale et al. (293) reported that the pH of the uterine fluid is lower than that of the oviduct. As already eluded to, the external pH of culture media formulated for pre-implantation embryos is commonly between 7.3 and 7.4 (depending on the CO_2 concentration used). Studies on the mouse embryo determined that development from the 2-cell stage to the blastocyst stage could occur in media with a pH range from 5.9 to 7.8 (66). Similarly, hamster 8-cell embryos could develop to the blastocyst stage in medium with an external pH range from 6.4 to 7.4 (289). In contrast, however, it has been shown that a transient exposure of zygotes and 2-cell mouse embryos to medium with elevated pH significantly reduced subsequent development to the blastocyst stage (294). Although these studies demonstrate that some embryos could develop to the blastocyst stage in a wide range of external pHs, the subsequent viability of these embryos following transfer is unknown. Consequently, there has been considerable work in the area of intracellular pH (pHi) and its role in regulating embryo development. It has been established that even relatively small fluctuations in pHi can significantly retard subsequent developmental competence. Fluctuations in either the acidic (84,295) or the alkaline (296) range can drastically reduce development. Even more significantly, it has recently been determined that mammalian oocytes and embryos for around 6 h following fertilization lack any functional transport systems to regulate pHi in either the acid (295) or the alkaline ranges (295,297). Therefore, care should be taken to avoid fluctuations in the pH of media during embryo manipulation and culture. This is especially relevant for oocytes that are stripped of their cumulus before an ICSI procedure. These oocytes and embryos immediately following the procedure do not appear to be able to regulate their ionic homeostasis. Amino acids are also known to increase the intrinsic buffering capacity of the embryo and reduce fluctuations in the pHi of the embryo. Therefore, as discussed earlier, addition of amino acids to the culture or handling medium can also help to reduce pHi fluctuations (26). It is also important to consider the relationship between the pH of the culture medium (pHo) and the pHi of the embryo; over a pHo range of 7.0–7.6, the pHi of the mouse zygote remained at 7.17. Rather than simply pHo affecting pHi directly, it is the presence of weak acids or bases in the culture medium which dramatically affect pHi (84).

The pH of a CO_2/bicarbonate buffered medium is not easy to quantitate. A pH electrode can be used, but one must be quick, and the same technician must take all readings to ensure consistency. Solid state probes are now available with a higher degree of accuracy. An alternative and highly accurate approach is to take samples of medium and measure the pH with a blood–gas analyzer. A simple and reliable method of checking the pH of a medium is to use color standards; however, this requires the presence of phenol red in the medium. The color standards use 0.067 M solutions of potassium phosphate and sodium phosphate. These solutions are then added together in varying quantities to produce solutions of the required pH. The preparation of such standards is shown in Table 14.3.

TABLE 14.3

Preparation of Color Standards for pH of Media

pH at 18°C	Solution A (mL)	Solution B (mL)
6.6	62.7	37.3
6.8	50.8	49.2
7.0	39.2	60.8
7.2	28.5	71.5
7.4	19.6	80.4
7.6	13.2	86.8

Note: Stock A: 9.08 g KH_2PO_4 (0.067 M), 10 mg phenol red in one liter of water. Stock B: 9.46 g Na_2HPO_4 (0.067 M), 10 mg phenol red in one liter of water. Measure the pH with meter, and adjust pH as required. i.e. add solution A to lower the pH (make more acidic), add solution B to increase the pH (make more alkali). The pH standards should be filter-sterilized and can then be kept for up to 6 months.

Temperature

Temperature fluctuations at the early cleavage stages have been demonstrated to decrease the subsequent development potential of embryos in vitro. Exposure of mouse zygotes to room temperature for just 5 minutes inhibited cleavage rates. Increasing the exposure time to 10 and 15 minutes further decreased cleavage rates and reduced development to the blastocyst stage such that blastocyst development was half of the control after 15 minutes at room temperature (294). Exposure of rabbit cleavage stage embryos to room temperature of 3 h also decreased cleavage rates as assessed by thymidine incorporation and development to morulae and blastocyst stages (298). Exposure of human oocytes to room temperature has been reported to induce damage to the meiotic spindle (299). It would therefore seem advisable to maintain a constant temperature of 37°C when handling human oocytes and embryos. Of note, when setting the temperature of any heated stage or tube warmer, it is imperative to establish the actual temperature of the medium within the dish/tube itself. For example, many dishes have lips of plastic on the bottom, so the actual base of the dish itself does not come into contact with the heated stage. Consequently, there will be an air gap, which will result in loss of thermal conductivity, and in such cases even though the temperature of the stage is 37°C, the actual temperature of the medium in the culture dish could be as low as 34°C.

Incubator/Incubation Chamber/Time-Lapse

As discussed above, for the development of human embryos in vitro it is important to maintain both the pH of the medium and temperature for optimal development. The choice and use of incubators is therefore paramount for the success of an IVF program. It has been documented that embryo development in vitro is increased by restricting the opening of an incubator (101). Furthermore, the use of IR sensor incubators which restore CO_2 levels within 2–3 minutes helps to alleviate the detrimental effect of repeated opening of the incubator, as opposed to incubators which employ thermo-couple CO_2 sensors, which are dependent upon incubator humidity to determine concentration, and hence are typically slow to recover. Alternatively, the use of modular incubator chambers (MICs) inside the incubator enable the gas phase that embryos are cultured in to remain constant. Mouse embryo development in MICs at the same gas phase as the incubator was significantly increased compared to development in the main chamber of the incubator (101). The use of incubators that have multiple chambers within the single incubator chamber can also reduce fluctuations in temperature and CO_2 levels induced by frequent door openings.

Alternatives to classic tissue culture incubators are mini incubators with constant flow chambers, which allow for direct heat transfer between the chamber and culture vessel. Such chambers also allow for a direct flow of pre-mixed gas, and therefore minimize changes in pH. More recently, such chambers

have evolved to include time-lapse capability, facilitating the constant monitoring of embryos without the need to remove them from their culture environment (300,301). Consequently, this approach has been shown to have inherent advantages for embryo development, by minimizing handling and variations in temperature and pH (192,302).

What is evident is that it is imperative to have sufficient numbers of incubator chambers to match the caseload. This is especially true when performing extended culture. It is important to consider the number of times an incubator will be opened in a day and to keep this to a minimum. It is advisable to have separate incubators for media equilibration and for embryo culture, thereby minimizing the amount of access to incubators containing embryos. It can also be useful to have a mixture of incubator chambers for overnight or longer term cultures, and bench-top models which recover quickly for manipulations such as denuding and ICSI.

Light

Several studies have investigated the effect of exposure to visible light on the development of mammalian pre-implantation embryos in vitro. Collection and culture of embryos from the hamster (303) and rabbit (298,304) under low illumination increased development and cleavage in vitro. Furthermore, exposure to light predisposed rabbit embryos to temperature stress, a further example of one stress predisposing the embryos to a second, culminating in compromised development. Exposure of hamster oocytes for 1 h to visible light prior to insemination disrupted the completion of meiosis and fertilization (305). In the human, implementation of an oocyte collection system employing low light at low oxygen concentration resulted in significantly increased rates of blastocyst formation of spare embryos and increased pregnancy and live birth weights when embryos were transferred on days 2–4 of culture (306). Therefore it would seem prudent to perform all oocyte and embryo collections and manipulations under low illumination.

Incubation Volume/Embryo Grouping

Within the lumen of the female reproductive tract the developing embryo is exposed to microlitre volumes of fluid (203). In contrast, the embryo grown in vitro is subject to relatively large volumes of medium of up to 1 mL (307). Consequently, any autocrine factor(s) produced by the developing embryo will be diluted and may therefore become ineffectual. It has been demonstrated in the mouse that blastocyst formation and cell number increase when embryos are grown in groups (up to 10) or reduced volumes (around 20 µL) (58,308–310). Similar results have been obtained with sheep (98) and cow embryos (311,312), and more recently positive effects of culturing embryos in groups has been reported for the human (313), although other clinical studies failed to observe any effects (314). Overall, it does appear that the pre-implantation mammalian embryo produces a factor(s) capable of stimulating development of both itself and surrounding embryos (Figure 14.6), therefore indicating that the number of embryos per drop and/or incubation volume are important parameters in determining IVF outcome. Furthermore, it has recently been reported that atmospheric oxygen impairs the ability of mouse embryos to develop during individual culture, with cleavage delay being induced, culminating in a resultant decrease in both inner cell mass and trophectoderm development in the blastocyst (191).

In order to culture in such reduced volumes (of 20–50 µL) an oil overlay is required. Although the use of an oil overlay is time-consuming, it prevents the evaporation of media, thereby reducing the harmful effects of increases in osmolality, and reduces changes in pH caused by a loss of CO_2 from the medium when culture dishes are taken out of the incubator for embryo examination.

Culture System

Optimization of embryo development in vitro is not only dependent upon the composition of the culture medium or media used, but is also affected by physical parameters, such as the incubation environment and gas phase. Therefore, it is important to consider a holistic analysis and the "culture system" when attempting to improve embryo culture media efficacy (Figure 14.7).

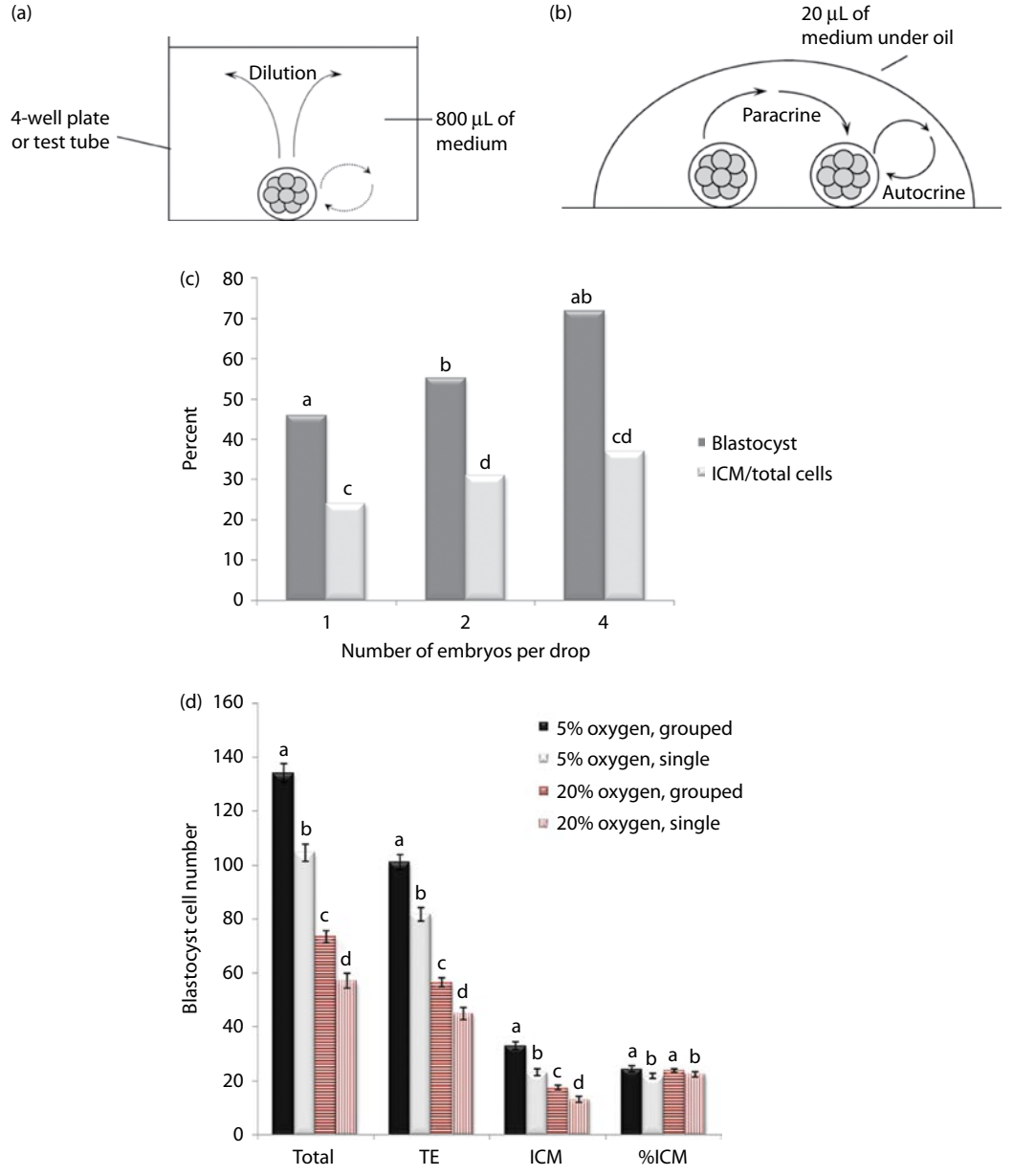

FIGURE 14.6 Effect of incubation volume and embryo grouping on embryo development and differentiation. (a) A single embryo cultured in a four-well plate or test-tube, any factor produced by the embryo will become ineffectual as a result of dilution. (b) Culture of embryos in reduced volumes and/or groups increases the effective concentration of embryo-derived factors, facilitating their action in either a paracrine or autocrine manner. (c) Effect of embryo grouping on bovine blastocyst development and differentiation. Bovine embryos were cultured either individually or in groups of two or four in 50 µL drops of medium. Like pairs are significantly different ($p < 0.05$). (d) Cell numbers on day 5 of mouse embryo culture in 5% or 20% oxygen, in groups of 10 or individually. Different letters represent significant differences between treatments; Total, TE, ICM <0.001, %ICM <0.05. (Data from [c] Ahern TJ, Gardner DK. *Theriogenology*. 1998;49:194; [d] Kelley RL, Gardner DK. *RBM Online*. 2016;33(5):537–49.)

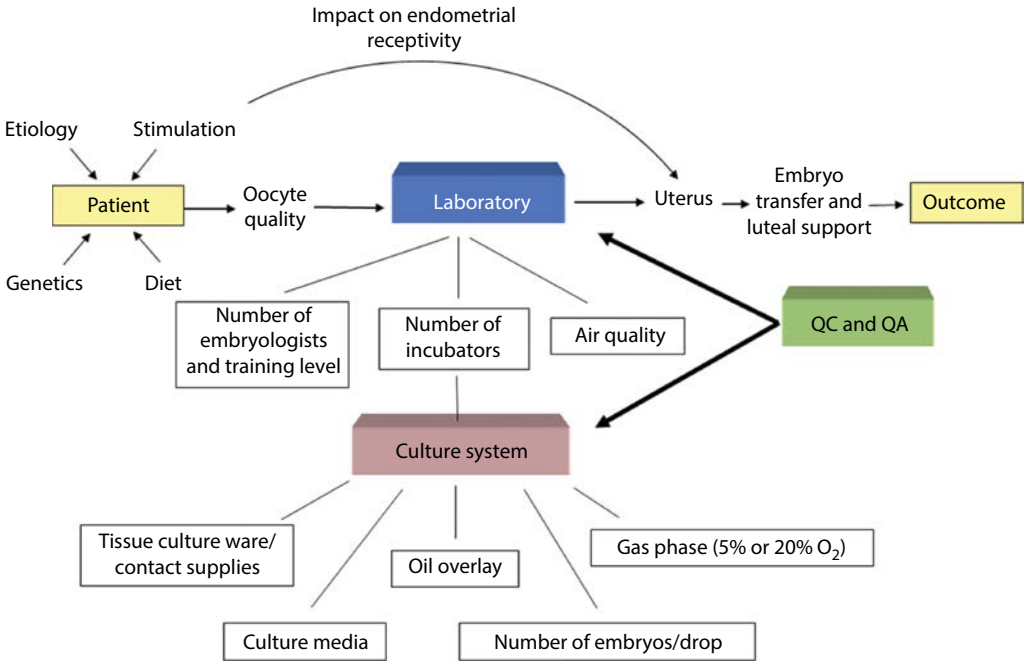

FIGURE 14.7 A holistic analysis of human IVF. This figure serves to illustrate the complex and interdependent nature of human IVF treatment. For example, the stimulation regimen used not only impacts oocyte quality, hence embryo physiology and viability (315), but can also affect subsequent endometrial receptivity (316–318). Furthermore, the health and dietary status of the patient can have a profound effect on the subsequent developmental capacity of the oocyte and embryo (143,319). The dietary status of patients attending IVF is typically not considered as a compounding variable, but growing data would indicate otherwise. In this schematic, the laboratory has been broken down into its core components, only one of which is the culture system. The culture system has in turn been broken down into its components, only one of which is the culture media. Therefore, it would appear rather simplistic to assume that by changing only one part of the culture system (i.e., culture media), that one is going to mimic the results of a given laboratory or clinic. A major determinant of the success of a laboratory and culture system is the level of quality control and quality assurance in place. For example, one should never assume that anything coming into the laboratory that has not been pre-tested with a relevant bioassay (e.g., mouse embryo assay) is safe merely because a previous lot has performed satisfactorily. Only a small percentage of the contact supplies and tissue culture ware used in IVF comes suitably tested. Therefore it is essential to assume that everything entering the IVF laboratory without a suitable pretest is embryo toxic until proven otherwise. In our program the 1-cell mouse embryo assay (MEA) is employed to prescreen every lot of tissue culture ware that enters the program, i.e., plastics that are approved for tissue culture. Around 25% of all such material fails the 1-cell MEA (in a simple medium lacking protein after the first 24 h). Therefore, if one does not perform QC to this level, one in four of all contact supplies used clinically could compromise embryo development. In reality many programs cannot allocate the resources required for this level of QC; and when embryo quality is compromised in the laboratory, it is the media that are held responsible, when in fact the tissue culture ware is more often the culprit. (Modified from Gardner DK, Lane M. *Reprod Biomed Online*. 2003;6(4):470–81 with permission from Reproductive Healthcare Ltd).

What Stage Should the Human Embryo Be Transferred?

It has been an accepted global practice to transfer embryos on day 2 (around the 4-cell stage) or on day 3 (around the 8-cell stage) of development (320). However, such cleavage stage embryos reside in the fallopian tube and not in the uterus (321). The significance of this observation is that in other mammalian species the transfer of cleavage stage embryos to the uterus results in lower pregnancy rates than are attained by the transfer of post compaction or blastocyst stage embryos (6,322). With the development of more effective culture media, it has become possible to culture human embryos to the blastocyst stage as

a matter of routine (323,324). So now we can ask the question, what stage of development is optimal for human embryos conceived through IVF?

The potential advantages of blastocyst culture and transfer in human IVF include

1. The synchronization of the embryo with the female tract (322,325), leading to increased implantation rates (326), therefore reducing the need for multiple embryo transfers.
2. The ability to assess embryo development and viability over extended culture. This can be achieved by both the identification of those embryos with little developmental potential, as manifest by slow development or degeneration in culture, and by the introduction of non-invasive tests of developmental potential to select the most viable embryos from within a cohort for transfer.
3. Minimize the exposure of the embryo to a hyperstimulated uterine environment.
4. Culture for an extra 2 to 3 days increases the time available between cleavage stage embryo biopsy and the time of transfer. This is of particular importance where the biopsied material has to be sent to a separate locale for analysis. However, this procedure is now utilized less with the move to trophectoderm biopsy.
5. Assessment of true embryo viability, i.e., assessing the embryo post genome activation (327).
6. Reduced uterine contractions on day 5 of embryo development, minimizing the chance that an embryo will be expelled from the uterus (328).
7. Increased ability to undergo cryopreservation (329–331).
8. The generation of blastocysts will facilitate the introduction of trophectoderm biopsy for the screening of genetic diseases. Trophectoderm biopsy represents the earliest form of genetic diagnosis of non-embryonic material (332).
9. Reduced pregnancy loss (333).

There is now considerable evidence that blastocyst transfer can be more successful than cleavage stage transfer. However, findings have not been universal, and this is likely due to the interactions of all of the components, from ovarian stimulation, culture media and system, oxygen levels, types of incubation system, training levels, and numbers of embryologists, as well as quality control. In a meta-analysis of prospective trials, in which equal numbers of embryos were transferred, it was concluded that "The best available evidence suggests that the probability of live birth after fresh IVF is significantly higher after blastocyst-stage embryo transfer as compared to cleavage-stage embryo transfer when equal number of embryos are transferred …." (334). Additionally, the most recent Cochrane report on blastocyst transfer showed a significant difference in live birth rate per couple favoring blastocyst transfer (335). In support of such analyses, from a model previously developed to determine which patients should have single embryo transfer, it was determined that pregnancy outcome was more favorable with day 5 than day 3 transfer (336). As well as the published prospective randomized trials, there are retrospective studies that have concluded that day 5 transfer exhibits significant benefits for human IVF in both non-selected and specific patient populations (323,324,337).

For patients having oocyte donation, blastocyst culture and transfer is the most effective course of treatment. Oocytes from donors generally represent a more viable cohort of gametes, as they tend to come from young, fertile women. Embryos derived from oocyte donors tend to reach the blastocyst stage at a higher frequency than those from IVF patients, and be of higher quality. It is possible to attain an implantation rate of >65% when transferring blastocysts to recipients whose mean age is over 40 (8,338). Such data not only reflect the competency of modern embryo culture systems, but emphasize the need to move to single embryo transfers, especially when performing day 5 transfers (339).

However, questions have recently been raised concerning cumulative pregnancy rates per retrieval, and whether the overall success of IVF is actually greater with cleavage stage transfer rather than when blastocyst transfer is being performed (340). Furthermore, the same authors performed a meta-analysis raising concerns about a possible increase in the rate of adverse perinatal outcomes with blastocyst transfer. A subsequent analysis of the literature utilized for this meta-analysis revealed that those studies

in which blastocyst transfer appeared linked to preterm delivery and large for gestational age were undertaken using atmospheric oxygen in the culture system, whereas those studies which reported benefits with blastocyst transfer had utilized 5% oxygen (280). Furthermore, previous analysis of cumulative pregnancy rates included a time period prior to the optimization of blastocyst culture systems, and the use of slow freezing for cryopreservation, as opposed to the significantly more effective vitrification methods currently used worldwide (341–343). Consequently, future analyses, based on data in which physiological oxygen is employed for embryo culture and where vitrification is used for cryopreservation, are predicted to err in favor of blastocyst transfer with regards to overall cycle efficacy and resultant child health. What is imperative, is the need for ongoing monitoring of the health and development of children born through all procedures introduced into clinical IVF.

Quality Control: Embryo Bioassay

The pre-implantation mouse embryo is the most widely used bioassay for medium components, culture media, and equipment used in clinical IVF. The evolution and efficacy of this approach has been well documented (344), and with the advent of time-lapse microscopy, has been further refined (345). Further, it is here proposed that through the use of time-lapse in a clinical setting, it is now feasible to utilize defined patient populations to monitor carefully the weekly performance of the clinical IVF laboratory, and to rapidly establish the effects of new batches of culture media, culture dishes, hormones, etc., which may impact embryo development.

Summary

In the 1990s a resurgence of research into mammalian embryo physiology and culture culminated in more suitable culture conditions for the human embryo conceived through IVF. In this chapter we have considered the types of media utilized in human IVF, and have examined all media components and their respective roles. We have placed the different culture media into the framework of the embryo culture system (Figure 14.7), each aspect of which needs to be optimized if the overall system is to work efficiently. Furthermore, it is important to consider that, by working in vitro, we introduce potentially harmful artifacts, such as temperature and pH shifts, the accumulation of ammonium, and the creation of unstirred layers around the embryo, which will alter the level and availability of nutrients. A failure to appreciate such matters could lead to compromised embryo development. A further theme throughout the chapter is the interactions of specific individual components of the embryo culture system, be they carbohydrates together with amino acids, or atmospheric oxygen together with ammonium or individual culture. Consequently, it is our hope that this chapter will not only help you in interpreting the literature, but, on a practical level, will assist in establishing and maintaining an effective culture system for your IVF laboratory.

REFERENCES

1. Streffer C, van Beuningen D, Molls M, Zamboglou N, Schulz S. Kinetics of cell proliferation in the preimplanted mouse embryo in vivo and in vitro. *Cell Tissue Kinet*. 1980;13(2):135–43.
2. Edwards RG. Causes of early embryonic loss in human pregnancy. *Hum Reprod*. 1986;1(3):185–98.
3. Bernardini L, Alam V, Asch RH, Balmaceda JP. Pregnancy and implantation rates in normal replacement versus stimulated cycles. *Hum Reprod*. 1993;8(11):1938–41.
4. Hartshorne GM, Edwards RG. Early embryo development. In: *Reproductive Endocrinology Surgery and Technology*, Adashi EY, Rosenwaks Z (Eds.). Philadelphia: Lippincott-Raven Publishers, 1996, 435.
5. Quinn P, Stone BA, Marrs RP. Suboptimal laboratory conditions can affect pregnancy outcome after embryo transfer on day 1 or 2 after insemination in vitro. *Fertil Steril*. 1990;53(1):168–70.
6. Bavister BD. Culture of preimplantation embryos: Facts and artifacts. *Hum Reprod Update*. 1995;1(2):91–148.
7. Gardner DK, Lane M. Culture and selection of viable blastocysts: A feasible proposition for human IVF? *Hum Reprod Update*. 1997;3(4):367–82.

8. Gardner DK. Dissection of culture media for embryos: The most important and less important components and characteristics. *Reprod Fertil Dev.* 2008;20(1):9–18.
9. Lane M, Maybach JM, Hooper K, Hasler JF, Gardner DK. Cryo-survival and development of bovine blastocysts are enhanced by culture with recombinant albumin and hyaluronan. *Mol Reprod Dev.* 2003;64(1):70–8.
10. Whitten WK, Biggers JD. Complete development in vitro of the pre-implantation stages of the mouse in a simple chemically defined medium. *J Reprod Fertil.* 1968;17(2):399–401.
11. Whitten WK. Culture of tubal mouse ova. *Nature.* 1956;177(4498):96–7.
12. Whittingham DG. Culture of mouse ova. *J Reprod Fertil Suppl.* 1971;14:7–21.
13. Edwards RG. Test-tube babies, 1981. *Nature.* 1981;293(5830):253–6.
14. Edwards RG, Steptoe PC, Purdy JM. Establishing full-term human pregnancies using cleaving embryos grown in vitro. *Br J Obstet Gynaecol.* 1980;87(9):737–56.
15. Quinn P, Kerin JF, Warnes GM. Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. *Fertil Steril.* 1985;44(4):493–8.
16. Carrillo AJ, Lane B, Pridman DD, Risch PP, Pool TB, Silverman IH et al. Improved clinical outcomes for in vitro fertilization with delay of embryo transfer from 48 to 72 hours after oocyte retrieval: Use of glucose- and phosphate-free media. *Fertil Steril.* 1998;69(2):329–34.
17. Chatot CL, Ziomek CA, Bavister BD, Lewis JL, Torres I. An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro. *J Reprod Fertil.* 1989;86(2):679–88.
18. Lawitts JA, Biggers JD. Joint effects of sodium chloride, glutamine, and glucose in mouse preimplantation embryo culture media. *Mol Reprod Dev.* 1992;31(3):189–94.
19. Whitten WK. The effect of progesterone on the development of mouse eggs in vitro. *J Endocrinol.* 1957;16(1):80–5.
20. Ham RG. An improved nutrient solution for diploid Chinese hamster and human cell lines. *Exp Cell Res.* 1963;29:515–26.
21. DeCherney AH. In vitro fertilization and embryo transfer: A brief overview. *Yale J Biol Med.* 1986;59(4):409–14.
22. Lopata A, Hay DL. The potential of early human embryos to form blastocysts, hatch from their zona and secrete HCG in culture. *Hum Reprod.* 1989;4(8 Suppl):87–94.
23. Meldrum DR, Chetkowski R, Steingold KA, de Ziegler D, Cedars MI, Hamilton M. Evolution of a highly successful in vitro fertilization-embryo transfer program. *Fertil Steril.* 1987;48(1):86–93.
24. Gardner DK, Leese HJ. Concentrations of nutrients in mouse oviduct fluid and their effects on embryo development and metabolism in vitro. *J Reprod Fertil.* 1990;88(1):361–8.
25. Gardner DK, Lane M, Calderon I, Leeton J. Environment of the preimplantation human embryo in vivo: Metabolite analysis of oviduct and uterine fluids and metabolism of cumulus cells. *Fertil Steril.* 1996;65(2):349–53.
26. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the mouse preimplantation embryo: Amino acids act as buffers of intracellular pH. *Hum Reprod.* 1998;13(12):3441–8.
27. Gardner DK. Changes in requirements and utilization of nutrients during mammalian preimplantation embryo development and their significance in embryo culture. *Theriogenology.* 1998;49(1):83–102.
28. Lane M, Gardner DK. Amino acids and vitamins prevent culture-induced metabolic perturbations and associated loss of viability of mouse blastocysts. *Hum Reprod.* 1998;13(4):991–7.
29. Gardner DK, Pool TB, Lane M. Embryo nutrition and energy metabolism and its relationship to embryo growth, differentiation, and viability. *Semin Reprod Med.* 2000;18(2):205–18.
30. Lane M, Gardner DK. Regulation of ionic homeostasis by mammalian embryos. *Semin Reprod Med.* 2000;18(2):195–204.
31. Lane M, Gardner DK. Lactate regulates pyruvate uptake and metabolism in the preimplantation mouse embryo. *Biol Reprod.* 2000;62(1):16–22.
32. Lane M. Mechanisms for managing cellular and homeostatic stress in vitro. *Theriogenology.* 2001;55(1):225–36.
33. Lane M, Gardner DK. Understanding cellular disruptions during early embryo development that perturb viability and fetal development. *Reprod Fertil Dev.* 2005;17(3):371–8.
34. Gardner DK, Lane M. Ex vivo early embryo development and effects on gene expression and imprinting. *Reprod Fertil Dev.* 2005;17(3):361.

35. Gardner DK, Lane M. Development of viable mammalian embryos in vitro: Evolution of sequential media. In: *Principles of Cloning*, Cibelli R, Lanza K, Campbell AK, West MD (Eds.). Chapter 9. Oxford: Elsevier Science, 2002, 187–213.
36. Gardner DK, Lane M. Mammalian preimplantation embryo culture. *Methods Molec Biol*. 2014;1092:167–82.
37. Gardner DK. Mammalian embryo culture in the absence of serum or somatic cell support. *Cell Biol Int*. 1994;18(12):1163–79.
38. Pool TB. Recent advances in the production of viable human embryos in vitro. *Reprod Biomed Online*. 2002;4(3):294–302.
39. Gardner DK, Lane M. Culture of viable mammalian embryos. In: *Prinicples of Cloning*, 2nd ed., Cibelli JLR, Campbell K, West MD (Eds.). San Diego: Academic Press, 2014, 63–84.
40. Lawitts JA, Biggers JD. Optimization of mouse embryo culture media using simplex methods. *J Reprod Fertil*. 1991;91(2):543–56.
41. Lawitts JA, Biggers JD. Culture of preimplantation embryos. *Methods Enzymol*. 1993;225:153–64.
42. Erbach GT, Lawitts JA, Papaioannou VE, Biggers JD. Differential growth of the mouse preimplantation embryo in chemically defined media. *Biol Reprod*. 1994;50(5):1027–33.
43. Ho Y, Wigglesworth K, Eppig JJ, Schultz RM. Preimplantation development of mouse embryos in KSOM: Augmentation by amino acids and analysis of gene expression. *Mol Reprod Dev*. 1995;41(2):232–8.
44. Gardner DK, Lane M. Amino acids and ammonium regulate mouse embryo development in culture. *Biol Reprod*. 1993;48(2):377–85.
45. Biggers JD, Racowsky C. The development of fertilized human ova to the blastocyst stage in KSOM(AA) medium: Is a two-step protocol necessary? *Reprod Biomed Online*. 2002;5(2):133–40.
46. Hardarson T, Bungum M, Conaghan J, Meintjes M, Chantilis SJ, Molnar L et al. Noninferiority, randomized, controlled trial comparing embryo development using media developed for sequential or undisturbed culture in a time-lapse setup. *Fertil Steril*. 2015;104(6):1452–9 e1–4.
47. Borland RM, Biggers JD, Lechene CP, Taymor ML. Elemental composition of fluid in the human Fallopian tube. *J Reprod Fertil*. 1980;58(2):479–82.
48. Borland RM, Hazra S, Biggers JD, Lechene CP. The elemental composition of the environments of the gametes and preimplantation embryo during the initiation of pregnancy. *Biol Reprod*. 1977;16(2):147–57.
49. Van Winkle LJ, Haghighat N, Campione AL. Glycine protects preimplantation mouse conceptuses from a detrimental effect on development of the inorganic ions in oviductal fluid. *J Exp Zool*. 1990;253(2):215–9.
50. Biggers JD, Lawitts JA, Lechene CP. The protective action of betaine on the deleterious effects of NaCl on preimplantation mouse embryos in vitro. *Mol Reprod Dev*. 1993;34(4):380–90.
51. Gardner DK, Sakkas D. Mouse embryo cleavage, metabolism and viability: Role of medium composition. *Hum Reprod*. 1993;8(2):288–95.
52. Lane M, Gardner DK. Differential regulation of mouse embryo development and viability by amino acids. *J Reprod Fertil*. 1997;109(1):153–64.
53. Wales RG. Effects of ions on the development of the pre-implantation mouse embryo in vitro. *Aust J Biol Sci*. 1970;23:421.
54. Bavister BD, Golden M. Alteration of extracellular cation concentrations and ratios in culture medium does not affect first cleavage division of hamster zygotes in vitro nor overcome the 'two-cell block'. *Reprod Fertil Dev*. 1989;1(3):231–6.
55. McKiernan SH, Bavister BD. Environmental variables influencing in vitro development of hamster 2-cell embryos to the blastocyst stage. *Biol Reprod*. 1990;43(3):404–13.
56. Roblero LS, Guadarrama A, Ortiz ME, Fernandez E, Zegers-Hochschild F. High potassium concentration and the cumulus corona oocyte complex stimulate the fertilizing capacity of human spermatozoa. *Fertil Steril*. 1990;54(2):328–32.
57. Roblero LS, Riffo MD. High potassium concentration improves preimplantation development of mouse embryos in vitro. *Fertil Steril*. 1986;45(3):412–6.
58. Wiley LM, Yamami S, Van Muyden D. Effect of potassium concentration, type of protein supplement, and embryo density on mouse preimplantation development in vitro. *Fertil Steril*. 1986;45(1):111–9.
59. Dumoulin JC, Evers JL, Michiels AH, Pieters MH, Bras M, Land JA et al. Modulation of embryonic Na(+)-K(+)-ATPase activity and mouse preimplantation development in vitro in media containing high concentrations of potassium. *Mol Reprod Dev*. 1993;36(3):320–7.

60. Anbari K, Schultz RM. Effect of sodium and betaine in culture media on development and relative rates of protein synthesis in preimplantation mouse embryos in vitro. *Molec Reprod Dev.* 1993;35(1):24–8.
61. Ducibella T, Anderson E. Cell shape and membrane changes in the eight-cell mouse embryo: Prerequisites for morphogenesis of the blastocyst. *Dev Biol.* 1975;47(1):45–58.
62. Lane M, Boatman DE, Albrecht RM, Bavister BD. Intracellular divalent cation homeostasis and developmental competence in the hamster preimplantation embryo. *Mol Reprod Dev.* 1998;50(4):443–50.
63. Lane M, Bavister BD. Calcium homeostasis in early hamster preimplantation embryos. *Biol Reprod.* 1998;59(4):1000–7.
64. Rout UK, Krawetz SA, Armant DR. Ethanol-induced intracellular calcium mobilization rapidly alters gene expression in the mouse blastocyst. *Cell Calcium.* 1997;22(6):463–74.
65. Quinn P. Enhanced results in mouse and human embryo culture using a modified human tubal fluid medium lacking glucose and phosphate. *J Assist Reprod Genet.* 1995;12(2):97–105.
66. Brinster RL. Studies On The Development Of Mouse Embryos In Vitro. I. The Effect Of Osmolarity And Hydrogen Ion Concentration. *J Exp Zool.* 1965;158:49–57.
67. Bavister BD, McKiernan SH. Regulation of hamster embryo development in vitro by amino acids. In: *Preimplantation Embryo Development*, Bavister BD (Ed.). New York: Springer-Verlag, 1992, 57–72.
68. Dawson KM, Collins JL, Baltz JM. Osmolarity-dependent glycine accumulation indicates a role for glycine as an organic osmolyte in early preimplantation mouse embryos. *Biol Reprod.* 1998;59(2):225–32.
69. Nichol R, Hunter RH, Gardner DK, Leese HJ, Cooke GM. Concentrations of energy substrates in oviductal fluid and blood plasma of pigs during the peri-ovulatory period. *J Reprod Fertil.* 1992;96(2):699–707.
70. Leese HJ, Barton AM. Production of pyruvate by isolated mouse cumulus cells. *J Exp Zool.* 1985;234(2):231–6.
71. Wales RG, Whittingham. The metabolism of specifically labelled lactate and pyruvate by two-cell mouse embryos. *J Reprod Fertil.* 1973;33(2):207–22.
72. Leese HJ, Hooper MA, Edwards RG, Ashwood-Smith MJ. Uptake of pyruvate by early human embryos determined by a non-invasive technique. *Hum Reprod.* 1986;1(3):181–2.
73. Hardy K, Hooper MA, Handyside AH, Rutherford AJ, Winston RM, Leese HJ. Non-invasive measurement of glucose and pyruvate uptake by individual human oocytes and preimplantation embryos. *Hum Reprod.* 1989;4(2):188–91.
74. Gott AL, Hardy K, Winston RM, Leese HJ. Non-invasive measurement of pyruvate and glucose uptake and lactate production by single human preimplantation embryos. *Hum Reprod.* 1990;5(1):104–8.
75. Gardner DK, Lane M, Stevens J, Schoolcraft WB. Noninvasive assessment of human embryo nutrient consumption as a measure of developmental potential. *Fertil Steril.* 2001;76(6):1175–80.
76. Biggers JD, Whittingham DG, Donahue RP. The pattern of energy metabolism in the mouse oocyte and zygote. *Proc Natl Acad Sci U S A.* 1967;58(2):560–7.
77. Conaghan J, Handyside AH, Winston RM, Leese HJ. Effects of pyruvate and glucose on the development of human preimplantation embryos in vitro. *J Reprod Fertil.* 1993;99(1):87–95.
78. Lane M, Gardner DK. Mitochondrial malate-aspartate shuttle regulates mouse embryo nutrient consumption. *J Biol Chem.* 2005;280(18):18361–7.
79. Cross PC, Brinster RL. The sensitivity of one-cell mouse embryos to pyruvate and lactate. *Exp Cell Res.* 1973;77(1):57–62.
80. Pomp D, Crister ES, Rutledge JJ. Lower sodium lactate in Whitten's medium improves in vitro developmental capacity of one-cell mouse embryos. *Theriogenology.* 1988;29:1019.
81. Gardner DK, Wale PL. Analysis of metabolism to select viable human embryos for transfer. *Fertil Steril.* 2013;99(4):1062–72.
82. Gardner DK. Lactate production by the mammalian blastocyst: Manipulating the microenvironment for uterine implantation and invasion? *Bioessays.* 2015;37(4):364–71.
83. Harvey AJ, Rathjen J, Gardner DK. Metaboloepigenetic Regulation of Pluripotent Stem Cells. *Stem Cells Int.* 2016;2016:1816525.
84. Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the preimplantation mouse embryo: Effects of extracellular pH and weak acids. *Mol Reprod Dev.* 1998;50(4):434–42.
85. Brinster RL. Studies on the development of mouse embryos in vitro. IV. Interaction of energy sources. *J Reprod Fertil.* 1965;10(2):227–40.
86. Brinster RL, Thomson JL. Development of eight-cell mouse embryos in vitro. *Exp Cell Res.* 1966;42(2):308–15.

87. Barbehenn EK, Wales RG, Lowry OH. The explanation for the blockade of glycolysis in early mouse embryos. *Proc Natl Acad Sci USA*. 1974;71(4):1056–60.
88. Barbehenn EK, Wales RG, Lowry OH. Measurement of metabolites in single preimplantation embryos; a new means to study metabolic control in early embryos. *J Embryol Exp Morphol*. 1978;43:29–46.
89. Biggers JD, Gardner DK, Leese HJ. Control of carbohydrate metabolism in preimplanation mammalian embryos. In: *Growth Factors in Mammalian Embryos*, Rosenblum IY, Heyner S (Eds.). Boca Raton: CRC Press, 1989, 19–36.
90. Leese HJ, Biggers JD, Mroz EA, Lechene C. Nucleotides in a single mammalian ovum or preimplantation embryo. *Anal Biochem*. 1984;140(2):443–8.
91. Schini SA, Bavister BD. Two-cell block to development of cultured hamster embryos is caused by phosphate and glucose. *Biol Reprod*. 1988;39(5):1183–92.
92. Thompson JG, Simpson AC, Pugh PA, Tervit HR. Requirement for glucose during in vitro culture of sheep preimplantation embryos. *Mol Reprod Dev*. 1992;31(4):253–7.
93. Takahashi Y, First NL. In vitro development of bovine one-cell embryos influence of glucose, lactate, amino acids and vitamins. *Theriogenology*. 1992;37:963–78.
94. Rosenkrans CF, Jr., Zeng GQ, GT MC, Schoff PK, First NL. Development of bovine embryos in vitro as affected by energy substrates. *Biol Reprod*. 1993;49(3):459–62.
95. Matsuyama K, Miyakoshi K, Fukui Y. Effects of glucose levels during in vitro culture in synthetic oviduct fluid medium on in vitro development of bovine oocytes matured and fertilized in vitro. *Theriogenology*. 1993;40:595–605.
96. Gardner DK, Lane M. The 2-cell block in CF1 mouse embryos is associated with an increase in glycolysis and a decrease in tricarboxylic acid (TCA) cycle activity: Alleviation of the 2-cell block is associated with the restoration of in vivo metabolic pathway activities. *Biol Reprod*. 1993;49(Suppl.1):152 (Abstract).
97. Barnett DK, Bavister BD. Inhibitory effect of glucose and phosphate on the second cleavage division of hamster embryos: Is it linked to metabolism? *Hum Reprod*. 1996;11(1):177–83.
98. Gardner DK, Lane M, Spitzer A, Batt PA. Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage in vitro in the absence of serum and somatic cells: Amino acids, vitamins, and culturing embryos in groups stimulate development. *Biol Reprod*. 1994;50(2):390–400.
99. Lane M, Gardner DK. Inhibiting 3-phosphoglycerate kinase by EDTA stimulates the development of the cleavage stage mouse embryo. *Mol Reprod Dev*. 2001;60(2):233–40.
100. Aoki K, Nakamura M, Namiki H, Okinaga S, Arai K. The effect of glucose and phosphate on mouse two-cell embryos to develop in vitro. *Zool Sci*. 1990;7:973–7.
101. Gardner DK, Lane M. Alleviation of the '2-cell block' and development to the blastocyst of CF1 mouse embryos: Role of amino acids, EDTA and physical parameters. *Hum Reprod*. 1996;11(12):2703–12.
102. Ludwig TE, Lane M, Bavister BD. Increased fetal development after transfer of hamster embryos cultured with glucose. *Biol Reprod*. 1998;58(Suppl 1):306.
103. Gardner DK, Harvey AJ. Blastocyst metabolism. *Reprod Fertil Dev*. 2015;27(4):638–54.
104. Aghayan M, Rao LV, Smith RM, Jarett L, Charron MJ, Thorens B et al. Developmental expression and cellular localization of glucose transporter molecules during mouse preimplantation development. *Development*. 1992;115(1):305–12.
105. Dan-Goor M, Sasson S, Davarashvili A, Almagor M. Expression of glucose transporter and glucose uptake in human oocytes and preimplantation embryos. *Hum Reprod*. 1997;12(11):2508–10.
106. Gardner DK, Leese HJ. The role of glucose and pyruvate transport in regulating nutrient utilization by preimplantation mouse embryos. *Development*. 1988;104(3):423–9.
107. Hogan A, Heyner S, Charron MJ, Copeland NG, Gilbert DJ, Jenkins NA et al. Glucose transporter gene expression in early mouse embryos. *Development*. 1991;113(1):363–72.
108. Jansen S, Esmaeilpour T, Pantaleon M, Kaye PL. Glucose affects monocarboxylate cotransporter (MCT) 1 expression during mouse preimplantation development. *Reproduction*. 2006;131(3):469–79.
109. Jansen S, Pantaleon M, Kaye PL. Characterization and regulation of monocarboxylate cotransporters Slc16a7 and Slc16a3 in preimplantation mouse embryos. *Biol Reprod*. 2008;79(1):84–92.
110. Rogers PAW, Murphy CR, Gannon BJ. Changes in the spatial organisation of the uterine vasculature during implantation in the rat. *J Reprod Fertil*. 1982;65:211–4.
111. Rogers PAW, Murphy CR, Gannon BJ. Absense of capillaries in the endometrium surrounding the implanting rat blastocyst. *Micron*. 1982;13:373–4.

112. Leese HJ. Metabolism of the preimplantation mammalian embryo. *Oxford Rev Reprod Biol*. 1991;13:35–72.
113. Gardner DK. The nutrition and energy metabolism of the preimplantation mouse embryo [Ph.D. Thesis]. York, UK: University of York, 1987.
114. Lane M, Gardner DK. Selection of viable mouse blastocysts prior to transfer using a metabolic criterion. *Hum Reprod*. 1996;11(9):1975–8.
115. Perkins JL, Goode L. Free amino acids in the oviduct fluid of the ewe. *J Reprod Fertil*. 1967;14(2):309–11.
116. Menezo Y. Amino constiuents of tubal and uterine fluids of the eostrous ewe: Comparison with blood serum and ram seminal fluid. In: *The Biology of Spermatazoa*, Hafez ES, Thibault C (Eds.). New York: Basel Press, 1972, 174.
117. Casslen BG. Free amino acids in human uterine fluid. Possible role of high taurine concentration. *J Reprod Med*. 1987;32(3):181–4.
118. Miller JG, Schultz GA. Amino acid content of preimplantation rabbit embryos and fluids of the reproductive tract. *Biol Reprod*. 1987;36(1):125–9.
119. Harris SE, Gopichandran N, Picton HM, Leese HJ, Orsi NM. Nutrient concentrations in murine follicular fluid and the female reproductive tract. *Theriogenology*. 2005;64(4):992–1006.
120. Van Winkle LJ. Amino acid transport in developing animal oocytes and early conceptuses. *Biochim Biophys Acta*. 1988;947(1):173–208.
121. Schultz GA, Kaye PL, McKay DJ, Johnson MH. Endogenous amino acid pool sizes in mouse eggs and preimplantation embryos. *J Reprod Fertil*. 1981;61(2):387–93.
122. Chatot CL, Tasca RJ, Ziomek CA. Glutamine uptake and utilization by preimplantation mouse embryos in CZB medium. *J Reprod Fertil*. 1990;89(1):335–46.
123. Gardner DK, Clarke RN, Lechene CP, Biggers JD. Development of a noninvasive ultramicrofluorometric method for measuring net uptake of glutamine by single preimplantation mouse embryos. *Gamete Res*. 1989;24(4):427–38.
124. Eagle H. Amino acid metabolism in mammalian cell cultures. *Science*. 1959;130(3373):432–7.
125. Dumoulin JC, Evers JL, Bakker JA, Bras M, Pieters MH, Geraedts JP. Temporal effects of taurine on mouse preimplantation development in vitro. *Hum Reprod*. 1992;7(3):403–7.
126. Dumoulin JC, Evers JL, Bras M, Pieters MH, Geraedts JP. Positive effect of taurine on preimplantation development of mouse embryos in vitro. *J Reprod Fertil*. 1992;94(2):373–80.
127. Bavister BD. Studies on the developmental blocks in cultured hamster embryos. In: *The Mammalian Preimplantation Embryo*, Bavister BD (Ed.). New York: Plenum, 1987, 219–49.
128. McKiernan SH, Clayton MK, Bavister BD. Analysis of stimulatory and inhibitory amino acids for development of hamster one-cell embryos in vitro. *Mol Reprod Dev*. 1995;42(2):188–99.
129. Thompson JG, Gardner DK, Pugh PA, McMillan WH, Tervit HR. Lamb birth weight is affected by culture system utilized during in vitro pre-elongation development of ovine embryos. *Biol Reprod*. 1995;53(6):1385–91.
130. Lane M, Gardner DK. Nonessential amino acids and glutamine decrease the time of the first three cleavage divisions and increase compaction of mouse zygotes in vitro. *J Assist Reprod Genet*. 1997;14(7):398–403.
131. Lane M, Gardner DK. Increase in postimplantation development of cultured mouse embryos by amino acids and induction of fetal retardation and exencephaly by ammonium ions. *J Reprod Fertil*. 1994;102(2):305–12.
132. Gardner DK, Lane M. Towards a single embryo transfer. *Reprod Biomed Online*. 2003;6(4):470–81.
133. Steeves TE, Gardner DK. Temporal and differential effects of amino acids on bovine embryo development in culture. *Biol Reprod*. 1999;61(3):731–40.
134. Bavister BD, Arlotto T. Influence of single amino acids on the development of hamster one-cell embryos in vitro. *Mol Reprod Dev*. 1990;25(1):45–51.
135. Gardner DK, Lane M. Culture Systems for human embryo. In: *Textbook of Assisted ReproductiveTechniques Laboratory and Clinical Perspectives*, 2nd ed., Gardner DK, Weissman A, Howles C, Shoham Z (Eds.). London and New York: Taylor and Francis, 2004, 211–34.
136. Li J, Foote RH. Effect of inositol and glycine with increasing sodium chloride and constant osmolality on development of rabbit embryos. *J Assist Reprod Genet*. 1995;12(2):141–6.
137. Mehta TS, Kiessling AA. Development potential of mouse embryos conceived in vitro and cultured in ethylenediaminetetraacetic acid with or without amino acids or serum. *Biol Reprod*. 1990;43(4):600–6.

138. Zhang X, Armstrong DT. Presence of amino acids and insulin in a chemically defined medium improves development of 8-cell rat embryos in vitro and subsequent implantation in vivo. *Biol Reprod*. 1990;42(4):662–8.
139. Devreker F, Winston RM, Hardy K. Glutamine improves human preimplantation development in vitro. *Fertil Steril*. 1998;69(2):293–9.
140. Barnes FL, Crombie A, Gardner DK, Kausche A, Lacham-Kaplan O, Suikkari AM et al. Blastocyst development and birth after in-vitro maturation of human primary oocytes, intracytoplasmic sperm injection and assisted hatching. *Hum Reprod*. 1995;10(12):3243–7.
141. Mitchell M, Cashman KS, Gardner DK, Thompson JG, Lane M. Disruption of mitochondrial malate-aspartate shuttle activity in mouse blastocysts impairs viability and fetal growth. *Biol Reprod*. 2009;80(2):295–301.
142. Lane M, Gardner DK. Removal of embryo-toxic ammonium from the culture medium by in situ enzymatic conversion to glutamate. *J Exp Zool*. 1995;271(5):356–63.
143. Gardner DK, Stilley K, Lane M. High protein diet inhibits inner cell mass formation and increases apoptosis in mouse blastocysts developed in vivo by increasing the levels of ammonium in the reproductive tract. *Reprod Fertil Devel*. 2004;16(1,2):190.
144. Zander DL, Thompson JG, Lane M. Perturbations in mouse embryo development and viability caused by ammonium are more severe after exposure at the cleavage stages. *Biol Reprod*. 2006;74(2):288–94.
145. Gardner DK, Hamilton R, McCallie B, Schoolcraft WB, Katz-Jaffe MG. Human and mouse embryonic development, metabolism and gene expression are altered by an ammonium gradient in vitro. *Reproduction*. 2013;146(1):49–61.
146. Virant-Klun I, Tomazevic T, Vrtacnik-Bokal E, Vogler AK, Krsnik M, Meden-Vrtovec H. Increased ammonium in culture medium reduces the development of human embryos to the blastocyst stage. *Fertil Steril*. 2006;85(2):526–8.
147. Wale PL, Gardner DK. Oxygen affects the ability of mouse blastocysts to regulate ammonium. *Biol Reprod*. 2013;89(3):75.
148. Biggers JD, McGinnis LK, Summers MC. Discrepancies between the effects of glutamine in cultures of preimplantation mouse embryos. *Reprod Biomed Online*. 2004;9(1):70–3.
149. Menezo YR, Guerin, P. Preimplantation embryo metabolism and embryo interaction with the in vitro environment. In: *Human Preimplantation Embryo Selection*, Elder K, Cohen J (Eds.). London: Informa Healthcare, 2007, 191–200.
150. Nakazawa T, Ohashi K, Yamada M, Shinoda S, Saji F, Murata Y et al. Effect of different concentrations of amino acids in human serum and follicular fluid on the development of one-cell mouse embryos in vitro. *J Reprod Fertil*. 1997;111(2):327–32.
151. Dumoulin JC, Land JA, Van Montfoort AP, Nelissen EC, Coonen E, Derhaag JG et al. Effect of in vitro culture of human embryos on birthweight of newborns. *Hum Reprod*. 2010;25(3):605–12.
152. Kleijkers SH, van Montfoort AP, Smits LJ, Viechtbauer W, Roseboom TJ, Nelissen EC et al. IVF culture medium affects post-natal weight in humans during the first 2 years of life. *Hum Reprod*. 2014;29(4):661–9.
153. Bolton VN, Hawes SM, Taylor CT, Parsons JH. Development of spare human preimplantation embryos in vitro: An analysis of the correlations among gross morphology, cleavage rates, and development to the blastocyst. *J In Vitro Fert Embryo Transf*. 1989;6(1):30–5.
154. Hardy K. Development of human blastocysts in vitro. In: Bavister BD (Ed.) *Preimplantation embryo development*. New York: Springer-Verlag, 1993, 184 p.
155. Kane MT. The effects of water-soluble vitamins on the expansion of rabbit blastocysts in vitro. *J Exp Zool*. 1988;245(2):220–3.
156. Tsai FC, Gardner DK. Nicotinamide, a component of complex culture media, inhibits mouse embryo development in vitro and reduces subsequent developmental potential after transfer. *Fertil Steril*. 1994;61(2):376–82.
157. Loutradis D, John D, Kiessling AA. Hypoxanthine causes a 2-cell block in random-bred mouse embryos. *Biol Reprod*. 1987;37(2):311–6.
158. Downs SM, Dow MP. Hypoxanthine-maintained two-cell block in mouse embryos: Dependence on glucose and effect of hypoxanthine phosphoribosyltransferase inhibitors. *Biol Reprod*. 1991;44(6): 1025–39.

159. Nureddin A, Epsaro E, Kiessling AA. Purines inhibit the development of mouse embryos in vitro. *J Reprod Fertil*. 1990;90(2):455–64.
160. Abramczuk J, Solter D, Koprowski H. The beneficial effect EDTA on development of mouse one-cell embryos in chemically defined medium. *Devel Biol*. 1977;61(2):378–83.
161. Fissore RA, Jackson KV, Kiessling AA. Mouse zygote development in culture medium without protein in the presence of ethylenediaminetetraacetic acid. *Biol Reprod*. 1989;41(5):835–41.
162. Mehta TS, Kiessling AA. The developmental potential of mouse embryos conceived in Ham's F-10 medium containing ethylenediaminetetraacetic acid. *Fertil Steril*. 1993;60(6):1088–93.
163. Hoshi M, Toyoda Y. Effect of EDTA on the preimplantation development of mouse embryos fetilized in vitro. *Jpn J Zootechnol Sci*. 1985;56:931–7.
164. Gardner DK, Lane MW, Lane M. Bovine blastocyst cell number is increased by culture with EDTA for the first 72 hours of development from zygote. *Theriogenology*. 1997;47:278.
165. Hewitson LC, Leese HJ. Energy metabolism of the trophectoderm and inner cell mass of the mouse blastocyst. *J Exp Zool*. 1993;267(3):337–43.
166. Otsuki J, Iwasaki T, Katada Y, Stato H, Furuhashi K, Tsuji Y et al. Grade and looseness of the inner cell mass may laed to the development of monochorionic diamniotic twins. *Fertil Steril*. 2016.
167. Nasr-Esfahani M, Johnson MH, Aitken RJ. The effect of iron and iron chelators on the in-vitro block to development of the mouse preimplantation embryo: BAT6 a new medium for improved culture of mouse embryos in vitro. *Hum Reprod*. 1990;5(8):997–1003.
168. Nasr-Esfahani MH, Johnson MH. How does transferrin overcome the in vitro block to development of the mouse preimplantation embryo? *J Reprod Fertil*. 1992;96(1):41–8.
169. Agarwal A, Aponte-Mellado A, Premkumar BJ, Shaman A, Gupta S. The effects of oxidative stress on female reproduction: A review. *Reprod Biol Endocrinol*. 2012;10:49.
170. Noda Y, Matsumoto H, Umaoka Y, Tatsumi K, Kishi J, Mori T. Involvement of superoxide radicals in the mouse two-cell block. *Mol Reprod Dev*. 1991;28(4):356–60.
171. Umaoka Y, Noda Y, Narimoto K, Mori T. Effects of oxygen toxicity on early development of mouse embryos. *Mol Reprod Dev*. 1992;31(1):28–33.
172. Payne SR, Munday R, Thompson JG. Addition of superoxide dismutase and catalase does not necessarily overcome developmental retardation of one-cell mouse embryos during in-vitro culture. *Reprod Fertil Dev*. 1992;4(2):167–74.
173. Lindenau A, Fischer B. Effect of oxygen concentration in the incubator's gas phase on the development of cultured preimplantation rabbit embryos. *Theriogenology*. 1994;41:889–98.
174. Liu Z, Foote RH. Effects of amino acids on the development of in-vitro matured/in-vitro fertilization bovine embryos in a simple protein-free medium. *Hum Reprod*. 1995;10(11):2985–91.
175. Legge M, Sellens MH. Free radical scavengers ameliorate the 2-cell block in mouse embryo culture. *Hum Reprod*. 1991;6(6):867–71.
176. Nasr-Esfahani MH, Johnson MH. Quantitative analysis of cellular glutathione in early preimplantation mouse embryos developing in vivo and in vitro. *Hum Reprod*. 1992;7(9):1281–90.
177. Gardiner CS, Salmen JJ, Brandt CJ, Stover SK. Glutathione is present in reproductive tract secretions and improves development of mouse embryos after chemically induced glutathione depletion. *Biol Reprod*. 1998;59(2):431–6.
178. Takahashi M, Nagai T, Hamano S, Kuwayama M, Okamura N, Okano A. Effect of thiol compounds on in vitro development and intracellular glutathione content of bovine embryos. *Biol Reprod*. 1993;49(2):228–32.
179. Lim J, Lious SS, Hansel W. Intracytoplasmic glutathione concentration and beta-mercaptoethanol in preimplantation development of bovine embryo. *Theriogenology*. 1996;46:429.
180. de Matos DG, Furnus CC, Moses DF, Martinez AG, Matkovic M. Stimulation of glutathione synthesis of in vitro matured bovine oocytes and its effect on embryo development and freezability. *Mol Reprod Dev*. 1996;45(4):451–7.
181. de Matos DG, Nogueira D, Cortvrindt R, Herrera C, Adriaenssens T, Pasqualini RS et al. Capacity of adult and prepubertal mouse oocytes to undergo embryo development in the presence of cysteamine. *Mol Reprod Dev*. 2003;64(2):214–8.
182. Yoshida M, Ishigaki K, Nagai T, Chikyu M, Pursel VG. Glutathione concentration during maturation and after fertilization in pig oocytes: Relevance to the ability of oocytes to form male pronucleus. *Biol Reprod*. 1993;49(1):89–94.

183. Caamano JN, Ryoo ZY, Youngs CR. Promotion of development of bovine embryos produced in vitro by addition of cysteine and beta-mercaptoethanol to a chemically defined culture system. *J Dairy Sci*. 1998;81(2):369–74.
184. Bilska A, Wlodek L. Lipoic acid—The drug of the future? *Pharmacol Rep*. 2005;57(5):570–7.
185. Lane M, Maybach JM, Gardner DK. Addition of ascorbate during cryopreservation stimulates subsequent embryo development. *Hum Reprod*. 2002;17(10):2686–93.
186. Andrae U, Singh J, Ziegler-Skylakakis K. Pyruvate and related alpha-ketoacids protect mammalian cells in culture against hydrogen peroxide-induced cytotoxicity. *Toxicol Lett*. 1985;28(2–3):93–8.
187. Kouridakis K, Gardner DK. Pyruvate in embryo culture embryo media acts as an antioxidant. Proc Fert Soc Aus, Melbourne, Victoria, 1995.
188. O'Fallon JV, Wright RW. Pyruvate revisited: A non-metabolic role for pyruvate in preimplantation embryo development. *Theriogenology*. 1995;43:288.
189. Li J, Foote RH, Simkin M. Development of rabbit zygotes cultured in protein-free medium with catalase, taurine, or superoxide dismutase. *Biol Reprod*. 1993;49(1):33–7.
190. Truong TT, Soh YM, Gardner DK. Antioxidants improve mouse preimplantation embryo development and viability. *Hum Reprod*. 2016;31(7):1445–54.
191. Kelley RL, Gardner DK. Combined effects of individual culture and atmospheric oxygen on preimplantation mouse embryos in vitro. *RBM Online*. 2016;33(5):537–49.
192. Wale PL, Gardner DK. The effects of chemical and physical factors on mammalian embryo culture and their importance for the practice of assisted human reproduction. *Hum Reprod Update*. 2016;22(1):2–22.
193. Magli MC, Gianaroli L, Fiorentino A, Ferraretti AP, Fortini D, Panzella S. Improved cleavage rate of human embryos cultured in antibiotic-free medium. *Hum Reprod*. 1996;11(7):1520–4.
194. Otoi T, Tachikawa S, Kondo S, Suzuki T. Effect of antibiotics treatment of in vitro fertilized bovine embryos to remove adhering bacteria. *J Vet Med Sci*. 1992;54(4):763–5.
195. Barnes D, Sato G. Methods for growth of cultured cells in serum-free medium. *Anal Biochem*. 1980;102(2):255–70.
196. Barnes D, Sato G. Serum-free cell culture: A unifying approach. *Cell*. 1980;22(3):649–55.
197. Damewood MD, Hesla JS, Schlaff WD, Hubbard M, Gearhart JD, Rock JA. Effect of serum from patients with minimal to mild endometriosis on mouse embryo development in vitro. *Fertil Steril*. 1990;54(5):917–20.
198. Abu-Musa A, Takahashi K, Kitao M. The effect of serum obtained before and after treatment for endometriosis on in vitro development of two-cell mouse embryos. *Fertil Steril*. 1992;57(5):1098–102.
199. Dokras A, Sargent IL, Redman CW, Barlow DH. Sera from women with unexplained infertility inhibit both mouse and human embryo growth in vitro. *Fertil Steril*. 1993;60(2):285–92.
200. Miller KA, Pittaway DE, Deaton JL. The effect of serum from infertile women with endometriosis on fertilization and early embryonic development in a murine in vitro fertilization model. *Fertil Steril*. 1995;64(3):623–6.
201. Deaton JL, Dempsey RA, Miller KA. Serum from women with polycystic ovary syndrome inhibits fertilization and embryonic development in the murine in vitro fertilization model. *Fertil Steril*. 1996;65(6):1224–8.
202. Menezo Y, Testart J, Perrone D. Serum is not necessary in human in vitro fertilization, early embryo culture, and transfer. *Fertil Steril*. 1984;42(5):750–5.
203. Leese HJ. The formation and function of oviduct fluid. *J Reprod Fertil*. 1988;82(2):843–56.
204. Dorland M, Gardner DK, Trounson A. Serum in synthetic oviduct fluid causes mitochondrial degeneration in ovine embryos. *Reprod Fertil (Abstr Ser)*. 1994;13:70.
205. Khosla S, Dean W, Brown D, Reik W, Feil R. Culture of preimplantation mouse embryos affects fetal development and the expression of imprinted genes. *Biol Reprod*. 2001;64(3):918–26.
206. Walker SK, Heard TM, Seamark RF. In vitro culture of sheep embryos without co-culture: Success and perspectives. *Theriogenology*. 1992;37:111–26.
207. Gardner DK. Development of serum-free media for the culture and transfer of human blastocysts. *Hum Reprod*. 1998;13(Suppl 4):218–25.
208. Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C et al. Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nat Genet*. 2001;27(2):153–4.

209. Killian JK, Nolan CM, Wylie AA, Li T, Vu TH, Hoffman AR et al. Divergent evolution in M6P/IGF2R imprinting from the Jurassic to the Quaternary. *Hum Mol Genet*. 2001;10(17):1721–8.
210. Menezo YJ, Chouteau J, Torello J, Girard A, Veiga A. Birth weight and sex ratio after transfer at the blastocyst stage in humans. *Fertil Steril*. 1999;72(2):221–4.
211. Holst N, Bertheussen K, Forsdahl F, Hakonsen MB, Hansen LJ, Nielsen HI. Optimization and simplification of culture conditions in human in vitro fertilization (IVF) and preembryo replacement by serum-free media. *J In Vitro Fert Embryo Transf*. 1990;7(1):47–53.
212. Flood LP, Shirley B. Reduction of embryotoxicity by protein in embryo culture media. *Mol Reprod Dev*. 1991;30(3):226–31.
213. Ashwood-Smith MJ, Hollands P, Edwards RG. The use of Albuminar 5 as a medium supplement in clinical IVF. *Hum Reprod*. 1989;4(6):702–5.
214. Khan I, Staessen C, Devroey P, Van Steirteghem AC. Human serum albumin versus serum: A comparative study on embryo transfer medium. *Fertil Steril*. 1991;56(1):98–101.
215. Staessen C, Van den Abbeel E, Carle M, Khan I, Devroey P, Van Steirteghem AC. Comparison between human serum and Albuminar-20 (TM) supplement for in-vitro fertilization. *Hum Reprod*. 1990;5(3):336–41.
216. Huisman GJ, Lo ANNM, Alberda AT, Leerentveld RA, Verhoeff A, Zeilmaker GH. Comparison of results obtained with human serum and a protein solution as a supplement for in vitro fertilization culture medium. *Fertil Steril*. 1992;58(3):637–9.
217. Adler A, Reing AM, Bedford JM, Alikani M, Cohen J. Plasmanate as a medium supplement for in vitro fertilization. *J Assist Reprod Genet*. 1993;10(1):67–71.
218. Pool TB, Martin JE. High continuing pregnancy rates after in vitro fertilization-embryo transfer using medium supplemented with a plasma protein fraction containing alpha- and beta-globulins. *Fertil Steril*. 1994;61(4):714–9.
219. Weathersbee PS, Pool TB, Ord T. Synthetic serum substitute (SSS): A globulin-enriched protein supplement for human embryo culture. *J Assist Reprod Genet*. 1995;12(6):354–60.
220. Desai NN, Sheean LA, Martin D, Gindlesperger V, Austin CM, Lisbonna H et al. Clinical experience with synthetic serum substitute as a protein supplement in IVF culture media: A retrospective study. *J Assist Reprod Genet*. 1996;13(1):23–31.
221. Tucker KE, Hurst BS, Guadagnoli S, Dymecki C, Mendelsberg B, Awoniyi CA et al. Evaluation of synthetic serum substitute versus serum as protein supplementation for mouse and human embryo culture. *J Assist Reprod Genet*. 1996;13(1):32–7.
222. Hunter RH. Modulation of gamete and embryonic microenvironments by oviduct glycoproteins. *Mol Reprod Dev*. 1994;39(2):176–81.
223. Hanson RW, Ballard FJ. Citrate, pyruvate, and lactate contaminants of commercial serum albumin. *J Lipid Res*. 1968;9(5):667–8.
224. Dyrlund TF, Kirkegaard K, Poulsen ET, Sanggaard KW, Hindkjær JJ, Kjems J et al. Unconditioned commercial embryo culture media contain a large variety of non-declared proteins: A comprehensive proteomics analysis. *Hum Reprod*. 2014;29(11):2421–30.
225. Morbeck DE, Paczkowski M, Fredrickson JR, Krisher RL, Hoff HS, Baumann NA et al. Composition of protein supplements used for human embryo culture. *J Assist Reprod Genet*. 2014;31(12):1703–11.
226. Gray CW, Morgan PM, Kane MT. Purification of an embryotrophic factor from commercial bovine serum albumin and its identification as citrate. *J Reprod Fertil*. 1992;94(2):471–80.
227. Batt PA, Gardner DK, Cameron AW. Oxygen concentration and protein source affect the development of preimplantation goat embryos in vitro. *Reprod Fertil Dev*. 1991;3(5):601–7.
228. McKiernan SH, Bavister BD. Different lots of bovine serum albumin inhibit or stimulate in vitro development of hamster embryos. *In Vitro Cell Dev Biol*. 1992;28A(3 Pt 1):154–6.
229. Kane MT. Variability in different lots of commercial bovine serum albumin affects cell multiplication and hatching of rabbit blastocysts in culture. *J Reprod Fertil*. 1983;69(2):555–8.
230. Leonard PH, Charlesworth MC, Benson L, Walker DL, Fredrickson JR, Morbeck DE. Variability in protein quality used for embryo culture: Embryotoxicity of the stabilizer octanoic acid. *Fertil Steril*. 2013;100(2):544–9.
231. Takatori S, Akutsu K, Kondo F, Ishii R, Nakazawa H, Makino T. Di(2-ethylhexyl)phthalate and mono(2-ethylhexyl)phthalate in media for in vitro fertilization. *Chemosphere*. 2012;86(5):454–9.
232. Akutsu K, Takatori S, Nakazawa H, Makino T. Detection of polybrominated diphenyl ethers in culture media and protein sources used for human in vitro fertilization. *Chemosphere*. 2013;92(7):864–9.

233. Bavister BD, Kinsey DL, Lane M, Gardner DK. Recombinant human albumin supports hamster in-vitro fertilization. *Hum Reprod*. 2003;18(1):113–6.
234. Bungum M, Humaidan P, Bungum L. Recombinant human albumin as protein source in culture media used for IVF: A prospective randomized study. *Reprod Biomed Online*. 2002;4(3):233–6.
235. Murakami M, Egashira A, Tanaka K, Mine C, Otsubo H, Kuramoto T. Perinatal outcomes for transfer of blastocysts vitrified and warmed in defined solutions with recombinant human albumin: 374 babies born after 898 embryo transfers. *J Assist Reprod Genet*. 2014;31(12):1605–10.
236. Zorn TM, Pinhal MA, Nader HB, Carvalho JJ, Abrahamsohn PA, Dietrich CP. Biosynthesis of glycosaminoglycans in the endometrium during the initial stages of pregnancy of the mouse. *Cell Mol Biol (Noisy-le-grand)*. 1995;41(1):97–106.
237. Gardner DK, Rodriegez-Martinez H, Lane M. Fetal development after transfer is increased by replacing protein with the glycosaminoglycan hyaluronan for mouse embryo culture and transfer. *Hum Reprod*. 1999;14(10):2575–80.
238. Kano K, Miyano T, Kato S. Effects of glycosaminoglycans on the development of in vitro-matured and -fertilized porcine oocytes to the blastocyst stage in vitro. *Biol Reprod*. 1998;58(5):1226–32.
239. Stojkovic M, Peinl S, Stojkovic P, Zakhartchenko V, Thompson JGE, Wolf E. High concentration of hyaluronic acid culture medium increases the survival rate of frozen/thawed in vitro produced bovine blastocysts. *Theriogenology*. 2001;55:317.
240. Dattena M, Mara L, Bin TA, Cappai P. Lambing rate using vitrified blastocysts is improved by culture with BSA and hyaluronan. *Mol Reprod Dev*. 2007;74(1):42–7.
241. Urman B, Yakin K, Ata B, Isiklar A, Balaban B. Effect of hyaluronan-enriched transfer medium on implantation and pregnancy rates after day 3 and day 5 embryo transfers: A prospective randomized study. *Fertil Steril*. 2008;90(3):604–12.
242. Bontekoe S, Heineman MJ, Johnson N, Blake D. Adherence compounds in embryo transfer media for assisted reproductive technologies. *Cochrane Db Syst Rev*. 2014;2:CD007421.
243. Wu JT. Metabolism of progesterone by preimplantation mouse blastocysts in culture. *Biol Reprod*. 1987;36(3):549–56.
244. Edirisinghe WR, Wales RG. Effect of parenteral administration of oestrogen and progesterone on the glycogen metabolism of mouse morulae--early blastocysts in vivo. *J Reprod Fertil*. 1984;72(1):67–73.
245. Edirisinghe WR, Wales RG. Influence of environmental factors on the metabolism of glucose by preimplantation mouse embryos in vitro. *Aust J Biol Sci*. 1985;38(4):411–20.
246. Khurana NK, Wales RG. Effects of oestradiol and progesterone on the metabolism of [U-14C]glucose by mouse morulae and early blastocysts in vitro. *J Reprod Fertil*. 1987;79(1):267–73.
247. Khurana NK, Wales RG. Effects of prostaglandins E-2 and F-2 alpha on the metabolism of [U-14C] glucose by mouse morulae-early blastocysts in vitro. *J Reprod Fertil*. 1987;79(1):275–80.
248. Valbuena D, Martin J, de Pablo JL, Remohi J, Pellicer A, Simon C. Increasing levels of estradiol are deleterious to embryonic implantation because they directly affect the embryo. *Fertil Steril*. 2001;76(5):962–8.
249. Karagenc L, Lane M, Gardner DK. Oestradiol, cyclodextrin-encapsulated 17beta-oestradiol and the oestradiol solubilizer 2-hydroxypropyl-beta-cyclodextrin all impair preimplantation mouse embryo development. *Reprod Biomed Online*. 2004;9(3):280–6.
250. Yohkaichiya T, Fukaya T, Hoshiai H, Yajima A. Improvement of mouse embryo development in vitro by prolactin. *Tohoku J Exp Med*. 1988;155(3):241–6.
251. Lavranos TC, Seamark RF. Addition of steroids to embryo-uterine monolayer co-culture enhances embryo survival and implantation in vitro. *Reprod Fertil Dev*. 1989;1(1):41–6.
252. Thouas GA, Dominguez F, Green MP, Vilella F, Simon C, Gardner DK. Soluble ligands and their receptors in human embryo development and implantation. *Endocr Rev*. 2015;36(1):92–130.
253. Martin KL, Barlow DH, Sargent IL. Heparin-binding epidermal growth factor significantly improves human blastocyst development and hatching in serum-free medium. *Hum Reprod*. 1998;13(6):1645–52.
254. Lighten AD, Moore GE, Winston RM, Hardy K. Routine addition of human insulin-like growth factor-I ligand could benefit clinical in-vitro fertilization culture. *Hum Reprod*. 1998;13(11):3144–50.
255. Hannan NJ, Paiva P, Meehan KL, Rombauts LJ, Gardner DK, Salamonsen LA. Analysis of fertility-related soluble mediators in human uterine fluid identifies VEGF as a key regulator of embryo implantation. *Endocrinology*. 2011;152(12):4948–56.

256. Binder NK, Evans J, Gardner DK, Salamonsen LA, Hannan NJ. Endometrial signals improve embryo outcome: Functional role of vascular endothelial growth factor isoforms on embryo development and implantation in mice. *Hum Reprod*. 2014;29(10):2278–86.
257. Ziebe S, Loft A, Povlsen BB, Erb K, Agerholm I, Aasted M et al. A randomized clinical trial to evaluate the effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) in embryo culture medium for in vitro fertilization. *Fertil Steril*. 2013;99(6):1600–9.
258. Karagenc L, Lane M, Gardner DK. Granulocyte-macrophage colony-stimulating factor stimulates mouse blastocyst inner cell mass development only when media lack human serum albumin. *Reprod Biomed Online*. 2005;10(4):511–8.
259. Kawamura K, Chen Y, Shu Y, Cheng Y, Qiao J, Behr B et al. Promotion of human early embryonic development and blastocyst outgrowth in vitro using autocrine/paracrine growth factors. *PLoS One*. 2012;7(11):e49328.
260. Cha J, Sun X, Dey SK. Mechanisms of implantation: strategies for successful pregnancy. *Nat Med*. 2012;18(12):11754–67.
261. Quinn P, Wales RG. Growth and metabolism of preimplantation mouse embryos cultured in phosphate-buffered medium. *J Reprod Fertil*. 1973;35(2):289–300.
262. Minhas BS, Randall GW, Dodson MG, Robertson JL. A limited exposure of murine oocytes and zygotes to phosphate buffered saline retards development in vitro. *Theriogenology*. 1989;31:229.
263. Mahadevan MM, Fleetham J, Church RB, Taylor PJ. Growth of mouse embryos in bicarbonate media buffered by carbon dioxide, hepes, or phosphate. *J In Vitro Fert Embryo Transf*. 1986;3(5): 304–8.
264. Good NE, Winget GD, Winter W, Connolly TN, Izawa S, Singh RM. Hydrogen ion buffers for biological research. *Biochemistry*. 1966;5(2):467–77.
265. Quinn P, Barros C, Whittingham DG. Preservation of hamster oocytes to assay the fertilizing capacity of human spermatozoa. *J Reprod Fertil*. 1982;66(1):161–8.
266. Behr BR, Stratton CJ, Foote WD, Knutzen V, Sher G. In vitro fertilization (IVF) of mouse ova in HEPES-buffered culture media. *J In Vitro Fert Embryo Transf*. 1990;7(1):9–15.
267. Mastroianni L, Jr, Jones R. Oxygen tension within the rabbit Fallopian tube. *J Reprod Fertil*. 1965;147:99–102.
268. Ross RN, Graves CN. O_2 levels in female rabbit reproductive tract. *J Anim Sci*. 1974;39:994.
269. Fischer B, Bavister BD. Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. *J Reprod Fertil*. 1993;99(2):673–9.
270. Maas DH, Storey BT, Mastroianni L, Jr. Oxygen tension in the oviduct of the rhesus monkey (Macaca mulatta). *Fertil Steril*. 1976;27(11):1312–7.
271. Pabon JE, Jr, Findley WE, Gibbons WE. The toxic effect of short exposures to the atmospheric oxygen concentration on early mouse embryonic development. *Fertil Steril*. 1989;51(5):896–900.
272. Quinn P, Harlow GM. The effect of oxygen on the development of preimplantation mouse embryos in vitro. *J Exp Zool*. 1978;206(1):73–80.
273. Li J, Foote RH. Culture of rabbit zygotes into blastocysts in protein-free medium with one to twenty per cent oxygen. *J Reprod Fertil*. 1993;98(1):163–7.
274. Thompson JG, Simpson AC, Pugh PA, Donnelly PE, Tervit HR. Effect of oxygen concentration on in-vitro development of preimplantation sheep and cattle embryos. *J Reprod Fertil*. 1990;89(2):573–8.
275. Wale PL, Gardner DK. Time-lapse analysis of mouse embryo development in oxygen gradients. *Reprod Biomed Online*. 2010;21(3):402–10.
276. Kirkegaard K, Hindkjaer JJ, Ingerslev HJ. Effect of oxygen concentration on human embryo development evaluated by time-lapse monitoring. *Fertil Steril*. 2013;99(3):738–44 e4.
277. Katz-Jaffe MG, Linck DW, Schoolcraft WB, Gardner DK. A proteomic analysis of mammalian preimplantation embryonic development. *Reproduction*. 2005;130(6):899–905.
278. Rinaudo PF, Giritharan G, Talbi S, Dobson AT, Schultz RM. Effects of oxygen tension on gene expression in preimplantation mouse embryos. *Fertil Steril*. 2006;86(4 Suppl):1252–65, 65 e1-36.
279. Wale PL, Gardner DK. Oxygen regulates amino Acid turnover and carbohydrate uptake during the preimplantation period of mouse embryo development. *Biol Reprod*. 2012;87(1):24.
280. Gardner DK. The impact of physiological oxygen during culture, and vitrification for cryopreservation, on the outcome of extended culture in human IVF. *Reprod Biomed Online*. 2016;32(2):137–41.

281. Meintjes M, Chantilis SJ, Douglas JD, Rodriguez AJ, Guerami AR, Bookout DM et al. A controlled randomized trial evaluating the effect of lowered incubator oxygen tension on live births in a predominantly blastocyst transfer program. *Hum Reprod*. 2009;24(2):300–7.
282. Nanassy L, Peterson CA, Wilcox AL, Peterson CM, Hammoud A, Carrell DT. Comparison of 5% and ambient oxygen during days 3–5 of in vitro culture of human embryos. *Fertil Steril*. 2010;93(2):579–85.
283. Waldenstrom U, Engstrom AB, Hellberg D, Nilsson S. Low-oxygen compared with high-oxygen atmosphere in blastocyst culture, a prospective randomized study. *Fertil Steril*. 2009;91(6):2461–5.
284. Bontekoe S, Mantikou E, van Wely M, Seshadri S, Repping S, Mastenbroek S. Low oxygen concentrations for embryo culture in assisted reproductive technologies. *Cochrane Db Syst Rev*. 2012;7:CD008950.
285. Christianson MS, Zhao Y, Shoham G, Granot I, Safran A, Khafagy A et al. Embryo catheter loading and embryo culture techniques: Results of a worldwide Web-based survey. *J Assist Reprod Genet*. 2014;31(8):1029–36.
286. Gardner DK, Lane M. Embryo culture systems. In: *Handbook of In Vitro Fertilization*, 2nd ed., Trounson A, Gardner DK (Eds.). Boca Raton, FL: CRC Press, 1999, 205–64.
287. Graves CN, Biggers JD. Carbon dioxide fixation by mouse embryos prior to implantation. *Science*. 1970;167(924):1506–8.
288. Garris DR. Uterine blood flow, pH, and pCO2 during nidation in the guinea pig: Ovarian regulation. *Endocrinology*. 1984;114(4):1219–24.
289. Carney EW, Bavister BD. Regulation of hamster embryo development in vitro by carbon dioxide. *Biol Reprod*. 1987;36(5):1155–63.
290. Halliden K, Li J, Carney EW, Foote RH. Increasing carbon dioxide from five percent to ten percent improves rabbit blastocyst development from cultured zygotes. *Mol Reprod Dev*. 1992;33:276.
291. Farrell PB, Foote RH. Beneficial effects of culturing rabbit zygotes to blastocysts in 5% oxygen and 10% carbon dioxide. *J Reprod Fertil*. 1995;103(1):127–30.
292. Maas DH, Storey BT, Mastroianni L, Jr. Hydrogen ion and carbon dioxide content of the oviductal fluid of the rhesus monkey (Macaca mulatta). *Fertil Steril*. 1977;28(9):981–5.
293. Dale B, Menezo Y, Cohen J, DiMatteo L, Wilding M. Intracellular pH regulation in the human oocyte. *Hum Reprod*. 1998;13(4):964–70.
294. FitzGerald-Scott L, Sundaram SG, Smith S. The relevance and use of mouse embryos bioassays for quality control in an assisted reproductive technology program. *Fertil Steril*. 1993;60:559.
295. Lane M, Baltz JM, Bavister BD. Na+/H+ antiporter activity in hamster embryos is activated during fertilization. *Dev Biol*. 1999;208(1):244–52.
296. Zhao Y, Baltz JM. Bicarbonate/chloride exchange and intracellular pH throughout preimplantation mouse embryo development. *Am J Physiol*. 1996;271(5 Pt 1):C1512–20.
297. Phillips KP, Baltz JM. Intracellular pH regulation by HCO_3^-/Cl^- exchanger activity appears following fertilization in the mouse. *Dev Biol*. 1999;208:392–405.
298. Fischer B, Schumacher A, Hegele-Hartung C, Beier HM. Potential risk of light and room temperature exposure to preimplantation embryos. *Fertil Steril*. 1988;50(6):938–44.
299. Pickering SJ, Braude PR, Johnson MH, Cant A, Currie J. Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. *Fertil Steril*. 1990;54(1):102–8.
300. Pribenszky C, Matyas S, Kovacs P, Losonczi E, Zadori J, Vajta G. Pregnancy achieved by transfer of a single blastocyst selected by time-lapse monitoring. *Reprod Biomed Online*. 2010;21(4):533–6.
301. Meseguer M, Herrero J, Tejera A, Hilligsoe KM, Ramsing NB, Remohi J. The use of morphokinetics as a predictor of embryo implantation. *Hum Reprod*. 2011;26(10):2658–71.
302. Meseguer M, Rubio I, Cruz M, Basile N, Marcos J, Requena A. Embryo incubation and selection in a time-lapse monitoring system improves pregnancy outcome compared with a standard incubator: A retrospective cohort study. *Fertil Steril*. 2012;98(6):1481–9 e10.
303. Umaoka Y, Noda Y, Nakayama T, Narimoto K, Mori T, Iritani A. Effect of visual light on in vitro embryonic development in the hamster. *Theriogenology*. 1992;38:1043–54.
304. Schumacher A, Fischer B. Influence of visible light and room temperature on cell proliferation in preimplantation rabbit embryos. *J Reprod Fertil*. 1988;84(1):197–204.
305. Hirao Y, Yanagimachi R. Detrimental effect of visible light on meiosis of mammalian eggs in vitro. *J Exp Zool*. 1978;206(3):365–9.

306. Noda Y, Goto Y, Umaoka Y, Shiotani M, Nakayama T, Mori T. Culture of human embryos in alpha modification of Eagle's medium under low oxygen tension and low illumination. *Fertil Steril*. 1994;62(5):1022–7.
307. Bolton VN, Cutting R, Clarke H, Brison DR. ACE consensus meeting report: Culture systems. *Hum Fertil (Camb)*. 2014;17(4):239–51.
308. Paria BC, Dey SK. Preimplantation embryo development in vitro: Cooperative interactions among embryos and role of growth factors. *Proc Natl Acad Sci USA*. 1990;87(12):4756–60.
309. Lane M, Gardner DK. Effect of incubation volume and embryo density on the development and viability of mouse embryos in vitro. *Hum Reprod*. 1992;7(4):558–62.
310. Salahuddin S, Ookutsu S, Goto K, Nakanishi Y, Nagata Y. Effects of embryo density and co-culture of unfertilized oocytes on embryonic development of in-vitro fertilized mouse embryos. *Hum Reprod*. 1995;10(9):2382–5.
311. Gardner DK, Lane MW, Lane M. Development of the inner cell mass in mouse blastocyst is stimulated by reducing the embryo: Incubation volume ratio. *Hum Reprod*. 1997;12(1):P-132.
312. Ahern TJ, Gardner DK. Culturing bovine embryos in groups stimulates blastocyst development and cell allocation to the inner cell mass. *Theriogenology*. 1998;49:194.
313. Ebner T, Shebl O, Moser M, Mayer RB, Arzt W, Tews G. Group culture of human zygotes is superior to individual culture in terms of blastulation, implantation and life birth. *Reprod Biomed Online*. 2010;21(6):762–8.
314. Rijnders PM, Jansen CA. Influence of group culture and culture volume on the formation of human blastocysts: A prospective randomized study. *Hum Reprod*. 1999;14(9):2333–7.
315. Hardy K, Robinson FM, Paraschos T, Wicks R, Franks S, Winston RM. Normal development and metabolic activity of preimplantation embryos in vitro from patients with polycystic ovaries. *Hum Reprod*. 1995;10(8):2125–35.
316. Simon C, Garcia Velasco JJ, Valbuena D, Peinado JA, Moreno C, Remohi J et al. Increasing uterine receptivity by decreasing estradiol levels during the preimplantation period in high responders with the use of a follicle-stimulating hormone step-down regimen. *Fertil Steril*. 1998;70(2):234–9.
317. Van der Auwera I, Pijnenborg R, Koninckx PR. The influence of in-vitro culture versus stimulated and untreated oviductal environment on mouse embryo development and implantation. *Hum Reprod*. 1999;14(10):2570–4.
318. Ertzeid G, Storeng R. The impact of ovarian stimulation on implantation and fetal development in mice. *Hum Reprod*. 2001;16(2):221–5.
319. Lane M, Robker RL, Robertson SA. Parenting from before conception. *Science*. 2014;345(6198):756–60.
320. Gardner DK. Improving embryo culture and enhancing pregnancy rate. In: *Female Infertility Therapy: Current Practise*, Shoham Z, Howles C, Jacobs H (Eds.). London: Martin Dunitz, 1998, 283–99.
321. Croxatto HB, Ortiz ME, Diaz S, Hess R, Balmaceda J, Croxatto HD. Studies on the duration of egg transport by the human oviduct. II. Ovum location at various intervals following luteinizing hormone peak. *Am J Obstet Gynecol*. 1978;132(6):629–34.
322. Walker KJ, Green MP, Gardner DK. Spatial asynchronous transfer of cleavage-stage mouse embryos to the uterus compromises fetal development. *Mol Reprod Dev*. 2015;82(2):80.
323. Marek D, Langley M, Gardner DK, Confer N, Doody KM, Doody KJ. Introduction of blastocyst culture and transfer for all patients in an in vitro fertilization program. *Fertil Steril*. 1999;72(6):1035–40.
324. Wilson M, Hartke K, Kiehl M, Rodgers J, Brabec C, Lyles R. Integration of blastocyst transfer for all patients. *Fertil Steril*. 2002;77(4):693–6.
325. van Os HC, Alberda AT, Janssen-Caspers HA, Leerentveld RA, Scholtes MC, Zeilmaker GH. The influence of the interval between in vitro fertilization and embryo transfer and some other variables on treatment outcome. *Fertil Steril*. 1989;51(2):360–2.
326. Buster JE, Bustillo M, Rodi IA, Cohen SW, Hamilton M, Simon JA et al. Biologic and morphologic development of donated human ova recovered by nonsurgical uterine lavage. *Am J Obstet Gynecol*. 1985;153(2):211–7.
327. Braude P, Bolton V, Moore S. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature*. 1988;332(6163):459–61.
328. Fanchin R, Ayoubi JM, Righini C, Olivennes F, Schonauer LM, Frydman R. Uterine contractility decreases at the time of blastocyst transfers. *Hum Reprod*. 2001;16(6):1115–9.

329. Veeck LL. Does the developmental stage at freeze impact on clinical results post-thaw? *Reprod Biomed Online*. 2003;6(3):367–74.
330. Veeck LL, Bodine R, Clarke RN, Berrios R, Libraro J, Moschini RM et al. High pregnancy rates can be achieved after freezing and thawing human blastocysts. *Fertil Steril*. 2004;82(5):1418–27.
331. Anderson AR, Weikert ML, Crain JL. Determining the most optimal stage for embryo cryopreservation. *Reprod Biomed Online*. 2004;8(2):207–11.
332. Fragouli E, Lenzi M, Ross R, Katz-Jaffe M, Schoolcraft WB, Wells D. Comprehensive molecular cytogenetic analysis of the human blastocyst stage. *Hum Reprod*. 2008;23(11):2596–608.
333. Papanikolaou V. Early pregnancy loss is significantly higher after day 3 single embryo transfer that after day 5 single blastocyst transfer in GnRH antagonist stimulated IVF cycles. *RBM online*. 2006;12(1):60–5.
334. Papanikolaou EG, Kolibianakis EM, Tournaye H, Venetis CA, Fatemi H, Tarlatzis B et al. Live birth rates after transfer of equal number of blastocysts or cleavage-stage embryos in IVF. *A systematic review and meta-analysis*. *Hum Reprod*. 2008;23(1):91–9.
335. Glujovsky D, Farquhar C, Quinteiro Retamar AM, Alvarez Sedo CR, Blake D. Cleavage stage versus blastocyst stage embryo transfer in assisted reproductive technology. *Cochrane Db Syst Rev*. 2016;6:CD002118.
336. Hunault CC, Eijkemans MJ, Pieters MH, te Velde ER, Habbema JD, Fauser BC et al. A prediction model for selecting patients undergoing in vitro fertilization for elective single embryo transfer. *Fertil Steril*. 2002;77(4):725–32.
337. Balaban B, Urman B, Alatas C, Mercan R, Aksoy S, Isiklar A. Blastocyst-stage transfer of poor-quality cleavage-stage embryos results in higher implantation rates. *Fertil Steril*. 2001;75(3):514–8.
338. Schoolcraft WB, Gardner DK. Blastocyst culture and transfer increases the efficiency of oocyte donation. *Fertil Steril*. 2000;74(3):482–6.
339. Gardner DK, Surrey E, Minjarez D, Leitz A, Stevens J, Schoolcraft WB. Single blastocyst transfer: A prospective randomized trial. *Fertil Steril*. 2004;81(3):551–5.
340. Maheshwari A, Hamilton M, Bhattacharya S. Should we be promoting embryo transfer at blastocyst stage? *Reprod Biomed Online*. 2016;32(2):142–6.
341. Li Z, Wang YA, Ledger W, Edgar DH, Sullivan EA. Clinical outcomes following cryopreservation of blastocysts by vitrification or slow freezing: A population-based cohort study. *Hum Reprod*. 2014;29(12):2794–801.
342. Roy TK, Bradley CK, Bowman MC, McArthur SJ. Single-embryo transfer of vitrified-warmed blastocysts yields equivalent live-birth rates and improved neonatal outcomes compared with fresh transfers. *Fertil Steril*. 2014;101(5):1294–301.
343. Wirleitner B, Schuff M, Stecher A, Murtinger M, Vanderzwalmen P. Pregnancy and birth outcomes following fresh or vitrified embryo transfer according to blastocyst morphology and expansion stage, and culturing strategy for delayed development. *Hum Reprod*. 2016;31(8):1685–95.
344. Gardner DK, Reed L, Linck D, Sheehan C, Lane M. Quality control in human in vitro fertilization. *Semin Reprod Med*. 2005;23(4):319–24.
345. Wolff HS, Fredrickson JR, Walker DL, Morbeck DE. Advances in quality control: Mouse embryo morphokinetics are sensitive markers of in vitro stress. *Hum Reprod*. 2013;28(7):1776–82.

15

Polar Body, Cleavage Stage and Trophectoderm Biopsy

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Introduction

Pre-implantation genetic diagnosis and screening (PGD/PGS) are tools for embryo selection aimed at the identification of not affected and/or euploid embryos in a cohort produced by a couple during an IVF treatment. PGD applies to any couple with a specific genetic susceptibility to conceive affected children (monogenic diseases or structural chromosomal abnormalities). PGD was theorized in the early 1990s (1) and its value was soon clear to the field. Nowadays, PGD could be considered a valuable and established reproductive option together with prenatal diagnosis for couples at risk of transmitting a genetic defect. PGS instead aims at the identification of de novo chromosomal abnormalities in the embryo.

A major barrier in human reproduction is in fact represented by aneuploidies originating during meiosis or in the first mitotic cycles of embryonic pre-implantation development. Specifically, aneuploidies in pre-implantation embryo can be considered the single most important factor for implantation failure, and stand for the main cause of miscarriages, while their incidence in newborns is relatively low, with around 0.3%, entailing mainly trisomies 13, 18, and 21 and sex chromosome abnormalities (2). Indeed, especially during the first stages of pre-implantation development, when cell cycle checkpoints are turned off and embryonic genome activation (EGA) has not yet occurred, no negative selection against chromosomally abnormal embryos is in place (3). For these reasons, embryonic aneuploidies are the main reasons the fall of a woman's fertility and the respective exponential increase in miscarriage rate from 35 years of age onwards (advanced maternal age [AMA] population) (4,5). Given that the mean age of women undergoing IVF cycles is constantly growing worldwide, the risk of transferring aneuploid embryos is indeed considerably high.

PGS was conceived in this scenario. Theoretically it entails the power to reduce both implantation failure and miscarriage rates by preventing aneuploid embryo transfer (ET). However, in its first version it failed to fulfill its promise, and this raised a long-lasting skepticism in the scientific community.

The concern resolved in the systematic review and meta-analysis published by Mastenbroek et al. (6). The authors investigated the clinical outcomes of randomized controlled trials (RCTs) that compared PGS to standard care IVF. At that time, the gold standard approach for PGS entailed 9-chromosome fluorescence in situ hybridization (FISH)-based analysis of blastomere(s) biopsy at the cleavage stage. No significant advantage for the patients derived from PGS, and especially in the AMA population it actually lowered the cumulative pregnancy chances. This was perceived as the fall of a fascinating theory and of a promising embryo selection approach.

Both the technical and biological reasons underlying this failure were deeply investigated, and different strategies were then used to implement new approaches. While the use of 9-chromosome FISH was strongly reduced in favor of higher resolution and more accurate comprehensive chromosome screening (CCS) technologies (e.g., quantitative polymerase chain reaction [qPCR], array-CGH [comparative genomic hybridization], array-SNP [single nucleotide polymorphisms], and nowadays next generation sequencing [NGS]) (7), the definition of a different biopsy stage is instead a still ongoing process.

The choice of a proper stage to conduct the biopsy is in fact a critical issue for both PGD and PGS. First of all, a clinically effective biopsy procedure should not compromise the embryo developmental/

implantation potential. This is a critical point, since it is crucial to avoid any reduction of the live birth rate per stimulation cycle when extra manipulation procedures are used in the laboratory. Secondly, the biopsied sample should reliably mirror the embryo genetic constitution. The cell sample that is going to be analyzed has to provide a reliable genetic diagnosis and clinical positive and negative predictive values. Thirdly, the clinical application of the approach should be supported by strong evidence and class one data produced through extensive preclinical and clinical validations. Eventually, the protocol should give the best results with its clinical application in terms of cost-effectiveness and laboratory workload. To this aim, the genetic analysis should ideally be performed on reproductively competent embryos only, to minimize costs and times.

Across the last decades, mainly two further strategies were investigated besides the extensively used blastomere biopsy, namely polar body (PB) biopsy from the oocyte and/or the zygote, and trophectoderm (TE) biopsy at the blastocyst stage. The aim of this chapter is to comprehensively describe all the advantages and disadvantages of these biopsy approaches based on available scientific evidence in relation to PGD/PGS application.

Cleavage Stage Biopsy

Cleavage stage biopsy entails the removal of a single cell from a day 3 embryo showing at least six blastomeres and less than 30% of fragmentation, as suggested by the ESHRE guidelines (8).

Zona pellucida drilling is required. It can be achieved using laser pulses (9), tyrode acid (10), or mechanically (11). Clinical outcomes after the use of the three methods are similar, as has been reported in several RCTs on sibling embryos (12–14). However, laser-assisted zona drilling is more standardized and reproducible than the other methods. In fact, it is less operator-dependent (14), less time-consuming, and requires a shorter learning curve. Nevertheless, there is a considerable rise in the temperature of the culture media that surrounds the embryo during the procedure (15), which was perceived as a concern. In this regard, Taylor et al. (16) clearly showed that this does not compromise either technical or clinical outcomes. Nonetheless, it seems that zona opening *per se* could affect the subsequent processes that lead to blastocyst formation. A slower developmental rate as well as an impairment of the hatching process can in fact occur, depending on the number and the size of the holes produced (17–20).

After zona pellucida opening, a Ca^{++}/Mg^{++}-free medium is commonly used in order to release cell–cell bonds. This leads to an easier withdrawal of the selected blastomere, with apparently also less stressing of the embryo (21). Theoretically this procedure should not impact subsequent blastulation, but this is still a debatable issue. In particular, some studies have claimed that Ca^{++} depletion has a negative effect upon compaction by impacting cytoskeleton remodeling and intercellular communication mechanisms (22–24).

Kirkegaard et al. (25) performed a control-matched analysis in time-lapse where they compared the further development of embryos that were biopsied at the cleavage stage versus control undisturbed embryos. They highlighted that blastomere biopsy impairs the hatching procedure and results in delayed and smaller blastocysts with thicker zona pellucida.

All these aspects together led to the hypothesis that cleavage stage biopsy can actually impact embryo reproductive competence. In this regard, Scott et al. (26) performed an outstanding and elegant randomized paired study. In this study they included double ETs of sibling top quality embryos where only one underwent blastomere biopsy. The other embryo was kept as control. No genetic analysis was performed before replacement, and both embryos were transferred in the same cycle. Whenever a single embryo implanted, DNA fingerprinting from the original biopsy was performed and compared to either fetal or neonatal DNA. Matching results identified the implanted embryo as the biopsied one, and vice versa non-matching results identified the control embryo as the one which led to the pregnancy. A striking 39% relative decrease in implantation rate was reported after blastomere biopsy. The authors could confidently state then that the cleavage stage biopsy approach actually compromises embryo reproductive competence.

In general, a lower blastocyst rate was also reported after blastomere biopsy with respect to the standard care (27–29). As a consequence, the use of cleavage stage biopsy is expected to result in a lower live birth rate per intention to treat analysis, regardless whether PGD or PGS cycles are considered.

Besides the impact of biopsy on embryo viability, blastomere biopsy suffers from other biological and technical issues mainly related to single cell analysis. Even though today we have effective genetic technologies available to analyze low DNA input samples, they still do not perform at best when used on a single cell, and suffer from technical artifacts. Firstly, artifacts such as preferential amplification of genomic regions or whole chromosomes, random loss of alleles (allele drop-out [ADO]), allele drop-in (ADI), and a higher rate of amplification failure (8,30) are all possible consequences of single cell analysis which can translate to a wrong assessment of the actual embryonic karyotype/genotype, and to a low global efficacy of the procedure. Secondly, all the molecular methods we can manage at present do not discriminate whether a cell is replicating its genome (S-phase of the cell cycle) or not (G1- or G2/M-phase), a further potential source of false positive (FP) diagnosis (31). These objective technical challenges of working on a single cell inevitably translate to a considerable reduction of clinical performance. Indeed, an inconclusive diagnosis rate is also considerably high when working on a single blastomere, usually around 10% per test performed (32).

It is important to highlight that the high FP diagnosis rate that can be obtained following a single blastomere analysis can explain the impressive over-estimation of the prevalence of chromosomal mosaicism that was reported in the last decade, namely the coexistence within the same embryo of cells with different karyotypes, at the cleavage stage (up to 70%) (33–40). The estimated rates that have been published to date are in fact disproportionate, especially if compared to the overall incidence of mosaicism in spontaneous conceptions (1.22%) and in IVF pregnancies (1.32%), where true mosaicism accounts only for 0.3%–0.44% (41). A more logical explanation of cell to cell inconsistency of diagnosis when multiple blastomeres are analyzed to assess mosaicism, and with the use of FISH, is indeed the high technical error rate rather than a biological variation.

The scarce predictive power on implantation that derives from blastomere analysis was clearly highlighted in an important study by Scott et al. (42). Here they assessed that the positive clinical predictive value of blastomere biopsy, namely the rate of embryos actually leading to a sustained implantation after a cleavage stage-based diagnosis of euploidy, is lower than 30%.

Nevertheless, a comprehensive evaluation of the clinical value of a strategy entailing CCS-based analysis of blastomere biopsy is still missing. Some studies are in fact in the pipeline (NCT01571076, NCT01950104 registered in www.clinicaltrials.gov) that will solve this last issue in the future.

In conclusion, blastomere biopsy seems to severely impact embryo developmental potential, as shown by Scott et al., and many technical issues reduce the reliability of its molecular analysis. Accordingly, the use of blastomere biopsy for PGD/PGS is highly questionable based on available evidence, since it can significantly lower the live birth rate per stimulation cycle due to both technical inconsistency and reduction of embryo viability.

Polar Bodies Biopsy

The failure of the cleavage stage biopsy approach led embryologists worldwide to pursue novel biopsy strategies. In this regards, PB biopsy was identified as a putative valuable alternative to blastomere biopsy. In the main, two reasons led to its investigation: the evidence that embryo aneuploidy rate sharply increases with advance maternal age (4,5) and the unique possibility to predict embryo karyotype through the analysis of the waste by-products of female meiosis. Furthermore, PB biopsy is the only possible approach in those countries where embryo biopsy is not allowed at any stage of pre-implantation development.

While for PGD application the absence of male genome clearly limits its use for monogenic disease analysis, this approach soon showed also limitations for aneuploidy testing purposes. The main study, which highlighted its biological and technical limitations, was published in 2013 (43). It was designed as a sequential biopsy of PBs, blastomere, and TE, from the same embryos associated with blinded aCGH analysis. The analysis led to a comprehensive view of chromosomal segregation patterns throughout pre-implantation development in AMA patients. The drawbacks of the PB approach for embryo aneuploidy testing were defined among the results of this study. Firstly, it was evident that the majority of the aneuploidies arise in meiosis II (MII). Accordingly, both PBs are required, thus doubling the efforts and costs of a PGS cycle. Secondly, paternally- and mitotically-derived aneuploidies, and meiotic errors corrected

in further steps of embryo development, are all missed when adopting this biopsy strategy. The most common event leading to misdiagnosis was ascertained as the premature separation of sister chromatids (PSSC) in meiosis I (MI), which is balanced in MII. As a consequence, the PB biopsy approach showed high FP and false negative (FN) diagnosis rates. In fact, on a per chromosome basis, its accuracy in predicting blastocyst chromosomal complement was reported as significantly lower than blastomere(s) analysis (61.7% and 86.4%, respectively; $p < 0.01$).

The evidence of a discrepancy between the karyotype predicted by PBs screening and the actual zygote one was also reported by Handyside et al. for 21.1% of cases (44). Christopikou et al. also reported 17% of FP results after the follow-up of PBs-based diagnosis in the resulting embryos (45).

MI PSSC later balanced in MII was shown by Forman et al. to lead to euploid embryos, mostly resulting in chromosomally normal children (46,47). This was clear evidence that PB biopsy strategy entails the risk of preventing the transfer of reproductively competent embryos.

In a recent paper, Ottolini et al. screened the female meiotic "trios" based on PB1, PB2, and oocyte genetic content. The authors performed this study on donated human oocytes, which were artificially activated through a Ca^{++} ionophore-based protocol, thus avoiding any contamination of paternal genome in the oocyte. Karyomapping was chosen as the technique for the molecular analysis. This study design provided a comprehensive view of female meiosis with a notable depth of analysis through the so-called "meiomap" (48). Besides showing that recombination rate is inversely proportional to oocyte aneuploidy rate, they described the whole range of segregation mechanisms in MI and MII. In particular, together with the already known mechanisms of MI and MII non-disjunction and PSSC, they illustrated the previously unidentified mechanism of "reverse segregation." Briefly, the PB1 shows a bivalency constituted by different chromatids that can either result in a normal oocyte (77% of cases) or in aneuploid one (23%), dependent upon the migration of chromatids during MII at the same or at the opposite pole. Reverse segregation, together with PSSC, accounts for the majority of the meiotic events. These segregation patterns can affect the ability to accurately predict the chromosomal complement in the resulting oocyte when performing the analysis on PBs. This is especially true when CCS methods with an insufficient depth of analysis are adopted for the analysis.

Another pitfall of the PB biopsy approach is shared with cleavage stage one, namely single cell analysis. In this regard, the inconclusive diagnosis rate due to amplification failure of either PB1 or PB2 accounts for almost 15% of cases in highly qualified centers (49).

However, from a clinical perspective we lack powerful RCTs to properly investigate the safety and/or efficiency of this approach (50). Discordant data have also been reported when dealing with the impact of PB biopsy upon further embryo development. Some papers in fact claimed that it does not affect oocyte/embryo quality parameters or neonatal outcomes (12,51–54), while others reported abnormal embryo development with respect to standard care, but again missing the pivotal data about implantation potential (55).

To summarize, the PB biopsy approach suffers from technical concerns about its diagnostic accuracy. Moreover, it is not a cost-effective strategy, since all oocytes/zygotes must be biopsied regardless of their future developmental potential (part of them will never get to the cleavage/blastocyst stage in order to be eventually transferred). Paternally-derived genome is missed from monogenic diseases diagnosis. However, this approach can still be considered feasible, since no clinical data from RCTs have been produced against it.

Two clinical trials aimed at investigating the clinical effectiveness of the PB biopsy approach have been registered on the website www.clinicaltrials.gov: the ESHRE ESTEEM RCT (NCT01532284) and a study by the Weill Medical College (Cornell University) (NCT01574404). Possibly, reliable evidence will derive from these studies in the coming years.

Trophectoderm Biopsy at the Blastocyst Stage

TE biopsy was theorized in 2004 by de Boer et al. (56), and the first pregnancies following this approach were reported one year later by the same group (57) and by Kokkali et al. (58).

This biopsy approach is probably perceived as more complicated and less reproducible and standardized than blastomere biopsy, which is still the biopsy method characterized by the highest expertise

worldwide. In particular, embryos reach the blastocyst stage in an asynchronous fashion (day 5–7 of pre-implantation development) and they are characterized by heterogeneous morphology, unlike cleavage stage embryos. However, several papers in the last decade showed no significant correlation between blastocyst aneuploidy rate with either embryo developmental rate or static/dynamic morphological evaluation (59–62). This suggests that any embryo that reaches to this stage should be biopsied, regardless of the conventional parameters of embryo grading.

Two methods to conduct blastocyst stage biopsy have been described in the literature (summarized in Figure 15.1). The first was published by McArthur et al. (57) and the second by Capalbo (59). The former method requires a hole in the zona pellucida at the cleavage stage, and then the embryo is left undisturbed up to the blastocyst stage (Figure 15.1a). A non-physiological hatching of few TE cells is thus expected, which makes the biopsy procedure relatively easy, but potentially impairs embryo development to the

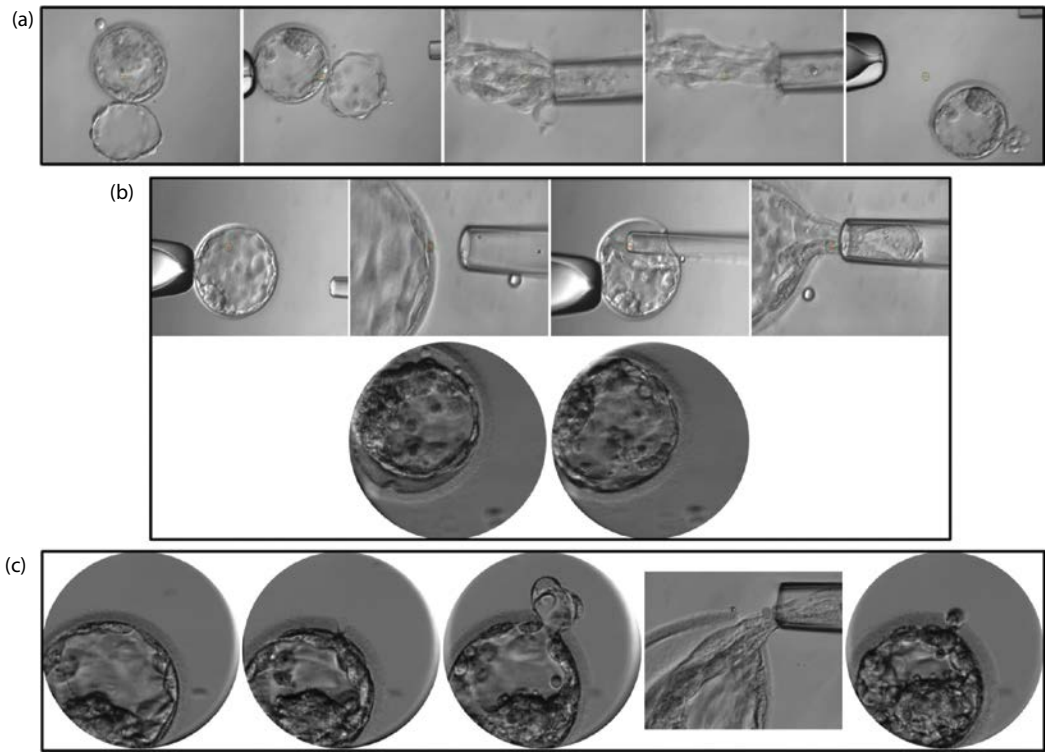

FIGURE 15.1 (a) Blastocyst biopsy method entailing the production of a hole in the zona pellucida at the cleavage stage. The embryo is removed from the incubator at the cleavage stage in order to drill through the zona pellucida with a few laser pulses. It is then put back into the incubator. This procedure would determine non-physiological cell hatching whenever the embryo reaches the blastocyst stage. A few laser pulses, together with a gentle suction, are then sufficient to remove the few trophectoderm cells that will compose the biopsy fragment. (b) Blastocyst biopsy method entailing simultaneous zona pellucida opening and trophectoderm biopsy. The fully-expanded blastocyst is positioned on the holding pipette so that the inner cell mass is kept as far as possible from the spot where the operator will target the laser pulses. Once the zona pellucida is opened, the biopsy operator starts detaching the blastocyst from it. The blastocyst collapses and the operator then chooses 5–10 trophectoderm cells, sucks them within the biopsy pipette and starts to slowly move towards the outer part of the blastocyst. The cellular junctions between the chosen cells and the body of the blastocyst are thus exposed. The operator targets them with few laser pulses. A gentle suction during the laser firing procedure is then sufficient to remove the biopsy fragment. The blastocyst gets back to full-expansion in 1–2 hours after biopsy. (c) Blastocyst biopsy entailing the production of a hole in the zona pellucida whenever full expansion is reached. The blastocyst is removed from the incubator on the morning of day 5 or day 6 and a hole is produced in the zona pellucida far from the inner cell mass. The blastocyst is then put back into the incubator. A few trophectoderm cells start to hatch from that hole during the following hours. When the hatching cells are sufficient to compose a proper biopsy fragment, the operator targets the cellular junctions between them and the body of the blastocyst with few laser pulses. A gentle suction during the firing procedure is sufficient then to remove the biopsy fragment.

blastocyst stage (25). Furthermore, the risk that the blastocyst will hatch starting from the inner cell mass (ICM) also exists, and this accounts for approximately one third of cases. According to the latter method, instead, the embryo is left undisturbed up to the fully-expanded blastocyst stage. Zona pellucida drilling and TE fragment retrieval are performed at the same time (Figure 15.1b). A physiological blastocyst expansion and zona pellucida thinning represents, in fact, an advantage of this protocol. Moreover, no extra stress at the cleavage stage is required, allowing a more physiological embryo growth to the blastocyst stage, as well as no additional operations in the laboratory. A third possibility also exists for operators still not confident with the simultaneous zona opening and TE fragment retrieval protocol. Namely, a hole in the zona (far from the ICM) can be made whenever the blastocyst reaches full expansion in the morning of day 5 or day 6. The blastocyst is then placed back into the incubator for a few hours until some TE cells start to herniate from the hole (Figure 15.1c). Following this alternative method, TE fragment retrieval results are technically similar to the protocol described by McArthur et al., but its putative drawbacks are avoided.

The paired randomized study by Scott et al. already mentioned in the "cleavage stage biopsy" paragraph represents again a landmark paper in this field (26). No significant impact in fact derived from the TE biopsy approach, conversely to that reported for blastomere biopsy. This evidence reassured embryologists worldwide about the safety of the procedure, and represented a milestone for the growing application of TE biopsy.

The reasons for a higher stress-tolerance of the embryo at this stage of pre-implantation development are mainly three: TE is the non-embryonic part of the blastocyst, EGA has already occurred, and a limited portion of the embryo biomass is removed (5–10 cells out of ≥200).

The 5–10 cells composing the biopsied fragment prevent the problems related to single cell analysis, and this leads to a more accurate and reliable downstream molecular analysis. However, the concerns about mosaicism persist beyond this. A non-random allocation of aneuploid cells solely to the TE was in fact proposed as a potential source of FP results (63–66). However, this hypothesis was not confirmed in available research studies investigating it, which showed an almost full concordance between ICM and TE chromosomal constitution (67–69). Indeed, TE-based molecular diagnosis can be considered diagnostic of the ICM genetic content. In this regard, we performed a study aimed at a clear definition of the incidence of mosaicism at the blastocyst stage and the risk of misdiagnosis related to it. At first, we designed and validated a method of ICM biopsy that ensured an almost total purity of the ICM and TE samples (69). Then, embryos diagnosed as aneuploid after a CGH-based analysis of a TE fragment were warmed, three further TE samples and the corresponding ICM were separately retrieved, and FISH-based analysis was conducted at the single cell level. The results highlighted that 79.1% of cases were represented by constitutional aneuploidies and 20.9% instead were mosaic. However, the real risk for misdiagnosis was found only in nearly 4% of the blastocysts, where a mosaic diploid/aneuploid constitution was observed. Furthermore, any time that mosaicism constituted more than 40% of the whole blastocyst, an aneuploid diagnosis was given by the first aCGH-based analysis. These data support that CCS techniques are sensible enough to recognize moderate/high grade mosaicism and prevent the transfer of the expected pathological embryos.

The prospective double-blinded non-selection study published by Scott et al. (42) and cited previously in this chapter was properly designed to compare the clinical effectiveness between the blastomere and TE biopsy methods. In particular, the positive clinical predictive value, namely the rate of embryos actually leading to a sustained implantation after a CCS-based diagnosis of euploidy, was reported as significantly higher after blastocyst stage biopsy (48.2%) than after the cleavage stage one (29.2%, $p = 0.0016$).

Also, an economic and logistic argument exists in favor of the implementation of blastocyst stage biopsy strategy in clinical practice: only developmentally-competent embryos reach this stage, since incompetent ones would have already stopped their growth previously during pre-implantation development, so avoiding the costs and time for the analysis of those embryos. However, concerns about blastocyst culture were also raised. In this regard, Glujovsky et al. (70) performed a Cochrane review of 12 RCTs, where they demonstrated that unscreened blastocyst transfer elicits better results than cleavage stage ET. Thus, given that an efficient culture system is available in the laboratory, no risk derives from embryo culture up to day 5, day 6, or even day 7.

There is also sufficient evidence showing that cryopreservation at the blastocyst stage is effective. Survival rate after biopsied blastocyst warming has in fact always been reported as ≥95% when a vitrification approach was used, and this is not affected by the TE biopsy procedure (7,57,71,72).

In this scenario, cycle segmentation was also theorized (73,74). According to this policy, oocyte/embryo cryopreservation is performed after ovarian stimulation and egg retrieval, while frozen ET is performed in a natural cycle. This leads to an OHSS-free clinic and perfectly suits euploid SET after a CCS-based blastocyst stage PGD/PGS strategy. Recently, we investigated the clinical outcomes in our practice between the years characterized by the gradual implementation of qPCR-based blastocyst stage PGS and frozen euploid single embryo transfer (SET) policy for the AMA patient population (72). We highlighted that the global pregnancy rate per oocyte retrieval, namely the efficacy of an IVF treatment, was kept constant, while the miscarriage rate and the multiple pregnancy rates were significantly reduced. These last two outcomes, together with a significantly higher pregnancy rate per transfer, underlined a higher efficiency due to the novel policy based on TE biopsy and qPCR analysis with frozen embryo transfer in a poor prognosis population of patients. Importantly, this study showed for the first time in the AMA population that the introduction of extended culture, blastocyst biopsy, vitrification, CCS analysis by qPCR, and frozen embryo transfer of euploid blastocyst does not compromise the cumulative live birth rate per treated patient.

Importantly, Forman et al., in two papers (75,76), also demonstrated that euploid SET after blastocyst stage CCS equals double untested blastocyst transfer in terms of pregnancy rate, meanwhile determining better obstetrical and perinatal outcomes due to the minimization of multiple pregnancies. This is a further pivotal advantage of this approach in PGD/PGS.

We recently investigated the accuracy and the reproducibility of our qPCR-based blastocyst stage biopsy protocol among seven embryologists from three different IVF centers. No significant influence was reported in both technical and clinical outcomes. Specifically, the amplification rate, qPCR data concurrence, and estimated mean number of retrieved TE cells, as well as ongoing implantation, biochemical, and miscarriage rates after euploid SET were equivalent among all the operators (77). These data may prompt a widespread implementation of CCS-based blastocyst stage PGS by highlighting it as a standardized and reproducible procedure when appropriate training is provided.

The number of papers dealing with CCS-based blastocyst stage biopsy application in clinical practice is growing impressively. Three RCTs have been performed so far, and all of them consistently show higher sustained implantation rate after the transfer of euploid blastocysts compared to the transfer of untested embryos. Recently, Dahdouh et al., in a review and in a meta-analysis, investigated these RCTs (78,79). They defined the approach as reliable, clinically valid, and efficient. Lee et al., in a review (80), also summarized all the studies focused on CCS-based PGS clinical application in both young and AMA patient populations.

Several more studies and RCTs aimed at investigating the clinical effectiveness of blastocyst stage biopsy strategy are in the pipeline (NCT01219283, NCT02032264, NCT02268786, NCT01977144, NCT01917240 registered at www.clinicaltrials.gov; ISRCTN81216689 registered at www.isrctn.com). Hopefully, they will provide novel proofs to support the further implementation of this approach in the future.

To conclude, blastocyst stage biopsy is the mostly validated strategy up to date to safely and accurately conduct PGD/PGS.

Conclusion and Future Perspectives

Fruitful evidence has been published in the last decade, which supports blastocyst stage implementation in PGD/PGS cycles. Meanwhile, blastomere biopsy has been identified as a harmful procedure as well as a method leading to an insufficient diagnostic performance. This last drawback is shared with the PB biopsy approach, although no reliable data have been produced that report negative clinical outcomes when this strategy is adopted (Figure 15.2).

The ESHRE PGD consortium data collections mirrored how this evidence influenced the application of the three biopsy methods in PGD/PGS. In particular, TE biopsy sharply increased from less than 1% of the total number of procedures performed in Europe in 2009–2010 (81) to almost 23% in 2012–2013 (81). PB biopsy has never been used for more than 10–15% of biopsy procedures, and it has recently been reported to account for less than 2% (82). Nevertheless, the application rate of blastomere biopsy dropped down from 90% to 75%, but it still represents the most commonly used technique in Europe (Figure 15.3).

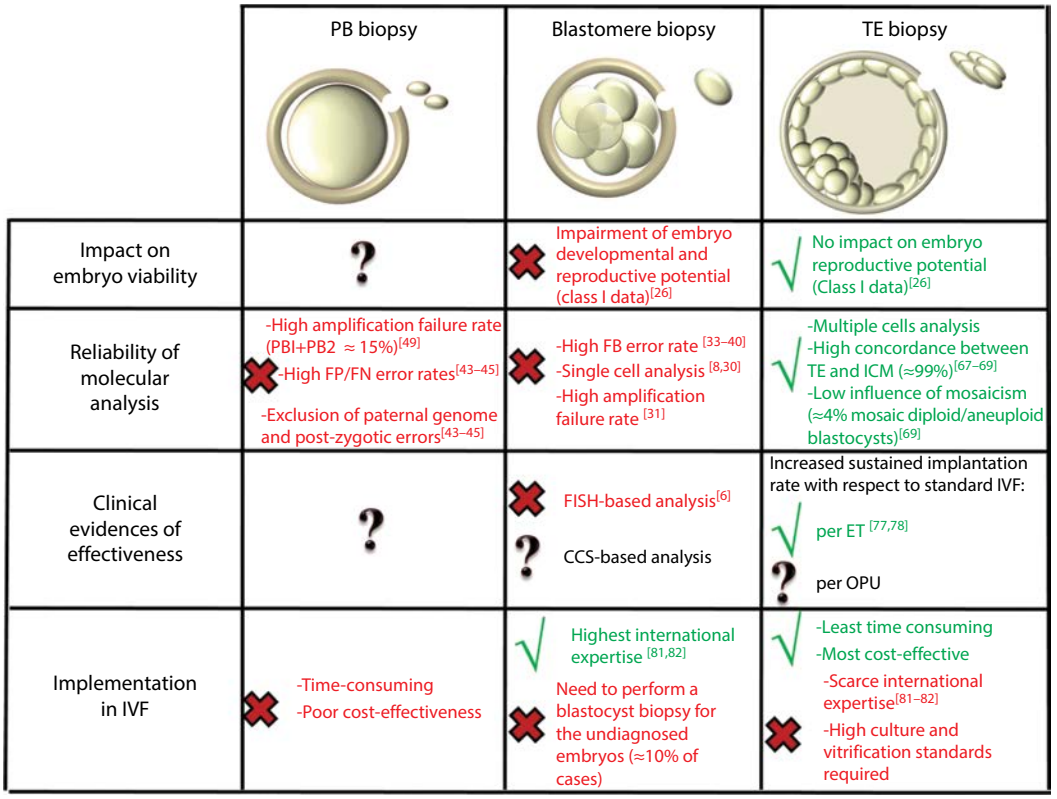

FIGURE 15.2 Comparison of polar body biopsy, blastomere biopsy, and trophectoderm biopsy approaches according to evidence of impact upon embryo viability, reliability of the downstream molecular analysis, clinical effectiveness, and implementation in IVF. PB, polar body; TE, trophectoderm; FP, false positive; FN, false negative; FISH, Fluorescent in situ hybridization; CCS, comprehensive chromosome screening; ET, embryo transfer; OPU, oocyte pick-up; red cross, negative aspect; green tick, positive aspect; question mark, aspect still under investigation.

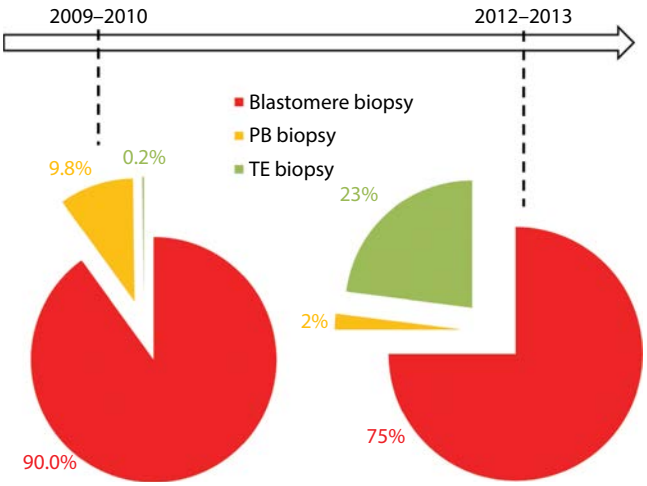

FIGURE 15.3 Data from ESHRE PGD consortium. The ESHRE PGD consortium data collection reported a sharp decrease in blastomere and PB biopsy approach between 2009–2010 and 2012–2013, compensated by a massive increase of TE biopsy implementation. PB, polar bodies; TE, trophectoderm

Possibly, the practice of CCS-based blastocyst stage biopsy is destined to grow further in the coming years, as a consequence of the future RCTs aimed at investigating its outcomes in terms of pregnancy rate per oocyte retrieval. In fact, if no adverse effects, but only advantages, are found for this approach, this would represent the ultimate evidence of its safety and effectiveness in IVF to significantly enhance embryo selection.

We have focused our attention mainly on PGS, but the concept of safety definitely applies to PGD as well, a practice that also requires a longer pathway, from molecular probe construction to diagnosis and higher costs for the whole treatment.

At present the highest implantation rate achievable by euploid SET is about 50% across the board for female age. Although this is a consistent rate, there is still room for improvement. Noninvasive methods to complement blastocyst stage aneuploidy screening are in fact under investigation. Novel information could arise in the next years to further uncover this unknown fertile field and increase our predictive power on implantation. However, static and dynamic blastocyst morphological evaluation in time-lapse, as well as proteomic/metabolomic screening of spent culture media, have not shown any significant clinical value to date (59,62,83,84).

In conclusion, it took decades to establish and prove PGS as an effective tool, despite the massive prevalence of aneuploidies in human embryos. Thus, we foresee an even more challenging pathway towards the definition of novel noninvasive biomarkers of embryonic competence. Nonetheless, this is a pathway that deserves to be followed, in order to further boost our predictive power on implantation beyond the level that is already guaranteed by euploidy when assessed by CCS-based TE biopsy analysis.

REFERENCES

1. Handyside AH, Kontogianni EH, Hardy K, Winston RM. Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. *Nature*. 1999;344(6268):768–70.
2. Nagaoka SI, Hassold TJ, Hunt PA. Human aneuploidy: Mechanisms and new insights into an age-old problem. *Nat Rev Genet*. 2012;13(7):493–504.
3. Los FJ, Van Opstal D, van den Berg C. The development of cytogenetically normal, abnormal and mosaic embryos: A theoretical model. *Hum Reprod Update*. 2004;10(1):79–94.
4. Heffner LJ. Advanced maternal age—How old is too old? *N Engl J Med*. 2004;351(19):1927–9.
5. Hassold T, Hunt P. To err (meiotically) is human: The genesis of human aneuploidy. *Nat Rev Genet*. 2001;2(4):280–91.
6. Mastenbroek S, Twisk M, van der Veen F, Repping S. Preimplantation genetic screening: A systematic review and meta-analysis of RCTs. *Hum Reprod Update*. 2011;17(4):454–66.
7. Capalbo A, Treff NR, Cimadomo D, Tao X, Upham K, Ubaldi FM et al. Comparison of array comparative genomic hybridization and quantitative real-time PCR-based aneuploidy screening of blastocyst biopsies. *Eur J Hum Genet*. 2015;23(7):901–6.
8. Harton G, Traeger-Syndinos J, Goossens V. Data from the ESHRE PGD consortium. *Human Reprod*. 2012;27(Suppl. 2):ii58.
9. Feichtinger W, Strohmer H, Fuhrberg P, Radivojevic K, Antinori S, Pepe G et al. Photoablation of oocyte zona pellucida by erbium-YAG laser for in-vitro fertilisation in severe male infertility. *Lancet*. 1992;339(8796):811.
10. Cohen J, Alikani M, Garrisi JG, Willadsen S. Micromanipulation of human gametes and embryos: Ooplasmic donation at fertilization. *Hum Reprod Update*. 1998;4(2):195–6.
11. Cohen J, Malter H, Elsner C, Kort H, Massey J, Mayer MP. Immunosuppression supports implantation of zona pellucida dissected human embryos. *Fertil Steril*. 1990;53(4):662–5.
12. Eldar-Geva T, Srebnik N, Altarescu G, Varshaver I, Brooks B, Levy-Lahad E et al. Neonatal outcome after preimplantation genetic diagnosis. *Fertil Steril*. 2014;102(4):1016–21.
13. De Vos A, Van Steirteghem A. Aspects of biopsy procedures prior to preimplantation genetic diagnosis. *Prenat Diagn*. 2001;21(9):767–80.
14. Geber S, Bossi R, Lisboa CB, Valle M, Sampaio M. Laser confers less embryo exposure than acid tyrode for embryo biopsy in preimplantation genetic diagnosis cycles: A randomized study. *Reprod Biol Endocrinol*. 2011;9:58.

15. Rink K, Delacrétaz G, Salathé RP, Senn A, Nocera D, Germond M et al. Non-contact microdrilling of mouse zona pellucida with an objective-delivered 1.48-microns diode laser. *Laser Surg Med*. 1996;18(1):52–62.
16. Taylor TH, Gilchrist JW, Hallowell SV, Hanshew KK, Orris JJ, Glassner MJ et al. The effects of different laser pulse lengths on the embryo biopsy procedure and embryo development to the blastocyst stage. *J Assist Reprod Genet*. 2010;27(11):663–7.
17. Malter HE, Cohen J. Blastocyst formation and hatching in vitro following zona drilling of mouse and human embryos. *Gamete Res.* 1989;24(1):67–80.
18. Cohen J, Feldberg D. Effects of the size and number of zona pellucida openings on hatching and trophoblast outgrowth in the mouse embryo. *Mol Reprod Dev.* 1991;30(1):70–8.
19. Schmoll F, Schneider H, Montag M, Wimmers K, Rink K, Schellander K. Effects of different laser-drilled openings in the zona pellucida on hatching of in vitro-produced cattle blastocysts. *Fertil Steril.* 2003;80(Suppl. 2):714–9.
20. Duncan FE, Stein P, Williams CJ, Schultz RM. The effect of blastomere biopsy on preimplantation mouse embryo development and global gene expression. *Fertil Steril*. 2009;91(4 Suppl.):1462–5.
21. Dumoulin JC, Bras M, Coonen E, Dreesen J, Geraedts JP, Evers JL. Effect of Ca^{2+}/Mg^{2+}-free medium on the biopsy procedure for preimplantation genetic diagnosis and further development of human embryos. *Hum Reprod*. 1998;13(10):2880–3.
22. Pratt HP, Ziomek CA, Reeve WJ, Johnson MH. Compaction of the mouse embryo: An analysis of its components. *J Embryol Exp Morphol*. 1982;70:113–32.
23. Clayton L, Stinchcombe SV, Johnson MH. Cell surface localisation and stability of uvomorulin during early mouse development. *Zygote*. 1993;1(4):333–44.
24. Sefton M, Johnson MH, Clayton L, McConnell JM. Experimental manipulations of compaction and their effects on the phosphorylation of uvomorulin. *Mol Reprod Dev.* 1996;44(1):77–87.
25. Kirkegaard K, Hindkjaer JJ, Ingerslev HJ. Human embryonic development after blastomere removal: A time-lapse analysis. *Hum Reprod*. 2012;27(1):97–105.
26. Scott RT Jr, Upham KM, Forman EJ, Zhao T, Treff NR. Cleavage-stage biopsy significantly impairs human embryonic implantation potential while blastocyst biopsy does not: A randomized and paired clinical trial. *Fertil Steril*. 2013;100(3):624–30.
27. Staessen C, Platteau P, Van Assche E, Michiels A, Tournaye H, Camus M et al. Comparison of blastocyst transfer with or without preimplantation genetic diagnosis for aneuploidy screening in couples with advanced maternal age: A prospective randomized controlled trial. *Hum Reprod*. 2004;19(12):2849–58.
28. Debrock S, Melotte C, Spiessens C, Peeraer K, Vanneste E, Meeuwis L et al. Preimplantation genetic screening for aneuploidy of embryos after in vitro fertilization in women aged at least 35 years: A prospective randomized trial. *Fertil Steril*. 2010;93(2):364–73.
29. Hardarson T, Hanson C, Lundin K, Hillensjö T, Nilsson L, Stevic J et al. Preimplantation genetic screening in women of advanced maternal age caused a decrease in clinical pregnancy rate: A randomized controlled trial. *Hum Reprod*. 2008;23(12):2806–12.
30. Brodie D, Beyer CE, Osborne E, Kralevski V, Rasi S, Osianlis T. Preimplantation genetic diagnosis for chromosome rearrangements—One blastomere biopsy versus two blastomere biopsy. *J Assist Reprod Genet*. 2012;29(8):821–7.
31. Van der Aa N, Cheng J, Mateiu L, Zamani Esteki M, Kumar P, Dimitriadou E et al. Genome-wide copy number profiling of single cells in S-phase reveals DNA-replication domains. *Nucleic Acids Res.* 2013;41(6):e66.
32. Traeger-Synodinos J, Coonen EC, De Rycke M, Moutou C, SenGupta S, Goossens V. Data from the ESHRE PGD consortium. ESHRE Abstract Book, Annual Meeting. Session 10: Data Reporting Session, June 30, 2014, Munich.
33. Coonen E, Derhaag JG, Dumoulin JC, van Wissen LC, Bras M, Janssen M et al. Anaphase lagging mainly explains chromosomal mosaicism in human preimplantation embryos. *Hum Reprod*. 2004;19(2):316–24.
34. Daphnis DD, Delhanty JD, Jerkovic S, Geyer J, Craft I, Harper JC. Detailed FISH analysis of day 5 human embryos reveals the mechanisms leading to mosaic aneuploidy. *Hum Reprod*. 2005;20(1):129–37.
35. Munné S, Sultan KM, Weier HU, Grifo JA, Cohen J, Rosenwaks Z. Assessment of numeric abnormalities of X, Y, 18, and 16 chromosomes in preimplantation human embryos before transfer. *Am J Obstet Gynecol*. 1995;172(4 Pt 1):1191–9.

36. Wells D, Delhanty JD. Comprehensive chromosomal analysis of human preimplantation embryos using whole genome amplification and single cell comparative genomic hybridization. *Mol Hum Reprod*. 2000;6(11):1055–62.
37. Voullaire L, Slater H, Williamson R, Wilton L. Chromosome analysis of blastomeres from human embryos by using comparative genomic hybridization. *Hum Genet*. 2000;106(2):210–7.
38. Bielanska M, Tan SL, Ao A. Chromosomal mosaicism throughout human preimplantation development in vitro: Incidence, type, and relevance to embryo outcome. *Hum Reprod*. 2002;17(2):413–9.
39. Baart EB, Van Opstal D, Los FJ, Fauser BC, Martini E. Fluorescence in situ hybridization analysis of two blastomeres from day 3 frozen-thawed embryos followed by analysis of the remaining embryo on day 5. *Hum Reprod*. 2004;19(3):685–93.
40. Munné S, Velilla E, Colls P, Garcia Bermudez M, Vemuri MC, Steuerwald N et al. Self-correction of chromosomally abnormal embryos in culture and implications for stem cell production. *Fertil Steril*. 2005;84(5):1328–34.
41. Huang A, Adusumalli J, Patel S, Liem J, Williams J 3rd, Pisarska MD. Prevalence of chromosomal mosaicism in pregnancies from couples with infertility. *Fertil Steril*. 2009;91(6):2355–60.
42. Scott RT Jr, Ferry K, Su J, Tao X, Scott K, Treff NR. Comprehensive chromosome screening is highly predictive of the reproductive potential of human embryos: A prospective, blinded, nonselection study. *Fertil Steril*. 2012;97(4):870–5.
43. Capalbo A, Bono S, Spizzichino L, Biricik A, Baldi M, Colamaria S et al. Sequential comprehensive chromosome analysis on polar bodies, blastomeres and trophoblast: Insights into female meiotic errors and chromosomal segregation in the preimplantation window of embryo development. *Hum Reprod*. 2013;28(2):509–18.
44. Handyside AH, Montag M, Magli MC, Repping S, Harper J, Schmutzler A et al. Multiple meiotic errors caused by predivision of chromatids in women of advanced maternal age undergoing in vitro fertilisation. *Eur J Hum Genet*. 2012;20(7):742–7.
45. Christopikou D, Tsorva E, Economou K, Shelley P, Davies S, Mastrominas M et al. Polar body analysis by array comparative genomic hybridization accurately predicts aneuploidies of maternal meiotic origin in cleavage stage embryos of women of advanced maternal age. *Hum Reprod*. 2013;28:1426–34.
46. Forman EJ, Treff NR, Stevens JM, Garnsey HM, Katz-Jaffe MG, Scott RT Jr et al. Embryos whose polar bodies contain isolated reciprocal chromosome aneuploidy are almost always euploid. *Hum Reprod*. 2013;28:502–8.
47. Scott RT Jr, Treff NR, Stevens J, Forman EJ, Hong KH, Katz-Jaffe MG et al. Delivery of a chromosomally normal child from an oocyte with reciprocal aneuploid polar bodies. *J Assist Reprod Genet*. 2012;6:533–7.
48. Ottolini CS, Newnham LJ, Capalbo A, Natesan SA, Joshi HA, Cimadomo D et al. Genome-wide maps of recombination and chromosome segregation in human oocytes and embryos show selection for maternal recombination rates. *Nat Genet*. 2015;47(7):727–35.
49. Geraedts J, Montag M, Magli MC, Repping S, Handyside A, Staessen C et al. Polar body array CGH for prediction of the status of the corresponding oocyte. Part I: Clinical results. *Hum Reprod*. 2011;26(11):3173–80.
50. Scott KL, Hong KH, Scott RT Jr. Selecting the optimal time to perform biopsy for preimplantation genetic testing. *Fertil Steril*. 2013;100(3):608–14.
51. Magli MC, Montag M, Köster M, Muzi L, Geraedts J, Collins J et al. Polar body array CGH for prediction of the status of the corresponding oocyte. Part II: Technical aspects. *Hum Reprod*. 2011;26(11):3181–5.
52. Montag M, Köster M, Strowitzki T, Toth B. Polar body biopsy. *Fertil Steril*. 2013;100(3):603–7.
53. Montag M, van der Ven K, Delacrétaz G, Rink K, van der Ven H. Laser-assisted microdissection of the zona pellucida facilitates polar body biopsy. *Fertil Steril*. 1998;69(3):539–42.
54. Hammoud I, Molina-Gomes D, Albert M, Bergere M, Bailly M, Wainer R et al. Are zona pellucida laser drilling and polar body biopsy safe for in vitro matured oocytes? *J Assist Reprod Genet*. 2010;27(7):423–7.
55. Levin I, Almog B, Shwartz T, Gold V, Ben-Yosef D, Shaubi M et al. Effects of laser polar-body biopsy on embryo quality. *Fertil Steril*. 2012;97(5):1085–8.
56. de Boer KA, Catt JW, Jansen RP, Leigh D, McArthur S. Moving to blastocyst biopsy for preimplantation genetic diagnosis and single embryo transfer at Sydney IVF. *Fertil Steril*. 2004;82(2):295–8.

57. McArthur SJ, Leigh D, Marshall JT, de Boer KA, Jansen RP. Pregnancies and live births after trophectoderm biopsy and preimplantation genetic testing of human blastocysts. *Fertil Steril*. 2005;84(6):1628–36.
58. Kokkali G, Vrettou C, Traeger-Synodinos J, Jones GM et al. Birth of a healthy infant following trophectoderm biopsy from blastocysts for PGD of beta-thalassaemia major. *Hum Reprod*. 2005;20(7):1855–9.
59. Capalbo A, Rienzi L, Cimadomo D, Maggiulli R, Elliott T, Wright G et al. Correlation between standard blastocyst morphology, euploidy and implantation: An observational study in two centers involving 956 screened blastocysts. *Hum Reprod*. 2014;29(6):1173–8.
60. Alfarawati S, Fragouli E, Colls P, Stevens J, Gutiérrez-Mateo C, Schoolcraft WB et al. The relationship between blastocyst morphology, chromosomal abnormality, and embryo gender. *Fertil Steril*. 2011;95(2):520–4.
61. Fragouli E, Alfarawati S, Spath K, Wells D. Morphological and cytogenetic assessment of cleavage and blastocyst stage embryos. *Mol Hum Reprod*. 2014;20(2):117–26.
62. Rienzi L, Capalbo A, Stoppa M, Romano S, Maggiulli R, Albricci L et al. No evidence of association between blastocyst aneuploidy and morphokinetic assessment in a selected population of poor-prognosis patients: A longitudinal cohort study. *Reprod Biomed Online*. 2015;30(1):57–66.
63. Johnson DS, Cinnioglu C, Ross R, Filby A, Gemelos G, Hill M et al. Comprehensive analysis of karyotypic mosaicism between trophectoderm and inner cell mass. *Mol Hum Reprod*. 2010;16(12):944–9.
64. Mottla GL, Adelman MR, Hall JL, Gindoff PR, Stillman RJ, Johnson KE. Lineage tracing demonstrates that blastomeres of early cleavage-stage human pre-embryos contribute to both trophectoderm and inner cell mass. *Hum Reprod*. 1995;10(2):384–91.
65. Evsikov S, Verlinsky Y. Mosaicism in the inner cell mass of human blastocysts. *Hum Reprod*. 1998;13(11):3151–5.
66. Magli MC, Jones GM, Gras L, Gianaroli L, Korman I, Trounson AO. Chromosome mosaicism in day 3 aneuploid embryos that develop to morphologically normal blastocysts in vitro. *Hum Reprod*. 2000;15(8):1781–6.
67. Northrop LE, Treff NR, Levy B, Scott RT Jr. SNP microarray-based 24 chromosome aneuploidy screening demonstrates that cleavage-stage FISH poorly predicts aneuploidy in embryos that develop to morphologically normal blastocysts. *Mol Hum Reprod*. 2010;16(8):590–600.
68. Fragouli E, Lenzi M, Ross R, Katz-Jaffe M, Schoolcraft WB, Wells D. Comprehensive molecular cytogenetic analysis of the human blastocyst stage. *Hum Reprod*. 2008;23(11):2596–608.
69. Capalbo A, Wright G, Elliott T, Rienzi L, Nagy ZP. FISH reanalysis of inner cell mass and trophectoderm samples of previously array-CGH screened blastocysts shows high accuracy of diagnosis and no major diagnostic impact of mosaicism at the blastocyst stage. *Hum Reprod*. 2013;28(8):2298–307.
70. Glujovsky D, Blake D, Farquhar C, Bardach A. Cleavage stage versus blastocyst stage embryo transfer in assisted reproductive technology. *Cochrane Db Syst Rev*. 2012;7:CD002118.
71. Cobo A, de los Santos MJ, Castellò D, Gámiz P, Campos P, Remohí J. Outcomes of vitrified early cleavage-stage and blastocyst-stage embryos in a cryopreservation program: Evaluation of 3,150 warming cycles. *Fertil Steril*. 2012;98(5):1138–46.e1.
72. Ubaldi FM, Capalbo A, Colamaria S, Ferrero S, Maggiulli R, Vajta G et al. Reduction of multiple pregnancies in the advanced maternal age population after implementation of an elective single embryo transfer policy coupled with enhanced embryo selection: Pre- and post-intervention study. *Hum Reprod*. 2015;30(9):2097–106.
73. Devroey P, Polyzos NP, Blockeel C. An OHSS-free clinic by segmentation of IVF treatment. *Hum Reprod*. 2011;26(10):2593–7.
74. Shapiro BS, Daneshmand ST, Restrepo H, Garner FC, Aguirre M, Hudson C. Matched-cohort comparison of single-embryo transfers in fresh and frozen-thawed embryo transfer cycles. *Fertil Steril*. 2013;99(2):389–92.
75. Forman EJ, Hong KH, Ferry KM, Tao X, Taylor D, Levy B et al. In vitro fertilization with single euploid blastocyst transfer: A randomized controlled trial. *Fertil Steril*. 2013;100(1):100–7.e1.
76. Forman EJ, Hong KH, Franasiak JM, Scott RT Jr. Obstetrical and neonatal outcomes from the BEST Trial: single embryo transfer with aneuploidy screening improves outcomes after in vitro fertilization without compromising delivery rates. *Am J Obstet Gynecol*. 2014;210(2):157.e1–6.
77. Capalbo A, Ubaldi FM, Cimadomo D, Maggiulli R, Patassini C, Dusi L et al. Consistent and reproducible outcomes of blastocyst biopsy and aneuploidy screening across different biopsy practitioners: A multicentre study involving 2586 embryo biopsies. *Hum Reprod*. 2016;31(1):199–208.

78. Dahdouh EM, Balayla J, García-Velasco JA. Impact of blastocyst biopsy and comprehensive chromosome screening technology on preimplantation genetic screening: A systematic review of randomized controlled trials. *Reprod Biomed Online*. 2015;30(3):281–9.
79. Dahdouh EM, Balayla J, García-Velasco JA. Comprehensive chromosome screening improves embryo selection: A meta-analysis. *Fertil Steril*. 2015;104(6):1503–12.
80. Lee E, Illingworth P, Wilton L, Chambers GM. The clinical effectiveness of preimplantation genetic diagnosis for aneuploidy in all 24 chromosomes (PGD-A): Systematic review. *Hum Reprod*. 2015;30(2):473–83.
81. Coonen E, De Rycke M, Kokkali G, Moutou C, SenGupta S, Traeger-Synodinos J et al. Data from the ESHRE PGD consortium. ESHRE Abstract Book, Annual Meeting. Session 9: Data Reporting Session, June 15, 2015, LISBON.
82. Moutou C, Goossens V, Coonen E, De Rycke M et al. ESHRE PGD Consortium data collection XII: Cycles from January to December 2009 with pregnancy follow-up to October 2010. *Hum Reprod*. 2014;29(5):880–903.
83. Kirkegaard K, Svane AS, Nielsen JS, Hindkjær JJ, Nielsen NC, Ingerslev HJ. Nuclear magnetic resonance metabolomic profiling of Day 3 and 5 embryo culture medium does not predict pregnancy outcome in good prognosis patients: A prospective cohort study on single transferred embryos. *Hum Reprod*. 2014;29(11):2413–20.
84. Hardarson T, Ahlström A, Rogberg L, Botros L, Hillensjö T, Westlander G et al. Non-invasive metabolomic profiling of Day 2 and 5 embryo culture medium: A prospective randomized trial. *Hum Reprod*. 2012;27(1):89–96.

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16

Pre-implantation Genetic Testing

Nathan R. Treff and Carmen Rubio

Introduction

New molecular biology based technologies have invigorated the field of pre-implantation genetic testing (PGT). Not only has testing improved to cover the analysis of aneuploidy for all 24 chromosomes (comprehensive chromosome screening), but methods to evaluate specific genetic loci for single gene disorders, segmental aneuploidy, mitochondrial DNA content, and mosaicism can also be performed in parallel from the same biopsy. In general, PGT has benefited from tremendous efforts in the field of human genetics, where the biotechnology industry has invested heavily in the development of new methodologies for the analysis of DNA. However, many obstacles to the adaptation of these methods to application in human embryonic DNA exist, including the extremely limited amount of DNA available from an embryo biopsy, as well as the underlying biology of chromosome malsegregation, particularly mitotic errors that may contribute to embryonic mosaicism. This chapter will provide a summary of new technologies and their capabilities, limitations, and evidence for utility from clinical trials.

Comprehensive Chromosomal Aneuploidy Screening

Aneuploidy is the most common genetic abnormality in humans. Large data sets from the application of comprehensive chromosome screening (CCS) demonstrate that over half of the embryos produced by in vitro fertilization (IVF) possess aneuploidy (1–3). In addition, every human chromosome is capable of contributing to aneuploidy in the embryo, an observation which necessitates the ability to screen all 24 chromosomes in parallel. The ability to do so largely stems from the development of methods for whole genome amplification (WGA). Many companies have entered into the development of WGA methods, some of which have been specifically created for application to single cells. This aspect of WGA is critical to application in PGT, where biopsy material can consist of single cells such as the polar body or blastomere, or very few cells, such as trophectoderm biopsied cells (approximately 5–10 cells). Although WGA is capable of amplifying DNA across the entire genome, it does have important limitations to consider, particularly when applied to the evaluation of chromosome copy number as well as individual loci. It is well established the WGA can introduce bias in the representation of the original quantity of DNA in a given sample (4). Often, this has been addressed with the analysis of many thousands of loci per chromosome in order to compensate for inconsistency at a single locus. Examples of platforms which use WGA as an initial step include comparative genomic hybridization arrays (aCGH), single nucleotide polymorphism microarrays (SNP arrays), and next generation sequencing (NGS) (Figure 16.1). An alternative to WGA is targeted multiplex polymerase chain reaction (PCR) which is currently used for quantitative real-time PCR and some NGS based methods of CCS.

aCGH

aCGH technology allows for the analysis of chromosome copy number variations in DNA from an embryo compared to a reference sample. DNA with different fluorescent labels is combined and then hybridized onto a slide (array) containing specific bacterial artificial chromosome (BAC) probes across

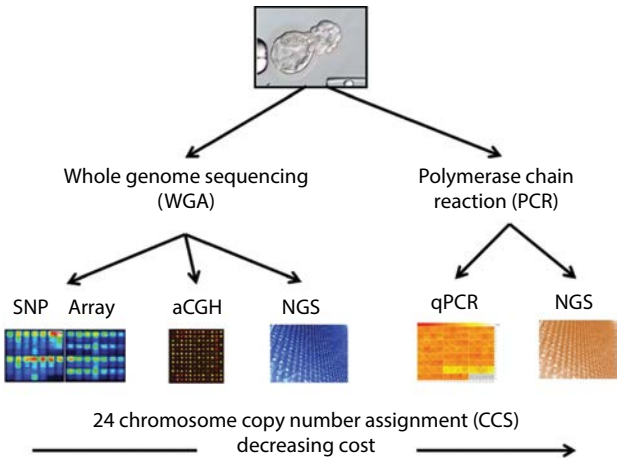

FIGURE 16.1 Methodologies for comprehensive chromosome screening. Contemporary methods involve performing a trophectoderm biopsy from the blastocyst stage embryo. Whole genome amplification can be performed and tested using either single nucleotide polymorphism (SNP) array, array comparative genomic hybridization (aCGH), or next generation sequencing (NGS). Alternatively, targeted polymerase chain reaction (PCR) can be performed followed by either quantitative PCR (qPCR) or NGS. Each method is arranged from left to right in order of decreasing cost.

the length of the chromosomes with 1 Mb coverage (Figure 16.2). Chromosome loss or gain is revealed by the color adopted by each spot after hybridization. Fluorescence intensity is detected using a laser scanner and specific software for data processing (5). The most commonly used protocol involves the use of reagents from Illumina (24sure®). WGA is performed using SurePlex WGA technology from Rubicon Genomics. After amplification, samples and reference DNAs are labeled with Cy3 and Cy5 fluorophores using random primers. The 24sure® microarray used for PGS is a single channel platform in which the test samples are labeled and hybridized against each other, and are compared to male and female DNA references which are hybridized in a different sub-array. In contrast, the 24sure+® arrays used for PGD are a dual channel platform in which the test and reference samples are hybridized onto the same sub-array (Illumina, USA). Practical resolutions for both platforms are 10 Mb and 6 Mb, respectively. Therefore, segmental imbalances below this resolution cannot be identified with these platforms. After labeling, the pairs of Cy3- and Cy5-labeled samples are combined and hybridized to the array. A dual-channel scanner reads fluorescence intensity signals to excite the Cy3 and Cy5 hybridized fluorophores and to record the emission signals (532/635 nm wavelength) at 10 µm resolution. With specific array software, scanned images are converted into clear profiles from which whole chromosome aneuploidy and sub-chromosomal structural imbalances can be analyzed. The data from each chromosome are plotted as green dots on a fused chart display, representing the data from the sample tested versus the reference DNA. For 24sure® microarrays the software compares each sample with both male and female references, whereas for 24sure+® microarrays, comparisons are made only with male reference DNA. The X chromosome log2 ratio (termed X-separation) is compared with the log2 ratios of each chromosome to highlight aneuploidy calls as gains (+) or losses (–). According to these comparisons, a normal sample is identified when there are no spots deviating from the reference DNA for any of the chromosomes shown on the plot. A sample is considered as abnormal with specific chromosome aneuploidies (full chromosome gains or losses), when the plot shows all the dots for these chromosomes within the upper or lower normal confidence lines defined by the reference DNA. The presence of aneuploidies for most of the chromosomes in the same specimen is interpreted as an abnormal chaotic pattern. Partial gains or losses for specific segments of a chromosome are considered as segmental aneuploidies when the size is higher than the resolution of the platform. aCGH has been successfully validated and applied in day 3 and blastocyst biopsies. Those studies comparing the aCGH result of the day 3 biopsy to the results of the FISH technique applied on the remaining cells of the day 5 embryos showed low false-positive rates, from 1.9% to 2.7% (6,7). Another blinded study, in which 109 embryos were reanalyzed using the same

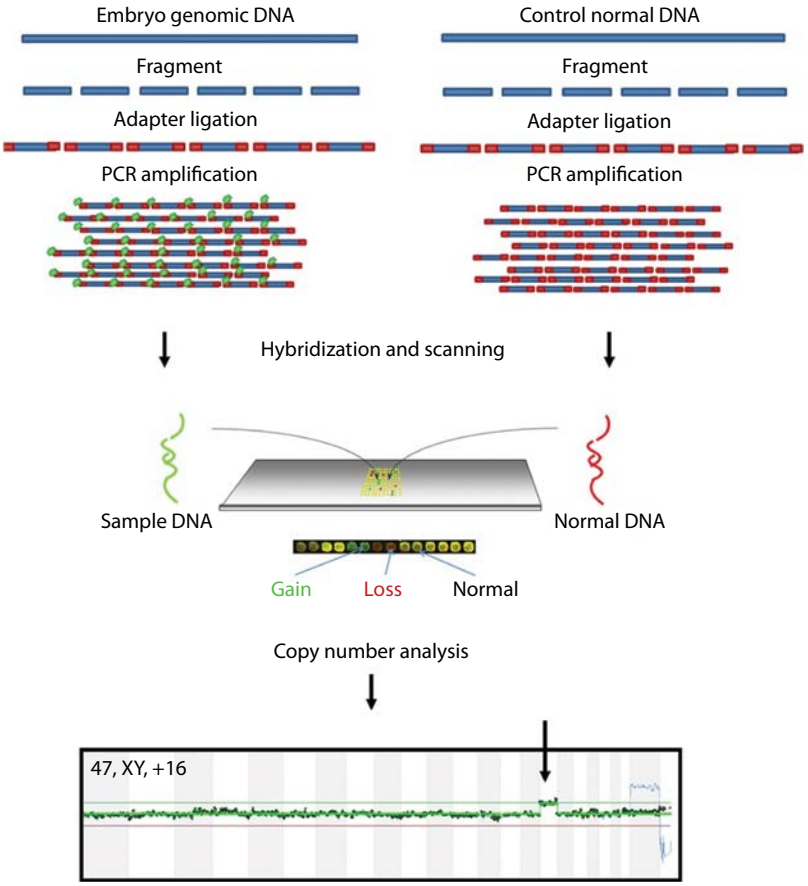

FIGURE 16.2 Array comparative genomic hybridization (aCGH) also begins with whole genome amplification (WGA) of embryonic DNA and control normal DNA. Here the control DNA is labeled with a red fluorophore and the embryonic DNA with a green fluorophore. The samples are mixed together and compete for hybridization to probes on an array. The resulting color at each locus interrogated is used to evaluate copy numbers. The example result shows a sample with an extra copy of chromosome 16 (indicated with an arrow) and a 47, XY, +16 karyotype.

aCGH technique used for the PGS analysis (PGS was performed on day 3 in 50 of the embryos, and on trophectoderm biopsies in the remaining 59 embryos). High confirmation rates per individual chromosome in both types of embryo biopsy (98.8% for day 3 versus 98.5% for trophectoderm) were shown, and these rates are also reflected in high confirmation rates of the PGS diagnosis (98% for day 3 versus 97.6% for trophectoderm) (8).

NGS

Next-generation sequencing (NGS) has been recently presented and validated as a new technique for the analysis of copy number variation in single cells and trophectoderm (9,10). Decrease in the cost of sequencing provides a promising platform for the study of not only aneuploidies, but also mitochondrial DNA or single gene disorders in a simultaneous analysis (11).

Some NGS protocols begin with the same WGA step as is performed with aCGH (Figure 16.3). This is followed by a barcoding procedure, in which the different samples are labeled with unique sequences, in a way that they can be mixed and sequenced together at the same time, but then bioinformatically separated back to the original embryo biopsy from which each sample originated. This pooling step has contributed to significantly reduce the cost of the technique and improve its potential for clinical application. The depth of sequencing is also an important aspect to consider, especially for the simultaneous

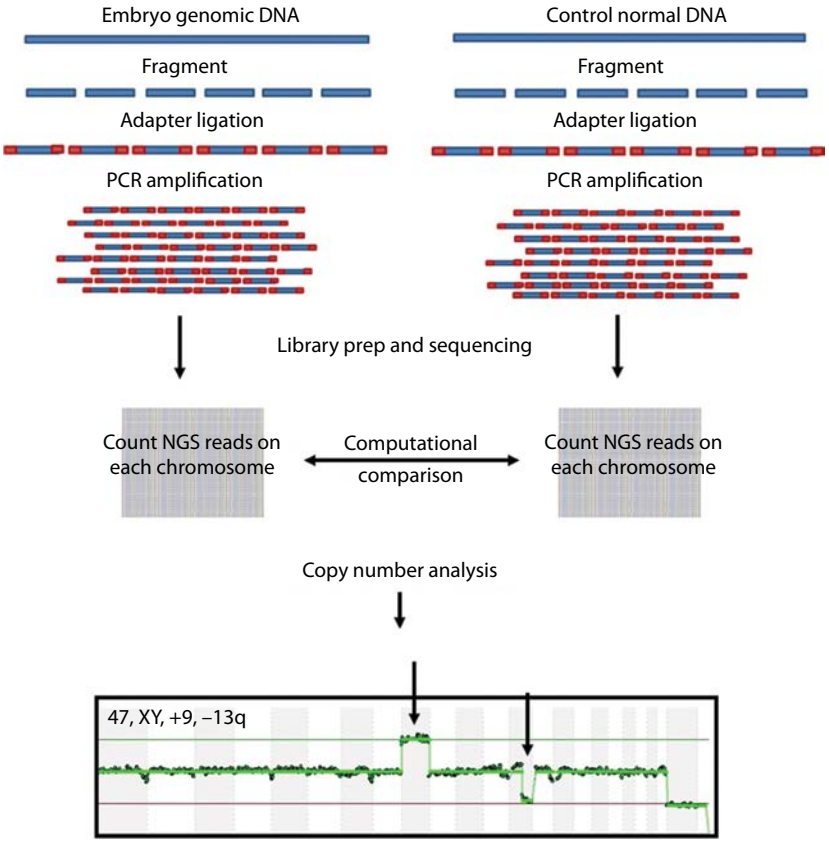

FIGURE 16.3 Some next generation sequencing methods begin with whole genome amplification (WGA). Sequencing can be performed on many different platforms which ultimately provide counts of sequencing "reads" across all 24 chromosomes. Relative read counts are established from an embryo biopsy when compared to data from known normal samples using standard bioinformatics tools (computational comparison). The example result shows a sample with an extra copy of chromosome 9 as well as a segmental deletion on chromosome 13 (indicated with arrows) and a karyotype of 47, XY, +9, −13q.

study of aneuploidies and single gene disorders, which would need high coverage within regions of interest. Two commercially available NGS platforms have been developed for PGS: VeriSeq PGS (Illumina) and ReproSeq PGS (Thermo Fisher Scientific).

The Illumina sequencing platform uses sequencing by synthesis. After WGA, DNA fragmentation and ligation using specific adapters is performed. Denaturing to single stranded DNA and immobilization at one end of a flow cell surface covered with adapters is then performed, followed by bridge amplification to form clusters which contain clonal DNA fragments. Before sequencing, the library splices into single strands with the help of a linearization enzyme, and the four nucleotides (ddATP, ddGTP, ddCTP, ddTTP) which contain different cleavable fluorescent dyes and a removable blocking group that complement the template one base at a time, and the signal can be captured by a charge-coupled device.

The Thermo Fisher Scientific platform uses semiconductor technology to detect the stepwise polymerization of the complementary strands during elongation by a DNA polymerase. When a nucleotide is incorporated into the DNA molecules by the polymerase, a proton is released changing the pH. These changes in turn induce a voltage potential in an adjacent sensor plate. By detecting the change in pH, the sequencer recognizes whether the nucleotide is added or not. Each time the chip is flooded with one nucleotide after another, if it is not the correct nucleotide, no voltage will be found; if there are two nucleotides added, there is double voltage detected.

In addition, NGS has been shown to detect lower levels of mosaicism in trophectoderm biopsies than aCGH (12). However, there are some concerns related to the accuracy in determining which embryos

reported as mosaics are truly mosaic (true positives), or whether some uniform euploid or aneuploidy embryos fall into the grey zone. Factors such as experimental noise related to the quality and quantity of the biological samples might impact in the dynamic range for different chromosomes. Recently, Greco et al. (13) have shown that embryos predicted to be mosaic can develop into healthy euploid newborns, suggesting that mosaic embryos could be considered for transfer in women who undergo CCS and have no euploid embryos available for transfer. The authors are cautious and state that additional clinical data must be obtained before this approach can be extended. Scott and Galliano (14) have discussed the need for balancing the risks of discarding a competent embryo versus transferring an embryo that may ultimately have a lower implantation potential, considering obstetrical and neonatal outcomes. Further research is needed to understand the relevance of mosaicism in the trophectoderm, as there are no studies related to the effect of different percentages of aneuploid cells in the blastocyst. In addition, more research on the false positive rate of predicting mosaicism is also needed, as many of the predictions made could be due to artifacts in the methodology of detection.

Another option in performing NGS based CCS involves a different type of preamplification where a single primer pair can be used. For example, Good Start Genetics has adapted a method originally published for use in prenatal testing, where the primers target a repetitive sequence found throughout the human genome (15). The sequence in between the primers is unique to specific chromosomes, allowing them to be counted based on alignment to individual chromosomes. The major advantage of this approach is the significant cost reduction relative to commercially available kits which involve WGA. However, one possible limitation is the reduced genome coverage that is necessary to capture putative segmental imbalances. A similar strategy has also been developed by the Treff laboratory (16), but with the use of Ion Torrent sequencing and a unique amplification methodology. This approach is currently under evaluation in a prospective blinded non-selection study and a randomized controlled trial.

qPCR

Another method for CCS involves quantitative real time PCR (qPCR) where 96 targets (four per chromosome) are co-amplified in a multiplex PCR reaction (preamplification) instead of WGA (17–20) (Figure 16.4). Individual primer pairs are then used in separate reactions to perform the quantitative PCR reaction on a 384-well plate. Different fluorophores can be used with TaqMan probes such that multiple targets can be quantified in the same reaction and increase the number of samples per plate that can be run. For example, using FAM, VIC, ABY, and JUN dyes allows for four embryo biopsies to be evaluated per 384-well plate, with each target getting quadruplicate tests. The throughput per instrument is actually better than NGS based strategies as a result of completing each plate within approximately 1 hour.

Unlike many other methods of CCS, qPCR has been evaluated in two randomized controlled trials (21,22) and has been assessed for accuracy using positive control cell lines (20). Cell lines can serve as an important control for establishing the validity of new tests. For example, Coriell Cell Repository has thousands of cell lines established, many of which have well-characterized aneuploidy for a variety of chromosomes. These cells can be easily obtained and grown in a standard incubator for use in assay development. Different numbers of cells can be extracted to model blastomere or trophectoderm biopsy cell quantities. Cells from different cell lines can be blinded, run through the assay, unblinded, and evaluated for consistency with the known cell line karyotypes. For example, when evaluating 72 samples from cell lines, qPCR demonstrated 97.6% consistency. Unfortunately, similar studies have not been published for many commercially available methods, including aCGH.

In addition to evaluating qPCR for consistency in cell lines, rebiopsies from embryos previously diagnosed as aneuploidy were also carefully evaluated. In this phase of development, embryos that had been given two consistent diagnoses from two separate biopsies using a quantitative SNP array approach (see below) were rebiopsied for analysis by qPCR. This design helps reduce the impact that embryonic mosaicism might have on evaluating the consistency of the assay. That is, inconsistency between multiple biopsies from the same embryo would be more likely from the technique itself (artifacts) as opposed to originating from biological variation due to mosaicsm. For example, qPCR demonstrated 98.6% consistency with the original SNP array-based prediction of aneuploidy. Unfortunately, similar studies have not been published for many commercially available methods, including aCGH.

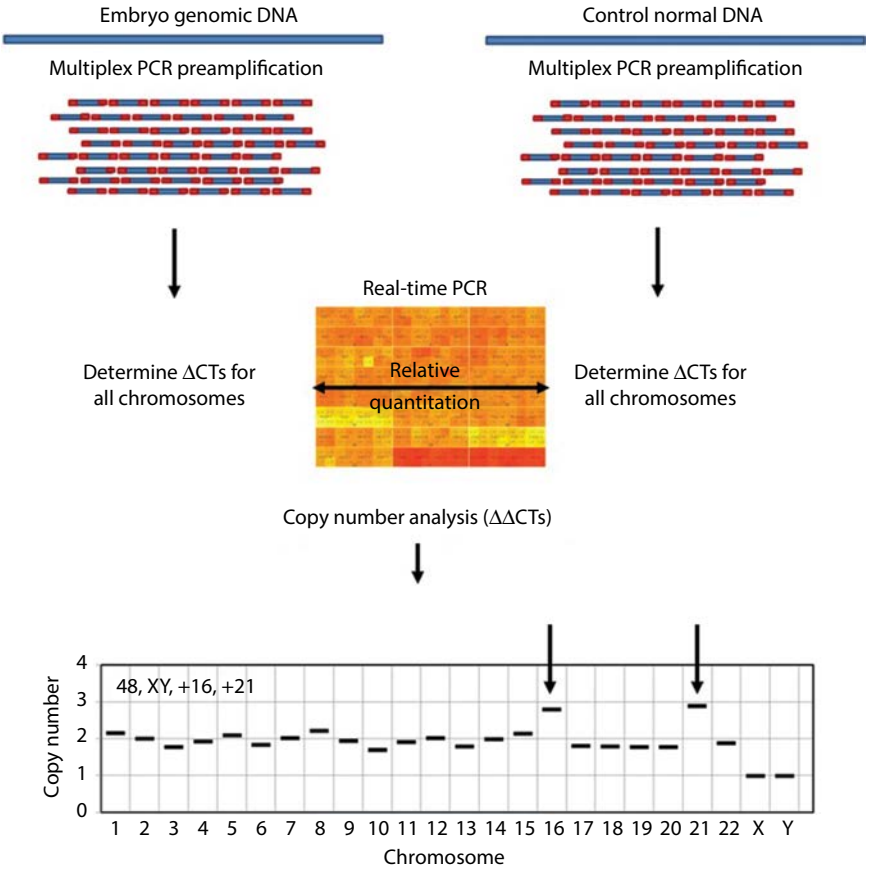

FIGURE 16.4 Quantitative real time PCR (qPCR) begins with targeted amplification of 96 loci across all 24 chromosomes. Relative threshold cycles (ΔCT) are compared between the embryo biopsy sample and known normal samples. The ΔΔCTs and copy number assignments are obtained using standard relative quantitation calculations. The example result shows a sample with an extra copy of chromosomes 16 and 21 (indicated with arrows) and a karyotype of 48, XY, +16, +21.

qPCR has also been evaluated in two randomized controlled trials to establish clinical validity. The first trial demonstrated significantly improved delivery rates per intention to treat (21). Sixty one of 72 cycles (85%) with qPCR CCS led to delivery, while 56 of 83 control cycles (68%) led to delivery ($p = 0.03$). In the second RCT, noninferiority of a single qPCR CCS euploid blastocyst compared to two unscreened was demonstrated (22). Fifty four of 89 patients (61%) delivered after transfer of a single euploid blast, while 56 of 86 patients (65%) delivered after transfer of two untested blasts ($p = 0.5$). More importantly, the twin rate went from 46% in the double unscreened embryo transfer group to 0% in the single euploid embryo transfer group. This study demonstrated that with CCS selection success rates are not compromised from single embryo transfer, and the major complication from IVF (multiples) can be eliminated.

SNP Array

There are a number of strategies for CCS that involve SNP array technology, including qualitative "Parental Support" (23) and "Karyomapping" (24), and quantitative based analyses. As with aCGH and some NGS methods, SNP array-based testing begins with WGA. In some cases, such as karyomapping, the initial WGA step involves multiple displacement amplification (MDA). MDA has been demonstrated to provide more accurate genotyping data compared to alternative WGA methods such as GenomePlex (Sigma) (4). Given that karyomapping relies on Mendelian inheritance rules and genotyping to make predictions for aneuploidy screening, the use of MDA is clearly the logical choice. However, karyomapping

is unable to identify all meiotic origins, such that many laboratories perform reflex testing with an alternative method to confirm euploidy diagnoses. Parental support also involves the use of genotyping data to make predictions of aneuploidy in the embryo. These examples of SNP array-based CCS indirectly assess the copy number of chromosomes using genotyping based data. In contrast, an alternative method using SNP array technology directly assesses the copy number state of each chromosome using signal intensities and GenomePlex based WGA (Figure 16.5). GenomePlex WGA4 has been shown to provide more accurate copy number assignments than MDA based testing (4).

Quantitative SNP array based CCS has also been evaluated for performance using a large number of aneuploid cell lines, indicating 98.6% consistency (25). In addition, a non-selection clinical trial demonstrated 96% predictive value of an aneuploid diagnosis for a negative clinical outcome. A randomized

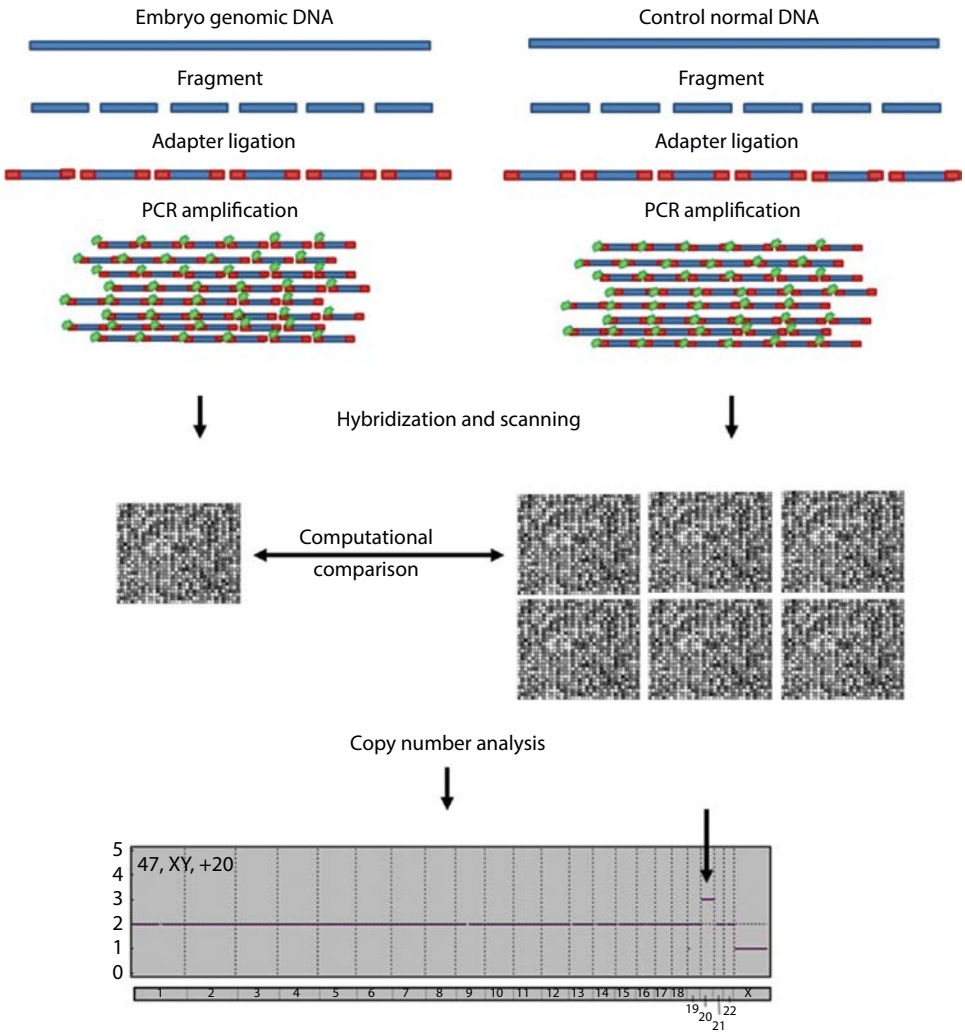

FIGURE 16.5 Whole genome amplification (WGA) and single nucleotide polymorphism (SNP) array analysis begins with fragmentation of the embryonic genomic DNA, adapter ligation, and PCR using a primer that is complementary to the adapter. Hybridization of labeled WGA DNA is performed using one sample per chip. The same procedure is used on multiple samples from control DNA known to be chromosomally normal and used as a reference to perform a comparison with an embryo biopsy sample. Copy number analysis is performed in order to assign a copy number state to each SNP interrogated on the array. The example result shows a sample with an extra copy of chromosome 20 (indicated with an arrow) and a 47, XY, +20 karyotype.

controlled trial was also performed and presented at the American Society for Reproductive Medicine, and indicated significant increase in success rates per intention to treat with the use of CCS (26).

Single Gene Disorder PGD

STRs

Preimplantation genetic diagnosis (PGD) has been mainly used to diagnose autosomal recessive, autosomal dominant, or X-linked single-gene disorders. Virtually any genetically inherited disease that can be diagnosed in adults can be identified in the embryo. Therefore, PGD could be applied to any genetic disease with a molecular diagnosis and/or defined marker linkage within a family. PGD is also offered to carriers of mutations predisposing to cancer or other late-onset diseases. This procedure has already been carried out for several diseases, including the common syndromes of genetic predisposition to colon and breast cancer (27–29). Despite the ethical and legal issues (30), PGD for cancer predisposition syndromes is a reality, and the number of cycles reported for this type of condition is increasing day to day (31).

A further indication is human leucocyte antigen (HLA) matching to select a healthy and HLA compatible embryo with an affected sibling (32). Hematopoietic stem cells from the umbilical cord or bone marrow from this new baby could be used as donor cells for an affected sibling (33). PGD for HLA typing alone is performed for acquired diseases, such as severe aplastic anemia or leukemias, or can be performed in conjunction with a single gene disorder, in order to select an embryo free of the inherited condition and HLA-matched to an affected sibling (34,35). This approach was applied for Fanconi anemia for the first time in 2001 (32), and nowadays is considered an established clinical procedure with documented positive outcomes (36).

Multiplex PCR

Multiplex PCR using targeted primers designed specifically for the mutation of interest combined with other primers for linked short tandem repeat (STR) markers has been the gold standard to perform PGD for monogenic disorders (37). The use of polymorphic markers requires a pre-PGD work-up, where DNA samples of the patients and other relatives allows identification of which alleles co-segregate with the mutation. The use of linked markers improves accuracy by minimizing potential errors caused by allele drop out (ADO) or contamination. The ADO phenomenon refers to the amplification failure of one of the two alleles, making a heterozygous locus appears homozygous, and potentially leading to misdiagnosis. Genotyping of the amplified products can be performed by means of different strategies such as amplification refractory mutation system (38), restriction enzyme digestion (39), real-time PCR (40), and minisequencing (41). In the minisequencing technique, a primer extension reaction is performed, permitting a quick and accurate detection of point mutations. The minisequencing primer is designed to anneal one base before the target site, and it will be elongated with only one dideoxynucleotide. The four different dideoxynucleotides are labeled with different fluorochromes, and the products can be analyzed on an automated DNA sequencing system.

The use of multiplex PCR for markers has become widespread in PGD for monogenic disorders (37,42) and HLA typing (43). The main advantage of multiplex PCRs exclusively for linked markers is that the protocols can be used for several couples, independently of the mutation they carry. This approach saves time and resources in pre-PGD work-up. However, the ability to use such indirect testing depends on the availability of appropriate family members to determine the "at risk haplotype."

WGA Amplification

The use of whole genome amplification (WGA) has been demonstrated to be a practical and efficient alternative to perform PGD (44). WGA amplifies the entire genome resulting in sufficient amplified DNA for several downstream applications. Multiple standard PCR assays need to be performed for haplotyping and the direct analysis of mutations in monogenic diseases, avoiding the necessity of optimizing

multiplex PCR protocols (45). Moreover, WGA allows combining PGD for single gene disorders or HLA typing with aCGH for the detection of chromosomal imbalances and aneuploidies using DNA from the same sample. These strategies have already been applied in several clinical programs, improving the clinical results (46).

However, it is well established that WGA methods yield relatively high ADO rates (4,47). This problem could be solved with the application of a sufficient number of linked markers to avoid misdiagnosis and with the use of trophectoderm biopsies instead of single cells, given that the former give lower ADO rates (46).

Next Generation Sequencing

Next generation sequencing (NGS) provides high throughput and base pair resolution data, providing the analysis of multiple genetic loci and samples from different couples simultaneously. Moreover, NGS, as Karyomapping, allows the combined evaluation of aneuploidy and single gene disorders from the same biopsy using a single platform. Several studies have been published showing the possibility of using NGS to test single cells (47,48). In 2013, Treff et al. (49,50) published a specific protocol to test DNA from a trophectoderm biopsy with NGS that was consistent with two conventional methodologies of PGD. The major concern relating to NGS technology is that an insufficient sequencing depth may result in false positive or negative identification of a mutation due to the presence of sequencing artifacts and ADO, respectively. Moreover, NGS has technical limitations in testing for dynamic mutations.

qPCR

One of the advantages of qPCR based CCS is the ability to add in additional primers which function under the same conditions for preamplification in order to target additional genomic loci. This approach has been reported for a specific case, demonstrating superiority to STR based approaches which failed to detect recombination (51). This is primarily due to the fact that more SNPs are present in the genome than STRs. More systematic development was also reported recently, and involved three phases of development including cell lines, consistency with three reference laboratories, and clinical performance. The approach involves a clinical work-up to define linked informative SNPs near the mutation, as well as the development of direct mutation TaqMan based allelic discrimination assays. Work-ups involve the use of family members and SNP genotyping arrays to define linked markers, and typically require less than four weeks to complete. Reliability of obtaining a diagnosis and ADO and locus amplification rates were all considerably better compared to previously developed methods, including karyomapping and STR based approaches.

Translocation PGD

Balanced structural chromosome rearrangements are the most frequent chromosome abnormalities in the general population, with a prevalence of 0.4% in the prenatal samples and 0.2% in newborns (52,53). The reproductive consequences for translocation and inversion carriers are the production of unbalanced gametes during meiosis because of abnormal segregation. Carriers of structural abnormalities have an increased risk of having fertility problems, recurrent miscarriages, and producing offspring with congenital abnormalities and mental retardation. It has been proven that PGD in these patients led to an improvement in their reproductive outcomes, reducing the time to achieve a successful live birth from 4–6 years to less than 4 months, and decreasing the incidence of miscarriage from more than 90% to less than 15% (54,55).

Until a few years ago the technique used to perform PGD in patients with structural chromosomal abnormalities in both polar body and interphase nuclei was FISH, despite only identifying imbalances of the translocated chromosomes. Some authors have described an interchromosomal effect (ICE), where the translocation chromosomes interfere with segregation of other chromosomes because of spindle disruption. To overcome these limitations, WGA approaches have been successfully used for translocation and aneuploidy screening, including SNP arrays (56–59), aCGH (60,61), and NGS (62–64).

Conclusions

New technologies for CCS in pre-implantation embryos have demonstrated the expected clinical benefit in multiple randomized controlled trials. WGA has been instrumental in the development of some of these new methodologies, and targeted PCR has also demonstrated capabilities beyond single gene disorder PGD, including CCS. Simultaneous analysis of aneuploidy and single gene disorders or translocations has now become standard practice. Future developments are expected for the use of NGS technology to investigate mitochondrial DNA, segmental aneuploidy, and mosaicism detection and quantitation.

REFERENCES

1. Franasiak JM, Forman EJ, Hong KH, Werner MD, Upham KM, Treff NR et al. The nature of aneuploidy with increasing age of the female partner: A review of 15,169 consecutive trophectoderm biopsies evaluated with comprehensive chromosomal screening. *Fertil Steril*. 2014;101:656–63e1.
2. Rabinowitz M, Ryan A, Gemelos G, Hill M, Baner J, Cinnioglu C et al. Origins and rates of aneuploidy in human blastomeres. *Fertil Steril*. 2012;97:395–401.
3. Fragouli E, Alfarawati S, Spath K, Jaroudi S, Sarasa J, Enciso M et al. The origin and impact of embryonic aneuploidy. *Hum Genet*. 2013;132:1001–13.
4. Treff NR, Su J, Tao X, Northrop LE, Scott RT Jr. Single-cell whole-genome amplification technique impacts the accuracy of SNP microarray-based genotyping and copy number analyses. *Mol Hum Reprod*. 2011;17:335–43.
5. Rodrigo L, Mateu E, Mercader A, Cobo AC, Peinado V, Milan M et al. New tools for embryo selection: Comprehensive chromosome screening by array comparative genomic hybridization. *Biomed Res Int*. 2014;2014:517125.
6. Gutierrez-Mateo C, Colls P, Sanchez-Garcia J, Escudero T, Prates R, Ketterson K et al. Validation of microarray comparative genomic hybridization for comprehensive chromosome analysis of embryos. *Fertil Steril*. 2011;95:953–8.
7. Mir P, Rodrigo L, Mercader A, Buendia P, Mateu E, Milan-Sanchez M et al. False positive rate of an arrayCGH platform for single-cell preimplantation genetic screening and subsequent clinical application on day-3. *J Assist Reprod Genet*. 2013;30:143–9.
8. Mir P, Mateu E, Mercader A, Herrer R, Rodrigo L, Vera M et al. Confirmation rates of array-CGH in day-3 embryo and blastocyst biopsies for preimplantation genetic screening. *J Assist Reprod Genet*. 2016;33:59–66.
9. Fiorentino F, Biricik A, Bono S, Spizzichino L, Cotroneo E, Cottone G et al. Development and validation of a next-generation sequencing-based protocol for 24-chromosome aneuploidy screening of embryos. *Fertil Steril*. 2014;101:1375–82e2.
10. Fiorentino F, Bono S, Biricik A, Nuccitelli A, Cotroneo E, Cottone G et al. Application of next-generation sequencing technology for comprehensive aneuploidy screening of blastocysts in clinical preimplantation genetic screening cycles. *Hum Reprod*. 2014;29:2802–13.
11. Fan J, Wang L, Wang H, Ma M, Wang S, Liu Z et al. The clinical utility of next-generation sequencing for identifying chromosome disease syndromes in human embryos. *Reprod Biomed Online*. 2015;31:62–70.
12. Ruttanajit T, Chanchamroen S, Cram DS, Sawakwongpra K, Suksalak W, Leng X et al. Detection and quantitation of chromosomal mosaicism in human blastocysts using copy number variation sequencing. *Prenat Diagn*. 2016;36:154–62.
13. Greco E, Minasi MG, Fiorentino F. Healthy babies after intrauterine transfer of mosaic aneuploid blastocysts. *N Engl J Med*. 2015;373:2089–90.
14. Scott RT Jr., Galliano D. The challenge of embryonic mosaicism in preimplantation genetic screening. *Fertil Steril*. 2016;105:1150–2.
15. Kinde I, Papadopoulos N, Kinzler KW, Vogelstein B. FAST-SeqS: A simple and efficient method for the detection of aneuploidy by massively parallel sequencing. *PLoS One*. 2012;7:e41162.
16. Treff NR, Tao X, Taylor D, Hong KH, Forman EJ, Scott RT. Development and validation of a next-generation sequencing (NGS)-based 24-chromosome aneuploidy screening system. *Fertil Steril*. 2013;100:S82.

17. Treff NR, Scott RT Jr. Four-hour quantitative real-time polymerase chain reaction-based comprehensive chromosome screening and accumulating evidence of accuracy, safety, predictive value, and clinical efficacy. *Fertil Steril*. 2013;99:1049–53.
18. Treff NR. qPCR-based CCS. Reprod Biomed Online. 2013;26:S5.
19. Treff N. Four hour 24 chromosome aneuploidy screening. *Reprod Biomed Online*. 2012;24:S37.
20. Treff NR, Tao X, Ferry KM, Su J, Taylor D, Scott RT Jr. et al. Development and validation of an accurate quantitative real-time polymerase chain reaction-based assay for human blastocyst comprehensive chromosomal aneuploidy screening. *Fertil Steril*. 2012;97:819–24e2.
21. Scott RT Jr., Upham KM, Forman EJ, Hong KH, Scott KL, Taylor D et al. Blastocyst biopsy with comprehensive chromosome screening and fresh embryo transfer significantly increases in vitro fertilization implantation and delivery rates: A randomized controlled trial. *Fertil Steril*. 2013;100:697–703.
22. Forman EJ, Hong KH, Ferry KM, Tao X, Taylor D, Levy B et al. In vitro fertilization with single euploid blastocyst transfer: A randomized controlled trial. *Fertil Steril*. 2013;100:100–7 e1.
23. Johnson DS, Gemelos G, Baner J, Ryan A, Cinnioglu C, Banjevic M et al. Preclinical validation of a microarray method for full molecular karyotyping of blastomeres in a 24-h protocol. *Hum Reprod*. 2010;25:1066–75.
24. Handyside AH, Harton GL, Mariani B, Thornhill AR, Affara NA, Shaw MA et al. Karyomapping: A universal method for genome wide analysis of genetic disease based on mapping crossovers between parental haplotypes. *J Med Genet*. 2010;47:651–8.
25. Treff NR, Su J, Tao X, Levy B, Scott RT Jr. Accurate single cell 24 chromosome aneuploidy screening using whole genome amplification and single nucleotide polymorphism microarrays. *Fertil Steril*. 2010;94:2017–21.
26. Schoolcraft WB, Surrey E, Minjarez D, Gustofson RL, Scott RT Jr., Katz-Jaffe MG. Comprehensive chromosome screening (CCS) with vitrification results in improved clinical outcome in women >35 years: A randomized control trial. *Fertil Steril*. 2012;98:S1.
27. Rechitsky S, Verlinsky O, Chistokhina A, Sharapova T, Ozen S, Masciangelo C et al. Preimplantation genetic diagnosis for cancer predisposition. *Reprod Biomed Online*. 2002;5:148–55.
28. Spits C, De Rycke M, Van Ranst N, Verpoest W, Lissens W, Van Steirteghem A et al. Preimplantation genetic diagnosis for cancer predisposition syndromes. *Prenat Diag*. 2007;27:447–56.
29. Jasper MJ, Liebelt J, Hussey ND. Preimplantation genetic diagnosis for BRCA1 exon 13 duplication mutation using linked polymorphic markers resulting in a live birth. *Prenat Diag*. 2008;28:292–8.
30. Clancy T. A clinical perspective on ethical arguments around prenatal diagnosis and preimplantation genetic diagnosis for later onset inherited cancer predispositions. *Fam Cancer*. 2010;9:9–14.
31. De Rycke M, Belva F, Goossens V, Moutou C, SenGupta SB, Traeger-Synodinos J et al. ESHRE PGD Consortium data collection XIII: Cycles from January to December 2010 with pregnancy follow-up to October 2011. *Hum Reprod* 2015;30:1763–89.
32. Verlinsky Y, Rechitsky S, Schoolcraft W, Strom C, Kuliev A. Preimplantation diagnosis for Fanconi anemia combined with HLA matching. *JAMA*. 2001;285:3130–3.
33. Grewal SS, Kahn JP, MacMillan ML, Ramsay NK, Wagner JE. Successful hematopoietic stem cell transplantation for Fanconi anemia from an unaffected HLA-genotype-identical sibling selected using preimplantation genetic diagnosis. *Blood*. 2004;103:1147–51.
34. Kuliev A, Rechitsky S, Verlinsky O, Tur-Kaspa I, Kalakoutis G, Angastiniotis M et al. Preimplantation diagnosis and HLA typing for haemoglobin disorders. *Reprod Biomed Online*. 2005;11:362–70.
35. Verlinsky Y, Rechitsky S, Sharapova T, Laziuk K, Barsky I, Verlinsky O et al. Preimplantation diagnosis for immunodeficiencies. *Reprod Biomed Online*. 2007;14:214–23.
36. Van de Velde H, De Rycke M, De Man C, De Hauwere K, Fiorentino F, Kahraman S et al. The experience of two European preimplantation genetic diagnosis centres on human leukocyte antigen typing. *Hum Reprod*. 2009;24:732–40.
37. Fiorentino F, Biricik A, Nuccitelli A, De Palma R, Kahraman S, Iacobelli M et al. Strategies and clinical outcome of 250 cycles of Preimplantation Genetic Diagnosis for single gene disorders. *Hum Reprod*. 2006;21:670–84.
38. Moutou C, Goossens V, Coonen E, De Rycke M, Kokkali G, Renwick P et al. ESHRE PGD Consortium data collection XII: Cycles from January to December 2009 with pregnancy follow-up to October 2010. *Hum Reprod*. 2014;29:880–903.

39. Spits C, Caignec CL, Rycke MD, Van Haute L, Van Steirteghem A, Liebaers I et al. Optimization and evaluation of single-cell whole-genome multiple displacement amplification. *Hum Mutat*. 2006;27:496–503.
40. Vrettou C, Traeger-Synodinos J, Tzetis M, Palmer G, Sofocleous C, Kanavakis E. Real-time PCR for single-cell genotyping in sickle cell and thalassemia syndromes as a rapid, accurate, reliable, and widely applicable protocol for preimplantation genetic diagnosis. *Hum Mutat*. 2004;23:513–21.
41. Fiorentino F, Magli MC, Podini D, Ferraretti AP, Nuccitelli A, Vitale N et al. The minisequencing method: An alternative strategy for preimplantation genetic diagnosis of single gene disorders. *Mol Hum Reprod*. 2003;9:399–410.
42. Dreesen JC, Jacobs LJ, Bras M, Herbergs J, Dumoulin JC, Geraedts JP et al. Multiplex PCR of polymorphic markers flanking the CFTR gene; a general approach for preimplantation genetic diagnosis of cystic fibrosis. *Mol Hum Reprod*. 2000;6:391–6.
43. Fiorentino F, Kahraman S, Karadayi H, Biricik A, Sertyel S, Karlikaya G et al. Short tandem repeats haplotyping of the HLA region in preimplantation HLA matching. *Eur J Hum Genet*. 2005;13:953–8.
44. Handyside AH, Robinson MD, Simpson RJ, Omar MB, Shaw MA, Grudzinskas JG et al. Isothermal whole genome amplification from single and small numbers of cells: A new era for preimplantation genetic diagnosis of inherited disease. *Mol Hum Reprod*. 2004;10:767–72.
45. Renwick P, Trussler J, Lashwood A, Braude P, Ogilvie CM. Preimplantation genetic haplotyping: 127 diagnostic cycles demonstrating a robust, efficient alternative to direct mutation testing on single cells. *Reprod Biomed Online*. 2010;20:470–6.
46. Rechitsky S, Pakhalchuk T, San Ramos G, Goodman A, Zlatopolsky Z, Kuliev A. First systematic experience of preimplantation genetic diagnosis for single-gene disorders, and/or preimplantation human leukocyte antigen typing, combined with 24-chromosome aneuploidy testing. *Fertil Steril*. 2015;103:503–12.
47. Navin N, Hicks J. Future medical applications of single-cell sequencing in cancer. *Genome Med*. 2011;3:31.
48. Wang J, Fan HC, Behr B, Quake SR. Genome-wide single-cell analysis of recombination activity and de novo mutation rates in human sperm. *Cell*. 2012;150:402–12.
49. Treff NR, Fedick A, Tao X, Devkota B, Taylor D, Scott RT Jr. et al. Evaluation of targeted next-generation sequencing-based preimplantation genetic diagnosis of monogenic disease. *Fertil Steril*. 2013;99:1377–84.
50. Treff NR, Forman EJ, Scott RT Jr. Next-generation sequencing for preimplantation genetic diagnosis. *Fertil Steril*. 2013;99:e17–8.
51. Gueye NA, Jalas C, Tao X, Taylor D, Scott RT Jr., Treff NR. Improved sensitivity to detect recombination using qPCR for Dyskeratosis Congenita PGD. *J Assist Reprod Genet*. 2014;31:1227–30.
52. Jacobs PA, Melville M, Ratcliffe S, Keay AJ, Syme J. A cytogenetic survey of 11,680 newborn infants. *Ann Hum Genet*. 1974;37:359–76.
53. Van Dyke D, Weiss L, Roberson J, Babu V. The frequency and mutation rate of balanced autosomal rearrangements in man estimated from prenatal genetic studies for advanced maternal age. *Amer J Hum Genet*. 1983;35:301–8.
54. Munne S, Sandalinas M, Escudero T, Fung J, Gianaroli L, Cohen J. Outcome of preimplantation genetic diagnosis of translocations. *Fertil Steril*. 2000;73:1209–18.
55. Verlinsky Y, Tur-Kaspa I, Cieslak J, Bernal A, Morris R, Taranissi M et al. Preimplantation testing for chromosomal disorders improves reproductive outcome of poor-prognosis patients. *Reprod Biomed Online*. 2005;11:219–25.
56. Treff NR, Tao X, Schillings WJ, Bergh PA, Scott RT Jr., Levy B. Use of single nucleotide polymorphism microarrays to distinguish between balanced and normal chromosomes in embryos from a translocation carrier. *Fertil Steril*. 2011;96:e58–65.
57. Treff NR, Northrop LE, Kasabwala K, Su J, Levy B, Scott RT Jr. Single nucleotide polymorphism microarray-based concurrent screening of 24 chromosome aneuploidy and unbalanced translocations in preimplantation human embryos. *Fertil Steril* 2010; 95: 1606–12 e1–2.
58. Tan YQ, Tan K, Zhang SP, Gong F, Cheng DH, Xiong B et al. Single-nucleotide polymorphism microarray-based preimplantation genetic diagnosis is likely to improve the clinical outcome for translocation carriers. *Hum Reprod*. 2013;28:2581–92.

59. van Uum CM, Stevens SJ, Dreesen JC, Drusedau M, Smeets HJ, Hollanders-Crombach B et al. SNP array-based copy number and genotype analyses for preimplantation genetic diagnosis of human unbalanced translocations. *Eur J Hum Genet*. 2012;20:938–44.
60. Alfarawati S, Fragouli E, Colls P, Wells D. First births after preimplantation genetic diagnosis of structural chromosome abnormalities using comparative genomic hybridization and microarray analysis. *Hum Reprod*. 2011;26:1560–74.
61. Vanneste E, Melotte C, Voet T, Robberecht C, Debrock S, Pexsters A et al. PGD for a complex chromosomal rearrangement by array comparative genomic hybridization. *Hum Reprod*. 2011;26:941–9.
62. Wang L, Cram DS, Shen J, Wang X, Zhang J, Song Z et al. Validation of copy number variation sequencing for detecting chromosome imbalances in human preimplantation embryos. *Biol Reprod*. 2014;91:37.
63. Vera-Rodriguez M, Michel CE, Mercader A, Bladon AJ, Rodrigo L, Kokocinski F et al. Distribution patterns of segmental aneuploidies in human blastocysts identified by next-generation sequencing. *Fertil Steril*. 2016;105:1047–1055 e2.
64. Bono S, Biricik A, Spizzichino L, Nuccitelli A, Minasi MG, Greco E et al. Validation of a semiconductor next-generation sequencing-based protocol for preimplantation genetic diagnosis of reciprocal translocations. *Prenat Diagn*. 2015;35:938–44.

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Oocytes and Embryo Cryopreservation

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Introduction

The cryopreservation of biological materials is the process by which they are subjected to cryogenic temperatures below zero enabling a complete stopping of biochemical reactions with the aim to preserve the viability of the cells. Freezing itself is lethal for living systems. However, the understanding of the mechanisms involved in the freezing process have proven highly useful in providing stable conditions necessary to preserve life in the frozen state. As a consequence, different cryopreservation protocols able to preserve life at subzero temperatures have been developed and are applied in different fields, including medicine. Assisted reproduction is no exception, since cryopreservation has been used for decades in this discipline, bringing greater flexibility to IVF treatments. Thus, efficient cryopreservation protocols have been developed for either spermatozoa or embryos at all developmental stages.

The first successful method for semen cryopreservation was described a long time ago, and consisted of a vitrification protocol including glycerol as cryoprotectant (1). On the other hand, the transfer of frozen-thawed embryos has been widely and successfully applied following the first pregnancy achieved with this strategy (2). Conversely, oocyte cryopreservation has proven much more challenging, traditionally producing insufficient results. Lately, vitrification has provided successful outcomes, enabling the establishment of egg-banks which are currently benefitting infertile patients as well as women who are seeking fertility preservation (FP) or oocyte recipients enrolled in egg-donation programs.

The aim of this chapter is to provide an overview of the main cryopreservation strategies and an appraisal of their efficacy when applied for different clinical indications in assisted reproduction technology (ART) practice.

Cryopreservation Protocols and Their Implications in the Survival of Oocytes and Embryos

The protocol for embryo cryopreservation was standardized 20 years ago and has been extensively applied with minimal modifications, providing successful outcomes since its introduction in 1985 (3). The method of choice has been the slow freezing procedure, in which the embryos are cooled at very slow rates after being treated with cryoprotectants at a relatively low concentration (1.0–1.5 M), thus limiting the toxic and osmotic damage. In this protocol, the dehydration of the cells and the diffusion of cryoprotectant agents (CPAs) into the cells takes place very slowly during a long time period. At the end, the procedure allows the equilibration of the extra- and intracellular fluids, which explains why this method is also known as "equilibrium freezing." When the process is completed, the extracellular liquid crystallizes, and intracellular ice formation cannot be completely avoided, this being one of the greatest shortcomings of this strategy. Extracellular ice formation leads to a considerable increase in the concentration of ions, macro-molecules, and other components in the remaining fluid, due to these molecules being excluded from the inner structure of the ice crystals. As a result, the hyperosmolarity produced can cause severe osmotic damage.

Noticeably, during slow freezing, the cells are exposed to what is known as chilling injury, which occurs between +15°C and −5°C and affects the lipids of the cell membrane and the microtubules of the meiotic spindle, and causes hardening of the zona pellucida (4). These effects have been mainly described in the case of the slow freezing of oocytes. The alteration in the lipid composition of the membrane causes irreversible damage (5) while the restoration of the meiotic spindle has been documented in humans (6).

The mechanisms by which intracellular ice formation causes damage are due to the aggregation of small crystals during thawing, becoming larger crystals, caused by high activation energy. These large crystals cause damage primarily by mechanical effects, destroying the cell structures. The deleterious effects of partial crystallization of cells and tissues depend on the cell type and on the number of cells. In a tissue, for example, the lysis of a number of cells can be compensated by those that survive, ultimately providing a functional tissue. That is the case for spermatozoa or ovarian cortex, or even embryos; but not in the case of oocytes, where it is "all or nothing" because the cell does or does not survive.

Intracellular ice formation is one of the causes responsible for the partial blastomere loss observed in a high proportion of embryos after slow freezing. It is well known that partial lysis in frozen thawed embryos results in an impaired implantation potential (7–9). Conventionally, survival after slow freezing was defined when 50% or more blastomeres survived the process. Due to the association between lower implantation and partial blastomere loss, it has been suggested that the efficiency of cryopreservation programs is more accurately assessed by the evaluation of the proportion of fully intact and partially lysed embryos recovered after the freezing/thawing process rather than the evaluation of survival (10).

During vitrification, ice formation is circumvented in both the intra- and the extracellular spaces, due to the direct conversion from liquid to a vitreous solid. The combination of the exposure to very high cryopretectant concentrations and very high cooling rates provokes a significant dehydration of the cell with a partial intracellular diffusion of CPA, thus the equilibrium between the intra- and extracellular compartments is not reached, explaining why this procedure is also known as "non-equilibrium freezing." The glass transition increases as the concentration of CPA increases. As a result a vitreous solid is formed. There are other factors than cryoprotectant concentration and cooling rate which are strongly involved in the attainment of efficient vitrification.

The complete absence of crystallization achieved with vitrification leads to an extremely high proportion of fully intact embryos of 95% (11), which may explain the comparable implantation rates observed when outcomes were evaluated between vitrified/warmed embryos vs fresh embryos belonging to the same morphological category (12). This finding and the increased survival rates achieved for embryos cryopreserved both at the cleavage and at the blastocyst stages represents an advantage of vitrification over slow freezing (13) which explains the current trend to switch to vitrification observed in many IVF centers.

The history of oocytes cryopreservation has been very different from that for embryos. A long series of failures and disappointing results followed the report of the first live birth after the slow freezing of human oocytes back in 1986 (14). Several factors may explain this lack of success, including oocyte size and shape, hydraulic conductivity patterns, the presence of highly sensitive structure such as the meiotic spindle, and the lipid composition of the cell membranes. The large amount of water which reduces the surface to volume ratio is responsible for the high sensitivity of oocytes to chilling injury and for the high susceptibility to intracellular ice formation shown by immature oocytes (15). These effects can be controlled to a different extent depending on the cryopreservation method applied.

Vitrification provides a delicate balance between multiple factors which ultimately leads to success. As explained above, during vitrification, ice formation is avoided by exposing the oocytes to high CPA concentrations when applying very high cooling rates (16). Thus, the probability of vitrification will rise by increasing the cooling rate and the viscosity of the solution and by lowering the volume containing the cells (16). Very high warming rates are also related to success due to the prevention of recrystallization during warming (17). Vitrification technology has evolved in the last few years, providing different devices, protocols, and methodologies that fulfill these requirements to varying degrees. Thus, open or closed systems, named due to the necessity of contact with liquid nitrogen in the former, or not in the latter, are available. Different combinations of cryoprotectants have also been described, as well as different osmotic agents and protein substitutes (4,18).

Chilling injury is efficiently circumvented during vitrification because the passage of the oocytes through the temperature range in which the chilling injury occurs (+15°C and −5°C) is completely avoided because the cells are just plunged into liquid nitrogen, passing from room temperature directly to −196°C. Due to the absence of chilling injury during vitrification, the depolimerization of the meiotic spindle does not occur, prior to or during the vitrification process. Initial studies have attributed a stabilizing effect of CPAs on tubulin fibers which has been confirmed by noninvasive studies (19). These studies have also shown that depolymerization occurs at warming during the dilution of cryoprotectants. Under these circumstances, the inner cryoprotectant is replaced by water during the rehydration, which occurs at room temperature, explaining why depolymerization takes place at this point. It has also been shown that the complete restoration of the spindle occurs depending on the incubation time post warming (19,20) and happens faster following vitrification than after slow freezing (19). These observations have counteracted the initial concerns related to the possible generation of unbalanced gametes after cryopreservation due to the disassembly of the meiotic spindle (21). The restoration of a functional meiotic spindle is supported by the findings showing comparable aneuploidy rates between embryos developed from vitrified and fresh oocytes from infertile patients (22), and by the analysis of perinatal outcomes of babies born from vitrified oocytes (23).

Safety Issues

Open systems have been questioned due to the hypothetical risk of cross contamination related to the direct contact with liquid nitrogen required for the vitrification step and during the storage period. Although there are no reports of cross-contamination after a cryo-transfer in ART, this concern has been strongly considered for many authors, and some country regulations have been developed considering this issue. A study aimed to assess the presence of viral sequences in different samples including follicular fluid, culture media, and liquid nitrogen used for vitrification and storage in seropositive patients for human immunodeficiency virus (HIV), hepatitis C virus (HCV), and hepatitis B virus (HBV) undergoing IVF cycles failed to detect the virus in all the samples analyzed (24). This is extremely interesting evidence for low or absent cross-contamination possibilities when using open vitrification systems, even when dealing with samples from seropositive women, who in some cases showed positive blood viral load. Even so there are some measures that can be taken to make the tool safer, such as the sterilization of liquid nitrogen (25,26), the storage in vapor-phase systems (27), or storage in a closed device even after open vitrification (28). Needless to say, the use of efficient closed devices circumvents any possibility of cross contamination while providing efficient outcomes both for embryos (29,30) and for oocytes (31,32). Although there is growing evidence on the comparable outcomes achieved with the used of closed versus open devices in cases of oocytes vitrification, there is still a lack of large prospective randomized trials demonstrating the harmlessness of closed devices, as is the case for open ones (33).

Clinical Outcome Following Vitrification

Embryo Vitrification

Embryo cryopreservation has become an essential therapeutic strategy in ART, allowing the opportunity to potentially achieve several pregnancies from a single controlled ovarian stimulation (COS) cycle. In this way, embryo storage is a necessary requisite to increase the cumulative outcomes. Furthermore, the availability of an efficient embryo cryopreservation program also allows the practice of single embryo transfer as a strategy to minimize the incidence of multiple pregnancy rates. These goals were traditionally accomplished in a relatively satisfactory way with the aid of the slow freezing procedure, but undoubtedly, nowadays vitrification has made a significant input to embryo cryopreservation programs, improving greatly the outcomes, and hence the scope of these programs. A meta-analysis published in 2008 confirmed the efficiency of embryo vitrification over the slow freezing procedure (34).

Prior to vitrification, the freezing at early cleavage stage rather than blastocyst stage was the most common approach (10), probably due to a lack of consistency after slow freezing of day-5–day-6 embryos,

combined with lower survival rates. Nowadays, the literature is profuse in reports showing successful outcomes after applying vitrification at all embryo developmental stages. Table 17.1 shows results from the most recent publications including data from the last 2 years showing high and similar outcomes between embryo developmental stages and different vitrification devices. Comparable survival, implantation and delivery rates were shown in a large series of cryotransfers of vitrified embryos from cleavage to blastocyst stage (11). In this study, high survival and delivery rates were achieved when vitrified/warmed expanded day-5 blastocysts were transferred. Interestingly, no artificial collapse of the blastocele was applied, clearly in contrast with other studies indicating the need to perform this strategy in order to achieve improved results (47,48). The rationale for applying the artificial collapse of the blastocele is to reduce the large cavity volume and thereby reduce the probability of ice formation within the cavity. A randomized trial addressed the convenience of artificial collapsing of the blastocele prior to vitrification when using a closed system for vitrification (47). We can speculate that the reason for the need for artificial shrinkage when using a closed device may be related to the higher probability of ice formation, which apparently is not the case for open systems, since results are not impaired after the vitrification of fully expanded blastocysts.

Nonetheless, it is worth mentioning that comparable results for early cleavage or blastocyst stage embryos in terms of survival and clinical outcome can be accomplished with both closed and open devices (Table 17.1). One more evidence of similar outcomes between both systems for embryo vitrification was provided by a study which analyzed the neonatal outcomes of babies born after the transfer of vitrified blastocysts using both devices (49).

Different vitrification approaches have also proven to be effective when considering biopsied or non-biopsied cleavage stage or blastocysts embryos, thus making vitrification a valuable complementary tool to preimplantational genetic screening (PGS) analysis (50,51). In our hands, blastocyst survival after trophoectoderm biopsy is 96.3%, and clinical outcomes after the transfer of chromosomally normal embryos indicates that vitrification does not affect their potential to implant (45.2% of implantation rate;

TABLE 17.1

Survival and Pregnancy Rate after Embryo Vitrification at Cleavage or Blastocyst Stage Using Open and/or Closed Devices

Author	Embryo Stage	Survival Rate (%)	Pregnancy Rate (%)	Observations
Liu et al. (35)	Cleavage (Day 3)	97.6	38.5 p	
Panagiotidis et al. (36)	Blastocyst	84.1	45.9	Open system
		82.1	42.4	Closed system
Chen et al. (37)	Blastocyst	98	47.6	Open system
		95.8	42.2	Closed system
Hashimoto et al. (38)	Blastocyst	96.9	46.8	Open system
		97	45.4	Closed system
Kang et al. (39)	Blastocyst	96.6	41.8	Single ET
		97.8	48.1	Double ET
Van Landuyt et al. (40)	Cleavage (Day 3)	94	–	20.7% IR
Muthukumar et al. (41)	Blastocyst	85.5	52.6	Day 5
		79.6	32.6	Day 6
Roy et al. (42)	Blastocyst	94.4	58.8	
Murakami et al. (43)	Blastocyst	98.7	51.5	HSA supplement
		98.9	56.0	rHA supplement
Levron et al. (44)	Cleavage (Day 2–3)	81.6	20.0	
Reed et al. (45)	Blastocyst	96.3	46.2	Non-biopsied
		97.6	58.2	Biopsied
Iwahata et al. (46)	Blastocyst	96.1	–	49.4% IR
		96.5	–	49.7% IR
Total		**96.3**	**45.9**	

Abbreviations: ET, Embryo Transfer; IR, Implantation rate; HAS, Human Serum Albumin; rHA, recombinant Human Albumin.

62.5% of clinical pregnancy rate; 54.2% of ongoing pregnancy rate), all these outcomes highlighting the utility of vitrification as an adjunct to PGS programs.

The availability of vitrification for embryo storage has also made possible the segmentation of IVF, leading to the so-called "freeze-all strategy" which has gained much attention lately. Controlled ovarian stimulation results in extremely high estrogen and progesterone levels that lead to an asynchrony between the embryo developmental stage and the endometrium, a situation which ultimately may impair the endometrial receptivity (52). As a result, delaying the embryo transfer to a different unstimulated cycle, in which the uterus has not been exposed to supra-physiological doses of reproductive hormones, would appear a reasonable solution. Moreover, the rationale of transferring the embryos in a more physiological, "non stimulated" endometrium, is for many, the reason why frozen embryo transfers produce better obstetric and perinatal outcomes as compared to fresh embryo transfers (53,54). In these studies different outcomes, such as the incidence of small for gestational age, was considerably lower after frozen embryo transfers as compared to fresh embryo transfers, although the incidence of large for gestational age was higher in the former group (55).

In addition to better neonatal outcomes, some studies have reported improved IVF results when all embryos are electively frozen for later transfer (52,56,57). These findings have led many to propose that the strategy be applied routinely in IVF. However, this evidence should be taken with caution, since some of the studies available have been criticized due to serious flaws in their design. In fact, in our routine practice we have found no evidence of improved IVF outcome after a freeze-all strategy in terms of ongoing pregnancy or live birth rates (36.2% vs 33.8%) in women with a normal response to ovarian stimulation (58). When adjustments were made for patient age and other variables likely to affect results, there was still no evidence of any impact of freezing. Although these findings do not support a change in IVF practice moving to a freeze-all strategy, there are some cases, such as patients at risk of ovarian hyperstimulation syndrome (59) or with elevated serum progesterone levels (60), etc., in which delaying the transfer would be the choice. What does appear certain currently is that more research is still needed to prove the validity of the approach (61).

Oocytes Vitrification

Vitrification has made possible egg-banking after decades of successive failures. Growing evidence for the efficiency and safety of female gamete vitrification has led both the American Society for Reproductive Medicine (ASRM) and European Society of Human Reproduction and Embryology (ESHRE) to not consider this technique experimental (62,63).

Table 17.2 summarizes the survival and clinical outcomes of human oocytes vitrification reports from the first pregnancy achieved in humans (64) to the present day (59). Although it is difficult to draw conclusions with such a great variety of protocols, devices, and types of oocytes (own/donated), it is interesting to attempt the following analysis. If we focus on donor oocytes, an overall survival rate of ~90% is observed and, interestingly, the great majority of studies used open devices, except for two studies from Stoop et al. and Papatheodorou et al. (31,32). Approximately a 60% clinical pregnancy rate has been achieved with donated vitrified oocytes in the literature (Table 17.2). Overall, the comparison between fresh and vitrified oocytes showed similar outcomes, as they were higher with vitrification when compared to slow freezing. When focusing on studies reporting data on own vitrified oocytes, a mean survival rate of 80% is observed with ~40% clinical pregnancy rate per transfer (Table 17.2).

Ovum donation programs via egg-banking have experienced radical improvements, especially in logistics, greatly facilitating the whole procedure. Among the greatest advantages of the availability of egg-banks in ovum donation programs is the lack of the need for synchronization between the donor and recipient, with no need for the common long waiting lists. Because of this, these banks make it possible to immediately provide oocytes that will be compatible to a couple once the endometrial preparation is completed. Quarantine period is also possible with egg-banks, in a fashion analogous to semen banks.

The efficiency of oocyte vitrification was demonstrated in a large clinical trial showing comparable results in terms of embryo development and clinical outcomes of vitrified versus fresh oocytes (33). In this large randomized clinical trial, the superiority of fresh donations over cryo-donations was not proven. Instead the non-inferiority of egg-banking strategy via vitrification was established (33).

TABLE 17.2

Human Oocytes' Vitrification, Survival and Clinical Outcomes of Different Studies from 1999–2015

Author	Study Design	Device	Own/ Donated	No. Cycles (No. Oocytes)	No. Warming Cycles (No. Oocytes)	Survival Rate (%)	Fertilization/ Inseminated (%)	IR (%)	CPR/ Transfer (%)	OPR/ Transfer (%)	LBR (%)
Kuleshova et al. (64)	Case report	OPS	Own-donated	4 (17)	4 (17)	64.7	45.4	33.3	33.3	33.3	1 (5.9)
Yoon et al. (65)	Case report	EM grid	Own	7 (90)	7 (90)	63.3	43.3[a]	9.4	42.9	42.9	
Yoon et al. (66)	Descriptive	EM grid	Own	34 (474)	34 (474)	68.6	71.7	6.4	21.4		7
Katayama et al. (67)	Case report	Cryotop	Own	6 (46)	6 (46)	94	91			33.3	
Kyono et al. (68)	Case report	Cryotop	Own	1 (5)	1 (5)	100	100	100	100		1
		SF	Own	1 (4)	1 (4)	25	100				
Kuwayama et al. (69)	Cohorts	Cryotop	Own	(64)	(64)	90.6	89.6		41.4		7 +3OPR
		SF	Own	(9)	(9)	22.2	0				
Yoon et al. (70)	Descriptive	EM grid	Own	28 (426)	30 (364)	85.1	77.4	14.2	43.3		5+ 7OPR
Selman et al. (71)	Descriptive	OPS	Own	6 (53)	6 (24)	75	77.7	21.4	33.3		
Antinori et al. (72)	Cohorts	Cryotop	Own	120 (463)	120 (330)	99.4	93	13.2	32.5	23.3	
		Fresh	Own	251 (1755)			96.7	10.3	28.6		
Lucena et al. (73)	Descriptive	Cryotop	Own	40 (370)	4 (28)	92.9	87		100		
		Cryotop	Donated	33 (337)	18 (131)	89.3	87.6		57.1		
Cobo et al. (74)	Cohorts	Cryotop	Donated	30 (231)	30 (231)	96.9	76.3	40.8	65.2	47.8	
		Fresh	Donated	30 (219)			82.2	100	100	100	
Chang et al. (75)	Cohorts: IVO	Cryotop	Donated	10 (240)	18 (137)	85.4	86.3	61.9	83.3	27.8	19 (13.9)
	Cohorts: IVM-MI	Cryotop	Donated			82.3	89.3				
	Cohorts: IVM-VG	Cryotop	Donated			79.3	60.8				
Sher et al. (76)	Descriptive	Cryoloop	Donated	16 (111)	19 (78)	96.1	90.7	61.3	81.2		17 (21.8)

(*Continued*)

TABLE 17.2 (*Continued*)

Human Oocytes' Vitrification, Survival and Clinical Outcomes of Different Studies from 1999–2015

Author	Study Design	Device	Own/ Donated	No. Cycles (No. Oocytes)	No. Warming Cycles (No. Oocytes)	Survival Rate (%)	Fertilization/ Inseminated (%)	IR (%)	CPR/ Transfer (%)	OPR/ Transfer (%)	LBR (%)
Chian et al. (77)	Cohorts: OS	Cryoleaf	Own	38 (463)	38 (463)	81.4	75.6	19.1	50[b]		15 (39.5)[c]
	Cohorts: IVM	Cryoleaf	Own	20 (215)	20 (215)	67.5	64.2	9.6	20[b]		4 (20.0)[c]
Nagy et al. (78)	Cohorts	Cryotop	Donated	20 (153)	20 (153)	89	87	55.3	75		26
		Fresh	Donated	9 (182)			75.3	47.4	55.6		
Cao et al. (79)	Randomized	Cryoleaf	Own-donated	(292)	(292)	91.8	67.9				
		SF	Own-donated	(123)	(123)	61	61.3				
Schoolcraft et al. (80)	Descriptive	Cryotop	Own	12 (160)	12 (160)	76.9	78.9	51.9	72.7		12 (7.5)
Fadini et al. (81)	Cohorts	Cryoleaf	Own	59 (285)	59 (285)	78.9	72.8	9.3	18.2		
		SF	Own	286 (1348)	286 (1348)	57.9	64.6	4.3	7.6		
Grifo and Noyes (82)	Cohorts	Cryotip	Own-donated	(163)	(163)	95.1	74.2				
		SF	Own-donated	(159)	(159)	88	84.3				
Cobo et al. (33)	Randomized	Cryotop	Donated	295 (3286)	295 (3286)	92.5	74.2	39.9	55.4	49.1	
		Fresh	Donated	289 (3185)			73.3	40.9	55.6	48.3	
Rienzi et al. (83)	Randomized sibling-oocytes	Cryotop	Own	40 (124)	40 (124)	96.8	79.2	20.4	38.5	30.8	
		Fresh	Own	40 (120)			83.3		43.2		
Kim et al. (84)	Descriptive	EM grid	Own	19 (483)	20 (395)	81	72.3	45.3	80[b]	65	20 (5.1)
Almodin et al. (85)	Cohorts sibling-oocytes	Vitri-inga	Own	46 (252)	46 (252)	84.9	80.8	14.9	45.6		
		Fresh	Own	79 (413)			81.4	21.3	51.9		
Ubaldi et al. (86)	Cohorts sibling-oocytes	Cryotop	Own	182 (1132)	115 (487)	89.7	85.4	16.1	31.5	25.2[d]	
		Fresh	Own	173 (511)			87.1	23.2	44.8	37.4[d]	

(*Continued*)

TABLE 17.2 (*Continued*)

Human Oocytes' Vitrification, Survival and Clinical Outcomes of Different Studies from 1999–2015

Author	Study Design	Device	Own/ Donated	No. Cycles (No. Oocytes)	No. Warming Cycles (No. Oocytes)	Survival Rate (%)	Fertilization/ Inseminated (%)	IR (%)	CPR/ Transfer (%)	OPR/ Transfer (%)	LBR (%)
Smith et al. (87)	Randomized	Cryotip	Own	48 (349)	48 (349)	80.5	76.9		37.5[e]		
		SF	Own	30 (238)	30 (238)	66.8	67.1		13.3[e]		
Noyes et al. (21)	Cohorts	Cryotip and cryolock	Own-donated		(167)	88	77.2				
		SF	Own-donated		(148)	85	89.7				
Trokoudes et al. (88)	Cohorts sibling-oocytes	Cryotop	Donated	36 (210)	36 (210)	91.4	84.4	24.7	55.6	47.2	17 (8.1)
		Fresh	Donated	36 (247)	41 (247)		86.6	25.6	48.8	43.9	17 (6.9)
García et al. (89)	Randomized	Cryolock	Donated	20 (283)	34 (283)	89.4	76.1	43.9	61.8		
		Fresh	Donated	58 (696)	85 (696)		87,5	42.9	60		
Paffoni et al. (90)	Cohorts	Cryotop	Own	53 (268)	53 (268)	82.8	73.0	13.4	26.4[e]		11 (4.1)
		Cryotip	Own	51 (261)	51 (261)	57.9	57.6	5.8	7.8[e]		3 (1.1)
Parmegiani et al. (28)	Cohorts sibling-oocytes	Cryotop + hermetical cryostorage	Own	31 (168)	31 (168)	89.9	84.9	17.1	35.5		7 (4.2)
		Fresh		31			88.3		13.3		
Cobo et al. (91)	Cohorts	Cryotop	Own	384 (1192)	384 (1192)	84.9	66.1	25			73 (6.1)
		Fresh	Own	587 (1170)			64.9	25.6			108 (6)
Stoop et al. (32)	Descriptive	CBS straw	Donated	14 (123)	20 (123)	90.2	77.5	33.3	50	45	
Forman et al. (22)	Randomized	Cryotop	Own	44 (294)	44 (294)	81.6	77.9			53,9	
		Fresh	Own	44 (294)			90.5			57.7	
García-Velasco et al. (92)	Descriptive: Non oncological	Cryotop	Own	725 (5498)	26 (191)	84.8			42.3[f]	30.7[g]	4 (2.1)

(*Continued*)

TABLE 17.2 (*Continued*)

Human Oocytes' Vitrification, Survival and Clinical Outcomes of Different Studies from 1999–2015

Author	Study Design	Device	Own/ Donated	No. Cycles (No. Oocytes)	No. Warming Cycles (No. Oocytes)	Survival Rate (%)	Fertilization/ Inseminated (%)	IR (%)	CPR/ Transfer (%)	OPR/ Transfer (%)	LBR (%)
	Oncological	Cryotop	Own	355 (2939)	4				25[f]	25[g]	1
Chang et al. (93)	Randomized sibling-oocytes	Cryotop	Own	22 (186)	22 (186)	79.6	66.6[a]	30.1	45.4		11 (5.9)
		Fresh	Own	22 (204)			75				
Papatheodorou et al. (31)	Randomized sibling-oocytes	Vitrisafe	Donated	75 (608)	75 (608)	90.9	73.4	10.1	28.0[b]	24.0[d]	18 (3)
		Vitisafe+ HSS	Donated	75 (598)	75 (598)	82.9	82.5	13.8	36.0[b]	33.3[d]	27 (4.5)
Solé et al. (94)	Randomized sibling-oocytes	Cryotop	Donated	99	99	85.6	78.2	34	53.5	44.4	42 (4.2)
		Fresh	Donated	99	99		80.7	33.3	47.5	39.4	38
Siano et al. (95)	Cohort sibling-oocytes	Cryotop		14 (83)	14 (83)	72 (86.7)	69.4	7 (25)	53.8		6 (46.1)[h]
		Fresh		14 (81)	14 (81)		78.2				
Martinez et al. (19)	Descriptive: Oncological	Cryotop	Own	375	11 (65)	60 (92.3)	46 (76.7)	7 (31.8)	6 (54.5)	4 (36.4)	4 (6.1)[h]
Cobo et al. (59)	Retrospective, observational	Cryotop	Donated	3146 (42152)	3610 (42152)	38087 (90.4)	26869 (71.2)	2220 (39)	1678 (48.4)	1382 (39.9)	1674 (4.0)

Abbreviations: IR, implantation rate; CPR, clinical pregnancy rate; OPR, ongoing pregnancy rate; LBR, live birth rate.

[a] 2PN/warmed oocyte.

[b] CPR/cycle started.

[c] LRB/cycle started.

[d] OPR/cycle.

[e] CPR/warming cycle.

[f] CPR/patient.

[g] OPR/patient.

[h] LBR/ET.

Others have reported their experience showing very similar outcomes (78,96). These outcomes mirror a study involving shared vitrified and fresh oocytes, which found similar developmental parameters and clinical outcomes between fresh and cryo-donations (88). Other authors evaluated the combined oocyte vitrification and embryo transfer strategy at the blastocyst stage of their ovum donation programs (89). Both blastocyst formation (41.3% vs 45.3%) and pregnancy rates (61.8% vs 60%) between vitrified and fresh oocytes were similar. Another prospective observational study involving 14 donors and 20 recipients reported a 90% survival rate and a 43.5% ongoing pregnancy rate (OPR) by considering "fresh" and cryo-transfers of surplus embryos (32). What is most interesting, and unlike most studies published to date, is that this work employed a closed vitrification system. In line with this, another study has shown comparable results between oocyte donation cycles conducted with vitrified oocytes using open versus closed systems (31). No differences were observed in terms of implantation (13.8% vs 10.1%), clinical pregnancy (36.0% vs 28.0%), ongoing pregnancy (33.3% vs 24.0%) and live birth (36.0% vs 24.0%) rates between the closed and open groups, although survival rate was statistically lower when the closed system was used (82.9% vs 91.0%, $p < 0.05$). Larger prospective randomized trials are needed to definitively confirm the lack of adverse impact of using a closed system.

Egg-banking in ovum donation has become one more standard procedure in our center. A very recent publication shows our experience after 6 years of systematic donation of vitrified oocytes, confirming the clinical outcomes reported previously, but also offering a complete picture of the scope of the technology (59). After analyzing more than 40,000 oocytes, we were able to confirm over 90% survival rate. Nevertheless, differential survival rates among donors or among different stimulation cycles from the same donor were also observed. Thus, a small proportion of cases in which survival was very low (10%–50%) or even 0% (in 1.4% of the cases) was detected (Table 17.3). In an attempt to develop a model to predict survival, it was found that no variable proved useful to that purpose. However, the awareness of the fact that in some cases unexplained low outcomes are achieved, and the low incidence of these adverse outcomes, is of interest to all who use egg-banking. The evaluation of this large series has confirmed our previous findings related to clinical outcome (Table 17.4).

Cumulative outcomes have been also confirmed as highly successful (close to 80% cumulative delivery rate after fresh embryo transfer plus three cryotransfers from one single donation). In these series, approximately 15 oocytes were needed to achieve a baby (oocyte to baby rate = 6.5%), which compares with previous reports on either ovum donation or infertile populations, thus confirming the validity of egg-banking (59). The probability of having a baby was also associated with the number of oocytes used, and increased greatly when adding few more oocytes to the number of oocytes consumed (Figure 17.1). To date, we have notification of nearly 6000 babies born (5989) after over 15,000 ovum donation cycles with vitrified oocytes (16,404), involving nearly 200,000 vitrified oocytes (187,647) in the IVI group (unpublished data), revealing the great scope of this approach. The health of infants and the obstetric evolution of the pregnancies conceived with vitrified oocytes are comparable to those observed in our population of children conceived with fresh oocytes, thus endorsing the safety of the technique (23).

TABLE 17.3

Distribution of Survival Rate in Warming Procedures Finally Not Donated Due to Low Survival

	No. Warming Procedures (%)	No. Surviving Oocytes/Total Warmed Oocytes	Mean Survival Rate/Warming Procedures (%)	Mean No. Surviving Oocytes/Warming Procedures
SV 0%	45 (31.5)	0/412	0,0	0,0
SV 10–15%	4 (2.8)	7/59	11.9 (3.6–20.1)	1.8 (1.7–1.9)
SV 16–20%	7 (4.9)	12/65	18.5 (9.0–27.9)	1.9 (1.8–2.0)
SV 21–30%	17 (11.9)	52/208	26.0 (19.1–30.9)	3.2 (3.2–3.3)
SV31–50%	62 (43.4)	254/602	42.2 (38.3–46.1)	4.1 (4.0–4.1)
SV54–67%	8 (5.6)	35/65	53.8 (41.7–66.0)	4.4 (4.3–4.5)
Total	143	362/1411	25.7 (23.4–27.9)	2.5 (2.5–2.5)

Source: Cobo A et al. *Fertil Steril*. 2015;104(6):1426–34.

TABLE 17.4

Clinical Outcome in an Ovum Donation after Six-Years of Egg-Banking Practice

		95% CI
No. cycles	3467	
MII oocytes injected/donation cycle (mean)	37725 (10.9)	10.7–11.1
Fertilization rate	26869 (71.2)	70.8–71.8
Number of fresh embryo transfers/donation cycle	3050/3467 (87.9)[a]	86.8–89.0
Number of embryos replaced (mean)	5695 (1.9)	1.8–1.9
Implantation rate (No. sacs/No. embryos transferred)	39.0 (2220/5695)	37.8–40.5
Clinical pregnancy rate/transfer	1678/3050 (55.0)	53.2–56.8
Clinical pregnancy rate/cycle	1678/3467 (48.4)	46.7–50.1
Clinical miscarriage	274 (16.3)	14.5–18.1
Ectopic Pregnancy	22 (1.3)	0.8–1.9
Ongoing pregnancy/transfer	1382 (45.3)	43.5–47.3
Ongoing pregnancy/cycle	1382 (39.9)	38.3–41.5
Delivery rate/donation cycle	1357/3467 (39.1)	37.5–40.7

Source: Cobo A et al. *Fertil Steril*. 2015;104(6):1426–34.

Note: Number in parentheses are percentages unless otherwise indicated.

[a] In 89 cases the fresh embryo transfer was deferred for a future cryotransfer.

Patients conducting IVF cycles with their own oocytes have also benefited from oocytes vitrification. A prospective randomized study conducted on sibling oocytes from typically infertile patients demonstrated comparable outcomes between vitrified and fresh oocytes (83). These authors also assessed cumulative outcomes in the same infertile population, showing that maternal age negatively impacts outcomes for both fresh and vitrified cycles; the implantation rate in patients older than 41 years was 12.2%, while it was 27.3% for patients younger than 34 years (86).

The scope of oocyte vitrification was also evaluated in a selected population of young fertile women, achieving 65% OPR and 20 live births, with a mean number of embryos transferred of 2.7 ± 0.7 and 9.1% implantation rate (84). These outcomes mirror the ones achieved with donors, due to the selected population, but do not correspond to the great majority of infertile patients, underlining the need for proper counseling in infertile patients. On the other hand, it is not surprising that lower outcomes are

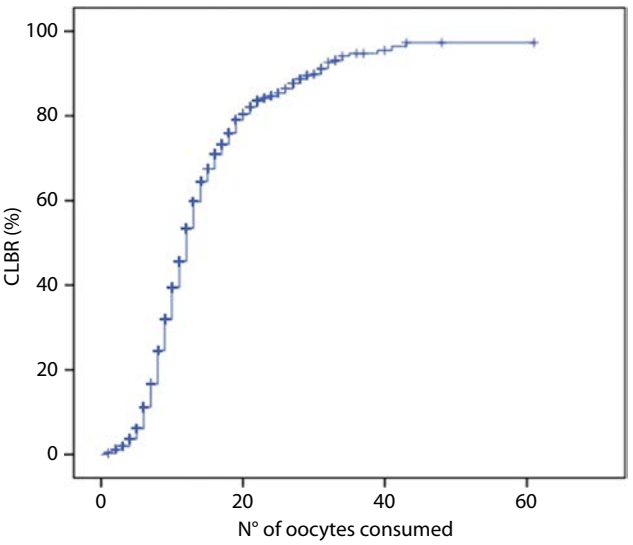

FIGURE 17.1 Kaplan–Meier curve for the cumulative delivery rate of at least one baby, depending on the total number of oocytes consumed. (From Cobo A et al. *Fertil Steril*. 2015;104(6):1426–34.)

achieved in older patients, which most probably is due to the compromise of oocyte quality rather than a direct effect of vitrification. Moreover, we could hypothesize that survival could serve as a filter, selecting the most capable gametes and discarding those with lower potential. The effects of age have also been revealed in a multicenter study in which, after confirming the reproducibility of outcomes between centers, age was revealed as one of the most determining factors for success, together with the number of oocytes and the embryo developmental stage at transfer (97).

The benefits of oocyte vitrification in two infertile populations include avoiding the risk of hyperstimulation (61) and low responders (LR) (99). We have addressed the benefits of accumulating oocytes from different COS cycles in low response patients, shown in terms of higher newborn rates per patient initiated as compared to standard-treated poor responder patients (99). Over 1000 vitrified oocytes obtained were accumulated by vitrification, leading to enhanced cohorts to be inseminated (mean 7.02 MII oocytes) (99).

Advanced maternal age LR patients (>40 years old with ≥6 metaphase II oocytes) undergoing PGS analysis also benefited from this strategy: implantation rate 24.4% vs 19.8% in vitrification versus fresh oocytes (100). A recent report has also shown that the strategy of accumulation in older patients requiring PGS can be conducted at MII oocytes or early cleavage embryos, showing similar advantages (101).

It is worth mentioning that the true advantage of this strategy lies in cost effective issues, rather than in the clinical benefit itself. The clinical outcome after performing several fresh cycles would be comparable to the outcome achieved after performing one accumulation cycle. However, the high dropout incidence in LR patients plays a key role in these cases, due to the limited capacity of couples to cope with consecutive failures. Instead patients quit, look for another clinic, or switch to ovum donation. On the other hand, the accumulation strategy may be of higher advantage from a cost-effective standpoint, since special packages considering successive COS and vitrification cycles and just one intracytoplasmic sperm injection (ICSI) procedure could result in a lower economic burden than is the case with the cost of consecutive entire IVF cycles. Moreover, as shown in our study, the cumulative outcome could be even higher in a great proportion of patients who accumulated oocytes, owing to the availability of surplus embryos to conduct additional embryo transfers within the same accumulation cycle (99).

Growing evidence of the efficiency of oocytes vitrification has encouraged practitioners to offer this option for fertility preservation (FP), either for cancer patients or due to other reasons. The change in motherhood trends means that women are often forced to choose between their profession and financial situation, among other social conditions, and the biological clock. Nowadays, with equal importance placed on their careers, women are at a disadvantage due to the well-known decline in fertility over 30 years of age. With more women deciding to delay motherhood, there is an increased interest in the availability of the current cryopreservation technologies in order to safeguard their options for the future. Hence, FP has become a new and growing branch of ART, with a significantly increasing demand. Data on outcomes for FP via egg-banking are still scarce, although interesting evidence is starting to appear.

A very recent report shows an exponential increase in the number of elective FP cycles, with a five-fold increase in the number of cases in the last few years (102). A 39% clinical pregnancy rate was achieved in the actual FP population analyzed, with a total number of 39 babies born. Nonetheless, when different comparisons were made among patients aged 35 years or older, outcomes were poorer in older patients, thus confirming the previous adverse effects seen in an aged infertile population as a consequence of age related fertility decline. The probability of having a baby was closely related to the number of oocytes consumed and was significantly lower when patients were 36 years or older. Thus, according to our data, 8–10 oocytes would be advisable in patients aged 35 or younger while specific evaluation should be done when they are 36 or older. Interestingly, according to our experience, 20% of patients vitrified their oocytes at an age of ≥40 years. This finding confirms the current tendency to decide for FP too late, highlighting our obligation to adequately inform women about their chances in order to avoid false expectations.

At present nearly 12,000 IVF cycles (11,785) involving the use of own vitrified oocytes (~73,000 oocytes) have been performed at our centers (2787 babies born of whom we have notification) accounting for a total of ~8000 children born from vitrified oocytes in our group.

In conclusion, vitrification has opened up new therapeutic options, allowing greater flexibility in the treatments, as well as the introduction of new ones, which undoubtedly has meant a revolution in ART.

REFERENCES

1. Polge C, Smith AU, Parkes AS. Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature*. 1949;164(4172):666.
2. Trounson A, Leeton J, Besanko M, Wood C, Conti A. Pregnancy established in an infertile patient after transfer of a donated embryo fertilised in vitro. *Br Med J (Clin Res Ed)*. 1983;286(6368):835–8.
3. Lassalle B, Testart J, Renard JP. Human embryo features that influence the success of cryopreservation with the use of 1,2 propanediol. *Fertil Steril*. 1985;44(5):645–51.
4. Vajta G, Kuwayama M. Improving cryopreservation systems. *Theriogenology*. 2006;65(1):236–44.
5. Ghetler Y, Yavin S, Shalgi R, Arav A. The effect of chilling on membrane lipid phase transition in human oocytes and zygotes. *Hum Reprod*. 2005;20(12):3385–9.
6. Cobo A, Perez S, De los Santos MJ, Zulategui J, Domingo J, Remohi J. Effect of different cryopreservation protocols on the metaphase II spindle in human oocytes. *Reprod Biomed Online* [Evaluation Studies]. 2008;17(3):350–9.
7. Edgar DH, Bourne H, Speirs AL, McBain JC. A quantitative analysis of the impact of cryopreservation on the implantation potential of human early cleavage stage embryos. *Hum Reprod*. 2000;15(1):175–9.
8. Van den Abbeel E, Camus M, Van Waesberghe L, Devroey P, Van Steirteghem AC. Viability of partially damaged human embryos after cryopreservation. *Hum Reprod*. 1997;12(9):2006–10.
9. El-Toukhy T, Khalaf Y, Al-Darazi K, Andritsos V, Taylor A, Braude P. Effect of blastomere loss on the outcome of frozen embryo replacement cycles. *Fertil Steril*. 2003;79(5):1106–11.
10. Edgar DH, Archer J, Bourne H. The application and impact of cryopreservation of early cleavage stage embryos in assisted reproduction. *Hum Fertil (Camb)*. 2005 Dec;8(4):225–30.
11. Cobo A, de los Santos MJ, Castello D, Gamiz P, Campos P, Remohi J. Outcomes of vitrified early cleavage-stage and blastocyst-stage embryos in a cryopreservation program: evaluation of 3,150 warming cycles. *Fertil Steril*. [Video-Audio Media]. 2012 Nov;98(5):1138–46 e1.
12. Zhang S, Tan K, Gong F, Gu Y, Tan Y, Lu C et al. Blastocysts can be rebiopsied for preimplantation genetic diagnosis and screening. *Fertil Steril*. [Research Support, Non-U.S. Gov't]. 2014 Dec;102(6):1641–5.
13. Chamayou S, Romano S, Alecci C, Storaci G, Ragolia C, Palagiano A et al. Oocyte vitrification modifies nucleolar remodeling and zygote kinetics-a sibling study. *J Assist Reprod Genet*. 2015;32(4):581–6.
14. Chen C. Pregnancy after human oocyte cryopreservation. *Lancet*. 1986;1(8486):884–6.
15. Mazur P. Principles of cryobiology. In: *Life in the Frozen State*, Fuller B, Lane M, Benson E, editors. New York: CRC Press; 2004. pp. 4–55.
16. Saragusty J, Arav A. Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. *Reproduction* [Review]. 2011;141(1):1–19.
17. Seki S, Mazur P. The dominance of warming rate over cooling rate in the survival of mouse oocytes subjected to a vitrification procedure. *Cryobiology* [Research Support, N.I.H., Extramural]. 2009;59(1):75–82.
18. Stoop D, Silber S, Cobo A. Fertility preservation for age-related fertility decline—authors' reply. *Lancet* [Comment Letter]. 2015;385(9967):507–8.
19. Martinez M, Rabadan S, Domingo J, Cobo A, Pellicer A, Garcia-Velasco JA. Obstetric outcome after oocyte vitrification and warming for fertility preservation in women with cancer. *Reprod Biomed Online* [Evaluation Studies Research Support, Non-U.S. Gov't]. 2014;29(6):722–8.
20. Bianchi V, Coticchio G, Fava L, Flamigni C, Borini A. Meiotic spindle imaging in human oocytes frozen with a slow freezing procedure involving high sucrose concentration. *Hum Reprod*. 2005;20(4):1078–83.
21. Noyes N, Knopman J, Labella P, McCaffrey C, Clark-Williams M, Grifo J. Oocyte cryopreservation outcomes including pre-cryopreservation and post-thaw meiotic spindle evaluation following slow cooling and vitrification of human oocytes. *Fertil Steril* [Comparative Study Evaluation Studies Research Support, Non-U.S. Gov't]. 2010;94(6):2078–82.
22. Forman EJ, Li X, Ferry KM, Scott K, Treff NR, Scott RT Jr. Oocyte vitrification does not increase the risk of embryonic aneuploidy or diminish the implantation potential of blastocysts created after intracytoplasmic sperm injection: A novel, paired randomized controlled trial using DNA fingerprinting. *Fertil Steril* [Randomized Controlled Trial Research Support, Non-U.S. Gov't]. 2012;98(3):644–9.
23. Cobo A, Serra V, Garrido N, Olmo I, Pellicer A, Remohi J. Obstetric and perinatal outcome of babies born from vitrified oocytes. *Fertil Steril* 2014;102(4):1006–15 e4.

24. Cobo A, Bellver J, de Los Santos MJ, Remohi J. Viral screening of spent culture media and liquid nitrogen samples of oocytes and embryos from hepatitis B, hepatitis C, and human immunodeficiency virus chronically infected women undergoing in vitro fertilization cycles. *Fertil Steril*. 2011.
25. Cobo A, Castellò D, Weiss B, Vivier C, De la Macorra A, Kramp F. Highest liquid nitrogen quality for vitrification process: Micro bacteriological filtration of LN2. 16th World Congress on In Vitro Fertilization; 6th World Congress on In Vitro Maturation, Tokyo, Japan, Abstract book. 2011:286.
26. Parmegiani L, Accorsi A, Cognigni GE, Bernardi S, Troilo E, Filicori M. Sterilization of liquid nitrogen with ultraviolet irradiation for safe vitrification of human oocytes or embryos. *Fertil Steril*. 2009.
27. Cobo A, Romero JL, Perez S, de los Santos MJ, Meseguer M, Remohi J. Storage of human oocytes in the vapor phase of nitrogen. *Fertil Steril* [Randomized Controlled Trial]. 2010;94(5):1903–7.
28. Parmegiani L, Cognigni GE, Bernardi S, Cuomo S, Ciampaglia W, Infante FE et al. Efficiency of aseptic open vitrification and hermetical cryostorage of human oocytes. *Reprod Biomed Online* [Randomized Controlled Trial]. 2011;23(4):505–12.
29. Van Landuyt L, Stoop D, Verheyen G, Verpoest W, Camus M, Van de Velde H et al. Outcome of closed blastocyst vitrification in relation to blastocyst quality: Evaluation of 759 warming cycles in a single-embryo transfer policy. *Hum Reprod* [Research Support, Non-U.S. Gov't]. 2011;26(3):527–34.
30. Vanderzwalmen P, Zech NH, Ectors F, Stecher A, Lejeune B, Vanderzwalmen S et al. Blastocyst transfer after aseptic vitrification of zygotes: An approach to overcome an impaired uterine environment. *Reprod Biomed Online* [Evaluation Studies]. 2012;25(6):591–9.
31. Papatheodorou A, Vanderzwalmen P, Panagiotidis Y, Prapas N, Zikopoulos K, Georgiou I et al. Open versus closed oocyte vitrification system: A prospective randomized sibling-oocyte study. *Reprod Biomed Online* [Randomized Controlled Trial Validation Studies]. 2013;26(6):595–602.
32. Stoop D, De Munck N, Jansen E, Platteau P, Van den Abbeel E, Verheyen G et al. Clinical validation of a closed vitrification system in an oocyte-donation programme. *Reprod Biomed Online*. 2012;24(2):180–5.
33. Cobo A, Meseguer M, Remohi J, Pellicer A. Use of cryo-banked oocytes in an ovum donation programme: A prospective, randomized, controlled, clinical trial. *Hum Reprod*. 2010;25(9):2239–46.
34. Loutradi KE, Kolibianakis EM, Venetis CA, Papanikolaou EG, Pados G, Bontis I et al. Cryopreservation of human embryos by vitrification or slow freezing: A systematic review and meta-analysis. *Fertil Steril* [Meta-Analysis Review]. 2008;90(1):186–93.
35. Liu SY, Teng B, Fu J, Li X, Zheng Y, Sun XX. Obstetric and neonatal outcomes after transfer of vitrified early cleavage embryos. *Hum Reprod*. 2013;28(8):2093–100.
36. Panagiotidis Y, Vanderzwalmen P, Prapas Y, Kasapi E, Goudakou M, Papatheodorou A et al. Open versus closed vitrification of blastocysts from an oocyte-donation programme: A prospective randomized study. *Reprod Biomed Online* [Comparative Study Randomized Controlled Trial]. 2013;26(5):470–6.
37. Chen Y, Zheng X, Yan J, Qiao J, Liu P. Neonatal outcomes after the transfer of vitrified blastocysts: closed versus open vitrification system. *Reprod Biol Endocrinol* [Research Support, Non-U.S. Gov't]. 2013;11:107.
38. Hashimoto S, Amo A, Hama S, Ohsumi K, Nakaoka Y, Morimoto Y. A closed system supports the developmental competence of human embryos after vitrification: Closed vitrification of human embryos. *J Assist Reprod Genet* [Research Support, Non-U.S. Gov't]. 2013;30(3):371–6.
39. Kang SM, Lee SW, Yoon SH, Kim JC, Lim JH, Lee SG. Comparison of clinical outcomes between single and double vitrified-warmed blastocyst embryo transfer according to the day of vitrification. *J Assist Reprod Genet*. 2013;30(6):779–85.
40. Van Landuyt L, Van de Velde H, De Vos A, Haentjens P, Blockeel C, Tournaye H et al. Influence of cell loss after vitrification or slow-freezing on further in vitro development and implantation of human Day 3 embryos. *Hum Reprod*. 2013;28(11):2943–9.
41. Muthukumar K, Kamath MS, Mangalaraj AM, Aleyamma T, Chandy A, George K. Comparison of clinical outcomes following vitrified warmed day 5/6 blastocyst transfers using solid surface methodology with fresh blastocyst transfers. *J Hum Reprod Sci*. 2013;6(1):59–64.
42. Roy TK, Brandi S, Tappe NM, Bradley CK, Vom E, Henderson C et al. Embryo vitrification using a novel semi-automated closed system yields in vitro outcomes equivalent to the manual Cryotop method. *Hum Reprod* [Research Support, Non-U.S. Gov't]. 2014;29(11):2431–8.
43. Murakami M, Egashira A, Tanaka K, Mine C, Otsubo H, Kuramoto T. Perinatal outcomes for transfer of blastocysts vitrified and warmed in defined solutions with recombinant human albumin: 374 babies born after 898 embryo transfers. *J Assist Reprod Genet*. 2014;31(12):1605–10.

44. Levron J, Leibovitz O, Brengauz M, Gitman H, Yerushalmi GM, Katorza E et al. Cryopreservation of day 2–3 embryos by vitrification yields better outcome than slow freezing. *Gynecol Endocrinol* [Evaluation Studies Observational Study]. 2014;30(3):202–4.
45. Reed ML, Said AH, Thompson DJ, Caperton CL. Large-volume vitrification of human biopsied and non-biopsied blastocysts: a simple, robust technique for cryopreservation. *J Assist Reprod Genet* [Clinical Study]. 2015;32(2):207–14.
46. Iwahata H, Hashimoto S, Inoue M, Inoue T, Ito K, Nakaoka Y et al. Neonatal outcomes after the implantation of human embryos vitrified using a closed-system device. *J Assist Reprod Genet* [Research Support, Non-U.S. Gov't]. 2015;32(4):521–6.
47. De Munck N, Petrussa L, Verheyen G, Staessen C, Vandeskelde Y, Sterckx J et al. Chromosomal meiotic segregation, embryonic developmental kinetics and DNA (hydroxy)methylation analysis consolidate the safety of human oocyte vitrification. *Mol Hum Reprod* [Research Support, Non-U.S. Gov't]. 2015;21(6):535–44.
48. Hiraoka K, Kinutani M, Kinutani K. Blastocoele collapse by micropipetting prior to vitrification gives excellent survival and pregnancy outcomes for human day 5 and 6 expanded blastocysts. *Hum Reprod* 2004;19(12):2884–8.
49. De Munck N, Santos-Ribeiro S, Stoop D, Van de Velde H, Verheyen G. Open versus closed oocyte vitrification in an oocyte donation programme: a prospective randomized sibling oocyte study. *Hum Reprod*. 2016 Feb;31(2):377–84.
50. Van Landuyt L, Verpoest W, Verheyen G, De Vos A, Van de Velde H, Liebaers I et al. Closed blastocyst vitrification of biopsied embryos: evaluation of 100 consecutive warming cycles. *Hum Reprod*. 2011;26(2):316–22.
51. Schoolcraft WB, Fragouli E, Stevens J, Munne S, Katz-Jaffe MG, Wells D. Clinical application of comprehensive chromosomal screening at the blastocyst stage. *Fertil Steril* [Evaluation Studies Research Support, Non-U.S. Gov't]. 2010;94(5):1700–6.
52. Roque M, Lattes K, Serra S, Sola I, Geber S, Carreras R et al. Fresh embryo transfer versus frozen embryo transfer in in vitro fertilization cycles: A systematic review and meta-analysis. *Fertil Steril* [Comparative Study Meta-Analysis Review]. 2013;99(1):156–62.
53. Maheshwari A, Pandey S, Shetty A, Hamilton M, Bhattacharya S. Obstetric and perinatal outcomes in singleton pregnancies resulting from the transfer of frozen thawed versus fresh embryos generated through in vitro fertilization treatment: A systematic review and meta-analysis. *Fertil Steril* [Comparative Study Meta-Analysis Review]. 2012Aug;98(2):368–77 e1–9.
54. Wennerholm UB, Soderstrom-Anttila V, Bergh C, Aittomaki K, Hazekamp J, Nygren KG et al. Children born after cryopreservation of embryos or oocytes: A systematic review of outcome data. *Hum Reprod*. 2009;24(9):2158–72.
55. Kato O, Kawasaki N, Bodri D, Kuroda T, Kawachiya S, Kato K et al. Neonatal outcome and birth defects in 6623 singletons born following minimal ovarian stimulation and vitrified versus fresh single embryo transfer. *Eur J Obstet Gynecol Reprod Biol*. 2012;161(1):46–50.
56. Shapiro BS, Daneshmand ST, Garner FC, Aguirre M, Hudson C, Thomas S. Evidence of impaired endometrial receptivity after ovarian stimulation for in vitro fertilization: A prospective randomized trial comparing fresh and frozen-thawed embryo transfer in normal responders. *Fertil Steril* [Comparative Study Randomized Controlled Trial Research Support, Non-U.S. Gov't]. 2011;96(2):344–8.
57. Shapiro BS, Daneshmand ST, Garner FC, Aguirre M, Hudson C, Thomas S. Evidence of impaired endometrial receptivity after ovarian stimulation for in vitro fertilization: A prospective randomized trial comparing fresh and frozen-thawed embryo transfers in high responders. *Fertil Steril* [Comparative Study Randomized Controlled Trial Research Support, Non-U.S. Gov't]. 2011;96(2):516–8.
58. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA*. 1977;74(12):5463–7.
59. Cobo A, Garrido N, Pellicer A, Remohi J. Six years' experience in ovum donation using vitrified oocytes: Report of cumulative outcomes, impact of storage time, and development of a predictive model for oocyte survival rate. *Fertil Steril* 2015;104(6):1426–34 e8.
60. Venetis CA, Kolibianakis EM, Bosdou JK, Tarlatzis BC. Progesterone elevation and probability of pregnancy after IVF: A systematic review and meta-analysis of over 60 000 cycles. *Hum Reprod Update* [Meta-Analysis]. 2013;19(5):433–57.

61. Drakopoulos P, Blockeel C, Stoop D, Camus M, de Vos M, Tournaye H et al. Conventional ovarian stimulation and single embryo transfer for IVF/ICSI. How many oocytes do we need to maximize cumulative live birth rates after utilization of all fresh and frozen embryos? *Hum Reprod* 2016;31(2):370–6.
62. Dondorp W, de Wert G, Pennings G, Shenfield F, Devroey P, Tarlatzis B et al. Oocyte cryopreservation for age-related fertility loss. *Hum Reprod* [Research Support, Non-U.S. Gov't]. 2012;27(5):1231–7.
63. The Practice Committees of the American Society for Reproductive Medicine and the Society for Assisted Reproductive Technology. Mature oocyte cryopreservation: A guideline. *Fertil Steril*. 2013;99(1):37–43.
64. Kuleshova L, Gianaroli L, Magli C, Ferraretti A, Trounson A. Birth following vitrification of a small number of human oocytes: Case report. *Hum Reprod*. 1999;14(12):3077–9.
65. Yoon TK, Chung HM, Lim JM, Han SY, Ko JJ, Cha KY. Pregnancy and delivery of healthy infants developed from vitrified oocytes in a stimulated in vitro fertilization-embryo transfer program. *Fertil Steril*. 2000;74(1):180–1.
66. Yoon TK, Kim TJ, Park SE, Hong SW, Ko JJ, Chung HM et al. Live births after vitrification of oocytes in a stimulated in vitro fertilization-embryo transfer program. *Fertil Steril* [Research Support, Non-U.S. Gov't]. 2003;79(6):1323–6.
67. Katayama KP, Stehlik J, Kuwayama M, Kato O, Stehlik E. High survival rate of vitrified human oocytes results in clinical pregnancy. *Fertil Steril*. 2003;80(1):223–4.
68. Kyono K, Nakajo Y, Kumagai S, Kyono K. Successful pregnancy and delivery after the transfer of a single blastocyst derived from a vitrified mature human oocyte. *Fertil Steril*. 2005;84(Suppl.1): S172.
69. Kuwayama M, Vajta G, Kato O, Leibo SP. Highly efficient vitrification method for cryopreservation of human oocytes. *Reprod Biomed Online*. 2005;11(3):300–8.
70. Yoon TK, Lee DR, Cha SK, Chung HM, Lee WS, Cha KY. Survival rate of human oocytes and pregnancy outcome after vitrification using slush nitrogen in assisted reproductive technologies. *Fertil Steril*. 2007;88(4):952–6.
71. Selman H, Angelini A, Barnocchi N, Brusco GF, Pacchiarotti A, Aragona C. Ongoing pregnancies after vitrification of human oocytes using a combined solution of ethylene glycol and dimethyl sulfoxide. *Fertil Steril*. 2006;86(4):997–1000.
72. Antinori M, Licata E, Dani G, Cerusico F, Versaci C, Antinori S. Cryotop vitrification of human oocytes results in high survival rate and healthy deliveries. *Reprod Biomed Online*. 2007;14(1):72–9.
73. Lucena E, Bernal DP, Lucena C, Rojas A, Moran A, Lucena A. Successful ongoing pregnancies after vitrification of oocytes. *Fertil Steril*. 2006;85(1):108–11.
74. Cobo A, Domingo J, Perez S, Crespo J, Remohi J, Pellicer A. Vitrification: An effective new approach to oocyte banking and preserving fertility in cancer patients. *Clin Transl Oncol*. 2008;10(5):268–73.
75. Chang CC, Shapiro DB, Bernal DP, Wright G, Kort HI, Nagy ZP. Human oocyte vitrification: In-vivo and in-vitro maturation outcomes. *Reprod Biomed Online*. 2008;17(5):684–8.
76. Sher G, Keskintepe L, Mukaida T, Keskintepe M, Ginsburg M, Agca Y et al. Selective vitrification of euploid oocytes markedly improves survival, fertilization and pregnancy-generating potential. *Reprod Biomed Online*. 2008;17(4):524–9.
77. Chian RC, Gilbert L, Huang JY, Demirtas E, Holzer H, Benjamin A et al. Live birth after vitrification of in vitro matured human oocytes. *Fertil Steril*. 2009;91(2):372–6.
78. Nagy ZP, Chang CC, Shapiro DB, Bernal DP, Elsner CW, Mitchell-Leef D et al. Clinical evaluation of the efficiency of an oocyte donation program using egg cryo-banking. *Fertil Steril* [Clinical Trial Research Support, Non-U.S. Gov't]. 2009;92(2):520–6.
79. Cao YX, Xing Q, Li L, Cong L, Zhang ZG, Wei ZL et al. Comparison of survival and embryonic development in human oocytes cryopreserved by slow-freezing and vitrification. *Fertil Steril* [Comparative Study Evaluation Studies]. 2009;92(4):1306–11.
80. Schoolcraft WB, Keller JL, Schlenker T. Excellent embryo quality obtained from vitrified oocytes. *Reprod Biomed Online*. 2009;19(6):820–3.
81. Fadini R, Brambillasca F, Renzini MM, Merola M, Comi R, De Ponti E et al. Human oocyte cryopreservation: Comparison between slow and ultrarapid methods. *Reprod Biomed Online*. [Comparative Study]. 2009;19(2):171–80.
82. Grifo JA, Noyes N. Delivery rate using cryopreserved oocytes is comparable to conventional in vitro fertilization using fresh oocytes: Potential fertility preservation for female cancer patients. *Fertil Steril*. 2010;93(2):391–6.

83. Rienzi L, Romano S, Albricci L, Maggiulli R, Capalbo A, Baroni E et al. Embryo development of fresh 'versus' vitrified metaphase II oocytes after ICSI: A prospective randomized sibling-oocyte study. *Hum Reprod* [Randomized Controlled Trial]. 2010;25(1):66–73.
84. Kim TJ, Laufer LR, Hong SW. Vitrification of oocytes produces high pregnancy rates when carried out in fertile women. *Fertil Steril* [Research Support, Non-U.S. Gov't]. 2010;93(2):467–74.
85. Almodin CG, Minguetti-Camara VC, Paixao CL, Pereira PC. Embryo development and gestation using fresh and vitrified oocytes. *Hum Reprod* [Comparative Study Evaluation Studies]. 2010;25(5):1192–8.
86. Ubaldi F, Anniballo R, Romano S, Baroni E, Albricci L, Colamaria S et al. Cumulative ongoing pregnancy rate achieved with oocyte vitrification and cleavage stage transfer without embryo selection in a standard infertility program. *Hum Reprod*. 2010;25(5):1199–205.
87. Smith GD, Serafini PC, Fioravanti J, Yadid I, Coslovsky M, Hassun P et al. Prospective randomized comparison of human oocyte cryopreservation with slow-rate freezing or vitrification. *Fertil Steril* [Comparative Study Randomized Controlled Trial]. 2010;94(6):2088–95.
88. Trokoudes KM, Pavlides C, Zhang X. Comparison outcome of fresh and vitrified donor oocytes in an egg-sharing donation program. *Fertil Steril*. 2011;95(6):1996–2000.
89. Garcia JI, Noriega-Portella L, Noriega-Hoces L. Efficacy of oocyte vitrification combined with blastocyst stage transfer in an egg donation program. *Hum Reprod*. 2011;26(4):782–90.
90. Paffoni A, Guarneri C, Ferrari S, Restelli L, Nicolosi AE, Scarduelli C et al. Effects of two vitrification protocols on the developmental potential of human mature oocytes. *Reprod Biomed Online*. 2011.
91. Cobo A, Castellò D, Viloria T, Campos P, Vallejo B, Remohí J. Vitrification of human oocytes in media containing Hydroxypropylcellulose (HPC), a non-protein supplement for cryopreservation media. *Hum Reprod*. 2012;(Suppl. ESHRE 28th Annual Meeting 2012):146.
92. Garcia-Velasco JA, Domingo J, Cobo A, Martinez M, Carmona L, Pellicer A. Five years' experience using oocyte vitrification to preserve fertility for medical and nonmedical indications. *Fertil Steril*. [Multicenter Study Research Support, Non-U.S. Gov't]. 2013;99(7):1994–9.
93. Chang CC, Elliott TA, Wright G, Shapiro DB, Toledo AA, Nagy ZP. Prospective controlled study to evaluate laboratory and clinical outcomes of oocyte vitrification obtained in in vitro fertilization patients aged 30 to 39 years. *Fertil Steril*. 2013;99(7):1891–7.
94. Sole M, Santalo J, Boada M, Clua E, Rodriguez I, Martinez F et al. How does vitrification affect oocyte viability in oocyte donation cycles? A prospective study to compare outcomes achieved with fresh versus vitrified sibling oocytes. *Hum Reprod*. 2013;28(8):2087–92.
95. Siano L, Engmann L, Nulsen J, Benadiva C. A prospective pilot study comparing fertilization and embryo development between fresh and vitrified sibling oocytes. *Conn Med* [Comparative Study Randomized Controlled Trial]. 2013;77(4):211–7.
96. Cobo A, Remohi J, Chang CC, Nagy ZP. Oocyte cryopreservation for donor egg banking. *Reprod Biomed Online*. 2011;23(3):341–6.
97. Rienzi L, Cobo A, Paffoni A, Scarduelli C, Capalbo A, Vajta G et al. Consistent and predictable delivery rates after oocyte vitrification: An observational longitudinal cohort multicentric study. *Hum Reprod* [Multicenter Study]. 2012;27(6):1606–12.
98. Herrero L, Pareja S, Losada C, Cobo AC, Pellicer A, Garcia-Velasco JA. Avoiding the use of human chorionic gonadotropin combined with oocyte vitrification and GnRH agonist triggering versus coasting: A new strategy to avoid ovarian hyperstimulation syndrome. *Fertil Steril*. 2011;95(3):1137–40.
99. Cobo A, Garrido N, Crespo J, Jose R, Pellicer A. Accumulation of oocytes: A new strategy for managing low-responder patients. *Reprod Biomed Online* [Controlled Clinical Trial]. 2012;24(4):424–32.
100. Milan M, Cobo AC, Rodrigo L, Mateu E, Mercader A, Buendia P et al. Redefining advanced maternal age as an indication for preimplantation genetic screening. *Reprod Biomed Online*. 2010;21(5):649–57.
101. Rodrigo L, Mateu E, Mercader A, Cobo AC, Peinado V, Milan M et al. New tools for embryo selection: Comprehensive chromosome screening by array comparative genomic hybridization. *Biomed Res Int*. 2014;2014: 517125.
102. Cobo A, Garcia-Velasco JA, Coello A, Domingo J, Pellicer A, Remohi J. Oocyte vitrification as an efficient option for elective fertility preservation. *Fertil Steril* [Multicenter Study Observational Study]. 2016;105(3):755–64 e8.

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18

Quality Control and Quality Assurance in the IVF Laboratory

David Mortimer and Sharon T. Mortimer

Introduction

It is a common misconception that quality control (QC) and quality assurance (QA) are simply interchangeable terms. In fact, QC and QA are distinct elements within a comprehensive systems approach to quality management—an approach that is critical in any modern IVF laboratory that is truly concerned about doing the best for its patients.

QC is the most basic level of quality management, concerned with controlling the quality of a product (or service) by identifying problems or defects. In the simplest terms it involves inspecting, testing, or checking to make sure the product meets expectations—which means that these expectations must be defined. QC is typically done at the end of the process (e.g., at the end of a production line, when a service is delivered, or after a test has been performed). If the product does not meet expectations, then it is considered to be a non-conforming product, and a "non-conformity" is declared, meaning that the product is rejected.

QA evolved from the realization that quality can be improved by looking "upstream," and is therefore aimed at preventing non-conformities from occurring. So while QA has QC at its core, it goes beyond the simple rejection of a product to consider all related activities or processes that might cause defects downstream, such as raw materials or other inputs, operator training, standard operating procedures, document control, and audits.

Nowadays, the emphasis on quality has widened to include systems thinking and management systems: Quality Management is a much broader philosophy that includes quality planning, not just QC and QA, but also Quality Improvement (QI), and extends even further into a systems approach. As a result, the overarching Quality Management System (QMS) considers all the various elements of the entire system. Within the ISO 9001 model, the QMS comprises five main aspects:

- The overall system and its component processes
- Management responsibility
- Resource management (physical, human, financial)
- Management of the service or product realization processes (each process that generates an output)
- Management of measurement, analysis, and improvement

For medical laboratories the principal international standard is ISO 15189:2012 *Medical laboratories—Requirements for quality and competence*. Only a small number of IVF laboratories are accredited to this standard, due to there being very few organizations capable of applying the standard to such specialized clinical service laboratories. However, a quick look at ISO 15189 and any accreditation scheme's standards will reveal close similarities in their expectations. For this reason, over the past two decades we have promoted the application of the principles of Total Quality Management (TQM) as a generalized philosophical basis for quality management in IVF laboratories (1).

TQM is founded on the three pillars of quality management, risk management, and systems management. Often quality management and risk management are two sides of the same coin, as revealed by the close similarity between Failure Modes and Effects Analysis (FMEA), the pro-active quality/risk management tool, and Root Cause Analysis (RCA), the retrospective troubleshooting tool. In any system, planning for quality must include planning to reduce risk in all its forms. For example, since the intention of QA is to improve quality by reducing the likelihood of a process going wrong, then it follows that we must minimize the risk of errors or faults occurring in the first place. Applying this combined type of approach requires a sound understanding of all of the aspects of a process; not just what the output is supposed to be, but what the inputs need to be to support a quality product, and also what factors control or influence the actual realization process itself.

Quality Management of the IVF Process

At the simplest level, the "IVF Laboratory" itself can be considered as a process (Table 18.1), but analyzing the IVF realization process step (where fertilization occurs in vitro) is confounded by the sheer number of component subprocesses and actions (process elements) that it includes. Achieving a full understanding of—and hence the ability to control, monitor, and improve—the realization process to increase quality and reduce risk, requires delving progressively deeper into the details to identify each single step individually. This is best achieved using one or more process mapping techniques.

When trying to understand what it takes to improve the quality of the IVF laboratory, we have found the most effective approach is to remember that everything that happens (or, perhaps more correctly, that *needs* to happen) in IVF is governed by the biology of the gametes and embryos.

Therefore, "all" that we need to do is provide optimal conditions for the gametes and embryos, and protect them from physiological stress and from adverse external factors. While it is certainly true that embryos are highly adaptable, any "adaptation" (e.g., to varying culture media formulations) costs energy—and this constitutes physiological stress (2). It is a general principle in cell culture that cellular stress can alter gene expression and/or regulation, and the untimely expression of heat shock proteins, for example, by an embryo would constitute a major metabolic stress. Suboptimal culture of

TABLE 18.1

A Simplified Overview of the IVF Laboratory as a Process

Process Step	Control Areas	Factors to be Controlled	Specific Requirements
Inputs	Gametes	Ovarian stimulation	Nuclear and cytoplasmic maturation
		Oocyte retrieval	Suction pressure, temperature
		Sperm collection and processing	Temperature, prevention of iatrogenic damage, senescence
	Contact materials	Oocyte retrieval needles	Bore, sterility, endotoxins
		Semen collection jars	Sterility, non-toxicity
		Plastic culture tubes & dishes	Sterility, non-toxicity
		Culture media	Formulations, sterility, endotoxins
Realization	Lab. environment	Air quality	Particulates & microbes
			Volatile organic compounds (VOCs)
		Light	Spectral sensitivity
	Culture system	Incubators	Temperature, pCO_2, pO_2, humidity, VOCs, calibration, malfunction
	Lab. processes	Methodology	Selection, SOPs
		Personnel	Training, skill, competence
		Insemination	Washed sperm concentration
		Handling and assessment conditions	Temperature, medium pH, and osmolarity
Outputs		Zygotes, embryos, blastocysts (depending on the time line)	Developmental competence

early cleavage stage mouse embryos has even been reported to adversely affect the behavior of resulting adult mice (3).

The importance of identifying, and thereby being able to minimize, chemical and physical factors that exert adverse effects on mammalian embryos during the practice of human IVF was recently emphasized by Wale and Gardner (4), who concluded that optimizing the in vitro environment involves far more than just considering culture media formulations. Consequently, embryologists must focus on the biochemical and biophysical requirements that govern all IVF laboratory procedures, as well as the design of the laboratory, the HVAC (heating, ventilation, and air conditioning) system, the engineering of the laboratory equipment, and the contact materials used—and use these principles to drive the laboratory's TQM system.

An IVF laboratory TQM system can be summarized as knowing and understanding what the *biology* needs (and hence what the *chemistry* and *physics* must be), and then using this knowledge to

- Define the requirements for the proper/safe handling of gametes and embryos (through *process design* and *optimization,* including *QC*)
- Elaborate and refine culture media formulations that are optimized for both gametes and embryos (human, not animal models) as appropriate
- *Engineer*—and *calibrate* and *maintain*—equipment to maintain the correct biophysical and biochemical conditions for each step of the process
- *Design* and *engineer* laboratory facilities to eliminate (or at least minimize as far as possible) all conditions or factors that could adversely affect gametes or embryos

But this is just the beginning: quality management is not a "one-off" activity. Having put systems in place they must be monitored continuously to ensure that they achieve—and continue to achieve—their intended purpose. Hence the final key element of an IVF laboratory QMS must be

- To *monitor* each process (and piece of equipment that performs a critical function) using *indicators* and relate them to *benchmarks* (i.e., *QA* and *QI*).

Indicators and Benchmarks

It is a basic tenet of quality management that one cannot control something unless it can be measured. To do this we need a series of *indicators*, with each measuring the outcome of a specific process or process element. Because there are so many process elements, we routinely select ones that are key to ensuring the overall process continues to function as expected, hence the term *Key Performance Indicators* or "KPIs."

Defining a KPI requires that it be

- Reliable—it must measure something that is useful, requiring definition of the exact process that is to be monitored
- Robust—extraneous effects must be minimized so that it measures only the intended process
- Routine— data collection must not involve a lot of extra work

If deriving KPIs involves too much extra effort, then there is a grave risk of them falling by the wayside; this is an excellent example of the old adage "work smarter not harder," and illustrates the crucial role of having a comprehensive and functional information system. Therefore, it is important to ensure that the KPIs selected can be measured as part of the normal daily routine of the laboratory.

On a regular basis KPIs are plotted on a Shewhart *control chart*, using the laboratory's own historical data to define the expected range of results (Figure 18.1). Once a laboratory has implemented its systems, then a series of, say, 6 monthly values for a KPI are collected and their mean and standard deviation (SD) calculated. Future values for the KPI should be expected to vary around the *control*

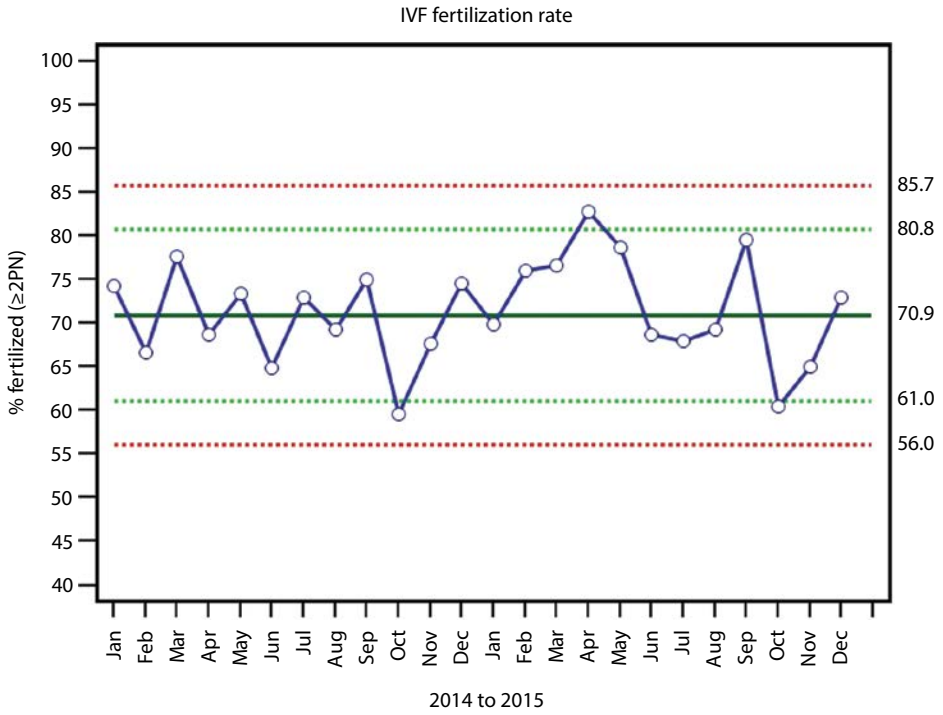

FIGURE 18.1 An example Shewhart Control Chart showing a laboratory's IVF fertilization rate (% fertilized oocytes with ≥2PNs) over the period 2014 through 2015. The control mean (70.9%) is based on data from the previous 6 months (July–December 2013); upper and lower warning limits are derived as the control mean ± 2SDs (95% range: 61.0%–81.8%); and upper and lower control limits are derived as the control mean ± 3SDs (99% range: 56.0%–85.7%). The figure was generated using MedCalc (www.medcalc.be).

mean, ideally staying within the *upper* and *lower warning limits* (i.e., the control mean ± 2 SDs, effectively the 95% range of historical performance). Deviations beyond the warning limit in the adverse direction should be investigated, as should three consecutive shifts in the adverse direction, to identify possible adverse influences or changes. If a KPI value deviates beyond the adverse *control limit* (calculated as the control mean ± 3 SDs, effectively the 99% range of historical performance), then careful investigation is mandatory. Deviations in the "good" direction are of far less concern—indeed, they are hoped for, since a systematic shift in that direction signifies an improvement in the process being monitored, and once this has been established the control mean and ranges should be recalculated so as to monitor the improved process into the future. At the same time, this is a good opportunity to "progress build" (rather than troubleshoot), to identify what has changed within the system that led to this improvement.

The other application of KPIs is to compare them against *benchmarks*. *Benchmarking* is the process—ideally continuous—of measuring performance against some external reference. *Internal benchmarking* might compare performance between units within a group or network, while *competitive benchmarking* would compare performance against the direct competition; *functional benchmarking* would allow comparison with national or world leaders, and *generic benchmarking* would use published consensus benchmarks as the reference.

Assuming that consensus benchmarks are derived from real world, ideally published, data, then all forms of benchmarking are expressions of the "proof of the possible." Unfortunately, there is a dearth of published benchmarks for the IVF laboratory. There was a set in the original *Instructions for Use* of the *Sydney IVF* sequential culture media system published by Cook (Nine Miles Plain, Qld, Australia), which were derived as rounded-down averages of the KPI values being achieved at Sydney IVF in late 1998–early 1999 (Table 18.2). However, the Sydney IVF culture system included not just the media but

TABLE 18.2

Example Benchmarks for IVF Laboratory Processes and Clinical Outcomes

Benchmark		Cook *Sydney IVF* System	Oozoa Biomedical Suggested
Oocytes	MII at stripping for ICSI	≥65%	≥80% (≥85%[a])
	GV at stripping for ICSI		<5%
Fertilization	IVF (as % of COCs inseminated)		>70%
	ICSI as % of oocytes injected		70%–75%
ICSI	Oocyte damage rate (% of oocytes injected)		<5% on Day 0 <10% on Day 1
Cleavage rate	2PNs that have cleaved on Day 2	≥95%	>95%
Early cleavage	Zygotes to 2-cell by 30 h p-i	50%–65%	>50%
	2-Cell to 4-cell by 42 h p-i	>75%	>75%
Embryo quality	High quality 4-cell embryos on Day 2		>45%
	High quality 8-cell embryos on Day 3		>40%[a]
	≥2 × 8-cell high quality embryos on Day 3		>50%[a]
Embryo development	Zygotes to blastocyst on Day 5	75%	>65%[a]
	Cycles with ≥1 blastocyst on day 5	90%	90%
	Utilization rate (embryos suitable for transfer or freezing on day of ET)		60% on Day 3 45% on Day 5
Embryo freezing	Cycles with embryos for freezing		40%[a]
Implantation	Fetal sac per Day 3 embryo transferred	≥25%	35%[a]
	Fetal sac per Day 5 blastocyst transferred		45%[a]
Clinical pregnancy rate	Day 3 ETs: women <37 yrs 2 embryos/ET	40%	Routine Day 5 ETs
	Day 5 ETs: women <36 yrs 1 blastocyst/ET		45%

Abbreviations: COC, cumulus-oocyte-complex; ET, embryo transfer; GV, germinal vesicle stage oocyte; MI, metaphase II; p-i, post-insemination.

[a] Denotes values for women aged <38 years.

also the use of the Cook MINC benchtop incubator with low pO_2 pre-mixed gas (as well as tube warmers and controlled environment workstations). At that time very few laboratories were using such incubators, and many were using ambient oxygen levels, with the result that the benchmarks were seen as unrealistic by many laboratories, and so were not included in later versions of the *Instructions for Use*.

Over the past 15 years we have evolved our own set of benchmarks (Table 18.2), and have assisted other laboratories and organizations to develop their own. At the Alpha congress in Antalya in May 2014 it was proposed that the development of an international set of benchmarks should be the subject of Alpha's fourth international consensus meeting, which took place in September 2016.

It is quite common to use reference groups to support meaningful comparison of results between laboratories or clinics. This might be achieved using a simple age qualifier, as for some of the values in Table 18.2 (women <38 years of age), or it could be a more complex description to identify a group of patients with "good prognosis" (Table 18.3).

Quality Control of the IVF Laboratory Environment

In terms of the actual IVF laboratory facility, critical parameters that should be monitored are primarily related to air quality, and obviously therefore depend on the standard to which the laboratory was designed and built. We typically specify that a clinical embryology laboratory be designed to achieve ISO Class 6 air quality (max. 102,000 particles ≥0.3 µm per m^3 and max. 35,200 particles ≥0.5 µm per m^3; equivalent to a US Federal Standard 209 Class 1000 clean room). This is with the purpose of making it easy to achieve and maintain ISO Class 7 on an ongoing basis—the equivalent to a typical operating theatre—(max. 352,000 particles ≥0.5 µm per m^3; equivalent to a US Federal Standard 209 Class 10,000

TABLE 18.3

An Example of Benchmarking Using a Reference Group

Benchmark	Data	Value
Pregnancies (ß-hCG positive)	67/105 cycles	64%
Clinical pregnancy rate	63/105 (≥1 fetal sac)	60%
Ongoing pregnancy rate	58/105 (≥1 fetal heart)	55%
Implantation rate	88 sacs from 210 embryos	42%
Multiple pregnancy rate	25/63 pregnancies	40% (all twins)

Note: Unpublished data from Dr ST Mortimer, Genesis Fertility Centre (Vancouver, Canada) for the calendar year 2002: based on women <37 years, first IVF cycle, 2 × 8-cell embryos per Day 3 ET.

clean room and EU Good Manufacturing Practice Grade B "in operation"/Grade C "at rest" air quality). Additional testing for airborne microbes is also needed, with an expectation of <10 cfu/m^3 in an air sample, and <5 cfu/4 h for a 90 mm diameter settle plate. Lastly—but probably most importantly—there also needs to be regular measurement of volatile organic compounds (VOCs), with total VOC levels (TVOCs) being below 100 ppb (5–7).

Regular measurements of air quality need to be made using a particle counter and TVOC analyzer, and the results logged and reported to the center's Quality Manual.

Clean room design also includes consideration of the number of air changes per hour (ACH) which, if too high (as might be expected in a typical operating theatre) can cause excessive cooling risk for the follicular fluid aspirated during oocyte retrievals (8). It is generally better to have the procedure room and IVF laboratory at a comfortable working temperature, rather than excessively warm in a misguided attempt to reduce the cooling risk to gametes and embryos (since the metaphase spindle of the MII oocyte undergoes depolymerization below 35°C [9,10]). Effective strategies for maintaining oocyte temperature are easily achieved without inflicting uncomfortable working conditions on everyone. Room temperature, and ideally also relative humidity, must also be measured and logged as part of ongoing quality management. Because there are effective control systems for the culture micro-environment, laboratory conditions should be comfortable for the embryologists—warm, humid rooms are uncomfortable to work in and will lead to distracted embryologists and to people rushing to finish work to get into a cooler, more pleasant environment. What is comfortable varies around the world, but around 22°C and 40% RH would be suitable conditions for many places.

Quality Control of IVF Laboratory Equipment

During the procurement of an item of equipment, the user (typically the IVF Laboratory Director) prepares a list of functional and operational specifications/selection criteria for the item, basically defining its suitability for purpose. Ideally, each manufacturer's *Design Qualification* (DQ), which describes the item's intended purpose, are then compared against these specifications to identify suitable contenders for further consideration. Unfortunately, all too often in IVF laboratories people just buy the same thing they were trained on, or "saw in so-and-so's lab," rather than undertaking this type of careful evaluation of how well a particular piece of equipment is suited to its intended purpose in their laboratory.

For example, many IVF laboratories still work in laminar flow cabinets where temperature control and pCO_2 control are far inferior to controlled environment "IVF Chamber" type workstations (see Reference 1 and Figure 18.2). Moreover, the use of tube warmers to keep follicular aspirates warm during egg retrieval procedures is gaining acceptance, but it has been a long time coming, considering how long we have known that cooling oocytes results in meiotic spindle depolymerization (11,12).

Once a piece of equipment has been delivered, an *Installation Qualification* (IQ) is performed by certified engineers (manufacturer or installation contractor) when it is first installed to ensure that it is operating in accordance with its design specifications. An *Operational Qualification* (OQ) is then performed

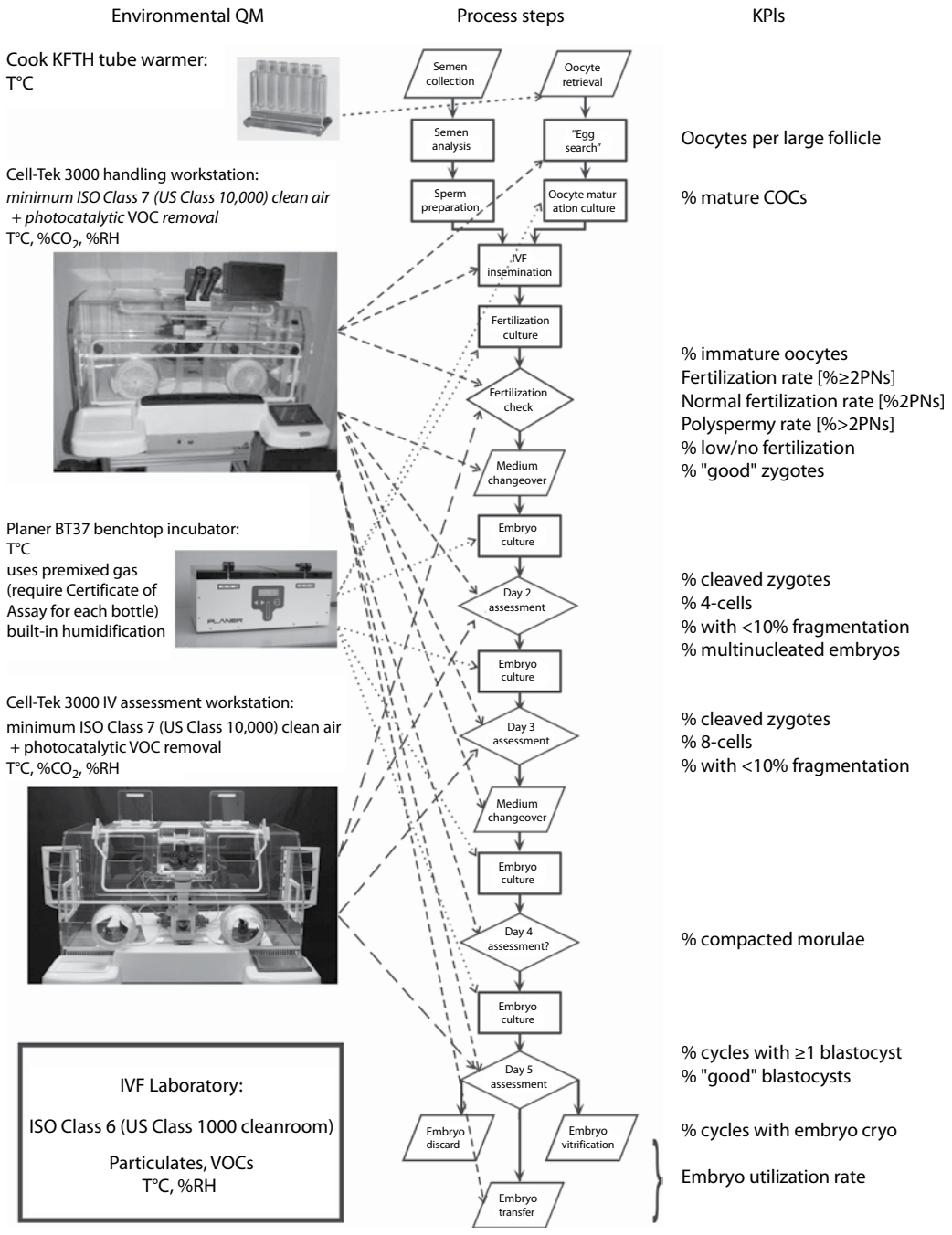

FIGURE 18.2 This figure summarizes many of the aspects of a modern IVF Laboratory's quality management system, integrating control of the laboratory environment, environmental control for the various laboratory processes, and monitoring processes and outcomes via KPIs. Cook K-FTH-1012 tube warmer from Cook Medical (Bloomington, IN, USA; www.cookmedical.com/products/wh_fth_webds/); Cell-Tek IVF workstations from Tek-Event (Round Corner, NSW, Australia; www.tekevent.com); BT37 benchtop incubator from Planer (Sunbury-on-Thames, UK; www.planer.com/products/incubators.html).

by certified engineers on a regular basis (usually annual, sometimes biannual or biennial) to establish that equipment is operating in accordance with its design specifications. Finally, an *Operational Verification*, which can be performed by a member of staff using appropriate calibrated instruments, is undertaken to verify that equipment continues to function within its required operational parameters. This is then repeated as often as the user considers necessary to be confident that the item continues to function according to defined requirements.

Routine preventative maintenance and servicing of equipment is referred to as (scheduled) *Repeat Operational Qualification* (ROQ), and must be performed by qualified engineers. If a piece of equipment is repaired (perforce by qualified engineers), then it must be returned with a report documenting its *Re-Qualification* (RQ)—including evidence such as certificates of calibration of the test equipment used during the RQ process.

An alternative to labor intensive Operational Verifications is the use of real-time monitoring systems that allow not just monitoring for alarm conditions but also provide regular logging (e.g., every 5 minutes) of critical operational parameters via separate sensors that are independent of the equipment itself (1,13), e.g., Planer's *ReAssure* system (see http://planer.com/planer-reassure.html).

Quality Control of IVF Contact Materials

Given the quality of contact materials now available for IVF use, with extensive manufacturer testing for sperm and embryo toxicity, combined with quality certifications such as CE marking, the case for in-house testing using sperm survival studies or mouse embryo assays (MEA) would seem to be hard to justify. This is especially so when one considers that it is not just a simple matter of running a test on some contact material(s) using some "home grown" mouse zygotes or "spare" "normal" patient sperm: to have validity within a formal QMS, in-house biocompatibility QC assays must themselves include comprehensive QC practices, including positive and negative controls. Consequently the cost of operating such a QC system becomes substantial, and very hard to justify—particularly in terms of cost-effectiveness—against a regulatory background of required manufacturer QC testing. For more than 15 years it has been our personal decision to purchase all contact materials only from trusted manufacturers/suppliers, and not operate in-house QC biocompatibility testing.

This position can certainly be supported for oocyte retrieval needles and embryo transfer catheters, and, by choosing to buy quality products in terms of plastic culture ware (e.g., BD Falcon "IVF" products, SparMED's *Oosafe*® range, Vitrolife *Labware* dishes, etc.), as well as handling devices such as MEA-tested glass Pasteur pipettes, "*Stripper*" or "*Flexipet*" (or similar) handling devices, and CE-marked/FDA-approved culture media, a laboratory can avoid substantial unnecessary costs. Probably the main products still tested by IVF laboratories are gloves—which, strictly speaking, are not actually contact materials. Consequently, it is our opinion that, today, this aspect of IVF laboratory QC can almost entirely rely on manufacturer testing.

Quality Control of IVF Laboratory Systems

KPIs are essential for evaluating the introduction of a technique or process, as minimum standards for proficiency, for monitoring ongoing performance within a QMS (for both IQC and EQA purposes), for benchmarking, and for quality improvement. However, for a KPI to have real value, it must fully and carefully define the biological or technical process that is to be monitored, identify the specific endpoint of interest for that process, identify relevant qualifiers (e.g., female partner's age), and identify potential confounders (including biological factors, clinical practices, and patient factors). It is also essential that the KPI's definition specifies the exact data to be collected, as well as how the KPI value is derived (calculation formulae, etc.).

The following list covers a reasonably comprehensive panel of KPIs for routine QC/QA of an ART program (i.e., covering clinical outcomes), and its laboratory, as well as some general operational KPIs for the laboratory services and the clinic as a whole (1,14).

Program KPIs

These should be broken down by female patient's age and procedure type (e.g., IVF, intracytoplasmic sperm injection [ICSI]), as well as perhaps for general patient type, e.g., infertility cases, oocyte donor cases, and PGD/S cases.

Pregnancy rates:

- Biochemical pregnancy rate (positive β-hCG)
- Clinical pregnancy rate (CPR: fetal sac at 7-week ultrasound)
- Ongoing pregnancy rate (OPR: fetal heart at 7-week ultrasound)
- Early pregnancy loss rate (EPLR: proportion of cases with positive β-hCG that fail to show a fetal sac).

Implantation rates: Calculated as the (total number of fetal sacs seen at 7-week ultrasound)÷(total number of embryos transferred to all patients in that age group and procedure type).

Multiple pregnancy rate: The proportion of pregnancies with >1 fetal sac at 7-week ultrasound.

Laboratory KPIs

Note that oocyte and embryo evaluations provide measures that are referred to as "grades": we strongly recommend not using the work "quality" as it can be very misleading, and be misunderstood by others.

- *Oocyte grade and/or maturity:* Although not actually a KPI of laboratory performance, this provides a description of the "raw material."
- *IVF fertilization rate:* Proportion of inseminated oocytes with ≥2PN the day after insemination; the proportion with 2PN can also be reported as the *IVF normal fertilization rate*, and those with >2PN as the *IVF polyspermy rate*.
- *ICSI fertilization rate:* Proportion of injected oocytes with 2PN the day after injection; this must be accompanied by the *ICSI damage rate*, calculated as the proportion of injected oocytes which degenerate during stripping, during microinjection, or immediately following the injection procedure (i.e., that are seen at the fertilization check). Ideally the ICSI damage rate should be available as the overall rate and as the three component elements.
- *Poor* and *failed fertilization rates:* Proportion of cycles in which <25% of inseminated/injected oocytes are fertilized, and in which no oocytes are fertilized.
- *Zygote grade.*
- *Cleavage rate:* Proportion of zygotes which cleave to become embryos.
- *Embryo development rates:* Proportions of cleaved embryos which are at
  - The 4-cell stage 2 days after insemination
  - The 8-cell stage 3 days after insemination
  - Good* blastocysts 5 days after insemination
- *Embryo fragmentation rate:* e.g., Proportion of Day 3 embryos with <5% fragmentation.
- *Embryo score or "grade":* e.g., Proportion of Day 3 embryos with high scores.
- *Embryo utilization rate:* Proportion of cleaved embryos which were transferred or cryopreserved, or at least in certain cases were deemed to be suitable for cryopreservation but might have been discarded electively.
- *Embryo cryosurvival rates:* See the Alpha consensus on these KPIs (18).

Laboratory Operations KPIs

- Number of each type of procedure performed each week or month
- Equipment malfunction reports

* For example, defined as "3BB" (15) or "2,11" (16,17).

- Equipment performance (e.g., amount of LN2 required to top up a storage dewar; amount of CO_2 or pre-mixed gas used by each incubator; incubator temperature; $\%CO_2$/$\%O_2$/humidity measurements)
- Rate of utilization of consumables

Efficiency KPIs

Can be used for the laboratory or other areas of clinic operations.

- Number of tests handled by individual operators
- Time lag between receipt of an inquiry and the response
- Proportion of patient records which are complete
- Number of telephone calls answered by a person, rather than by voicemail
- Average delay between completion of a test and the publication of results

Best Practice KPIs

- Nonconformity reports
- Treatment complications
- Infection and accident reports
- Number of comments received per month (both positive and negative)

Financial KPIs

- Comparison of service fees with those of other centers
- Comparison of the cost of performing a procedure and the revenue it generates
- Accounts payable and accounts receivable balances
- Number of patient referrals per month

Figure 18.2 summarizes many of the aspects of a modern IVF laboratory's quality management system, integrating control of the laboratory environment, environmental control for the various laboratory processes, and monitoring processes and outcomes via KPIs.

Assessing Competency

There is often confusion in what a "benchmark" is. For some a benchmark is a "minimum standard," while for others it is—more correctly—an "aspirational goal" defining what they would like to achieve. *Minimum performance values* define criteria for basic competency: if a laboratory cannot achieve at least this result for something, then it should not be doing it, should stop doing it, or should be stopped from doing it. *Aspirational goals*, on the other hand, define best practice, i.e., what a laboratory would like to achieve—although such targets must always be realistic. Clearly for quality improvement purposes the aspirational goal sense is the important one, but the minimum standard sense can be used when troubleshooting or reviewing a laboratory for performance improvement opportunities. In the Alpha consensus on oocyte and embryo cryopreservation, both values are provided for each stage being cryopreserved, and separately for slow freezing and vitrification (18).

If an extensive set of KPIs and benchmarks were to be available for all aspects of the IVF laboratory's processes then they could be used as objective definitions of what "competency" should be when training new embryologists—or for ensuring ongoing maintenance of competency ("MoComp") for experienced embryologists, e.g., for ongoing QA purposes or when returning from maternity leave. This need was clearly identified during the *Alpha Consensus on the Professional Status of the Clinical Embryologist* held in Antalya in May 2014 (19).

Conclusions

This overview of QC and QA in the IVF laboratory has demonstrated that quality is a fundamental, core aspect of laboratory management, how laboratory systems need to be designed with quality inherent at the lowest level, and how quality cannot be an after thought or "add-on," like a coat of varnish. A comprehensive QMS is not just crucial for effective day-to-day operational management of the IVF laboratory, it is also critical for creating an environment that facilitates troubleshooting. Furthermore, KPIs and benchmarks should not just be seen as reporting tools for QC and QA, or targets for QI; they can also be used within embryologist training programs, to define, document, and monitor competency as the field of clinical embryology evolves into what Bob Edwards envisioned.

REFERENCES

1. Mortimer ST, Mortimer D. *Quality and Risk Management in the IVF Laboratory*, 2nd ed. Cambridge: Cambridge University Press, 2014.
2. Gardner DK and Wale PL. Analysis of metabolism to select viable human embryos for transfer. *Fertil Steril.* 2013;99:1062–1072.
3. Ecker DJ, Stein P, Xu Z, Williams CJ, Kopf GS, Bilker WB, Abel T, Schultz RM. Long-term effects of culture of preimplantation mouse embryos on behavior. *Proc Nat Acad Sci USA.* 2004;101:1595–1600.
4. Wale PL, Gardner DK. The effects of chemical and physical factors on mammalian embryo culture and their importance for the practice of assisted human reproduction. *Hum Reprod Update,* 2016;22:2–22.
5. Esteves SC, Bento FC. Air quality control in the ART laboratory is a major determinant of IVF success. *Asian J Androl.* 2016;18:496–9.
6. Heitmann RJ, Hill MJ, James AN, Schimmel T, Segars JH, Csokmay JM, Cohen J, Payson MD. Live births achieved via IVF are increased by improvements in air quality and laboratory environment. *Reprod Biomed Online.* 2015;31:364–371.
7. Morbeck DE. Air quality in the assisted reproduction laboratory: A mini-review. *J Assist Reprod Genet.* 2015;32:1019–1024.
8. Mortimer D. A critical assessment of the impact of the European Union Tissues and Cells Directive (2004) on laboratory practices in assisted conception. *Reprod Biomed Online.* 2005;11:162–176.
9. Wang W-H, Meng L, Hackett RJ, Odenbourg R, Keefe DL. Limited recovery of meiotic spindles in living human oocytes after cooling-rewarming observed using polarized light microscopy. *Hum Reprod.* 2001;16:2374–2378.
10. Wang W-H, Meng L, Hackett RJ, Odenbourg R, Keefe DL. Rigorous thermal control during intracytoplasmic sperm injection stabilizes the meiotic spindle and improves fertilization and pregnancy rates. *Fertil Steril.* 2002;77:1274–1277.
11. Pickering SJ, Braude PR, Johnson MH, Cant A, Currie J. Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. *Fertil Steril.* 1990;54:102–108.
12. Almeida PA, Bolton VN. The effect of temperature fluctuations on the cytoskeletal organisation and chromosomal constitution of the human oocyte. *Zygote.* 1995;3:357–365.
13. Mortimer D, Di Berardino T. To alarm or monitor? A cost-benefit analysis comparing laboratory dial-out alarms and a real-time monitoring system. *Alpha Newslett.* 2008;39:7p.
14. Mayer JF, Jones EL, Dowling-Lacey D, Nehchiri F, Muasher S, Gibbons W, Oehninger S. Total quality improvement in the IVF laboratory: Choosing indicators of quality. *Reprod Biomed Online.* 2003;7:695–699.
15. Gardner DK, Schoolcraft WB. In vitro culture of human blastocysts. In *Towards Reproductive Certainty: Fertility and Genetics Beyond*, Jansen R, Mortimer D (Eds.). London: Parthenon Publishing, 1999, 378–388.
16. Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology. The Istanbul consensus workshop on embryo assessment: Proceedings of an expert meeting. *Reprod Biomed Online.* 2011;22:632–646. (simultaneous publication).
17. Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology. The Istanbul consensus workshop on embryo assessment: Proceedings of an expert meeting. *Hum Reprod.* 2011;26:1270–1283. (simultaneous publication).

18. Alpha Scientists in Reproductive Medicine. The Alpha consensus meeting on cryopreservation key performance indicators (KPIs) and benchmarks: Proceedings of an expert meeting. *Reprod Biomed Online*. 2012;25:146–167.
19. Alpha Scientists in Reproductive Medicine. The Alpha consensus meeting on the professional status of the clinical embryologist: Proceedings of an expert meeting. *Reprod Biomed Online*. 2014;30:451–461.

19

Oocyte Donation

Irene Woo and Richard J. Paulson

Introduction

Egg donation is a standard component of care offered by infertility specialists. Initially intended as therapy for women with premature ovarian failure, or those with heritable genetic defects, the use of egg donation has been extended to women with a variety of defects in egg production or function. The annual number of donor oocyte cycles has significantly increased in the past decade; the North American Society for Assisted Reproductive Technology (SART) registry reported 19,847 cycles in 2012 (1). Specifically, the largest group of women now undergoing treatment with egg donation represents those with age-related decline in fertility. Furthermore, techniques learned from egg donation, including endometrial preparation and embryo–endometrial synchrony, have been applied to other infertility treatments, including frozen embryo transfer and oocyte cryopreservation.

History

The first human egg donation was reported in 1983. At the time of ovulation, a fertile egg donor was inseminated with the recipient partner's sperm followed by uterine lavage on the 5th day after the luteinizing hormone (LH) peak. The recovered embryo was then transferred to the infertile patient. The technique resulted in a pregnancy and subsequent delivery of a child genetically similar to the donor (2). Unfortunately, this technique, known as "ovum transfer," was inefficient (3). Attempts to enhance the efficiency of the process by superovulating the donors were unsuccessful and led to retained pregnancies in the donors (4).

The first use of standard in vitro fertilization (IVF) methodology for egg donation was also reported in 1983; however, this pregnancy ended as a miscarriage (5). Subsequently, Lutjen et al. reported a successful pregnancy in a woman with ovarian failure. The donated eggs were fertilized in vitro and the resulting embryos were transferred to the recipient's uterus, which was prepared with a combination of oral estradiol and vaginal progesterone (6). This landmark event substantiated the observation that exogenous estrogen and progesterone could reliably produce a receptive endometrium. In women with residual ovarian function, donor and recipient cycles could thus be synchronized with gonadotropin-releasing hormone (GnRH) agonists (7), and the recipient endometrium stimulated with exogenous steroids (8,9). These essential principles have remained the key components of the current practice of egg donation.

Indications

Egg donation is a therapy which allows women to become pregnant when their own eggs are not capable of producing a pregnancy. Common indications for egg donation include cases of poor egg quality inferred from multiple failed cycles of conventional IVF or in women of advanced reproductive age with associated diminished ovarian reserve. Oocyte donation is also indicated in cases of premature ovarian failure secondary to chemotherapy in healthy cancer survivors, follicle-stimulating hormone (FSH) receptor defects, or other causes. Patients with gonadal dysgenesis are appropriate candidates for

egg donation. Those with Turner's syndrome may conceive, but are at higher risk of obstetrical complications (10). Other patients may choose egg donation in an attempt to avoid passing heritable genetic diseases to their children, although pre-implantation genetic diagnosis (PGD) is increasingly being applied in these situations. In certain social situations, such as same-sex male couples, use of donor oocytes and gestational surrogates is also indicated.

Prognostic Indicators

Donor Age

Since female fertility hinges on the age of the egg, the chronological age of the egg donor also likely plays a role in the overall success of the process. Currently, the mean donor age is approximately 26–28 years (11). In a recent large population study, the highest live birth rate in recipients was achieved in cycles with donors aged ≤34 years, while recipients with donors aged 35–39 were significantly less likely to achieve a live birth (12). Whereas prior fertility in a donor does not appear to play a role, previous successful cycles may improve the prognosis in subsequent ones (13).

Recipient Age

Recipient age per se does not appear to decrease the probability of pregnancy success with egg donation (14). Indeed, it was the observation that the age of the uterus does not play a role in the probability of the initiation of pregnancy that led to the conclusion that the age-related decline in human fertility was primarily due to egg age (15,16). At the heart of this observation are the physiological observations that uterine blood flow and the endometrial response to exogenous steroids do not appear to diminish with age (17). In a large clinical study, similar rates of pregnancy and live birth rates were achieved among fresh oocyte recipients aged <35, 35–39, 40–44, and ≥45 years (12), confirming that uterine conditions have less effect on outcomes than oocyte quality.

It is not yet definitively established if pregnancy success with oocyte donation after the age of 50 remains unaltered. In our experience (18,19) no decline has been observed. An analysis of data derived from the national registry suggested a modest decline in live birth rates after 45, with still poorer outcomes after age 50 (20,21). However, the absolute rates of live births were relatively high throughout the entire range of oocyte recipient ages (21).

Recipient Factors

The cause of infertility does not appear to play a role in the success of egg donation (13,19,22). However, uterine pathology such as Asherman's syndrome or previous radiation therapy may negatively impact uterine receptivity (23). Analogously, uterine fibroids, specifically submucosal fibroids or fibroids that distort the cavity, have been shown to decrease markers of endometrial receptivity and embryo implantation rates in oocyte donation models, and therefore should be resected (24). All hydrosalpinges should be removed, as these have been shown to decrease implantation rates and increase the risk of infection as well as ectopic pregnancy in recipients of egg donation (25). If salpingectomy is not possible, interruption of the oviduct in a location proximal to the utero-tubal junction also appears to prevent reflux of hydrosalpingeal fluid and thus to mitigate the adverse effects of the hydrosalpinx (26). Additionally, because the incidence of uterine pathology increases with the chronological age of the recipient, older recipients may require more careful screening for these factors. Obesity has been associated with decreased chances of pregnancy in women undergoing IVF with autologous oocytes; however, its effect on oocyte donation models have been variable (27). A recent systematic review noted no negative impact of recipient body mass index (BMI) on embryo implantation, miscarriage, or live births (28). Neither endometriosis nor adenomyosis in recipients appear to negatively impact embryo implantation (29,30). Pinopod expression, a marker of uterine receptivity, appears to be the same in recipients with and without endometriosis (31). Heavy tobacco smoking has also been shown to reduce uterine receptiveness, with significantly lower pregnancy rates in oocyte donation models, and therefore patients should be advised to quit smoking (32).

Repetitive Cycles

Among recipients with a normal uterine cavity and normal endometrial development, lack of success in a cycle does not negatively impact the outcome in subsequent cycles, and pregnancy rates appear to remain unaltered on a per-cycle basis (33). However, in one series, patients who conceived during their first cycle of egg donation were more likely to conceive in subsequent cycles (34). Cycle fecundability also appears to be unaltered by the number of cycles that the donor has undergone. The donor response to gonadotropin therapy, oocyte quantity and quality, and pregnancy rates has been reported to be unimpaired after successive stimulation cycles (35).

Donor Screening

The American Society for Reproductive Medicine (ASRM) publishes and periodically updates its guidelines for donor screening (36). While these guidelines endeavor to encompass information from the U.S. Centers for Disease Control and Prevention (CDC), the U.S. Food and Drug Administration (FDA), and the American Association of Tissue Banks (AATB), individual state practice requirements may differ. Oocyte donor screening usually includes a personal and sexual history and physical examination, with the intent of excluding those women at high risk for HIV, other sexually transmitted diseases, transmissible spongiform encephalopathy, or recent confirmed or suspected West Nile Virus infection (36,37). For a complete list of screening questions and exclusions, see "Uniform Donor Application" at www.sart.org. The FDA also requires the following laboratory tests be performed within 30 days of oocyte collection: serum screening for syphilis, hepatitis B and C, and HIV-1 and HIV-2. *Neisseria gonorrhoeae*, chlamydia, and blood type and Rh factor are also recommended. Psychological screening should include a family, sexual, substance use, and psychiatric history, information on educational background, and assessment of stability, motivation to donate, life stressors and coping skills, and interpersonal relationships. The counselor should confirm that the donor has been informed of the medical treatment, discuss the psychological risks of egg donation, and evaluate for signs of coercion.

Genetic screening should include a history, with a specific focus on any major Mendelian disorders, major malformations due to multifactorial causes, or known karyotypic abnormalities in the potential donor or in any of her first degree relatives. A screening test for cystic fibrosis is recommended in all donors, and heterozygotes may be included as a donor if the recipient is aware and the recipient's partner tests negative (38). Specific ethnic groups known to be at high risk for carrying certain recessive genes should undergo additional screening tests. It is not appropriate to screen donors for adult onset conditions (such as cancer predisposition, Huntington disease, etc.) without full consent of donor including formal genetic counseling (36).

Recipient Screening

As with donors, the ASRM periodically updates its guidelines for the screening of recipients (36). Routine screening includes a medical and reproductive history, physical examination, and standard preconception testing and counseling. These include blood type and Rh factor, rubella and varicella titers (with vaccination if not immune), along with infectious disease screening. Recipients should undergo counseling regarding the potential psychological implications of becoming parents as a result of egg donation (39).

The uterus should be examined by ultrasound and the cavity evaluated and pathology corrected prior to embryo transfer. Evaluation of the endometrial cavity can be achieved by saline-injection sonography (40), hysteroscopy, or hysterosalpingography (HSG). The advantage of HSG lies in its ability to visualize the fallopian tubes and thus detect the presence of hydrosalpinges.

Furthermore, potential recipients over the age of 45 should undergo thorough medical evaluation (including cardiovascular testing) and a high-risk obstetric consultation due to concerns for obstetrical complications in the very advanced maternal age women. As a special group, patients with Turner's syndrome should undergo echocardiography because they are thought to have as high as a 2% risk of aortic rupture or dissection with a risk of death during pregnancy (10).

Recipient Practice Cycle

During the early years of egg donation, all recipients underwent practice cycles to ensure an adequate response of the recipient's endometrium to exogenous steroids. Whereas this practice has become somewhat controversial, in that some programs feel that it is not necessary (41), our program continues to rely on the information the cycle provides. In a practice cycle, recipients undergo a regimen of exogenous estrogen and progesterone, which is identical to the actual recipient cycle. Our practice utilizes the regimen depicted in Table 19.1. Endometrial thickness is noted at the beginning of the cycle and prior to the initiation of progesterone. On the 7th day of progesterone administration, endometrial thickness is again documented, and the recipient undergoes a practice embryo transfer as well as endometrial biopsy. We have found that ultrasound measurement of endometrial thickness does not always correlate with biopsy findings (42). The biopsy may document a lack of adequate estrogen priming or out-of-phase endometrium (43). When practice cycles provide information on adjusting timing of embryo transfer, improved pregnancy rates have been noted (43).

In contrast, programs that no longer require endometrial biopsies rely on information from studies indicating that an in-phase endometrial biopsy does not predict pregnancy, and an out-of-phase biopsy does not necessarily predict failure (44).

Stimulation

Controlled ovarian hyperstimulation in oocyte donors is achieved in a manner similar to that of conventional IVF. As with conventional IVF, the use of antagonists in donor cycles also appears to reduce the amount of gonadotropins required and duration of treatment (45). Studies comparing agonists and antagonists in oocyte donation cycles have not shown a significant difference in pregnancy or live birth rates achieved with either protocol (46). In oocyte donation cycles, an antagonist protocol also has the advantage of the option to use gonadotropin-releasing hormone (GnRH) agonist as an alternative to HCG for oocyte maturation trigger. Due to its long half life, human chorionic gonadotropin (HCG) is associated with a high risk of ovarian hyperstimulation syndrome (OHSS) (47).

In our clinic, donors are most commonly prescribed a regimen of oral contraceptives pills (OCPs) prior to stimulation in order to coordinate with the recipient cycle. Oral contraceptives are begun on day 3 of menses, and stopped four days prior to anticipated stimulation start date. Donors are evaluated approximately one day after stopping OCPs to confirm down-regulation by ultrasound evaluation of the ovaries and endometrium and by measurement of serum estradiol. Stimulation is then performed using human menopausal gonadotropins alone or in conjunction with recombinant follicle stimulating hormone (FSH). Patients return on the fifth day of stimulation for an ultrasound and measurement of serum estradiol. The GnRH antagonist is given either as a multiple-dose fixed protocol (starting on the sixth day of stimulation) or as a flexible protocol (starting when the leading follicle reaches approximately 14 mm in size), depending on provider preference. Once two follicles achieve 18 mm in size, ovulation is triggered with a GnRH agonist, as previously discussed above.

Donor cycles require vigilance on the part of the nursing staff to ensure adherence to the protocol and prevention of undesired pregnancy. Donors should be encouraged to abstain from intercourse from the

TABLE 19.1

Standard Estrogen and Progesterone Replacement Regimen

	Micronized Estradiol (Oral Administration)	Micronized Progesterone (Vaginal Administration)
Days 1–5	1 mg BID	
Days 6–9	2 mg BID	
Days 10–14	2 mg TID	
Days 15–28 (Through 13th week of gestation)	2 mg BID	200 mg TID

Source: USC Fertility's internal regimen for estrogen and progesterone replacement, 2017.

onset of stimulation until the following menses, and prescribed some form of contraceptives to initiate with menses.

Donor Safety

Serious complications of oocyte donation are relatively rare. Less than 1% of donors experience events requiring hospitalization or emergent intervention during or following aspiration as a result of any cause, including severe OHSS, reactions to anesthesia, pelvic infection, and intraperitoneal hemorrhage (48). OHSS is virtually eliminated with the use of GnRH agonist triggers with an antagonist protocol as previously described. Furthermore, donors have the advantage of avoiding pregnancy-related exacerbations of OHSS (49). In our practice, donors are also evaluated in our office two days after follicle aspiration. If necessary, additional intravenous fluids (1000–2000 mL of normal saline) may be administered. Rarely, paracentesis may be required in cases of severe OHSS (50).

Little is known about the effect of egg donation on future health and fertility.

In the past, there has been some concern that high doses of fertility drugs for extended periods of time may increase the risk of gynecologic cancers, including breast and ovarian cancers. However, large studies have shown no increase in ovarian cancer risk between subfertile women treated for their infertility with ART and subfertile women not treated for their infertility (51), or when compared to women in the general population (52). Data on breast cancer risk following fertility treatment has been more controversial, with some studies showing a minimal increased risk (53) while others show no increased risk when compared to other subfertile women (54). However, there has been very little data on cancer risks in oocyte donors, who, in general, are young and healthy and do not suffer from subfertility. Given the absence of clear evidence, ASRM recommends limiting the number of stimulated cycles for a given oocyte donor to six (48).

Recipient Cycle

The goal of the recipient cycle is to prepare the endometrium for implantation and to synchronize endometrial progress with embryo development. Early in the history of oocyte donation, it was demonstrated that in-phase endometrium could be obtained with a variable estrogen and progesterone regimen, designed to mimic the natural cycle (55). Regardless of the specifics of the regimen, it is now clear that E2 and P are the only hormones needed to generate a receptive endometrial environment (56). Therefore, preparation of the recipient endometrium with estrogen and progesterone occurs in an identical fashion to the recipient practice cycle (Table 19.1).

Women with ovarian failure can initiate a cycle at any time, whereas those with residual ovarian function may undergo pituitary down-regulation with a GnRH agonist to avoid untimely uterine bleeding associated with fluctuating steroid levels. A variety of E2 preparations are available that utilize different routes, as well as duration of administration. Oral E2 is the most commonly utilized. However, it is extensively metabolized by the liver and converted to estrone (E1) and estrone sulfate (E1S), weak estrogens with lower binding affinity for the estrogen receptors when compared to E2. Parenteral routes: transdermal, IM, or vaginal, can bypass this hepatic first-pass effect. Although transdermal absorption of E2 may be highly variable, it produces a stable steady-state level of serum E2 (56). Vaginal E2 administration results in high serum levels and even higher endometrial tissue levels due to selective uptake by the endometrium of vaginally administered steroids (57,58), and is therefore usually reserved for patients when the other routes are not effective.

Estradiol therapy can also be provided in a fixed or variable regimen with comparable efficacy. In our clinic, we increase the E2 to mimic the natural pattern of E2 levels in the circulation (see Table 19.1). Fixed regimens using 4 mg or 8 mg per day without down-regulation have been used to achieve an endometrial thickness >6 mm in an average of 5–7 days with good pregnancy rates (59). Prolonged duration of E2 therapy does not appear to have a negative impact as E2 administration of 4 to 5 weeks has yielded successful pregnancies (57). However, a Cochrane review and a 2013 systematic review noted insufficient data to support one particular protocol for endometrial preparation over another (60,61).

Following adequate estrogen priming of the endometrium, it is progesterone that prepares the endometrium for implantation. Estradiol stimulates both endometrial proliferation and the induction of progesterone receptors. Thus, an endometrial biopsy taken during a practice cycle, which shows no progestational effect, has, in our experience, generally been reflective of inadequate estrogen priming rather than inadequate progesterone delivery to the endometrium. This effect may be observed in women after long episodes of amenorrhea, such as occurs in agonadal women in the absence of estrogen stimulation, or after prolonged hormonal replacement with combination estrogen and progesterone replacement in which menstrual sloughing does not take place. It is tempting to speculate that prolonged continuous progesterone stimulation of the endometrium produces a profound down-regulation of estrogen receptors, and that these patients may also require priming prior to attaining an appropriate response.

To achieve luteinization of the endometrium, progesterone is started in the recipient either on the evening of the day of donor oocyte retrieval or one day after (60). There is a "threshold level" of serum progesterone that must be achieved for an orderly luteinization of an adequately primed endometrium. A precise cut off value, however, has not yet been established, though it is generally accepted to be >5 ng/mL (56).

Progesterone delivery modes are more complex than those of estradiol, and for the purpose of endometrial preparation are primarily administered by the intramuscular or vaginal routes. Oral progesterone is ineffective because it is rapidly metabolized in the liver. In addition, progesterone is susceptible to metabolism by 5α-reductase in the skin, making transdermal delivery even more impractical due to the large size of the patch that would be required to provide adequate serum levels of the hormone.

Advantages of the intramuscular route of administration include higher serum progesterone levels and reassurance that the progesterone has been appropriately administered. Many programs in the United States still utilize this mode of delivery with a dose of 50–100 mg daily. Even lower doses of 25–50 mg per day results in luteal phase serum levels of progesterone, a secretory endometrium, and good pregnancy rates (62).

Vaginal progesterone has generally been formulated in three ways: capsules containing micronized progesterone powder, a silastic ring, which gradually releases progesterone, and a cream formulation. Vaginal micronized progesterone inserts rapidly disintegrate and the released progesterone is absorbed by the vaginal epithelium. Compared to intramuscular administration of progesterone, vaginal progesterone achieves a lower maximum serum progesterone level, but higher endometrial tissue levels, and reaches a steady state in a shorter amount of time (63). For this reason, we have all but abandoned the intramuscular route, reserving it only for those patients who dislike the use of vaginal suppositories. Most patients prefer the vaginal route over the pain and inconvenience of daily intramuscular injections (64).

Results from Europe on the use of a vaginal ring containing 1 g of natural progesterone, which provides continuous release of 10–20 nmol/L of progesterone for 90 days, has been favorable (65). In the United States, Crinone, formulated as a cream, and administered vaginally twice daily has been shown to result in in-phase biopsies and similar pregnancy rates to those achieved with intramuscular progesterone (66).

Oocyte Cryopreservation and Embryo Transfer

The ASRM guidelines for oocyte donor cycles recommend using the age of the donor to determine the appropriate number of embryos to transfer, and for donors age <35 years to strongly consider elective single embryo transfers (eSET) in order to reduce the rates of multiples (67). Given large number of oocytes are normally retrieved in an oocyte donor cycle, the excess oocytes and embryos may be cryopreserved, as in conventional IVF.

The benefit springing from the improved cryopreservation technology is its potential application towards commercial "egg banks." These entities would theoretically be able to provide recipients of egg donation with more choices in selecting a donor, more flexibility in timing, and potentially lower cost. As of 2012, 600 clinical pregnancies were achieved with frozen donor oocytes from seven commercial

egg banks identified in the United States, with still an estimate of 3130 oocytes from 294 donors stored for future use (68).

Pregnancy and Neonatal Outcomes

Pregnancy and live birth rates after oocyte donation have risen consistently. In the 2012 SART report, fresh donor oocyte cycles achieved a live birth rate of 56.4%, compared to frozen donor oocyte cycles (37%), fresh autologous cycles (36.3%) and frozen autologous cycles (37.5%) (Figure 19.1)(1)

However, oocyte donation cycles are associated with significantly higher rates of multiple gestations (34%) compared to autologous cycles (27%) and baseline population risk (3%) (1,11,69). Pregnancy loss after visualization of fetal heart motion has been reported to occur in 5.7% of singleton pregnancies after oocyte donation (70). In multiple gestation, the risk of fetal loss appears to correlate with the number of gestational sacs, however, miscarriage rates (loss of all gestational sacs) do not appear to be increased, and most losses occur prior to the ninth week gestation (71). The probability of spontaneous absorption of one or more embryos is approximately 19%–28% (70,71). Elective multi-fetal reduction from twins to singletons (72) and triplets to twins have been shown to improve perinatal outcomes in terms of gestational age and birth weight.

In addition to risk of multiples, oocyte donation in itself is an independent risk factor for pregnancy complications, mainly pre-eclampsia (Table 19.2) (73). Many studies have shown oocyte donation increases risk for pre-eclampsia even when controlling for ICSI (74), twins (75), maternal age (76), and history of chronic hypertension (77). One hypothesis proposed is that it may be due to inadequate immunoprotection of the fetoplacental unit (77,78).

Perinatal mortality and neonatal malformation rates do not appear to differ significantly from the general population. A recent large study noted good perinatal outcomes in donor oocyte cycles, with good perinatal outcome defined as singleton live born at ≥37weeks and ≥2500 g (11). Some studies have noted infants conceived after oocyte donation may be at higher risk for low birth weight and preterm births (Table 19.3) (79–81,83). However, after controlling for pre-eclampsia, neonatal outcomes were similar between oocyte donation cycles and traditional IVF (73,84). In a long-term follow-up study of children up to 5 years of age, conceived with oocyte donation, all were healthy. Growth and development was similar to children in the general population (85).

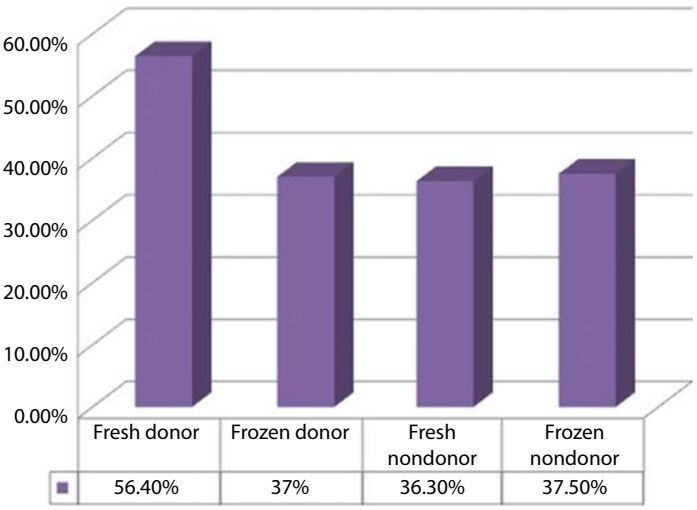

FIGURE 19.1 Percentage of transfers resulting in live births. (From [CDC] CfDCaP. 2012 Assisted Reproductive Technology National Summary Report.: Available at: http://www.cdc.gov/art; 2012.).

TABLE 19.2

Prevalence of Antenatal and Delivery Complications during Oocyte Donation

Singletons	Pregnancy Induced Hypertension (%)	Gestational Diabetes Mellitus (%)	First trimester Vaginal Bleeding (%)	Preterm Delivery (%)	Cesarean Section (%)	Pre-Eclampsia (%)
Sheffer-Mimouni et al. (79) ($n = 134$)	27.6	23.9	43.3	14.9	72	–
Abdalla et al. (80) ($n = 105$)	21	–	–	13	–	–
Soderstrom-Anttila et al. (81) ($n = 39$)	29	–	–	13	51	–
Malchau et al. (73) ($n = 215$)	15.8	–	–	–	59.5	9.8
Levron et al. (77) ($n = 139$)	25	16	–	9 (<34 wks)	85	9.3
Stoop et al. (82) ($n = 148$)	17	7.5	21.8	11.6	50	10.2
All Pregnancies						
Soderstrom-Anttila et al. (81) ($n = 51$)	31	12	53	30	57	–
Pados et al. (83) ($n = 52$)	32.7	–	34.6	1.9	63.5	–
Van Dorp et al. (84) ($n = 110$)	46.9	–	–	–	53.5	25.7
Tranquilli et al. (74) ($n = 26$)	11.5	3.8	7.5	50	–	19.2
Le Ray et al. (76) ($n = 104$)	–	–	–	29.7	61.4	19.2
Stoop et al. (82) ($n = 205$)	19.1	7.4	20.6	19.1	58.9	11.8

TABLE 19.3

Prevalence of Neonatal Complications in Singleton Pregnancies after Oocyte Donation

Study	Perinatal Mortality (%)	Neonatal Malformations (%)	Low Birth Weight (%)	Small for Gestational Age (%)
Sheffer-Mimouni et al. (79) ($n = 134$)	0	2.2	14.9	7.6
Remohi et al. (86) ($n = 188$)	2.7	1	–	–
Pados et al. (83) ($n = 52$)	1.7	0	–	–
Soderstrom-Anttila et al. (81) ($n = 41$)	3.3	5.1	10	5
Abdalla et al. (80) ($n = 105$)	–	–	18	15
Van Dorp et al. (84) ($n = 61$)	3.3	3.3	8.3	–
Malchau et al. (73) ($n = 244$)	–	10.3 ($n = 215$)	10.7	6.6
Stoop et al. (82) ($n = 148$)	2	–	8.8	–

Advanced Reproductive Age

Pregnancies can be achieved even in women over the age of 50 using donor oocytes and with the appropriate endometrial preparation (18,87). However, with the ability to facilitate pregnancies in advanced maternal age, medical and ethical concerns should also be addressed.

Pre-cycle screening of women of advanced reproductive age is extensive and, in addition to a thorough medical evaluation, should include cardiovascular testing (Table 19.4). All recipients,

TABLE 19.4

Screening Tests for Prospective Recipients of Oocyte Donation Over the Age of 45 Years

Medical
Complete blood count
Blood chemistry panel
Thyroid-stimulating hormone
Fasting cholesterol panel
Glucose tolerance test
Coagulation parameters
Urinalysis
Papanicolaou test
Mammogram
Chest roentgenogram
Treadmill and baseline electrocardiogram
Colonoscopy (if over 50)
Reproductive
Transvaginal ultrasound
Endometrial biopsy and practice embryo transfer (day 21 of practice cycle)
Hysterosalpingogram or saline injection sonography
Infectious disease screen
HIV
HTLV I/II
VDRL
HBsAg
Hep C Ab
Preconceptual and psychosocial counseling

particularly menopausal women, should also undergo a practice hormone replacement cycle of estrogen and progesterone, with an endometrial biopsy to confirm adequate estrogen priming and subsequent luteinization of endometrial lining.

Information about obstetrical and perinatal outcomes in the very advanced maternal age is limited. The incidence of gestational diabetes, pre-eclampsia, and cesarean section rate appears to be increased in this older age group (Table 19.5) (88,89). However, in a study of 77 post-menopausal women 50 years or older, we found that pregnancy rates, multiple gestation rates, and spontaneous abortion rates were

TABLE 19.5

Obstetrical Outcomes after Oocyte Donation to Women of Advanced Reproductive Age

Study (*n* = Number of Women)	Age of Subjects	Preeclampsia (%)	Gestational Diabetes (%)	Cesarean Section Rate (%)
Paulson et al. (90) (*n* = 77)	≥50	35	20	78
Porreco et al. (92) (*n* = 50)	>45	42	8	64
Borini et al. (93) (*n* = 34)	≥50	22	11	–
Sauer et al. (94) (*n* = 162)	≥45	5.4[a]	8	64.8
Antinori et al. (95) (*n* = 1150)	≥45	1.1[b]	0.8	75
Jackson et al. (88) (*n* = 120)	≥45	–	–	70.8
Kort et al. (89) (*n* = 101)	≥50	Hypertensive disorders –23%	8.9	81
Le Ray et al. (76) (*n* = 104)	≥43	19.2	7.7	61.4

[a] Gestational hypertension 10.8%.
[b] Gestational hypertension 11.8%.

similar to those of younger recipients (90). Other studies have also noted similar obstetrical risk between donor oocyte recipients in advanced maternal age and younger recipients (76,89,91). In a recent study, sub-analysis comparing women ≥45 years who conceived via donor oocyte cycles or autologous cycles, both groups had similar rates of postpartum hemorrhage, cesarean section, fetal gestational age, and birth weights (88). Therefore, it appears that oocyte donation in itself does not further increase obstetrical risks in advanced maternal age women.

It has been argued that egg donation to post-menopausal females may result in greater gender equality and reproductive freedom. Counter arguments have focused on the impact on the children and concerns regarding the physical and parenting capabilities of these older parents (96). In a study comparing parenting stress and physical functioning in women who conceived after the age of 50 years compared to women in their 30s, advanced maternal age was not found to reduce parenting capacity (97).

The most recent ASRM ethics committee opinion regarding this issue has proposed that in appropriately screened patients between 50 and 54 years of age without any persistent psychological, medical, or obstetrical concerns, it is reasonable to offer oocyte donation services (98).

Summary

Oocyte donation is a logical extension of the technology of IVF, designed to overcome blocks to fertility caused by oocyte problems. The details of an oocyte donation cycle are very similar to those of standard IVF. Stimulation of the donor is similar to standard IVF, yet may be simpler, since poor responder protocols are rarely needed, and concerns about hyperstimulation are essentially eliminated with an antagonist protocol with gonadotropin agonist trigger. Preparation of the recipient endometrium and synchronization between donor and recipient are also achieved with relative ease. Donors as well as recipients should be medically screened and offered psycho-social counseling to address issues of third-party parenting. Since oocyte donation bypasses most oocyte problems, it has increasingly been applied to the age-related block to conception in women of advanced reproductive age. It is possible to establish pregnancies in menopausal women over 50 years of age although this practice is somewhat controversial. Obstetric outcomes after oocyte donation appear similar to those after other assisted reproductive technologies, with a possible increase in the incidence of hypertensive disorders of pregnancy.

REFERENCES

1. CDC. *2012 Assisted Reproductive Technology National Summary Report*.: Available at: http://www.cdc.gov/art; 2012.
2. Bustillo M, Buster JE, Cohen, SW, Hamilton F, Thorneycroft IH, Simon JA et al. Delivery of a healthy infant following nonsurgical ovum transfer (letter). *JAMA*. 1984;251:889.
3. Bustillo M, Buster JE, Cohen SW et al. Nonsurgical ovum transfer as a treatment on infertile women. Preliminary experience. *JAMA*. 1984;251:1171.
4. Sauer MV, Anderson RE, Paulson RJ. A trial of superovulation in ovum donors undergoing uterine lavage. *Fertil Steril*. 1989;51:131.
5. Trounson A, Leeton J, Besanko M, Wood C, Conti A. Pregnancy established in an infertile patient after transfer of a donated embryo fertilised in vitro. *Br Med J*. 1983;286:835.
6. Lutjen P, Trounson A, Leeton J, Findlay J, Wood C, Renou P. The establishment and maintenance of pregnancy using in vitro fertilization and embryo donation in a patient with primary ovarian failure. *Nature*. 1984;307:174.
7. Devroey P, Smitz J, Camus M, Wisanto A, Deschacht J, Van Waesberghe L et al. Synchronization of donor's and recipient's cycles with GnRH analogues in an oocyte donation programme. *Hum Reprod*. 1989;4:270.
8. Sauer MV, Paulson RJ, Macaso TM, Francis-Hernandez M, Lobo RA. Establishment of a nonanonymous donor oocyte program: Preliminary experience at the University of Southern California. *Fertil Steril*. 1989;52:433.
9. Sauer MV, Stein AL, Paulson RJ, Moyer DL. Endometrial responses to various hormone replacement regimens in ovarian failure patients preparing for embryo donation. *Int J Gynecol Obstet*. 1991;35:61.

10. Hagman A, Loft A, Wennerholm UB, Pinborg A, Bergh C, Aittomäki K et al. Obstetric and neonatal outcome after oocyte donation in 106 women with Turner syndrome: A Nordic cohort study. *Hum Reprod*. 2013;28(6):1598–1609.
11. Kawwass JF, Monsour M, Crawford S, Kissin DM, Session DR, Kulkarni AD et al. Trends and outcomes for donor oocyte cycles in the United States, 2000–2010. *JAMA*. 2013;310(22):2426–34.
12. Wang YA, Farquhar C, Sullivan EA. Donor age is a major determinant of success of oocyte donation/recipient programme. *Hum Reprod*. 2012;27(1):118–125.
13. Harris SE, Faddy M, Levett S, Sharma V, Gosden R. Analysis of donor heterogeneity as a factor affecting the clinical outcome of oocyte donation. *Hum Fertil*. 2002; 5, 193.
14. Paulson RJ. Does the age of the recipient influence the probability of pregnancy among recipients of oocyte donation? *Fertil Steril*. 2014;101(5):1248–1249.
15. Sauer MV, Paulson RJ, Lobo RA. A preliminary report on oocyte donation extending reproductive potential to women over 40. *N Engl J Med*. 1990;323:1157.
16. Navot D, Bergh, PA, Williams MA, Garrisi GJ, Guzman I, Sandler B et al. Poor oocyte quality rather than implantation failure as a cause of age-related decline in female fertility: *Lancet*. 1991;337:1375.
17. Sauer MV, Miles RA, Dahmoush L, Paulson RJ, Press M, Moyer D et al. Evaluating the effect of age on endometrial responsiveness to hormone replacement therapy: A histologic, ultrasonographic, and tissue receptor analysis. *J Assist Reprod Genet*. 1993;10:47.
18. Sauer MV, Paulson RJ, Lobo RA, Pregnancy after age 50: Application of oocyte donation to women after natural menopause. *Lancet*. 1993;341:321.
19. Paulson RJ, Hatch IE, Lobo RA, Sauer MV. Cumulative conception and live birth rates after oocyte donation: Implications regarding endometrial receptivity. *Hum Reprod*. 1997;12(4):835–9.
20. Toner JP, Grainger DA, Frazier LM. Clinical outcomes among recipients of donated eggs: An analysis of the U.S. national experience, 1996–1998. *Fertil Steril*. 2002;78(5):1038–45.
21. Yeh JS, Steward RG, Dude AM, Shah AA, Goldfarb JM, Muasher SJ. Pregnancy outcomes decline in recipients over age 44: An analysis of 27,959 fresh donor oocyte in vitro fertilization cycles from the Society for Assisted Reproductive Technology. *Fertil Steril*. 2014;101(5):1331–6.
22. Garcia-Velasco JA, Isaza V, Caligara C, Pellicer A, Remohí J, Simón C. Factors that determine discordant outcome from shared oocytes. *Fertil Steril*. 2003;80:54.
23. Teh WT, Stern C, Chander S, Hickey M, Teh WT, Stern C, Chander S, Hickey M. The impact of uterine radiation on subsequent fertility and pregnancy outcomes. *Biomed Res Int*. 2014;2014:482968.
24. Shokeir T, El-Shafei M, Yousef H, Allam AF, Sadek E. Submucous myomas and their implications in the pregnancy rates of patients with otherwise unexplained primary infertility undergoing hysteroscopic myomectomy: A randomized matched control study. *Fertil Steril*. 2010;94(2):724–9.
25. Cohen MA, Lindheim SR, Sauer MV. Hydrosalpinges adversely affect implantation in donor oocyte cycles. *Hum Reprod*. 1999;14:1087.
26. Johnson N, van Voorst S, Sowter MC, Strandell A, Mol BW et al. Surgical treatment for tubal disease in women due to undergo in vitro fertilisation. *Cochrane Db Syst Rev*. 2010(1):Cd002125.
27. Soares SR, Velasco JA, Fernandez M et al. Clinical factors affecting endometrial receptiveness in oocyte donation cycles. *Fertil Steril*. 2008;89(3):491–501.
28. Jungheim ES, Schon SB, Schulte MB, DeUgarte DA, Fowler SA, Tuuli MG. IVF outcomes in obese donor oocyte recipients: A systematic review and meta-analysis. *Hum Reprod*. 2013;28(10):2720–7.
29. Pellicer A, Navarro J, Bosch E, Garrido N, Garcia-Velasco JA, Remohí J et al. Endometrial quality in infertile women with endometriosis. *Ann N Y Acad Sci*. 2001;943:122.
30. Martinez-Conejero JA, Morgan M, Montesinos M, Fortuño S, Meseguer M, Simón C et al. Adenomyosis does not affect implantation, but is associated with miscarriage in patients undergoing oocyte donation. *Fertil Steril*. 2011;96(4):943–50.
31. Zenke U, Chetkowski RJ. Transfer and uterine factors are the major recipient-related determinants of success with donor eggs. *Fertil Steril*. 2004;82(4):850–6.
32. Soares SR, Simon C, Remohi J, Pellicer A. Cigarette smoking affects uterine receptiveness. *Hum Reprod*. 2007;22(2):543–7.
33. Paulson RJ, Hatch IE, Lobo RA, Sauer MV. Cumulative conception and live birth rates after oocyte donation: Implications regarding endometrial receptivity. *Hum Reprod*. 1997;12:835.
34. Spandorfer SD, Moomjy M, Davis OK, Barmat LI, Cholst I, Rosenwaks Z. Oocyte donation: Does a previous attempt affect a subsequent attempt? *Fertil Steril*. 1998;70:222.

35. Jain A, Robins JC, Williams DB, Thomas MA. The effect of multiple cycles in oocyte donors. *Am J Obstet Gynecol*. 2005;192(5):1382–4.
36. Practice Committee of American Society for Reproductive Medicine; Practice Committee of Society for Assisted Reproductive Technology. Recommendations for gamete and embryo donation: A committee opinion. *Fertil Steril*. 2013;99(1):47–62.
37. Practice Committee of American Society for Reproductive Medicine; Practice Committee of Society for Assisted Reproductive Technology. American Society for Reproductive Medicine/Society for Assisted Reproductive Technology position statement on West Nile Virus. *Fertil Steril*. 2005;83(2):527–8.
38. Zenke U, Chetkowski, R.J. Inclusion of heterozygotes for cystic fibrosis in the egg donor pool. *Fertil Steril*. 2002;78:557.
39. American Society for Reproductive Medicine. Psychological assessment of gamete donors and recipients. *Fertil Steril*. 2004;82(Suppl 1):S18–9.
40. Lindheim SR, Sauer MV. Upper genital-tract screening with hysterosonography in patients receiving donated oocytes. *Int J Gynecol Obstet*. 1998;60:47.
41. Jun SH, Hornstein MD. Is there a role for preparatory cycle in ovum donation recipients? *Curr Opin Obstet Gynecol*. 2006;18(3):333–7.
42. Sauer MV, Paulson RJ, Moyer DL. Assessing the importance of endometrial biopsy prior to oocyte donation. *J Assist Reprod Genet*. 1997;14:125.
43. Potter DA, Witz CA, Burns WN, Brzyski RG, Schenken RS. Endometrial biopsy during hormone replacement cycle in donor oocyte recipients before in vitro fertilization-embryo transfer. *Fertil Steril*. 1998;70(2):219–21.
44. Jun SH, Racowsky C, Fox JH, Hornstein MD. The role of preparatory cycles in ovum donation recipients: A retrospective study. *J Assist Reprod Genet*. 2004;21:377.
45. Sauer MV, Paulson RJ, Lobo RA. Comparing the clinical utility of GnRH antagonist to GnRH agonist in an oocyte donation program. *Gynecol Obstet Invest*. 1997;43:215.
46. Bodri D, Vernaeve V, Guillen JJ, Vidal R, Figueras F, Coll O. Comparison between a GnRH antagonist and a GnRH agonist flare-up protocol in oocyte donors: A randomized clinical trial. *Hum Reprod*. 2006;21(9):2246–51.
47. Erb TM, Vitek W, Wakim AN. Gonadotropin-releasing hormone agonist or human chorionic gonadotropin for final oocyte maturation in an oocyte donor program. *Fertil Steril*. 2010;93(2):374–378.
48. Repetitive oocyte donation: A committee opinion. *Fertil Steril*. 2014;102(4):964–6.
49. Morris RS, Paulson RJ. Ovarian hyperstimulation syndrome: Classification and management. *Contemp Obstet Gynecol*. 1994;39:43.
50. Aboulghar MA, Mansour RT, Serour GI. Ultrasonically guided vaginal aspiration of ascites in the treatment of severe ovarian hyperstimulation syndrome. *Fertil Steril*. 1990;53:933.
51. Venn A, Watson L, Bruinsma F, Giles G, Healy D. Risk of cancer after use of fertility drugs with in-vitro fertilisation. *Lancet*. 1999;354(9190):1586–90.
52. Siristatidis C, Sergentanis TN, Kanavidis P, Trivella M, Sotiraki M, Mavromatis I. Controlled ovarian hyperstimulation for IVF: Impact on ovarian, endometrial and cervical cancer—A systematic review and meta-analysis. *Hum Reprod Update*. 2013;19(2):105–23.
53. Reigstad MM, Larsen IK, Myklebust TÅ, Robsahm TE, Oldereid NB, Omland AK et al. Risk of breast cancer following fertility treatment—A registry based cohort study of parous women in Norway. *Int J Cancer*. 2015;136(5):1140–8.
54. Lerner-Geva L, Rabinovici J, Olmer L, Blumstein T, Mashiach S, Lunenfeld B. Are infertility treatments a potential risk factor for cancer development? Perspective of 30 years of follow-up. *Gynecol Endocrinol*. 2012;28(10):809–14.
55. Navot D, Laufer N, Kopolovic J, Rabinowitz R, Birkenfeld A, Lewin A et al. Artificially induced endometrial cycles and establishment of pregnancies in the absence of ovaries. *N Engl J Med*. 1986;314:806.
56. Paulson RJ. Hormonal induction of endometrial receptivity. *Fertil Steril*. 2011;96(3):530–5.
57. Tourgeman DE, Slater CC, Stanczyk FZ, Paulson RJ. Endocrine and clinical effects of micronized estradiol administered vaginally or orally. *Fertil Steril*. 2001;75:200.
58. Tourgeman DE, Gentzchein E, Stanczyk FZ, Paulson RJ. Serum and tissue hormone levels of vaginally and orally administered estradiol. *Am J Obstet Gynecol*. 1999;180(6 Pt 1):1480–3.
59. Shapiro H, Cowell C, Casper RF. The use of vaginal ultrasound for monitoring endometrial preparation in a donor oocyte program. *Fertil Steril*. 1993;59:1055.

60. Glujovsky D, Pesce R, Fiszbajn G, Sueldo C, Hart RJ, Ciapponi A. Endometrial preparation for women undergoing embryo transfer with frozen embryos or embryos derived from donor oocytes. *Cochrane Db Syst Rev*. 2010(1):Cd006359.
61. Groenewoud ER, Cantineau AE, Kollen BJ, Macklon NS, Cohlen BJ. What is the optimal means of preparing the endometrium in frozen-thawed embryo transfer cycles? A systematic review and meta-analysis. *Hum Reprod Update*. 2013;19(5):458–70.
62. Tavaniotou A, Smitz J, Bourgain C, Devroey P. Comparison between different routes of progesterone administration as luteal phase support in infertility treatments. *Hum Reprod Update*. 2000;6:139.
63. Paulson RJ, Collins MG, Yankov VI. Progesterone pharmacokinetics and pharmacodynamics with 3 dosages and 2 regimens of an effervescent micronized progesterone vaginal insert. *J Clin Endocrinol Metab*. 2014;99(11):4241–9.
64. Yanushpolsky E, Hurwitz S, Greenberg L, Racowsky C, Hornstein M. Crinone vaginal gel is equally effective and better tolerated than intramuscular progesterone for luteal phase support in in vitro fertilization-embryo transfer cycles: A prospective randomized study. *Fertil Steril*. 2010;94(7):2596–9.
65. Zegers-Hochschild F, Balmaceda JP, Fabres C, Alam V, Mackenna A, Fernández E et al. Prospective randomized trial to evaluate the efficacy of a vaginal ring releasing progesterone for IVF and oocyte donation. *Hum Reprod*. 2000;15:2093.
66. Gibbons WE, Toner JP, Hamacher P et al. Experience with a novel vaginal progesterone preparation in a donor oocyte program. *Fertil Steril*. 1998;69:96.
67. Criteria for number of embryos to transfer: A committee opinion. *Fertil Steril*. 2013;99(1):44–6.
68. Quaas AM, Melamed A, Chung K, Paulson RJ. Egg banking in the United States: Current status of commercially available cryopreserved oocytes. *Fertil Steril*. 2013;99(3):827–31.
69. Kissin DM, Kulkarni AD, Mneimneh A, Warner L, Boulet SL, Crawford S et al. Embryo transfer practices and multiple births resulting from assisted reproductive technology: An opportunity for prevention. *Fertil Steril*. 2015;103(4):954–61.
70. Legro RS, Wong IL, Paulson RJ, Lobo RA, Sauer MV. Multiple implantation after oocyte donation: A frequent but inefficient event. *Fertil Steril*. 1995;63:849.
71. Rodriguez-Gonzalez M, Serra V, Garcia-Velasco JA, Pellicer A, Remohí J. The "vanishing embryo" phenomenon in an oocyte donation programme. *Hum Reprod*. 2002;17:798.
72. Haas J, Mohr Sasson A, Barzilay E, Mazaki Tovi S, Orvieto R, Weisz B. Perinatal outcome after fetal reduction from twin to singleton: To reduce or not to reduce? *Fertil Steril*. 2015;103(2):428–32.
73. Malchau SS, Loft A, Larsen EC, Aaris Henningsen AK, Rasmussen S, Andersen AN et al. Perinatal outcomes in 375 children born after oocyte donation: A Danish national cohort study. *Fertil Steril*. 2013;99(6):1637–43.
74. Tranquilli AL, Biondini V, Talebi Chahvar S, Corradetti A, Tranquilli D, Giannubilo S. Perinatal outcomes in oocyte donor pregnancies. *J Matern Fetal Neonatal Med*. 2013;26(13):1263–7.
75. Sekhon LH, Gerber RS, Rebarber A, Saltzman DH, Klauser CK, Gupta S et al. Effect of oocyte donation on pregnancy outcomes in in vitro fertilization twin gestations. *Fertil Steril*. 2014;101(5):1326–30.
76. Le Ray C, Scherier S, Anselem O, Marszalek A, Tsatsaris V, Cabrol D. Association between oocyte donation and maternal and perinatal outcomes in women aged 43 years or older. *Hum Reprod*. 2012;27(3):896–901.
77. Levron Y, Dviri M, Segol I, Yerushalmi GM, Hourvitz A, Orvieto R et al. The "immunologic theory" of preeclampsia revisited: A lesson from donor oocyte gestations. *Am J Obstet Gynecol*. 2014;211(4):383.e381–385.
78. van der Hoorn ML, van Egmond A, Swings GM, van Beelen E, van der Keur C, Tirado-González I et al. Differential immunoregulation in successful oocyte donation pregnancies compared with naturally conceived pregnancies. *J Reprod Immunol*. 2014;101–102:96–103.
79. Sheffer-Mimouni G, Mashiach S, Dor J, Levran D, Seidman DS. Factors influencing the obstetric and perinatal outcome after oocyte donation. *Hum Reprod*. 2002;17:2636.
80. Abdalla HI, Billett A, Kan AK, Baig S, Wren M, Korea L et al. Obstetric outcome in 232 ovum donation pregnancies. *Br J Obstet Gynaecol*. 1998;105:332.
81. Soderstrom-Anttila V, Tiitinen A, Foudila T, Hovatta O. Obstetric and perinatal outcome after oocyte donation: Comparison with in-vitro fertilization pregnancies. *Hum Reprod*. 1998;13:483.
82. Stoop D, Baumgarten M, Haentjens P, Polyzos NP, De Vos M, Verheyen G et al. Obstetric outcome in donor oocyte pregnancies: A matched-pair analysis. *Reprod Biol Endocrinol*. 2012;10:42.

83. Pados G, Camus M, Van Steirteghem A, Bonduelle M, Devroey P. The evolution and outcome of pregnancies from oocyte donation. *Hum Reprod*. 1994;9:538.
84. van Dorp W, Rietveld AM, Laven JS, van den Heuvel-Eibrink MM, Hukkelhoven CW, Schipper I. Pregnancy outcome of non-anonymous oocyte donation: A case-control study. *Eur J Obstet Gynecol Reprod Biol*. 2014;182:107–12.
85. Soderstrom-Anttila V, Sajaniemi N, Tiitinen A et al. Health and development of children born after oocyte donation compared with that of those born after in-vitro fertilization, and parents' attitudes regarding secrecy. *Hum Reprod*. 1998;13:2009–15.
86. Remohi J, Gartner B, Gallardo E, Yalil S, Simón C, Pellicer A. Pregnancy and birth rates after oocyte donation. *Fertil Steril*. 1997;67:717.
87. Sauer MV, Paulson RJ, Lobo RA. Reversing the natural decline in human fertility: An extended clinical trial of oocyte donation to women of advanced reproductive age [see comment]. *JAMA*. 1992;268:1275. [erratum appears in JAMA, 269, 476, 1993].
88. Jackson S, Hong C, Wang ET Alexander C, Gregory KD, Pisarska MD. Pregnancy outcomes in very advanced maternal age pregnancies: The impact of assisted reproductive technology. *Fertil Steril*. 2015;103(1):76–80.
89. Kort DH, Gosselin J, Choi JM, Thornton MH, Cleary-Goldman J, Sauer MV. Pregnancy after age 50: Defining risks for mother and child. *Am J Perinatol*. 2012;29(4):245–50.
90. Paulson RJ, Boostanfar R, Saadat P, Mor E, Tourgeman DE, Slater CC et al. Pregnancy in the sixth decade of life: Obstetric outcomes in women of advanced reproductive age. *JAMA*. 2002;288(18):2320–3.
91. Krieg SA, Henne MB, Westphal LM. Obstetric outcomes in donor oocyte pregnancies compared with advanced maternal age in in vitro fertilization pregnancies. *Fertil Steril*. 2008;90(1):65–70.
92. Porreco RP, Harden L, Gambotto M, Shapiro H. Expectation of pregnancy outcome among mature women. *Am J Obstet Gynecol*. 2005;192:38.
93. Borini A, Bafaro G, Violini F, Bianchi L, Casadio V, Flamigni C. Pregnancies in post-menopausal women over 50 years old in an oocyte donation program. *Fertil Steril*. 1995;63:258.
94. Sauer MV, Paulson RJ, Lobo RA. Oocyte donation to women of advanced reproductive age: Pregnancy results and obstetrical outcomes in patients 45 years and older. *Hum Reprod*. 1996;11:2540.
95. Antinori S, Gholami GH, Versaci C, Cerusico F, Dani L, Antinori M. Obstetric and prenatal outcome in menopausal women: A 12-year clinical study. *Reprod Biomed Online*. 2003;6:257.
96. Shufaro Y, Schenker JG. The risks and outcome of pregnancy in an advanced maternal age in oocyte donation cycles. *J Matern Fetal Neonatal Med*. 2014;27(16):1703–9.
97. Steiner AZ, Paulson RJ. Motherhood after age 50: An evaluation of parenting stress and physical functioning. *Fertil Steril*. 2005;87:1327–32.
98. Ethics Committee of the American Society for Reproductive Medicine. Oocyte or embryo donation to women of advanced age: A committee opinion. *Fertil Steril*. 2013;100(2):337–40.

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Endometrial Receptivity

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Introduction

The crossroad of assisted reproduction and genomics confronts us with intriguing questions and new avenues of diagnosis in precision medicine. The endometrial factor has been historically neglected due to the absence of real knowledge about its impact in infertility and implantation failure, as well as the lack of objective diagnosis for endometrial receptivity. The concept of the window of opportunity as a prerequisite for successful embryo implantation and pregnancy was first described in early ancient civilization. Soranus (98–117 AD), a great Greek scholar, in his book *Soranus' Gynecology*, gives a classical description of the fertile period in natural cycles, commencing a couple of days after the menstrual bleeding (1). Little was added until the era of Hippocrates (460–377 BCE), which had a lasting fascination, where he brought a rationalistic perspective with relevance to many infertility treatments in his books: *Diseases of Women*, *Nature of women*, and *The generating seed and the nature of child* (2). The medieval physicians followed this concept until the twentieth century, when diagnostic methods began to emerge (e.g., Huhner's postcoital test, Ascheim Zondek test) followed by the discovery of human chorionic gonadotropin (HCG) in 1962, a cardinal step in assisted reproduction (3,4).

The history of endometrial histology dates back to the diligent efforts of Noyes et al. in the 1950s, when the first diagnostic evaluation for the endometrial factor as a method of endometrial dating in natural cycles was achieved. This commendable observation in a setting with limited modern technology, inspiring both for its perseverance and scientific insight by this group, was the crux of the knowledge utilized until recently (5). In this chapter, we aim that our readers will be able to better understand the complex field of endometrial receptivity, the pros and cons of the diagnostic techniques clinically used today, and future perspectives.

Endometrial Receptivity, the "Window of Implantation"

The endometrium can be considered as the lifeline of mammalian reproduction. Human endometrium is a hormonally regulated organ with outstanding plasticity that undergoes cyclic morphological and functional changes due to the effect of the hypothalamic–pituitary–ovarian axis in preparation for the arrival of the embryo. The period when the endometrium becomes maximally receptive to the attachment of the human embryo in the mid-secretory phase is termed the receptive endometrium, or the window of implantation (WOI). The terms "uterine receptivity" and "fertile window" are other synonyms used in the scientific literature. The perception of endometrial receptivity and the existence of a WOI were first suggested by the scholarly works of Hertig and Rock in 1956 (5,6).

The WOI is a limited period of time that is synchronized with the developing embryo, a notion deciphered from ovum donation models in the 1990s (7). In relation to this, a population study evaluating natural conceptions demonstrated the concept that the human embryo implants 8–10 days after ovulation, and any implantation beyond the normal receptive endometrial phase resulted in early pregnancy loss (8). However, in this important contribution, the day of ovulation was defined on the basis of changes

in urinary excretion of the estradiol metabolite estrone 3-glucuronide and the progesterone metabolite pregnanediol 3-glucuronide, which were measured by radioimmunoassay. The authors developed an algorithm to identify the day of ovulation on the basis of the ratio of these urinary hormone metabolites, and claimed the test was similar to measurement of the luteinizing hormone (LH) peak (8). Now, 26 years later, the method proposed by the authors to time ovulation was never clinically adopted, and we recognize the limitations of the use of LH measurement in urine or even in blood to predict ovulation (9). Every LH surge does not result in ovulation, and some women may have multiple peaks or a plateau in the same menstrual cycle (9,10). Nevertheless, the clinical community has since assumed that the endometrium in all patients becomes receptive during the indicated time frame (7–9 days after ovulation), regardless of individual characteristics or the hormonal treatment received (natural cycles, controlled ovarian stimulation, etc.). Due to the use of direct molecular diagnosis of endometrial receptivity based on the transcriptomic signature in the last decade, we now know that the WOI is personalized, and the duration might last from 12 hours to 2 days; moreover, in 30% of patients it is displaced, ranging from progesterone +4 days to progesterone +7 days in a hormone replacement cycle (11–16).

Except for the receptive period, the endometrium remains hostile to embryo implantation in the rest of the phases, rendering it non-receptive. Failure of the endometrium to attain a receptive status is an important cause of infertility (17,18), and this is a challenge to infertility specialists due to lack of a reliable diagnostic test. Despite assisted reproductive technologies (ART) promising a substantial ray of hope to such patients, hormonal treatment by itself may disturb the endometrial profile, owing to a non-receptive endometrium secondary to premature secretory changes or dys-synchronous glandular and stromal differentiation (19–22).

Biomarkers of Endometrial Receptivity

The best marker of a receptive endometrium is the successful implantation of the blastocyst itself (23,24). At the morphological level, the human endometrium is composed of epithelial, stromal, vascular, and immune cell compartments. The endometrial epithelium is the first mediator in the dialogue between the embryo and the endometrium. The initial adhesion process at the time where the ectoplasmic projections called pinopodes appear was earlier claimed to be important in establishing the diagnosis of receptive endometrium (25). However, in recent years the significance of pinopodes as markers of endometrial receptivity markers has been disputed (26,27). The decidual reaction in humans is a conceptus-independent process occurring by default immediately after the closure of the WOI, and is essential for coordinated trophoblastic invasion and placental formation, lest results in abnormal implantation (28). It is characterized by ultrastructural modifications in fibroblast-like endometrial stromal cells into larger and rounded decidual cells, as well as growth and development of secretory glandules. The secretion of specific molecules such as prolactin (PRL) and the insulin-like growth factor binding protein-1 (IGFBP-1), and extracellular matrices such as laminin, type IV collagen, fibronectin, and heparin sulphate proteoglycan play an important role in embryo-endometrial dialogue. Several biomarkers of endometrial receptivity such as cell adhesion molecules (integrins, cadherins, selectins, immunoglobulins, mucins, cytokines), leukemia inhibitory factor (LIF), interleukins (IL-6, IL-11), growth factors (VEGF, CSF, TGFβ), and prostaglandins have been unraveled, and many are still being identified. However, no explicit clinically applicable receptivity marker/s has been identified yet.

Endometrial immune cells have an essential role in implantation and pre-implantation processes, to allow invasion and maintenance of the fetal semi-allograft (29); however, there is limited understanding of their functional mechanism. The most widely studied are the uterine natural killer (uNK) cells and macrophages, and dendritic and T cells (30,31). Increased levels of pro-inflammatory T helper cells (Th)-1 and cytokines (LIF, IL-6, IL-8, TNFα) are present in the early stages of implantation (32,33). uNK cells are the most abundant immune cells in the endometrium during the WOI and late-secretory phase (34,35), but the role of these cells in implantation and pregnancy remains elusive (36). uNK cells are $CD56^{bright,}$ a phenotype exhibited by only 10% of blood cells, the majority of which are $CD56^{dim}$ $CD16^{+ve}$, and possible roles in the regulation of trophoblast invasion and angiogenesis (37,38) have been suggested. uNK cells infiltrate the endometrium on LH+3 and accumulate around spiral arterioles and decidualized

stroma, being important in regulation of the invasion of the cytotrophoblast (36,39). Deregulation of uNK cell has been associated with reproductive disorders such as unexplained infertility (40), recurrent pregnancy loss (41), and endometriosis (42).

In light of these studies, uNK cells as biomarkers of endometrial receptivity during the mid-secretory and late-secretory phase of the menstrual cycle have been battled through in recent years (43,44). However, isolated uNK cell count alone did not have any significant correlation with pregnancy outcome, and conflicting reports in uNK and peripheral natural killer (pNK) cell studies are seen in women with infertility and recurrent miscarriage versus controls (45,46). In a recent study (43), the prognostic value of uNK cell count was significantly increased when combined with histological dating, but limitations of this classical approach have been overwhelming (47–49). Additionally, it has been difficult to associate NK cells with reproductive failure in women, or to develop them as clinical targets, so the prognostic value of measuring pNK or uNK cell parameters remains uncertain.

Assessment of Endometrial Receptivity

Rigorous observations of clinical parameters of the menstrual cycle, such as first day of bleeding, characteristics of the cervical mucus, or basal body temperature measurement, were widely used until the twentieth century, to determine the day of ovulation and therefore the period of receptivity. Nevertheless, all of these assessment techniques are considered obsolete nowadays.

Trans-vaginal ultrasound assessment of the endometrium has been popular globally in clinical practice as a noninvasive diagnostic, if not definitive, test for endometrial receptivity, measuring the endometrial thickness, echogenicity, and uterine artery blood flow indices. However, it is unclear how helpful these measurements are, as they do not reflect the molecular maturity of the endometrium (50,51). A trilaminar endometrial pattern and a thickness between 6–12 mm are indirect evidence for a receptive endometrium (52–54). Endometrial thickness <6 mm is considered "atrophic," while thickness >12 mm is indicative of hyperplasia. Recently a three dimensional (3D) approach of measuring the endometrial thickness as well as volume, uterine artery blood flow, and endometrial tissue vascular flow has been proposed to give a better understanding of endometrial receptivity (55). Nonetheless, a meta-analysis of 14 studies concluded that although a relationship between endometrial thickness and pregnancy exists, the endometrial receptivity potential is more intricate than a single measurement of endometrial thickness (56).

Histological endometrial dating by Noyes et al. (57) has also been criticized in recent years because of the subjectivity of the observer and the limitations of the criteria to discriminate between fertility and infertility (47–49). Gradually, new techniques have outstripped this popular diagnostic method.

Many studies focused on a single molecule or a family of specific molecules such as growth factors (epidermal growth factor, transforming growth factor TGF-α), integrins, cadherins, selectins, and the immunoglobulin superfamily as magic bullets (58), but with limited clinical significance. Mucins (59), cytokines (60), monoamine oxidase A (61), LIF (62), and cyclins E and p 27 (63) have been deeply studied as well. Integrins are a family of transmembrane glycoproteins that act as receptors for extracellular matrix ligand osteopontin (OPN) and contribute to cell migration and signal transduction (64). The E-tegrity test based on the presence of β3 integrin in endometrial biopsies taken between days 20 and 24 of the menstrual cycle (7–14 days after the LH surge) was proposed to diagnose luteal phase defects, unexplained infertility, endometriosis, and hydrosalpinx (65). However, the use of this test as a diagnostic method did not spread due to high variability between menstrual cycles and poor clinical relevance of its results (66,67). Dubowy et al. developed the Endometrial Function Test, based on the expression of cyclins E and p27 (63). It was based on the presence or absence of these cyclins in specific parts of the endometrial cells during receptivity acquisition.

The Quest for the Transcriptomic Signature of Endometrial Receptivity

The search for biochemical and molecular markers was accelerated with the emergence of the -omics sciences in the twenty-first century (68). The classical "-omic" approach deals with the analyses of

biological samples in a physiological context with a holistic perspective. In the stream of this enlightenment, a whole genome compendium of biological data can be extracted through the study of genes (genomics), gene expression/transcriptome profiling (transcriptomics), quantification and presence of proteome (proteomics or secretomics), lipidome (lipidomics), interactome (interactomics), and many more (http://omics.org/index.php/Omics_classification). Transcriptomics has usually been studied with microarray analysis, and is still at the present time a stable technology offering a global view among hundreds of genes differentially expressed throughout the menstrual cycle, in search of an endometrial receptivity signature (69–71). This platform also allows gene expression characterization at the mRNA level of a cell population, giving rise to a sample-specific molecular profile, thus representing new disease phenotypes or functional characteristics (72).

Most of the early studies in this field have compared the expression profile during the WOI versus different menstrual cycle stages: receptive versus pre-receptive stage (11,73–78); receptive versus proliferative stage (79–81), and receptive versus post-receptive stage (78,82,83), or the search of the complete luteal phase (20). Surplus knowledge has been accumulating in the last decade (13,84,85). However, there has been low consistency between results of different research groups due to inherent experimental design, timing of the endometrial sampling, tissue processing, and the absence of consistent guidelines for data presentation (16,83,85). Overall, the results suggest that most genes are up-regulated in the receptive (mid-secretory) stage compared to the pre-receptive (early-secretory) stages (11,75,77,79,86,87). The products involved in cell metabolism, transport, and germ cell migration and negative cell-proliferation characterize the early secretory/pre-receptive phase. Thus, an increase in metabolism is highly consistent with the nature of the early secretory endometrium being functionally active coinciding with the dynamic molecular preparations before the arrival of embryo (88). Interestingly, as part of the endometrial receptivity preparation, an activation of responses to stress, defense, humoral immunity, and innate immunity can be substantiated (88–90), being consistent with the immune response/tolerance during embryonic implantation. Key up-regulated genes involved in these processes include glycodelin, which decreases maternal immune response to the implanting embryo (91) and CXCL14, a chemokine that acts as a major recruitment stimulus for immune cells during the WOI (88), as well as chemotaxis of natural killer cells to cluster around epithelial glands (92), and IL-15 involved in uNK cell proliferation and differentiation (93) from peripheral blood CD16 (-) NK cells (94). Also, another set of overexpressed genes during the receptive phase are involved in protecting the endometrium and/or the embryo, as happens with metallothioneins and GPXs (antioxidants), which protect them from free radicals, heavy metals, and oxidative damage.

Endometrial Receptivity Analysis (ERA)

With the aim to build-up the knowledge in the quest for the transcriptomic signature of endometrial receptivity, endometrial biopsies were taken from fertile women at the proliferative (9–12 days of the menstrual cycle), pre-receptive (between LH+1 and LH+5), receptive (LH+7), and post-receptive (LH+9–LH+11) stages (83). A total of 238 differentially expressed genes were found in the endometrial cycle. Of these, 134 genes represent a specific transcriptomic signature of the receptive phase. Based on this finding, a reliable transcriptomic signature of endometrial receptivity was developed, which forms the basis of endometrial receptivity analysis (ERA) (11). This molecular tool consists initially of a customized microarray containing those 238 genes related to receptivity, coupled to a computational predictor with a specificity of 88.57% and a sensitivity of 99.76%. The accuracy and reproducibility of ERA is superior to histological dating as a diagnostic method for endometrial receptivity (12). The test analyzes an endometrial biopsy taken 7 days after the LH surge in natural cycles (LH+7) or after 5 days following progesterone administration (P+5) in hormone replacement therapy cycles (HRT). Once the mRNA has been hybridized in the customized array, the computational predictor classifies the set of gene expression values in one of the endometrial stages (proliferative, pre-receptive, receptive, post-receptive). It was expected that all the women would be in receptive phase at these cycle days (LH+7 or P+5), but after ERA clinical application, some women showed a displaced WOI, being advanced or delayed (Figure 20.1) when compared to the standard WOI (14).

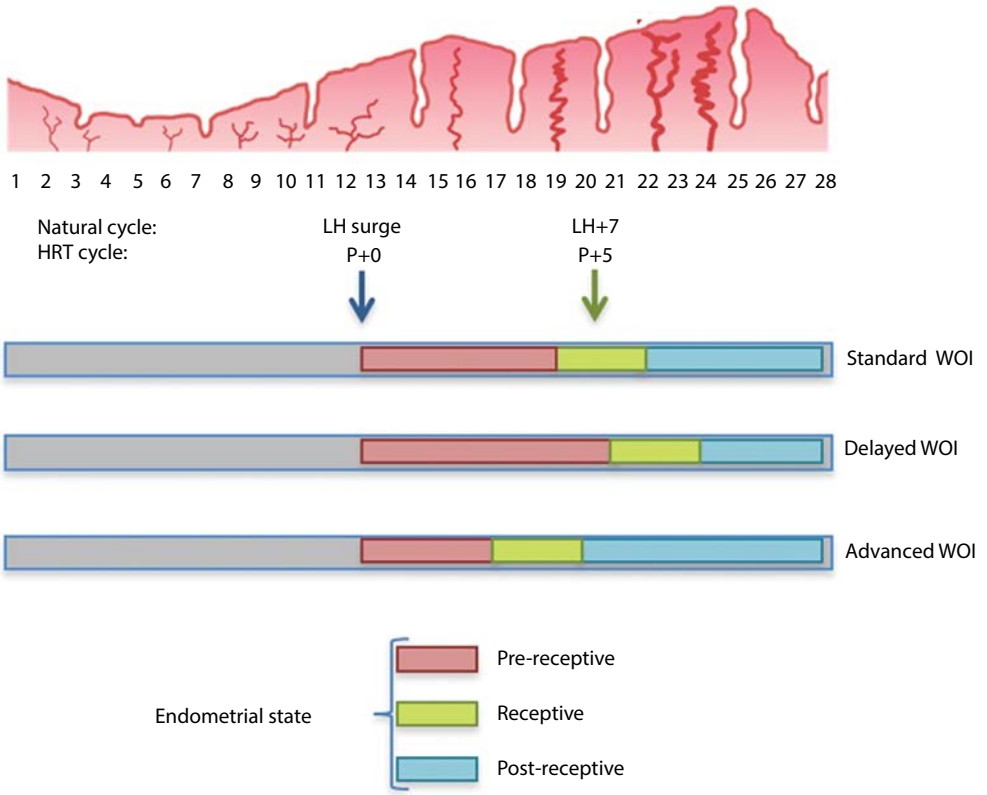


FIGURE 20.1 Endometrial profile related to the receptivity status, illustrating a standard window of implantation (WOI) against a delayed and an advanced WOI. LH: luteinizing hormone; FSH: follicle-stimulating hormone; HRT: hormone replacement therapy; P: progesterone.

Clinical Application of ERA

The identification of WOI displacements provides a new clinical concept: the personalization of the embryo transfer (pET), illustrated in Figure 20.2. This is performed on the specific recommended day according to the ERA result (14,95). In a prospective interventional multicenter clinical trial, comparing 85 recurrent implantation failure (RIF) patients versus 25 control patients with one or no previous failed cycles, one out of four RIF patients had a displaced WOI. Of these, 84% had a delayed WOI, while 16% had an advanced one (14). In order to validate where the personalized WOI (pWOI) was located, a second ERA test was performed in 18 women following the recommendation of the first one. A receptive result was obtained in 15 patients. Subsequently, pET was performed at the proposed pWOI, obtaining 50% pregnancy rate and 39% implantation rate in these patients with severe RIF of endometrial origin. These data reveal the clinical relevance of detecting displaced WOI to solve implantation failures due to desynchronized embryo transfers, avoiding an unnecessary economical and emotional impact on patients (14,15). Thus, this customized molecular diagnostic test has been instrumental in identifying the pWOI and in guiding clinicians to pET (Figures 20.3 and 20.4). This exciting finding is now being explored in an international RCT to determine the endometrial receptivity during fertility screening in reproductive care. (The ERA as a diagnostic guide for personalized embryo transfer. ClinicalTrials.gov Identifier: NCT01954758.)

Defective endometrial receptivity secondary to benign gynecological conditions, e.g., endometrial polyps, endometriosis, thin endometrium, endometrial atrophy, and hydrosalpinx, challenge the management of infertility (96). In recent years, data on genomic profile in these conditions has begun to

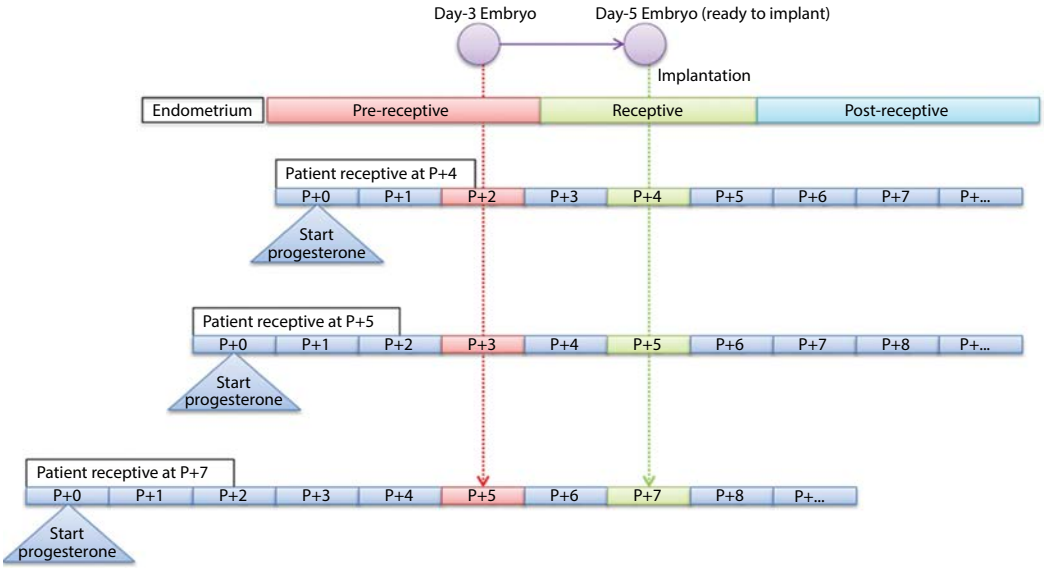


FIGURE 20.2 Personalized embryo transfer (pET) synchronizing embryo stage with receptive endometrium. The figure shows the specific day to perform an embryo transfer according to the endometrial receptive status. Day-5 embryo should be transferred on the day in which the endometrium is receptive, and a day-3 embryo two days earlier regarding this moment. Three examples are shown, with three patients being receptive at P+4, P+5, or P+7 respectively. P: progesterone.

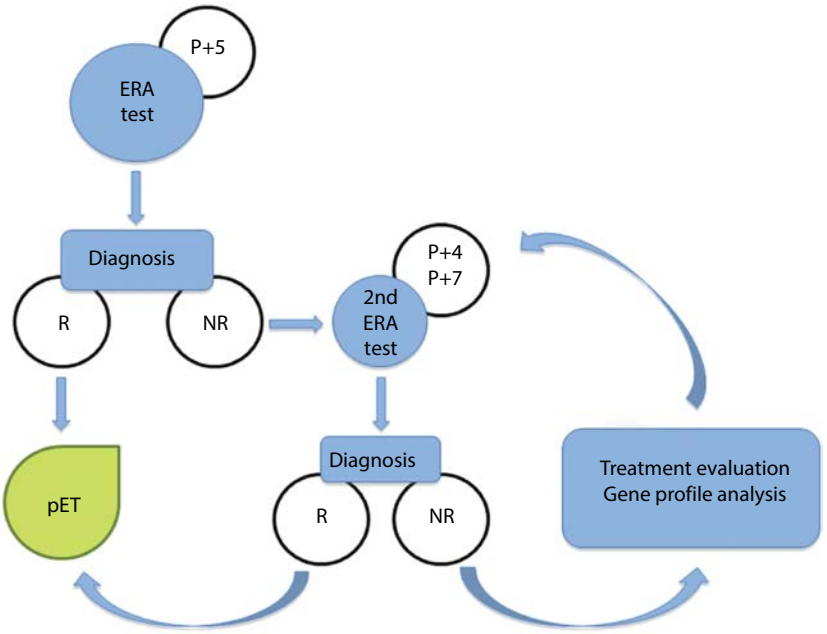


FIGURE 20.3 Decision tree of the Endometrial Receptivity Array in a hormone replacement therapy (HRT) cycle. First endometrial biopsy is performed after five full days of progesterone (P) impregnation. If result is receptive (R), a personalized embryo transfer (pET) is performed in the subsequent cycle. If the result is non-receptive (NR), the endometrium could be either in the pre-receptive or post-receptive phase, and a new biopsy will be recommended on a specific day in order to locate when the window of implantation opens. If the result is R, a pET is performed in the subsequent cycle. If the result is NR again, gene profile analysis and treatment evaluation is performed, and a new biopsy is recommended to guide fertility clinicians to plan for pET on a specific day or call for a change in the treatment.

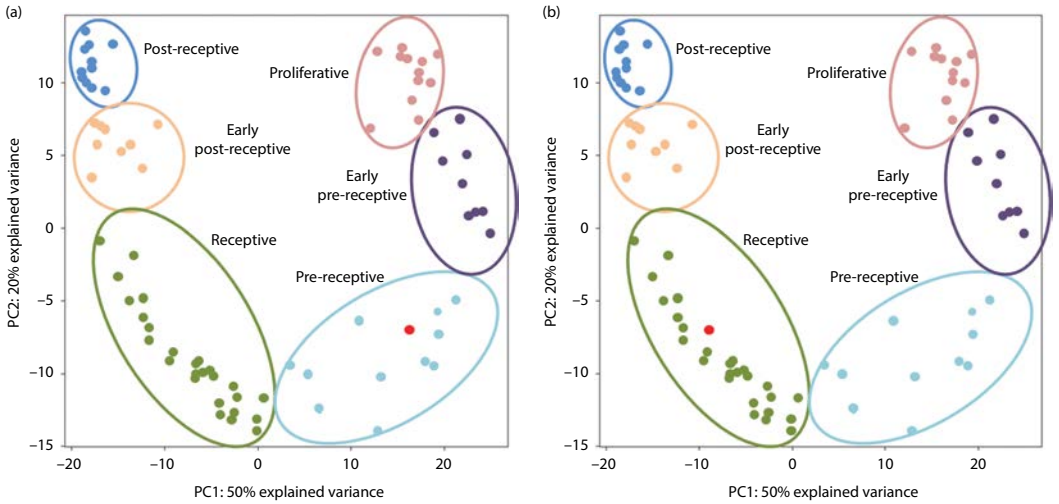


FIGURE 20.4 Evolution of endometrial profile in a patient with a displaced window of implantation. (a) Endometrial biopsy taken at P+5 (red spot) shows a pre-receptive profile compared with the control samples used to train the ERA predictor. (b) In the same patient, endometrial biopsy shows a receptive profile after two more days with progesterone, P+7 (red spot).

accumulate. Our group has consistent clinical data demonstrating that endometriosis is not detrimental to embryo implantation in ovum recipients (97–99). Endometrial markers, however, have been reported to be significantly different in eutopic endometrium between women with endometriosis compared to women without endometriosis (100), and further association between altered expression of several markers and impaired embryo implantation of endometrial origin has been proposed in women with endometriosis (65,96,101–103). Recently, a prospective, multicenter interventional trial assessing the endometrial receptivity gene signature using the ERA test in patients with different stages of endometriosis confirmed that endometrial receptivity gene signature during the WOI does not vary significantly among different stages of endometriosis patients versus controls (104).

New Perspectives in Human Endometrial Receptivity Assessment

Unlike the genome, the proteome, and the metabolome itself, are dynamic, complex, and variable. It reflects closely the cellular function and depends on the developmental stage of the cells, reflecting also the impact of environmental stimuli. New developments in mass spectrometry have increased the number of studies related to the profile of proteins expressed in the endometrial tissue and secreted to the endometrial fluid (105).

The endometrial fluid is a complex biological fluid secreted by the endometrial glands that provides nutrients for blastocyst survival and constitutes the microenvironment where the embryo–endometrial dialog occurs prior to implantation (106). The advantage of working with endometrial fluid is that it can be collected easily and painlessly by aspiration using noninvasive methods (91). Furthermore, uterine secretions are less complex than endometrial tissues at the protein repertoire level, and can serve as biomarkers for endometrial function and research. It has been demonstrated that the endometrial fluid obtained by trans-cervical aspiration immediately prior to embryo transfer does not affect the reproductive outcome within the same cycle (107). Although aspiration of endometrial fluid is a safe method, sometimes the material obtained is not sufficient for analysis, or can be diluted due to washing, making the results difficult to interpret (108).

The composition of endometrial secretion varies during the menstrual cycle, revealing three different protein patterns that are typical of the equivalent phases of the menstrual cycle: intermediate phase, proliferative phase, and secretory phase. The results present characteristic "families" of protein bands corresponding to 63 proteins, some of which are identified by their molecular weight (109).

Studies in animal models have shown the importance of lipids at the time of embryo implantation, allowing the identification and characterization of different lipids during the receptive phase. Lipid molecules such as endocannabinoids, lysophosphatidic acid, and prostaglandins (PGs) have been described during embryo implantation, that correlate to the target organ-Cox2 specific deficiency (110–112). A defective endometrial prostaglandin synthesis has been linked to repetitive implantation failure in assisted reproduction patients (113).

In recent years the lipidomic signature of prostaglandins PGE2 and PGF2α in endometrial fluid has been found to be a noninvasive biomarker of endometrial receptivity (114). These results are relevant for three reasons: first, to prove that the endometrial fluid is suitable for lipid analysis in humans, a possibility never investigated before; second, illustrating how lipid profile changes in endometrial fluid along the menstrual cycle by opening the possibility of developing noninvasive methods to determine the endometrial receptivity; and, finally, because the information can be immediately generated, providing the possibility of embryo transfer within 24 hours after the test is performed (115). Currently, clinical validation for this system is underway (ClinicalTrials.gov Identifier: NCT02189369).

Changes in the expression of epigenetic modulators such as DNA methyltransferases or changes in the markers of global acetylation in histones throughout the menstrual cycle suggest that epigenetic regulation may be relevant to control endometrial gene expression during implantation (116). Therefore, understanding the epigenetic control of implantation and diseases such as endometriosis could lead to the design of new therapeutic strategies.

In recent years, microRNAs have been investigated for their potential use as endometrial receptivity markers (117,118). MicroRNAs are short (19–22 nucleotides), highly conserved sequences of non-coding RNA that regulate the expression of 50% of endogenous genes on the human genome with relevance in cellular differentiation, proliferation, and apoptosis (119–121). They can be secreted by both endometrial and embryonic cells, and incorporated into exosomes or associated with proteins that protect them from degradation. In recent years, several microRNAs have been identified (122–124) that regulate epithelial cell proliferation and differentiation in the endometrium (125). In context with this, a human endometrial receptivity gene, the insulin growth factor-binding protein related 1 (IGFBP-rP1), in which a microRNA is localized in the glandular epithelium and endometrial cells, has been identified (126). In another study, 12 microRNAs of the MIR family were identified in the secretory endometrium, confirming that microRNAs may suppress cell proliferation (81). Additional evidence was provided by Altmae et al. (127) in endometrial biopsy samples from the pre-receptive (LH+2) and receptive phases (LH+7) from healthy fertile women in natural cycles, where, in a subset of microRNAs, has-miR-30b, has-miR-494, and has-miR-923 were down-regulated in the receptive endometrium. These findings parallel the results in previous studies comparing natural versus stimulated cycles (128). Recent studies have demonstrated the active participation of microRNAs secreted by the blastocyst in the implantation process, allowing their use as biomarkers of implantation potential, or as targets to treat implantation failure and infertility (129,130). A novel cell-to-cell communication mechanism that involves hsa-miR-30d and its associated exosome, secreted by maternal endometrium, has recently been identified (131). According to this study, both exosome and free hsa-miR-30d are taken up by the embryo trophectoderm cells from the endometrial fluid, then incorporated into the RISC complex to exert gene regulation under physiological conditions, thereby resulting in modifications of the transcriptome and embryo adhesion. To summarize, the evidence provided above suggest that endometrial microRNAs have a potential role to regulate genes involved in the endometrial receptivity and early gestation, although the exact role still needs to be delineated.

New perspectives in endometrial assessment are difficult to predict, but certainly the next mandatory technological step would be the use of sequencing technologies. This knowledge has to be integrated into a system biology approach in order to achieve a holistic understanding of endometrial receptivity. It is within the realm of possibility that within the next few years, endometrial defects and abnormal maternal–embryo synchrony will be diagnosed and treatment tailored to a person's unique genetic profile, a concept fast emerging as 'personalized medicine' in reproductive health. This would guide fertility specialists, molecular biologists, and genetic counselors to craft a lifelong health maintenance strategy tailored to a person's unique genetic constitution.

Diving in deeper, and incorporating endometrial assessment into personalized medicine, assisted reproduction techniques (ART) will evolve, shifting the emphasis from reaction to detection, prescribing

customized fertility. This would assist in eliminating trial-and-error inefficiencies that inflate health care costs, demotivate patients, and reduce the time, enabling personalized embryo transfer (pET) with faster outcome feedback to aspiring mothers.

REFERENCES

1. Soranus, Temkin O. *Soranus' Gynecology.* Baltimore: Johns Hopkins University Press, 1956, xlix, 258 pp.
2. King H. *Hippocrates' Woman Reading the Female Body in Ancient Greece*. London; New York: Routledge; 1998, xvi, 322 pp.
3. Gemzell CA. Induction of ovulation with human pituitary gonadotrophins. *Fertil Steril*. 1962;13:153–68.
4. Gemzell CA, Roos P, Loeffler FE. The clinical use of pituitary gonadotrophins in women. *J Reprod Fertil*. 1966;12(1):49–64.
5. Noyes RW, Hertig, AT, Rock, J. Dating of endometrial biopsy. *Fertil Steril*. 1950;1:3–17.
6. Hertig AT, Rock J, Adams EC. A description of 34 human ova within the first 17 days of development. *Am J Anat*. 1956;98(3):435–93.
7. Navot D, Scott RT, Droesch K, Veeck LL, Liu HC, Rosenwaks Z. The window of embryo transfer and the efficiency of human conception in vitro. *Fertil Steril*. 1991;55(1):114–8.
8. Wilcox AJ, Baird DD, Weinberg CR. Time of implantation of the conceptus and loss of pregnancy. *N Engl J Med*. 1999;340(23):1796–9. Epub 1999/06/11. eng.
9. Direito A, Bailly S, Mariani A, Ecochard R. Relationships between the luteinizing hormone surge and other characteristics of the menstrual cycle in normally ovulating women. *Fertil Steril*. 2013;99(1):279–85.
10. Park SJ, Goldsmith LT, Skurnick JH, Wojtczuk A, Weiss G. Characteristics of the urinary luteinizing hormone surge in young ovulatory women. *Fertil Steril*. 2007;88(3):684–90.
11. Diaz-Gimeno P, Horcajadas JA, Martinez-Conejero JA, Esteban FJ, Alama P, Pellicer A et al. A genomic diagnostic tool for human endometrial receptivity based on the transcriptomic signature. *Fertil Steril*. 2011;95(1):50–60, e1–15.
12. Díaz-Gimeno P, Ruiz-Alonso M, Blesa D, Bosch N, Martínez-Conejero JA, Alamá P et al. The accuracy and reproducibility of the endometrial receptivity array is superior to histology as a diagnostic method for endometrial receptivity. *Fertil Steril*. 2013 2//;99(2):508–17.
13. Garrido-Gomez T, Ruiz-Alonso M, Blesa D, Diaz-Gimeno P, Vilella F, Simon C. Profiling the gene signature of endometrial receptivity: Clinical results. *Fertil Steril*. 2013;99(4):1078–85.
14. Ruiz-Alonso M, Blesa D, Diaz-Gimeno P, Gomez E, Fernandez-Sanchez M, Carranza F et al. The endometrial receptivity array for diagnosis and personalized embryo transfer as a treatment for patients with repeated implantation failure. *Fertil Steril*. 2013;100(3):818–24.
15. Ruiz-Alonso M, Galindo N, Pellicer A, Simon C. What a difference two days make: "Personalized" embryo transfer (pET) paradigm: A case report and pilot study. *Hum Reprod*. 2014;29(6):1244–7.
16. Ulbrich SE, Groebner AE, Bauersachs S. Transcriptional profiling to address molecular determinants of endometrial receptivity--lessons from studies in livestock species. *Methods*. 2013;59(1):108–15.
17. Edwards RG. Human uterine endocrinology and the implantation window. *Ann NY Acad Sci*. 1988;541:445–54. Epub 1988/01/01. eng.
18. Edwards RG. Clinical approaches to increasing uterine receptivity during human implantation. *Hum Reprod*. 1995;10(Suppl 2):60–6. Epub 1995/12/01. eng.
19. Bourgain C, Devroey P. The endometrium in stimulated cycles for IVF. *Hum Reprod Update*. 2003;9(6):515–22.
20. Kolibianakis E, Bourgain C, Albano C, Osmanagaoglu K, Smitz J, Van Steirteghem A et al. Effect of ovarian stimulation with recombinant follicle-stimulating hormone, gonadotropin releasing hormone antagonists, and human chorionic gonadotropin on endometrial maturation on the day of oocyte pick-up. *Fertil Steril*. 2002;78(5):1025–9.
21. Kolibianakis EM, Devroey P. The luteal phase after ovarian stimulation. *Reprod Biomed Online*. 2002;5(Suppl 1):26–35.
22. Tavaniotou A, Albano C, Smitz J, Devroey P. Effect of clomiphene citrate on follicular and luteal phase luteinizing hormone concentrations in in vitro fertilization cycles stimulated with gonadotropins and gonadotropin-releasing hormone antagonist. *Fertil Steril*. 2002;77(4):733–7.
23. Klentzeris LD. The role of endometrium in implantation. *Hum Reprod*. 1997;12(11 Suppl):170–5.

24. Younis JS, Simon A, Laufer N. Endometrial preparation: Lessons from oocyte donation. *Fertil Steril*. 1996;66(6):873–84.
25. Murphy CR. Understanding the apical surface markers of uterine receptivity—Pinopods—Or uterodomes? *Hum Reprod*. 2000;15(12):2451–4. English.
26. Lessey BA. Assessment of endometrial receptivity. *Fertil Steril*. 2011;96(3):522–9.
27. Quinn CE, Casper RF. Pinopodes: A questionable role in endometrial receptivity. *Hum Reprod Update*. 2009;15(2):229–36.
28. Tanaka T, Wang C, Umesaki N. Autocrine/paracrine regulation of human endometrial stromal remodeling by laminin and type IV collagen. *Int J Mol Med*. 2008;22(5):581–7.
29. Robertson SA. Control of the immunological environment of the uterus. *Rev Reprod*. 2000;5(3):164–74.
30. Gnainsky Y, Dekel N, Granot I. Implantation: Mutual activity of sex steroid hormones and the immune system guarantee the maternal-embryo interaction. *Semin Reprod Med*. 2014;32(5):337–45.
31. Dekel N, Gnainsky Y, Granot I, Racicot K, Mor G. The role of inflammation for a successful implantation. *Am J Reprod Immunol*. 2014;72(2):141–7.
32. Dominguez F, Yanez-Mo M, Sanchez-Madrid F, Simon C. Embryonic implantation and leukocyte transendothelial migration: Different processes with similar players? *FASEB J*. 2005;19(9):1056–60.
33. Van Sinderen M, Menkhorst E, Winship A, Cuman C, Dimitriadis E. Preimplantation human blastocyst-endometrial interactions: The role of inflammatory mediators. *Am J Reprod Immunol*. 2013;69(5):427–40.
34. Bulmer JN, Longfellow M, Ritson A. Leukocytes and resident blood cells in endometrium. *Ann NY Acad Sci*. 1991;622:57–68.
35. Salamonsen LA, Woolley DE. Menstruation: Induction by matrix metalloproteinases and inflammatory cells. *J Reprod Immunol*. 1999;44(1–2):1–27.
36. Moffett-King A, Entrican G, Ellis S, Hutchinson J, Bainbridge D. Natural killer cells and reproduction. *Trends Immunol*. 2002;23(7):332–3.
37. Dosiou C, Giudice LC. Natural killer cells in pregnancy and recurrent pregnancy loss: Endocrine and immunologic perspectives. *Endocr Rev*. 2005;26(1):44–62.
38. Bulmer JN, Williams PJ, Lash GE. Immune cells in the placental bed. *Int J Dev Biol*. 2010;54(2–3):281–94.
39. King A, Hiby SE, Gardner L, Joseph S, Bowen JM, Verma S et al. Recognition of trophoblast HLA class I molecules by decidual NK cell receptors--a review. *Placenta*. 2000;21(Suppl A):S81–5.
40. Sacks G, Yang Y, Gowen E, Smith S, Fay L, Chapman M. Detailed analysis of peripheral blood natural killer cells in women with repeated IVF failure. *Am J Reprod Immunol*. 2012;67(5):434–42.
41. Karami N, Boroujerdnia MG, Nikbakht R, Khodadadi A. Enhancement of peripheral blood CD56(dim) cell and NK cell cytotoxicity in women with recurrent spontaneous abortion or in vitro fertilization failure. *J Reprod Immunol*. 2012;95(1–2):87–92.
42. Giuliani E, Parkin KL, Lessey BA, Young SL, Fazleabas AT. Characterization of uterine NK cells in women with infertility or recurrent pregnancy loss and associated endometriosis. *Am J Reprod Immunol*. 2014;72(3):262–9.
43. Liu B, Mariee N, Laird S, Smith J, Li J, Li TC. The prognostic value of uNK cell count and histological dating in the mid-luteal phase of women with reproductive failure. *Eur J Obstet Gyn R B*. 2014;181:171–5.
44. Ledee N, Petitbarat M, Rahmati M, Dubanchet S, Chaouat G, Sandra O et al. New pre-conception immune biomarkers for clinical practice: Interleukin-18, interleukin-15 and TWEAK on the endometrial side, G-CSF on the follicular side. *J Reprod Immunol*. 2011;88(2):118–23.
45. Tang AW, Alfirevic Z, Quenby S. Natural killer cells and pregnancy outcomes in women with recurrent miscarriage and infertility: A systematic review. *Hum Reprod*. 2011;26(8):1971–80.
46. Matteo MG, Greco P, Rosenberg P, Mestice A, Baldini D, Falagario T et al. Normal percentage of CD56bright natural killer cells in young patients with a history of repeated unexplained implantation failure after in vitro fertilization cycles. *Fertil Steril*. 2007;88(4):990–3.
47. Sharkey AM, Smith SK. The endometrium as a cause of implantation failure. *Best Pract Res Clin Obstet Gynaecol*. 2003;17(2):289–307.
48. Myers ER, Silva S, Barnhart K, Groben PA, Richardson MS, Robboy SJ et al. Interobserver and intraobserver variability in the histological dating of the endometrium in fertile and infertile women. *Fertil Steril*. 2004;82(5):1278–82.
49. Coutifaris C, Myers ER, Guzick DS, Diamond MP, Carson SA, Legro RS et al. Histological dating of timed endometrial biopsy tissue is not related to fertility status. *Fertil Steril*. 2004;82(5):1264–72.

50. Alcazar JL. Three-dimensional ultrasound assessment of endometrial receptivity: A review. *Reprod Biol Endocrinol*. 2006;4:56.
51. Merce LT, Barco MJ, Bau S, Troyano J. Are endometrial parameters by three-dimensional ultrasound and power Doppler angiography related to in vitro fertilization/embryo transfer outcome? *Fertil Steril*. 2008;89(1):111–7.
52. Oliveira JB, Baruffi RL, Mauri AL, Petersen CG, Borges MC, Franco JG, Jr. Endometrial ultrasonography as a predictor of pregnancy in an in-vitro fertilization programme after ovarian stimulation and gonadotrophin-releasing hormone and gonadotrophins. *Hum Reprod*. 1997;12(11):2515–8.
53. Zhang X, Chen CH, Confino E, Barnes R, Milad M, Kazer RR. Increased endometrial thickness is associated with improved treatment outcome for selected patients undergoing in vitro fertilization-embryo transfer. *Fertil Steril*. 2005;83(2):336–40.
54. McWilliams GD, Frattarelli JL. Changes in measured endometrial thickness predict in vitro fertilization success. *Fertil Steril*. 2007;88(1):74–81.
55. Ledee N. Uterine receptivity and the two and three dimensions of ultrasound. *Ultrasound Obstet Gynecol*. 2005;26(7):695–8.
56. Momeni M, Rahbar MH, Kovanci E. A meta-analysis of the relationship between endometrial thickness and outcome of in vitro fertilization cycles. *J Hum Reprod Sc*. 2011;4(3):130–7.
57. Noyes RW, Hertig AT, Rock J. Dating the endometrial biopsy. *Am J Obstet Gynecol*. 1975;122(2):262–3. Epub 1975/05/01. eng.
58. Giudice LC, Saleh W. Growth factors in reproduction. *Trends Endocrinol Metab*. 1995;6(2):60–9.
59. Meseguer M, Aplin JD, Caballero-Campo P, O'Connor JE, Martin JC, Remohi J et al. Human endometrial mucin MUC1 is up-regulated by progesterone and down-regulated in vitro by the human blastocyst. *Biol Reprod*. 2001;64(2):590–601.
60. Tabibzadeh S, Sun XZ. Cytokine expression in human endometrium throughout the menstrual cycle. *Hum Reprod*. 1992;7(9):1214–21.
61. Henriquez S, Tapia A, Quezada M, Vargas M, Cardenas H, Rios M et al. Deficient expression of monoamine oxidase A in the endometrium is associated with implantation failure in women participating as recipients in oocyte donation. *Mol Hum Reprod*. 2006;12(12):749–54.
62. Brinsden PR, Alam V, de Moustier B, Engrand P. Recombinant human leukemia inhibitory factor does not improve implantation and pregnancy outcomes after assisted reproductive techniques in women with recurrent unexplained implantation failure. *Fertil Steril*. 2009;91(4 Suppl):1445–7.
63. Dubowy RL, Feinberg RF, Keefe DL, Doncel GF, Williams SC, McSweet JC et al. Improved endometrial assessment using cyclin E and p27. *Fertil Steril*. 2003;80(1):146–56.
64. Lessey BA, Castelbaum AJ, Buck CA, Lei Y, Yowell CW, Sun J. Further characterization of endometrial integrins during the menstrual cycle and in pregnancy. *Fertil Steril*. 1994;62(3):497–506.
65. Lessey BA, Castelbaum AJ, Sawin SW, Buck CA, Schinnar R, Bilker W et al. Aberrant integrin expression in the endometrium of women with endometriosis. *J Clin Endocrinol Metab*. 1994;79(2):643–9.
66. Creus M, Ordi J, Fabregues F, Casamitjana R, Ferrer B, Coll E et al. alphavbeta3 integrin expression and pinopod formation in normal and out-of-phase endometria of fertile and infertile women. *Hum Reprod*. 2002;17(9):2279–86.
67. Ordi J, Creus M, Quinto L, Casamitjana R, Cardesa A, Balasch J. Within-subject between-cycle variability of histological dating, alpha v beta 3 integrin expression, and pinopod formation in the human endometrium. *J Clin Endocrinol Metab*. 2003;88(5):2119–25.
68. Quackenbush J. Microarray analysis and tumor classification. *N Engl J Med*. 2006;354(23):2463–72.
69. Bersinger NA, Wunder DM, Birkhauser MH, Mueller MD. Gene expression in cultured endometrium from women with different outcomes following IVF. *Mol Hum Reprod*. 2008;14(8):475–84.
70. Margalioth EJ, Ben-Chetrit A, Gal M, Eldar-Geva T. Investigation and treatment of repeated implantation failure following IVF-ET. *Hum Reprod*. 2006;21(12):3036–43.
71. Kao LC, Germeyer A, Tulac S, Lobo S, Yang JP, Taylor RN et al. Expression profiling of endometrium from women with endometriosis reveals candidate genes for disease-based implantation failure and infertility. *Endocrinology*. 2003;144(7):2870–81.
72. Nevins JR, Potti A. Mining gene expression profiles: Expression signatures as cancer phenotypes. *Nature Rev Genet*. 2007;8(8):601–9.

73. Carson DD, Lagow E, Thathiah A, Al-Shami R, Farach-Carson MC, Vernon M et al. Changes in gene expression during the early to mid-luteal (receptive phase) transition in human endometrium detected by high-density microarray screening. *Molec Hum Reprod*. 2002;8(9):871–9. Epub 2002/08/30. eng.
74. Franchi A, Zaret J, Zhang X, Bocca S, Oehninger S. Expression of immunomodulatory genes, their protein products and specific ligands/receptors during the window of implantation in the human endometrium. *Mol Hum Reprod*. 2008;14(7):413–21. Epub 2008/06/06. eng.
75. Haouzi D, Assou S, Mahmoud K, Tondeur S, Reme T, Hedon B et al. Gene expression profile of human endometrial receptivity: Comparison between natural and stimulated cycles for the same patients. *Hum Reprod*. 2009;24(6):1436–45.
76. Mirkin S, Arslan M, Churikov D, Corica A, Diaz JI, Williams S et al. In search of candidate genes critically expressed in the human endometrium during the window of implantation. *Hum Reprod*. 2005;20(8):2104–17.
77. Riesewijk A, Martin J, van Os R, Horcajadas JA, Polman J, Pellicer A et al. Gene expression profiling of human endometrial receptivity on days LH+2 versus LH+7 by microarray technology. *Molec Hum Reprod*. 2003;9(5):253–64. Epub 2003/05/03. eng.
78. Tseng LH, Chen I, Chen MY, Yan H, Wang CN, Lee CL. Genome-based expression profiling as a single standardized microarray platform for the diagnosis of endometrial disorder: An array of 126-gene model. *Fertil Steril*. 2010;94(1):114–9.
79. Borthwick JM, Charnock-Jones DS, Tom BD, Hull ML, Teirney R, Phillips SC et al. Determination of the transcript profile of human endometrium. *Molec Hum Reprod*. 2003;9(1):19–33. Epub 2003/01/17. eng.
80. Kao LC, Tulac S, Lobo S, Imani B, Yang JP, Germeyer A et al. Global gene profiling in human endometrium during the window of implantation. *Endocrinology*. 2002;143(6):2119–38. Epub 2002/05/22. eng.
81. Kuokkanen S, Chen B, Ojalvo L, Benard L, Santoro N, Pollard JW. Genomic profiling of microRNAs and messenger RNAs reveals hormonal regulation in microRNA expression in human endometrium. *Biol Reprod*. 2010;82(4):791–801.
82. Critchley HO, Robertson KA, Forster T, Henderson TA, Williams AR, Ghazal P. Gene expression profiling of mid to late secretory phase endometrial biopsies from women with menstrual complaint. *Am J Obstet Gynecol*. 2006;195(2):406 e1–16.
83. Horcajadas JA, Pellicer A, Simon C. Wide genomic analysis of human endometrial receptivity: New times, new opportunities. *Hum Reprod Update*. 2007;13(1):77–86.
84. Altmae S, Esteban FJ, Stavreus-Evers A, Simon C, Giudice L, Lessey BA et al. Guidelines for the design, analysis and interpretation of "omics" data: Focus on human endometrium. *Hum Reprod Update*. 2014;20(1):12–28.
85. Ruiz-Alonso M, Blesa D, Simon C. The genomics of the human endometrium. *Biochim Biophys Acta*. 2012;1822(12):1931–42.
86. Haouzi D, Dechaud H, Assou S, De Vos J, Hamamah S. Insights into human endometrial receptivity from transcriptomic and proteomic data. *Reprod Biomed Online*. 2012;24(1):23–34.
87. Horcajadas JA, Minguez P, Dopazo J, Esteban FJ, Dominguez F, Giudice LC et al. Controlled ovarian stimulation induces a functional genomic delay of the endometrium with potential clinical implications. *J Clin Endocrinol Metab*. 2008;93(11):4500–10.
88. Talbi S, Hamilton AE, Vo KC, Tulac S, Overgaard MT, Dosiou C et al. Molecular phenotyping of human endometrium distinguishes menstrual cycle phases and underlying biological processes in normo-ovulatory women. *Endocrinology*. 2006;147(3):1097–121.
89. Giudice LC. Application of functional genomics to primate endometrium: Insights into biological processes. *Reprod Biol Endocrinol*. 2006;4(Suppl 1):S4.
90. Simmen FA, Simmen RC. Orchestrating the menstrual cycle: Discerning the music from the noise. *Endocrinology*. 2006;147(3):1094–6.
91. Aghajanova LS, C. Horcajadas, JA. Are favourite molecules of endometrial receptivity still in favour? *Expert Rev Obstet Gynecol*. 2008;3:487–501.
92. Mokhtar NM, Cheng CW, Cook E, Bielby H, Smith SK, Charnock-Jones DS. Progestin regulates chemokine (C-X-C motif) ligand 14 transcript level in human endometrium. *Mol Hum Reprod*. 2010;16(3):170–7.

93. Okada H, Nakajima T, Sanezumi M, Ikuta A, Yasuda K, Kanzaki H. Progesterone enhances interleukin-15 production in human endometrial stromal cells in vitro. *J Clin Endocrinol Metab.* 2000;85(12):4765–70.
94. Kitaya K, Yamaguchi T, Honjo H. Central role of interleukin-15 in postovulatory recruitment of peripheral blood CD16(-) natural killer cells into human endometrium. *J Clin Endocrinol Metab.* 2005;90(5):2932–40.
95. Blesa D, Ruiz-Alonso M, Simon C. Clinical management of endometrial receptivity. *Semin Reprod Med*. 2014;32(5):410–3.
96. Revel A. Defective endometrial receptivity. *Fertil Steril*. 2012;97(5):1028–32.
97. Budak E, Garrido N, Soares SR, Melo MA, Meseguer M, Pellicer A et al. Improvements achieved in an oocyte donation program over a 10-year period: Sequential increase in implantation and pregnancy rates and decrease in high-order multiple pregnancies. *Fertil Steril*. 2007;88(2):342–9.
98. Diaz I, Navarro J, Blasco L, Simon C, Pellicer A, Remohi J. Impact of stage III-IV endometriosis on recipients of sibling oocytes: Matched case-control study. *Fertil Steril*. 2000;74(1):31–4.
99. Simon C, Gutierrez A, Vidal A, de los Santos MJ, Tarin JJ, Remohi J et al. Outcome of patients with endometriosis in assisted reproduction: Results from in-vitro fertilization and oocyte donation. *Hum Reprod*. 1994;9(4):725–9.
100. May KE, Villar J, Kirtley S, Kennedy SH, Becker CM. Endometrial alterations in endometriosis: A systematic review of putative biomarkers. *Hum Reprod Update*. 2011;17(5):637–53.
101. Daftary GS, Troy PJ, Bagot CN, Young SL, Taylor HS. Direct regulation of beta3-integrin subunit gene expression by HOXA10 in endometrial cells. *Mol Endocrinol*. 2002;16(3):571–9.
102. Taylor HS, Bagot C, Kardana A, Olive D, Arici A. HOX gene expression is altered in the endometrium of women with endometriosis. *Hum Reprod.* 1999;14(5):1328–31.
103. Wei Q, St Clair JB, Fu T, Stratton P, Nieman LK. Reduced expression of biomarkers associated with the implantation window in women with endometriosis. *Fertil Steril*. 2009;91(5):1686–91.
104. Garcia-Velasco JA, Fassbender A, Ruiz-Alonso M, Blesa D, D'Hooghe T, Simon C. Is endometrial receptivity transcriptomics affected in women with endometriosis? A pilot study. *Reprod Biomed Online*. 2015;31(5):647–54.
105. Casado-Vela J, Rodriguez-Suarez E, Iloro I, Ametzazurra A, Alkorta N, Garcia-Velasco JA et al. Comprehensive proteomic analysis of human endometrial fluid aspirate. *J Proteome Res*. 2009;8(10):4622–32.
106. Thouas GA, Dominguez F, Green MP, Vilella F, Simon C, Gardner DK. Soluble ligands and their receptors in human embryo development and implantation. *Endocr Rev.* 2015;36(1):92–130.
107. van der Gaast MH, Beier-Hellwig K, Fauser BC, Beier HM, Macklon NS. Endometrial secretion aspiration prior to embryo transfer does not reduce implantation rates. *Reprod Biomed Online*. 2003;7(1):105–9.
108. Olivennes F, Ledee-Bataille N, Samama M, Kadoch J, Taupin JL, Dubanchet S et al. Assessment of leukemia inhibitory factor levels by uterine flushing at the time of egg retrieval does not adversely affect pregnancy rates with in vitro fertilization. *Fertil Steril*. 2003;79(4):900–4.
109. Lessey BA, Killam AP, Metzger DA, Haney AF, Greene GL, McCarty KS, Jr. Immunohistochemical analysis of human uterine estrogen and progesterone receptors throughout the menstrual cycle. *J Clin Endocrinol Metab.* 1988;67(2):334–40. Epub 1988/08/01. eng.
110. Lim H, Paria BC, Das SK, Dinchuk JE, Langenbach R, Trzaskos JM et al. Multiple female reproductive failures in cyclooxygenase 2-deficient mice. *Cell*. 1997;91(2):197–208.
111. Maccarrone M, DeFelici M, Klinger FG, Battista N, Fezza F, Dainese E et al. Mouse blastocysts release a lipid which activates anandamide hydrolase in intact uterus. *Mol Hum Reprod*. 2004;10(4):215–21.
112. Mizugishi K, Li C, Olivera A, Bielawski J, Bielawska A, Deng CX et al. Maternal disturbance in activated sphingolipid metabolism causes pregnancy loss in mice. *J Clin Invest*. 2007;117(10):2993–3006.
113. Achache H, Tsafrir A, Prus D, Reich R, Revel A. Defective endometrial prostaglandin synthesis identified in patients with repeated implantation failure undergoing in vitro fertilization. *Fertil Steril.* 2010;94(4):1271–8.
114. Berlanga O, Bradshaw HB, Vilella-Mitjana F, Garrido-Gomez T, Simon C. How endometrial secretomics can help in predicting implantation. *Placenta*. 2011;32 (Suppl 3):S271–5.
115. Vilella F, Ramirez L, Berlanga O, Martinez S, Alama P, Meseguer M et al. PGE2 and PGF2alpha concentrations in human endometrial fluid as biomarkers for embryonic implantation. *J Clin Endocrinol Metab.* 2013;98(10):4123–32.

116. Yamagata Y, Asada H, Tamura I, Lee L, Maekawa R, Taniguchi K et al. DNA methyltransferase expression in the human endometrium: Down-regulation by progesterone and estrogen. *Hum Reprod*. 2009;24(5):1126–32.
117. Li R, Qiao J, Wang L, Li L, Zhen X, Liu P et al. MicroRNA array and microarray evaluation of endometrial receptivity in patients with high serum progesterone levels on the day of hCG administration. *Reprod Biol Endocrinol*. 2011;9:29.
118. Galliano D, Pellicer A. MicroRNA and implantation. *Fertil Steril*. 2014;101(6):1531–44.
119. Ambros V. MicroRNA pathways in flies and worms: Growth, death, fat, stress, and timing. *Cell*. 2003;113(6):673–6.
120. Bartel DP. MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell*. 2004;116(2):281–97.
121. Lai EC. microRNAs: Runts of the genome assert themselves. *Curr Biol*. 2003;13(23):R925–36.
122. Creighton CJ, Benham AL, Zhu H, Khan MF, Reid JG, Nagaraja AK et al. Discovery of novel microRNAs in female reproductive tract using next generation sequencing. *PLoS One*. 2010;5(3):e9637.
123. Evans GE, Martinez-Conejero JA, Phillipson GT, Simon C, McNoe LA, Sykes PH et al. Gene and protein expression signature of endometrial glandular and stromal compartments during the window of implantation. *Fertil Steril*. 2012;97(6):1365–73 e1–2.
124. Evans GE, Phillipson GT, Sin IL, Frampton CM, Kirker JA, Bigby SM et al. Gene expression confirms a potentially receptive endometrium identified by histology in fertile women. *Hum Reprod*. 2012;27(9):2747–55.
125. Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nature Cell Biol*. 2008;10(5):593–601.
126. Dominguez F, Avila S, Cervero A, Martin J, Pellicer A, Castrillo JL et al. A combined approach for gene discovery identifies insulin-like growth factor-binding protein-related protein 1 as a new gene implicated in human endometrial receptivity. *J Clin Endocrinol Metab*. 2003;88(4):1849–57.
127. Altmae S, Martinez-Conejero JA, Esteban FJ, Ruiz-Alonso M, Stavreus-Evers A, Horcajadas JA et al. MicroRNAs miR-30b, miR-30d, and miR-494 regulate human endometrial receptivity. *Reprod Sci*. 2013;20(3):308–17.
128. Sha AG, Liu JL, Jiang XM, Ren JZ, Ma CH, Lei W et al. Genome-wide identification of micro-ribonucleic acids associated with human endometrial receptivity in natural and stimulated cycles by deep sequencing. *Fertil Steril*. 2011;96(1):150–5 e5.
129. Capalbo A, Ubaldi FM, Cimadomo D, Noli L, Khalaf Y, Farcomeni A et al. MicroRNAs in spent blastocyst culture medium are derived from trophectoderm cells and can be explored for human embryo reproductive competence assessment. *Fertil Steril*. 2015;105(1):225–35.
130. Rosenbluth EM, Shelton DN, Wells LM, Sparks AE, Van Voorhis BJ. Human embryos secrete microRNAs into culture media—A potential biomarker for implantation. *Fertil Steril*. 2014;101(5):1493–500.
131. Vilella F, Moreno-Moya JM, Balaguer N, Grasso A, Herrero M, Martinez S et al. Hsa-miR-30d, secreted by the human endometrium, is taken up by the pre-implantation embryo and might modify its transcriptome. *Development*. 2015;142(18):3210–21.

21

Embryo Transfer

Giulia Mariani and José Bellver

Introduction

Historically, the embryo transfer (ET) technique was viewed as an unimportant variable in the outcome of an assisted reproductive technology (ART) cycle. Nowadays, ET efficiency is considered one of the main factors that may affect embryo implantation (1). To date there is great variability regarding many aspects of this procedure (2); only after standardizing an ET protocol will it be possible to maximize IVF success. In fact, today, in the era of evidence-based medicine, every step taken in the whole process should not be left to the clinician's personal preference, but should follow a consensual protocol.

Trans-Cervical Embryo Transfer Technique

In general, the most frequently used approach is the trans-cervical intra-uterine transfer. Other techniques, such as ultrasound-guided trans-myometrial transfer or embryo intra-Fallopian transfer (EIFT), have been described as alternatives to the trans-cervical approach in case of extremely difficult transfers by conventional methods. Because of their higher invasiveness, they are currently seldom used.

ET is typically undertaken with the woman lying in the dorsal lithotomy position, without sedation. The cervix, exposed by the insertion into the vagina of a sterile speculum, is cleaned from cervical mucus in a variety of ways avoiding embryotoxic chemicals. A transfer catheter loaded with embryo(s) is passed through the cervical canal and then into the uterus; the embryo(s) is deposited inside the uterine cavity by applying pressure on the attached syringe. The procedure is typically carried out under abdominal ultrasound guidance with a full bladder. Finally, the transfer catheter is withdrawn and immediately checked by the embryologist in case any embryos are retained, or to check for the presence of mucus and blood (Figure 21.1).

The transfer is defined as easy if the catheter passage occurs easily through the cervical canal, without the use of any other instrumentation. It is considered difficult if blood or mucus are present on the catheter, if resistance is encountered during the catheter insertion, if the procedure is time consuming, or requires cervical manipulation, or a shift to a harder catheter, or reloading of embryos.

Factors That Can Influence the Success of Embryo Transfer

Multiple variables have been identified that might influence the pregnancy rate after ET.

Preparation Prior to Embryo Transfer

Clinician Experience

It has been shown that the "physician factor" has an important impact on the outcome of an in-vitro fertilization (IVF) cycle. Even within the same IVF program, there is a great variability in the success rate

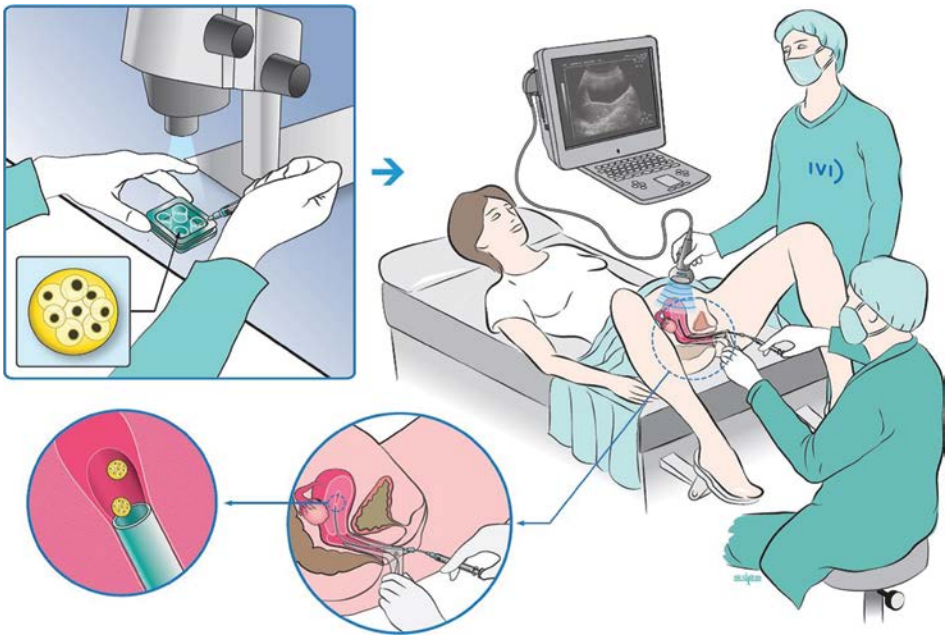


FIGURE 21.1 Schematic representation of a trans-cervical embryo transfer under trans-abdominal ultrasound guidance.

dependent on different physician performance (3). The lower provider experience, the lower clinical pregnancy rates (4); physicians reach the same results as experienced staff only after a tailored training (5).

Trial Transfer

Trial transfer can be done at different times during an ART cycle: in the first appointment, before starting ovarian stimulation, at the time of oocyte retrieval or just before the "real" transfer. The choice of timing of a trial transfer does not affect embryo implantation or pregnancy rates (6). The main advantage offered by a mock transfer is the reduction in the incidence of difficult transfers that might impact the clinical results (7). In fact, it allows choice of the most suitable ET catheter for each patient and assessment of the path of the cervical canal, uterine depth, and position, as well as the degree of difficulty in entering the uterine cavity (8).

When the trial transfer is performed before stimulation, a catheter is passed blindly through the cervical canal and into the uterine cavity to assess uterine length and direction; if a difficulty is found, ultrasound may be used.

It has been shown that the uterine position and depth may vary between the mock transfer done before starting ovarian stimulation, and the real embryo transfer, limiting the role of trial transfer in fresh ET (9,10).

In order to make the assessment of the uterine cavity as accurate as possible, it is better to perform the trial transfer closer to the time of the real ET. In this case the catheter should just be passed through the internal cervical os without advancing it in the uterine cavity, to prevent any possible endometrial damage (11). If this procedure is easy to do, the catheter can be removed, and the transfer catheter loaded with the embryos can then be inserted. To avoid the additional trauma by the passage of two different catheters, another procedure has been proposed, also known as afterloading, which consists of using the outer sheath of the catheter inserted into the cervical canal as a guide to advance the soft inner catheter containing the embryo(s) (12).

Aspiration of Cervical Mucus

In order to reduce the incidence of retained embryos and cervical contamination, routine cervical cleaning of mucus is recommended before performing the ET. Removal of cervical mucus has been associated

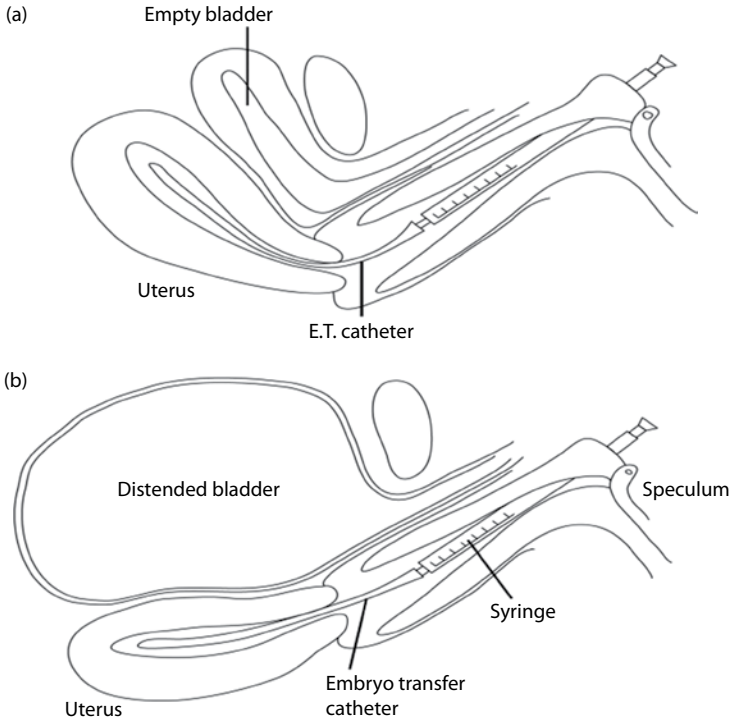


FIGURE 21.2 Schematic representation of embryo transfer in an anteverted uterus. Note the uterine–cervical angle change with an empty bladder (a), and with a full bladder (b). (Adapted from Lewin A et al. *J Assist Reprod Genet*. 1997;14:32–4.)

with an increase in pregnancy rates (13), but this result is not universally accepted (14). Cervical mucus can be removed with a sterile cotton swab, with gentle irrigation with saline or transfer medium, and it can also be aspirated using a sterile syringe attached to a transfer catheter.

Repletion of the Bladder

Traditionally, ultrasound-guided ET was performed with a full bladder to prevent a traumatic and difficult transfer. With a full bladder, the utero-cervical angle is passively straightened, making the insertion of the catheter into the uterine cavity simpler, especially for the severely anteverted uterus (Figure 21.2) (15).

Bladder distension appears to be a beneficial factor in optimizing the outcome of the ET procedure (16). However, a recent review concluded that there is no evidence of a statistically significant change in pregnancy outcomes when performing ET with a full bladder compared to with an empty bladder (14).

The main drawbacks reported are patient discomfort due to bladder distension, the waiting time necessary for filling of the bladder, and the patient's psychological distress due to the need for an immediate post-transfer micturation, which interferes with bed rest (16). In addition, the full bladder can cause a difficult visualization of the endometrium, especially in obese patients and in the presence of uterine retroversion or abnormalities (17), and can increase uterine contractility reducing the chances of successful ET (7).

Technical Aspects Related to the Embryo Transfer Catheter

Type of Catheter

It is known that a strong correlation exists between an easy transfer and pregnancy outcome: the ideal catheter is the one that allows a more atraumatic insertion into the uterine cavity, with an adequate flexibility to follow its natural curvature.

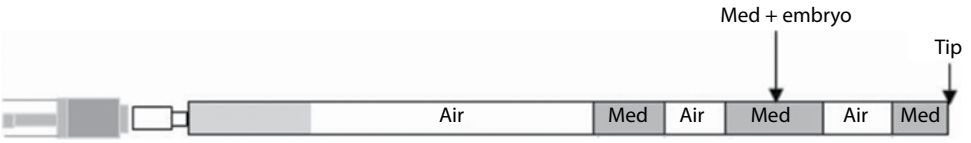


FIGURE 21.3 Schematic representation of the "three-drop catheter loading technique." (Adapted from Madani T, Jahangiri N. Increasing pregnancy by improving embryo transfer techniques. In: Advances in Embryo Transfer, Wu B (Ed.). InTech, 2012, under Open Access from http://www.intechopen.com/books/howtoreference/advances-in-embryo-transfer/advances-in-embryo-transfer)

Many authors have analyzed the relationship that exists between the type of catheter and ART outcomes (18,19). Soft catheters, such as Edwards–Wallace (Marlow Technologies, Willoughby, Ohio) or Cook (Cook Ob/Gyn, Inc., Bloomington, Indiana), should be considered as the first-choice catheter. They are associated with higher pregnancy rates than firm catheters such as Frydman (Laboratoire CCD, Paris, France), Tom Cat (Kendell Health Care, Hampshire, Massachusetts), TDT (Tight Difficult Transfer) (Laboratoire CCD, Paris, France), Tefcat (Kendell Health Care, Hampshire, Massachusetts), or Rocket (Rocket Medical, Watford, United Kingdom) (18,19).

Despite the known benefits in producing less cervical and endometrial damage during the ET procedure, the use of soft catheters leads to a greater chance of failure in passing through the cervical canal, and therefore it may increase the need for cervical manipulation (8). So if, on the one hand, softer catheters represent the ideal, working very well in easy transfers, on the other hand they are associated with a relatively higher incidence of difficult transfers and insertion failure rate.

To improve soft catheter performance and provide a better visualization during an ultrasound-guided ET, the use of echogenic catheters has been promoted (20). The echogenicity can be extended along the entire length of the catheter or confined to the catheter tip.

In conclusion, at present a variety of ET catheters are available, but soft and echogenic catheters are those recommended to perform the best ET (18–20).

Catheter Loading Technique

After performing the mock transfer and selecting the suitable catheter for ET, the embryo loading process can be started. The embryo is loaded by a clinical embryologist from the culture medium into the catheter using different methods. Two main catheter-loading techniques exist: the air–fluid and fluid-only methods (21). In the standard air–fluid model, two air bubbles are created on both sides of the medium drop containing the embryo ("three-drop technique") (Figures 21.3 and 21.4). In the fluid-only model, the embryos are loaded in the catheter without any air brackets or bubbles (21). A modified air–fluid method has been proposed, with the aim of reducing air bubble movements at the time of embryo transfer, using only one air bubble at the tip of the embryo transfer catheter (22). Indeed, when introducing a small amount of air bubble into the uterine cavity the embryos seem to retain their place of deposition at the time of ET.

On the one hand, the presence of air bubbles in the catheter is considered a contributing factor to the techniques' success, by protecting the embryo from cervical mucus and trauma or from accidental discharge before entering the endometrial cavity (23), and by identifying more clearly the culture medium with embryos during the ultrasound-guided embryo transfer (24). On the other hand, it is considered a non-physiological factor that adversely affects embryo implantation (25). To date there is no evidence about the superiority of the air–fluid or fluid-only methods during embryo loading (21).

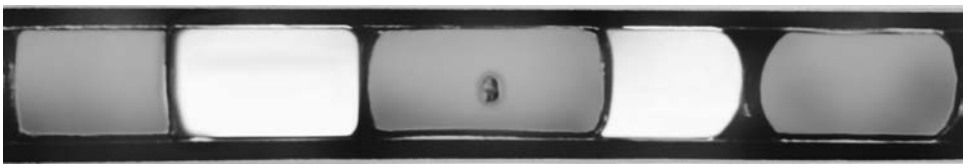


FIGURE 21.4 Embryo transfer catheter loaded with a blastocyst placed between two air bubbles.

Timing

Long time intervals, especially of more than 120 seconds, impact negatively on pregnancy rates (26), probably due to the embryo vulnerability to exposure to some environmental factors. However, considering only good quality embryos, a prolonged duration of transfer, up to 7.5 minutes, has not been shown to affect the outcome of the cycle (27).

Embryo Transfer Procedure

Embryo Deposition

There is no consensus in the scientific literature on the best site where the embryos should be deposited in the cavity. Traditionally, the embryos were placed close to the uterine fundus, with a high risk of ectopic pregnancy (28). Subsequently, it has been shown that a higher pregnancy rate is achieved when the transfer catheter tip is placed at a distance of 15–20 mm from the fundus (29,30).

Considering the variability in endometrial cavity length between patients, it has been suggested that the best way to select the transfer location is by taking into account the depth of the uterine cavity, and not a fixed reference point such as the distance from the fundus (31).

Recently, greater attention has been given to assessing the position of the air bubbles deposited with the embryo at the time of transfer, because they are considered to reflect the actual embryo position (Figure 21.5) (32). In contrast with the results from the catheter tip studies, the majority of the air bubble studies suggest that pregnancy rates are higher when the bubbles are closer to the fundus (33,34). So pregnancy rate seems to be associated with the distance between fundal endometrial surface and air bubbles, with the highest rates registered at a distance of <10 mm from the fundus (30).

Ultrasound Guidance

Traditionally, embryos have been placed into the uterine cavity blindly using the "clinical touch" method, based exclusively on the clinician's tactile senses. Performing a blind embryo transfer can lead to inadvertent contact with the uterine fundus, stimulating miometrial–endometrial contractility and damaging the endometrium, and to the inaccurate identification of the right site when depositing the embryos.

To enhance transfer efficiency, ultrasound (US)-guided embryo transfer has been devised. Today it is widely demonstrated that US guidance leads to a significant improvement in clinical pregnancy rates compared with clinical touch alone (35–38). In fact, US guidance during ET reduces the incidence of difficult transfers (39). It may also help to confirm the position of the catheter tip near the uterine fundus (36),

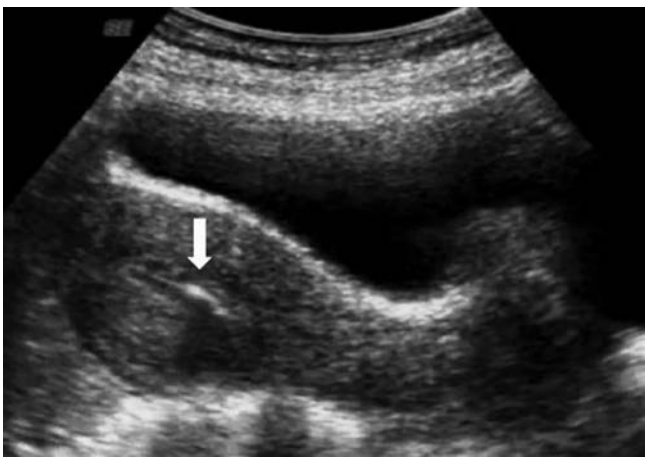


FIGURE 21.5 Trans-abdominal ultrasonographic visualization of the echogenic air bubbles (arrow) within the uterine cavity after transfer.

especially in cases of distorted pelvic anatomy, and thus it allows the proper placement of the embryos in the cavity (29). Furthermore, using US it is possible to observe the introduction of air bubbles into the uterus (1) and their persistence after catheter removal. Finally, US may help to minimize contamination of the catheter tip with blood and mucus (40), and decrease the chance of traumatizing the fundus and thus stimulating uterine contractions (41). From a psychological point of view, the US direct visualization of embryo placement allows both patient and her partner to have the opportunity to be involved in the procedure.

The main disadvantages of US guidance are the need for more time, space, equipment, a trained ultrasound second operator, and a full bladder, in many cases responsible for the patient's discomfort or cramps (42). Another possible drawback is the potential endometrium damage caused by the movement of the catheter required in some cases to identify the catheter tip (43). However, this movement is not required with the use of the new echogenic catheters. Finally, we have to consider that the need for a US machine and a second operator leads to higher costs (38).

Some authors proposed trans-vaginal (TV) US-guided embryo transfer (44). To perform a TV ultrasound, a full bladder is not required, and a TV probe offers a clearer delineation of the catheter tip than a trans-abdominal probe. The obvious main disadvantage is the technical difficulty of performing the US and the transfer procedure at the same time (44). Trans-abdominal and trans-vaginal US seem to be similarly effective in terms of overall pregnancy, clinical pregnancy, live birth, and implantation rates (44).

There are studies suggesting a role of three-dimensional (3D) ultrasound in US-guided embryo transfers (45). The need for enhanced US technique is related to the fact that the two-dimensional scan shows the catheter in a different position from the real one in 20% of cases (45).

Volume and Type of Transfer Media

Concerning the quantity of fluid used for ET, while extremely low volumes of culture media in the catheter (<10 μL) are associated with reduced implantation and pregnancy rates (46), higher volumes of media (40 μL) enhance the rate of implantation and pregnancy (47). Very large transfer volumes (>60 mL), associated with large air bubbles in the catheter, may result in a failed transfer, with the expulsion of embryos from the cervical canal (48).

Adherence compounds have been added to the embryo transfer medium with the purpose of increasing the chance of the embryo adhering to the uterus, improving pregnancy and live-birth rates. There is no evidence suggesting the superiority of one transfer media in comparison to another.

The role of fibrin sealant and hyaluronic acid (HA) added to the transfer media in improving the process of implantation is still controversial. A recent Cochrane Library review found no evidence that fibrin sealant increases pregnancy rates (49). Moderate-quality evidence suggests a beneficial treatment effect, with an increase in the number of live births, using a HA-enriched transfer medium (49).

Presence of Bacteria in the Cervix or on the Catheter Tip

Endometrial subclinical infection related to cervical contamination during ET procedure has been considered as a possible factor causing implantation failure. Contamination of the catheter tip and endometrial cavity by cervical flora is associated with a lower pregnancy rate (14). For this reason, the importance of cleaning the cervix and vagina with saline solution has been suggested, with the aim of reducing bacterial contamination. The use of vaginal antiseptics at the time of ET is highly discouraged for their potential embryo toxicity. The administration of antibiotics before or at ET is still a subject of controversy: it has been proven to reduce upper genital tract microbial contamination, but without increasing clinical pregnancy rates (50). Due to the lack of proven benefit, antibiotics administration is not a recommended procedure for ET.

Post-Transfer Aspects

Catheter Removal

After a gentle injection of the embryos, maintaining a constant pressure on the plunger of the syringe until the catheter is completely withdrawn from the uterus is a crucial maneuver, necessary to minimizing the

risk of retained embryos. Furthermore, the outer and inner sheaths should be removed simultaneously, and the transfer catheter should be slowly withdrawn (51). It has been investigated if the time interval before catheter removing constitutes a measure affecting IVF success, but no difference in pregnancy rate has been observed between women with immediate catheter withdrawal versus a delayed one (52).

Difficult Transfer

A time-consuming ET that requires a firmer catheter, extra manipulation, or additional instrumentation leads to reduced chances of pregnancy (53). The most common reasons that make a transfer difficult are cervical stenosis or a large degree of anteversion/retroversion or anteflexion/retroflexion of the uterus (54). The main factors strongly related to transfer difficulty are endometrial trauma, presence of blood on the catheter, and the stimulation of uterine contractions by intense manipulation or touching of the fundus.

It can be reasonably concluded that in the case of a complicated embryo transfer, it would be better to freeze all the embryos and postpone the transfer once the complications are resolved.

Contamination of the Catheter Tip with Blood or Mucus

The presence of blood on the catheter after ET may be a marker of a difficult or traumatic transfer associated with a poor chance of pregnancy (55). If blood is floating inside the endometrial cavity, it can interfere with embryo deposition and implantation.

The presence of mucus on the ET catheter is also considered a variable that may negatively affect ART success, decreasing implantation and pregnancy rates (56). In fact, mucus on the transfer catheter has been correlated with an increased incidence of retained embryos at ET, causing the mechanical blockage of the catheter opening, and favoring embryo adhesion to the catheter at the time of withdrawal (57). Furthermore, an excess of cervical mucus may displace the embryos from their original proper site within the uterine cavity (13). Another risk is the contamination of the catheter and uterus by cervical flora (13).

Retained or Expelled Embryos

About 3.9% of all transfers are complicated by retained embryos (58). Immediate retransfer of embryos found in the transfer catheter does not seem to reduce embryo implantation and pregnancy rates (58). To optimize the transfer procedure and to avoid the eventuality of re-aspirating the embryos once discharged into the uterus, maintaining the pressure on the plunger of the syringe until complete withdrawal of the catheter is strongly recommended (59). Another important precaution to minimize retained embryos is a slow withdrawal of the catheter after deposition.

Expulsion of embryos toward the cervical canal following their transfer into the uterine cavity is known to be one of the factors associated with unsuccessful ET (7). About 15% of the transferred embryos are found outside the uterine cavity after ET (48), but almost certainly the number of lost embryos is greater than we know, because cervical or speculum controls are not routinely performed (7).

Embryo expulsion may result due to different factors such as local pressure changes during ET (60), variations in injection speed (61), negative pressure produced by rapid withdrawal of the catheter (62), and uterine contractions induced by uterine fundus contact or cervical manipulation (63).

To avoid negative pressure produced by removing the catheter, an alternative ET technique, which consists in pushing 0.2 mL of air into the catheter immediately after ET, has been described (62). This modified ET method has led to statistically significant increases in implantation and pregnancy rates (62).

Uterine Contractions

US imagery using high-resolution vaginal probes has permitted a noninvasive and objective analysis of the possible consequences of uterine contractile activity on fertilization and embryo implantation processes (64). To offer more reliable US measurements of uterine contractions, an alternative method using US 3D-derived technique has been developed (65). Uterine contractions come from the junctional

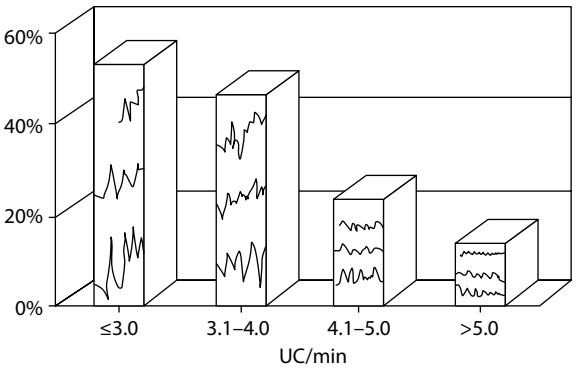


FIGURE 21.6 While the frequency of uterine contractions (UC) per minute increases, clinical pregnancy rates progressively decrease. (From Fanchin R et al. *Hum Reprod.* 1998;13:1968–74, with permission.)

zone of the myometrium (63). Intense myometrial–endometrial contractile activity adversely affects ET outcome; the frequency of uterine contractions at the time of embryo transfer inversely correlates with clinical pregnancy rates and implantation rates (Figure 21.6) (65,66).

In fact, an intense uterine peristalsis might expel the embryo out of the uterine cavity, resulting in implantation failure or ectopic pregnancy. Indeed, to optimize embryo transfer outcome it is recommended to avoid maneuvers that might cause uterine contractions, such as touching the uterine fundus, using rigid catheters, prolonged presence of the embryo transfer catheter in the endometrial cavity, intensive cervical manipulation, or excessive movement of the catheter tip.

A significant negative correlation has been observed between plasma progesterone levels on the day of embryo transfer and uterine contraction frequency, probably due to the relaxing properties of progesterone on the uterus (67,68).

A relationship has also been registered between supraphysiological estradiol concentrations from ovarian stimulation and uterine contraction frequency; high estradiol levels are a stimulating factor for uterine contractions at the time of transfer (69), maybe through increased oxytocin receptor gene expression in the myometrium (70).

Drugs

Many drugs have been tested as a way to reduce uterine contractility.

Oxytocin, which plays an important role in many reproductive functions, has been recognized to be involved in the genesis of myometrial activity and, thereby, in the success of the fertilization and implantation process. Intravenous infusion of oxytocin antagonist before ET has proven to be effective in reducing myometrial activity and increasing endometrial perfusion, thus promoting implantation by preventing early embryo expulsion (71). However, a randomized double blind study has shown that live birth rate was not significantly higher in women receiving Atosiban infusion (Tractocile; Ferring Arzneimittel, Kiel, Germany), an oxytocin antagonist, around the time of embryo transfer, compared to women receiving placebo (72).

Bed Rest

Post-transfer bed rest is still a subject of controversy; some recommend bed rest for variable periods of time immediately after the embryo transfer, others discourage it because of its unproven efficacy in improving ongoing pregnancy rates (73).

The recommendation for bed rest derives from the belief that reduced physical activity and the supine position will improve embryo retention within the uterine cavity compared with immediate mobilization. A large majority of patients believe that physical activity may have a negative effect on ART outcomes, but to date it has not been demonstrated that bed rest can increase the success of ART (74). Furthermore,

TABLE 21.1

Protocol for Embryo Transfer Performance

- Perform a trial transfer
- Ultrasound guidance
- Routine removal of cervical mucus
- Use of a soft catheter
- Minimize the time interval between loading the embryos and the transfer into the uterus
- Minimun ejection speed
- Deposition of embryos at 15–20 mm from fundus
- Slow withdrawl of the transfer catheter
- Check the catheter for retained embryos, blood, or mucus
- No bed rest

bed rest seems to have a bad effect on ET outcome (75). Surely every patient should be treated according to her needs, bearing in mind that restriction of physical activity after ET may increase levels of stress and anxiety.

In conclusion, the performance of an ET is essential to IVF success, and every step is summarized in Table 21.1.

REFERENCES

1. Phillips JA, Martins WP, Nastri CO, Raine-Fenning NJ. Difficult embryo transfers or blood on catheter and assisted reproductive outcomes: A systematic review and meta-analysis. *Eur J Obstet Gynecol Reprod Biol*. 2013;168:121–8.
2. Christianson MS, Zhao Y, Shoham G, Granot I, Safran A, Khafagy A et al. Embryo catheter loading and embryo culture techniques: Results of a worldwide web-based survey. *J Assist Reprod Genet*. 2014;31:1029–36.
3. Angelini A, Brusco GF, Barnocchi N, El-Danasouri I, Pacchiarotti A, Selman HA. Impact of physician performing embryo transfer on pregnancy rates in an assisted reproductive program. *J Assist Reprod Genet*. 2006;23:329–32.
4. Desparoir A, Capelle M, Banet J, Noizet A, Gamerre M, Courbiere B. Does the experience of the provider affect pregnancy rates after embryo transfer? *J Reprod Med*. 2011;56:437–43.
5. Dessolle L, Freour T, Barriere P, Jean M, Ravel C, Daraï E et al. How soon can I be proficient in embryo transfer? Lessons from the cumulative summation test for learning curve (LC-CUSUM). *Hum Reprod*. 2010;25:380–6.
6. Katariya KO, Bates GW, Robinson RD, Arthur NJ, Propst AM. Does the timing of mock embryo transfer affect in vitro fertilization implantation and pregnancy rates? *Fertil Steril*. 2007;88:1462–4.
7. Schoolcraft WB, Surrey ES, Gardner DK. Embryo transfer: Techniques and variables affecting success. *Fertil Steril*. 2001;76:863–70.
8. Mansour R, Aboulghar M, Serour G. Dummy embryo transfer: A technique that minimizes the problems of embryo transfer and improves the pregnancy rate in human in vitro fertilization. *Fertil Steril*. 1990;54:678–81.
9. Henne MB, Milki AA. Uterine position at real embryo transfer compared with mock embryo transfer. *Hum Reprod*. 2004;19:570–2.
10. Miller KL, Frattarelli JL. The pre-cycle blind mock embryo transfer is an inaccurate predictor of anticipated embryo transfer depth. *J Assist Reprod Genet*. 2007;24:77–82.
11. Tiras B, Cenksoy PO. Practice of embryo transfer: Recommendations during and after. *Semin Reprod Med*. 2014;32:291–6.
12. Neithardt AB, Segars JH, Hennessy S, James AN, McKeeby JL. Embryo afterloading: A refinement in embryo transfer technique that may increase clinical pregnancy. *Fertil Steril*. 2005;83:710–4.
13. Eskandar MA, Abou-Setta AM, El-Amin M, Almushait MA, Sobande AA. Removal of cervical mucus prior to embryo transfer improves pregnancy rates in women undergoing assisted reproduction. *Reprod Biomed Online*. 2007;14:308–13.
14. Derks RS, Farquhar C, Mol BWJ. Techniques for preparation prior to embryo transfer. *Cochrane Db Syst Rev*. 2009;4:CD007682.

15. Lewin A, Schenker JG, Avrech O, Shapira S, Safran A, Friedler S. The role of uterine straightening by passive bladder distension before embryo transfer in IVF cycles. *J Assist Reprod Genet*. 1997;14:32–4.
16. Abou-Setta AM. Effect of passive uterine straightening during embryo transfer: A systematic review and meta-analysis. *Acta Obstet Gynecol*. 2007;86:516–22.
17. Lorusso F, Depalo R, Bettocchi S, Vacca M, Vimercati A, Selvaggi L. Outcome of in vitro fertilization after transabdominal ultrasound-assisted embryo transfer with a full or empty bladder. *Fertil Steril*. 2005;84:1046–8.
18. Abou-Setta AM, Al-Inany HG, Mansour RT, Serour GI, Aboulghar MA. Soft versus firm embryo transfer catheters for assisted reproduction: A systematic review and meta-analysis. *Hum Reprod*. 2005;20:3114–21.
19. Buckett WM. A review and meta-analysis of prospective trials comparing different catheters used for embryo transfer. *Fertil Steril*. 2006;85:728–34.
20. Coroleu BA, Barri P, Carreras O, Belil I, Buxaderas R, Veiga A et al. Effect of using an echogenic catheter for ultrasound-guided embryo transfer in an IVF programme: A prospective, randomized, controlled study. *Hum Reprod*. 2006;21:1809–15.
21. Abou-Setta AM. Air fluid versus fluid-only models of embryo catheter loading: A systematic review and meta-analysis. *Reprod BioMed Online*. 2007;14:80–4.
22. Allahbadia G, Gandhiji G, Kadam K, Virk S, Kaur K. A prospective randomized comparison of two different embryo transfer catheter loading techniques. *Fertil Steril*. 2005;84:S114–5.
23. Tiras B, Korucuoglu U, Polat M, Saltik A, Zeyneloglu HB, Yarali H. Effect of air bubble localization after transfer on embryo transfer outcomes. *Eur J Obstet Gynecol Reprod Biol*. 2012;164:52–4.
24. Zinger M, Wrona DA, Desanti R, Miller CE, Miller CE. Movement of intrauterine air bubble during embryo transfer (ET) catheter withdrawal is not a prognostic indicator. *Fertil Steril*. 2004;82:S63.
25. Krampl E, Zegermacher G, Eichler C, Obruca A, Strohmer H, Feichtinger W. Air in the uterine cavity after embryo transfer. *Fertil Steril*. 1995;63:366–70.
26. Matorras R, Mendosa R, Exposito A, Rodriguez-Escudero FJ. Influence of the time interval between embryo catheter loading and discharging on the success of IVF. *Hum Reprod*. 2004;19:2027–30.
27. Ciray HN, Tosum S, Hacifazlioglu O, Mesut A, Bahceci M. Prolonged duration of transfer does not affect outcome in cycles with good embryo quality. *Fertil Steril*. 2007;87:1218–21.
28. Pope CD, Cook EK, Arny M, Novak A, Grow DR. Influence of embryo transfer depth on in vitro fertilization and embryo transfer outcomes. *Fertil Steril*. 2004;81:51–8.
29. Coroleu B, Barri PN, Carrera O, Martínez F, Parriego M, Hereter L et al. The influence of the depth of the embryo replacement into the uterine cavity on implantation rates after IVF: A controlled, ultrasoundguided study. *Hum Reprod*. 2002;17:341–6.
30. Cenksoy PO, Ficicioglu C, Yesiladali M, Akcin OA, Kaspar C. The importance of the length of uterine cavity, the position of the tip of the inner catheter and the distance between the fundal endometrial surface and the air bubbles as determinants of the pregnancy rate in IVF cycles. *Eur J Obstet Gynecol Reprod Biol*. 2014;172:46–50.
31. Frankfurter D, Trimarchi JB, Silva CP, Keefe DL. Middle to lower uterine segment embryo transfer improves implantation and pregnancy rates compared with fundal embryo transfer. *Fertil Steril*. 2004;81:1273–7.
32. Friedman BE, Lathi RB, Henne MB, Fisher SL, Milki AA. The effect of air bubble position after blastocyst transfer on pregnancy rates in IVF cycles. *Fertil Steril*. 2011;95:944–7.
33. Lambers MJ, Dogan E, Lens JW, Schats R, Hompes PG. The position of transferred air bubbles after embryo transfer is related to pregnancy rate. *Fertil Steril*. 2007;88:68–73.
34. Saravelos SH, Wong AW, Chan CP, Kong GW, Cheung LP, Chung CH et al. Assessment of the embryo flash position and migration with 3D ultrasound within 60 min of embryo transfer. *Hum Reprod*. 2016;31:591–6.
35. Abou-Setta AM, Mansour RT, Al-Inany HG, Aboulghar MM, Aboulghar MA, Serour G. Among women undergoing embryo transfer, is the probability of pregnancy and live birth improved with ultrasound guidance over clinical touch alone? A systemic review and meta-analysis of prospective randomized trials. *Fertil Steril*. 2007;88:333–41.
36. Buckett WM. A meta-analysis of ultrasound-guided versus clinical touch embryo transfer. *Fertil Steril*. 2003;80:1037–41.

37. Brown J, Buckingham K, Abou-Setta AM, Buckett W. Ultrasound versus "clinical touch" for catheter guidance during embryo transfer in women. *Cochrane Db Syst Rev.* 2010;1:CD006107.
38. Teixeira DM, Dassunc LA, Vieira CVR, Barbosa MA, Coelho Neto MA, Nastri CO et al. Ultrasound guidance during embryo transfer: A systematic review and meta-analysis of randomized controlled trials. *Ultrasound Obstet Gynecol*. 2015;45:139–48.
39. Matorras R, Urquijo E, Mendoza R, Corcóstegui B, Expósito A, Rodríguez-Escudero FJ. Ultrasound-guided embryo transfer improves pregnancy rates and increases the frequency of easy transfers. *Hum Reprod*. 2002;17:1762–6.
40. Alvero R, Hearns-Stokes RM, Catherino WH, Leondires MP, Segars JH. The presence of blood in the transfer catheter negatively influences outcome at embryo transfer. *Hum Reprod*. 2003;18:1848–52.
41. Lesny P, Killick SR, Tetlow RL. Embryo transfer and uterine junctional zone contraction. *Hum Reprod Update*. 1999;5:87–8.
42. Martins A, Baruffi R, Mauri A, Petersen C, Oliveira JB, Contart P et al. Ultrasound guidance is not necessary during easy embryo transfers. *J Assist Reprod Genet*. 2004;21:421–5.
43. Garcia-Velasco J, Isaza V, Martinez-Salazar J, Landazábal A, Requena A, Remohí J et al. Transabdominal ultrasoundguided embryo transfer does not increase pregnancy rates in oocyte recipients. *Fertil Steril*. 2002;78:534–9.
44. Porat N, Boehnlein LM, Schouweiler CM, Kang J, Lindheim SR. Interim analysis of a randomized clinical trial comparing abdominal versus transvaginal ultrasound-guided embryo transfer. *J Obstet Gynaecol Res*. 2010;36:384–92.
45. Letterie GS. Three-dimensional ultrasound-guided embryo transfer: A preliminary study. *Am J Obstet Gynecol*. 2005;192:1983–8.
46. Ebner T, Yaman C, Moser M, Sommergruber M, Pölz W, Tews G. The ineffective loading process of the embryo transfer catheter alters implantation and pregnancy rates. *Fertil Steril*. 2001;76:630–2.
47. Montag M, Kupka M, van der Ven K, van der Ven H. Embryo transfer on day 3 using low versus high fluid volume. *Eur J Obstet Gyn R B*. 2002;102:57–60.
48. Poindexter ANI, Thompson DJ, Gibbons WE, Findley WE, Dodson MG, Young RL. Residual embryos in failed embryo transfer. *Fertil Steril*. 1986;46:262–7.
49. Bontekoe S, Heineman MJ, Johnson N, Blake D. Adherence compounds in embryo transfer media for assisted reproductive technologies. *Cochrane Db Syst Rev*. 2014;2:CD007421.
50. Kroon B, Hart RJ, Wong BM, Ford E, Yazdani A. Antibiotics prior to embryo transfer in ART. *Cochrane Db Syst Rev*. 2012;3:CD008995.
51. Mansour RT, Aboulghar MA. Optimizing the embryo transfer technique. *Hum Reprod*. 2002;17:1149–53.
52. Martinez F, Coroleu B, Parriego M, Carreras O, Belil I, Parera N et al. Ultrasound-guided embryo transfer: Immediate withdrawal of the catheter versus a 30 second wait. *Hum Reprod*. 2001;16:871–4.
53. Singh N, Gupta P, Mittal S, Malhotra N. Correlation of technical difficulty during embryo transfer with rate of clinical pregnancy. *J Hum Reprod Sci*. 2012;5:258–61.
54. Lass A, Abusheikha N, Brinsden P. The effect of difficult embryo transfer on the outcome of IVF. *Hum Reprod*. 1999;14:2417–20.
55. Tiras B, Korucuoglu U, Polat M, Saltik A, Zeyneloglu HB, Yarali H. Effect of blood and mucus on the success rates of embryo transfers. *Eur J Obstet Gyn R B*. 2012;165:239–42.
56. Moragianni VA, Cohen JD, Smith SE, Schinfeld JS, Somkuti SG, Lee A et al. Effect of macroscopic or microscopic blood and mucus on the success rates of embryo transfers. *Fertil Steril*. 2010;93:570–3.
57. Visschers BA, Bots RS, Peeters MF, Mol BW, van Dessel HJ. Removal of cervical mucus: Effect on pregnancy rates in IVF/ICSI. *Reprod Biomed Online*. 2007;15:310–5.
58. Lee HC, Seifer DB, Shelden RM. Impact of retained embryos on the outcome of assisted reproductive technologies. *Fertil Steril*. 2004;82:334–7.
59. Hearns-Stokes RM, Miller BT, Scott L, Creuss D, Chakraborty PK, Segars JH. Pregnancy rates after embryo transfer depend on the provide at embryo transfer. *Fertil Steril*. 2000;74:80–6.
60. Grygoruk C, Sieczynski P, Pietrewicz P, Mrugacz M, Gagan J, Mrugacz G. Pressure changes during embryo transfer. *Fertil Steril*. 2011;95:538–41.
61. Groeneveld E, de Leeuw B, Vergouw C, Visser OW, Lambers MJ, Heymans MW et al. Standardization of catheter load speed during embryo transfer: Comparison of manual and pump-regulated embryo transfer. *Reprod Biomed Online*. 2012;24:163–9.

62. Madani T, Ashrafi M, Jahangiri N, Abadi AB, Lankarani N. Improvement of pregnancy rate by modification of embryo transfer technique: A randomized clinical trial. *Fertil Steril*. 2010;94:2424–6.
63. Lesny P, Killick SR, Tetlow RL, Robinson J, Maguiness SD. Uterine junctional zone contractions during assisted reproduction cycles. *Hum Reprod Update*. 1998;4:440–5.
64. Abramowicz JS, Archer DF. Uterine endometrial peristalsis—A transvaginal ultrasound study. *Fertil Steril*. 1990;54:451–4.
65. Fanchin R, Righini C, Olivennes F, Taylor S, de Ziegler D, Frydman R. Uterine contractions at the time of embryo transfer alter pregnancy rates after in-vitro fertilization. *Hum Reprod*. 1998;13:1968–74.
66. Zhu L, Che HS, Xiao L, Li YP. Uterine peristalsis before embryo transfer affects the chance of clinical pregnancy in fresh and frozen-thawed embryo transfer cycles. *Hum Reprod*. 2014;29:1238–43.
67. Fanchin R, Ayoubi JM, Righini C, Olivennes F, Schönauer LM, Frydman R. Uterine contractility decreases at the time of blastocyst transfers. *Hum Reprod*. 2001;16:1115–9.
68. Zhu L, Li Y, Xu A. Influence of controlled ovarian hyperstimulation on uterine peristalsis in infertile women. *Hum Reprod*. 2012;27:2684–9.
69. Ayoubi JM, Epiney M, Brioschi PA, Fanchin R, Chardonnens D, de Ziegler D. Comparison of changes in uterine contraction frequency after ovulation in the menstrual cycle and in in vitro fertilization cycles. *Fertil Steril*. 2003;79:1101–5.
70. Richter ON, Kubler K, Schmolling J, Kupka M, Reinsberg J, Ulrich U. Oxytocin receptor gene expression of estrogen-stimulated human myometrium in extracorporeally perfused non-pregnant uteri. *Hum Reprod*. 2004;10:339–46.
71. Moraloglu O, Tonguc E, Var T, Zeyrek T, Batioglu S. Treatment with oxytocin antagonists before embryo transfer may increase implantation rates after IVF. *Reprod Biomed Online*. 2010;21:338–43.
72. Ng EY, Li RH, Chen L, Lan VT, Tuong HM, Quan S. A randomized double blind comparison of atosiban in patients undergoing IVF treatment. *Hum Reprod*. 2014;29:2687–94.
73. Küçük M. Bed rest after embryo transfer: Is it harmful? *Eur J Obstet Gyn R B*. 2013;167:123–6.
74. Abou-Setta AM, Peters LR, D'Angelo A, Sallam HN, Hart RJ, Al-Inany HG. Post-embryo transfer interventions for assisted reproduction technology cycles. *Cochrane Database Syst Rev*. 2014;8:CD006567.
75. Gaikwad S, Garrido N, Cobo A, Pellicer A, Remohi J. Bed rest after embryo transfer negatively affects in vitro fertilization: A randomized controlled clinical trial. *Fertil Steril*. 2013;100:729–35.

22

Multiple vs. Singleton Births: Consequences and Prevention

Ernesto Bosch

Introduction

Infertility treatment has historically been related to multiple pregnancies because of the use of exogenous gonadotropins to stimulate follicular development in the ovary. Back in 1971, Atlay and Pennington described the incidence of multiple pregnancies in ovulation induction cycles in a series of 2369 pregnancies, from which 7.9% multiple pregnancies occurred: 6.9% twins, 0.5% triplets, 0.3% quadruplets, and 0.13% quintuplets (1).

The implication of ovarian stimulation became evident in Sampson et al.'s review in 1983; these authors estimated the rate of multiple births in natural cycles after artificial insemination (AI) at 1.8% as opposed to 11% for having practiced AI in a stimulated cycle (2). Part of the problem was traditionally due to general experience demonstrating (still the case) that ovarian stimulation improves AI outcomes (3). Apart from the high prevalence of twin pregnancies, high-order multiple pregnancies have been observed quite frequently (4).

For similar reasons, in vitro fertilization (IVF) has also contributed to increase the development of multiple pregnancies. Despite using natural cycles to obtain and inseminate mature oocytes in initial IVF development stages (5), first experiences evidenced that controlling ovarian stimulation (COS), obtaining a variable number of embryos in laboratories, and transferring several embryos improved the results in pregnancy-rate terms (6). In 1985 there were warnings about problems with multiple pregnancies in IVF, and although it was stated that transferring multiple embryo transfer remains a major advance in the success of IVF and ET (7) certainly it has become one of the major obstacles.

Complications of Multiple Pregnancies

Evidently this rise in multiple pregnancy incidence led to a significant increase in pregnancy and neonatal pathology. Initially the problem of multiple pregnancies was a barely relevant one, as assisted reproduction centers were few and far between, their welfare capacity was not high, and pregnancy rates were relatively low.

In the late 1980s and early 1990s, studies began to be published which had analyzed the determining factors of multiple pregnancies, and proposed preventive or corrective measures for them, at least for high-order cases.

In 1990 the results of a review carried out in the United Kingdom were published. It included 1267 pregnancies with 1581 offspring born after IVF or gamete intrafallopian tube transfer (GIFT) (8). This review showed that the rate of multiple pregnancies was 23% (1% in the general population), 24% of preterm births (6% in the general population), and 32% of babies born weighing less than 2500 g (7% in the general population). Perinatal mortality was double that of the general population, due to a high frequency of multiple births. The conclusion drawn was that multiple pregnancies were observed more frequently after assisted reproduction treatments, which was the main determining factor for complications of pregnancies and fetal health at time of birth (9).

Around a decade ago, Scandinavian countries were pioneers in considering single-embryo transfer as the usual practice in IVF cycles. An exhaustive review of the incidence and complications of twin pregnancies after IVF/ICSI carried out by Pinborg et al. (10) demonstrated that, in perinatal outcome terms, gestational age was on average 3 weeks less in twin pregnancies than in singleton ones, with a mean birth weight of 800–1000 g less. Besides, the prematurity risk was 10-fold higher and the very prematurity risk was 7-fold higher. Regarding obstetric complications, pre-eclampsia incidence was 2.4-fold higher, the risk of having to be hospitalized during pregnancy was 3.5-fold higher, and the likelihood of requiring sick leave during pregnancy was 6.8-fold higher. The incidence of ending pregnancy by cesarean section was 2–3-fold higher, and it was 3.8 times more likely that newborns had to be hospitalized in Neonate Intensive Care Units, with a mean stay of 9 more days. The risk of perinatal mortality was double. On the whole, medical expenditure incurred by twin pregnancies was 5-fold higher than by singleton pregnancies.

A more recent population study was carried out in Australia (11), which included more than 233,000 births between 1993 and 2003, and a subsequent follow-up until 2008. It revealed that prenatal mortality in twin pregnancies was 3.4-fold higher, and 9.6-fold higher in higher-order multiple pregnancies, while neonatal mortality was 6.4- and 36.7-fold higher, respectively. It indicated that the risk of prematurity was 18.7-fold higher in twin pregnancies, and no less than 525.1-fold higher in high-order multiple pregnancies. This study also demonstrated an exponential increase in hospital expenditure per newborn during the first 5 years of life, which it estimated at $2730 for singleton births, $8993 for twin births, and $24411 for high-order multiple pregnancies. Figure 22.1 graphically illustrates the differences for various complications between singleton and twin pregnancies.

Multiple pregnancy incidence varies from country to country, depending on policies on the number of embryos transferred. According to the latest data published by the European Society of Human Reproduction and Embryology (ESHRE) Register (12), the European multiple pregnancy mean is around 19.6%. The countries with the highest multiple pregnancy incidence are Bulgaria, Macedonia, and Montenegro, while Sweden and Holland have the lowest figures (Figure 22.2).

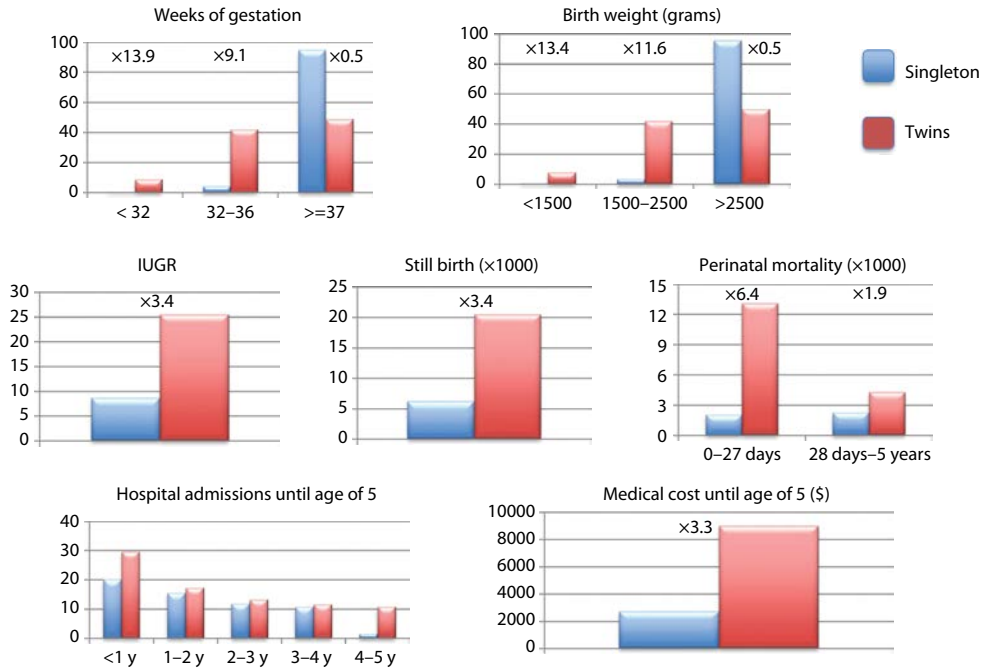


FIGURE 22.1 Obstetrical and perinatal complications of singleton vs twin pregnancies observed retrospectively in a population cohort study using individually linked birth, hospital, and death records among 233,850 infants born in Western Australia between October 1993 and September 2003, and followed up to September 2008. IUGR: Intrauterine growth retardation. (Data from Chambers GM et al. *JAMA Pediatr.* 2014;168:1045–53.)

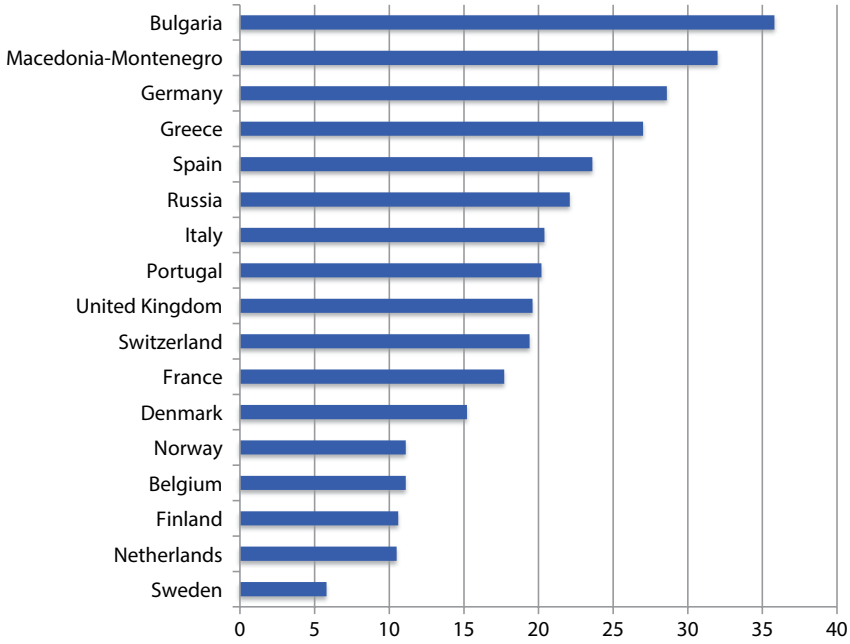


FIGURE 22.2 Incidence (%) of twin pregnancies after IVF in Europe (2010). (Data from Kupka MS et al.; European IVF-Monitoring Consortium, for the European Society of Human Reproduction and Embryology. *Hum Reprod.* 2014;29:2099–113.)

Toward Single-Embryo Transfers

Generalized single-embryo transfer in the general population lowers the pregnancy rate per embryo transfer (13). However, advances made in the efficacy of embryo cryopreservation techniques have allowed elective fresh single-embryo transfer (eSET), followed by a transfer of a previously cryopreserved embryo if the fresh embryo transfer failed. This achieves similar results, in possible pregnancy terms, to the transfer of two fresh embryos, but with the logical reduction in twin pregnancy (13% for DET to 0–2% for SET × 2) (14).

In general terms, eSET is recommended for patients with a good prognosis; that is, young patients with a good quality embryo cohort. Fertility societies in different countries have published their recommendations for selecting patients for eSET (15–17) but only a small proportion of patients follow these recommendations, despite such efforts. Deviations are inevitable, as clinics consider each patient's particular characteristics to estimate the multiple pregnancy risk (18–21). Consequently, single-embryo transfer is still not a widespread practice nowadays (22). Clinical decisions are made in order to reduce the likelihood of a multiple pregnancy, but without lowering the pregnancy rate. This objective can be met by employing validated predictive models that use specific data from patients' medical records about their response to a certain treatment cycle and data on embryo development parameters. In recent years, Instituto Valenciano de Infertilidad(IVI) has developed two predictive models of multiple pregnancy, one for own oocyte cycles and another for donor oocyte cycles.

Predictive Model of Multiple Pregnancy in Own Oocyte Cycles

In 2009, IVI-Valencia completed 418 first IVF cycles with own oocytes, in which transfers were made of one embryo or two on day 3 of embryo development. Two embryos were transferred in 371 cycles (88.8%), while only one was electively transferred in 47 cycles (11.2%). The outcome was 221 pregnancies

(52.9%), of which 72 (32.6%) were twin pregnancies. There were 138 term pregnancies (33.0% of the transfers), of which 34 (24.6%) were twin pregnancies.

According to these data, a predictive model of multiple pregnancy was constructed. To this end, a collaboration agreement was reached with the North American firm Univfy©, which specializes in developing such tools. During the 2005–2009 period, 1560 cycles were analyzed in which two embryos had been transferred and at least one live newborn had been born. Those cycles in which three IVF cycles or more had been done in patients were excluded.

Of the 1560 cycles included, 1359 corresponded to the 2005–2008 period, and a predictive model was constructed with them. The 201 cycles from 2009 were used for internal validation purposes (23). The following variables were included while developing the model:

- Baseline—previous reproductive history, including previous cycles, age, body mass index (BMI), ovarian reserve parameters (basal follicle stimulating hormone [FSH], antral follicle count), and infertility etiology.
- Response to IVF treatment—total doses of gonadotropins, stimulation protocol, stimulation duration, endometrial thickness, and estradiol (E2) on the day of human chorionic gonadotrophin (hCG).
- Laboratory parameters—sperm motility (%), number of oocytes, number of embryos, number of embryos with 8, 7, 6, etc., cells.

Table 22.1 provides the weight of all these factors for the likelihood of a multiple pregnancy if two embryos were transferred and pregnancy was achieved. The resulting predictive model obtained an area under the curve of the receiving operating characteristics curve (AUC-ROC) of 0.69, which implied a 13% increase compared with the predictive capacity of the age factor (AUC-ROC = 0.61). Applying the model showed that 43.7% of the cases with a twin pregnancy probability was higher than that estimated when considering only age, was lower in 30.2%, and a twin pregnancy estimation obtained by applying the predictive model and prediction according to age coincided in only 26.1% of the cases. Table 22.2 shows the percentage of patients in each twin pregnancy risk interval. In 40.8% of the cases, the twin pregnancy risk was 35% or above. In order to apply the predictive model as a pilot experiment in clinical practice, it was decided that all the patients with a risk above 35% should be advised to undergo single-embryo transfer. Figure 22.3 provides an example of a twin pregnancy risk report.

The pilot experiment included 83 patients with the same inclusion criteria as those included when developing the model. Of these, embryo transfers were completed in 79. In 41 cases (49.4%), the multiple pregnancy risk was above 35%. Of these, 18 (43.9%) opted for single-embryo transfer. Of the 42 patients (50.6%) whose risk of a twin pregnancy equaled or was above 35%, 13 (31%) also opted for single-embryo transfer. Thus, in 31 patients (39.2%) all the patients underwent single-embryo transfer, which represented a very marked increase compared to the previous incidence when the model was applied (11.2%). The pregnancy rate was 60.8% (48/79), and the multiple pregnancy rate was 22.9% (11/48), which is almost ten points less than that obtained for this population before applying the model.

TABLE 22.1

Relative Impact of Each Predictive Factor on the Total Estimation of Multiple Gestation after IVF with Own Oocytes

Predictive Factor	Multiple Gestation Prediction (%)
Age	19
Sperm account	6
Body mass index	2
Ovarian reserve	4
Embryo quality	37
Ovarian response	19
Etiology and reproductive background	13

TABLE 22.2

Percentage of Patients with a Given Risk of Twin Pregnancy after IVF in Case of Double Embryo Transfer

% of Patients	Twin Pregnancy Risk
0.5	≥60%
1.0	≥55%
1.5	≥50%
4.5	≥45%
18.4	≥40%
40.8	≥35%
66.7	≥30%
85.1	≥25%
94.0	≥20%
99.0	≥15%
100	≥10%
100	≥5%
0	>0%

Note: As shown, 100% of patients showed a risk of 10% or higher. In 66.7% of the patients, the risk of twins was equal or above to 30%. In 18.4% of the patients, the risk of twins was of 40% or higher.

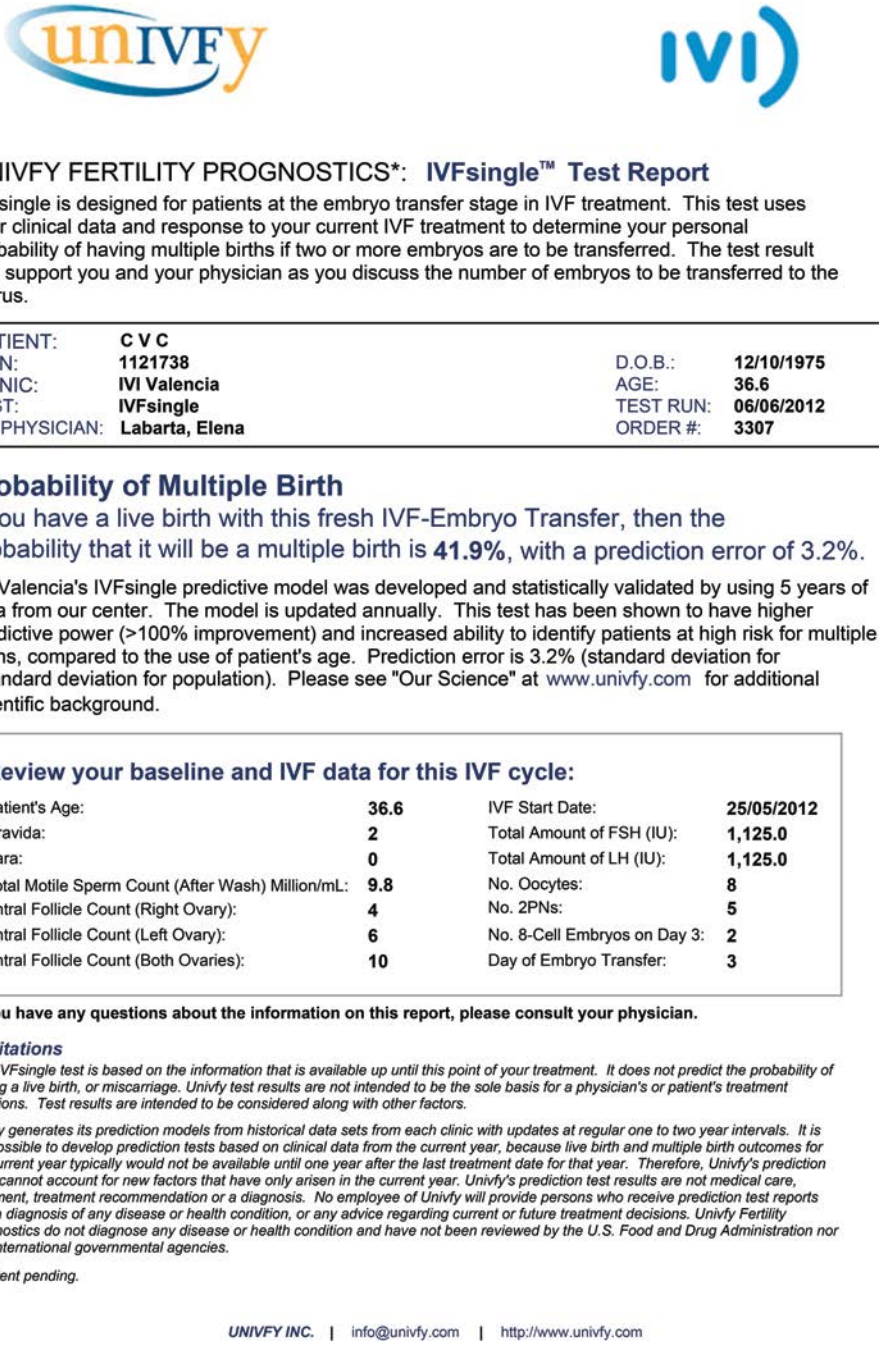
The conclusions reached with this pilot experiment were considered positive as it managed to significantly lower the twin pregnancy rate (32.6% to 22.9%, which is a relative reduction of 29.8%) without lowering the overall pregnancy rate (52.9% vs 60.8%). It was noteworthy that the use of the predictive model with these characteristics raised more awareness of the problem among doctors and patients. This was particularly reflected in the patients whose risk was lower than 35%, in whom the single-embryo transfer option increased to 31% when they knew about their personalized risk of a twin pregnancy, despite this group not showing a proactive attitude to eSET.

Currently, the predictive model of multiple pregnancy with own oocytes has extended to all treatment types, including blastocyst transfer, frozen embryo transfer, and cycles with a pre-implantation diagnosis, and has become a routine application. In 2014, the twin pregnancy rate in cycles with own oocytes at IVI Valencia was 22%, which did not affect the pregnancy rate, and it reduced to 19% in the first half of 2015. As the twin pregnancy rate lowers progressively and does not affect the pregnancy rate, trust in single-embryo transfers will grow, and the marked objective of obtaining a twin pregnancy rate of around 15%, set when the project started, may be met.

Predictive Model of Multiple Pregnancy in Donor Oocyte Cycles

In the donor oocyte context, high pregnancy rates are accomplished given the combination of transferring good quality embryos and the highly receptive endometrium of receiving patients. Consequently, the probability of multiple pregnancy increases if more than one embryo is transferred, which has been described as being between 15% and 45% (24–26). It is also important to bear in mind that these patients' more advanced age entails a significantly higher incidence of obstetric and perinatal complications (27–29).

Some authors have suggested that single-embryo transfer should be the usual practice in donor oocyte cycles, as the accumulated pregnancy rate after transferring fresh embryos and frozen embryos individually is similar between single- and double-embryo transfers, but the multiple pregnancy rate evidently reduces (30), as do obstetric risks and perinatal complications. Yet the usual reasons why patients request the transfer of two embryos, which tend to be age, impact of previously failed cycles, and particularly the economic and emotional cost that requiring more cycles entails, are even more present in donor oocyte cycles.



UNIVFY FERTILITY PROGNOSTICS*: **IVFsingle™ Test Report**

IVFsingle is designed for patients at the embryo transfer stage in IVF treatment. This test uses your clinical data and response to your current IVF treatment to determine your personal probability of having multiple births if two or more embryos are to be transferred. The test result can support you and your physician as you discuss the number of embryos to be transferred to the uterus.

PATIENT:	C V C		
MRN:	1121738	D.O.B.:	12/10/1975
CLINIC:	IVI Valencia	AGE:	36.6
TEST:	IVFsingle	TEST RUN:	06/06/2012
IVF PHYSICIAN:	Labarta, Elena	ORDER #:	3307

Probability of Multiple Birth

If you have a live birth with this fresh IVF-Embryo Transfer, then the probability that it will be a multiple birth is **41.9%**, with a prediction error of 3.2%.

IVI Valencia's IVFsingle predictive model was developed and statistically validated by using 5 years of data from our center. The model is updated annually. This test has been shown to have higher predictive power (>100% improvement) and increased ability to identify patients at high risk for multiple births, compared to the use of patient's age. Prediction error is 3.2% (standard deviation for (standard deviation for population). Please see "Our Science" at www.univfy.com for additional scientific background.

Review your baseline and IVF data for this IVF cycle:

Patient's Age:	36.6	IVF Start Date:	25/05/2012
Gravida:	2	Total Amount of FSH (IU):	1,125.0
Para:	0	Total Amount of LH (IU):	1,125.0
Total Motile Sperm Count (After Wash) Million/mL:	9.8	No. Oocytes:	8
Antral Follicle Count (Right Ovary):	4	No. 2PNs:	5
Antral Follicle Count (Left Ovary):	6	No. 8-Cell Embryos on Day 3:	2
Antral Follicle Count (Both Ovaries):	10	Day of Embryo Transfer:	3

If you have any questions about the information on this report, please consult your physician.

Limitations

This IVFsingle test is based on the information that is available up until this point of your treatment. It does not predict the probability of having a live birth, or miscarriage. Univfy test results are not intended to be the sole basis for a physician's or patient's treatment decisions. Test results are intended to be considered along with other factors.

Univfy generates its prediction models from historical data sets from each clinic with updates at regular one to two year intervals. It is not possible to develop prediction tests based on clinical data from the current year, because live birth and multiple birth outcomes for the current year typically would not be available until one year after the last treatment date for that year. Therefore, Univfy's prediction tests cannot account for new factors that have only arisen in the current year. Univfy's prediction test results are not medical care, treatment, treatment recommendation or a diagnosis. No employee of Univfy will provide persons who receive prediction test reports with a diagnosis of any disease or health condition, or any advice regarding current or future treatment decisions. Univfy Fertility Prognostics do not diagnose any disease or health condition and have not been reviewed by the U.S. Food and Drug Administration nor any international governmental agencies.

** Patent pending.*

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FIGURE 22.3 Example of report of risk estimation of twin pregnancy after IVF with own oocytes as provided by Univy©.

The prediction model was linked to the electronic chart so the likelihood of pregnancy and twin pregnancy according to the number of embryos transferred could be estimated in real time (Figure 22.4).

A database was obtained from 13,386 donor oocyte cycles from 13 IVI Group clinics between January 2007 and December 2010. The cycles in which two fresh embryos were transferred and evolutionary pregnancy was achieved were included. This provided an analysis of 3189 procedures. The number of

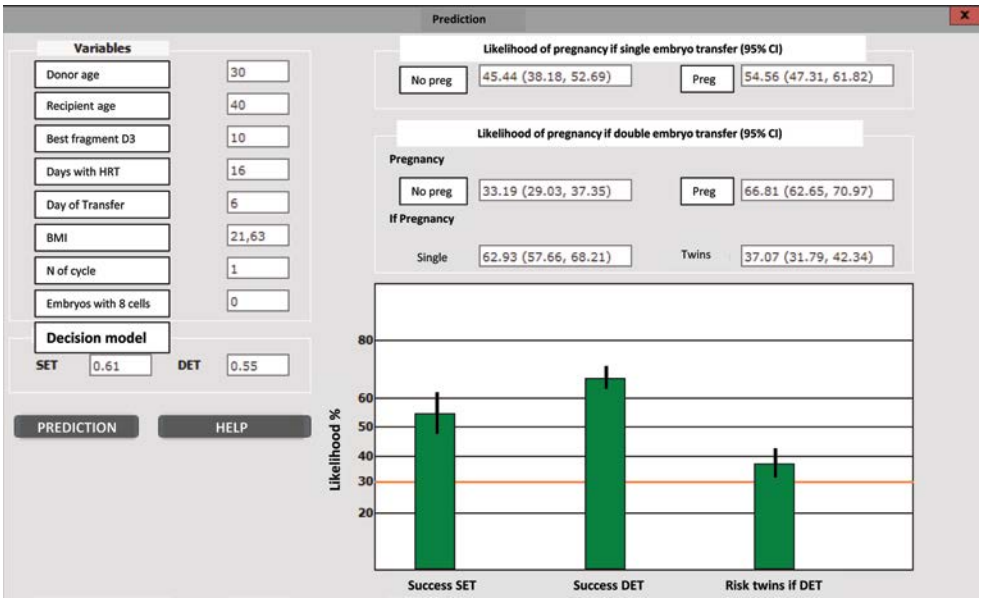

FIGURE 22.4 Prediction of pregnancy and twin pregnancy in oocyte donation cycles developed for IVI electronic chart. Known variables are introduced in the corresponding fields (upper left side), and when "Prediction" is required, results are displayed automatically by the system (right side of the panel), in order to help patients and clinicians in the decision process for the number of embryos to transfer.

analyzed variables consisted in a package of 51 variables, which contemplated the data of the donor, the receiving patient, and the laboratory. The laboratory data included oocyte, sperm, and embryo quality parameters. Embryo quality was defined by the number of cells present on day 3 of embryo development and the degree of fragmentation.

The quality of these data was evaluated by an automated control procedure (31), which allowed information to be obtained about the distribution of variables, extreme values, and lost or inconsistent data, before applying statistical modeling.

The multiple pregnancy risk was obtained by constructing a logistic regression model. Of the 3189 cycles included, 2392 were randomly selected and used to construct the model, while 797 were employed for internal validation purposes. This process was repeated up to 1000 times to increase the consistency of the results.

In a first phase, a univariate analysis was done with each variable included to identify those variables that had a relevant relation with the dependent variable, including those with a value $p < 0.25$ (32). In a second phase, a multivariate logistical regression model was constructed. In the third phase, all the possible second-order interactions were evaluated to obtain the baseline model. Finally, as with previous studies that have shown that the receiving patient's age and her BMI are related to the end result of treatment with donor oocytes (33–36), these two variables were included in the model, which gave the definitive model.

The final result is presented by the accuracy rate (ACC) and the AUC-ROC, with the 95% confidence interval (Table 22.3) (37). The twin pregnancy rate in our population included in the analysis was 42.7%, with a 15%–80% range. In 81.8% of cases, the probability of a twin pregnancy was between 30% and 50%. Only 1.3% of the cycles gave a probability below 30%, and it was equal to or over 50% in 16.8% of the cases (Figure 22.5).

The variables that were closely associated with the multiple pregnancy risk were day of transfer ($p = 0.008$) and embryo cohort quality, defined as the number of embryos with eight cells on day 3 ($p < 0.001$). The number of patient's cycles ($p = 0.021$) and endometrial preparation treatment duration ($p = 0.006$) related inversely to the twin pregnancy risk.

The resulting AUC-ROC was 0.59 (0.56–0.62) and the accuracy rate (ACC) was 0.59 (0.57–0.60). In accordance with these data, if all the patients with an estimated twin pregnancy risk that exceeded 40% had a single embryo transferred, the twin pregnancy rate would lower by half. If this measure was

TABLE 22.3

Results of Predictive Model of Twin Pregnancy in Oocyte Donation

Model	AUC	95% CI	ACC	95% CI
Prevalence	0.5	–	0.5	–
Basal model	0.59	(0.55–0.62)	0.59	(0.57–0.60)
Basal model + age	0.58	(0.55–0.62)	0.58	(0.57–0.60)
Basal model + BMI	0.58	(0.55–0.61)	0.58	(0.57–0.60)
Basal model + age + BMI	0.58	(0.55–0.62)	0.58	(0.57–0.60)

Abbreviations: AUC, area under the ROC curve; ACC, accurate rate; 95% CI, 95% confidence interval; BMI, body mass index.

Note: The basal model included: The number of oocyte donation cycle of the patient, the number of 8 cells embryos available on day 3, the lowest embryo fragmentation on day 3, and the number of days with HRT before the donation. Donor and recipient's age and recipient's body mass index were added to the basal model, but no improvement in the predictive capability was observed.

applied to a risk over 35%, the risk of twins would reduce by two thirds. If it was applied to a risk over 30%, two embryos would be transferred in only 7% of the cycles, which would lead to the practical elimination of twin pregnancies.

Since this predictive model has been applied, a highly significant reduction in the twin rate has been achieved in our donor oocyte program, which currently lies at about 20%. If its use continues, user trust (both doctors and patients) will grow, and it will lead to a progressive reduction in twin pregnancy rates without lowering pregnancy rates.

Conclusions—Key Aspects

- The post-IVF twin pregnancy rate in Spain is above the mean European rate. Given the higher incidence of obstetric and perinatal complications of these cases, the emotional and economic cost of such a situation is very high.
- Assisted reproduction centers wish to offer their patients the best possible results in pregnancy terms.

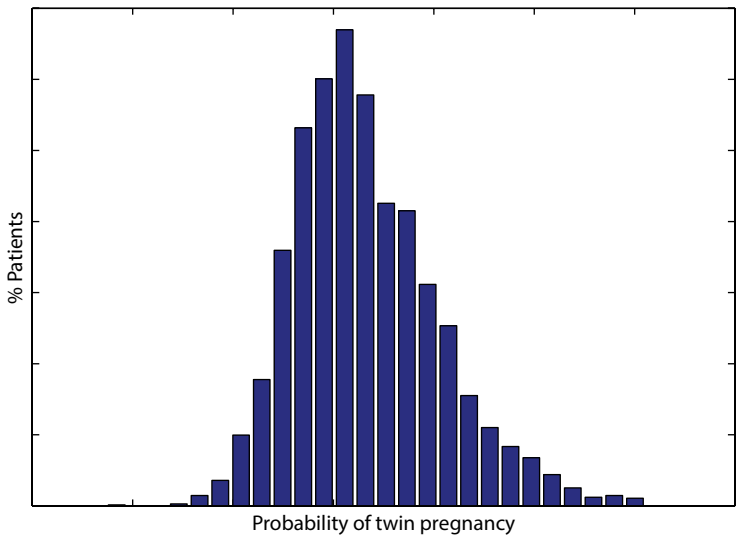

FIGURE 22.5 Probability of twin pregnancy in oocyte donation in IVI. The "x" axis shows the probability of twin pregnancy in case of double embryo transfer. The "y" axis shows the percentage of patients for each risk category.

- Patients also place pressure on professionals to optimize pregnancy rates by transferring two embryos. So it is necessary to adopt measures that can lower twin pregnancy rates without compromising pregnancy rates.
- To achieve this, the use of predictive models is essential, as they allow the personalized twin pregnancy risk to be estimated in each case, and to advise transferring a single embryo to those cases in whom this risk is very high. IVI Valencia has developed two predictive models for such cases, whose preliminary results are very promising.
- All this, along with raising awareness in doctors and patients, must lead us to the progressive reduction in twin pregnancy rates, but at the same time maintaining high overall pregnancy rates.

REFERENCES

1. Atlay R, Pennington G. The use of clomiphene citrate and pituitary gonadotropin in succesive pregnancies. The Sheffield quadruplets. *Am J Obstet Gynecol.* 1971;109:402–7.
2. Sampson J, Alexander N, Fulgham D, Burry K. Gender after artificial induction of ovulation and artificial insemination. *Fertil Steril.* 1983;40:481–4.
3. Melis G, Paoletti A, Strigini F, Fabris F, Canale D, Fioretti P. Pharmacologic induction of multiple follicular development improves the success rate of artificial insemination with husband's semen in couples with male-related or unexplained infertility. *Fertil Steril.* 1987;47:441–5.
4. Friedman A. Sextuplet pregnancy after human menopausal gonadotropins superovulation and intrauterine insemination. A case report. *J Reprod Med.* 1990;35:113–5.
5. Steptoe P, Edwards R. Birth after reimplantation of a human embryo. *Lancet.* 1978;2:366.
6. Kerin J, Warner G, Quinn P, Jeffrey R, Godfrey B, Broom T et al. The effect of clomid induced superovulation on human follicular and luteal function for extracorporeal fertilization and embryo transfer. *Clin Reprod Fertil.* 1983;2:129–42.
7. Gronow M, Martin M, McBain J, Wein P, Speirs A, Lopata A. Aspects of multiple embryo transfer. *Ann NY Acad Sci.* 1985;442:381–6.
8. Births in Great Britain resulting from assisted conception, 1978–87. MRC working party on children conceived by in vitro fertilisation. *BMJ.* 1990;300:1229–33.
9. Beral V, Doyle P, Tan S, Mason B, Campbell S. Outcome of pregnancies resulting from assisted conception. *Br Med Bull.* 1990;46:753–68.
10. Pinborg A. IVF/ICSI twin pregnancies: Risks and prevention. *Hum Reprod Update.* 2005;11:575–93.
11. Chambers GM, Hoang VP, Lee E, Hansen M, Sullivan EA, Bower C, Chapman M. Hospital costs of multiple-birth and singleton-birth children during the first 5 years of life and the role of assisted reproductive technology. *JAMA Pediatr.* 2014;168:1045–53.
12. Kupka MS, Ferraretti AP, de Mouzon J, Erb K, D'Hooghe T, Castilla JA et al. Assisted reproductive technology in Europe: Results generated from European registers by ESHRE. *Hum Reprod.* 2014;29:2099–113.
13. Pandian Z, Marjoribanks J, Ozturk O, Serour G, Bhattacharya S. Number of embryos for transfer following in vitro fertilisation or intra-cytoplasmic sperm injection. *Cochrane Database Syst Rev.* 2013;(7):CD003416.
14. Thurin A, Hausken J, Hillensjö T, Jablonowska B, Pinborg A, Strandell A, Bergh C. Elective single-embryo transfer versus double-embryo transfer in in vitro fertilization. *N Engl J Med.* 2004;351:2392–402.
15. Cutting R, Morroll D, Roberts SA, Pickering S, Rutherford A, BFS and ACE. Elective single embryo transfer: Guidelines for practice British Fertility Society and Association of Clinical Embryologists. *Hum Fertil (Camb).* 2008;11:131–46.
16. Gunby J, Bissonnette F, Librach C, Cowan L, IVF Directors Group of the Canadian Fertility and Andrology Society. Assisted reproductive technologies (ART) in Canada: 2007 results from the Canadian ART Register. *Fertil Steril.* 2011;95:542–7.
17. Practice Committee of Society for Assisted Reproductive Technology, Practice Committee of American Society for Reproductive Medicine. Elective single-embryo transfer. *Fertil Steril.* 2012;97:835–84.
18. Gleicher N, Oleske DM, Tur-Kaspa I, Vidali A, Karande V. Reducing the risk of high-order multiple pregnancy after ovarian stimulation with gonadotropins. *N Engl J Med.* 2000;343:2–7.

19. Gleicher N, Barad D. Twin pregnancy, contrary to consensus, is a desirable outcome in infertility. *Fertil Steril*. 2009;91:2426–31.
20. Chervenak FA, McCullough LB, Rosenwaks Z. Ethical dimensions of the number of embryos to be transferred in in vitro fertilization. *J Assist Reprod Genet*. 2001;18:583–7.
21. Jungheim ES, Ryan GL, Levens ED, Cunningham AF, Macones GA, Carson KR et al. Embryo transfer practices in the United States: A survey of clinics registered with the Society for Assisted Reproductive Technology. *Fertil Steril*. 2010;94:1432–36.
22. Voelker R. Researchers in Canada call for policy to mandate single-embryo transfer in IVF. *JAMA*. 2011;305:1848.
23. Banerjee P, Choi B, Shahine LK, Jun SH, O'Leary K, Lathi RB, Westphal LM, Wong WH, Yao MW. Deep phenotyping to predict live birth outcomes in in vitro fertilization. *Proc Nat Acad Sci USA A*. 2010;107:13570–5.
24. Budak E, Garrido N, Soares SR, Melo MA, Meseguer M, Pellicer A, Remohí J. Improvements achieved in an oocyte donation program over a 10-year period: Sequential increase in implantation and pregnancy rates and decrease in high-order multiple pregnancies. *Fertil Steril*. 2007;88:342–9.
25. Clua E, Tur R, Coroleu B, Boada M, Barri PN, Veiga A. Analysis of factors associated with multiple pregnancy in an oocyte donation programme. *Reprod Biomed Online*. 2010;21:694–9.
26. Kawwass JF, Monsour M, Crawford S, Kissin DM, Session DR, Kulkarni AD, Jamieson DJ, National ART Surveillance System (NASS) Group. Trends and outcomes for donor oocyte cycles in the United States, 2000–2010. *JAMA*. 2013;11:2426–34.
27. Soares SR, Troncoso C, Bosch E, Serra V, Simón C, Remohí J, Pellicer A. Age and uterine receptiveness: Predicting the outcome of oocyte donation cycles. *J Clin Endocrinol Metab*. 2005;90:4399–404.
28. Simchen MJ, Shulman A, Wiser A, Zilberberg E, Schiff E. The aged uterus: Multifetal pregnancy outcome after ovum donation in older women. *Hum Reprod*. 2009;24:2500–3.
29. Le Ray C, Scherier S, Anselem O, Marszalek A, Tsatsaris V, Cabrol D, Goffinet F. Association between oocyte donation and maternal and perinatal outcomes in women aged 43 years or older. *Hum Reprod*. 2012;27:896–901.
30. Clua E, Tur R, Coroleu B, Boada M, Rodríguez I, Barri PN, Veiga A. Elective single-embryo transfer in oocyte donation programmes: Should it be the rule? *Reprod Biomed Online*. 2012;25:642–8.
31. Sáez C, Martínez-Miranda J, Robles M, García-Gómez JM. Organizing data quality assessment of shifting biomedical data. *Stud Health Technol Inform*. 2012;180:721–5.
32. Mickey J, Greenland S. A study of the impact of confounder selection criteria on effect estimation. *Am J Epidemiol*. 1989;129:125–37.
33. Soares SR, Velasco JA, Fernandez M, Bosch E, Remohí J, Pellicer A, Simón C. Clinical factors affecting endometrial receptiveness in oocyte donation cycles. *Fertil Steril*. 2008;89:491–501.
34. Bellver J, Rossal LP, Bosch E, Zúñiga A, Corona JT, Meléndez F, Gómez E, Simón C, Remohí J, Pellicer A. Obesity and the risk of spontaneous abortion after oocyte donation. *Fertil Steril*. 2003;79:1136–40.
35. Bellver J, Melo MA, Bosch E, Serra V, Remohí J, Pellicer A. Obesity and poor reproductive outcome: The potential role of the endometrium. *Fertil Steril*. 2007;88:446–51.
36. Bellver J, Pellicer A, García-Velasco JA, Ballesteros A, Remohí J, Meseguer M. Obesity reduces uterine receptivity: Clinical experience from 9,587 first cycles of ovum donation with normal weight donors. *Fertil Steril*. 2013;100:1050–8.
37. Brodersen KH, Ong CS, Stephan KE, Buhmann JM. The balanced accuracy and its posterior distribution. In Proceedings of ICPR. 2010, Canada, 2010, 3121–4.

23

Monitoring and Evaluation of Infertility Treatments: Is There a Priority for Monitoring of Cognitive Function in Children Born from ART and Other Treatments?

Michael J. Davies, Vivienne M. Moore, Tassia K. Oswald, and Alice R. Rumbold

Introduction

The purpose of this chapter is to introduce the reader to the intentions behind monitoring and evaluation, and highlight how monitoring processes can be used to identify knowledge gaps, and guide innovation for improvements in clinical outcomes. For the present chapter, we focus on the often overlooked long-term outcome of intellectual function among children born after ART. As the assessment of intellectual function has a long latency compared to other developmental outcomes, such as structural birth defects, it is a useful example to demonstrate how innovation outstrips available data to inform clinical decision making, and how routine information systems may be inadequately prepared to monitor complex long-term outcomes. In this sense, intellectual function can be used as an example that could be generalized to other health conditions where there is a long latency between treatment and outcomes, such as metabolic health.

Purposes of a Reproductive Health Monitoring System

Monitoring systems operate at various administrative levels within the health system, ranging from quality control processes within individual pieces of equipment within a laboratory to integrated patient systems management within clinics, hospitals, governments, and across international jurisdictions. For example, the International Committee Monitoring Assisted Reproductive Technologies (ICMART) (1) reports broad patterns of ART practice across 50 countries, with a particular focus on trends across years. For the present discussion, we can focus on a national jurisdiction, as this is often the level at which broad regulation operates, either by government or professional societies.

Examples of the range of purposes of a national human reproductive monitoring system include the capacity to:

- Describe and develop reproductive health indicators
- Report accurate and reliable estimates on the frequency of outcomes
- Create the evidence base for regulatory oversight of clinical practice
- Guide the development of training programs
- Inform the timely and appropriate provision of care to patients
- Relate patterns of risk and change in risk overtime to characteristics of the patient population and the treatments received
- Provide data for hypothesis generation and testing in etiological research
- Provide aggregate data for assessing the burden of disease and attributable factors

- Inform policy development and debate
- Inform resource allocation
- Provide a mechanism for tracking interventions over time

These purposes can be considered as part of an iterative self-regulating loop of continuous refinement into which a new technology can be inserted (2). This process is particularly important where the rate of innovation is rapid, and where the costs of either the investment in technology and treatment, or the consequences of adverse outcomes is significant. All three are the case with ART. These technologies share a number of features with other "breakthrough" technologies where there is little extant knowledge on which to base decision making, and more importantly, where the probability of an outcome also changes over time as a result of innovation, creating a significant lag function between practice and outcome. One implication of this complexity is that the assessable outcomes in the laboratory or clinic are quite distant from the outcomes in the created individual, particularly years or decades later, possibly even for generations. This points to a new imperative to more clearly consider ART within the structures of mainstream medicine in which there is greater synthesis of information systems between clinic and routine health collections, including registries for births and deaths, hospitalizations, and data collections for enduring and important outcomes, such as educational attainment.

ART and Adverse Outcomes

There is now a substantial body of literature demonstrating that children conceived with infertility treatments are at increased risk of poor perinatal outcomes (preterm birth, low birth weight, neonatal morbidity) and congenital anomalies, compared with naturally conceived children, even after adjustment for confounders such as maternal age (3–5). For congenital anomalies, there is also accumulating evidence that prevalence is elevated in children conceived with noninvasive infertility treatments (NIFT), such as ovulation induction (6). This is of relevance, as noninvasive treatments performed outside an ART clinic are rarely, if ever, recorded in a mandated surveillance system.

A further complication for monitoring outcomes after infertility treatment relates to the difficulty of recording potential sources of risk in sufficient detail to permit the appropriate attribution of risk to a source. This is important where, for example, there has been a secular change in the patient population over time that alters treatment outcomes, or where a treatment is associated with an indication for that specific treatment. With regards to developmental anomalies after ART, attributing increased occurrence of health problems in offspring entirely to treatment is not appropriate, as children conceived naturally to subfertile couples also appear to have compromised health. For example, in a systematic review of 17 studies, Messerlian et al. (7) found that women who conceived naturally with a long time to pregnancy had an increased risk of preterm birth (<37 weeks) and low birth weight (<2500 g). A smaller literature shows that children born to subfertile couples have an excess of congenital anomalies (8). Thus, it is likely that subfertility is, to some degree, responsible for the above increases in risks in children conceived with medical assistance, although we should be mindful that this population may also be using non-monitored infertility treatments. A further limitation in monitoring systems is the relatively short-term observation and reporting cycles, such that until recently, adequate data have not been available to evaluate longer-term outcomes (in adolescence or adulthood) (9), and even studies of short-term outcomes have been criticized as suffering from poor study design and data limitations (10,11).

Nevertheless, there are now numerous studies from routine reporting systems, individual studies, and systematic reviews indicating elevated multiple pregnancy rates, impaired perinatal outcomes, and increased risks of congenital anomalies in children born from assisted conception. We can now extend our horizons to consider new outcomes, as with the increasing sophistication of data linkage capability, monitoring systems can efficiently examine the impact of ART on not just anatomical features but also on functional characteristics of development.

It is important to attend to *intellectual impairment* that may occur even in the absence of overt conditions, such as cerebral palsy, or where there may be decrements within the normal range. It is important to assess outcomes in adolescence, as impairments in complex cognitive functioning may only emerge

as children reach this period of development and, conversely, deficits detected early may not predict later performance (12). A practical consequence of such impairment is that children perform poorly at school, in turn affecting many life opportunities (13).

In the following section we consider the question as to whether *assisted conception affects intellectual development of the children, and whether there is variation by treatment, and what do we need to have in place to examine this question?*

We have undertaken a systematic search for relevant studies with objective outcome assessment. We identified 29 studies including children assessed at age 5 years or older, summarized in Table 23.1 (some

TABLE 23.1

Studies Assessing Cognitive and Learning Abilities in Children Aged ≥5 Years

No.	Lead Author	Sample Size	Child Age (Yrs)	Main Measures
1	Golombok (14)	AC: 68, C: 39	4–8	Pictorial Scale of Perceived Competence and Social Acceptance
2	Golombok (15)	AC: 224, C: 120	4–8	Pictorial Scale of Perceived Competence and Social Acceptance
3	Levy-Shiff (16)	AC: 51, C: 51	9–10	WPPSI-R
4	Golombok (17)	AC: 34, SC: 38	12	Social adjustment inventory for children and adolescents
5	Golombok (18)	AC: 37, C: 91	11–12	CAFE schedule
6	Golombok (19)	AC:196, C: 102	11–12	CAFE schedule
7	Strömberg (20)	AC: 5680, C: 15,397	1–14	Neurological diagnoses on national register
8	Leslie (21)	AC: 177, C:110	5	WPPSI-R
9	Place (22)	AC: 118, C:59	5	WPPSI-R
10	Neri (23)	AC:101 C: 57	5	WPPSI-R
11	Ponjaert-Kristoffersen (24)	AC: 300, C: 260	5	WPPSI-R
12	Ponjaert-Kristoffersen (25)	AC: 935, C:488	5	WPPSI-R
13	Leunens (26)	AC: 151, C: 153	8	WPPSI-R
14	Belva (27)	AC: 150, C:147	8	Neurological examination
15	Knoester (28)	AC: 169, C: 85	5–8	Revised Amsterdam child intelligence test
16	Leunens (29)	AC: 109, C: 90	10	WPPSI-R
17	Wagenaar (30)	AC: 233, C: 233	8–18	Standardized school achievement tests
18	Goldbeck (31)	AC: 69, C: 0	5–10	Kaufman Assessment Battery for Children
19	Golombok (32)	AC: 26, C:56	18	CAFE schedule
20	Ludwig (33)	AC: 276, C: 273	5.5	Kaufman Assessment Battery for Children
21	Mains (34)	AC: 423, C: 372	8–17	Standardized school achievement tests
22	Carson (35)	AC: 263, C: 6244	3–5	British Ability Scales (2nd Edition)
23	Gucuyner (36)	AC: 70, C: 92	5	Standford-Binet Intelligence Scale Form
24	Ozbaran (37)	AC: 35, C: 35	2–13	WISC-R
25	Bay (11)	AC: 33,139, C: 555,828	8–17	Clinical diagnoses in the Danish Psychiatric Central Research Register
26	Sandin (38)	AC: 30,959, C: 2,510,166	1.5–28	Clinical diagnosis of mental retardation (IQ < 70) on national patient register
27	Bay (39)	AC: 71, C:1577	5	WPPSI-R
28	D'haeseleer (40)	AC: 20, C: 0	3–10	WPPSI-R
29	Winter (41)	AC: 96, C: 48	5–6	WPPSI-III

Abbreviations: AC, assisted conception; C, comparison group; CAFE, Child and Adolescent Functioning and Environment; WPPSI-R, Weschler Preschool and Primary Scales of Intelligence—Revised; WISC, Weschsler Intelligence Scale for Children.

groups repeated across publications). Fourteen studies (Nos 3–7, 16–19, 21, 24–26, and 28) provided information on children from 10 years of age. However, as outlined below, few conclusions can be drawn from this literature due to methodological limitations.

Among the five studies that did not have major limitations, three (Nos 7, 25, and 26) reported poorer cognitive ability (including a greater risk of mental retardation) among assisted-conception children compared to spontaneously conceived peers. Each of these utilized national health registers of conditions requiring admission to hospital or outpatient services. A degree of detection bias (favoring naturally-conceived children) may have occurred in these studies, as assisted conception children have more contact with the health system. Importantly, these three studies could only assess impairments for which the nature and/or severity meant that the health system was accessed. One further study (No. 21) reported better performance among assisted-conception children, and one (No. 24) reported no differences between comparison groups.

Major methodological weaknesses:

- The majority of studies have small samples (median size of the assisted conception group is 150), thus would only be able to detect large differences between groups.
- Most studies are subject to selection bias, with 21 (Nos 1–6, 9, 11–20, 22–23, 27, and 29) *excluding children at higher risk of adverse developmental outcomes* based on factors such as preterm birth, low birth weight, other perinatal complications, and multiple pregnancy. Children with neurological or intellectual disability were specifically excluded in 12 studies (Nos 1–2, 4–6, 15, 18–19, 22–23, 27, and 29). While it can sometimes be appropriate to stratify results by these factors, not obtaining information on a complete group of children exposed to treatment limits the depiction and knowledge of outcomes.
- Twelve studies (Nos 2, 8–9, 11, 12–17, 20, and 28) had substantial differences in participation between assisted-conception and comparison groups (differential response rates and/or differential attrition).

Many studies (Nos 7, 10, 18, 21, 23, 24, 26, and 28) failed to adequately consider confounding by family background.

Nine studies (Nos 1, 2, 4–6, 17, 19, 22, and 27) included a comparison group of children conceived naturally to parents with subfertility. However, all specifically excluded children at high risk of adverse developmental outcomes or with manifest neurological impairment. Three studies (Nos 22, 25, and 26) reported outcomes separately for less invasive techniques. Two (Nos 22 and 26) found no significant differences in outcome between NIFT and spontaneous conceptions. A further study (No. 25) reported a small increase in the risk of disorders of psychological development in children conceived with ovulation induction. None of these studies directly compared ART and NIFT children.

Questions on Treatment Options and Outcome Variability in Health

To what extent do the following procedures affect the health and development of offspring born after assisted conception: (a) IVF vs ICSI; (b) fresh vs frozen embryo transfer?

With ICSI, fertilization occurs by the direct injection of a single spermatozoon into the oocyte cytoplasm. ICSI was originally developed as a treatment for severe male-factor infertility. However, its use for non-male-factor infertility has grown substantially: data from the United States indicated use for non-male-factor infertility was 15% of cycles in 1996 but had increased to 67% by 2012 (42).

Although the fertilization rate is significantly higher for ICSI cycles than for IVF, this does not result in a higher live birth rate, even when male-factor infertility is taken into account (42). This could reflect poor technical skill or suboptimal in vitro conditions, but it is also possible that it is due to gamete manipulation.

Studies directly comparing *cognitive abilities* in IVF versus ICSI children are scant. Of the studies listed in Table 23.1, eight (Nos 8–10, 12, 15, 18, 21, and 26) included a comparison of IVF and ICSI but, as noted earlier, five excluded children at risk of poor development. The three studies that did not do

so found no difference between IVF and ICSI (Nos 10 and 21); and ICSI children at increased risk of mental retardation (No. 26). Only three studies concerned children aged ≥10 years (Nos 18, 21, and 26) and in all but one (No. 26) the ICSI group was small.

Turning to cryopreservation, which permits surplus embryos from ovarian stimulation for use in a later treatment cycle, and thereby facilitates single embryo transfer, we now have a course of care with successive single embryo transfers from which we can calculate cumulative success and adverse events. A recent systematic review of randomized controlled trials evaluating frozen versus fresh embryo transfer cycles found a higher rate of ongoing pregnancy and lower rate of miscarriage after frozen cycles (43).

The freeze/thaw technique raises important questions about safety, although improvements in some outcomes for frozen embryos are biologically plausible through selection pressures on embryo survivorship and the introduction of a time lag to allow the maternal endometrium to recover from ovarian hyperstimulation (the latter supported by recent findings of Roy et al.) (44).

There have been three systematic reviews (45–47) of studies evaluating *perinatal outcomes* following freeze/thaw cycles. The reviews indicate that with frozen embryo transfer, antepartum hemorrhage may be reduced. Compared to fresh embryos, frozen embryos had reduced perinatal mortality and similar or better outcomes in terms of birth weight and prematurity. Five studies published since have confirmed the reduction in prematurity and low birth weight for frozen embryos; however, there were increased risks of post-term birth, and large for gestational age (5,48–50). The apparent benefits of cryopreservation, at least in the short-term, have led to calls for reproductive technology clinics to adopt a "freeze-all" policy (43). Currently, impediments include patient preference for fresh cycles, legislative restrictions on freezing, and extra training required (51). There is ongoing debate as to whether the differences observed at birth are due to differences in characteristics of couples undergoing fresh and frozen embryo transfer (45).

Studies comparing the *cognitive abilities* of children conceived using frozen compared with fresh embryo transfer are lacking (46). We located only two. One found no differences in school performance test scores, but included only 82 children exposed to cryopreservation as an embryo (No. 21). The other (No. 26) found no difference in mental retardation (IQ <70) but, being a registry-based study drawing on hospitalization and outpatient records, could not investigate more subtle outcomes. As a consequence, we perceive there to be an urgent need to consider more complex treatment strategies for their intellectual outcomes in offspring.

Summary

Assisted reproductive treatments are remarkable scientific breakthroughs and have benefited many couples worldwide. Advances in the field over 30 years are testimony to consistent striving for improvement and innovation. Concerted efforts have been made to address safety of treatments. The literature presented in the previous section, despite its limitations in relation to the specific questions we posed, demonstrates a commitment to gain knowledge about the health and development of children conceived with medical assistance. Nevertheless, assessment of cognitive or learning abilities is beleaguered with problems affecting all studies involving medium- to long-term follow up. Again, registry-based studies provide a way to overcome many limitations of bespoke cohort studies, and several have now been published (11,20,38). The current limitation in this area lies in what is captured in available registries, which is typically health service use relating to specific or severe problems. In addition, information about the cause of infertility is either absent (20,38) or restricted to certain treatment groups only (e.g., IVF/ICSI) (52). Research delineating the roles of parental subfertility and specific treatment strategies is also warranted (53), particularly as intellectual function is highly socially contextual.

The above also illustrates why the questions we pose are unlikely to be addressed with trials. Studies with a focus on clinical pregnancy are short and often do not need to be large. Pooling and follow-up of trials should be considered, but it will be years before sufficient children reach adolescence.

Consulting the list of purposes listed at the beginning of the chapter, we argue that surveillance systems have a primary focus on the regulatory and quality functions. Expanding the range of purposes, for instance, addressing etiological or basic research questions, will face a series of challenges including

data harmonization, developing a common nomenclature, and of course resource allocation. A common objective for these purposes is to develop an internationally accepted and continually updated set of definitions, which would be used for analysis across platforms of increasing scale, including internationally, to ensure the continual development of effective, efficient, and increasingly safe infertility treatments. ART is also unique in that the technical innovations used on one patient impact fundamentally on a hypothetical person yet to exist. Hence, we are dealing with a range of interests which need to be imbedded with the decision making, and thereby monitoring within feedback hierarchies. Access to data from a well-defined population over sufficient time for groups is therefore required, including detailed clinical records concerning clinic-based treatment for infertility and information on developmental anomalies, and standardized assessments of educational performance.

REFERENCES

1. Mansour R, Ishihara O, Adamson GD, Dyer S, de Mouzon J, Nygren KG et al. International committee for monitoring assisted reproductive technologies world report: Assisted reproductive technology 2006. *Hum Reprod*. 2014;29(7):1536–51.
2. Donabedian A. Evaluating the quality of medical care. 1966. *Milbank Q*. 2005;83(4):691–729.
3. Hansen M, Bower C. The impact of assisted reproductive technologies on intra-uterine growth and birth defects in singletons. *Semin Fetal Neonatal Med*. 2014;19(4):228–33.
4. Henningsen AA, Gissler M, Skjaerven R, Bergh C, Tiitinen A, Romundstad LB et al. Trends in perinatal health after assisted reproduction: A Nordic study from the CoNARTaS group. *Hum Reprod*. 2015;30(3):710–6.
5. Marino JL, Moore VM, Willson KJ, Rumbold A, Whitrow MJ, Giles LC et al. Perinatal outcomes by mode of assisted conception and sub-fertility in an Australian data linkage cohort. *PLoS One*. 2014;9(1):e80398.
6. Davies MJ, Moore VM, Willson KJ, Van Essen P, Priest K, Scott H et al. Reproductive technologies and the risk of birth defects. *N Engl J Med*. 2012;366(19):1803–13.
7. Messerlian C, Maclagan L, Basso O. Infertility and the risk of adverse pregnancy outcomes: A systematic review and meta-analysis. *Hum Reprod*. 2013;28(1):125–37.
8. Zhu JL, Basso O, Obel C, Bille C, Olsen J. Infertility, infertility treatment, and congenital malformations: Danish national birth cohort. *BMJ*. 2006;333(7570):679.
9. Wilson CL, Fisher JR, Hammarberg K, Amor DJ, Halliday JL. Looking downstream: A review of the literature on physical and psychosocial health outcomes in adolescents and young adults who were conceived by ART. *Hum Reprod*. 2011;26(5):1209–19.
10. Schieve LA, Rasmussen SA, Reefhuis J. Risk of birth defects among children conceived with assisted reproductive technology: Providing an epidemiologic context to the data. *Fertil Steril*. 2005;84(5):1320–4.
11. Bay B, Mortensen EL, Hvidtjorn D, Kesmodel US. Fertility treatment and risk of childhood and adolescent mental disorders: Register based cohort study. *BMJ*. 2013;347:f3978.
12. Bracken BA, Walker KC. The utility of intelligence tests for preschool children. In: *Contemporary Intellectual Assessment: Theories, Tests and Issues*, Flanagan DP, Genshaft JL, Harrison PL (Eds.). New York: Guilford Press, 2007, 484–502.
13. Gerber PJ. The impact of learning disabilities on adulthood: A review of the evidenced-based literature for research and practice in adult education. *J Learn Disabil*. 2012;45(1):31–46.
14. Golombok S, Cook R, Bish A, Murray C. Families created by the new reproductive technologies: Quality of parenting and social and emotional development of the children. *Child Dev*. 1995;66(2):285–98.
15. Golombok S, Brewaeys A, Cook R, Giavazzi MT, Guerra D, Mantovani A et al. The European study of assisted reproduction families: Family functioning and child development. *Hum Reprod*. 1996;11(10):2324–31.
16. Levy-Shiff R, Vakil E, Dimitrovsky L, Abramovitz M, Shahar N, Har-Even D et al. Medical, cognitive, emotional, and behavioral outcomes in school-age children conceived by in-vitro fertilization. *J Clin Child Psychol*. 1998;27(3):320–9.
17. Golombok S, MacCallum F, Goodman E. The "test-tube" generation: Parent-child relationships and the psychological well-being of in vitro fertilization children at adolescence. *Child Dev*. 2001;72(2):599–608.

18. Golombok S, Brewaeys A, Giavazzi MT, Guerra D, MacCallum F, Rust J. The European study of assisted reproduction families: the transition to adolescence. *Hum Reprod*. 2002;17(3):830–40.
19. Golombok S, MacCallum F, Goodman E, Rutter M. Families with children conceived by donor insemination: A follow-up at age twelve. *Child Dev*. 2002;73(3):952–68.
20. Stromberg B, Dahlquist G, Ericson A, Finnstrom O, Koster M, Stjernqvist K. Neurological sequelae in children born after in-vitro fertilisation: A population-based study. *Lancet*. 2002;359(9305):461–5.
21. Leslie GI, Gibson FL, McMahon C, Cohen J, Saunders DM, Tennant C. Children conceived using ICSI do not have an increased risk of delayed mental development at 5 years of age. *Hum Reprod*. 2003;18(10):2067–72.
22. Place I, Englert Y. A prospective longitudinal study of the physical, psychomotor, and intellectual development of singleton children up to 5 years who were conceived by intracytoplasmic sperm injection compared with children conceived spontaneously and by in vitro fertilization. *Fertil Steril*. 2003;80(6):1388–97.
23. Neri QV, Tanaka N, Wang A, Katagiri Y, Takeuchi T, Rosenwaks Z et al. Intracytoplasmic sperm injection. Accomplishments and qualms. *Minerva Ginecol*. 2004;56(3):189–96.
24. Ponjaert-Kristoffersen I, Tjus T, Nekkebroeck J, Squires J, Verte D, Heimann M et al. Psychological follow-up study of 5-year-old ICSI children. *Hum Reprod*. 2004;19(12):2791–7.
25. Ponjaert-Kristoffersen I, Bonduelle M, Barnes J, Nekkebroeck J, Loft A, Wennerholm UB et al. International collaborative study of intracytoplasmic sperm injection-conceived, in vitro fertilization-conceived, and naturally conceived 5-year-old child outcomes: Cognitive and motor assessments. *Pediatrics*. 2005;115(3):e283–9.
26. Leunens L, Celestin-Westreich S, Bonduelle M, Liebaers I, Ponjaert-Kristoffersen I. Cognitive and motor development of 8-year-old children born after ICSI compared to spontaneously conceived children. *Hum Reprod*. 2006;21(11):2922–9.
27. Belva F, Henriet S, Liebaers I, Van Steirteghem A, Celestin-Westreich S, Bonduelle M. Medical outcome of 8-year-old singleton ICSI children (born >or = 32 weeks' gestation) and a spontaneously conceived comparison group. *Hum Reprod*. 2007;22(2):506–15.
28. Knoester M, Helmerhorst FM, Vandenbroucke JP, van der Westerlaken LA, Walther FJ, Veen S. Perinatal outcome, health, growth, and medical care utilization of 5- to 8-year-old intracytoplasmic sperm injection singletons. *Fertil Steril*. 2008;89(5):1133–46.
29. Leunens L, Celestin-Westreich S, Bonduelle M, Liebaers I, Ponjaert-Kristoffersen I. Follow-up of cognitive and motor development of 10-year-old singleton children born after ICSI compared with spontaneously conceived children. *Hum Reprod*. 2008;23(1):105–11.
30. Wagenaar K, Ceelen M, Van Weissenbruch MM, Knol DL, Delemarre-van de Waal HA, Huisman J. School functioning in 8- to 18-year-old children born after in vitro fertilization. *Eur J Pediatr*. 2008;167(11):1289–95.
31. Goldbeck L, Gagsteiger F, Mindermann I, Strobele S, Izat Y. Cognitive Development of Singletons Conceived by Intracytoplasmic Sperm Injection or In vitro Fertilization at Age 5 and 10 years. *J Pediatr Psychol*. 2009;34(7):774–81.
32. Golombok S, Owen L, Blake L, Murray C, Jadva V. Parent-child relationships and the psychological well-being of 18-year-old adolescents conceived by in vitro fertilisation. *Hum Fertil*. 2009;12(2):63–72.
33. Ludwig A, Katalinic A, Thyen U, Sutcliffe AG, Diedrich K, Ludwig M. Neuromotor development and mental health at 5.5 years of age of singletons born at term after intracytoplasmatic sperm injection ICSI: Results of a prospective controlled single-blinded study in Germany. *Fertil Steril*. 2009;91(1):125–32.
34. Mains L, Zimmerman M, Blaine J, Stegmann B, Sparks A, Ansley T et al. Achievement test performance in children conceived by IVF. *Hum Reprod*. 2010;25(10):2605–11.
35. Carson C, Kelly Y, Kurinczuk JJ, Sacker A, Redshaw M, Quigley MA. Effect of pregnancy planning and fertility treatment on cognitive outcomes in children at ages 3 and 5: Longitudinal cohort study. *BMJ*. 2011;343:d4473.
36. Gucuyener K, Arhan E, Soysal AS, Ergenekon E, Turan O, Onal E et al. Language assessment of non-handicapped twins at 5 years of age. *Padiatr Int*. 2011;53(6):944–9.
37. Ozbaran B, Kose S, Ardic UA, Erermis S, Ergin HK, Bildik T et al. Psychiatric evaluation of children born with assisted reproductive technologies and their mothers: A clinical study. *Arch Neuropsych*. 2011;50(1):59–64.
38. Sandin S, Nygren KG, Iliadou A, Hultman CM, Reichenberg A. Autism and mental retardation among offspring born after in vitro fertilization. *JAMA*. 2013;310(1):75–84.

39. Bay B, Mortensen EL, Kesmodel US. Fertility treatment and child intelligence, attention, and executive functions in 5-year-old singletons: A cohort study. *BJOG*. 2014;121(13):1642–51.
40. D'Haeseleer E, Vanden Meerschaut F, Bettens K, Luyten A, Gysels H, Thienpont Y et al. Language development of children born following intracytoplasmic sperm injection (ICSI) combined with assisted oocyte activation (AOA). *Int J Lang Commun Disord*. 2014;49(6):702–9.
41. Winter C, Van Acker F, Bonduelle M, Desmyttere S, De Schrijver F, Nekkebroeck J. Cognitive and psychomotor development of 5- to 6-year-old singletons born after PGD: A prospective case-controlled matched study. *Hum Reprod*. 2014;29(9):1968–77.
42. Boulet SL, Mehta A, Kissin DM, Warner L, Kawwass JF, Jamieson DJ. Trends in use of and reproductive outcomes associated with intracytoplasmic sperm injection. *JAMA*. 2015;313(3):255–63.
43. Roque M, Lattes K, Serra S, Sola I, Geber S, Carreras R et al. Fresh embryo transfer versus frozen embryo transfer in in vitro fertilization cycles: A systematic review and meta-analysis. *Fertil Steril*. 2013;99(1):156–62.
44. Roy TK, Bradley CK, Bowman MC, McArthur SJ. Single-embryo transfer of vitrified-warmed blastocysts yields equivalent live-birth rates and improved neonatal outcomes compared with fresh transfers. *Fertil Steril*. 2014;101(5):1294–301.
45. Maheshwari A, Pandey S, Shetty A, Hamilton M, Bhattacharya S. Obstetric and perinatal outcomes in singleton pregnancies resulting from the transfer of frozen thawed versus fresh embryos generated through in vitro fertilization treatment: A systematic review and meta-analysis. *Fertil Steril*. 2012;98(2):368–77.
46. Wennerholm UB, Soderstrom-Anttila V, Bergh C, Aittomaki K, Hazekamp J, Nygren KG et al. Children born after cryopreservation of embryos or oocytes: A systematic review of outcome data. *Hum Reprod*. 2009;24(9):2158–72.
47. Pinborg A, Wennerholm UB, Romundstad LB, Loft A, Aittomaki K, Soderstrom-Anttila V et al. Why do singletons conceived after assisted reproduction technology have adverse perinatal outcome? Systematic review and meta-analysis. *Hum Reprod Update*. 2013;19(2):87–104.
48. Wennerholm UB, Henningsen AK, Romundstad LB, Bergh C, Pinborg A, Skjaerven R et al. Perinatal outcomes of children born after frozen-thawed embryo transfer: A Nordic cohort study from the CoNARTaS group. *Hum Reprod*. 2013;28(9):2545–53.
49. Ishihara O, Araki R, Kuwahara A, Itakura A, Saito H, Adamson GD. Impact of frozen-thawed single-blastocyst transfer on maternal and neonatal outcome: An analysis of 277,042 single-embryo transfer cycles from 2008 to 2010 in Japan. *Fertil Steril*. 2014;101(1):128–33.
50. Schwarze JE, Crosby JA, Zegers-Hochschild F. Effect of embryo freezing on perinatal outcome after assisted reproduction techniques: Lessons from the Latin American Registry of Assisted Reproduction. *Reprod Biomed Online*. 2015;31(1):39–43.
51. Evans J, Hannan NJ, Edgell TA, Vollenhoven BJ, Lutjen PJ, Osianlis T et al. Fresh versus frozen embryo transfer: Backing clinical decisions with scientific and clinical evidence. *Hum Reprod Update*. 2014;20(6):808–21.
52. Bay B, Mortensen EL, Kesmodel US. Assisted reproduction and child neurodevelopmental outcomes: A systematic review. *Fertil Steril*. 2013;100(3):844–53.
53. Pinborg A, Henningsen AK, Malchau SS, Loft A. Congenital anomalies after assisted reproductive technology. *Fertil Steril*. 2013;99(2):327–32.

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