

# Fundamentals of Male Infertility

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# Foreword

It is always intriguing to write a foreword for a special groundbreaking book. It is inspiring that this book deals in a clear no-nonsense way with the sometimes controversial fundamentals of male infertility. In the past male infertility had been vexed by specious treatments and even IVF was problematic. I have had yearly transatlantic collaboration with Dr. S. Silber on this subject since 1979. We met every year many times somewhere in the world to solve the problems of male infertility. For 40 years we accomplished many advances in the field.

This book is fascinating because it honestly and clearly describes the science of male infertility and all the possible treatments which have been proposed during these last 40 years. The surprise of the book is that the reader will be guided into the correct approach for every controversial issue. There are many myths in male infertility. The crucial point of this book is to explain the science of male infertility and focus on truly valid and proven effective treatment. The author tries to find the truth, debunk myths, and explain the correct efficacious treatment. Dr. S. Silber is very open in personally explaining the solid reasons for his approach.

There are certainly many treatments for male infertility which are not proven to be helpful. This book opens the avenue to the correct approach, exposing dubious and irrelevant treatments.

One example of a completely irrelevant approach is the operative intervention to cure varicocele. Millions of males have undergone this surgery without any positive effect. There is no good evidence in favor of this operation. We can hope that this book will inform the reader and eliminate this useless and meddlesome operation.

Even when we cannot treat the male, we can treat his gametes. The discovery of in vitro fertilization with ICSI has been a key milestone in the treatment of female and also male infertility. Furthermore, the advent of vitrification for freezing eggs and embryos and individual single sperm, the genetic control of spermatogenesis, and even how to make spermatozoa from skin cells will inspire all of us.

Dr. Silber explains very clearly how intracytoplasmic sperm injection (ICSI) has changed the world of male infertility treatment. Only one sperm is needed to obtain a healthy pregnancy. The most inspiring thinking of the book is related to the fact that even males with extreme oligospermia do have the same chance to initiate a

viable pregnancy as the male with completely normal sperm. In the past, males with azoospermia were hopelessly sterile. But after testicular sperm extraction (TESE) the quality of sperm is sufficient to obtain normal pregnancies with ICSI.

This book analyzes the basic science and treatment of male factor infertility with Dr. Silber's characteristic honesty and clear thinking. It describes finally what works and what doesn't work. It will inspire most readers who wish to understand male infertility better.

Brussels, Belgium  
2018

Paul Devroey

# Historical Preface

I hope this book will give you a rational and up-to-date explanation of both the science and clinical treatment of male infertility. Of course this includes endocrinology, and genetics. But modern male infertility treatment began historically with microsurgery. My 47-year history of developing a rational approach to male infertility began in 1970 when I was just a urology resident at the University of Michigan in Ann Arbor, not at all interested in infertility. That was before the incredible advances in andrology. But our modern understanding and treatment of the male developed originally with the popularization of microsurgery. So it would be useful to start with a historical introduction to male reproductive microsurgery.

My initial goal was just to understand compensatory renal hypertrophy, i.e., why when you remove one kidney, the other kidney compensates, and renal function of the remaining kidney is almost doubled. To answer this question, I decided in 1969 to transplant extra kidneys into animals, to see if the reverse would occur, i.e., would the kidneys “hypotrophy”? I did not want the experiment to be hampered by allogenic rejection, and the largest animal inbred enough to avoid rejection was the rat. So I developed in my earliest papers in the early 1970s a whole field of microvascular surgery in urology [1, 2]. With the help and instruction of a brilliant janitor at the University of Michigan animal lab, Jimmy Crudop, I transplanted extra kidneys from one Lewis rat into another Lewis rat using donor renal artery and vein anastomosis to the recipient aorta and vena cava. The rat renal artery was only 0.3 mm in diameter and the aorta was only 1.0 mm in diameter. Such microscopic surgery at the time seemed almost unbelievable. I thus unintentionally became a pioneer in microsurgery, purely in order to study renal physiology, not infertility (Figs. 1, 2, 3, 4, 5, and 6). It was subsequently only as a result of this effort that the modern field of male infertility treatment evolved. For historic reasons, therefore, it is appropriate to give a short history of urologic microsurgery.

We found that the kidneys did not reduce in size, but rather these animals had two times the renal function with four kidneys than controls with two kidneys [3–6]. On the other hand, when we removed one kidney from a rat, so that the other kidney doubled in size, and we transplanted two of those hypertrophied kidneys back into one animal, they reduced back to their original size. Renal function reverted quickly

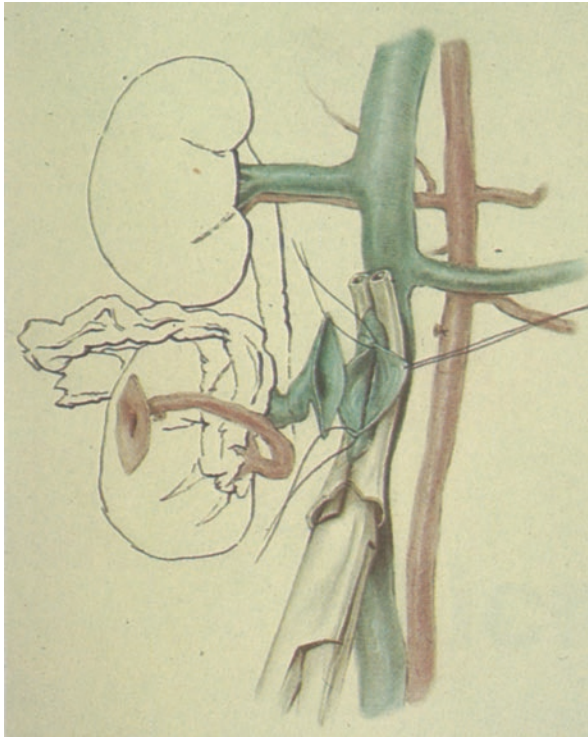


**Fig. 1** Microvascular surgery in rat: end-to-side microvascular anastomosis of renal vein to vena cava (1 mm diameter)

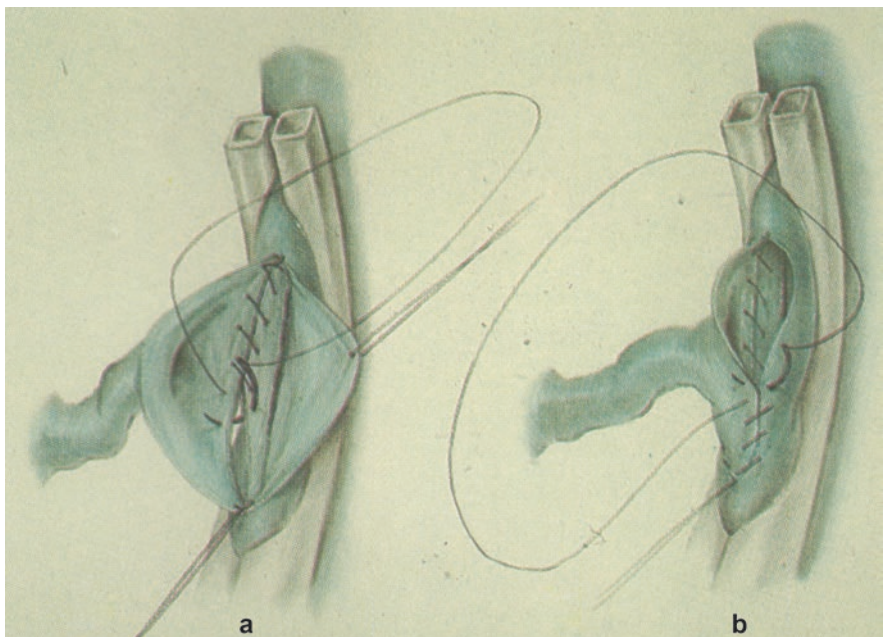


**Fig. 2** Microvascular end-to-side anastomosis in rat renal vein to vena cava (1 mm)

**Fig. 3** Intravenous  
pyelogram of rat showing  
three functioning kidneys

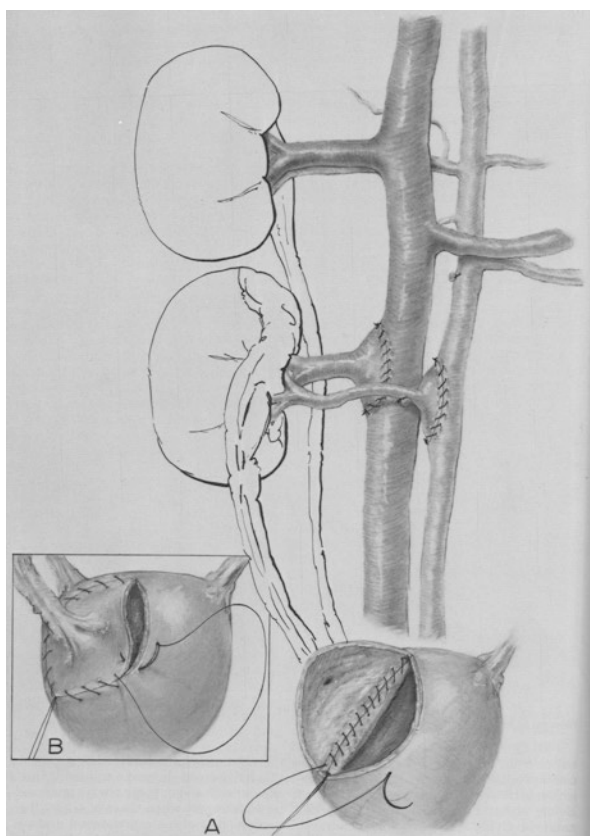


**Fig. 4** Ureteral bladder anastomosis in rat

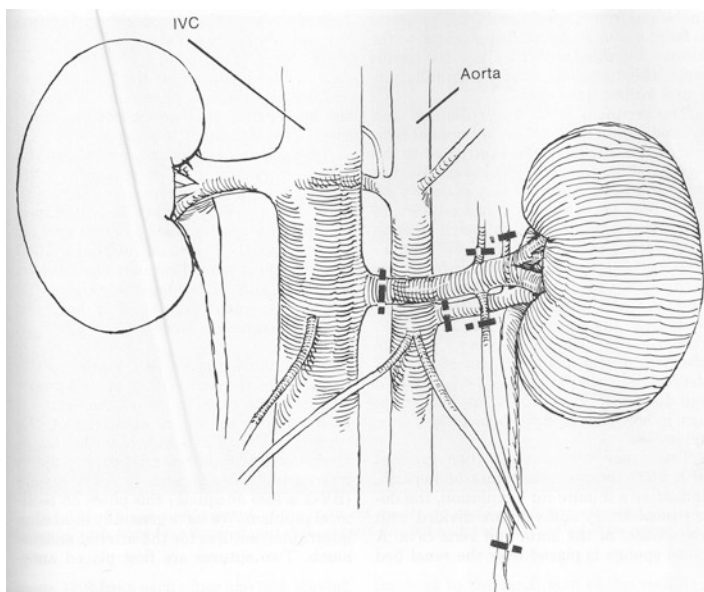


**Fig. 5** Using the timer end-to-end anastomosis of rat renal artery and vein and ureter, we could do experiments that left the donor alive

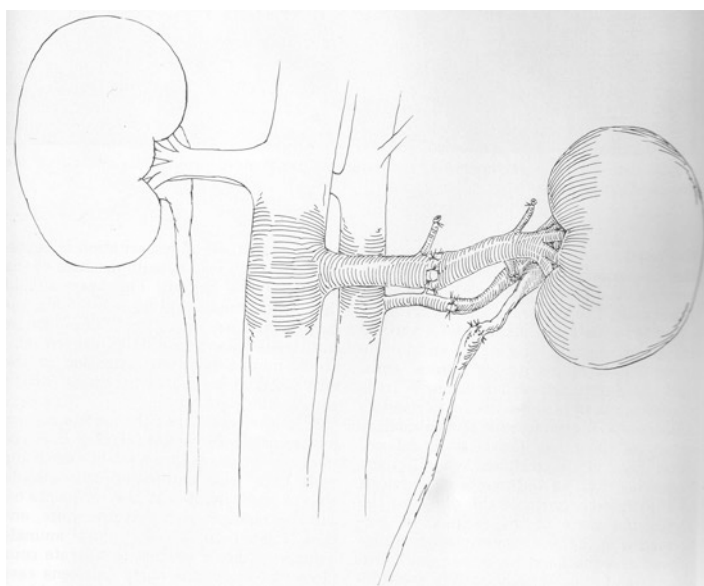


**Fig. 6** Completed end-to-end anastomosis of rat renal artery (1/3 mm), renal vein (1 1/2 m), and ureter (1/3 mm)

from twice normal to normal. This research led to many advances in renal transplantation in the 1970s: the prevention of renal failure after kidney transplantation by salt loading the rats before inducing prolonged ischemia times, prolonged hydration and osmotic loading prior to surgery, the limits of warm and cold ischemic time a kidney (or ovary or testis) could tolerate before permanent loss of function, and preventing allograft rejection using prior properly timed injection of donor antigens [7–9]. Thus when I left Ann Arbor, Michigan, and Melbourne, Australia (1969–1975), I was known as a kidney transplanter and microvascular surgeon. But I had no idea I would ever be an infertility doctor.

While in Melbourne, I worked with Bob Fowler and Douglas Stephens, who were very unhappy with their popular “Fowler-Stephens” operation for intra-abdominal cryptorchid testis, because of the high frequency of ischemic testis atrophy. So we developed the microvascular testis autotransplant procedure, using the same microvascular technique on human spermatic artery and vein that Jimmy Crudop and I had developed for renal artery and vein in rats (Figs. 7, 8, 9, and 10)

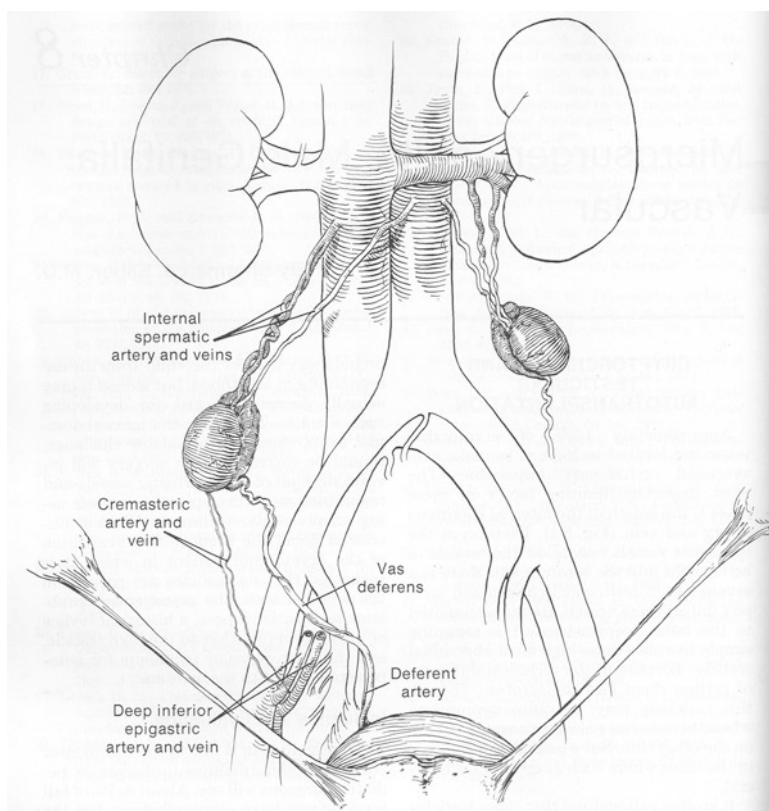


**Fig. 7** Anatomy in human for microvascular testis autotransplant for high cryptorchid abdominal testis

**Fig. 8** Photograph of typical intra-abdominal cryptorchid testis in a child that could not be brought down into the scrotum without dividing the spermatic vessels

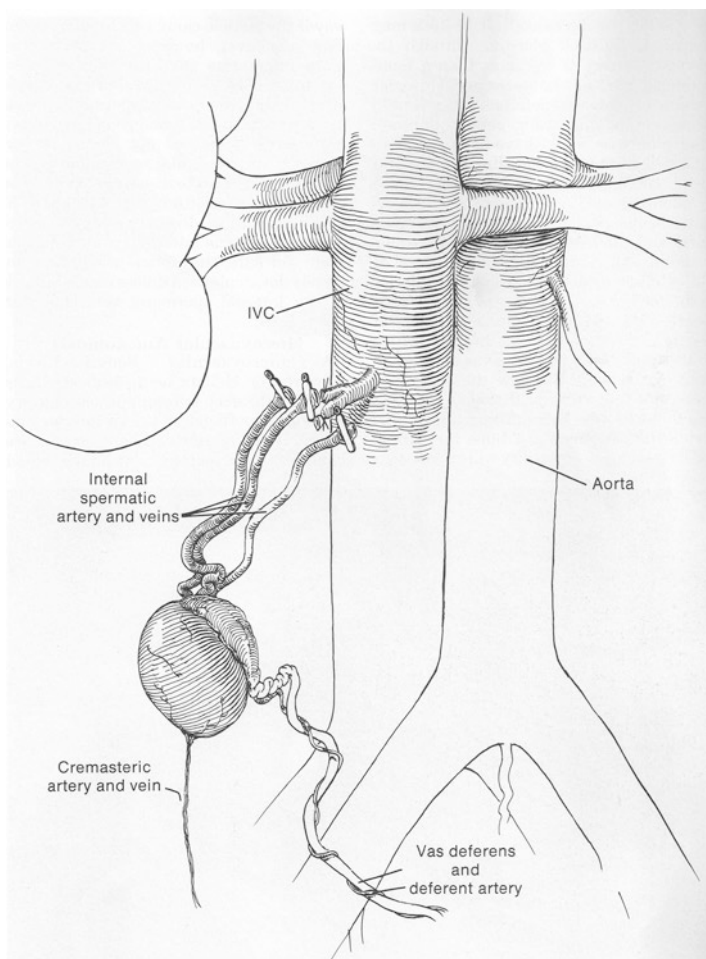


**Fig. 9** Division of spermatic (1/2 mm) vessels to bring down high cryptorchid testis

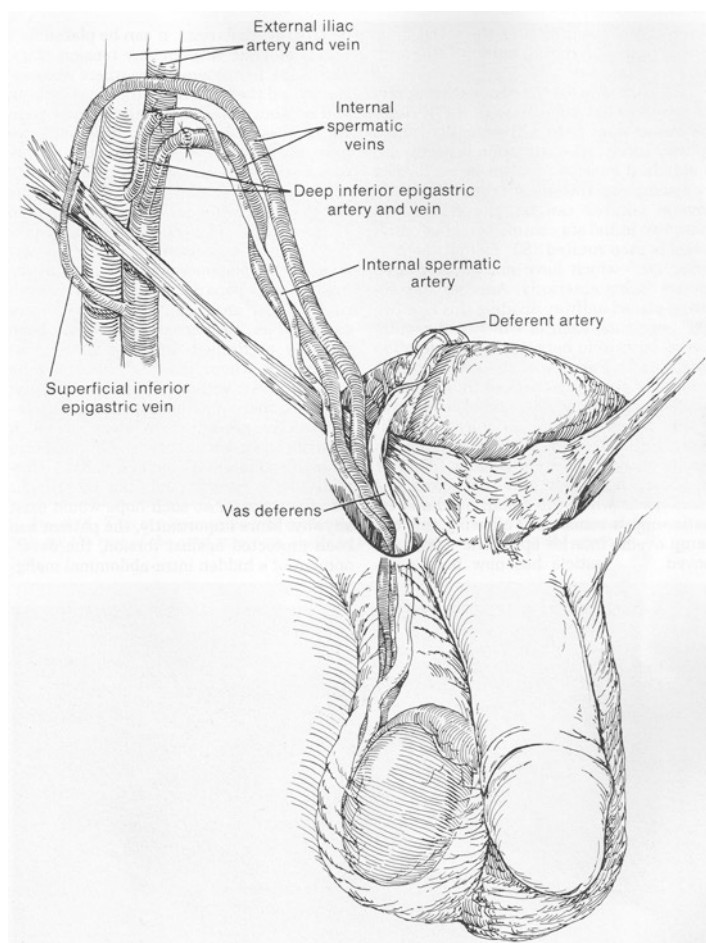


**Fig. 10** Completed re-anastomosis of spermatic vessels (1/3 mm) to bring down intra-abdominal testes

[10]. We divided the spermatic artery and vein of these young boys, which otherwise would keep the testis tethered up high, and we re-anastomosed them to the inferior epigastric artery and vein. This allowed the intra-abdominal testis otherwise high up near the kidney, to be safely brought down to the scrotum (Figs. 11, 12, 13, and 14). Fowler and Stephens adopted this procedure and indeed gave me credit for it. But many urologists who were not microvascular surgeons continued to use only the more destructive Fowler-Stephens operation (which Bob Fowler and Douglas Stephens abhorred).

Before leaving Melbourne in 1975, I worked with the famed plastic surgeon Ian Taylor to perform one of the first human free microvascular skin grafts. I had taught Ian Taylor how to do microvascular anastomosis in rats in Peter Morris's lab in

**Fig. 11** This micro-arteriogram demonstrates the arterial and venous vasculature of the human testis. To anastomose such a tiny spermatic artery to the larger inferior epigastric needed a modified end-to-side approach

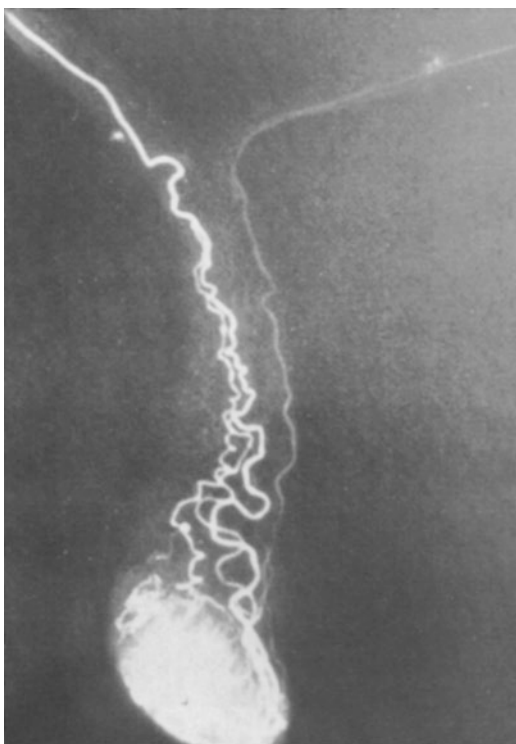


**Fig. 12** A picture of the completed anastomosis in the first human testis transplant of the spermatic artery spatulated to the inferior epigastric artery. Our hearts pounded when we removed the clamps, and good pulsatile blood flow was observed

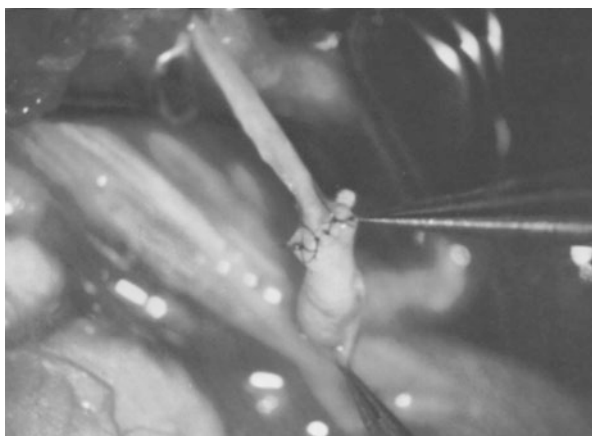


**Fig. 13** The first large free groin skin flap microvascular transfer by the author and Ian Taylor in Melbourne, Australia

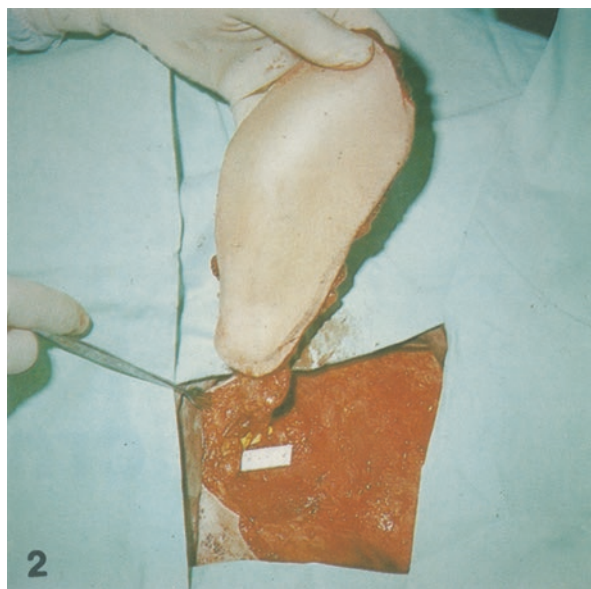
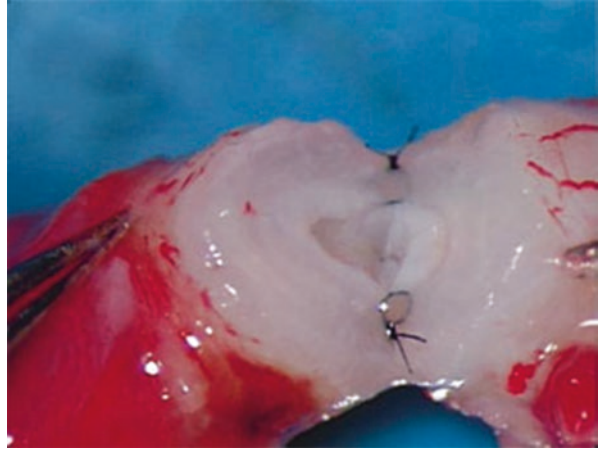


**Fig. 14** The author and Dr. Ian Taylor in 1975 performing world's first free microvascularized groin and bone flap, using techniques honed in the rat lab

**Fig. 15** The original two-layer microsurgical re-anastomosis of vas (vasovasostomy)



Melbourne, and Ian had worked out the anatomy of the skin of the groin. So together we performed the world's first free huge vascularized skin flap and bone graft operations, which revolutionized reconstructive plastic surgery.

In 1978, using these same microsurgery techniques we performed the first human testis transplant, from an identical twin with two normal testes to his brother who was born with anorchia [11]. These testis transplants worked perfectly, and resulted in five healthy babies from spontaneous pregnancy. This microsurgery was praised at the AUA (American Urological Association) meeting that year by the great Dr. Willard Goodwin of UCLA urology fame. But I never dreamed of the impact which these techniques would have on vasectomy reversal. In fact I considered that to be just an insignificant fun thing to do, but not very important. I honestly thought that no one who had a vasectomy would ever want it reversed (Fig. 15). So the original papers I wrote on microsurgery to reverse vasectomy seemed to me to be trivial. But I guess they weren't.

After presentation of a live telecast on closed circuit TV at the American College of Surgeons meeting in San Francisco in October of 1975, with a team of urology discussants it became the main story on the front page of *The New York Times*. This was in 1975, 3 years before IVF. It was shocking to the world that we could get such high pregnancy rates in otherwise azoospermic men by simply doing a better microsurgical anastomosis [12]. It had been thought erroneously before this that the consistent failure of vasectomy reversal was due to sperm antibodies, which has subsequently been disproven [13–15]. The cause of the consistent failure of vasectomy reversal was not auto-immunity, but rather just mechanical failure to re-establish accurate continuity.

There was however great controversy in the late 1970s. Some called vasectomy reversal "immoral." They would actually argue at urology meetings, "if a man has had a vasectomy, it is unethical to reverse it." Others said "you don't need a microscope," and some even apocryphally claimed they already were doing this. Others thought we were lying, the same as the British Medical Society thought of Steptoe and Edward's IVF in 1978. Many questioned if we actually performed a two-layer

anastomosis of this tiny structure. We used 10-0 nylon interrupted stitches for the inner mucosa, and 9-0 for the outer muscle. At this time, 45 years ago, urologists had no idea what a 10-0 nylon suture was. Some who resisted using a microscope claimed that two layers were not necessary. But eventually everyone finally accepted that microsurgical two-layer anastomosis was the proper procedure for vasovasostomy. Nonetheless, I suffered extreme criticism because of fear of a future that many could not accept. Only Bruce Stewart of the Cleveland Clinic supported me in the beginning. But eventually most urologists in the world adopted this procedure.

However, we noted in my 1977 papers that if there was no sperm in the vas fluid at the time of vasovasostomy, the operation always failed, despite a perfect anastomosis. Furthermore the testis biopsy, despite no sperm in the vas fluid, always showed normal spermatogenesis. Also if there was a sperm granuloma at the vasectomy site, there was always the sperm in the vas fluid, and virtually all vasovasostomies then were successful [16–18]. In fact we recommended “open-ended” vasectomy to ensure the formation of sperm granuloma as early as 1978. We were promptly ridiculed for this suggestion, but we were, and still are, correct [19].

Because of this observation, we explored the epididymis in men with no sperm in the vas fluid in the vas fluid—remember, spermatogenesis is always normal in these men (Fig. 16)—and found pressure-induced secondary epididymal extravasation and blowouts, and thus, secondary epididymal obstruction was found in all of these cases with no sperm in the vas fluid [20]. Until then, it was not generally appreciated that there was always increased pressure buildup after vasectomy. This resulted in

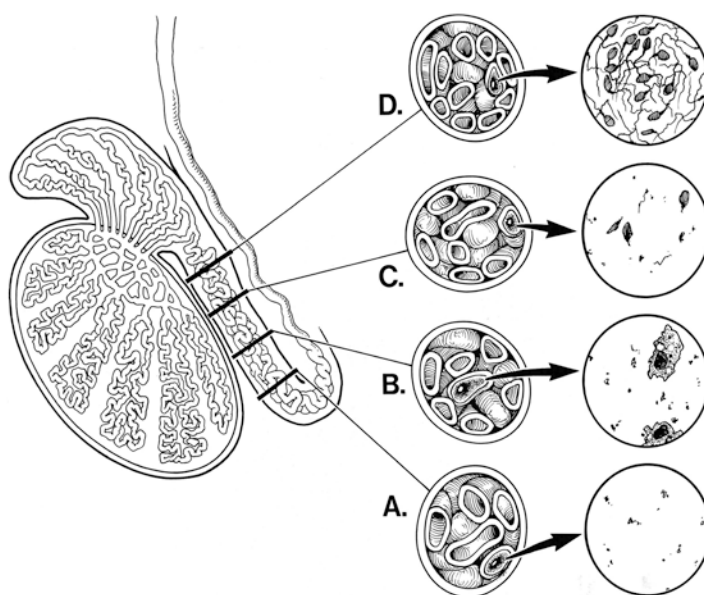


**Fig. 16** Transecting the epididymis proximally in men who had no sperm in the vas fluid always revealed epididymal secondary obstruction (and normal spermatogenesis)

extravasation in the epididymis and secondary epididymal blockage. Furthermore if the vasectomy were formed loosely, so that there was sperm leakage at the vasectomy site, there was less pressure buildup, and there was no epididymal obstruction. So in those cases microsurgical vasovasostomy was almost always successful. At that time we found that the longer the duration of time since vasectomy the greater was the incidence of epididymal blowouts, due to a longer interval of increased pressure. Poor results were not caused by damage to the testis owing to a long duration of obstruction, although to this day patients are erroneously being told this. We actually wrote the original papers that showed success rates for vasovasostomy were better if the vasectomy was less than 10 years before the reversal. However this was not due to testicular damage.

So for cases where there was no sperm in the fluid we invented the “specific tubule” vasoepididymostomy [21]. The technique of vasoepididymostomy for obstructive azoospermia previously was to make a gross fistula between the vas deferens, and an opening in the epididymal tunica, with terribly low success (Fig. 17). But when we discovered that failure of vasectomy reversal was due to

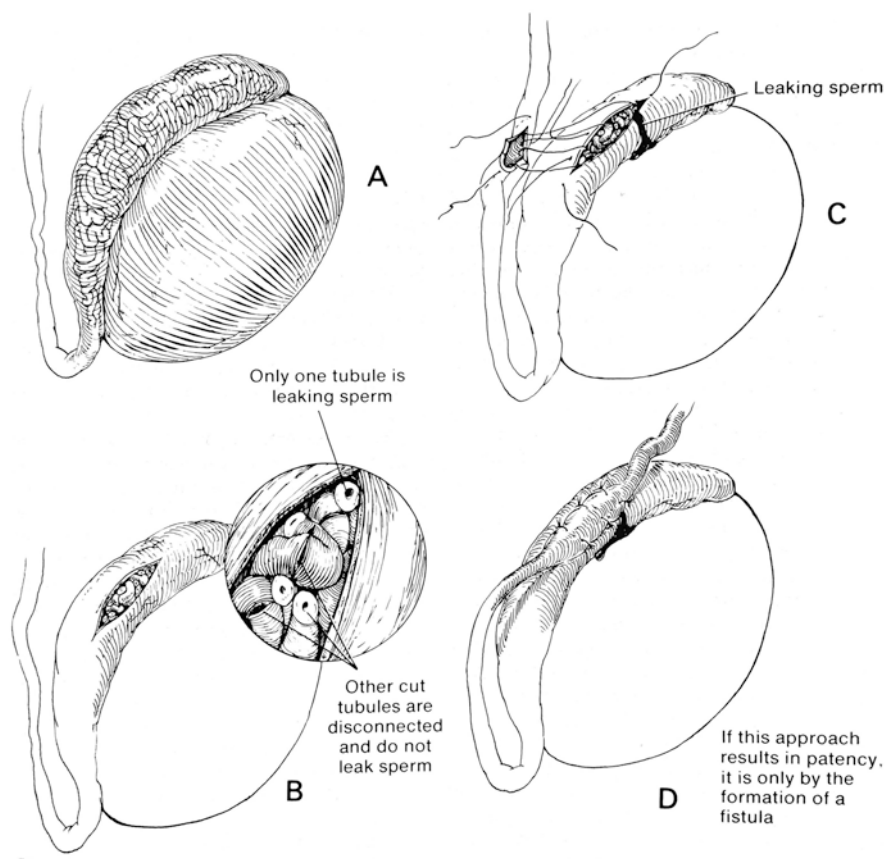


**Fig. 17** The old method of vasoepididymostomy before the author's single specific tubule method in 1978

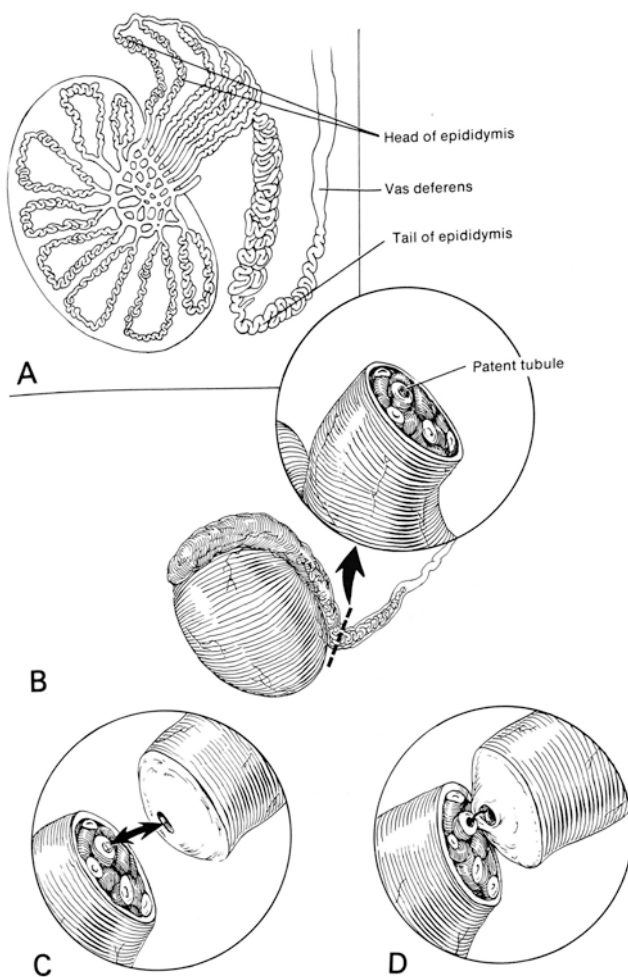


**Fig. 18** The author's end to end specific tubule vasoepididymostomy

secondary epididymal obstruction, we realized that we needed to achieve better results with vasoepididymostomy. So in 1978 we developed a microsurgical procedure for anastomosing the inner mucosa of the vas deferens to the very delicate specific tubule of the epididymis proximal to the site of obstruction [21]. The results were astoundingly as good as with vasovasostomy when there was sperm in the vas fluid (Fig. 18). This again was at first greeted with disbelief and initially disdain. Most urologists still shy away from this approach even today, but our success rate argues in favor of it [22]. Originally we transected the epididymis serially and proximally until we were beyond the obstruction, as evidenced by the finding of good quality sperm (Fig. 17). This was an end-to-end anastomosis. Eventually we switched to an end-to-side anastomosis but retained our original concept of anasto-

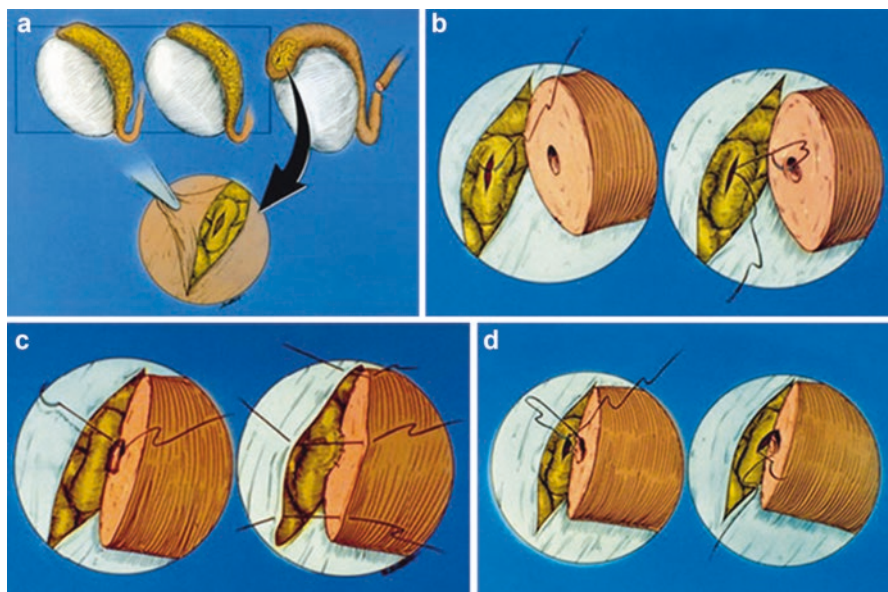


**Fig. 19** (a–d) Modified end-to-side vas to epididymostomy

mosis to the specific epididymal tubule, disbanding the previous concept of just creating a fistula (Fig. 19a–d).

However there were cases of obstructive azoospermia where there was no vas (CBVAD, or congenital absence of vas), for which we developed the MESA procedure first with conventional IVF in 1986, and then with ICSI in 1992 (Fig. 20). Again, remarkably, pregnancy and live baby rates were no different from couples with normal sperm counts in their ejaculate [23, 24–31]. Then even more remarkably in Brussels, when there was no epididymis at all, we reported in 1993 that testicular nonmotile sperm with ICSI resulted in normal pregnancy and live baby rates [25, 32]. We coined that procedure TESE (testicular sperm extraction). We still have the original napkin from the surgeon’s lounge in Brussels where Paul Devroey wrote this down when we coined the eponym “TESE” [24–27, 33]. We also changed the basic science concept of epididymal function. It had been thought that the most motile sperm would be the most distal that had transversed through most of the epididymis, but we discovered that in obstructive azoospermia, the opposite was true, and only found good motility in the most proximal sperm. Then in 1994, we found that even with azoospermic men who had no obstruction (and seemingly no spermatogenesis at all), there were often (over 50%) a very tiny number of rare sperm in the testis, which were adequate for successful ICSI with normal pregnancy [25, 34]. This was based on our original papers of quantitative histologic exam of testis biopsy specimens in the 1970s [35].

We then used these early micro-TESE patients who had nonobstructive azoospermia to map and sequence the Y chromosome, beginning in 1992 at the Page

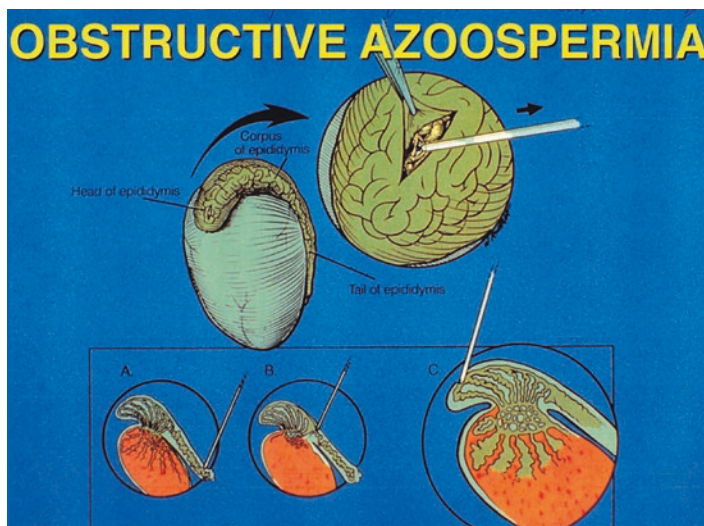


**Fig. 20** For MESA, where there was no vas, we found that the most viable motile sperm were proximal, nondistal as most had previously thought

lab at MIT, and thereby discovered genes on the Y that control spermatogenesis and are deleted in infertile men [36]. This was the now famous DAZ gene (we in fact coined the term DAZ). From there the Page lab discovered autosomal DAZL on chromosome 3. As it now turns out DAZL is the major ancestral universal gene that “licenses” early embryonic stem cells to become germ cells, either sperm or in fact eggs. DAZ, or DAZL, turns out to be the most common key to spermatogenesis and oogenesis in most animals going very far back in the phylogenetic tree.

With our development of microsurgical TESE, we discovered the simplest way to avoid adhesions, which obviously applies to tubal microsurgery in the female as well. Whenever we had to re-operate on these men with nonobstructive azoospermia, to retrieve more sperm, there were never any adhesions in the tunica albuginea space. It was as though there had never been any previous surgery in his scrotum. We realized that this was an affirmation within the scrotum of the principles of minimal tissue trauma, perfect hemostasis, and pulsatile irrigation with heparinized saline promulgated by Gomel and Winston in tubal microsurgery. There was no commercial adhesion prevention product that could compare with just keeping the tissue constantly wet with heparinized saline, and using micro-bipolar forces for hemostasis with no tissue damage.

As important as was prevention of adhesions was how to avoid testis tissue damage with microsurgery, despite total exploration of the testis to find those rare sperm in an azoospermic male. We avoided intra-testicular swelling and subsequent testis atrophy by stopping all bleeding with micro-bipolar forceps and closing the tunica albuginea (which has no elasticity) with 9-0 nylon interrupted sutures, rather than the prevailing running stitch with 4-0 vicryl. This prevents a purse string reduction

in testis volume and subsequent pressure atrophy. Furthermore, we studied the location of spermatogenic stem cells (SSCs) and realized that the surgeon does not have to “dig into” the testis to find the rare sperm. One can stay on the periphery of the seminiferous tubules and nonetheless sample every anatomic lobule. If there is a SSC anywhere within the testis, you will find sperm at the periphery of the tubule where it loops around. Thus you avoid any internal testis damage (as can occur with a deeply invasive “microdissection” technique).

Varicocelectomy has been a very popular operation for infertile men since 1952. I am no longer an advocate of that procedure (even though for many urologists it is still very popular). However, I did make it a much safer procedure in 1979 by being the first to suggest a microsurgical approach, thus sparing damage to the tiny spermatic artery [37]. Prior to that, there was a 5% incidence of painful hydrocele, and often testicular atrophy or a reduction (rather than increase) in spermatogenesis because of spermatic artery damage. In addition, recurrence of the varicocele, because of failure to ligate every spermatic vein, was common. Although before 1979 no one used a microsurgical approach to varicocelectomy, now everyone does. However, the vast majority of infertility doctors, who are not urologists, do not believe in varicocelectomy. They feel that when men undergo this procedure, waiting for the sperm count to improve, the wife’s eggs are simply getting older. My views on this will be explained and documented in detail in this book.

Most recently, we have turned our attention to culturing and freezing spermatogonial stem cells (SSCs) in order to transplant them back after sterilizing chemotherapy in prepubertal boys with cancer. We have analyzed with single cell analysis of gene transcription revealing the genomics of spermatogenesis, and identified the heretofore elusive human SSCs. We can easily freeze a TESE specimen from a prepubertal boy today, and then when he is an adult, culture and amplify the number of SSCs, and then transplant them later into his azoospermic testis. Such spectacular progress in andrology was made possible focusing on microsurgery, genetics, and cell culture. In addition, by understanding the culturing (and freezing) of SSCs, and determining the entire transcriptome of spermatogenesis, there will be great progress in the future for male infertility treatment. For example, we can culture and amplify the SSCs from a TESE specimen, then inject them back into the rete testis of azoospermic men, and thereby increase their spermatogenesis. Furthermore, in mice it is possible to generate sperm (and eggs) from stem cells derived from a skin biopsy.

It is important for andrologists to understand not only male fertility but also female. For example, we performed the first successful frozen ovary transplant in 2004 in the United States [38]. We have a series of many healthy babies from these procedures in women who had been menopausal for more than 5 years, either from POF or from cancer treatments [39–41]. As a result of scientific study of this remarkable series of human ovary transplants, we have deciphered the duration of time in the human between primordial follicle recruitment and ovulation, as well as the heretofore mysterious control of that oocyte recruitment. Furthermore, by studying the development of human oocytes from the skin cells of our identical twins discordant for premature ovarian failure, we are currently determining at what embryonic stage PGC (primordial germ cell) specification occurs in the human

embryo. Furthermore, we are on the brink of being able to make SSCs (thus normal sperm) from IPS cells derived from skin. This will be explained in detail in the last section of this book. In the future we will actually be able to make sperm in azoospermic men from skin.

So it has been a long, almost half-century journey from my first microsurgical experiments in rats to remarkable advances in the understanding and treatment of human infertility, both in the male and the female. In fact our results with vasectomy reversal, tubal reversal, and vasoepididymostomy are still stunning, despite the great ease now of microsurgery for MESA, TESE, and IVF which evolved from those original studies in the early 1970s in rats [22, 42–44]. The new advances for male infertility amazingly now are ICSI, freezing and transplantation of SSCs, as well as deciphering the entire genetic transcriptome of spermatogenesis, and making sperm from a man's skin via IPS cell transformation. The objective of this book is to give you the fundamentals necessary for rationally and successfully dealing with male infertility in a leading edge manner.

St. Louis, MO

Sherman Silber

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**Part I**  
**Basic Science of the Testis**

# Chapter 1

## Testis Development, Embryology, and Anatomy



The adult testis consists of a gamete portion, which produces sperm, and an endocrine portion, which secretes testosterone [1–8]. Spermatogenesis occurs in the gamete portion, inside the seminiferous tubules where spermatozoa are generated and supported by Sertoli cells. Androgen (testosterone) is produced by Leydig cells in the interstitium in between the seminiferous tubules (Figs. 1.1 and 1.2). This androgen production is also crucial for spermatogenesis. However, it is only the local intratesticular androgen that promotes spermatogenesis. Exogenous androgen suppresses the pituitary and so turns off spermatogenesis.

Embryonically, the fetal testis starts out as an undifferentiated gonad before 6 weeks, being just a mass of mesoderm that could become either testis or ovary. In males the undifferentiated gonad will differentiate into the somatic elements of the testis after 6 weeks. But prior to this time, the urogenital tract is identical in both sexes (Figs. 1.3, 1.4, and 1.5). Genetic males and genetic females start out with both Wolffian and Müllerian duct systems, as well as an undifferentiated gonad.

After 6 weeks the Müllerian duct in the male regresses under the influence of AMH secreted by the testis, and the Wolffian duct becomes more prominent because of secretion of testosterone by the fetal testis. The gonad's somatic cells are derived from the mesoderm of the urogenital ridges. However, the primordial germ cells (PGCs) originate outside the area of the presumptive gonad and are initially identifiable in the epiblast and then yolk sac [6]. These cells arise from the epiblast just after 7–8 days of embryo development and then migrate to the gonad at the gonadal ridge in front of the spine, where they undertake gametogenesis (Fig. 1.6a, b). It is crucial that while the rest of the somatic embryo cells are rapidly differentiating, these PGCs remain undifferentiated “outside” of the embryo proper, in the yolk sac. By 6 weeks, however, most primordial germ cells (PGCs) have finally migrated to the gonad and are in close association with the adjacent somatic gonadal cells. These primordial germ cells can become either oocytes or spermatogonial stem cells (SSCs) depending on whether the gonadal differentiation has been male (testis) or female (ovary).

**Fig. 1.1** Seminiferous tubule with Sertoli cells but no spermatogenesis. Note the prominent blue standing nucleolus in every Sertoli cell nucleus, and note that the cytoplasm is indistinct and the cell is amoeboid in character

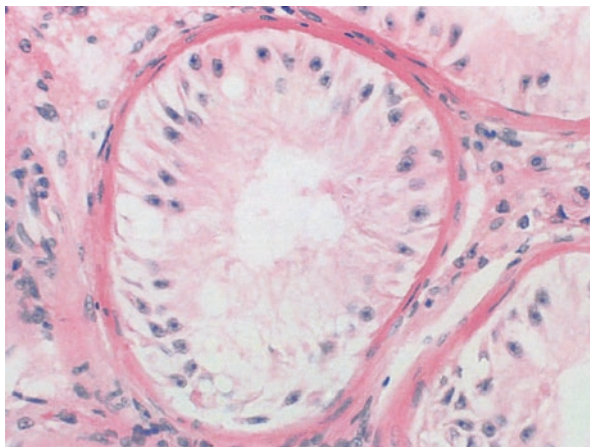
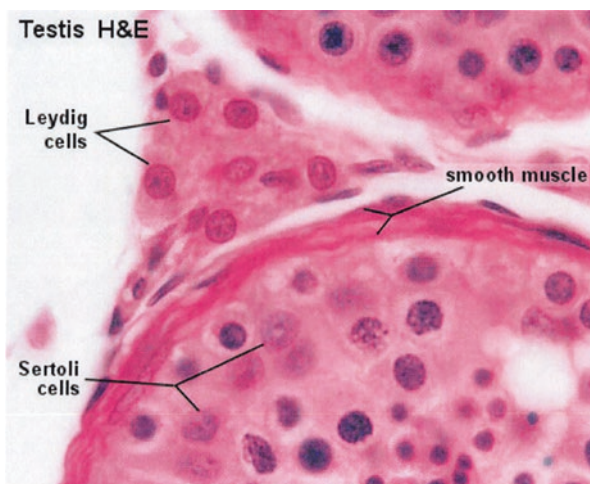


**Fig. 1.2** Spermatogenesis is taking place in this seminiferous tubule and is supported by the Sertoli cells. Note that the Leydig cells are in the interstitium which is in between the seminiferous tubules



The testis at 6 weeks develops from a primitive gonad on the medial surface of the embryonic mesonephros at the dorsal ridge. Sex chords (destined to become seminiferous tubules in the male) are no different from the follicles in the female gonad (Figs. 1.4 and 1.5). During the 16th week, the sex cords become U-shaped, and their ends anastomose on the periphery of the testis under the tunica albuginea and drain centrally into the rete testis [5–8]. This is a critical anatomic feature that allows safe successful microsurgical testicular sperm extraction (TESE) for azoospermic men (Figs. 1.7, 1.8, 1.9a–c, 1.10, and 1.11).

Primordial germ cells can be recognized in the 6–8-day-old human epiblast (Fig. 1.6a, b). At the beginning of the 4th week of human embryonic development, germ cells begin to migrate from the yolk sac (essentially outside of the embryo) to the gonadal ridges. The primordial germ cells begin basically “outside” of the embryo proper, so they will not differentiate, while the rest of the fetus is rapidly

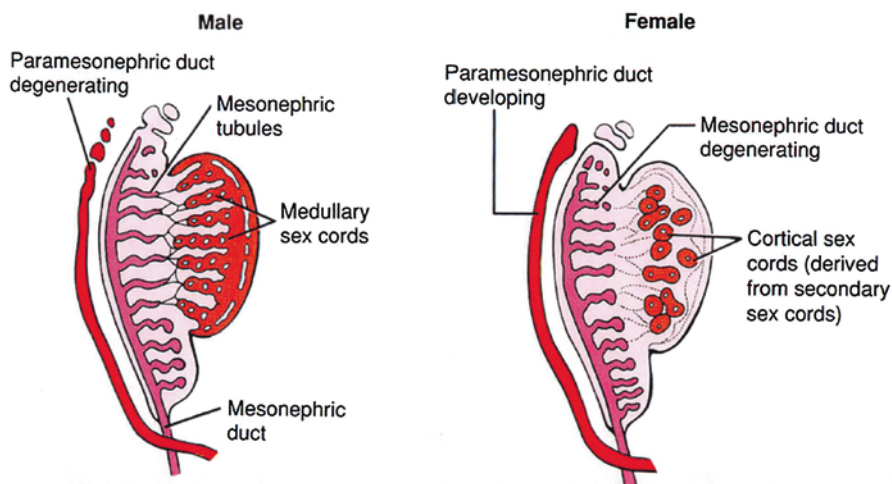


**Fig. 1.3** Early differentiation of the testis and ovary. Note the sex cords which in the male become seminiferous tubules and in the female become ovarian follicles. The Sertoli cells of the testis are homologous with the granulosa cells of the ovarian follicles

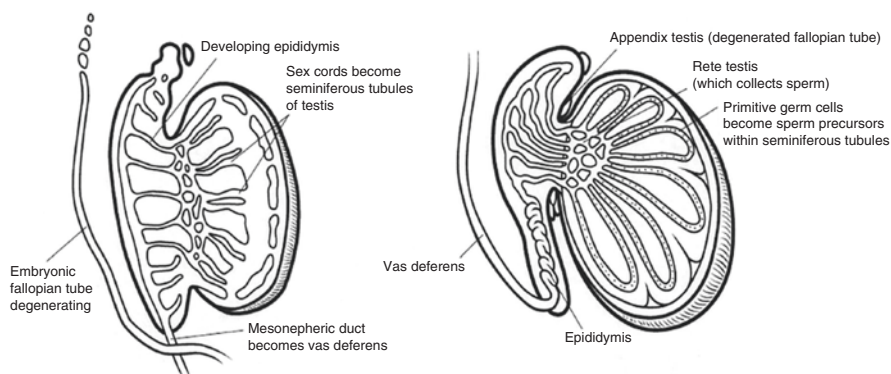


**Fig. 1.4** Primitive germ cells become sperm precursors in seminiferous tubules of embryonic testis

differentiating (Fig. 1.6). They only migrate to the gonadal ridge when the somatic cells of the primitive gonad have formed. It is important for these committed germ cells to be outside the somatic differentiation of the rest of the fetus. The formation of the gonadal blastema is completed during the 5th week of human embryogenesis and thereafter becomes either the testis or the ovary, depending on the sex-determining gene *SRX*. Before this time, the primitive, undifferentiated gonad in both male and female is composed of three distinct cell types: entering germ cells, supporting epithelial cells that give rise to either testicular Sertoli cells or to ovarian

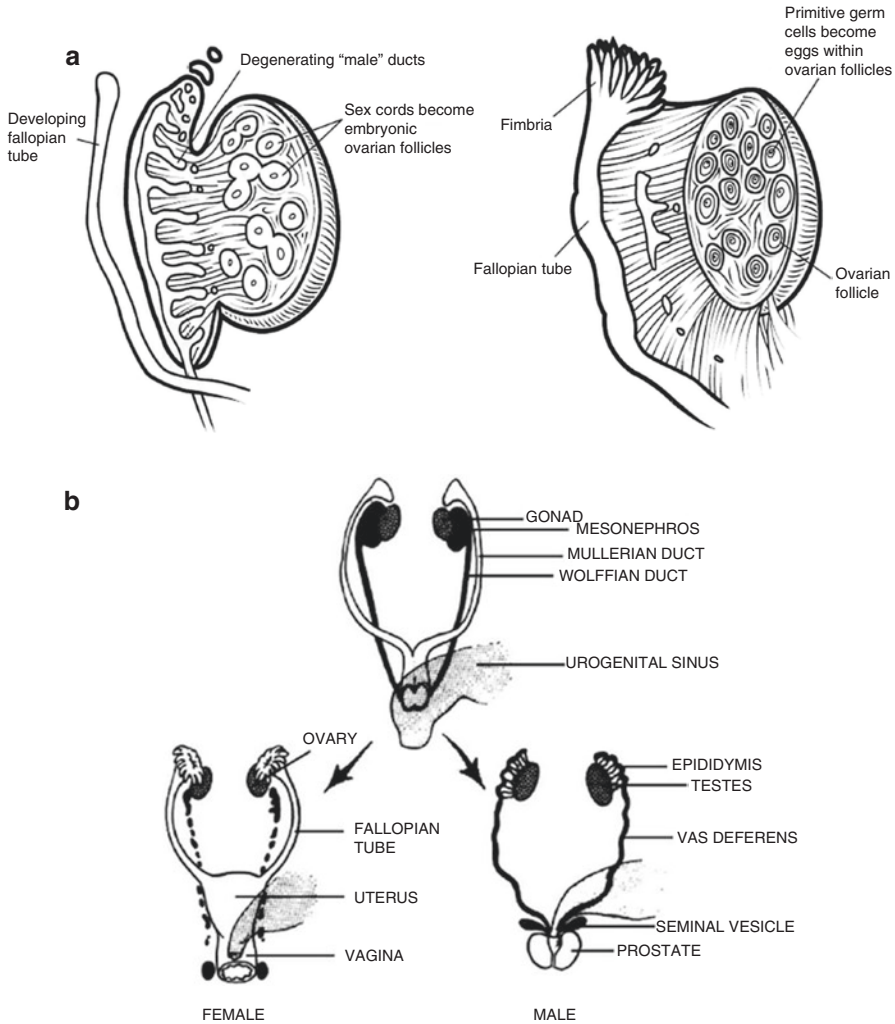


**Fig. 1.5** (a) Primitive germ cells become eggs in embryonic ovarian follicles. (b) The Müllerian ducts and Wolffian ducts lie parallel to each other in the fetus before sexual differentiation after 6 weeks. Then the Müllerian ducts regress in the male under the influence of AMH secreted by the testis, and the Wolffian duct enhances under the effect of fetal testosterone

granulosa cells, and stromal (interstitial) cells derived from the mesenchyme of the gonadal ridge. The Sertoli cells of the male equate to granulosa cells of the female.

Sex differentiation is determined by whether the primitive gonad becomes a testis or an ovary, and that is determined by the presence of the SRY gene on the Y chromosome. All placental mammals have an XX female/XY male sex-determining system. The SRY gene is responsible for the transformation of the undifferentiated gonad to a differentiated testis. The sex-determining gene (SRY) is located on the

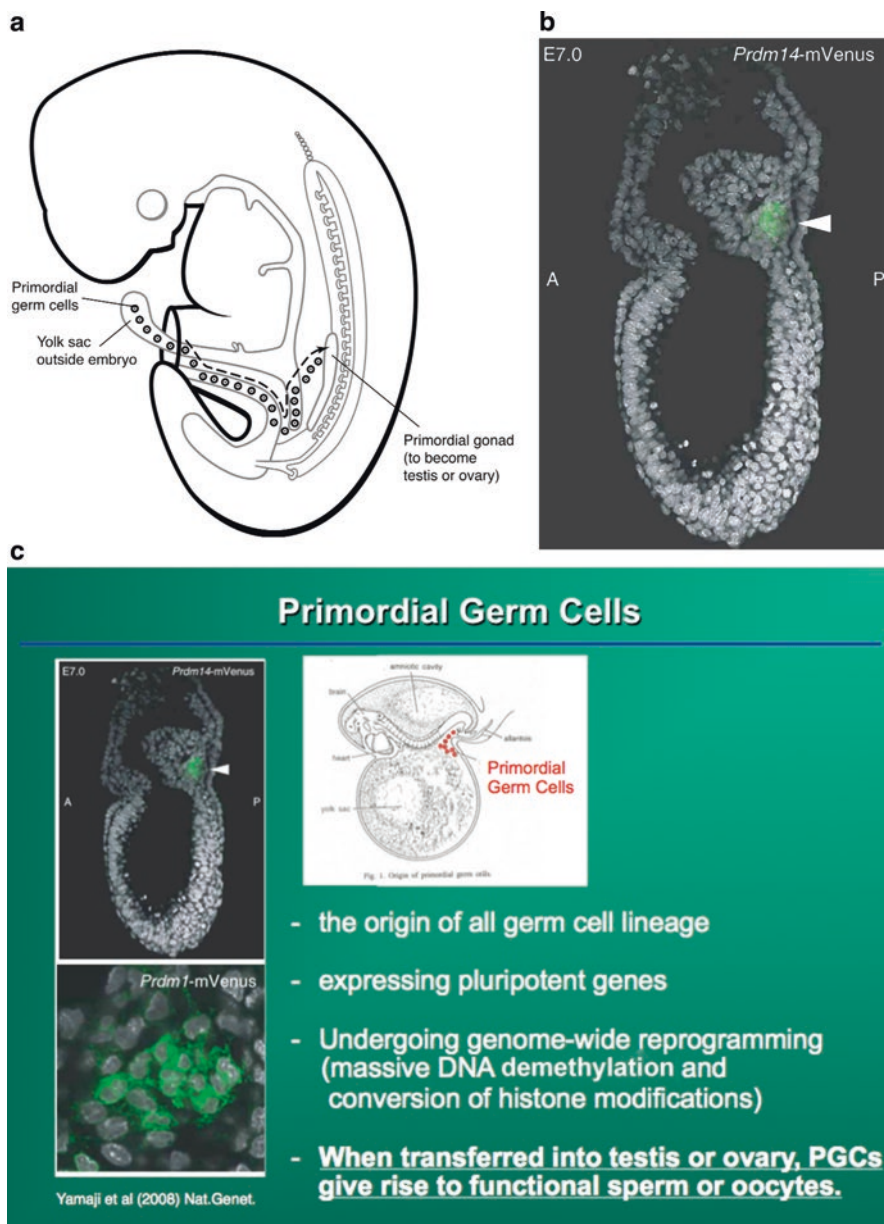


**Fig. 1.6** (a) Three-week-old human embryo: migration of primordial germ cells (future sperm or eggs) to future testis or ovary. (b) Mouse epiblast where PGCs arise

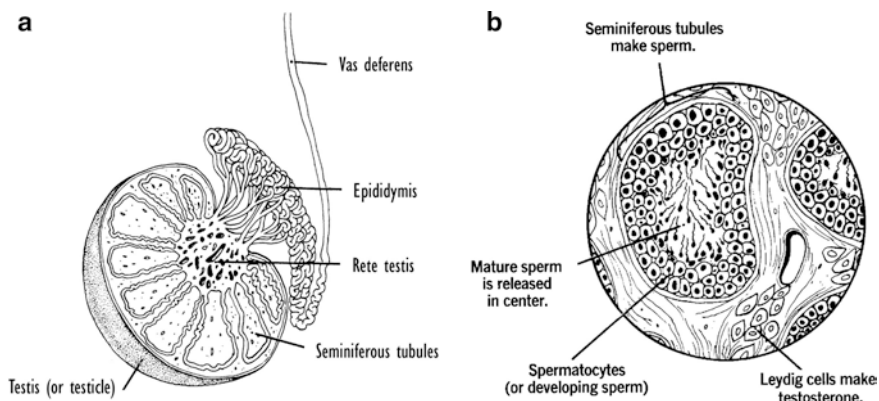


**Fig. 1.7** (a) The testicle with U-shaped seminiferous tubules forming a U-curve at the peripheral and drawing into the rete testis internally. (b) The sperm factory (testicle biopsy) sperm being manufactured in the tubules, while testosterone is produced by Leydig cells in the interstitium

**Fig. 1.8** Testis anatomy. Note that 300 anatomic lobules (one side section) drain into the rete testis and from there into vasa efferentia that then drain into the caput epididymis which eventually leads to the vas deferens



short arm of Y chromosome (Yp) and is the first gene involved in the differentiation process and is the initiator of the downstream cascade of the very many testis-forming genes that are actually located on autosomes [9, 10]. These multiple downstream genes determine the development of the testis from the undifferentiated gonad. The SRY on the Y chromosome is just a trigger. The actual development of the undifferentiated gonad into testis is controlled by many autosomal genes, downstream from the initial SRY trigger.

In the female, when the primordial germ cells (PGCs) reach the genital ridge, they are engulfed in retinoic acid which upregulates the gene *stra8*, which initiates meiosis [11, 12]. In the male fetus, however, the Sertoli cell protects the primordial germ cell from retinoic acid, and so the germ cells of the male do not enter meiosis and become instead spermatogonial stem cells (SSCs), which in the postpubertal

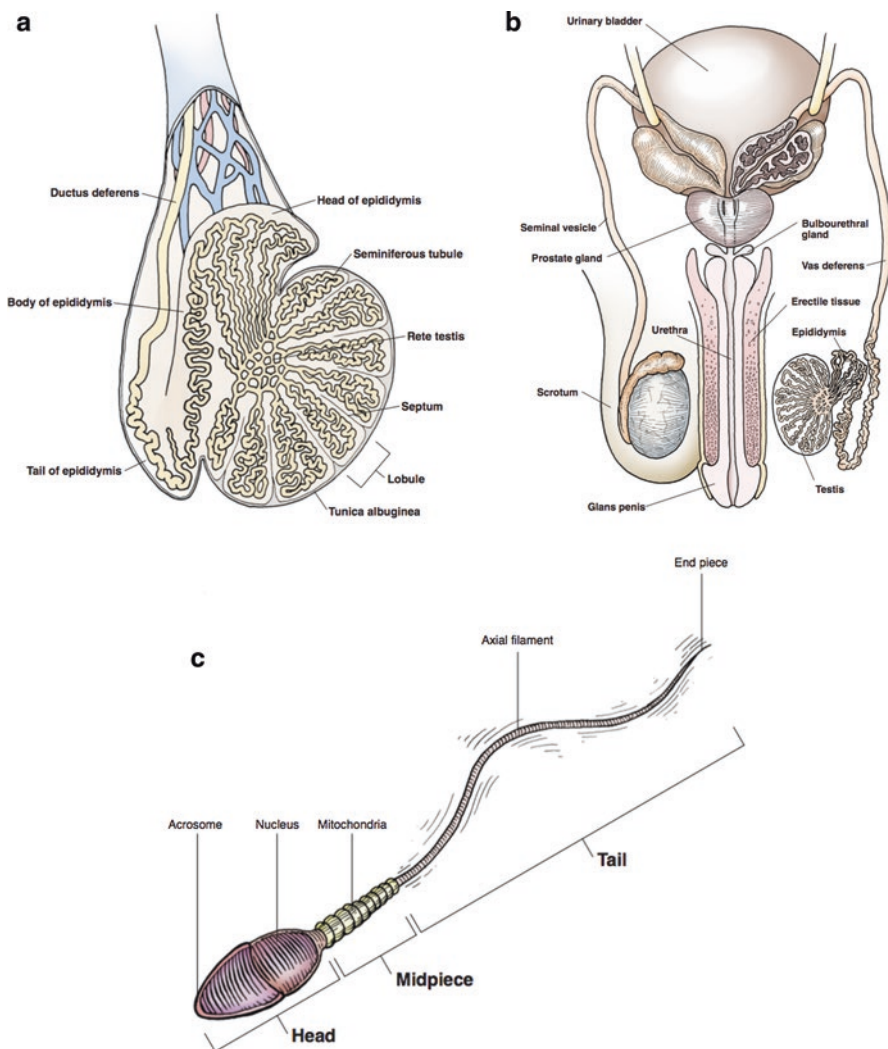


**Fig. 1.9** (a) Section through the testis showing the organization of how the tubules form a U-shape at the periphery. Note: that means that any peripheral testis biopsy or TESE procedure will not cause obstruction to the seminiferous tubules. But a deep biopsy will cause obstructive damage. (b) Male sex organs: overview showing the whole system. (c) Anatomy of a single normal mature spermatozoon (sperm). Note the importance of the acrosome at the head like a battering ram for egg penetration and the intense condensation of mitochondria at the mid-piece to provide energy for sperm mobility

male initiate spermatogenesis. In the female, meiosis is arrested in early prophase by the primordial follicle in the ovarian cortex under the influence of cortical stromal tissue pressure and the gene Fox 3 going intranuclear. The female thus cannot generate more oocytes, even though the male can continue spermatogenesis indefinitely. The meiosis and development of the oocyte is held in check by the primordial

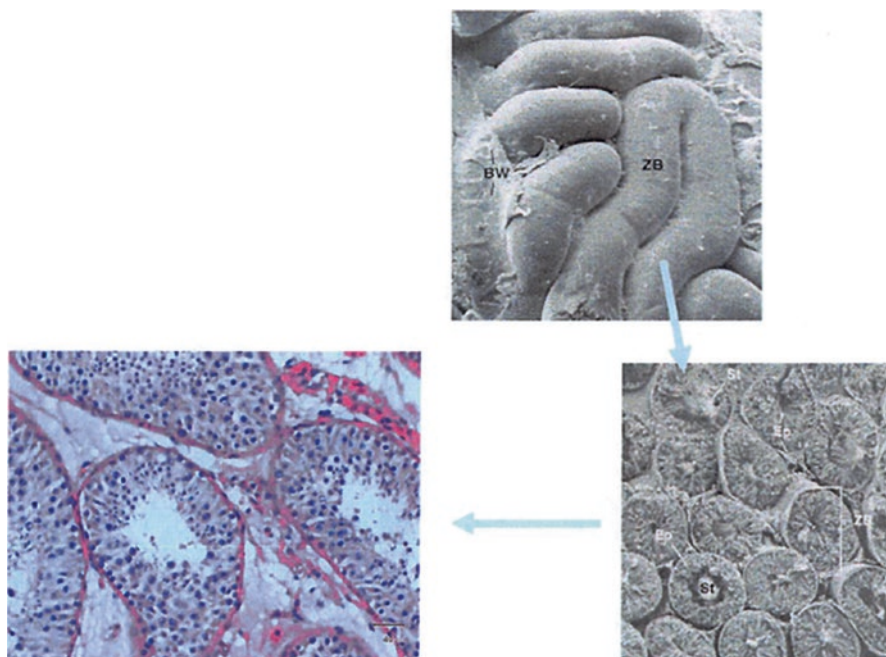


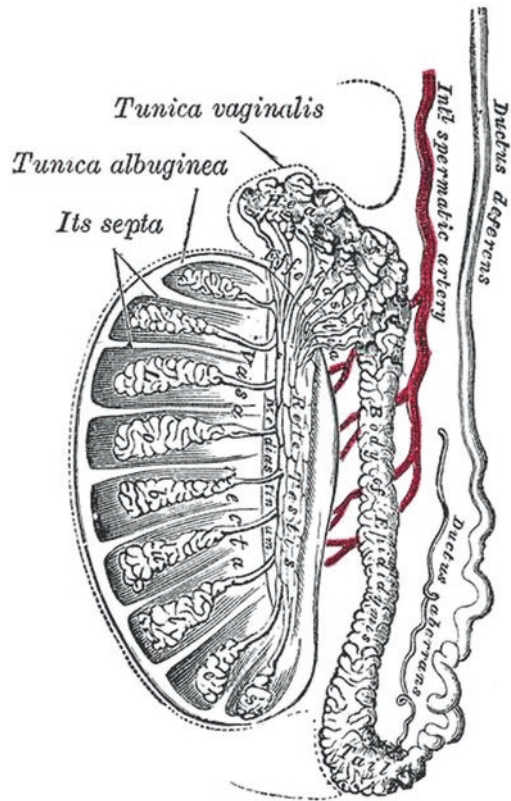
**Fig. 1.10** Testis anatomy from a sagittal view of the testis, epididymis, and vas deferens to a scanning E/M view of the seminiferous tubules and to a high power histology and a low power histology of the normal seminiferous tubule making sperm

follicle which prevents all the eggs from proceeding at once through meiosis, thus being completely all gone by the time of birth. Without the arrest of meiosis caused by the primordial follicle, all of the oocytes would quickly be gone [13, 14].

So what is the reason for this clumsy convoluted system for oocytes to begin meiosis immediately and then be arrested immediately and only released gradually throughout life, and eventually the female does run out of eggs while the male keeps making new sperm indefinitely? The answer to this question is beautiful. Most mutations in human populations originate during sperm production because of inevitable replication errors [15]. Too many such mutations can cause havoc in the population, but it is the basis of all evolution. The oocyte does not go through replication and therefore stabilizes the species against too many mutations. Without the germ cell mutations in testis, there would be no evolution. Without the ovary, our species would quickly destabilize.

Why should the oocyte begin meiosis and then be “locked” for a lifetime, while sperm are constantly produced by spermatogonial stem cells? What is the benefit to the species of such a dichotomy? The benefit of this dichotomy between oogenesis and spermatogenesis is that most of the mutations that occur in a species over many years occur during spermatogenesis in the testis, as errors. The oocyte is spared that risk by not having to undergo recurrent mitosis. But of course the oocyte unfortu-

**Fig. 1.11** A simplified diagram of the testis and epididymis showing the tubules within the septa drawing into the vasa efferentia and from there into the epididymis



nately ages, causing female infertility [16]. These two mechanisms are wedded to each other evolutionarily. Without the mutations caused by spermatogenesis and thus constant germ cell error prone duplication, there would be no evolution. However, without the “locking” of the oocyte in meiosis and avoidance of constant duplication errors, as occurs in sperm, the species would have no stability.

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## Chapter 2

# Descent of the Fetal Testis from the Abdomen to the Scrotum



In most mammals the testes migrate from their original site in the abdomen alongside the lumbar spine down into the scrotum [1, 2]. Exceptions are elephants, whales, and rhinoceros, whose testes remain in the abdomen. In most mammals, however, including humans, they pass through the abdominal wall into the evagination of the peritoneum that forms the scrotum (Figs. 2.1, 2.2, and 2.3). The descent of the testis occurs in two morphologically and hormonally distinct phases termed as transabdominal and inguinoscrotal phases. During the first phase, the testis remains anchored to the retroperitoneal inguinal area by the swollen gubernaculum, which prevents its ascent as the fetus enlarges. The gubernaculum is a cylindrical structure attached to the inguinal canal. During the third trimester of development, the testis slips down the posterior abdominal wall dragging its neurovascular bundle and descends from the inguinal area into the scrotum guided by the gubernaculum (Fig. 2.4). By birth, or shortly thereafter, the testis has moved into its definitive extra-abdominal location and is covered by the processus vaginalis of the peritoneum (Fig. 2.5).

Cryptorchidism (undescended testis) is associated with impaired spermatogenesis, because spermatogenesis cannot occur at the warmer intra-abdominal temperature (Fig. 2.4). That is why the testis must be “outside” the body in order for germ cell development to occur. The “pampiniform plexus” of scrotal veins cool the warmer arterial blood from the abdomen as it enters the scrotal testis. Androgens are still produced when the testis does not descend properly, but spermatogenesis is suppressed by the warmer intra-abdominal temperature. The purpose of the scrotum is to allow a cooler temperature for spermatogenesis. Only a few mammals, like the whale, hippopotamus, and elephant, can maintain spermatogenesis at abdominal temperature.

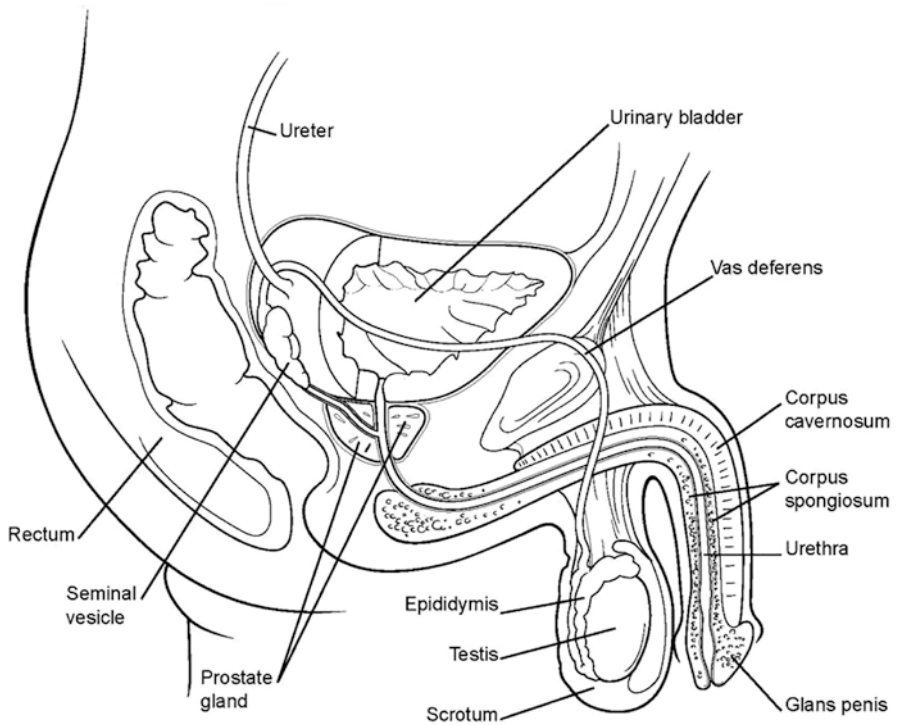


**Fig. 2.1** Male sex organs: side view showing the path of what was an intra-abdominal fetal testis to a scrotal position

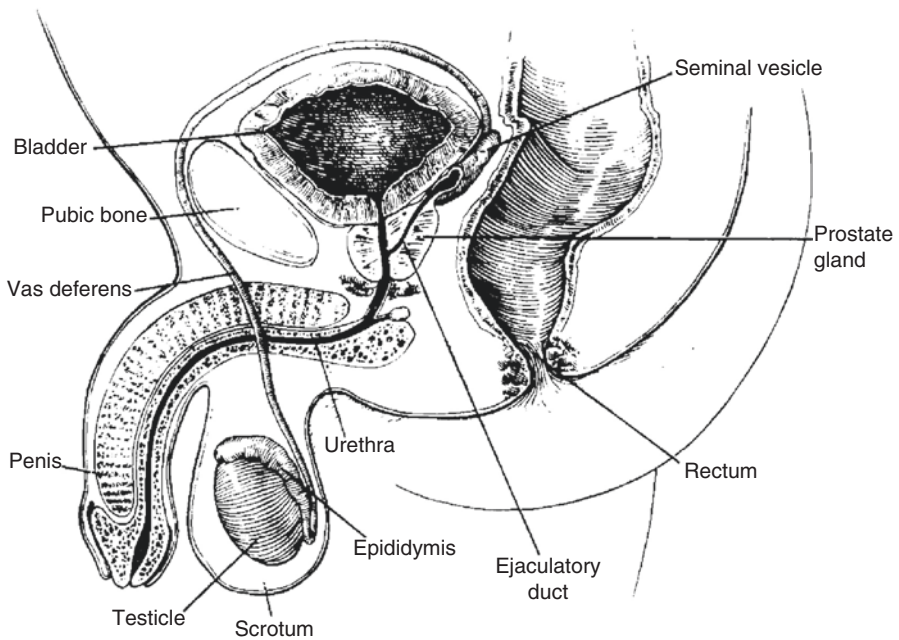


**Fig. 2.2** Male reproductive organs (side view). A more detailed drawing of the entire male reproductive anatomy

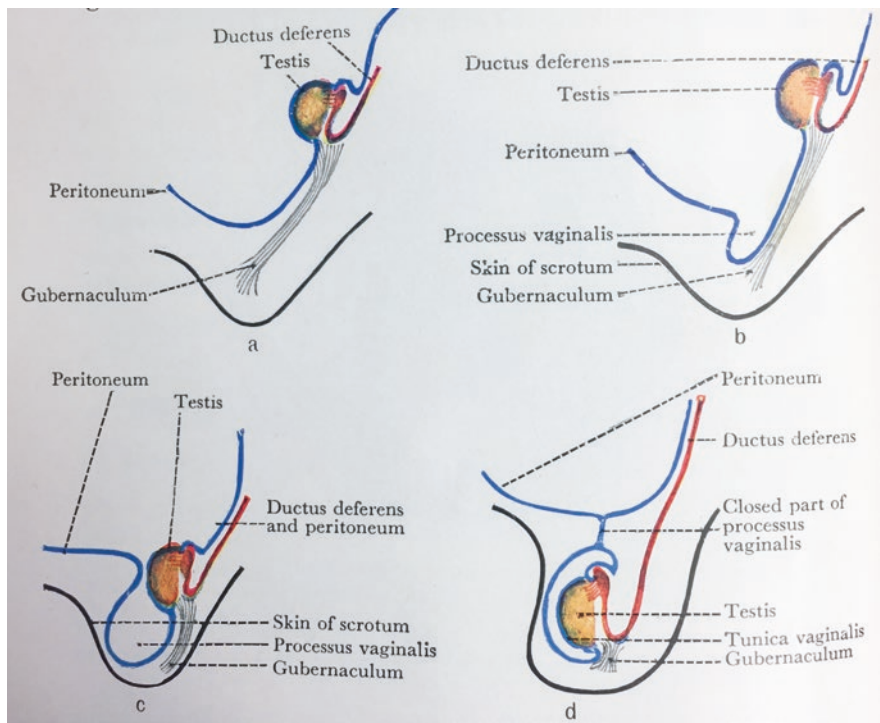
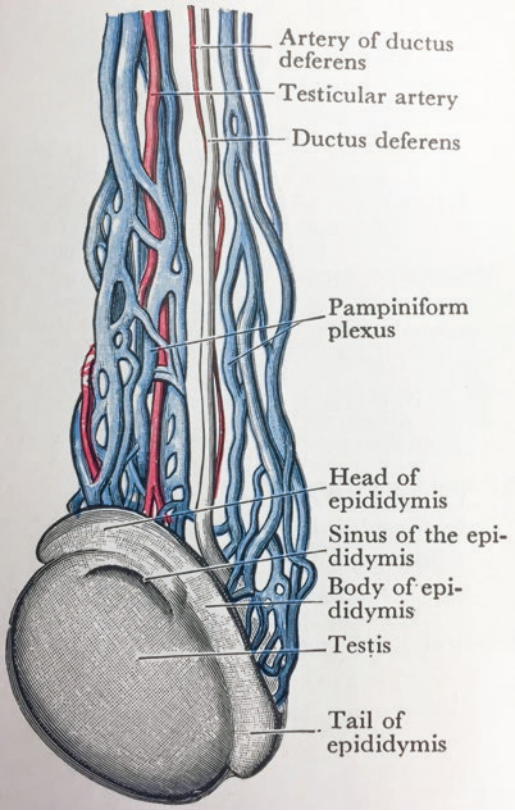


**Fig. 2.3** Diagrams illustrating the descent of the testis and derivation of the tunica vaginalis from the peritoneal lining of abdomen

**Fig. 2.4** Diagram of the left spermatic cord showing how the venous pampiniform plexus cools blood coming to the testis



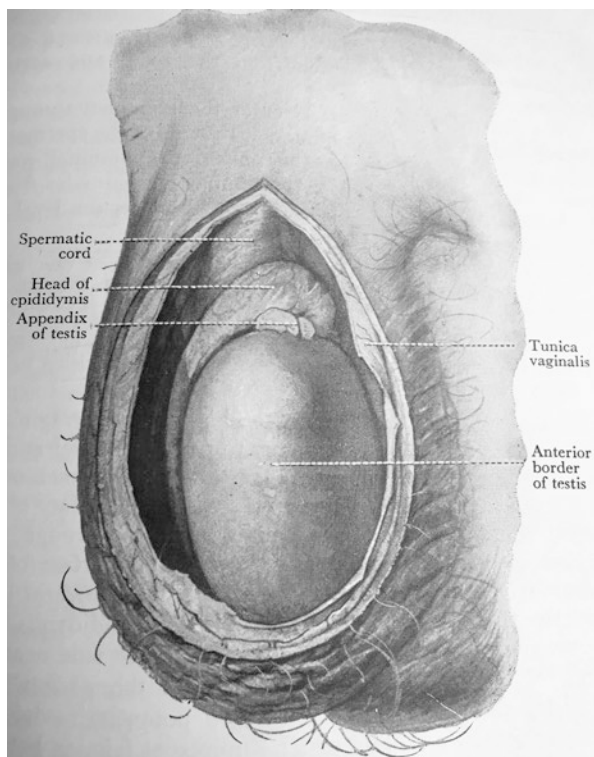


**Fig. 2.5** Testis and epididymis within the tunica vaginalis showing that the hydrocele sac of the scrotum is just an extension of the peritoneal cavity which developed from the descent of the testis

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## Chapter 3

# Adult Testis Anatomy



To review, the human testis lies within the scrotum. The “sex cords” of the early embryo become follicles in the female ovary and seminiferous tubules in the male testes (Figs. 1.3, 1.4, and 1.5). The Sertoli cells in the seminiferous tubules are equivalent to the granulosa cells in the female follicle. The Leydig cells in the interstitium between the tubules are equivalent to the theca cells in the ovary. The testis structure is surprisingly similar to the ovary (Figs. 1.3, 1.4, and 1.5). The average testicular volume is 20 mL but varies. The right testis is usually 10% larger than the left. Normal longitudinal length of the testis is approximately 4.5–5.1 cm. The average weight of human testis is 15–19 g. The tunica albuginea of the testis in the male is exactly equivalent to the cortex of the ovary in the female, and in the female, it has “hitch-hiked” the very important function of regulating primordial follicle suppression and recruitment. It is the densest and toughest connective tissue in the body. It is the denseness and toughness of this tunica albuginea that in the male protect the exposed testis but in the female controls the rate of primordial follicle recruitment [1].

Normally, there is a small amount of fluid between the tunica vaginalis (the peritoneum) and the tunica albuginea [2]. Hydrocele is just an excessive accumulation of this fluid between these two layers (Fig. 2.5). Hydrocele can either represent an “indirect hernia” caused by failure of the process vaginalis to close off when the testis descends, or it can result from inflammatory congestive secretions from the enclosed tunica (Fig. 2.3).

All men have a small structure near the head of the epididymis called the appendix testis, which is the Müllerian duct remnant that has regressed (Fig. 3.1a, b). But if you look closely, you can amazingly see the regressed, tiny fallopian tubes, uterus, and upper vagina (if it were to be a female). The reason that these Müllerian duct embryonic structures regress in the male fetus is the direct secretion of AMH (anti-Müllerian hormone) by the fetal testis. At the same time, secretion of testosterone from the fetal testis promotes development of the parallel Wolffian (or mesonephric) duct, which becomes the epididymis, vas deferens, and prostate gland (Fig. 1.5b).

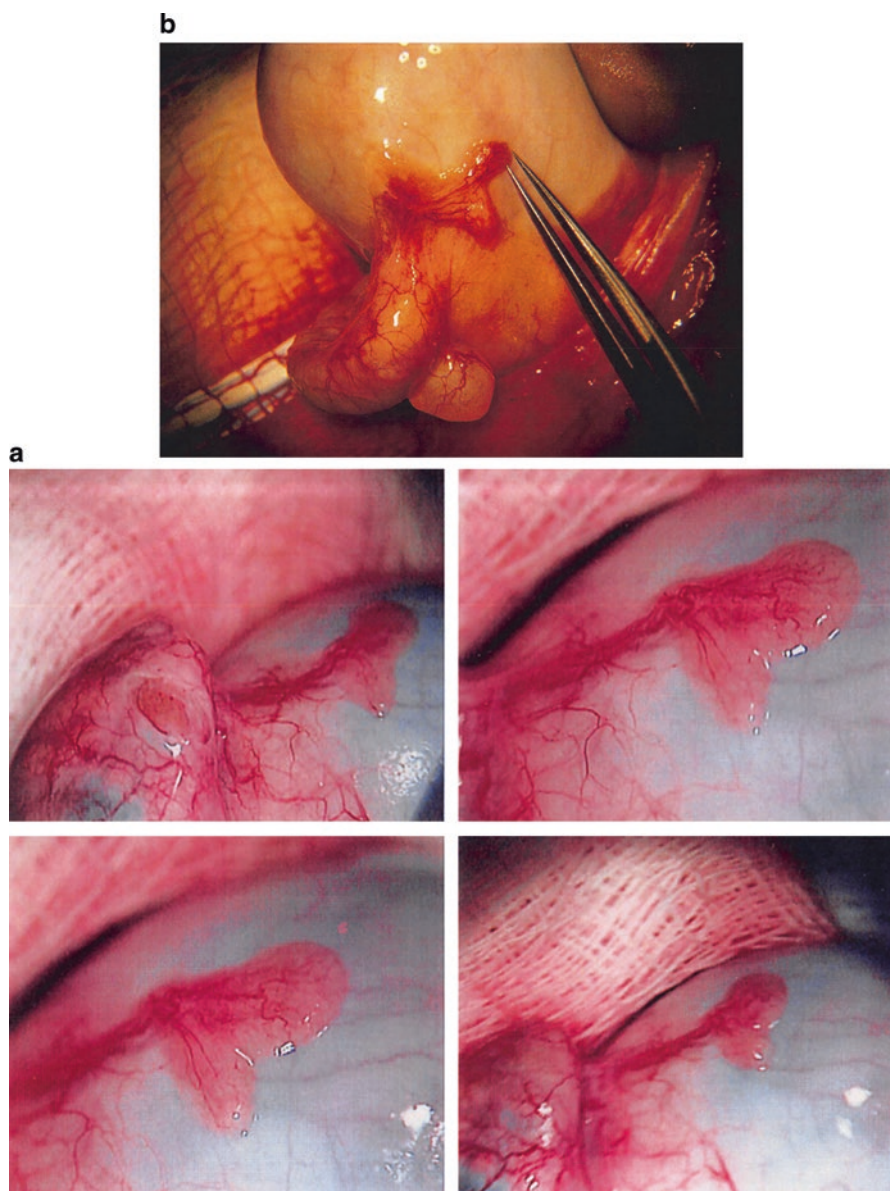


**Fig. 3.1** (a) The appendix testis is the remnant of the regressed Müllerian duct in the adult male. Note that you can see the rudimentary uterus and fallopian tubes. (b) Four different views of the Müllerian duct remnant showing the uterine artery, the uterus, and fallopian tubes in the adult scrotum of the male

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## Chapter 4

# Structure of the Seminiferous Tubules



The testis is made up of the seminiferous tubules, where the spermatozoa develop, and interstitial cells (Leydig cells), where testosterone is produced. The seminiferous tubules are long U-shaped tubules which form their U-turn at the periphery of the testis as both distal ends drain toward the central superior and posterior regions of the testis into the rete testis, which has a flat cuboidal epithelium (Figs. 1.4, 1.7, 1.8, and 1.9). This is important clinically, because a testis biopsy at the periphery, no matter how extensive, will cause no obstruction, but a testis biopsy in the center will cause severe obstruction and damage. The rete testis prevents the passage of fluid from the epididymis backward into the seminiferous tubules. This valve-like effect of the rete testis explains why vasectomy causes pressure buildup and epididymal blowouts, but does not affect the testis or spermatogenesis. The seminiferous tubules loop peripherally toward the tunica albuginea (Figs. 1.4, 1.8, and 1.9). The rete testis lies along the epididymal edge of the testis and coalesces in the superior portion of the testis, to form 5–10 vasa efferent ductules. These vasa efferentia leave the testis and travel a short distance to enter the head or caput region of the epididymis. These vasa efferentia also protect the testis by rapidly moving their ciliated cells forward, forcing fluid forward into the epididymis, preventing backflow. The seminiferous tubules are arranged in anatomic lobules each containing between one and four tubules (Figs. 1.8, 1.9, and 1.11). Understanding this anatomic arrangement is key to performing microsurgical TESE for azoospermic patients.

## Chapter 5

# Blood Supply to the Testis



The spermatic artery (same as ovarian artery in the female) divides into two branches just above the testis (Figs. 2.4, 2.5, and 5.1a). One of them supplies the epididymis, and the other branch enters the testis posteriorly, descends to the inferior pole, and turns back superiorly along the anterior surface. The testes receive their blood supply from three vessels actually, from the testicular, cremasteric, and deferential arteries. The testicular artery is the primary testis blood supply; it arises from the abdominal aorta just inferior to the origin of the renal arteries and courses through the retroperitoneum and through the inguinal canal and cord. When it reaches the testicular surface, it courses along the surface of the testis. The testicular artery pierces the tunica albuginea at the posterior aspect of the superior pole, courses down to the inferior pole, and then ascends along the anterior surface, just under the tunica albuginea, giving off several branches that course into the testicular parenchyma [1–3]. There are several important clinical applications of this unique blood supply. The cold venous blood returning from “outside the body” encircles the arterial blood supply and cools like a radiator the warm arterial blood coming to the testis. Also, note how easy it would be to infarct the testis if you were to do a biopsy in the center rather than the periphery.

The venous and arterial anatomy of the testis is important because of the controversial role of varicocele in male infertility as well as the risk to blood supply from TESE (testis sperm extraction) procedures (Figs. 5.1a–d, 5.2). The veins emerging from the testis form a dense network of intercommunicating branches known as the pampiniform plexus which extends through the scrotum and into the spermatic cord. The arteries supplying the testis pass through this plexus of veins in route to the testis. The scrotal venous blood leaving the testis thus (33 °C) cools the arterial blood coming from the abdomen by a countercurrent heat exchange mechanism. After traversing the inguinal canal, the venous plexus disperses into veins that follow the arterial supply of the testis. The right testicular vein empties into the inferior vena cava. In contrast, the left testicular vein normally enters the left renal vein. The reason that a left varicocele is so common in upright humans is that the left spermatic vein enters into the left renal vein instead of into the vena cava, resulting

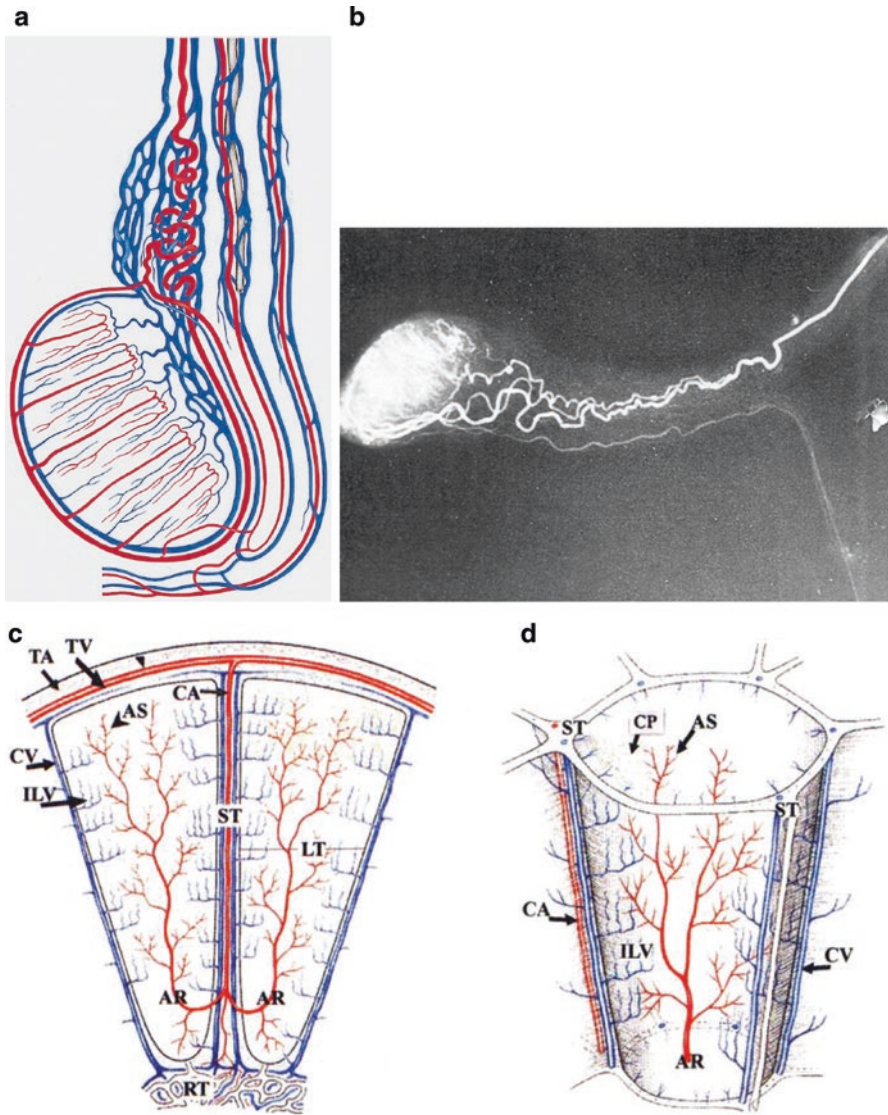


**Fig. 5.1** (a) This is a diagram showing the details of the arterial supply and venous drainage of the testis. The spermatic artery joins the vasal artery and the cremasteric artery to circle the surface of the testis, and then dives down to the mediastium and sends end arteries back up into the lobules. (b) This is an arteriogram showing the very thin microscopic spermatic artery feeding the testis and the large spermatic veins returning blood. (c) Detailed drawing of how the blood supply to the testis and arteries first dives down from the surface, and turns around to supply an entire lobule. (d) In 3-D, one can see how an incision on the surface for TESE is safe, but dissecting deep into the lobule is destructive

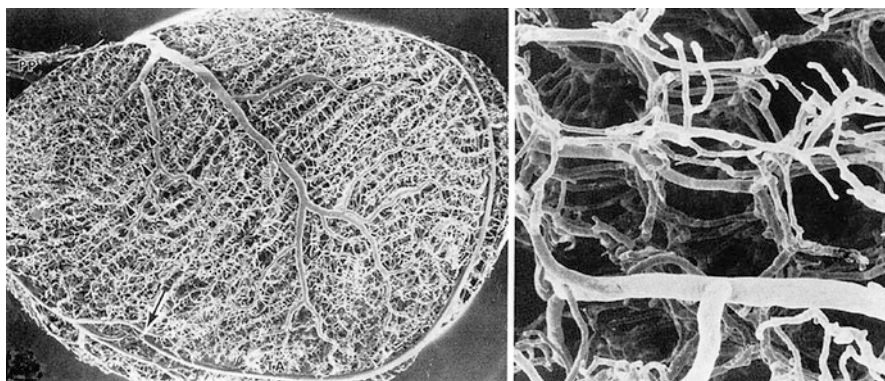


**Fig. 5.2** Testis—intratesticular blood vessels—actual macroscopic view

frequently in defective valves at the renal vein and spermatic vein junction. The arterial anatomy of the testis is often misunderstood by urologists attempting to dissect the seminiferoustubles to retrieve sperm (Fig. 5.1a–d). Dissection deep into the testis will disrupt the tiny end arteries that dive into the lobule.

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## Chapter 6

# Histology of the Testis and Spermatogenesis



Spermatogenesis in the human can be divided into cycles based on the six stages of spermatid maturation from round spermatid to the elongated sperm with a tail (Figs. 6.1, 6.2, 6.3, and 6.4). There are six distinct cellular associations of spermatogonia and spermatocytes in each of these stages of the cycle of spermatogenesis. In mice and rats and most animals, these stages pass like a wave along the seminiferous tubule, and at any given point along the tubule at any time, you only see one of these stages, meaning there may be no sperm or elongated spermatids seen, even though spermatogenesis is normal, and you are seeing only one stage at any given point [1–10]. However, in the human there is no such obvious wave, and the cellular associations of various stages or all stages of spermatogenesis may very well be in any location of the tubule you biopsy (Figs. 6.5a, b and 6.6). At any point in the human seminiferous tubules, you do not have to look for a specific wave to find sperm. This chaotic distribution of the stages of spermatogenesis in the human is very important for clinical performance of TESE as will be explained in Part II of this book.

The most readily apparent cells at any point are spermatogonia, pachytene, spermatocytes, and elongated spermatids [11–13]. Spermatogonia are the early sperm precursors that are located around the inside of the periphery of the seminiferous tubule and that give rise to the various stages of spermatogenesis leading to the spermatozoa that are released eventually toward the center of the tubule. Spermatogenesis thus proceeds from the inner perimeter of the tubule toward the center. All of the steps of spermatogenesis are located within these amoeboid-like Sertoli cells, which nourish the spermatogonia, the spermatocytes, and the maturing sperm (Figs. 1.1, 6.5b and 6.7a). These amoeboid-like Sertoli cells are the equivalent in the male of the granulosa cells in the ovary. Spermatogonial stem cells (SSCs) give rise to the spermatogonia and are self-replicating, unlike in the female, where no new oocytes are produced after initial fetal ovarian and oogonia development.

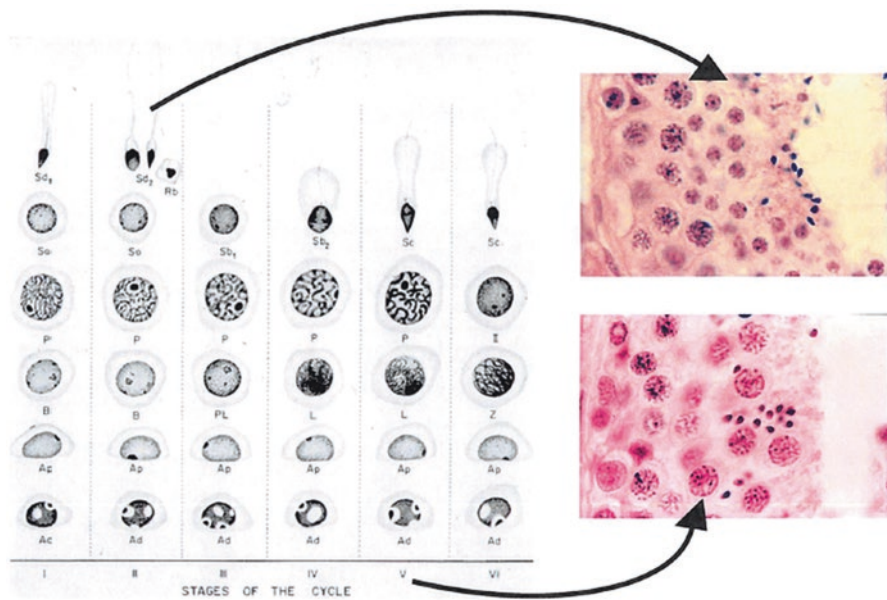


**Fig. 6.1** The six specific stages of spermatogenesis in the human occur randomly, not as a wave, but mixed together everywhere in the seminiferous tubules. Here you can see stages 2 and 5 located together. It is only in stages 3 and 4 that you will not see elongated sperm heads with tails. But you will not see these stages isolated from each other, as would be the case in other species

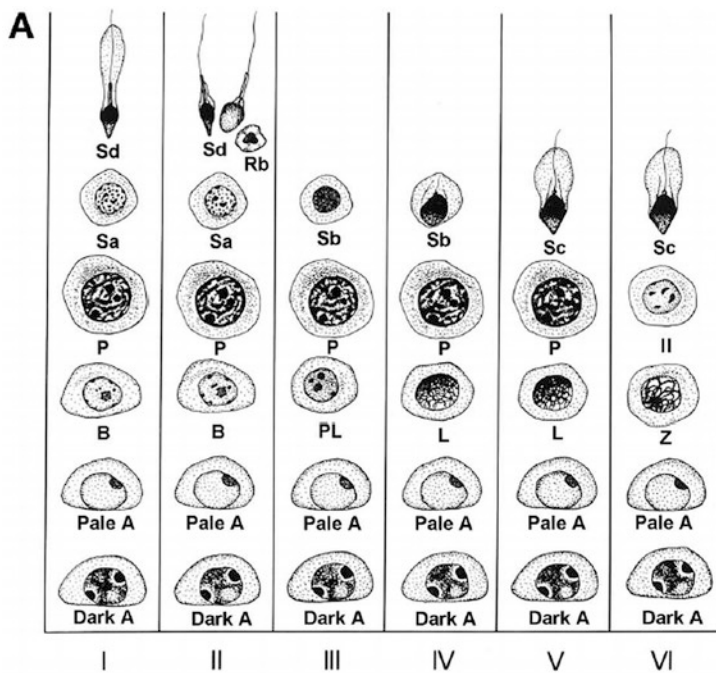


**Fig. 6.2** Stages of spermatogenesis—a more detailed drawing. Note that these six stages are based upon cellular associations that are always seen together and depict the steps in the whole journey from spermatogonia to spermatocytes, to the evanescent secondary spermatocyte, and then to the round spermatid and finally to the haploid maturing sperm

A

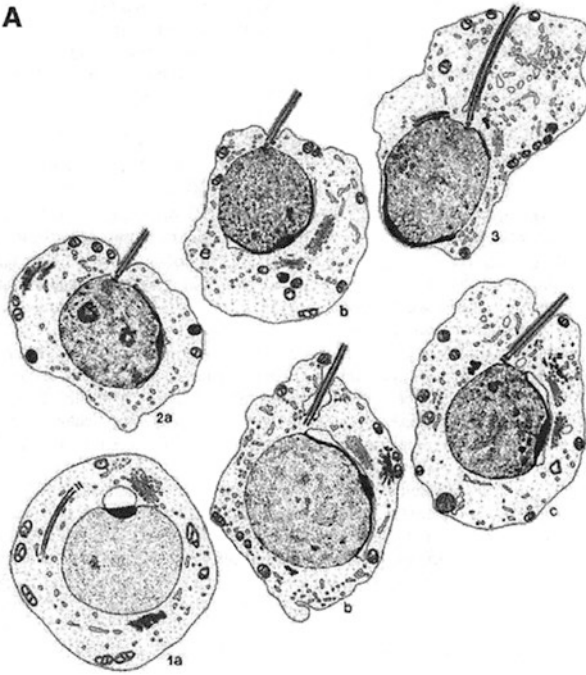


**Fig. 6.3** Drawings of the stages of spermiogenesis after the second meiotic division has occurred. Prior to the formation of the tail, the round spermatid can always be recognized by the prominent acrosomal vesicle. As the acrosomal vesicle recedes, the tail begins to form

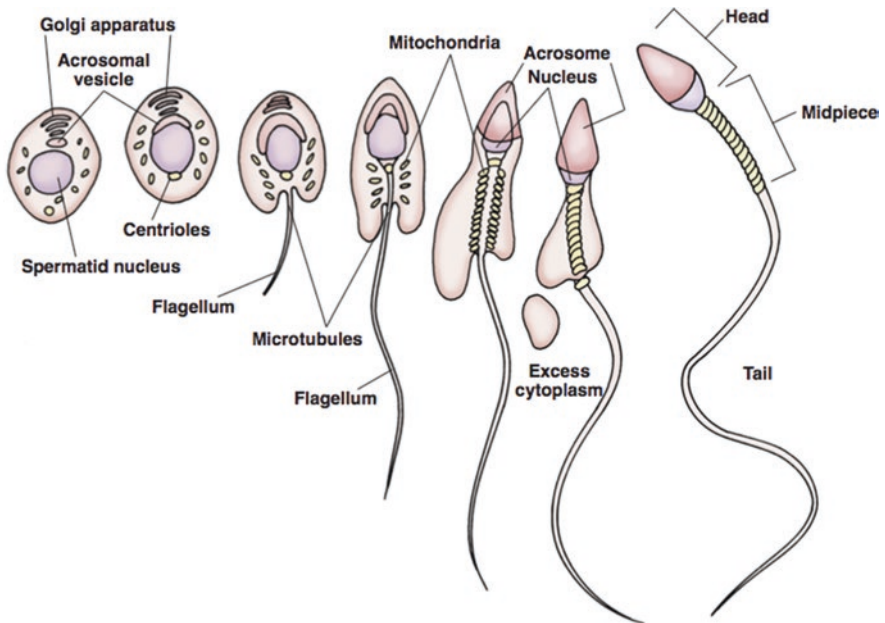


**Fig. 6.4** Another figurative depiction of the maturing of the haploid sperm from the round spermatid. Note that in the human there is no such thing as round spermatid arrest. So it makes no sense to look for round spermatids in TESE when you find no mature sperm. Spermatogenesis maturation arrest in the human is always at the spermatocyte (4N) level

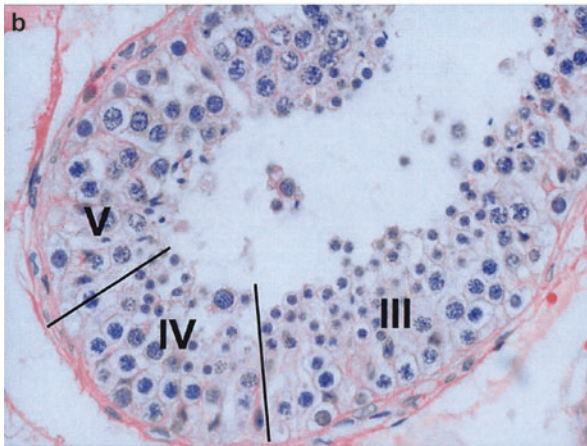
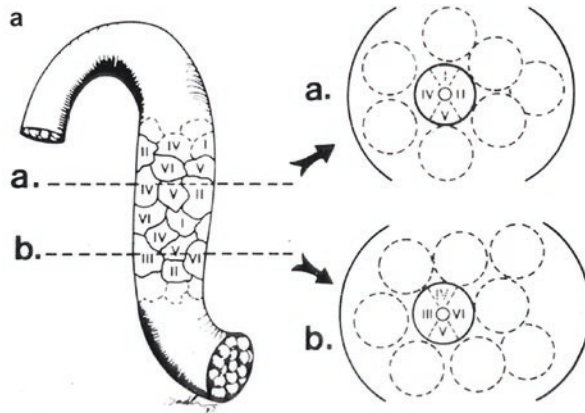


**Fig. 6.5** (a) Spermatogenesis in the human does not occur in an orderly wave, but rather in a mixed arrangement in every seminiferous tubule cross section. (b) Cross section of a tubule with normal spermatogenesis which shows three different stages in one area

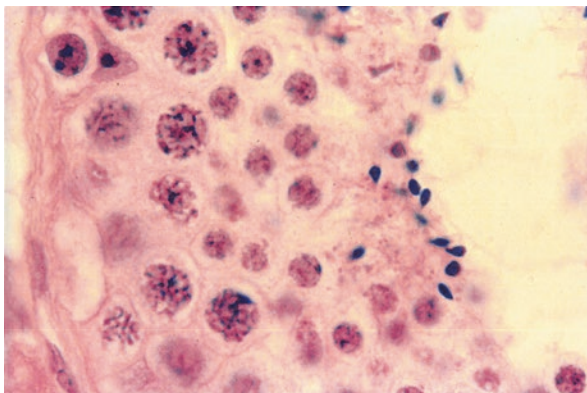


**Fig. 6.6** Normal spermatogenesis—here noted at stages 1 and 2, you can see the most mature, condensed spermatids, which look like sperm with tails at TESE. The reason you rarely see tails on histology sections of normal spermatogenesis is that these sections are only 5 μm thick. So you will see the sperm head, but the very thin tail will usually not be seen. However of course it should be readily seen on TESE under Hoffman optics

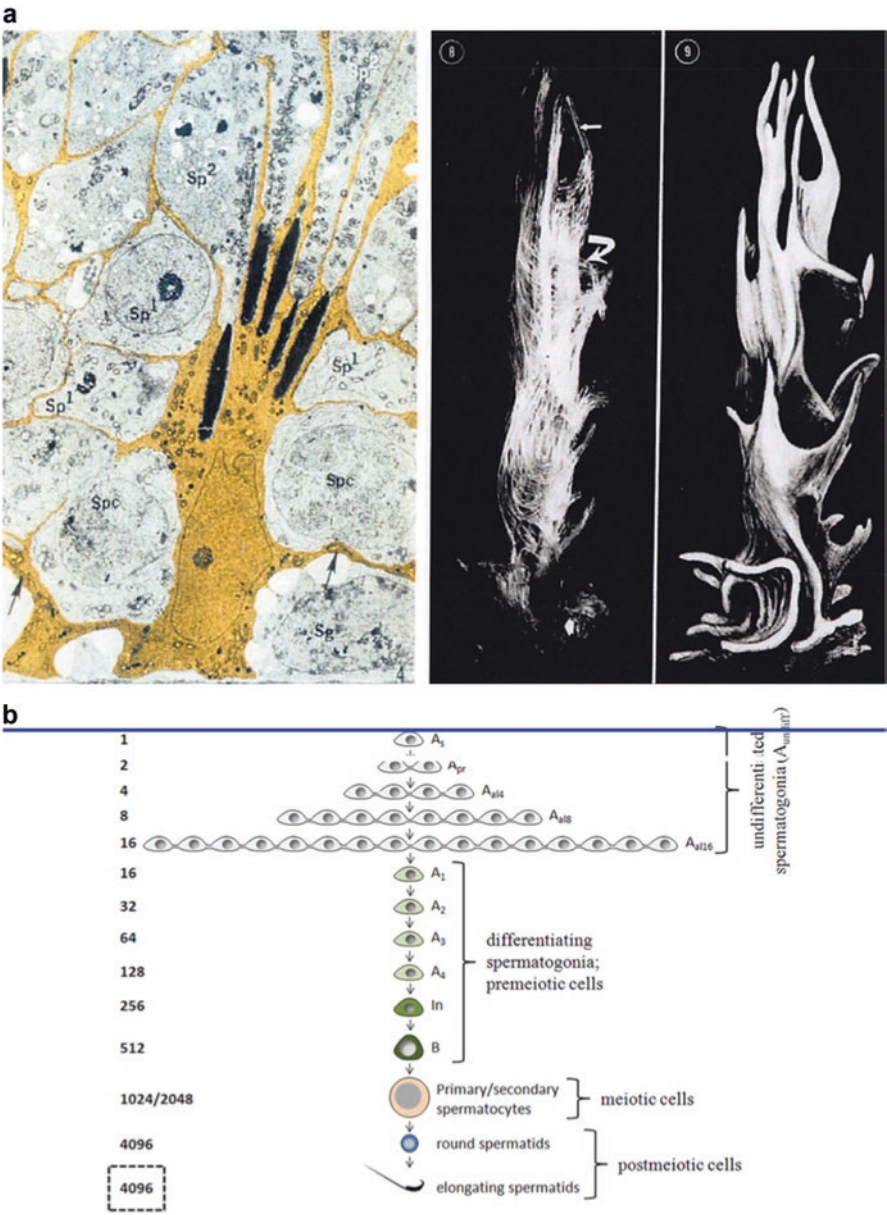


**Fig. 6.7** (a) Testis—Sertoli cells shown graphically to demonstrate their elusive amoeboid character. (b) Spermatogenesis. In theory one A-single spermatogonium having undergone differentiation division can give rise to 4096 spermatozoa. In reality this number is lower due to apoptosis. On the left the value in the column refers to the number of a specific germ cell type at the same level

Spermatogonia histology in the humans is not as clear as in the more closely studied mouse. In the mouse, there are “paired” spermatogonia, which are progenitor, and “aligned” spermatogonia, all of which do not replicate themselves and just produce sperm (Fig. 6.7b). The spermatogonial stem cells (SSCs) just stay on the basement membrane and give rise to spermatogonia [14].

The SSCs make “progenitor” cells which then make differentiating spermatogonia. It is the SSCs that continually renew themselves but at a much slower rate of cell division. It is after chemotherapy when the pale spermatogonia are destroyed, that surviving dark spermatogonia give rise to the pale spermatogonia and recovery of spermatogenesis. In the human, all we can see are “dark” spermatogonia and “pale” spermatogonia. It is the pale spermatogonia which make sperm and the dark spermatogonia replicate. SSCs are located within the population of dark spermatogonia.

The spermatogonial stem cells (SSC's) make spermatogonia, but they also make more stem cells. The slow rate of division of the SSCs (i.e., the spermatogonial stem cells) helps minimize the occurrence of mutations in spermatogenesis. However, there are enough continual mitotic divisions in the SSCs and the more rapidly dividing progenitor spermatogonia to create a *de novo* mutation load of 2 new mutations in the sperm per year, or 60 new mutations by the time the man is 45, or even as many as 100 new sperm mutations by the time the man is 65 years old. This does not happen with eggs.

In women, there are no further mitotic divisions after the initial development of oogonia, which are arrested in meiosis at the early fetal stage. Therefore older men will have more sperm mutations than younger men and much more than in women.

So there are six specific stages of spermatogenesis in the human testis (Figs. 6.1 and 6.2). These six stages of spermatogenesis each last 16 days. The developing sperm precursors have to pass through at least four cell layers. Therefore the complete duration of time to make a mature sperm is at least 64 days or longer. The complete cycle will obviously be longer, but it is not clear how much longer. This is important in trying to time recovery of normal sperm production after cessation of a patient taking testosterone supplements or for vasectomy reversal and vas-epididymostomy patients and for patients after initiation of gonadotropin therapy for hypogonadotropic hypogonadism or even after stopping chemotherapy for cancer.

All spermatogonia are mitotic but at different rates. Dark staining A spermatogonia proliferate least, and some few may be SSCs, which self-renew. Pale staining A spermatogonia make type B spermatogonia, which proliferate the most. SSCs are the spermatogonial stem cells that self-renew and make the progenitor and type B spermatogonia, which are responsible for going into meiosis and making spermatocytes, which eventually complete meiosis to make spermatozoa. SSCs make progenitor spermatogonia of course, but they are unique in that they self-renew, to make new SSCs. Progenitor spermatogonia divide at the fastest rate, cannot self-renew, and eventually are committed after such proliferation to make differentiated spermatozoa. After radiation or chemotherapy, pale A spermatogonia are depleted,

and if there is any recovery of spermatogenesis, it is from slowly dividing dark A spermatogonia or the SSCs.

Progenitor spermatogonia are transitional between SSCs and differentiating type B spermatogonia. Type A spermatogonia (progenitors) never actually produce spermatocytes or therefore sperm. They only amplify themselves or produce type B spermatogonia that differentiate into spermatocytes and spermatozoa. But all pale type A spermatogonia, despite self-replication, are eventually committed to becoming sperm.

It is important that SSCs are separate cells from the progenitor type A or the type B spermatogonia in that SSCs are very slowly dividing. The mutations that occur in the rapidly dividing spermatogonia are not cumulative. Only the mutations in the SSCs are cumulative over the man's lifetime. The slower mitosis rate of the SSCs therefore lowers to some degree the rate of mutation generation, even though still the great majority of human mutations occur in the testis rather than the ovary, during spermatogenesis.

There is an intriguing philosophical ramification of this mutation generation in spermatogenesis: the sperm's problem is that it is constantly making errors during cell cycle duplication. The oocyte's problem, as it avoids the mutation generating result of duplication, is that it ages. Thus the oocyte genetically stabilizes the species, while the sperm allows for evolution by encouraging the acquisition of a mutation load. These two opposing mechanisms have to strike a balance with each other. Men remain fertile as they age but threaten the genetic stability of the species, whereas women become infertile as they age but genetically stabilize the species.

To understand spermatogenesis completely will require a full understanding of meiosis, which will be detailed at the end of this section.

However, to simplify, it is not until the spermatocyte stage that meiosis begins, and homologous chromosomes line up with each other (Fig. 6.8). On histologic inspection, you can easily see the condensing chromatin characteristic of spermatocytes. These homologous chromosomes have all undergone the beginning of a replication, but the chromosomes do not yet split completely and are held together still by the centromere, which has not split. So at this spermatocyte stage, the cells have twice the normal complement of DNA (4N).

These "like" chromosomes then separate from each other to form the very briefly appearing secondary spermatocyte (which is 2N). At this stage the chromatids quickly separate, forming the haploid round spermatid (1N), which then elongates, condenses, and grows a tail, becoming the completed sperm (Figs. 1.9c and 6.1 and 6.4) [15]. Once the haploid round spermatid is formed, it normally will grow the tail and elongate and condense its chromatin into what becomes the head of the sperm. This round spermatid, though not a fully formed sperm, is capable of normal fertilization if injected into the egg. Round spermatids can be definitively identified by the presence of an acrosomal vesicle, but not all round spermatids have an acrosomal vesicle visible.

The spermatogonia gave rise to the spermatocytes which finish meiosis to form spermatids and mature sperm. It is not until the spermatocyte stage that meiosis begins, where homologous chromosomes line up with each other. On histologic

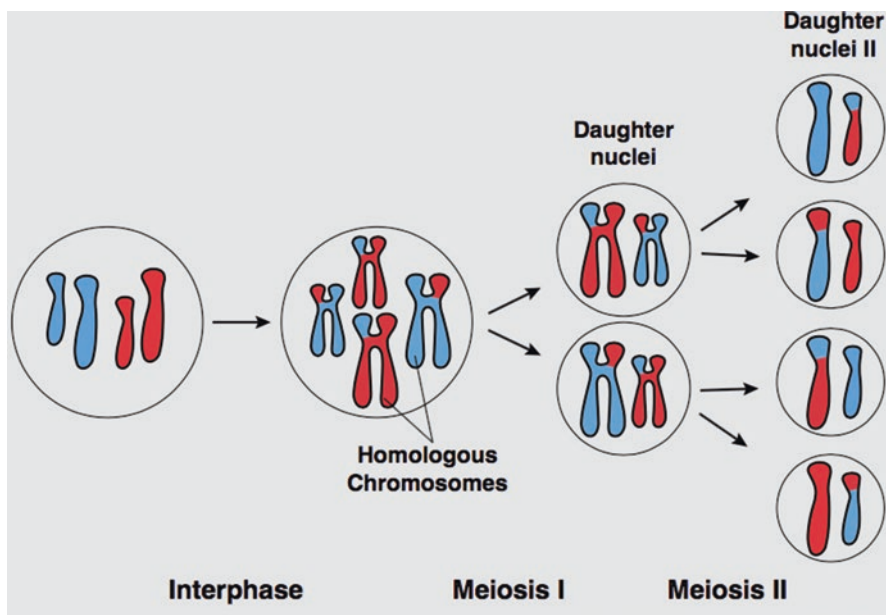


**Fig. 6.8** Meiosis in spermatogenesis

inspection, you can easily see the condensing chromatin representing the development of spermatocytes (Figs 6.1, 6.5b, and 6.6). These “like” chromosomes have all undergone the beginning of mitosis, but the chromosomes do not yet split completely and are held together still by the centromere, which has not split. So, at this spermatocyte stage, the cells are tetraploid, not diploid or haploid. These “like” chromosomes then separate from each other to form the very briefly appearing secondary spermatocyte (which is  $2N$ ). Then at this stage the chromatids quickly separate, completing the early mitosis stage of meiosis forming the haploid round spermatid, which then elongates, condenses, and grows a tail, becoming the mature sperm. In the human, it would be unusual to find a round spermatid in the absence of a mature sperm. It is important clinically to know that maturation arrest in the human most commonly occurs at the spermatocyte stage. However, occasionally (4%) it is at the round spermatid stage.

There was some confusion about spermatids in many IVF centers when we first introduced TESE (testis sperm extraction) for nonobstructive azoospermia. Under Hoffman optics in the IVF lab, the Sertoli cell nucleus (with a prominent nucleolus) has been mistakenly thought to be a round spermatid with its acrosomal vesicle (see Chap. 2). However in most cases, if there are round spermatids, there will be normal sperm found in the testis. That is because arrest of development at the round spermatid stage in human cases of nonobstructive azoospermia is fairly uncommon rare (Figs. 6.3 and 6.4). Maturation arrest in the human is usually at the spermatocyte stage, but very occasionally can be at the round spermatid stage.

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## Chapter 7

# Hormonal Regulation of Spermatogenesis



The hypothalamus, the pituitary, and the testes form an integrated feedback system responsible for the correct secretion of male hormone and for normal spermatogenesis. The testes require stimulation by the pituitary gonadotropins, luteinizing hormone (LH), and follicle stimulation hormone (FSH), which are secreted in response to the pulsatile secretion of hypothalamic gonadotropin-releasing hormone (GnRH) [1, 2]. GnRH has a direct connection to the posterior lobe of the pituitary gland, and so it stimulates FSH and LH release by a direct apocrine effect. It is only permissive and does not participate in the feedback loop.

FSH is only partly responsible for spermatogenesis. Without the effect of LH, spermatogenesis is severely hampered. Testosterone, secreted by the Leydig cells in response to LH, stimulates sperm production and virilization (along with dihydrotestosterone) and also feeds back to the pituitary to inhibit both FSH and LH [3–6]. Therefore men, who take testosterone to increase their muscle mass for bodybuilding and athletics, have no spermatogenesis because of this feedback loop. FSH stimulates Sertoli cells to support spermatogenesis and to secrete inhibin B that negatively also inhibits FSH secretion.

It is important to note that with only FSH stimulation, the testis produces very low numbers of poor-quality sperm. FSH is just necessary for initiating completion of spermatogenesis. LH is required for both quality and quantity of sperm production. Testosterone inhibits both LH and FSH and thus turns off spermatogenesis. But FSH alone is not adequate for fertility nor is LH alone adequate.

The testis consists of the seminiferous tubules and, interspersed between them, Leydig cells in the interstitial space. The seminiferous tubules, containing germ cells, are lined by a layer of Sertoli cells coated by and sitting on the lamina propria. The Sertoli cells are amoeboid in character and support the developing spermatocytes and spermatids by enveloping them (Figs. 6.6 and 6.7). The lamina propria consists of the basal membrane covered by peritubular cells (fibroblasts). The main

component of the interstitial space are the Leydig or interstitial cells. Spermatogonial stem cells are located in the basement membrane of the seminiferous tubule. This is essential to know when trying to culture spermatogonial stem cells.

When doing a TESE procedure to obtain spermatogonial stem cells, it is different from trying to find sperm clinically. When trying to find sperm in a TESE procedure, one discards the solid tissue after dissection. But that “solid” tissue is where the spermatogonial stem cells are located. That is, they are located inside the basement membrane of the seminiferous tubule.

The Leydig cells in the interstitial space produce testosterone. Testosterone is converted to estradiol by aromatase (or to dihydrotestosterone by 5 $\alpha$ -reductase). In the ovary the pathway for estrogen production. In the male there actually is estrogen production in the testis but to a far lesser degree obviously than the ovary. Estrogen originates from testosterone (which is converted to estrogen by aromatase). So I often like to say humorously that the ovary is just a more advanced form of testis, because it has were aromatase and converts testosterone to estrogen. In fact there is no estrogen production in the absence of testosterone.

The Sertoli cells are circumferentially located on the basement membrane, and their apex is oriented toward the lumen of the seminiferous tubules [7]. It is an amoeboid type cell which engulfs and nourishes the spermatogonial cells of the seminiferous tubules. In the 6-week fetus, the Sertoli cells protect the primordial germ cells (PGCs) from retinoic acid, so that they do not enter meiosis as happens to germ cells in the fetal ovary.

Spermatocytes and spermatids first appear at puberty and are supported and nourished by the Sertoli cells. Spermatogenesis beyond spermatogonia does not begin until the pubertal release of FSH and LH from the pituitary. Premeiotic transitions including spermatogonia differentiation and meiotic initiation are coordinated by RA from Sertoli (somatic) cells. Once germ cells enter meiosis, pachytene spermatocytes produce RA to coordinate the two postmeiotic transitions (spermatid elongation and sperm release). In combination, meiotic (pachytene spermatocytes) and somatic cells (Sertoli cells) produce retinoic acid and coordinate spermatogenesis. Sertoli cells support spermatogenesis and nutrition to germ cells and the release of elongated mature sperm into the seminiferous tubule (Figs. 1.1, 1.2, 1.10, and 6.6).

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## Further Reading

Endo T, Freinkman E, de Rooij DG, Pafe DC (2017) Periodic production of retinoic acid by meiotic and somatic cells coordinates four transitions in mouse spermatogenesis. *Proc Natl Acad Sci U S A* 114:E10132–E10141

## Chapter 8

# Meiosis

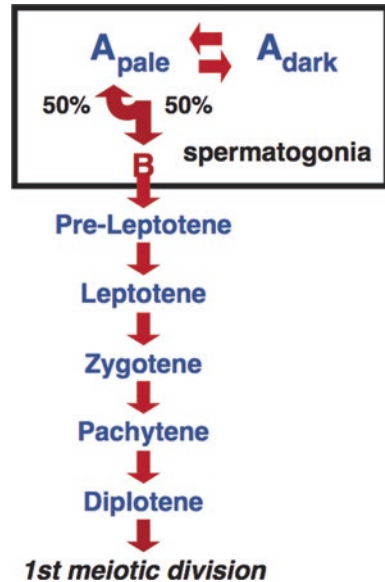


Meiosis, which in the male is initiated in puberty by the secretion of FSH and LH, can be confusing. So here is a simple explanation (Figs. 6.8 and 8.1). First, each chromosome begins a mitosis, but the mitotic division does not complete because the two divided chromosomes remain held together by the centromere that does not divide. They are called chromatids. DNA content doubles but the number of chromosomes remains the same. Thus meiosis always begins with a mitosis that does not get completed. The divided chromosomes are still held together by their centromere. These cells are the primary spermatocytes; they progressively show the nuclear features that identify meiosis I stages of leptotene, zygotene, pachytene, and diplotene (Fig. 8.1). During meiosis I, after the incomplete mitosis has occurred, homologous chromosomes pair with each other in an organized way and recombine after that initial partial division. But the mitotic aspect of meiosis I is not complete, as the centromeres do not divide, and all four “chromatids” then recombine. This represents the spermatocytes all the way from leptotene to zygotene to pachytene. After the pachytene stage, then the homologous chromosomes separate from each other forming secondary spermatocytes. But the sister chromatids still have not yet separated. Secondary spermatocytes have a very brief existence as the sister chromatids then divide immediately (thus completing their initial mitotic division), and this is the second meiotic division resulting in spermatocytes and sperm.

The “purpose,” or function, of meiosis is not just to diversify genes as is taught in biology courses. It is very clearly necessary, via homologous recombination, to repair DNA defects in the germ cells that occur with aging. In fact the whole function of germ cells is to counter aging and to immortalize the DNA. This will be discussed more deeply in the last section of this book.

In the male, unlike in the female, FSH and LH jointly and continuously control this meiosis in a continuous, noncyclic fashion. Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are glycoproteins consisting of alpha and beta polypeptide chains (A and B subunits). Once synthesized, LH and FSH are stored in granules in the pituitary. GnRH induces granule exocytosis and hormonal release into the circulation of FSH and LH. LH exerts its influence on the Leydig cells,

**Fig. 8.1** Meiosis in spermatogenesis



stimulating the production of steroids, mainly testosterone. The Sertoli cells have receptors for FSH and testosterone. It is therefore believed that both hormones support the initiation of spermatogenesis and are both needed for the maintenance of quantitatively normal spermatogenesis. Intratesticular androgen, not circulating androgen, is crucial for normal spermatogenesis. FSH, on the other hand, binds to FSH receptors on Sertoli cells and initiates signal transduction events that ultimately lead to the production of inhibin B, which is a marker of Sertoli cell activity. Inhibin B and testosterone, in turn, regulate pituitary FSH secretion.

This dual hormonal dependence for normal spermatogenesis can be appreciated in hypogonadotropic hypogonadism males as well as in the “fertile eunuch syndrome.” In men with hypogonadotropic hypopituitarism, giving HCG alone will restore spermatogenesis up to the pachytene spermatocyte stage, before separation of homologous chromosomes which would complete meiosis I. However, FSH is necessary for the completion of meiosis I and the continuation of spermatogenesis. On the other hand, as in the case of the fertile eunuch, FSH alone will result in a low amount of spermatogenesis and poor-quality sperm. LH is required in order to have good spermatogenesis in such patients. Thus both FSH and LH are critical for normal spermatogenesis.

## Part II

# Evaluation and Treatment of Male Infertility

There have been many years of debate over the causes and therapy of male infertility. Many treatments have been strongly advocated for male infertility during the past four decades, such as clomiphene citrate, testosterone, human menopausal gonadotropin, human chorionic gonadotropin, corticosteroids (for sperm antibodies), cold wet athletic supporters, vitamins, and even more recently popularly marketed nutritional supplements, without any documented evidence of effectiveness [1]. Even the varicocelectomy operation has come into serious question [2–6]. It is becoming clear that most spermatogenic defects in humans are actually genetic in origin and clearly impervious to improvement with any therapy [7–10]. Furthermore, the development of intracytoplasmic sperm injection (ICSI) as an effective therapy for all cases of male infertility has caused a major reassessment and critical analysis of the diagnostic and therapeutic approaches to male infertility [11]. Finally even the definition of male infertility can be very cloudy, as we know that even with very low sperm counts men can successfully impregnate a very young fertile woman [12]. In this chapter I will debunk many of the myths about male infertility and its treatment, and I will elaborate what really works and what doesn't.

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## Chapter 9

# Evaluation of Male Infertility



The first and most important test for men still remains the semen analysis (sperm count). However, a poor semen analysis, or a low sperm count, does not rule out natural conception, and a normal sperm count does not necessarily mean that the husband's sperm can fertilize his wife's eggs. Men with extremely low sperm counts often have no difficulty impregnating their wives, and yet in a small percentage of in vitro fertilization (IVF) cycles in which the semen analysis is completely normal, there is no fertilization [1–3]. Very often what seems superficially to be a “male factor” probably is really either a female factor or unknown factor.

In fact, how many sperm and what quality of sperm are necessary for a man to be fertile is not at all a simple question. Forty years ago, it was thought that a sperm count of less than 40 million spermatozoa per milliliter meant that the husband was infertile and the urologist gave such couples a poor prognosis for pregnancy. When the wife did get pregnant, this happy result was usually attributed to whatever (in truth ineffective) treatment was actually being administered to the so-called infertile husband.

Acknowledging that low sperm counts can be compatible with fertilization, the World Health Organization (WHO) in 1992 issued reduced “normal values” for semen analysis that included a sperm concentration of greater than 20 million per milliliter and a total sperm count of 40 million per ejaculate, 50% of sperm exhibiting forward progressive motility and 30% with normal morphology [4]. However, even this new, lower table of normal values has been appropriately attacked as very misleading and still implied a fallacious threshold concept for male fertility, above which the man is fertile and below which he is infertile [1, 4, 5]. When a couple has been unable to achieve a pregnancy during a certain period of time (e.g., 1 or 2 years), all we really know is that the couple is infertile. The important question is, to what extent is the husband's “deficient” or “abnormal” sperm count contributing to (or not affecting) the couple's infertility?

9.1 How to Interpret the Semen Analysis

The correlation of sperm count with fertility was originally presented in the classic article by MacLeod and Gold in 1951. These authors studied sperm counts in 1000 “fertile” and 1000 “infertile” couples (Table 9.1) [6]. Their results indicated that in a fertile population, the vast majority of men had sperm counts  $>40 \times 10^6/\text{mL}$ . Only 17% had sperm counts  $<40 \times 10^6/\text{mL}$ . Only 5% of fertile men had sperm counts  $<20 \times 10^6/\text{mL}$ . This distribution would suggest that a normal count is  $>40 \times 10^6$  spermatozoa/mL, and this had been the assumption for many decades.

Rehan et al. in 1975 reported results similar to MacLeod and Gold [7]. In 1300 fertile men, the percentages were remarkably similar to those of MacLeod and Gold, with only 7% of fertile men having sperm counts  $<20 \times 10^6/\text{mL}$ . Eighty-three percent of fertile men had sperm motility of grades 3 and 4, but what perhaps has not been adequately emphasized is that 17% of fertile men had very poor sperm motility of grades 1 and 2. Similarly, 86% of fertile men had greater than 40% motile sperm, but 14% had less than 40% motile sperm and 4% of fertile men had sperm motility of less than 20%. Neither of these early studies addressed the possibility that low sperm counts, like high sperm counts, might occur at either end of the bell-shaped population curve and might perhaps be unrelated to the man’s fertility.

David et al. in 1979 reported on sperm counts in almost 3000 infertile men with a lop-sided control group of only 190 fertile men (Table 9.2) [8]. The frequency distribution of sperm counts in fertile and infertile men obtained by these authors is shown in Table 9.2 and is similar to that of MacLeod and Gold in 1951. Thus, the inference remained strong that a sperm count of more than  $40 \times 10^6/\text{mL}$  indicates a much greater likelihood of fertility. That sperm count actually may not correlate closely with the man’s fertility was first proposed in 1974. Nelson and Bunge reported among 386 fertile men that low sperm counts are compatible with fertility and that a sperm count of less than  $20 \times 10^6$  or less than  $40 \times 10^6/\text{mL}$  does not

**Table 9.1** Frequency distribution of sperm counts in 1000 fertile men and 1000 infertile men [6]

| Sperm count ( $10^6/\text{mL}$ ) | Fertile men (%) | Infertile men (%) |
|----------------------------------|-----------------|-------------------|
| <20                              | 5               | 16                |
| 20–39                            | 12              | 13                |
| 40–59                            | 12              | 11                |
| >60                              | 71              | 60                |

**Table 9.2** Frequency distribution of sperm counts in 190 fertile men and 2889 infertile men [8]

| Sperm count ( $10^6/\text{mL}$ ) | Fertile men (%) | Infertile men (%) |
|----------------------------------|-----------------|-------------------|
| <20                              | 6.9             | 28                |
| 20–39                            | 9.5             | 16.4              |
| 40–59                            | 14.7            | 13.6              |
| >60                              | 69              | 41.3              |

indicate a “male factor” [9]. In 1977, Zukerman et al. reported on several thousand fertile men who had a semen analysis performed prior to vasectomy [3, 10]. Twenty-three percent of these fertile men had sperm counts less than  $20 \times 10^6/\text{mL}$  and only 40% had sperm counts greater than  $60 \times 10^6/\text{m}$ .

I have reviewed sperm count and motility indices in men following vasovasostomy whose wives became pregnant in comparison with those whose wives did not become pregnant (Table 9.3). The distribution of sperm counts, percentage motility, and total motile sperm per ejaculate was quite similar in both groups [11, 12]. Twelve percent of the patients who had successful vasovasostomy and whose partners became pregnant had total motile sperm counts per ejaculate of less than  $10 \times 10^6$ . In fact, the extensive comparison by Jouannet et al. of spontaneous pregnancy rates in infertile couples with varying sperm parameters showed results similar to my long-term follow-up of patients who have had vasovasostomy performed: above a count of  $5 \times 10^6$  sperm, the difference in pregnancy rate related to differences in sperm count is not dramatic [13].

Nonetheless, although a low sperm count and a low sperm motility do not necessarily indicate infertility in any particular couple, control studies have shown that lower motile sperm counts are still associated with lower spontaneous conception rates over the course of time in couples who are infertile. Schoysman and Gerris in 1983 studied the spontaneous pregnancy rate over the course of time in 1327 oligozoospermic couples (Table 9.4) [14]. When the motile sperm count was less than 1 million (mL) (even as low as 100,000/mL) despite no treatment of either the husband or wife, in 5 years, there was a 4% spontaneous pregnancy rate, and in 12 years, the wives of 9% of these couples spontaneously conceived.

**Table 9.3** Frequency distribution of motile sperm count and pregnancy rates after vasovasostomy in men whose wives did or did not become pregnant [11, 12]

| Total motile sperm count (10 <sup>6</sup> /ejaculate) | Total patients (frequency distribution) | No pregnant (frequency distribution) | Pregnancy rate |
|-------------------------------------------------------|-----------------------------------------|--------------------------------------|----------------|
| 0–10                                                  | 32 (12%)                                | 25 (11%)                             | 78%            |
| 10–20                                                 | 31 (12%)                                | 27 (12%)                             | 87%            |
| 20–40                                                 | 32 (12%)                                | 30 (13%)                             | 94%            |
| 40–80                                                 | 79 (31%)                                | 68 (30%)                             | 86%            |
| >80                                                   | 84 (33%)                                | 78 (34%)                             | 93%            |
| Totals                                                | 258 (100%)                              | 228 (100%)                           | 88%            |

**Table 9.4** Pregnancy rates in 1327 men with oligozoospermia % pregnancy [14]

| Motile sperm count (10 <sup>6</sup> /mL) | 5 years | 12 years |
|------------------------------------------|---------|----------|
| 0.1–1                                    | 3.9     | 8.7      |
| 1–5                                      | 11.9    | 26.6     |
| 5–10                                     | 22.1    | 34.3     |
| 10–15                                    | 45.0    | 58.5     |
| 15–20                                    | 68.6    | 82.0     |

When the motile sperm count was between  $5$  and  $10 \times 10^6$ , 22% conceived within 5 years and 34% within 12 years. When the motile sperm count was between  $15$  and  $20 \times 10^6$ , 69% conceived within 5 years and 82% within 12 years.

Baker et al. (1986) constructed a life-table pregnancy curve for infertile couples with varying degrees of oligozoospermia comparing them to various fertile control populations (Fig. 9.1) [15–18]. Pregnancy rates were compared for couples with a sperm count of less than  $5 \times 10^6$ /mL,  $5$ – $20 \times 10^6$ /mL, greater than  $20 \times 10^6$ /mL with less than 60% motile, and greater than  $20 \times 10^6$  with 60% motile. These four groups were compared graphically with the life-table conception rate of a donor insemination group, Vessey's women discontinuing the intrauterine device, and the spontaneous pregnancy rate reported in 1953 by MacLeod and Gold for fertile couples [16–18].

Again, quite remarkably, with less than  $5 \times 10^6$  spermatozoa/mL regardless of motility, the pregnancy rate at 2 years was 26% (Fig. 9.1). When the sperm count

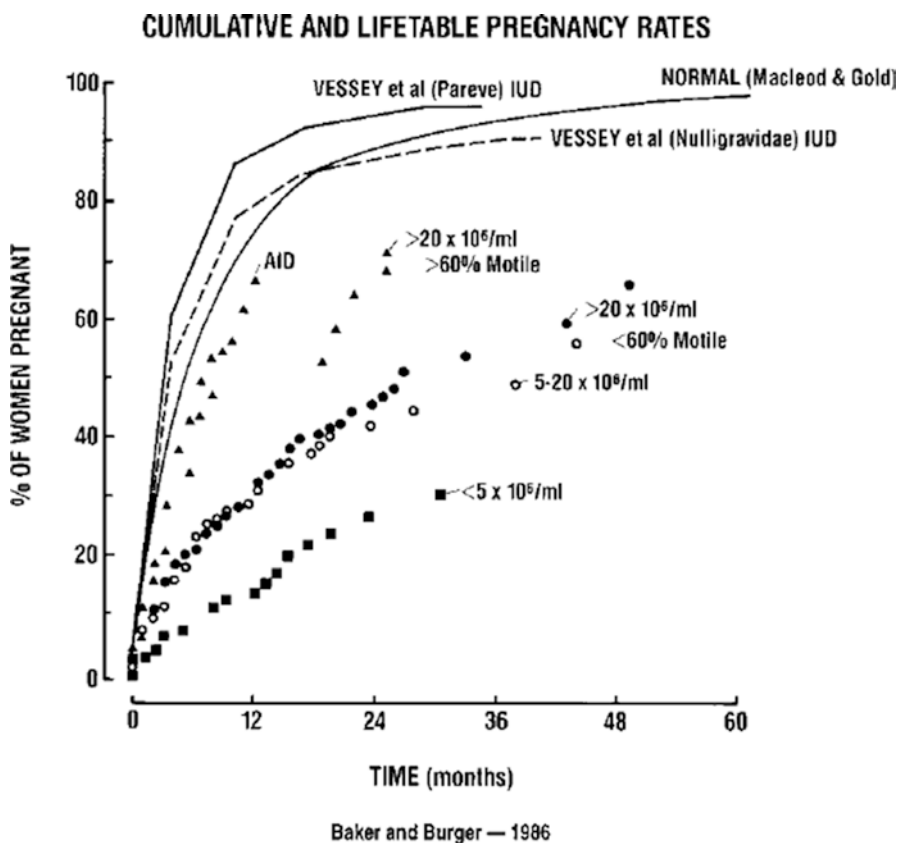


**Fig. 9.1** Cumulative and life-table pregnancy rates (From: Baker HWG, Burger HG. Male infertility in reproductive medicine. In: Steinberger E, Frajese G, Steinberger A, eds. Reproductive Medicine. New York: Raven, 1986:187–197)

was greater than 5 up to less than  $20 \times 10^6$ / mL, the pregnancy rate at 2 years was 42%. When the sperm count was greater than  $20 \times 10^6$ /mL with less than 60% motile, the pregnancy rate at 2 years was similar to the results obtained when the count was  $5\text{--}20 \times 10^6$ . When the sperm count was greater than  $20 \times 10^6$  and the motility greater than 60%, the pregnancy rate at 2 years was 63%. When any of these pregnancy rates is compared with that of donor insemination or to otherwise fertile couples discontinuing an intrauterine device, it is clear that no matter how high the sperm count, the subsequent pregnancy rate of couples attending a fertility clinic is lower than that of a normal control population. Furthermore, even though women may become spontaneously pregnant with extremely low sperm counts, nonetheless, among infertile couples, a higher motile sperm count does increase the chance for spontaneous conception.

9.2 Male Infertility and the Female Factor

The major variable in the oligospermic couple’s chances for pregnancy is not the sperm count, but rather the wife and most specifically her age. Before the era of intracytoplasmic sperm injection (ICSI), when donor insemination was performed for couples with both azoospermia and severe oligospermia, the pregnancy rate was always higher in the wives of men with azoospermia than in the wives of men with oligospermia [19]. Thus, a man with severe oligospermia might have initiated a pregnancy even with his small number of spermatozoa if the woman herself did not also have reduced fertility [11]. Thus, when couples with oligospermia in the pre-ICSI era would opt for donor sperm, the pregnancy rate was lower than with azoospermic men because the wife was more likely to be infertile herself. As Schoysman said, “The subfertile female reveals the subfertile male” [14].

Hargreave and Elton studied the spontaneous pregnancy rate in couples with varying degrees of oligospermia in relation to the duration of prior infertility [20]. Men with an extremely low sperm count successfully impregnated their wives without any treatment if the duration of prior infertility was only 1 year (Table 9.5).

**Table 9.5** Mathematical model to predict a couple’s fertility for given sperm concentration and motility and allowing for duration of involuntary infertility: percentage chance of conception for the next year [20](Hargreave and Elton 1983)

|                                                            | Motile density | Duration of infertility (months) |    |    |    |
|------------------------------------------------------------|----------------|----------------------------------|----|----|----|
|                                                            |                | 12                               | 24 | 48 | 96 |
| Azoospermia sperm present<br>(millions of motile sperm/mL) | 0              | 0                                | 0  | 0  | 0  |
|                                                            | 0.5            | 16                               | 12 | 9  | 6  |
|                                                            | 1              | 25                               | 19 | 14 | 9  |
|                                                            | 2              | 34                               | 26 | 19 | 13 |
|                                                            | 5              | 36                               | 28 | 21 | 14 |
|                                                            | 10+            | 37                               | 28 | 21 | 14 |

If the duration of prior infertility was much longer, then the outlook with oligospermia was much worse. In fact, the most critical factors that determined pregnancy prognosis, in couples with oligospermia, were the age of the wife and the duration of prior infertility, more so even than the sperm count.

In an extensive study of the various factors affecting pregnancy rate with sperm retrieval and ICSI in men with azoospermia, neither the quality of the sperm nor the site of retrieval had any effect on the pregnancy rate. The only factors that affected implantation and pregnancy rate were the age of the wife and her ovarian reserve [21]. According to Nieshlag, in his carefully controlled studies of couples undergoing varicocelectomy or being deferred for counseling, the treatment of the varicocele and the sperm count had no effect on the pregnancy rate. The only factor that was judged to be significant was the age of the wife when the duration of prior infertility was equivalent [22, 23]. In fact, according to Collins' studies, the woman's age is the single most important determinant of the couple's fertility [24].

In the era of IVF, it became clear that couples with reduced or abnormal standard semen parameters have lower fertilization rates (e.g., 68% vs. 23%), which leads to lower pregnancy rates [25]. However, it was still impossible to predict from the semen analysis which couples with reduced semen parameters would have normal fertilization and which would have reduced or no fertilization. A lower transfer rate, fewer embryos, and lower pregnancy rates were obtained in couples with abnormal semen parameters, but there was no way to predict which couples with reduced semen parameters would fertilize and which couples would not [26].

### 9.3 Sperm Morphology

Frustration with the inability of the ordinary semen analysis to accurately predict fertility of the couple and a clear absence of a threshold value below which one can definitely determine that the man is infertile have led to the introduction of many other more specialized tests to evaluate sperm function. One of the simplest of these tests is the "strict criteria" evaluation of sperm morphology [27]. The WHO has for years defined the lower limit of normal for morphology of sperm in the semen analysis as 30% [1]. This parameter has not been very successful in predicting fertility (Fig. 9.2a, b) [5]. However, the simple categories of normal (oval-headed), amorphous (irregular-headed), tapered-headed, and small-headed sperm have now been replaced by strict criteria [27, 28]. The "strict criteria" method of determining morphology specifically measures the length and width of the oval spermatozoa head to a more exacting degree, and a sperm head could only be called "normal" if it fits within this narrow range (2.5–3.5  $\mu\text{m}$  wide and 5–6  $\mu\text{m}$  long). The acrosome had to represent 40% or more of the sperm head, and other perhaps less important measurements of the mid-piece (1  $\mu\text{m}$  wide and 7.5–9  $\mu\text{m}$  long) and tail of the sperm (45- $\mu\text{m}$  long and uncoiled) had to be "strictly" applied. With these strict criteria, it was suggested that the lower limit of normal was 14% rather than 30%. Those with less than 4% normal morphology by strict criteria had only a 7.6% fertilization rate

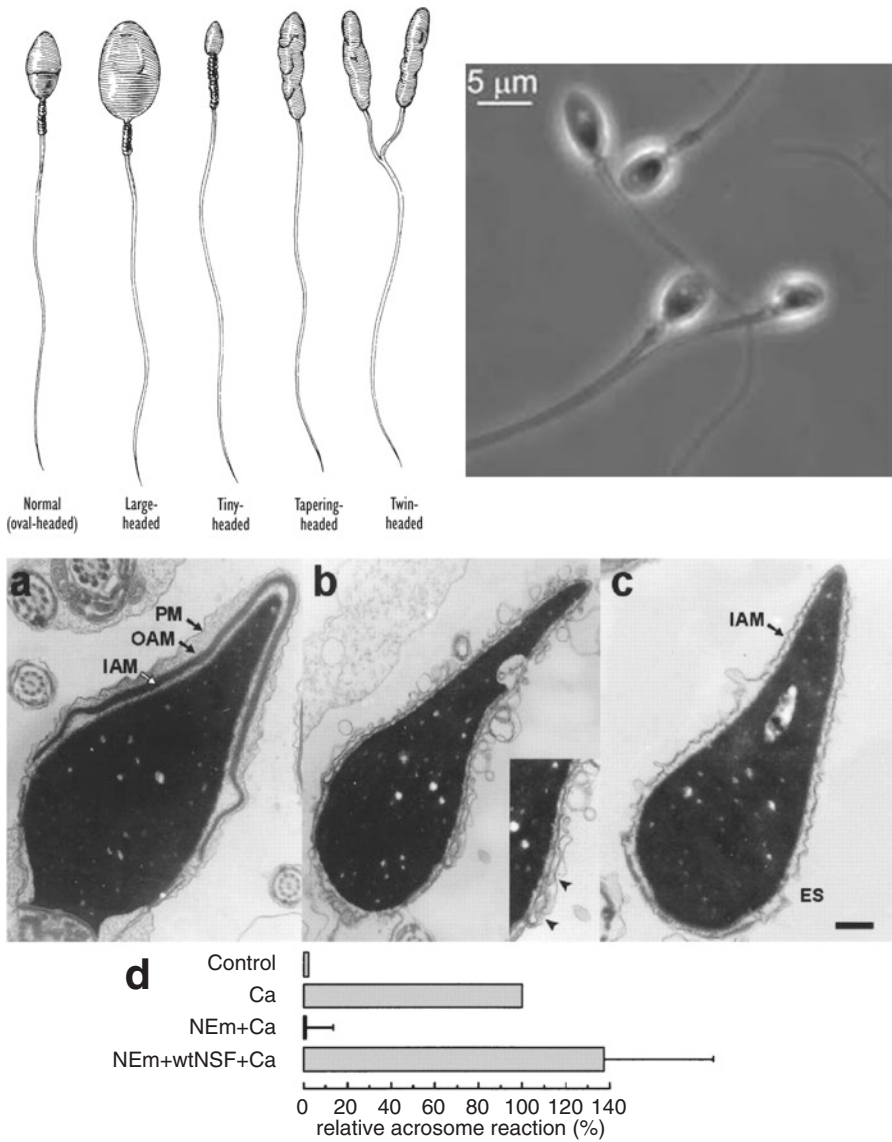


**Fig. 9.2** Normal and abnormal morphology of sperm. (a) Under 800× magnification (IMSI), detailed sperm morphology reveals a perfectly oval shaped head and a “growing” acrosomal cap. (b) Under 5000 electron microscopy (E/M), a normal non-senescent sperm head is noted to have no “sperm DNA fragmentation”

with IVF, those with 4–14% normal forms by strict criteria had a 64% fertilization rate with IVF, and those with greater than 14% normal morphology by strict criteria had a 91% fertilization rate [29]. However, this simple system has not really been perfectly consistent either [28, 30].

The concept of trying to predict fertilization by the strict evaluation of sperm morphology has been enthusiastically embraced in theory but has not worked well in practice. The original criteria that defined normal sperm were based on an esthetically pleasing oval shape [31, 32]. What really matters, however, in assessment of morphology for predicting fertilization capability of sperm is (1) whether the acrosome can function properly in zona binding and zona penetration and (2) whether the abnormal morphology is related to any basic DNA defect in the sperm head. The strict criteria approach to assessing morphology was an effort to apply the metric standards first established by WHO regarding sperm head length and width and then to exclude more sperm from that normal category because of subtle abnormalities in sperm head shape and staining properties [33–36]. Some would argue that whether you use the WHO criteria (which are objective and metric) or the strict criteria, the same basic methodology is used, and it is just a matter of whether a smaller number of sperm can be considered normal because of various subtle differences in shape and staining [4, 29].

The theoretic basis for the predictability of fertility by evaluation of sperm morphology by strict criteria is that it is indirectly indicative of acrosomal function, which is necessary for sperm binding to the zona pellucida and penetration through the zona pellucida. Sperm with abnormally shaped heads do not bind to the zona and cannot penetrate the egg [37–40]. This is a sound theoretic foundation for reliance on morphology. Nonetheless, even with evaluation of morphology by strict criteria, the range of what is found in fertile and infertile men still only represents spectra with no clear threshold [28, 30]. It is logical to expect that unless there is truly 100% abnormal morphology (which is extremely rare), strict morphology suffers from the same dilemma of all the other sperm parameters in the semen analysis [13]. Results of strict morphology evaluation are certainly related to fertilization rate in vitro, but patients with poor morphology do fertilize, and at least 25% of patients who do not fertilize have perfectly normal morphology by the strictest criteria [37–40]. Thus, there still seems to be no easy way to eliminate the possibility of a man being infertile despite a normal semen analysis or that he may be fertile despite an abnormal semen analysis.

## 9.4 Zona Binding, Sperm Penetration, and In Vitro Fertilization

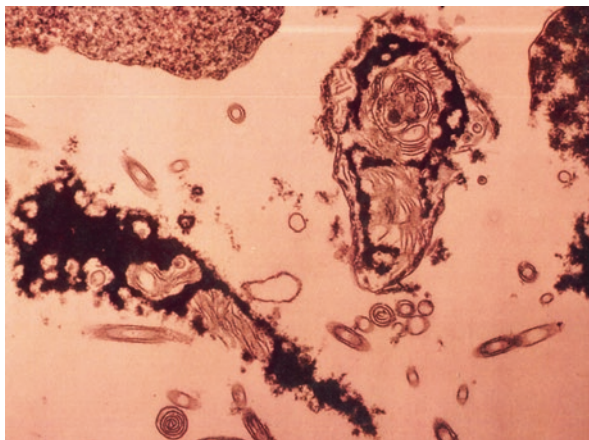
Because failure of fertilization is unexplained in at least 25% of patients, Liu and Baker made an extensive study of the sperm of patients with unexplained “failed fertilization” in IVF who had otherwise completely normal semen parameters, including normal morphology by strict criteria [37–40]. They noted that (1) sperm

with abnormal morphology did not bind to or penetrate the zona pellucida and (2) sperm with normal morphology did bind to the zona pellucida but, in cases of failed fertilization, did not penetrate it. A failure of the zona-induced sperm acrosome reaction thus explained the failure of fertilization in men with otherwise normal semen parameters. General “acrosome reaction” assays that are not induced by zona binding are unphysiologic, and, therefore, it is no surprise that they are of no predictive value [41]. They have nothing to do with how a sperm fertilizes an egg, which begins with the zona-induced acrosome reaction. The studies by Liu and Baker thus seemed to eliminate a great deal of confusion about sperm testing problems and provided an explanation for unexplained failed fertilization and also clarified why and how sperm morphology affects fertility [42]. Human spermatozoa must first bind to the zona pellucida to fertilize the egg, and they do this with an intact normal sperm head that has not yet undergone the acrosome reaction. Once the sperm head is bound to the zona pellucida, the zona becomes an efficient inducer of the acrosome reaction, which then allows the sperm to penetrate through it. Sperm with normal morphology that are capable of binding to the zona pellucida, but then cannot penetrate, have a specific failure of the zona-induced acrosome reaction. Therefore, however sound the rationale, for strict morphology, much like the rest of the semen parameters, it provides no assurance of whether the sperm can or cannot fertilize.

## 9.5 Other Various Tests of Sperm Function

Many other tests of sperm function have been developed in an effort to solve this enigma of “male factor,” and most have enjoyed temporary popularity, only eventually to be discarded. The hamster egg sperm penetration assay, the cervical mucous sperm penetration assay (as well as the simpler postcoital test), computerized sperm motility analysis, hemizona binding assay, sperm DNA fragmentation, etc. were all developed because of the apparent inadequacy of the routine semen analysis [43]. Most of these tests have fallen into disfavor either because they yielded no greater information than the standard semen analysis (or sperm morphology evaluation) or because they involved a great deal of equipment and expense that could not be justified by what, at best, was a controversial and debatable effectiveness. Many such tests, as the sperm DNA fragmentation test, are purely driven by desire for profit (Fig. 9.3). “Sperm DNA fragmentation” is just a fancy new term for what we described in 1975 while doing vasectomy reversal. When sperm (which have a limited lifetime) die, their chromatin deteriorates and fragments. This is readily seen on E/M (electron microscopy). It also can be detected indirectly with electrophoresis. All sperm samples will have some nonmotile sperm that are senescent and therefore have “sperm DNA fragmentation.” It is probably the diverse population of spermatozoa in the semen of each man that makes such testing problematic because most infertile men who do not have azoospermia represent spectra of fertility. The development of IVF and ICSI and the lack of reliability of semen analysis in providing

**Fig. 9.3** Old sperm obstructed in the distal epididymis



prognostic information to predict fertilization led to the proliferation of all of these more complicated and expensive functional tests for spermatozoa. However, most clinicians today favor the use only of routine semen analysis with morphology and motility assessment, recognizing full well its limitations [44–46].

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# Chapter 10

## Treatment of Male Infertility



### 10.1 Critique of Classic Andrologic Treatments

With the arrival of ICSI, Devroey has argued that none of these complex andrologic tests are of great importance any longer anyway [1]. For the most part, treatment of male infertility, prior to IVF and ICSI, has been authority based and not evidence-based. It is highly doubtful whether the fertility of any male with oligospermia, or oligoasthenoteratospermia, can be improved by any treatment whatsoever, including antiestrogens such as Clomid and Tamoxifen, androgens, gonadotropins, or even varicocelectomy [1–10]. It has been argued that with the exception of an occasional testicular cancer that may be detected, even physical examination has no impact on therapeutic results for oligoasthenoteratospermia [10].

This anti-andrologic sounding drift of opinion has angered many andrologists and urologists. But instead of recoiling and defending outmoded andrology, it would be better to innovate and join a more scientific future for andrology, such as making sperm from IPS cells or just freezing testis tissue of prepubertal boys with cancer, and then culturing those cells until they survive but the leukemic cells die, and then transferring those cancer-free cells back. Also just the huge molecular field of searching for the genomic variants that affect spermatogenesis is the future for andrology.

For example, with varicocelectomy, Baker et al. found that couples who underwent varicocelectomy, as well as couples who did not undergo varicocelectomy, had a conception rate within 1 year of approximately 30% and by 2 years of approximately 45% (Fig. 10.1). Nieschlag in his varicocele control study of 125 infertile couples found that 25% of couples with varicocele who did not undergo varicocelectomy became pregnant within 1 year and a similar percent that had undergone varicocelectomy became pregnant within 1 year (Fig. 10.2).

WHO published a compendium graph of sperm counts in a single man over 120 weeks of testing (Fig. 10.3). You can see his sperm count went as high as 170 million and as low as 2 million/mL. Thus with only placebo given when his count

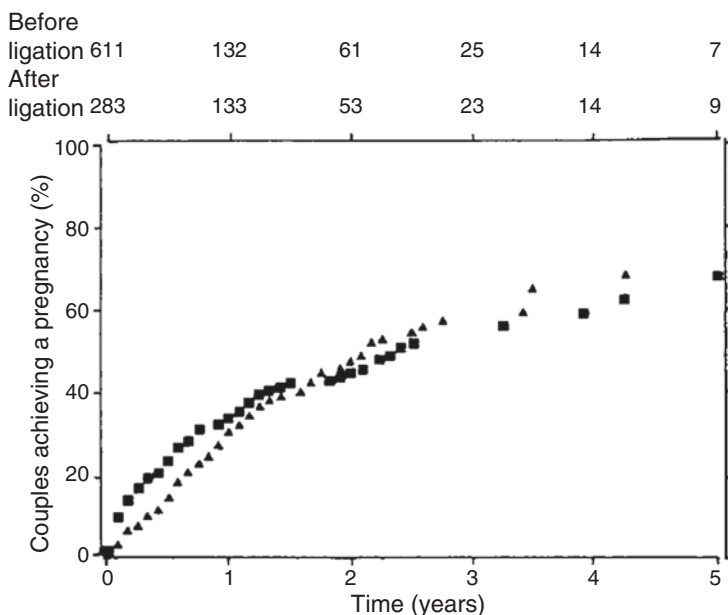


**Fig. 10.1** Life table curves of pregnancy rates for before ligation (f) and after ligation (°) groups. The number of patients initially and those followed up to the end of each year is shown at top of figure. Symbols indicate those months in which the life table changed, that is, when pregnancies occurred. Although some patients were followed up for more than 5 years (those in before ligation group for maximum of 92 months, after ligation group for 108 months), the longest duration of follow-up to pregnancy was 60 months. There was no significant difference between the two curves by log-rank test (Baker HWG, Burger HG, de Kretser DM, et al. Testicular vein ligation and fertility in men with varicoceles. Reprinted with permission from Br Med J. 1985;291:1678–1680)

was low, you would have thought he was helped by the placebo. But if the placebo was given when his count was high, you might have thought he was hurt by the placebo. Therefore, it is impossible to assess the effectiveness of any of the popularly advocated treatments for male infertility during the last 40 years without rigorously controlled studies [2, 3, 10–12].

There is probably no subject that is more controversial in the area of male infertility than varicocele. Most non-urologist infertility specialists around the world are extremely skeptical of the role of varicocele or varicocelectomy in the treatment of male infertility, despite the fact that most urologists are enthusiasts. The directors of most assisted reproductive technology programs view the enthusiasm with which urologists approach varicocelectomy as a potential impediment to the couple that is getting older and often does not have much time left for having good pregnancy rates with assisted reproductive technology. They feel that these couples are being inappropriately delayed in obtaining assisted reproductive technology with the hope that varicocelectomy will solve their problem. Often during that time, years are wasted while the woman becomes older.

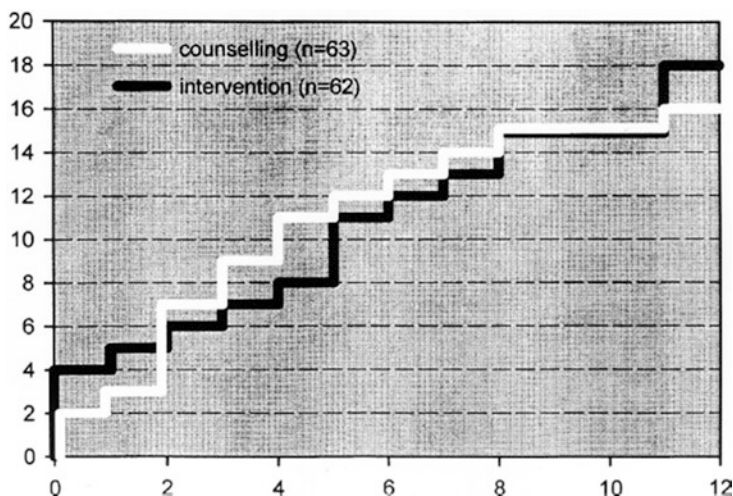


**Fig. 10.2** Life-table analysis of pregnancy rate in patients with varicocele who underwent counselling only, and who underwent varicocelectomy, and showing no difference in pregnancy rate. (From: Nieschlag E, Hertle L, Fischedick A, et al. Update on treatment of varicocele: Counseling as effective as occlusion of the vena spermatica. *Hum Reprod.* 1998;13:2147–2150. Also from: Nieschlag E, Hertle L, Fischedick A, et al. Treatment of varicocele: Counseling as effective as occlusion of the vena spermatica. *Hum Reprod.* 1995;10:347–353)

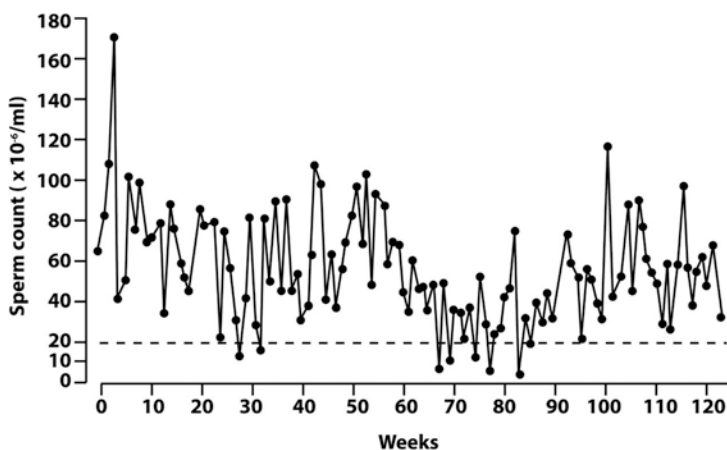


**Fig. 10.3** Variation of weekly sperm count over a 2-year period in a single, normal male volunteer

There are quite a few controlled studies that show no effect of varicocelectomy on male infertility. These papers are generally given much greater credence by infertility specialists who are not urologists [2–6, 8, 10, 13–16]. The only “controlled” studies that favor varicocelectomy were extremely flawed by obvious patient selection. Nonetheless, these flawed studies are the ones which urologists

often quote to support their enthusiasm for varicocelectomy. One study involved 455 patients undergoing varicocelectomy with only 19 controls [17]. Another study involved 1500 men with infertility who underwent varicocelectomy and only 47 controls who did not [18]. Finally, the third controlled “study” involved 238 couples who were separated from the original WHO study of more than 7000 couples. Of these 238 couples selected from the original 7000, only 45 were actually studied and the remaining 193 were dismissed from the study for a variety of reasons. The other 7000 or more WHO study participants discontinued participation in the study because of protocol deviations [2, 3, 19]. Thus, the evidence in favor of varicocelectomy for male-factor infertility is very thin. Even the claim that semen parameters are improved by varicocelectomy is much weakened by the failure of most papers to consider the variability of semen analysis in infertile men and its regression toward the mean [12, 20–22].

Let me explain “regression toward the mean” because it confuses so many studies in male infertility (Fig. 10.3). Whenever there is extreme mathematical instability of a variable (such as is the case with sperm count), if you start out with a low-end value, anything you do will raise it closer to the mean. If you start out with a high-end value, anything you do will lower it to the mean. So look at the WHO graph of variation in sperm count of one student (Fig. 10.3). If you give anything to that student when the count is low, it will appear to improve with the next count. But if you give anything to him when the count is high, it will go down and it will appear that you have hurt him.

A meta-analysis of all the published controlled trials of various treatments of male infertility fails to support any conventional treatment for male infertility with the exception of the rare cases of Kallman syndrome and hypopituitarism [9]. The few properly controlled studies of various treatments for male infertility (including Clomid, gonadotropin, and varicocelectomy) failed to provide any solid evidence-based support [1–4, 12, 20–23]. It is easy to be deluded into thinking that whatever treatment we apply to the male, including vitamin C, erythromycin, or Proxeed, is actually having an impact because of the relatively high pregnancy rate in a control group undergoing no treatment at all. Therefore, it is easy to incorrectly think that our treatment was effective just because a pregnancy occurs. It is also easy to be deluded into thinking the sperm count has increased, because careful longitudinal studies of semen analysis in untreated patients often appear to increase because of this phenomenon known as “regression toward the mean” [22]. Whenever one measures a test result that is extremely variable, such as semen analysis, with the same patient performed at different times, the purely mathematic phenomenon of regression toward the mean will make it appear that a patient who initially consulted because of a low sperm count will appear over the course of time to have an improvement without any treatment at all. This phenomenon was recognized as early as the original study of MacLeod and Gold [24] and was mathematically elucidated with carefully controlled longitudinal trials by Baker in 1985, which serve as a model for evaluating ineffective treatments for male infertility that are mistakenly advocated with misguided enthusiasm [12, 20–22, 25–29].

## 10.2 Intracytoplasmic Sperm Injection (ICSI) for Male Infertility

In the face of previously miserable results in both the diagnosis and treatment for various causes of male infertility, the development of ICSI has been a *deus ex machina* (Fig. 10.4). Any type of male-factor infertility can be treated simply and effectively by type in full ICSI [7, 30, 31]. The most severe cases of oligoasthenoatozoospermia have resulted in the same pregnancy rates with ICSI as with normal sperm from those cases of couples undergoing conventional IVF [32, 33]. Neither severe morphologic defects nor the tiniest number of spermatozoa (even “pseudo-azoospermia”) had any negative effect on the pregnancy rate with ICSI (Table 10.1). It appeared that all of the characteristics of the sperm, whether low numbers, poor motility, or abnormal morphology and deficient zona-induced acrosome reaction, had no impact on the fertilizing capacity of the sperm or the delivery of healthy offspring. Furthermore, the source of spermatozoa and the cause of the sperm defect appear to have no significant effect on the success of the procedure, whether the spermatozoa was from the epididymis, fresh or frozen, testicular, ejaculated, or from the testicles of men with severe defects in spermatogenesis [32–34].

The only exception was absolute immotility of ejaculated sperm, which is extremely rare. In a man who has the appearance of absolutely no motility in any of the sperm, in most instances, a careful search will find occasional weakly twitching sperm, and again the success rate with such sperm is no different than in men with normal semen parameters undergoing IVF. In fact, it is not the immotility of the sperm that has any negative effect on the results, but rather the nonviability. Completely nonmotile sperm, which are viable, are still capable of normal fertilization and pregnancy rates. There is also the rare case of 100% round-headed sperm, i.e., sperm with no acrosome. They also will result in normal babies using ICSI, but the oocyte must be artificially activated with calcium ionophore.

## 10.3 How Is ICSI Done and IUI (Intrauterine Insemination)

### 10.3.1 Details of the ICSI Technique (Figs. 10.4a–d, 10.5a–c)

1. Lower the injection pipette into the edge of a PVP droplet, and gently aspirate a small amount of PVP, without sperm, into the injection pipette.
2. Then use the micromanipulator to maneuver the ICSI needle to capture a motile sperm.
3. Gently aspirate the sperm and move the pipette over the upper middle portion of the sperm tail, and crush the sperm tail. Repeat this until the sperm is immobile. Then aspirate the sperm into the tip of pipette, tail first.
4. Move the microscope stage to visualize the droplet containing the mature (M-II) oocyte to be injected.

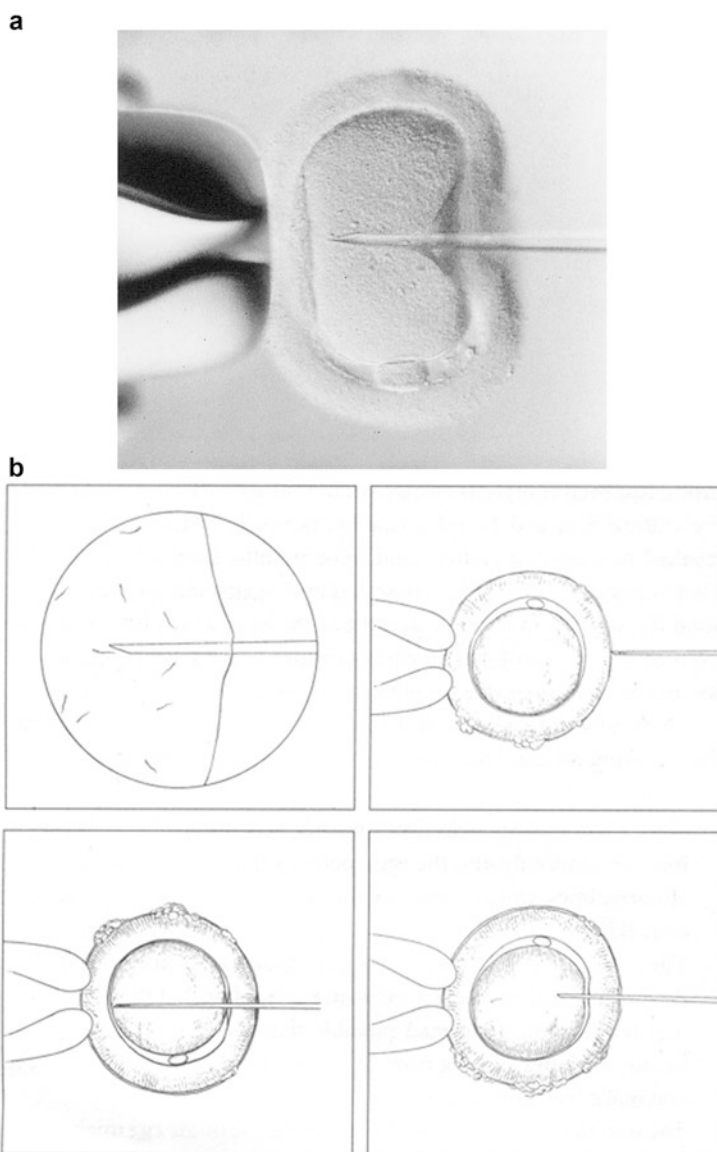


**Fig. 10.4** Intracytoplasmic sperm injection has completely revolutionized treatment of male infertility. It was introduced at approximately the same time that there was a greater awareness among reproductive clinicians that none of the popular conventional treatments for male infertility were consistent with evidence-based control studies. (From: Silber SJ. Intracytoplasmic sperm injection (ICSI) today: A personal review. *Hum Reprod.* 1998,13:208–218). (a–d) Diagram of ICSI procedure: picking up a weak infertile sperm using a micropipette, preparing to inject the sperm into the egg

**Table 10.1** Results of intracytoplasmic sperm injection using ejaculated spermatozoa categorized according to sperm quality [33]

|                            | No. of cycles | 2PN (%) | Transfer (%) | Clinical pregnancies (%) |
|----------------------------|---------------|---------|--------------|--------------------------|
| <i>Sperm count (total)</i> |               |         |              |                          |
| “0”                        | 57            | 58      | 86           | 25                       |
| >0–1 × 10 <sup>6</sup>     | 97            | 64      | 96           | 26                       |
| >1–5 × 10 <sup>6</sup>     | 128           | 70      | 96           | 22                       |
| >5 × 10 <sup>6</sup>       | 684           | 71      | 93           | 30                       |
| <i>Motility (%)</i>        |               |         |              |                          |
| 0*                         | 12            | 10      | 42           | 0                        |
| 0                          | 54            | 69      | 87           | 13                       |
| >0–5                       | 19            | 68      | 100          | 32                       |
| >5–50                      | 479           | 70      | 88           | 31                       |
| >50                        | 337           | 74      | 95           | 26                       |
| <i>Morphology</i>          |               |         |              |                          |
| 0                          | 48            | 68      | 88           | 31                       |
| >1–3                       | 125           | 70      | 96           | 33                       |
| >4–13                      | 307           | 71      | 94           | 26                       |
| >14                        | 203           | 75      | 95           | 29                       |

\*No significant difference CNS1 except for 0% motility

- Lower the holding pipette perpendicular to the zona pellucida of the oocyte and apply gentle suction to stabilize the oocyte.
- Move the injection pipette so the tip of the pipette is at 3 o'clock to the oocyte. Use the injection pipette to manipulate the oocyte so the polar body is at right angles to the bevel of the injection pipette and focus on the oocyte membrane.
- Adjust the microinjection pipette so it is in focus with the oocyte, move sperm to the tip of the pipette, and gently push the needle through the zona pellucida and then through the oocyte membrane.
- Aspirate a small amount of oocyte cytoplasm into the ICSI pipette in order to break the membrane and then expel both the cytoplasm and sperm back into the center of the oocyte.
- Withdraw the injection pipette, release the suction to the oocyte, raise both injection holders, and repeat all this until all M-II oocytes in the dish have been injected.

### 10.3.2 Pitfalls of Sperm Injection (ICSI) (Fig. 10.5b, c)

#### 10.3.2.1 Avoiding Damage to the Egg Nucleus and Spindle

If the injection pipette were to damage the egg's nucleus, i.e., the spindle, the egg would degenerate, no differently than if a sword were placed through an animal's heart. Positioning the egg in such a way that the nucleus is at a 12 o'clock or 6 o'clock position ensures that the penetrating needle can't do any damage as it goes

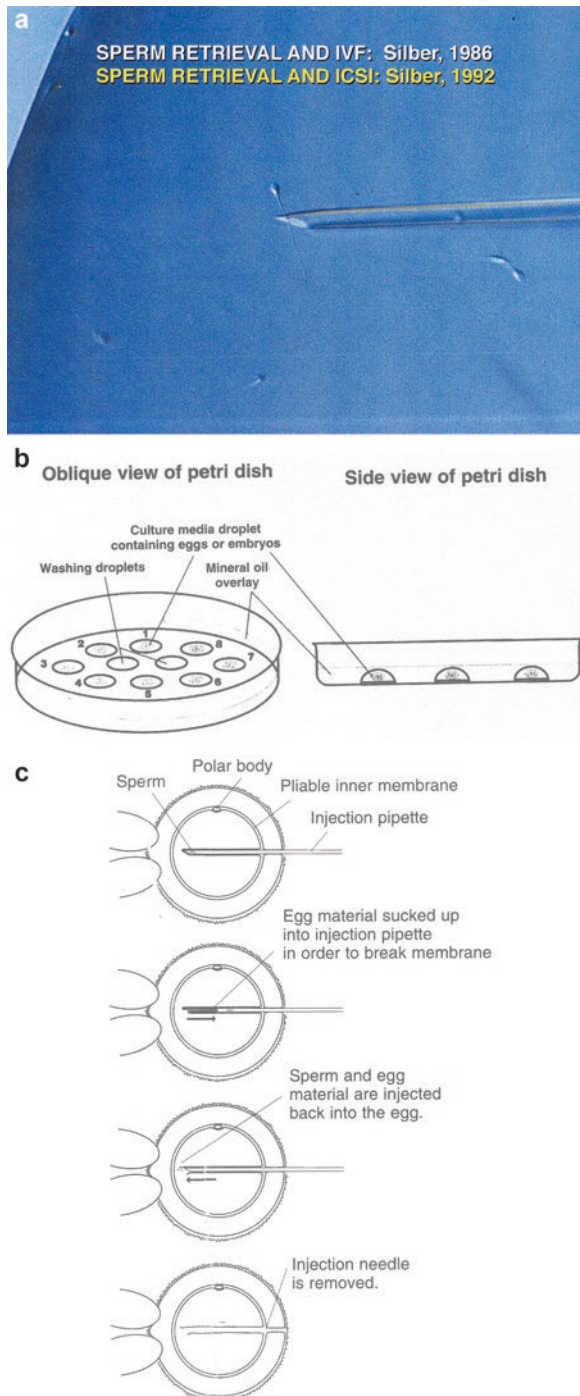


**Fig. 10.5** (a) Sperm retrieval and ICSI. (b) Diagram of petri dish setup for ICSI. (c) Diagram of the steps of ICSI. (d) The ovary is visualized with transvaginal ultrasound probe. (e) The oocyte is then aspirated through a needle attached to the ultrasound guide. (f) With the most simple sperm wash technique,

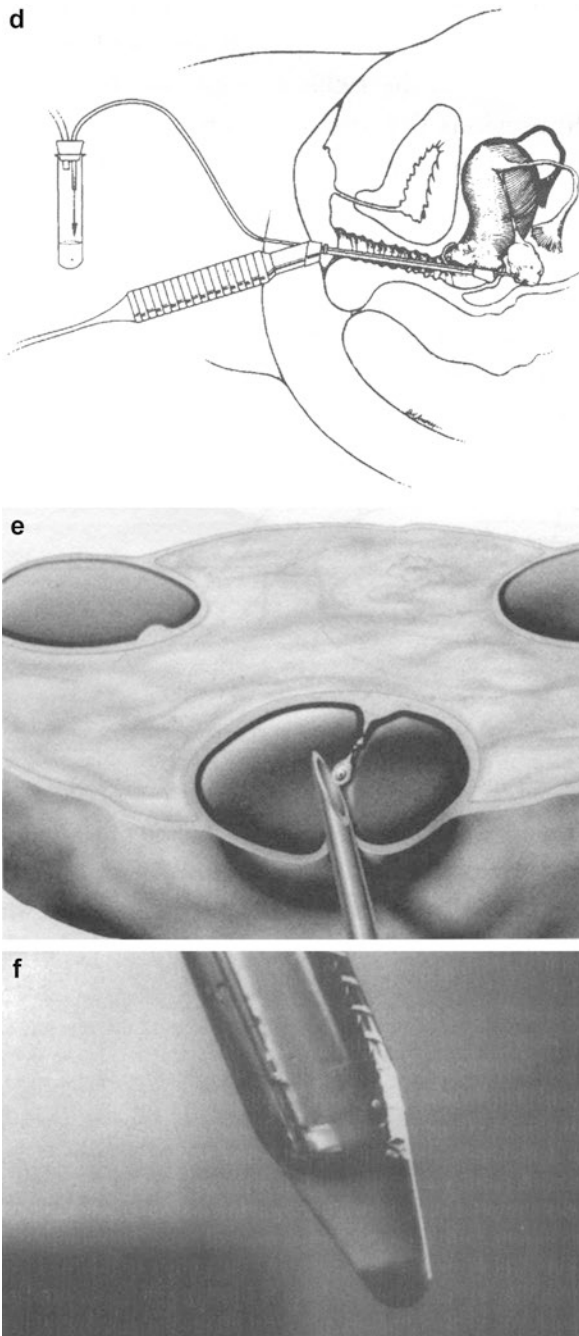


**Fig. 10.5** (continued) the semen is just mixed with media and centrifuged. Then the pellet is resuspended in fresh media. (g) Active sperm can then just be allowed to “swim up” from the centrifuged pellet into fresh media. (h) With the “density gradient” method, semen is layered on top of a gradient of various concentrations of Percoll, and after being centrifuged, only the best sperm reach the bottom. (i) Normal development to blastocyst stage 5–6 days after ICSI

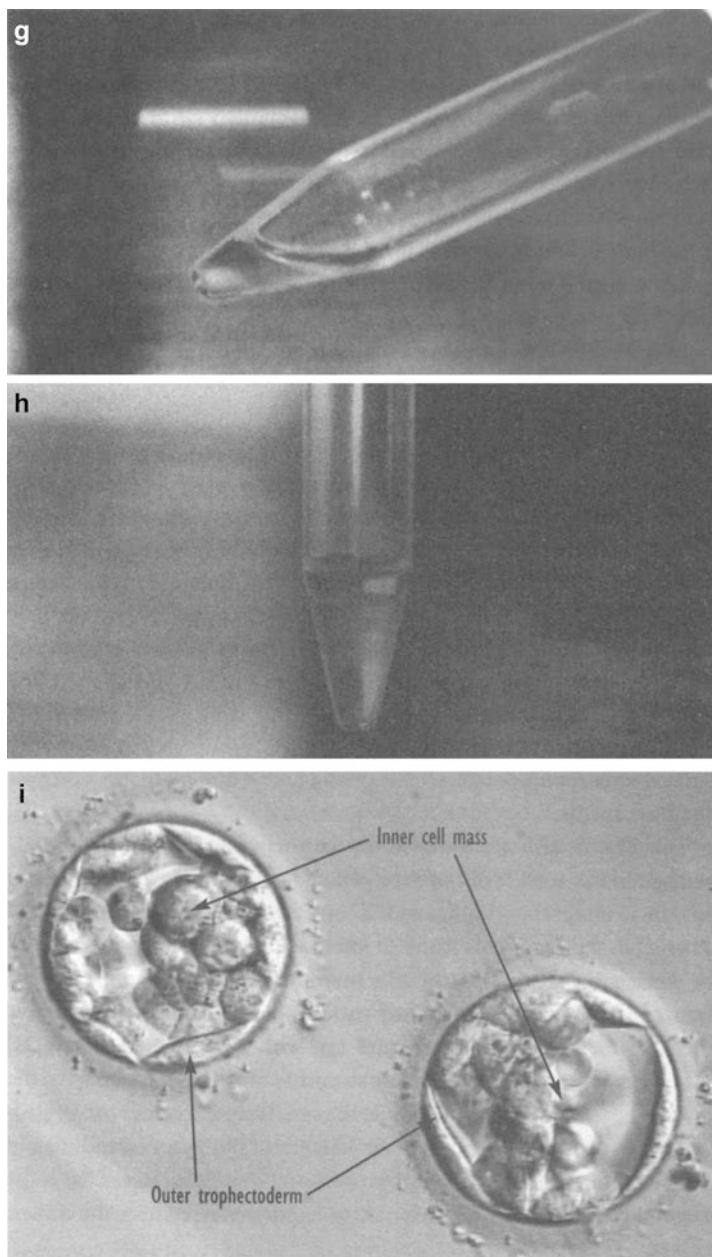


**Fig. 10.5** (continued)

through. However, it is almost impossible to see the nucleus in a mature egg that is ready for fertilization using Hoffman optics. That is why the polar body is placed at the 12 o'clock or 6 o'clock position, so that the egg's invisible nucleus (which is usually right next to the polar body but just inside the egg membrane) will usually be free from harm.

### **10.3.2.2 The Membrane Is Invaginated by the Injection Pipette but Not Broken**

The egg membrane is so incredibly pliable that once the tough zona pellucida is entered, the continuing insertion of the pipette does not break this membrane easily. Because the egg membrane is so elastic, one might think that the sperm is being injected into the egg substance, when in truth it is still outside the egg's membrane. Several minutes later, after the injection pipette is removed and the invaginated egg membrane comes back to its normal position, you can then easily see the sperm sitting outside the egg in the subzonal space without having actually penetrated into the cytoplasm. Thus, in order to achieve fertilization by direct injection into the egg, some of the egg's substance (cytoplasm) actually has to be sucked into the injection pipette until one can see the membrane pop, indicating it is broken. This is all performed under 400 times magnification. Only then can the sperm be injected into the egg. Without this delicate maneuver, the egg will not be fertilized.

### **10.3.2.3 Catching the Sperm**

Probably the most difficult aspect of the ICSI procedure is catching the sperm in the pipette. If you ever went fishing in a trout stream and saw some trout or salmon hovering around a pool, and if you were tempted to try to reach out and just grab these fish instead of using proper fishing techniques, you would then realize how incredibly difficult it must be to pick up a sperm. One time, while fishing in Alaska, one of my sons was extremely impressed when he saw a brown bear come down to the stream and casually pick up a salmon in its claws, eat it in one quick bite, and then go on to pick up another salmon, seemingly effortlessly. My son decided to try to dispense with all the complicated and expensive fishing gear necessary to trick a fish into biting the hook and simply reached down to grab the fish that was right in front of him. After hours of frustration, he looked up at me, looked over at the bears, and said, "Boy, they're really good."

In order to make it easier to catch the sperm, we first should slow them down by putting them in a very viscous solution of polyvinylpyrrolidone (PVP). This is a thick liquid plastic that was used for years as a plasma expander for patients who had lost blood. Even the most infertile, miserable sperm move at a speed that makes picking them up with a micropipette and micromanipulator difficult. Sometimes, however, the sperm are so weak that they don't require PVP. Even so, you still need PVP because otherwise the fluid moves so quickly in the tiny pipette that delicate control

of the rate of injection (so as to avoid damage to the egg) is difficult. Thus, there are two reasons to put the sperm in PVP: to slow them down so they are easier to catch and to have a thick fluid that gives better control of the rate of injection into the egg.

#### **10.3.2.4 Problem of the Sperm Swimming Around Inside the Egg Substance**

With conventional *in vivo* fertilization, the moment that the sperm fuses with the egg membrane, the tail is immediately immobilized so that the sperm can no longer move. However, when a sperm is injected via ICSI into the substance of the egg, it is not immobilized. Left alone, it would swim around quite happily in the egg and thus destroy it. Therefore, the sperm must be paralyzed before they are inserted into the egg by ICSI. This is accomplished by picking up the sperm in the PVP droplet, lining it up perpendicular to the micromanipulation pipette, and then actually crushing the tail of the sperm between the pipette and the bottom of the petri dish (see Fig. 10.5). This is really a remarkable sight. Imagine picking up a tadpole like creature by the tail, which is as thin as 1/15,000 of an inch, and then crushing that tiny tail between the pipette and the bottom of the dish, all with finely tuned micromanipulator dials. Once the tail has been crushed, the sperm cannot move. When it is injected into the egg's substance, the sperm can then properly fertilize the egg because it cannot wiggle around within the egg and damage it.

There is another reason that the sperm's tail must be broken before it is injected into the egg. In normal fertilization the egg will not begin to develop into a dividing embryo unless it is first activated by the sperm's release of enzymes. After ICSI this activation will not occur unless the sperm's membrane is broken mechanically. This allows the sperm's enzymes to make the egg's membrane permeable to calcium, and that is what starts the fertilization process.

### ***10.3.3 Preparing the Eggs and the Sperm for ICSI***

#### **10.3.3.1 Eggs**

Egg retrieval is carried out by ultrasound-guided needle puncture 36 h after the injection of HCG (as with standard IVF), and the eggs are placed in a petri dish to be prepared for the ICSI procedure (Fig. 10.5d, e). Normally, eggs are surrounded by a gooey, cumulus mass of gelatinous material, as well as a tighter layer of granulosa cells closely attached to the zona pellucida. This gelatinous mass makes it impossible to handle the egg or even to see the interior of the cytoplasm very well. These outer vestments of the egg must be cleaned off first. With standard IVF this step is not necessary because the sperm and the eggs are simply put together in a droplet in the petri dish, the sperm manages to work its way through this gooey mass on its own (like in real life), and the next day it is easily cleaned off. But with ICSI,

the cleaning has to be performed before the injection procedure on the same day the eggs are retrieved.

The eggs are cleaned using an enzyme called hyaluronidase followed by aspiration the eggs in and out of glass pipettes that are about the same size as the diameter of the egg. The eggs must be put in the hyaluronidase mixture for only half a minute, to avoid damaging them. After that initial chemical action of the hyaluronidase, the outer gooey mass and granulosa cells can then be dissected off micromechanically by sucking the eggs in and out of these pipettes many times over a course of several minutes. After the eggs are properly washed, they are placed into a tiny microdrop-let of media in a petri dish. Only eggs that are M-II, i.e., that have extruded the first polar body, can be injected with sperm.

### **10.3.3.2 Preparation of the Sperm (Which Is the Same as for IUI, Intrauterine Insemination)**

There are many methods of washing the sperm and obtaining the purest fraction of the best-quality sperm. We tried all of these different methods in our early work with ICSI. However, it is now apparent that the method of sperm preparation has no major importance. Individual laboratories' convenience and preference is all that matters in how they prepare the sperm for putting it in the petri dish for ICSI, because there is absolutely no need to depend upon sperm physiology in order to achieve a proper fertilization with ICSI.

However, for IUI, the sperm simply has to be washed so that the sperm are out of the semen. Semen is actually "toxic" both to sperm (amazingly) but also to the woman if placed into her uterus.

Spermatozoa must be separated from the semen ("sperm washing") in order to use them either for IVF, ICSI, or IUI. Semen is actually a toxic medium. It is the cervical mucous (which is alkaline) that protects sperm from the acid environment of the vagina. The spermatozoa must be able to permeate the female's cervical mucous which actually physiologically "washes" the sperm out of the semen after ejaculation. But the semen ironically is otherwise a toxic medium for the sperm and in fact would be very toxic to the female if it were to get into her uterus, and it would actually prevent fertilization even with IVF.

Sperm can be washed with any of four methods. With the classic "swim-up," media is just layered over the semen, and active sperm literally swim up out of the semen. The semen can also just be mixed in culture medium and centrifuged. Then the pellet is resuspended in fresh culture medium (Fig. 10.5f). This is the simplest approach, but does not concentrate the best-quality sperm. Also the active sperm can just be allowed to "swim up" from the pellet into fresh media (Fig. 10.5g). The semen can be layered over density gradients like Percoll and centrifuged. Only the most motile sperm can then pass through the various density gradients interphase. It is like a feather that is not twitching gets stuck, but a twitching dense body passes through (Fig. 10.5h). Finally a small aliquot of sperm can be placed at one end of an "M"-shaped tunnel, and the best swim to the other end.

However, there is one warning: The only requirement is that for the sperm's DNA to result in fertilization, the sperm must be alive. The slightest motility, even a rare, occasional, barely observable twitch, is all that is necessary to verify that the sperm is still alive. If the sperm have truly died (most commonly from "old age"), then the DNA rapidly deteriorates so that normal fertilization is then impossible, even with ICSI.

Washing sperm for IUI (intrauterine insemination) is an extremely common procedure often attempted many times before IVF or ICSI. It has a low success rate, despite being so popular (because it is so easy) and in fact may be no better than timed intercourse. The long history of IUI and its origins to me is very revealing as I was part of it. In 1983, most infertility doctors were feeling precariously unable to use what was then the "new" technique of in vitro fertilization (IVF). Doctors from the USC Medical Center in Los Angeles aroused a swarm of IVF wannabes by showing how they can enter the IVF world with IUI, have washed sperm in a simple centrifuge and then this washed sperm could be inserted through a readily available infant "feeding tube" into the uterus and achieve pregnancy rates equivalent to IVF. These boasts were frankly fraudulent. Nonetheless IUI remains part of the standard, accepted armamentarium for male infertility even though ICSI is far superior. In fact a critical backward look shows that timed intercourse is just as effective (or even more effective) as IUI. IUI survived through hype and tradition because it was thought to be better than intercourse. I doubt that.

One reason that IUI is so ineffective compared to ICSI-IVF is that after ovulation, the egg in the female is only good for about 8 h. Yet after intercourse the sperm in the female should be good for 2–3 days. So you will often get the timing wrong with IUI. With intercourse at last the sperm is stored in the cervical mucous and not immediately depleted, as it is with IUI.

### 10.3.3.3 The Pipettes Used for Injection

The glass pipettes used for ICSI are ultrafine microscopic "needles" made by placing very tiny, thin-walled glass capillary tubes into a computer-controlled microelectrode "puller." These pipettes are now readily available commercially. The glass of this tiny pipette is heated, and the two ends of the pipette are pulled apart in a carefully computerized fashion in order to give a precisely determined outer and inner diameter of microscopic dimensions. There are two main types of pipettes. One is the holding pipette, with which the egg is held by suction during the procedure. This pipette is much larger than the injection pipette. The injection pipettes are obviously much finer and very sharp. They have a diameter of about 1/5000 of an inch. After the injection pipette is "pulled," it is placed in a microgrinder whetstone, and its tip is carefully ground to a 50-degree angle. The pipette is then bent with a heated forge under a microscope at an angle of about 35°, to make it easier for it to reach over and into the petri dish. The outer diameter at the tip is about 6 or 7 mm, and the inner diameter is about 4–5 mm (to accommodate the width of the sperm head). These glass injection and holding pipettes are extremely delicate. Any

accidental touch of the tip will break them instantly and will require going to all the trouble of making another one.

#### **10.3.3.4 Picking Up the Sperm, Breaking the Tail, and Injecting It into the Egg**

Moving sperm will always eventually find their way to the periphery of the microdroplet and swim along the outer edge. One can then micromechanically bring the glass injection pipette down to the edge of the microdroplet and pick up individual sperm, one at a time, as they swim along this edge. When the sperm are extremely weak and few, we use the principle of microfluidics to separate out the few good sperm for injection. We just create an M-shaped line of culture media in the bottom of the petri dish and place the droplet of semen or washed sperm at one end. The best sperm then eventually swim out and negotiate their way around all these angles to reach the other end of the M. It is then easy to pick these best sperm up and crush the tail.

The egg is then picked up with the holding micropipette and held in place while the injection pipette is used to rotate the egg so that the polar body is at the 12 o'clock position. The injection micropipette is then manipulated to enter the egg at the three o'clock position. The pliable inner membrane does not break easily. Some of the egg material actually has to be sucked into the injection pipette in order to break the membrane. The instant that the membrane is broken, the sperm and the tiny amount of egg cytoplasm that has been sucked up in the needle are then injected back into the egg, and the injection needle is removed from the egg.

#### **10.3.3.5 Fertilization and Cleavage**

After all of the eggs in the injection dishes have had a single sperm inserted into them, they are placed into a separate microdroplet of a culture dish, which is put back into the incubator. This culture dish will be the home for the egg over the 3–6 days as it grows and develops, before it is put back into the patient. Within 4–6 h of the sperm injection, if the egg was successfully fertilized, the second polar body will extrude, indicating that the second meiotic division has been initiated by the penetration of the sperm. Within 14–20 h, we will be able to see the nucleus of the egg and the nucleus of the sperm clearly juxtaposed. This is called the two-pronuclear (2PN) egg, and it is the absolute indication that successful fertilization has taken place. The normal laboratory routine is to check each of the injected eggs early in the morning on day 1 after injection and to look for 2PN fertilization. On average, whether with regular IVF or with ICSI, 60–70% of the eggs will exhibit 2PN fertilization. This 2PN fertilization is normal, which means that there is a single set of chromosomes from the mother and a single set of chromosomes from the father. Then by day 5 or day 6, blastocyst will have developed (Fig. 10.5i).

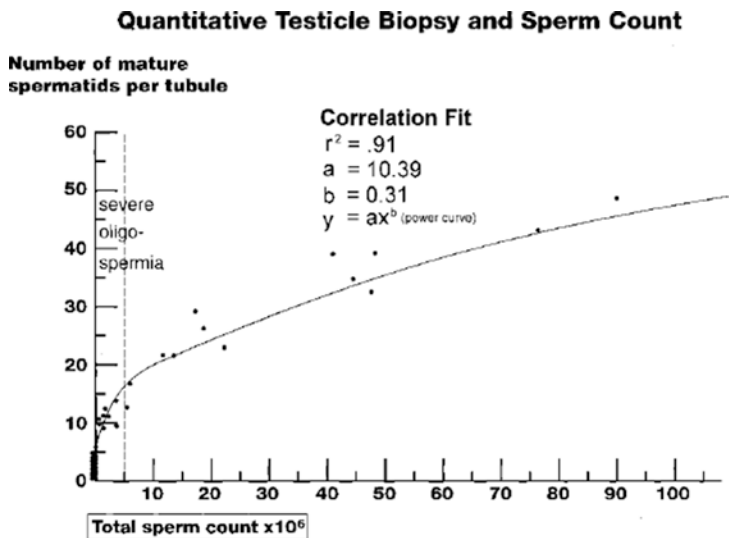


**Fig. 10.6** An exponential curve relating sperm count in the ejaculate to the average number of mature spermatids seen in each seminiferous tubule. A threshold of three to six mature spermatids per tubule had to be exceeded for sperm to appear in the ejaculate. (From: Silber SJ, Nagy Z, Devroey P, et al. Distribution of spermatogenesis in the testicles of azoospermic men: The presence or absence of spermatids in the testes of men with germinal failure. *Hum Reprod.* 1997;12:2422–2428)

It was immediately apparent that ICSI was the answer for which we were all searching for so many decades. The treatment of oligoasthenoteratospermia in the present era is simply ICSI with ejaculated sperm, and, for the most part, the diagnostic dilemmas of oligospermia have been made largely irrelevant by ICSI. It quickly became apparent however that ICSI would be equally successful in combination with sperm retrieval techniques even for the treatment of azoospermia (Fig. 10.6) [7, 32, 34–40].

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# Chapter 11

## Azoospermia



Oligospermia, as we have mentioned, is generally refractory to urologic treatment and requires ICSI. ICSI has revolutionized the management of oligoasthenospermia. However, azoospermia is an even bigger hurdle. Thus, we will spend a lot of time on this subject, since there is now good treatment for both obstructive and even nonobstructive azoospermia.

Approximately 20% of couples are infertile, and approximately 25% of all infertile couples have a low sperm count [1–3]. It is guessed that about 0.5–2% of infertile couples have azoospermia [3]. Thus, azoospermia represents approximately 2–8% of the cases of male infertility. One can therefore estimate that approximately 1 out of every 500 men or so in the population (excluding those who have had a vasectomy) has azoospermia. Approximately 25% of men who have previously undergone vasectomy (perhaps over 30 million in the USA alone) become remarried and then wish to have children again [4]. Thus, there is a huge population of infertile men around the world who have azoospermia.

We classify azoospermia as “obstructive” (OA) and “nonobstructive” (NOA). Obstructive azoospermia includes patients who have had a vasectomy, patients with congenital absence of the vas deferens, those who have had accidental surgical interruption of the vas or epididymis during a hernia or hydrocele operation, or patients who have primary epididymal blockage from previous infections. They all have normal spermatogenesis in the testes. Even before the arrival of ICSI, all of these patients, with the exception of congenital absence of the vas, were amenable to microsurgical repair [5–17]. For obstructive azoospermia, ICSI simply adds a new dimension in those who have failed with reconstructive attempts or those with congenital absence of the vas (which is not reconstructable) can now also have children [18–20]. In fact, because of ICSI, virtually any man with obstructive azoospermia can now father his own child, with the only limitation being the fertility of the wife [18]. In this section of the chapter, I will elaborate on how to go about evaluating and treating the azoospermic man with ICSI.

## 11.1 Evaluation of the Azoospermic Man

The diagnosis of obstructive versus nonobstructive azoospermia should be quite simple. However, it is sometimes approached in a confusing way that can lead to misjudgments, such as attempting to perform a vasoepididymostomy on a patient who has no obstruction. If the diagnosis is obstructive azoospermia (OA), the management is quite different than if it is nonobstructive (NOA). Adherence to a few simple principles will avoid these difficulties and allow a proper preoperative decision to be made: if a patient has a testicle biopsy that shows normal spermatogenesis and if he has azoospermia, his infertility must be caused by obstruction. Everything else is superfluous. If in addition to these two criteria he also has a palpable vas deferens on physical examination and a semen volume greater than 1 mL, he is a candidate for surgical exploration and probable vasoepididymostomy. All other data are irrelevant.

But occasionally a pathologist may make a reading of normal spermatogenesis when it really is incomplete maturation arrest because he did not make a quantitative reading.

A normal follicle-stimulating hormone (FSH) level does not necessarily indicate normal spermatogenesis or obstruction. In fact, more commonly it indicates maturation arrest and nonobstructive azoospermia. The serum FSH level correlates most closely with the total number of spermatogonia and less well with the number of mature spermatids or the sperm count [21–23]. The most common diagnosis for patients with azoospermia and a normal serum FSH level is maturation arrest, not obstruction. Follicle-stimulating hormone level is in the normal range because the total number of spermatogonia and spermatocytes in these cases is normal. It is true that an increased FSH level usually means inadequate spermatogenesis associated with Sertoli cell only, but even this axiom is not always true. Thus, endocrine evaluations are only modestly helpful in the diagnosis of obstruction. You do not want to operate on a man expecting to do a vasoepididymostomy when the diagnosis of obstruction is incorrect. One useful clue is that the FSH must be in the very low range of normal to indicate normal spermatogenesis. In the moderately normal range, FSH is just as likely to mean maturation arrest and nonobstructive azoospermia.

A vasogram should be performed only as part of the operative procedure for correcting obstruction. It should not be used to make a diagnosis or to determine the need for surgery. Performing a vasogram as an isolated diagnostic procedure creates many problems. First, a scrotal exploration is not needed to ascertain that the vas is present; that should be easily discernible by physical examination or scan. Second, unless performed as part of a careful microsurgical procedure, any injection or transection of the vas in performing a vasogram could result in obstruction where originally there was none. Third, the vasogram data are not necessary for preoperative planning. Most importantly, the test indicates nothing about the epididymis and can lead to a false-positive diagnosis of obstruction as well as a false-negative diagnosis of no obstruction. If a diagnosis of obstruction is certain, based on testicle biopsy

and sperm count, the most logical time to perform a vasogram is at the time of vaso-epididymostomy, once the vas is transected, to make sure that the vas empties distally into the ejaculatory duct and prostatic urethra. It is not necessary to know this information ahead of time. I find that scrotal ultrasound is silly and yields no more information than a physical exam.

Physical examination of the epididymis and testes, as well as a history or lack of history of infection, can be misleading as well. Testicles that produce a normal amount of sperm may be relatively small, and those that produce no sperm (that have maturation arrest) may often be large. Historic data can be similarly confusing. At least 50% of our patients who were found to have epididymal obstruction from inflammatory causes had no previous history of clinical epididymitis. We must assume that whatever infection caused their epididymal obstruction must have been subclinical.

In conclusion, most of the ancillary medical information that we routinely consider in male fertility evaluation is irrelevant to the question of whether the patient has obstruction. The physical examination is only relevant in that if a vas deferens is not palpable (i.e., congenital absence of the vas), then no surgical anastomosis can be planned. With that exception, the history and physical examination, serum FSH, luteinizing hormone, testosterone levels, and vasography are irrelevant to the diagnosis.

## 11.2 Diagnostic Testicle Biopsy

The open technique for diagnostic testicle biopsy, which we recommend, is very simple, should cause no pain, and should be a quick outpatient procedure using local anesthesia only with essentially no pain afterward, because of a special local anesthetic combination I will describe. The spermatic cord is injected with approximately 10 mL local anesthetic via a 25-gauge needle just distal to the external inguinal ring. Then an additional 10 mL of local anesthetic is injected over the anterior scrotal skin in the area where a 1.5 cm incision is made down to the tunica vaginalis, which is then opened, exposing the tunica albuginea. With this method, a small “window” is created through which the testis can be visualized or even extruded painlessly.

For local anesthesia, I now recommend a very remarkable long-acting mixture of ropivacaine 30 cc, Toradol 30 mg, and morphine 10 mg. This will provide almost 3 days of anesthesia to the local operative area. This anesthetic combination is amazing. It really makes most surgery painless post-op. It is based on the finding that morphine has local receptors in most tissues that are in fact more profound than its effect on the brain. The same is true of anti-inflammatory analgesics. Indeed they bind to these local receptors and give prolonged anesthesia.

Small pieces of testicular tissue are excised from the peripheral millimeter of testis surface and placed in Zenker's (or Bouin's) fixative with an atraumatic “no touch” technique comprising every anatomic lobule peripherally. This is a

thoroughly painless clinical procedure (except for the initial injection of local anesthetic). In the testis periphery, we will sample every lobule and do no harm. The patient is able to get up and walk away immediately afterward with no more pain than if he had had a vasectomy performed. There is absolutely no need for a general anesthetic.

Needle biopsy is another alternative, but it is no less painful than the open biopsy as just described, and the open biopsy always yields a sufficient number of seminiferous tubules (at least 20 cross sections) to perform an adequate quantitative analysis. Needle biopsy cannot yield this amount unless performed multiple times, which is ironically much more invasive and dangerous than the open biopsy technique. The open biopsy only looks for peripheral tissue, and if you remember the looped anatomy described earlier in this chapter, you cannot cause obstruction or vascular damage. However, multiple needle biopsies into the center of the testis can cause great damage. The biopsy must be of adequate quality to determine: (1) does the patient have normal spermatogenesis and, therefore, obstruction that may be amenable to microsurgical repair or (2) if he has nonobstructive azoospermia, will TESE provide a good or poor prognosis?

Many testis biopsies are fixed incorrectly in formalin or so traumatized by handling as to create artifacts and absurd readings like “sloughing and disorganization” [23–26]. Testicle biopsy has been used by most clinicians in a non-quantitative manner only, which has severely limited its usefulness and has led to many errors in interpretation [27–30]. A simplified quantitative evaluation of the testicle biopsy is based on the normal histology and kinetics of spermatogenesis in the human [31]. The rate, or speed, of spermatogenesis in humans, or in any species, is constant for any variety of sperm counts, high or low. Reduced sperm production is always caused by lower numbers of sperm and not by a diminished rate of sperm production. Therefore, the daily quantity of sperm being produced for the ejaculate by the testicle is reflected quite accurately by the testicle biopsy. Thus, testicle biopsies of patients with both oligospermia and normal sperm counts have been found to be predictive of mean sperm count in the ejaculate. Patients with severe oligospermia after a strictured vasovasostomy have normal spermatogenesis. A testicle biopsy should clarify whether blockage or just poor spermatogenesis is causing the poor semen quality and indeed for azoospermic cases, whether there is any mature sperm production at all (Fig. 11.1).

The testicle biopsy is performed bilaterally, and at least 20 seminiferous tubules are included in the count on each side. The mature spermatids (the oval cells with dark, densely stained chromatin) and large pachytene spermatocytes are the easiest to count. Previous studies have shown that these cells have the greatest correlation with sperm count and are the easiest ones to recognize. All of the steps of spermatogenesis from spermatogonia through leptotene, zygotene, pachytene spermatocytes, and early spermatids are observed, of course, but what is most important clinically is the number of mature spermatids in a minimum of 20 tubules, divided by the number of tubules (Fig. 11.2, b).

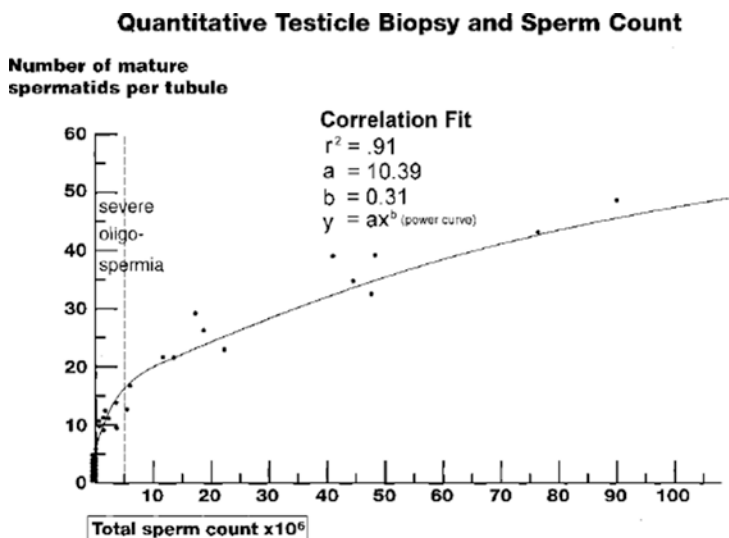


**Fig. 11.1** An exponential curve relating sperm count in the ejaculate to the average number of mature spermatids seen in each seminiferous tubule. A threshold of three to six mature spermatids per tubule had to be exceeded for sperm to appear in the ejaculate. (From: Silber SJ, Nagy Z, Devroey P, et al. Distribution of spermatogenesis in the testicles of azoospermic men: The presence or absence of spermatids in the testes of men with germinal failure. *Hum Reprod.* 1997;12:2422–2428)

Using an exponential curve (Fig. 11.1), the number of mature spermatids per tubule can be used to predict the anticipated sperm count. In the absence of obstruction, the correlation is remarkably close. For example, if the patient has 40 mature spermatids per tubule, the sperm count should be just less than 60 million/mL; if there are 45 mature spermatids per tubule, the sperm count should be more than 80 million. The patient with a sperm count of 3 million would be expected to have only 6 to 10 mature spermatids per tubule. This portion of the chapter is emphasizing the use of testis biopsy to diagnose obstruction with normal spermatogenesis. Later, we will describe in detail the histology of a testis with nonobstructive azoospermia.

Frequently, patients undergo an attempt at vasoepididymostomy inappropriately, because the pathology report incorrectly indicates “normal spermatogenesis.” Such readings often are not quantitative, but rather a qualitative impression of the pathologist that tubules are filled with spermatocytes and some mature sperm. If the biopsy shows thick tubules with large numbers of spermatocytes but only two or three mature spermatids per tubule, obstruction is not the cause of the patient’s “azoospermia.” Such patients require TESE with ICSI for nonobstructive azoospermia caused by maturation arrest (Fig. 11.2c–e). Some clinicians have attempted to use the serum FSH level to monitor the amount of spermatogenesis: a normal FSH level in a patient with azoospermia would supposedly indicate obstruction. Unfortunately, this correlation is poor [22]. Patients with maturation arrest causing azoospermia

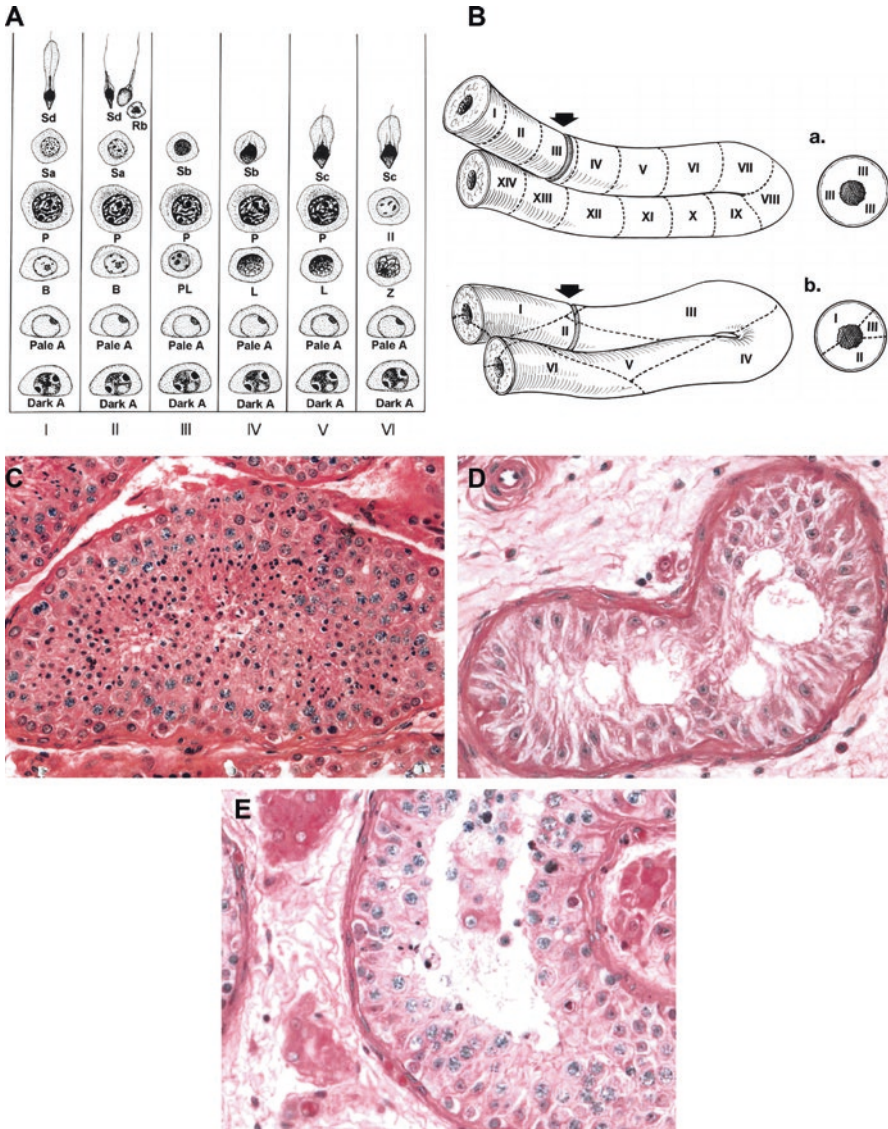


**Fig. 11.2** (a) The stages of spermatogenesis in the testicle (from Silber SJ. *Reproductive Infertility Microsurgery in the Male and Female*. Baltimore, MD: Williams & Wilkins and Waverly Press Inc., 1984). Drawings of the progression of stages of spermatogenesis in the rat seminiferous tubule (A) and in the human seminiferous tubule. (b) In most animals, there is a wave of spermatogenesis going in an orderly manner down the seminiferous tubule. In the human, however, there is a mosaic arrangement of the six stages of spermatogenesis (from: Silber SJ. *Reproductive Infertility Microsurgery in the Male and Female*. Baltimore, MD: Williams & Wilkins and Waverly Press Inc., 1984). (c) Normal spermatogenesis means there are many oval plank staining sperm heads. (d) Sertoli cell only is associated with a high FSH does not always mean azoospermia. (e) Maturation arrest is associated with a normal FSH because there are so many spermatogenic cells, but there are few to no sperm

usually have a normal FSH level. The FSH level correlates most closely with the total number of spermatogonia and with the testicular volume but not with the number of mature sperm (Fig. 11.2c–e).

Ironically, it is the scattered mosaic arrangement of the various stages of spermatogenesis in the human seminiferous tubule (as opposed to the orderly wave moving across the tubule in most other species) that makes quantifying the human testicular biopsy so simple. In rats or mice, a cut through any particular seminiferous tubule shows only one particular stage (Fig. 11.2). In humans, a cut through any area of the testicle reveals a scattered array of all the various stages of spermatogenesis (Fig. 11.9b). Thus, in humans, unlike most other animals, it requires a very small number of only 20 seminiferous tubules for a good statistical sample of the total range of spermatogenesis in the entire testicle.

### 11.3 Microsurgical Vasovasostomy

The majority of patients who have had their vasectomy nowadays are found to have secondary pressure-induced epididymal blockage (whether caused by “blowouts” or by inspissation) in addition to blockage of the vas. This results in absence of sperm in the vas fluid, which correlates with a zero success rate no matter how good the vasovasostomy technique [10–13, 16, 32]. This problem of secondary epididymal obstruction has become much more common (at a much earlier date after vasectomy than previously because of tighter seal at the vasectomy site) since the adoption of “better” techniques for vasectomy that result in no occult leakage of sperm at the vasectomy site [4, 14, 33]. When microsurgery with vasovasostomy was first popularized in the mid-1970s, sperm was present in the vas fluid in the majority of patients, and vasovasostomy (if performed microscurgically and accurately) resulted in remarkably high success rates (Figs. 11.3, 11.4, 11.5, and 11.6) [10–14]. However, in the late 1980s and 1990s, the “improved” vasectomy techniques being used by most urologists

**Fig. 11.3** Microsurgical two-layer anastomosis of vas deferens



**Fig. 11.4** Sample the vas fluid microscopically

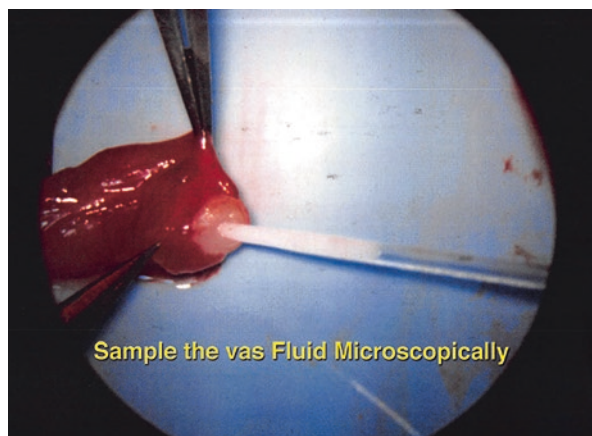


**Fig. 11.5** Normal long-tailed sperm in vas fluid indicates no secondary epididymal blockage

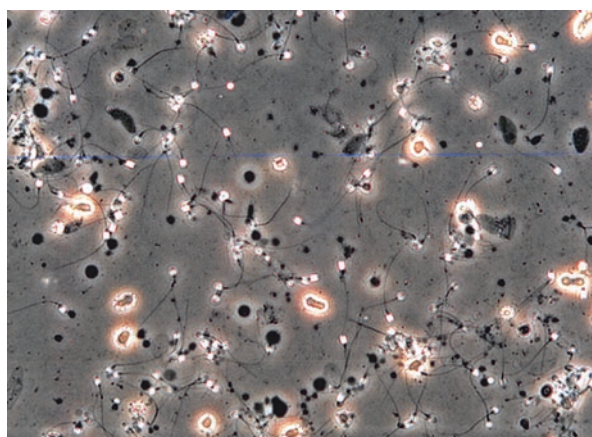
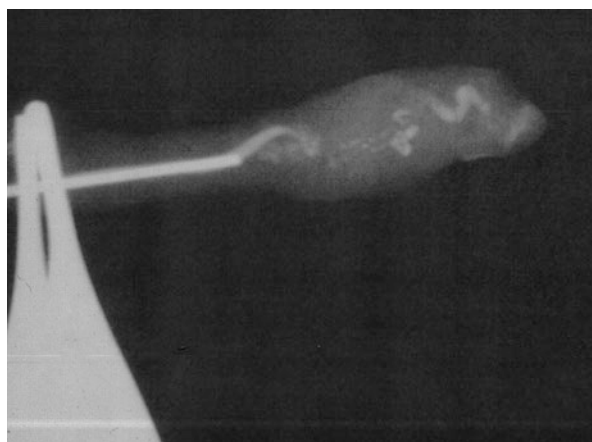


**Fig. 11.6** Microsurgical correction of partial obstruction of a non-microsurgical attempt at vasovasostomy



allowed no occult leakage of vas fluid and a lower incidence of sperm granuloma at the vasectomy site. Ironically, this has created a much earlier occurrence of secondary epididymal obstruction from pressure buildup proximal to the vasectomy site.

In fact, today, 85% of patients who have had even a recent vasectomy will have epididymal blowouts and have zero success with vasovasostomy. Thus, in the modern era, without addressing the problem of secondary epididymal blockage when performing a vasectomy reversal, the results with microsurgical anastomosis of the vas are likely to be very poor. However, with specific tubule microanastomosis of the vas to the epididymis, first described by me in 1978, this problem of secondary epididymal blockage can be circumvented [9, 16] (Fig. 11.7a, b). In fact, the only way vasectomy reversal can be recommended in preference to sperm retrieval and ICSI today is if a high success rate can be achieved. Although vasovasostomy is very successful if normal sperm are found in the vas fluid, it cannot succeed if there is no sperm in the vas fluid [5, 6, 9, 15, 16] (Figs. 11.3 and 11.8). That would require vasoepididymostomy. Unless the urologist is well trained in microsurgery of the epididymis, he is better off not attempting any reconstructive procedure, not even vasovasostomy, because of the high likelihood that there will be epididymal damage indicated by the finding of no sperm in the vas fluid.

Thus, there are four major considerations for vasectomy reversal: (1) techniques for obtaining a reliable reanastomosis of the vas deferens (with modern microsurgical techniques, accurate reanastomosis should be achievable in almost every instance), (2) the detrimental secondary effects of vasectomy (pressure-induced epididymal damage), (3) microsurgical bypass of this secondary epididymal obstruction, and (4) freezing of epididymal sperm for later use with ICSI as a backup if the reversal operation should fail [34].

## 11.4 Technique of Vasovasostomy

A microscope is necessary for the accuracy of the operation. Loupes can, at best, provide  $\times 2.5$  to  $\times 4$  magnification [10, 12, 13, 35–43]. Visualization of the inner lumen of the vas deferens for easy and accurate placement of stitches requires  $\times 16$  magnification. Other advantages of a microscope are that the depth of focus is clear, the light is constantly supplied directly to the patient, and the instrument rests on a stand and is immobile. The operators can move their heads or necks from time to time without disturbing the steadiness of the view of the subject. The microsurgical technique of vasovasostomy in two layers is crucial for a nonstrictured anastomosis and is depicted in the figure from our original publication [7, 8, 10] (Fig. 11.8).

To make a perfect two-layer vasovasostomy, use 10-0 nylon interrupted sutures for the inner layer (4–6) and 9-0 nylon interrupted for the outer layer. The main job of the assistant is to continually irrigate with pulsatile heparinized saline. The surgeon can then easily see the field despite tiny bleeders, and he can stop them with micro-bipolar forceps. Success also depends on freeing up the vas deferens sufficiently so that there is no tugging or tension on the anastomosis. As long as there is no tension and the mucosal layer is perfectly approximated, success should be about 98%.

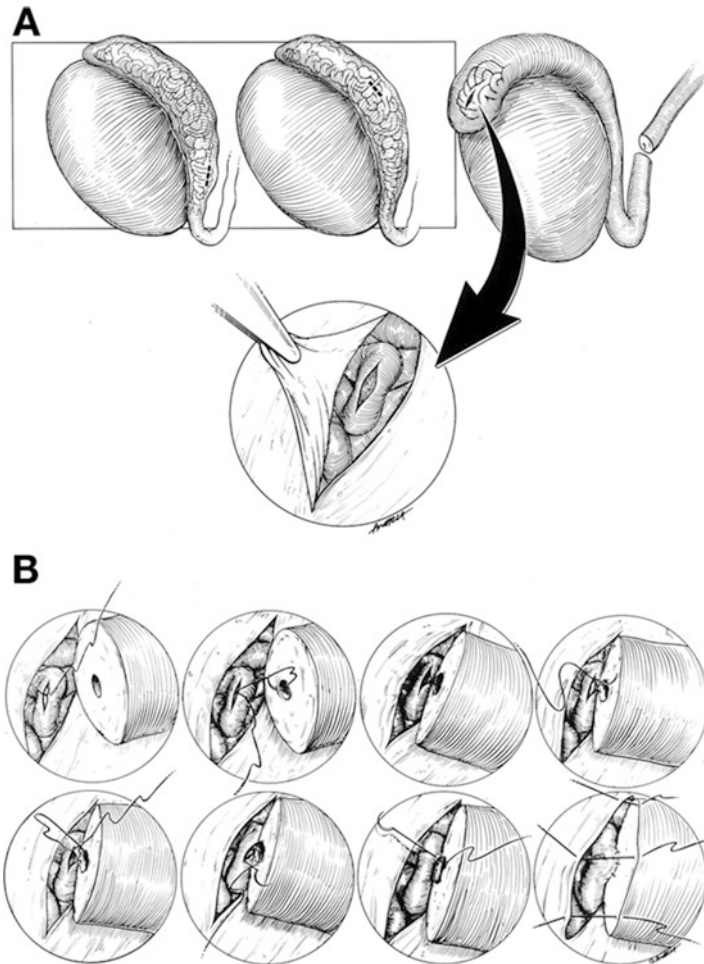


**Fig. 11.7** (a) The microsurgical-specific tubule vasoepididymostomy technique requires first transection of the vas and then locating of the distal-most site that is proximal to the area of blockage in the epididymis, freeing the tubule, and making a tiny longitudinal slit for aspirating sperm and subsequent anastomosis. (b) A depiction of the eight stages of the microsurgical-specific tubule anastomosis of the vas to the epididymis, bypassing epididymal blockage

## 11.5 Results of Vasovasostomy

The sperm count and quality should tend to improve gradually with time so long as there is no secondary epididymal pressure-induced blockage. If the anastomosis is strictured, however, the count may increase briefly but then eventually reduce to oligospermia or azoospermia. If the patient is still azoospermic 3 months or more after vasovasostomy, then either the vas anastomosis or the epididymis is obstructed.

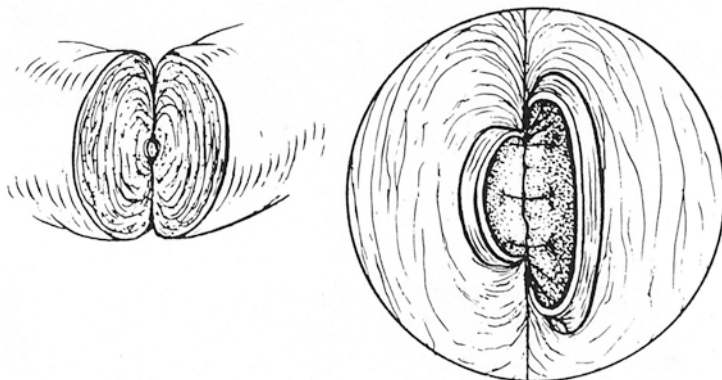


**Fig. 11.8** An original depiction of the two-layer microscopic vasovasostomy, using interrupted 10-0 nylon mucosal sutures to anastomose the undilated abdominal side lumen of the vas to the dilated testicular side lumen of the vas with no leakage and no stricturing (from: Silber SJ. Microscopic technique for reversal of vasectomy. *Surg Gynecol Obstet.* 1976;143:630–631)

More than 98% of patients with sperm in the vas fluid at the time of vasovasostomy have sperm in the ejaculate postoperatively [5]. Eighty-two percent of their wives conceived spontaneously, and this is dependent only on the fertility of the wife. Most of the confusion in the literature about vasovasostomy stems from the lack of documentation of preoperative sperm quality in the vas fluid, sparse observations of the epididymal ductal system, poor testis biopsy studies among men who have undergone vasectomy, and the failure to bypass secondary epididymal blockage.

The group on whom we operated participated in such a careful study. Seminal fluid was sampled from the testicular side of the obstructed vas from each patient at the time of reanastomosis. The age of the patient, and his wife, time since vasectomy, type of vasectomy, and area in which it was performed were correlated to subsequent sperm count and pregnancy of the partner. Appearance and quantity of the vas fluid as well as sperm morphology (electron and light microscopy), quantity, and motility were recorded and correlated with postoperative results. The distribution of sperm counts among patients with a patent vas postoperatively does not bear a statistically significant difference from that among control populations of fertile men [3–14]. The sperm count postoperatively was closely correlated with the results of quantitative testis biopsy.

The woman's age was the critical determinant of pregnancy rate (Table 11.1). These data led us to conclude that spermatogenesis is not substantially harmed by obstruction and that failure to achieve fertility after an accurate vasovasostomy is caused by dilatation and then perforation of the epididymal duct (or inspissation) with subsequent secondary epididymal obstruction (Fig. 11.9). We reported subsequently detailed results on 4000 men undergoing vasectomy reversal in the *Journal of Andrology* with similar results in this large following series. But 85% of them required vasoepididymostomy [44–46].

**Table 11.1** Ability to establish a pregnancy as affected by age of female partner (Silber and Grotjan 2004)

| Wife's age               | Number of patients | Patients establishing a pregnancy | %    |
|--------------------------|--------------------|-----------------------------------|------|
| <30                      | 770                | 725                               | 94.2 |
| 30–35                    | 610                | 554                               | 90.8 |
| 36–40                    | 278                | 228                               | 82.0 |
| ≥40                      | 72                 | 44                                | 61.1 |
| All ages                 | 1730               | 1551                              | 89.7 |
| Probability $P < 0.0001$ |                    |                                   |      |

Patients with no viable intact sperm in the vas fluid rarely had successful vasovasostomy, as measured by sperm in the ejaculate. All men with sperm granulomas had abundant, morphologically normal sperm in the vas fluid. Even when the vasectomy had been performed more than 10 years previously, none of these men had poor quality sperm. The presence of a sperm granuloma at the vasectomy site represents persistent and continual leakage of sperm, which alleviates the deleteriously high intravasal and epididymal pressure that otherwise always occurs after vasectomy. The high pregnancy rate with vasovasostomy was only achieved in couples whose husband had abundant sperm in the vas fluid at the time of the reversal procedure.

## 11.6 Microsurgical Vasoepididymostomy

The microsurgical-specific tubule technique of vasoepididymostomy was first described in 1978 as end-to-end and subsequently modified to end-to-side [8, 9, 12, 13, 15, 16, 25, 41–46]. The tunica vaginalis is opened, and the testis and epididymis are everted from the hydrocele sac. The dilated epididymal tubule is usually approximately 0.2 mm in diameter. The epididymal duct is extraordinarily delicate with a wall thickness of approximately 30 microns. Earlier nonmicroscopic approaches made a deep longitudinal incision into the outer epididymal tunic, cutting through what looks like as many as 10 or 20 tiny tubules. Then, a fistula was created by suturing the vas to the epididymal tunic. The results were terrible.

The proper approach for reestablishment of continuity of the ductal system is to perform a specific anastomosis between the inner lumen of the vas deferens and the epididymal tubule (Fig. 11.7a, b). Our original approach was end-to-end, but now an end-to-side approach is preferred. The requirements for success and results are the same (Figs. 11.9, 11.10, 11.11, 11.12, and 11.13). The results with this approach to vasoepididymostomy are similar to those of vasovasostomy when there is sperm in the vas fluid [5, 6]. The objective is to explore the epididymis more and more proximally (usually mid to proximal corpus) until we get beyond the secondary obstruction and find good quality motile sperm and perform specific tubule anastomosis at that level [46].

The first 10-0 nylon inner mucosal suture is placed at the 6 o'clock position for easy visualization. Then subsequent mucosal sutures are placed up to 3 o'clock and

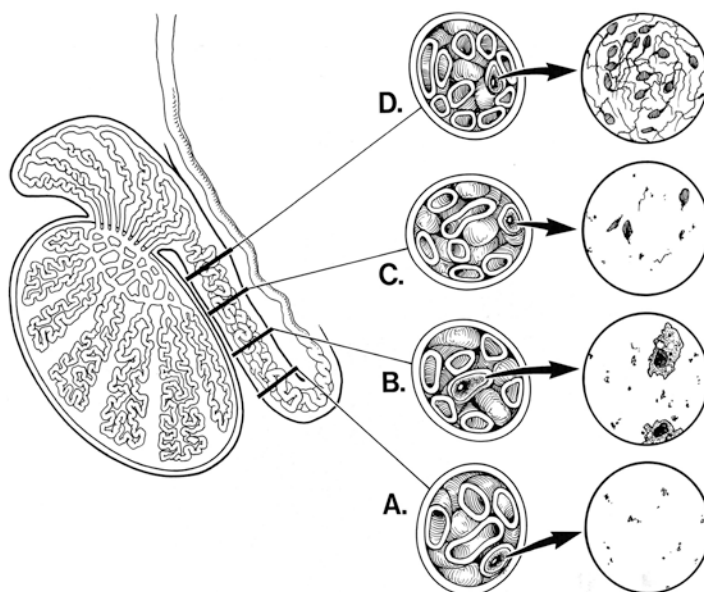


**Fig. 11.9** A diagram of the serial transection of the epididymis in a patient who had undergone vasectomy but has no sperm in the vas fluid. At some point proximally in the epididymis, secondary epididymal obstruction is bypassed, and then normal motile sperm are seen (from: Silber SJ. Reproductive Infertility Microsurgery in the Male and Female. Baltimore, MD: Williams & Wilkins and Waverly Press, Inc., 1984)

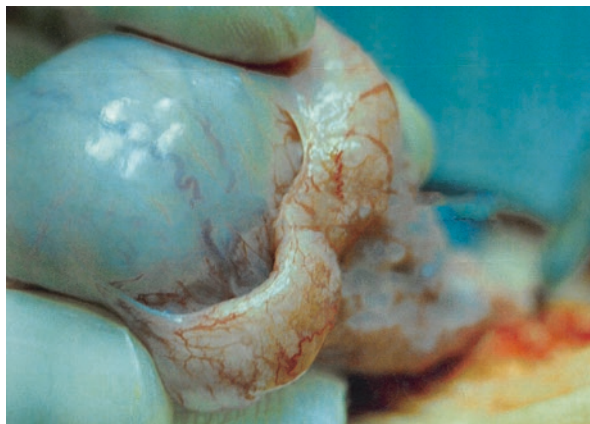


**Fig. 11.10** Secondary epididymal obstruction resulting from pressure-induced "blowouts" or inspissation caused by intranasal pressure after vasectomy

**Fig. 11.11** Epididymis distal to blowouts—no sperm

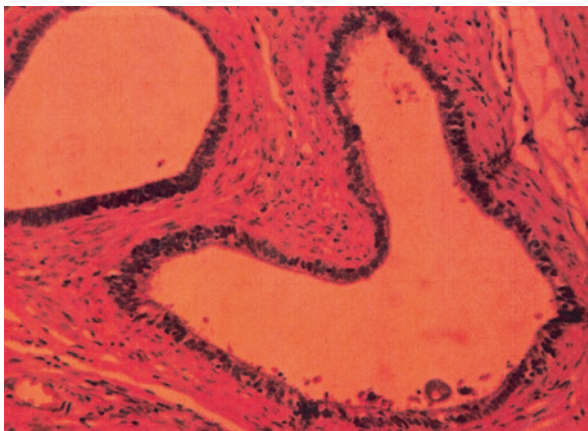


**Fig. 11.12** Epididymis proximal to blowouts—many sperm

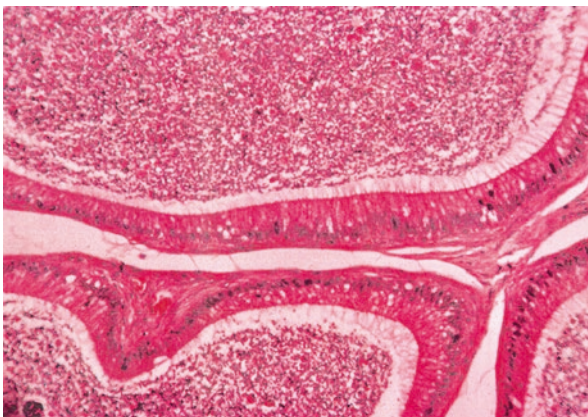
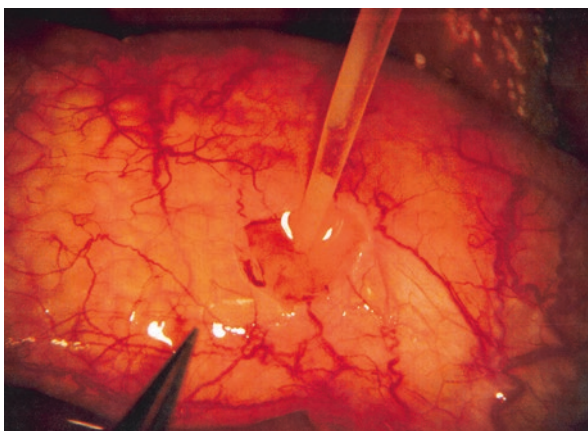


**Fig. 11.13** Aspiration of epididymal fluid to determine the site of blockage



9 o'clock, and finally to 12 o'clock (Figs. 11.10, 11.11, 11.12, 11.13, 11.14, 11.15, 11.16, 11.17, 11.18, 11.19, 11.20, 11.21, 11.22, and 11.23). Then an outer layer of 9-0 nylon interrupted is placed for strength. Using this technique, even cases of epididymal blockage can be successfully repaired with good results depending strictly on the age of the wife (Table 11.1).

**Fig. 11.14** Making a longitudinal slit in epididymal tubule for end-to-side anastomosis. (a) The surgeon makes longitudinal openings in the epididymal tubule worst more proximal until good sperm is found. (b) The first 10-0 nylon suture is placed at the 6 o'clock position. The rest of the mucosal sutures are then placed proximally

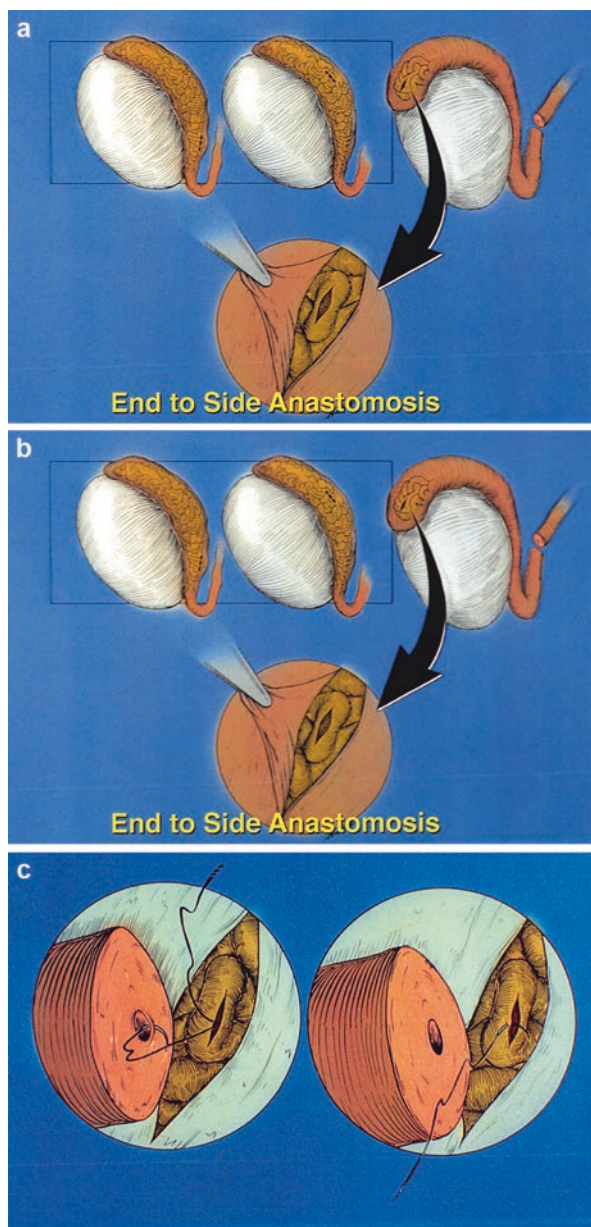


**Fig. 11.15** First three mucosal sutures for end-to-side anastomosis have been placed posteriorly starting at the 6 o'clock position and then marching up to the 3 o'clock and 9 o'clock position

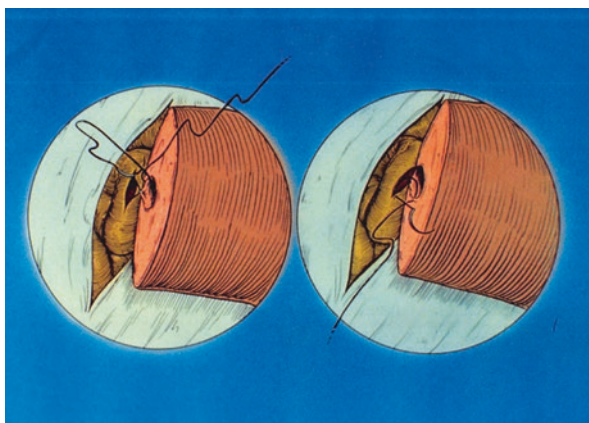


**Fig. 11.16** Second mucosal stitch 10-0 nylon of vas to epididymal tubule

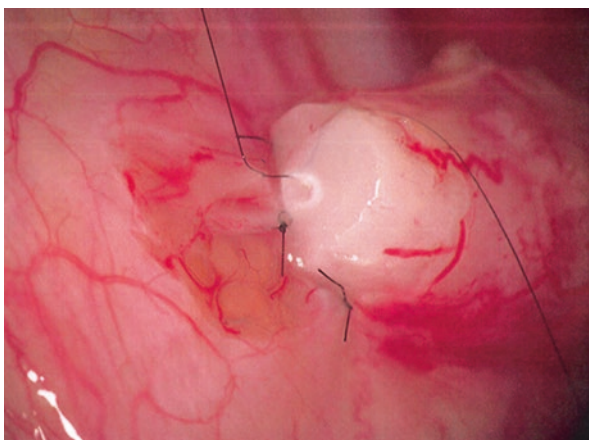


**Fig. 11.17** Third mucosal stitch 10-0 nylon of vas to epididymal tubule

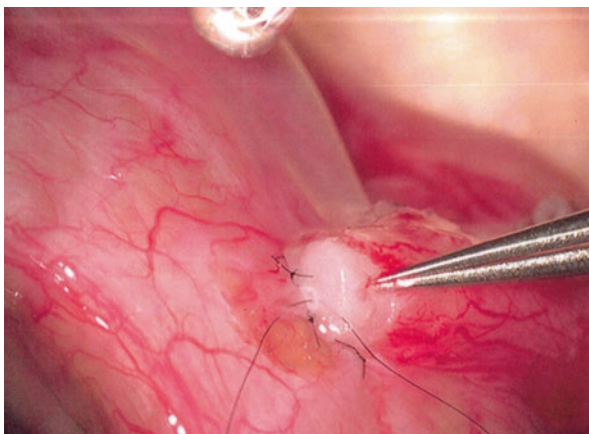


**Fig. 11.18** Opening in vasa efferentia proximal to the caput epididymis



**Fig. 11.19** First mucosal structure vas efferentia



**Fig. 11.20** Second mucosal suture vas to vas efferentia

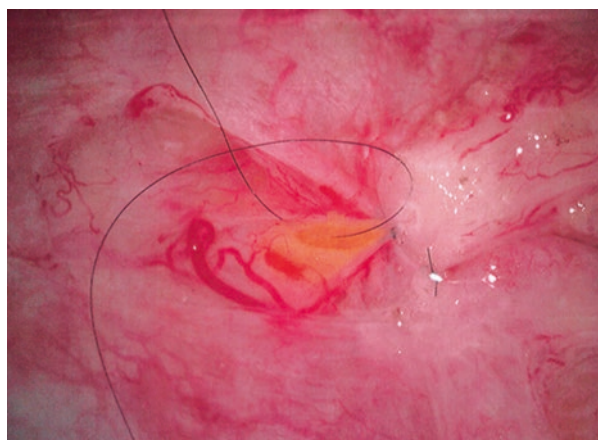


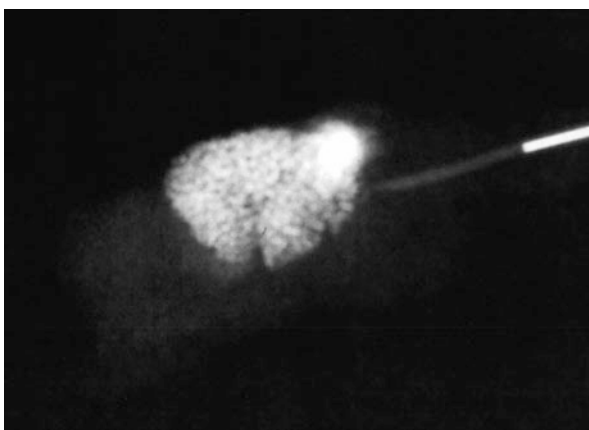
**Fig. 11.21** Third mucosal suture vas to vasa efferentia



**Fig. 11.22** Completed vasa efferentia anastomosis



**Fig. 11.23** Vasogram showing successful vasoepididymostomy with specific anastomosis of vas lumen to epididymal tubule



## 11.7 Congenital Absence of Vas

If the male has congenital bilateral absence of the vas deferens, both he and his partner should have cystic fibrosis mutation analysis performed prior to microsurgical epididymal sperm harvesting or testicular tissue extraction coupled with ICSI. The vast majority of these CBVAD patients do not have cystic fibrosis. Most are carriers of one allele of a serious CF gene mutation and on the other allele a weak mutation. So they do not have CF, but they do have CBVAD. Clinical cystic fibrosis (CF) has a frequency of 1:1600 people of Northern European descent. It is an autosomal recessive disorder with a carrier frequency of 1:20. In clinical CF, the predominant pathophysiologic consequences are obstructive pulmonary disease and exocrine pancreatic failure. These lead to chronic respiratory infection and pulmonary decline as well as malnutrition and pancreatitis if untreated. Nearly all males with clinical CF will also have absence of the vas deferens bilaterally and experience obstructive azoospermia. However, most men with CBVAD do not have the disease CF even though their CBVAD is caused by mutations in their CF gene.

Here is how that works. The cystic fibrosis gene is located on chromosome 7q31.2 and encodes for a protein termed the cystic fibrosis transmembrane conductance regulator (CFTR) (<http://www.genet.sickkids.on.ca/cftr>). CFTR is a 1480-amino-acid membrane-bound chloride channel helping to regulate sodium/chloride transport and therefore extracellular osmolality which helps, in part, to determine the fluidity of mucus and other epithelial tubal secretions. The total CFTR pool is derived from both the maternal and paternal allele.

The combination of mutations inherited determines the clinical expression of disease. If both the maternal and paternal CFTR mutations are “severe,” then most likely clinical CF will result because the total CFTR pool is grossly deficient either quantitatively or qualitatively (<5% normal physiologic levels). However, if at least one of the two mutations is less severe or “mild,” then the phenotypic expression of those two mutations will be less pronounced as the total CFTR pool is less severely affected either quantitatively or qualitatively. In these circumstances, the mildest manifestation of CFTR mutations will be exhibited, namely, congenital bilateral absence of the vas deferens in the male, CBVAD absence of variable segments of the epididymis, and absence/aplasia/dysplasia of the seminal vesicles. The caput of the epididymis is always present as this is derived from a different embryologic precursor than the corpus or the cauda, which are mesonephric.

Some men with CBAVD may have mild respiratory or pancreatic illness or a prior history of pneumonia, bronchitis, or sinusitis, and it is the recognition that they have mutations in CFTR that leads to the reclassification of those illnesses as a part of the cystic fibrosis mutation spectrum. However, most men with CBAVD will have no symptoms. There have been over 1500 abnormalities defined in the CF genes, including single-nucleotide changes, small insertions or deletions (both exonic and intronic), and large rearrangements. In a male with CBAVD, there is usually a common severe mutation on one allele. However, the other allele is certain to have a very mild mutation, such as the “T5-allele” [26, 47]. Interestingly it takes

only a small reduction in CFTR protein to cause CBVAD, but it requires near total reduction in CFTR protein to cause the actual disease of CF. Congenital absence of the vas deferens occurs in approximately 1% of infertile couples [24]. Until 1986, it was a frustrating and dismal problem. Since the first successful use of epididymal sperm aspiration and IVF for congenital absence of the vas deferens was reported, ICSI has now made it possible for all these men to have children [18, 35, 48–50]. In fact, with ICSI, the pregnancy rate with epididymal sperm retrieval (microsurgical epididymal sperm aspiration) is only related to female factors [18, 48, 49].

## 11.8 MESA: Microsurgical Sperm Aspiration with ICSI

The men undergo a simple “window” scrotal exploration using local anesthesia immediately after their female partners undergo oocyte aspiration. Using  $\times 10$  to  $\times 40$  magnification with an operating microscope, a 0.3 cm incision is made with microscissors into the epididymal tunic to expose the epididymal tubules in the most proximal portion of the congenitally blind-ending epididymis. Sperm are aspirated with a micropipette with a tuberculin syringe directly from the opening in the epididymal tubule. The specimens are immediately diluted in HEPES-buffered medium, and a tiny portion is examined for motility and quality of progression. If sperm motility is absent or poor, another aspiration is made 0.5 cm more proximally. Sperm are obtained from successively more proximal regions until progressive motility is found (Figs. 11.24, 11.25, 11.26, 11.27, 11.28, 11.29, 11.30, 11.31, and 11.32).

Motile sperm are usually not obtained until the most proximal portion of the epididymis or vasa efferentia is reached. This is exactly the opposite of what you would expect in the nonobstructed epididymis. Normally in a nonobstructed state, sperm gradually obtain motility as they mature during passage through the epididymis. However, shockingly in the obstructed epididymis, the distal sperm have died of senescence, and only the proximal sperm are viable in most circumstances (Figs. 11.30 and 11.32). The distal epididymal sperm demonstrated on electron microscopy (E/M) show these changes as “DNA fragmentations.” In fact the now popular term of “DNA fragmentation” is just sperm senescence. Older sperm have died of old age, and then their chromatin just degrades and fragments. Almost all sperm samples have some such senescent sperm. Once the area of motile sperm is found, an aliquot of epididymal fluid is used for ICSI, and the remainder is frozen. It is very difficult to get the best proximal motile sperm with blind needle aspiration.

The present state of the art appears to be that there are virtually no cases of obstructive azoospermia that cannot be successfully treated with sperm retrieval methods and ICSI, so long as the female does not have insurmountable problems herself. For obstructive azoospermia, we prefer to use epididymal sperm, although testicular sperm works almost as well. We have found higher pregnancy and delivery rates with proximal epididymal sperm than testis sperm, as well as higher with

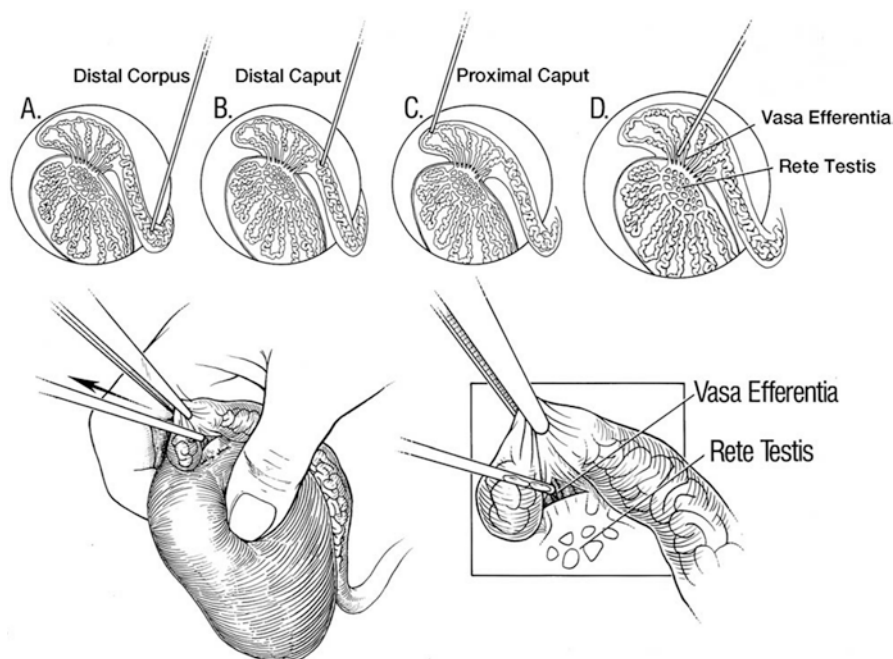


**Fig. 11.24** A depiction of microsurgical epididymal sperm aspiration beginning at the distal corpus (a) and moving proximally to the distal caput, the proximal caput, and the vasa efferentia (b–d). With obstructive azoospermia, there is an inversion of the usual physiologic location of greatest and least sperm motility. With obstruction, the most motile sperm are always the most proximal. Distal sperm, because of senescence, are the least motile (from: Silber SJ. Congenital absence of the vas deferens. *N Engl J Med.* 1990;323:1788–1792)

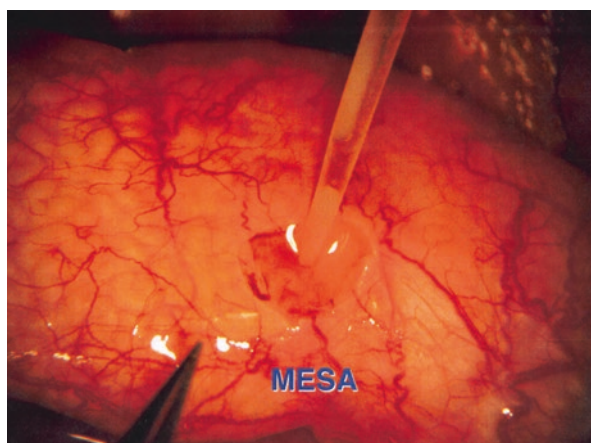


**Fig. 11.25** Microsurgical aspiration of sperm for CBVAD

**Fig. 11.26** 10× view of obstructed epididymal tubule



**Fig. 11.27** 25× view of obstructed epididymal tubule

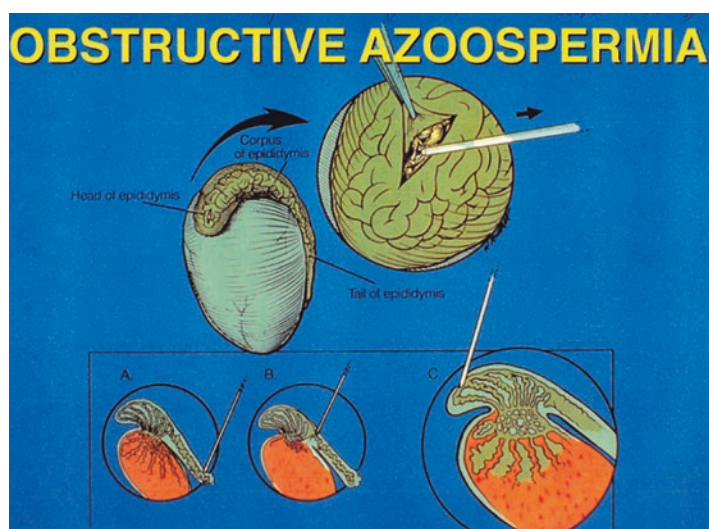
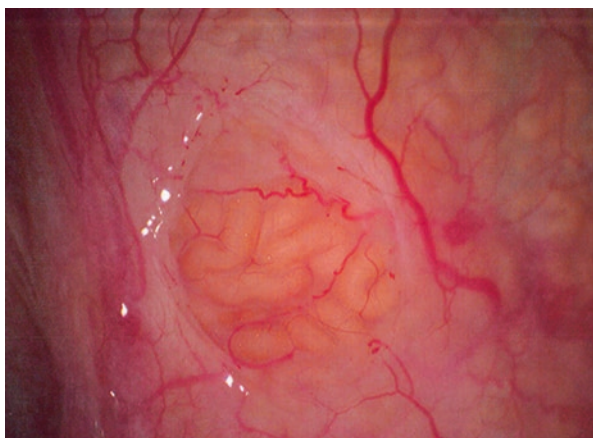


**Fig. 11.28** Sampling epididymal fluid from distal to proximal to evaluate motility

**Fig. 11.29** With obstructive azoospermia, the most distal sperm are the oldest and therefore the poorest motility. The most proximal sperm ironically are the best motility because they have been more recently produced

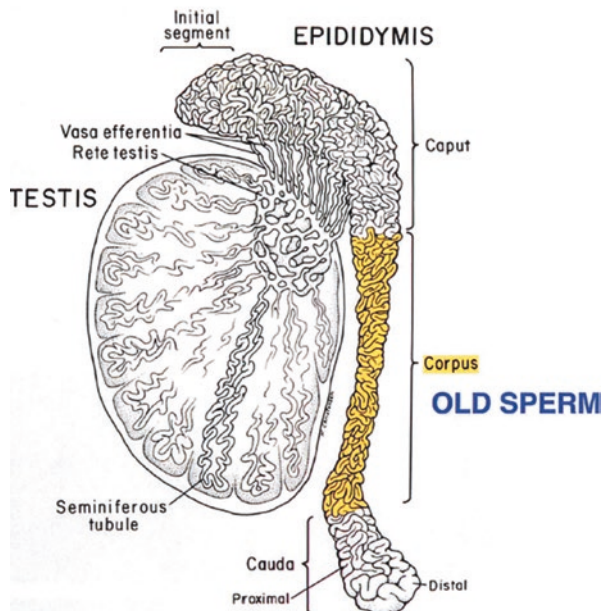
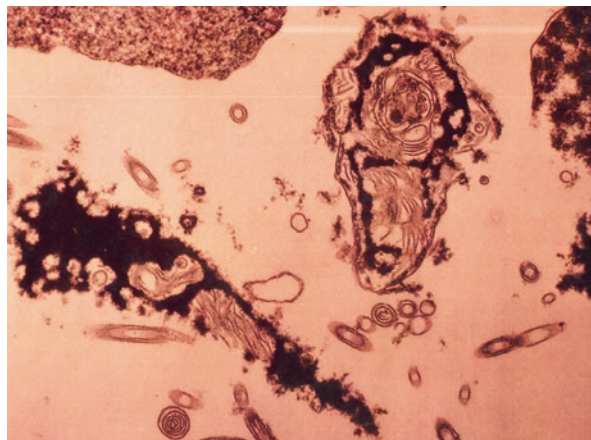


**Fig. 11.30** Old sperm obstructed in the distal epididymis



proximal then distal epididymal sperm. The advantage of epididymal sperm as a first choice is that it freezes so easily and represents such a simple, clean, easy, indefinite supply of sperm for the laboratory to use for that particular patient without any need for future invasive procedures. However, epididymal sperm is only successful if one goes proximal enough to retrieve motile sperm. Distal non-motile sperm is senescent and will not work even with ICSI.

Fertilization and live babies can be produced by conventional (non-ICSI) IVF with epididymal sperm [26, 51]. However, the results are very much poorer than

**Fig. 11.31** In the obstructed state, contrary to expectations in the nonobstructed state, proximal sperm are the most viable and the most mobile

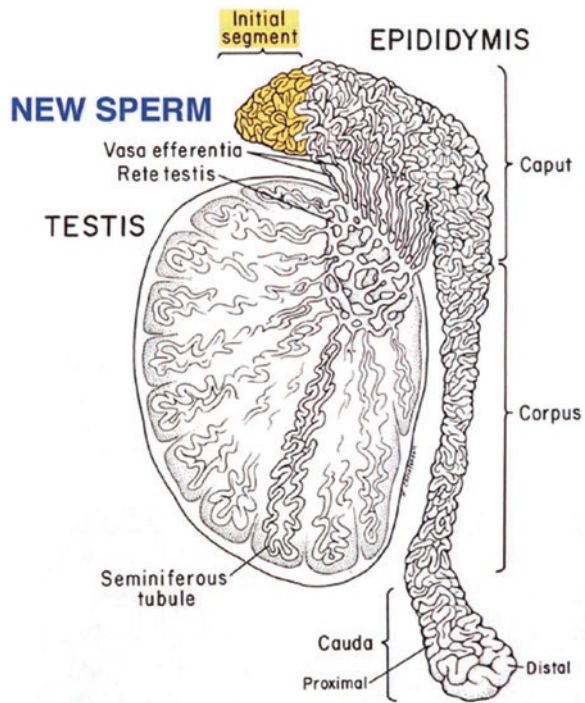
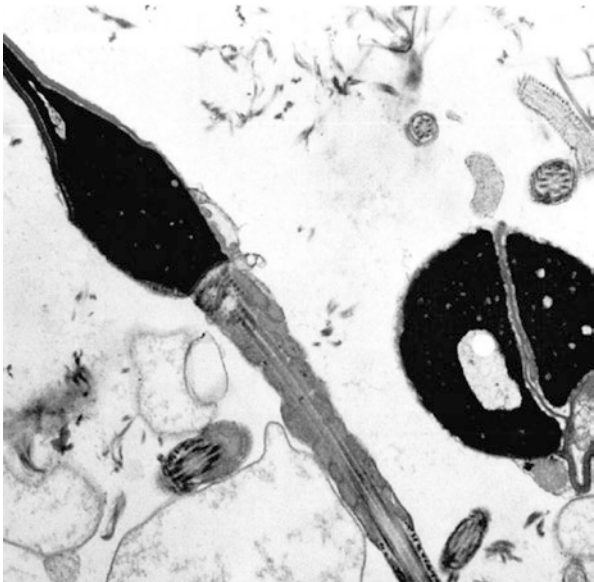


**Fig. 11.32** Motile live new sperm in proximal epididymal tubule. From the same obstructed epididymis as in Fig. 11.30, proximal sperm here show no DNA fragmentation have good motility



with ICSI, as we reported over 20 years ago. We also reported about 20 years ago that with ICSI, success is strictly related to the age of the wife (Tables 11.2 and 11.3) [48].

There have been many trivial debates about how to best collect epididymal or testicular sperm from patients with azoospermia for ICSI. The reader can decide what works best in his or her own particular setting, but our preference is as follows:

For obstructive azoospermia, there is usually some epididymis present no matter how severe the defect. In these instances, we prefer microsurgical epididymal sperm aspiration (MESA). We perform all sperm retrieval using local anesthesia without sedation. Although the approach is microsurgical and careful, it is an outpatient procedure performed with minimal postoperative discomfort. The spermatic cord is first grasped between the thumb and forefinger by the urologist, a manner quite similar to performing vasectomy. The cord is then infiltrated with anesthesia. This produces anesthesia of the testicle but not of the scrotum. Then, just like with testis biopsy, the anterior scrotal skin is infiltrated with a 25-gauge needle along a proposed 1-cm to 2-cm incision line. Once the tunica vaginalis line is entered, the epididymis and testicle are exposed and brought into the field of an operating microscope. The patient, indeed, can watch the whole procedure on a video monitor and should be wide-awake and comfortable.

The advantage of epididymal sperm retrieval performed in this way is the huge number of the most motile sperm that can readily be obtained from the most proximal duct and frozen for an unlimited number of future ICSI cycles. There is often only one specific area of the proximal epididymis where motile sperm can be retrieved, and this can be found more easily through microsurgery than via a blind needle stick (which, in truth, is more painful than this microsurgical epididymal sperm aspiration procedure).

**Table 11.2** Obstructive azoospermia: effect of age of wife (Silber et al. 1994)

| Age of wife (years) | No. of cycles (% of total) | No. of eggs at MII | No. of 2PN oocytes (% of MII eggs) | No. delivered pregnancies per cycle (% per cycle) | Implantation rate % (per embryo) |
|---------------------|----------------------------|--------------------|------------------------------------|---------------------------------------------------|----------------------------------|
| <30                 | 50 (27%)                   | 735                | 392 (53%)                          | 22 (44%)                                          | 22%                              |
| 30–36               | 87 (47%)                   | 1111               | 610 (55%)                          | 30 (34%)                                          | 19%                              |
| 37–39               | 24 (13%)                   | 207                | 113 (55%)                          | 3 (12%)                                           | 4%                               |
| 40+                 | 25 (13%)                   | 281                | 147 (52%)                          | 1 (4%)                                            | 7%                               |
| All ages            | 186 (100%)                 | 2334               | 1262 (54%)                         | 56 (30%)                                          | 16.2%                            |

**Table 11.3** Comparison of MESA with ICSI to conventional MESA with IVF in a similar patient population (Silber et al. 1995)

| Cycles           | Mature eggs | 2PN | Fertilization rate | Transfers   | Pregnancy rate (delivered) |
|------------------|-------------|-----|--------------------|-------------|----------------------------|
| <i>IVF-MESA</i>  |             |     |                    |             |                            |
| 67               | 1427        | 98  | 7%                 | 13/67 (19%) | 3/67 (4.5%)                |
| <i>ICSI-MESA</i> |             |     |                    |             |                            |
| 33               | 431         | 201 | 47%                | 31/33 (94%) | 12/33 (36.3%)              |

For nonobstructive azoospermia, epididymal sperm can never be retrieved because the walls are collapsed, and there is no obstruction to allow epididymal sperm collection to take place. Nonetheless, for nonobstructive azoospermia, an open testicular biopsy performed using the microscope can still be accomplished in the same way using the same type of local anesthetic with the patient wide-awake and with minimum postoperative discomfort.

### 11.9 Testicular Sperm Extraction (TESE) for Nonobstructive Azoospermia (NOA)

Soon after introducing sperm retrieval for obstructive azoospermia, we made the observation that even in men with the most severe spermatogenic defects, nonobstructive azoospermia causing complete lack of sperm in the ejaculate, there were often a minute number of sperm sparsely present in an extensive testicular biopsy, and these occasional testicular sperm could be used for ICSI [19, 21, 23–25, 49, 52, 53]. We called this procedure testicular sperm extraction (TESE). The resultant pregnancy rate is no different than if the patient had normal spermatogenesis (Tables 11.4–11.10). The pregnancy rate and live baby rate are very slightly better

**Table 11.4** Ejaculated sperm pregnancy rates for ejaculated sperm in 1849 consecutive cases (Silber et al. 2015)

| Age      | <2 million sperm | 2–5 million sperm | 6–20 million sperm | >20 million sperm | Donor sperm | Overall      |
|----------|------------------|-------------------|--------------------|-------------------|-------------|--------------|
| <30      | 61/121—50%       | 22/39—56%         | 29/59—49%          | 94/162—58%        | 19/35—54%   | 225/416—54%  |
| 30–35    | 102/247—41%      | 53/94—56%         | 61/127—48%         | 161/322—50%       | 27/68—40%   | 404/858—47%  |
| 36–40    | 51/118—43%       | 18/46—39%         | 28/73—38%          | 70/185—38%        | 14/34—41%   | 181/456—40%  |
| >40      | 6/42—14%         | 3/13—23%          | 4/19—21%           | 8/39—21%          | 0/6—0%      | 21/119—18%   |
| Overall* | 220/528—42%      | 96/192—50%        | 122/278—44%        | 333/708—47%       | 60/143—42%  | 831/1849—45% |

\*NS for horizontal rows for rows >35 is <.001

**Table 11.5** Sperm found per TESE for NOA (Silber et al. 2015)

|                | Number of cases sperm found | Percent |
|----------------|-----------------------------|---------|
| MA             | 42                          | 44%     |
| SCO            | 45                          | 32%     |
| SCO/MA         | 3                           | 27%     |
| Klinefelter    | 11                          | 48%     |
| Male Turner    | 3                           | 100%    |
| Cryptorchidism | 10                          | 83%     |
| Post chemo     | 6                           | 28%     |
| Total          | 207/444                     | 47%     |

**Table 11.6** Ejaculated sperm delivery rates for ejaculated sperm in 1849 consecutive ICSI cycles (Silber et al. 2015)

| Age       | <2 million sperm | 2–5 million sperm | 6–20 million sperm | >20 million sperm | Donor sperm | Overall      |
|-----------|------------------|-------------------|--------------------|-------------------|-------------|--------------|
| <30       | 45/121—37%       | 20/39—51%         | 20/59—34%          | 67/162—41%        | 17/35—49%   | 169/416—41%  |
| 30–35     | 74/247—30%       | 35/94—37%         | 39/127—31%         | 114/322—35%       | 20/68—29%   | 282/858—33%  |
| 36–40     | 25/118—21%       | 11/46—24%         | 17/73—23%          | 46/185—25%        | 9/34—26%    | 108/456—24%  |
| >40       | 2/42—5%          | 0/13—0%           | 1/19—5%            | 4/39—10%          | 0/6—0%      | 7/119—6%     |
| Overall * | 146/528—28%      | 66/192—34%        | 77/278—28%         | 231/708—33%       | 46/143—32%  | 566/1849—31% |

\*NS for horizontal rows for rows &gt;35 is &lt;.001

**Table 11.7** ICSI pregnancy rates for azoospermia patients by sperm source (testis versus epididymis) (Silber et al 2015)

| Age      | MESA OA fresh | MESA OA frozen | TESE OA fresh | TESE NOA fresh and frozen | Overall     |
|----------|---------------|----------------|---------------|---------------------------|-------------|
| <30      | 26/40—65%     | 12/28—43%      | 9/24—38%      | 24/49—49%                 | 71/141—50%  |
| 30–35    | 24/47—51%     | 52/79—66%      | 18/42—43%     | 18/66—27%                 | 112/234—48% |
| 36–40    | 8/20—40%      | 23/48—48%      | 8/24—33%      | 15/36—42%                 | 54/128—42%  |
| >40      | 0/4—0%        | 8/14—57%       | 1/4—25%       | 0/3—0%                    | 9/25—36%    |
| Overall* | 58/111—52%    | 95/169—56%     | 36/94—38%     | 57/154—37%                | 246/528—47% |

**Table 11.8** ICSI delivery rates for azoospermia patients by sperm source (testis versus epididymis) (Silber et al. 2015)

| Age      | MESA OA fresh | MESA OA frozen | TESE OA fresh | TESE NOA fresh and frozen | Overall     |
|----------|---------------|----------------|---------------|---------------------------|-------------|
| <30      | 23/40—58%     | 11/28—39%      | 6/24—25%      | 13/49—27%                 | 53/141—38%  |
| 30–35    | 16/47—34%     | 40/79—51%      | 14/42—33%     | 11/66—17%                 | 81/234—35%  |
| 36–40    | 3/20—15%      | 15/48—31%      | 3/24—13%      | 10/36—28%                 | 31/128—24%  |
| >40      | 0/4—0%        | 2/14—14%       | 0/4—0%        | 0/3—0%                    | 3/25—8%     |
| Overall* | 42/111—38%    | 68/169—40%     | 23/94—24%     | 34/154—22%                | 167/528—32% |

**Table 11.9** Van Weyly et al. (2015)

|                                                 | MESA <i>N</i> = 280 | TESE <i>N</i> = 94 | <i>P</i> -value |
|-------------------------------------------------|---------------------|--------------------|-----------------|
| Female age, mean (95% CI)                       | 33.3 (32.7–33.9)    | 33.8 (32.7–34.8)   | 0.45            |
| Spermatozoa retrieved, <i>N</i> (%)             |                     |                    |                 |
| Less than 0.1 × 10 <sup>6</sup>                 | 213 (76%)           | 88 (94%)           | <0.001          |
| 0.1 × 10 <sup>6</sup> up to 1 × 10 <sup>6</sup> | 35 (13%)            | 6 (6%)             |                 |
| More than 0.1 × 10 <sup>6</sup>                 | 32 (11%)            | 0                  |                 |
| <i>Fresh or frozen sperm, N (%)</i>             |                     |                    |                 |
| Fresh sperm                                     | 112 (40%)           | 80 (85%)           | <0.001          |
| Frozen sperm                                    | 168 (60%)           | 14 (15%)           |                 |
| Oocytes, mean (95% CI)                          | 13.9 (12.9–14.9)    | 13.0 (11.3–14.6)   | 0.32            |
| Embryos, mean (95% CI)                          | 6.9 (6.4–7.4)       | 6.3 (5.3–7.3)      | 0.26            |
| Number of embryos transferred mean (95% CI)     | 3.4 (3.2–3.6)       | 3.5 (3.1–3.8)      | 0.68            |
| Implantation rate, mean (95% CI)                | 0.22 (0.19–0.26)    | 0.15 (0.10–0.20)   | 0.037           |
| Clinical pregnancy, <i>N</i> (%)                | 132 (47%)           | 29 (30%)           | 0.005           |
| Miscarriage, <i>N</i> (%)                       | 23 (8%)             | 6 (6%)             | 0.57            |
| Live birth, <i>N</i> (%)                        | 109 (39%)           | 23 (24%)           | 0.011           |
| Multiple pregnancy, <i>N</i> (%)                | 33 (30%)            | 10 (43%)           | 0.21            |

**Table 11.10** Van Weyly et al. (2015)

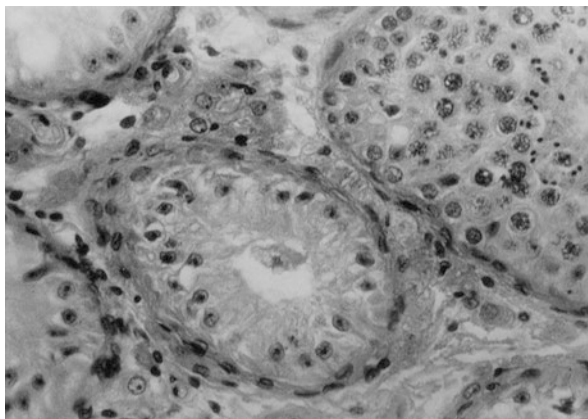
| Screening parameters      | Univariable logistic analysis |           |                 | Multivariable logistic analysis |           |                 |
|---------------------------|-------------------------------|-----------|-----------------|---------------------------------|-----------|-----------------|
|                           | OR unadjusted                 | 95% CI    | <i>P</i> -value | OR adjusted                     | 95% CI    | <i>P</i> -value |
| MESA versus TESE          | 2.00                          | 1.22–3.29 | 0.01            | 1.82                            | 1.05–3.67 | 0.01            |
| Female age                | 0.92                          | 0.88–0.97 | <0.001          | 0.93                            | 0.90–0.97 | 0.007           |
| Number of oocytes         | 1.07                          | 1.04–1.10 | <0.001          | 1.06                            | 1.03–1.09 | <0.001          |
| Fresh versus frozen sperm | 0.73                          | 0.48–1.11 | 0.14            | 0.72                            | 0.43–1.22 | 0.22            |

Note: There was no indication for interaction between MESA/TESE and whether fresh or frozen sperm was used

with proximal epididymal and ejaculated sperm than testis sperm. But this difference is small.

This approach was based on a quantitative study of spermatogenesis dating back to the late 1970s [25, 26, 54–56]. Examination of the testicular histology of men with azoospermia, with oligospermia, and with normal sperm counts showed that the number of sperm in the ejaculate is directly correlated to the number of mature spermatids found quantitatively in the testis. The average mature spermatid count per tubule in a large number of tubules is predictive of the sperm count in the ejaculate. Intriguingly, however, many patients with complete azoospermia have been found to have a few mature spermatids in their testis histology (Figs. 11.33–11.38). These studies of quantitative spermatogenesis, in the late 1970s and early 1980s, provided the theoretic basis for our efforts to extract sperm, however few, from men with azoospermia caused by Sertoli cell only or maturation arrest and allowed for

**Fig. 11.33** This is a histologic section of testicle biopsy in a patient with Sertoli cell only, increased FSH, and occasional tubules with normal spermatogenesis. Upper right-hand tubule exhibits normal spermatogenesis, but all of the other tubules are Sertoli cell only (from: Silber SJ, Johnson L, Verheyen G, et al. Round spermatic injection. Fertil Steril. 2000;73:897–900)



## Degrees of Azoospermia

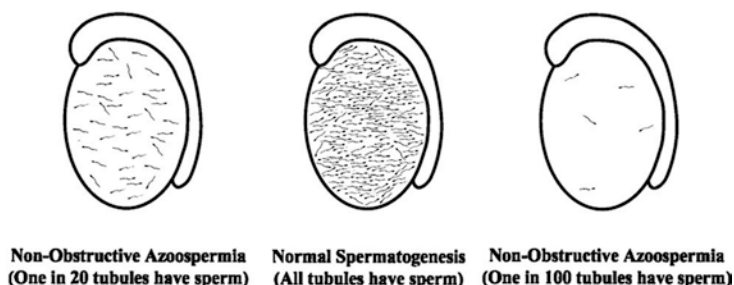
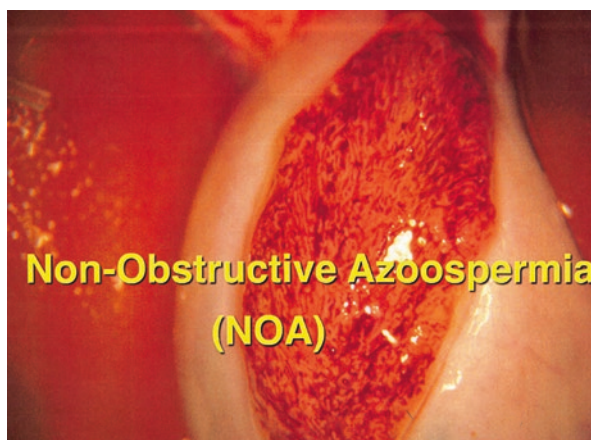


**Fig. 11.34** Various “degrees” of azoospermia. Normal spermatogenesis (center drawing) is associated with obstructive azoospermia. With nonobstructive azoospermia, TESE may be easy as in the drawing depicted on the left or difficult as depicted in the drawing on the right

**Fig. 11.35** Nonobstructive azoospermia (NOA), in this case caused by Sertoli cell only (SCO). Note the very thin appearance of the tubules, because there are no spermatogenic cells inside of them



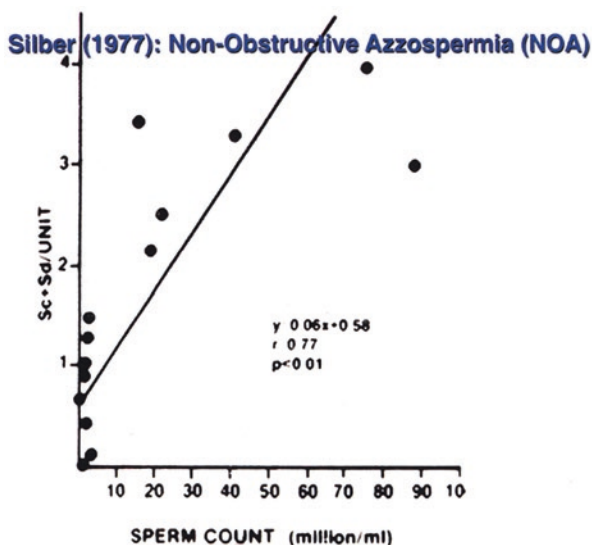
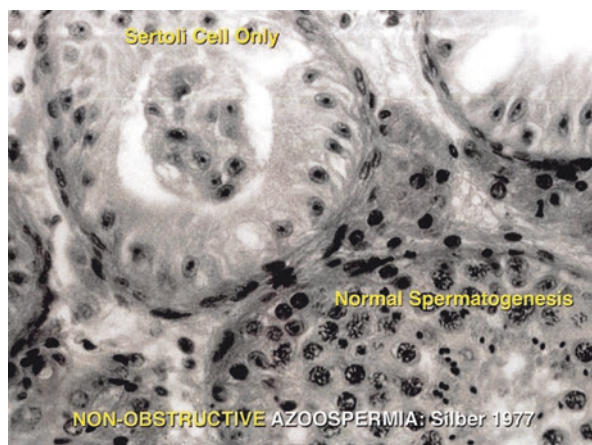


**Fig. 11.36** Some sperm in tiny numbers exist in nonobstructive azoospermia (NOA), first discovered by us in 1977, but not realizing its impact until ICSI in 1994

**Fig. 11.37** Nonobstructive azoospermia. A tubule with normal spermatogenesis surrounded by a sea of Sertoli cell only syndrome



the use of these few sperm for ICSI. An extremely diminished quantity of sperm production in the testis will result in absolute absence of sperm in the ejaculate even though there are some sperm being produced in the testicle. With severely reduced spermatogenesis, there is simply not enough sperm quantitatively to surpass the low threshold of sperm production needed for any sperm to actually spillover into the ejaculate.

Thus, severe oligospermia, which is readily treated with ICSI, is just a quantitative variant of azoospermia, and there is some minute presence of spermatogenesis in almost half of azoospermic men depending on the patient population

## Graph depicting quantitative testicle biopsy and sperm count

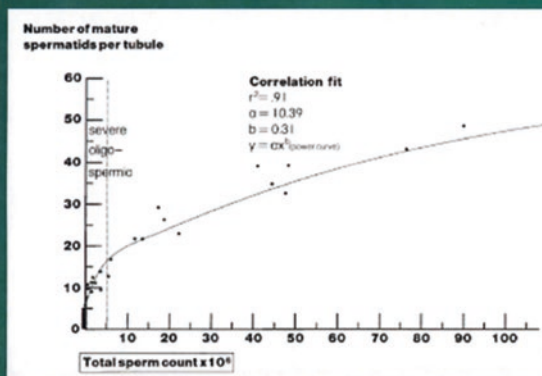
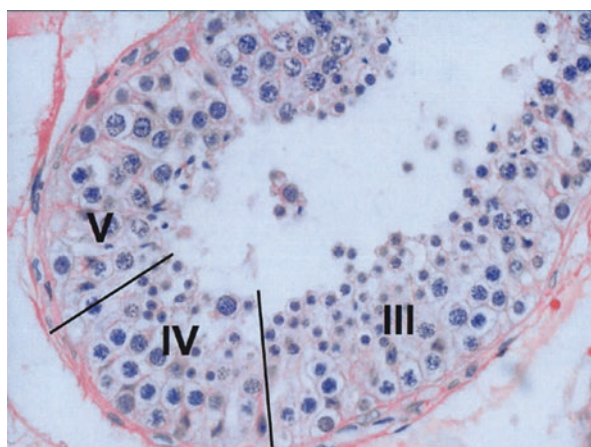


**Fig. 11.38** Graph depicting quantitative testicle biopsy and sperm count, much later showing an exponential relationships of sperm seen on a two-dimensional histologic section and total sperm production, which is volumetric (not very dimensional)

**Fig. 11.39** Three different stages of spermatogenesis in one tubular cross section are typical and unique to human spermatogenesis. This variation in stages including all stages of spermatogenesis in any area of our seminiferous tubule (with no wave to look for) makes human TESE for NOA much easier



(Figs. 11.33–11.38). The amount of spermatogenesis, however, is below the threshold necessary for these few sperm to spill into the ejaculate [23]. There is no adequate test to predict whether there will be sperm found at TESE (Fig. 11.39). The reason is that in the normal testis, hundreds of millions of sperm are produced every day. Yet with nonobstructive azoospermia looking for 3–15 sperm represents a millionfold decline from normal sperm production but will still allow a successful TESE (Figs. 11.35–11.47). Comparison of open micro-TESE to needle biopsy

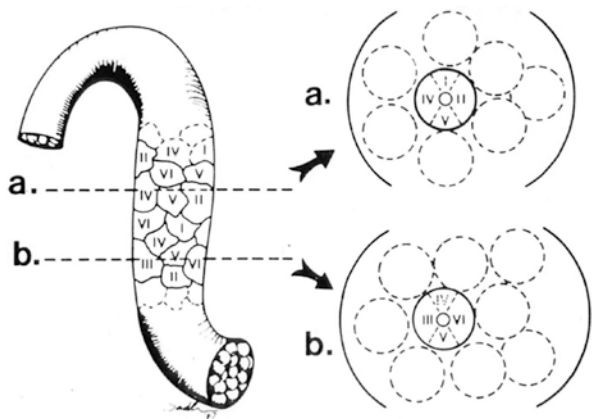


**Fig. 11.40** No “wave” of stages of spermatogenesis in the human

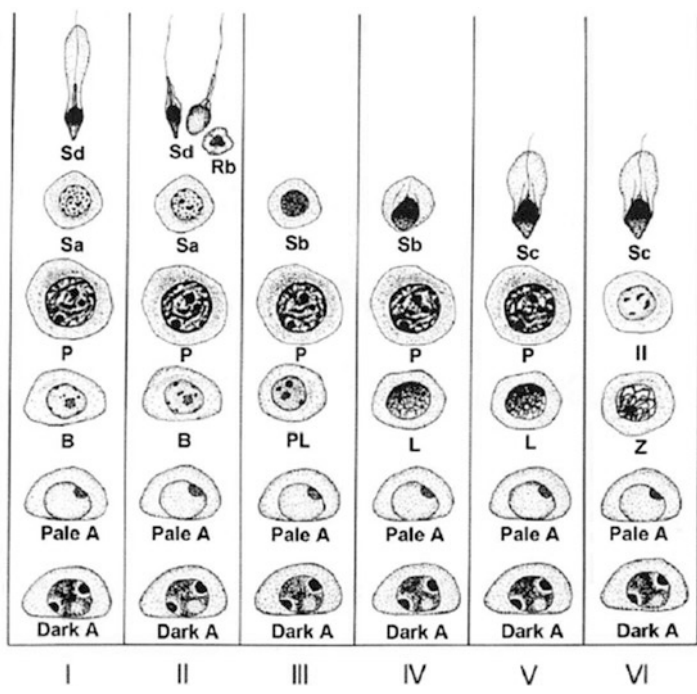


**Fig. 11.41** The six stages of spermatogenesis cycles in the human testis. Although human spermatogenesis from stage to stage is quite orderly and precise, the location of these stages in the tubule is completely mixed up. Ironically, that allows for an easier method for TESE

**Fig. 11.42** Normal spermatogenesis. If this patient is obstructed and needs TESE for ICSI, only a small incision is needed because anywhere you look there will be many sperm

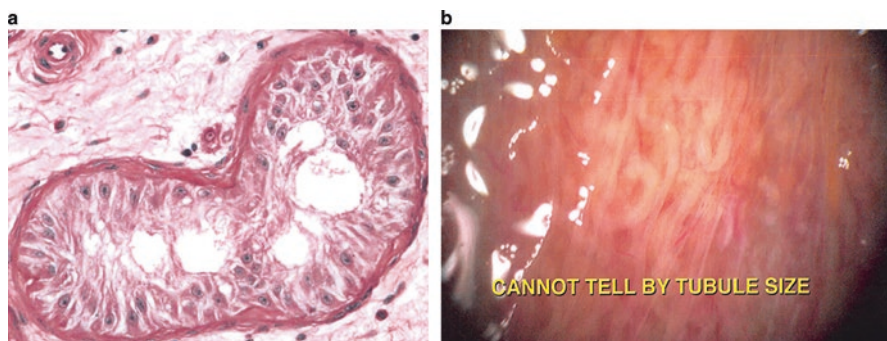
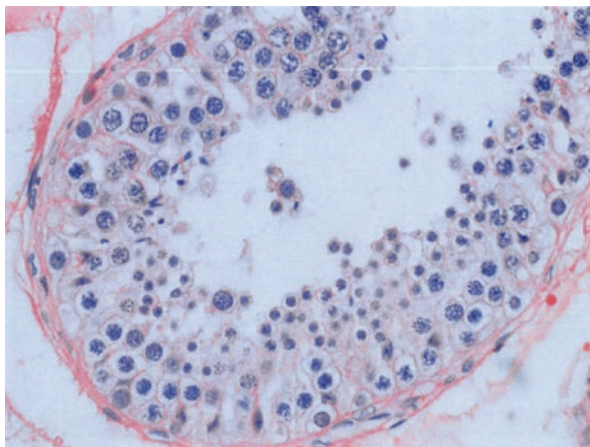


**Fig. 11.43** (a, b) Sertoli cell only. In this case, one has to search for the rare tubule that has a spermatogenesis. Anywhere you look in the periphery you will find sperm, without having to dissect deeply

**Fig. 11.44** Partial spermatogenic maturation arrest. In such a case at TESE the tubules are not thin, because they are filled with spermatocytes and spermatogonia. The problem is there are very few if any mature sperm or spermatids. So these seminiferous tubules all look normal and indeed the FSH is normal. There are few tubules with mature sperm, and so this is called “incomplete or partial” maturation arrest

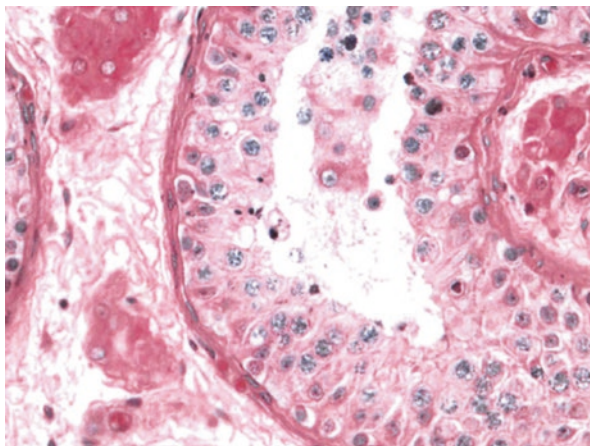


**Fig. 11.45** Complete Spermatocytic maturation arrest. In this case, again at TESE, all the tubules look like normal spermatogenesis. But there are no mature sperm or spermatids anywhere in this testis, only spermatogonia and spermatocytes

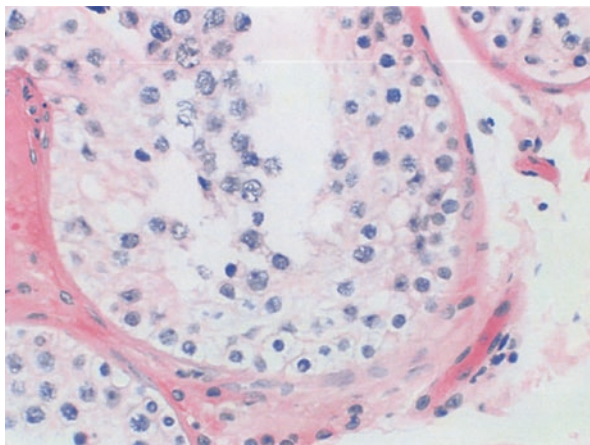
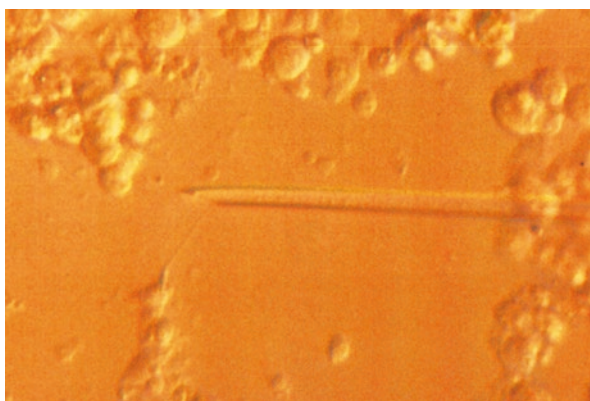


**Fig. 11.46** Picking up a rare sperm in a TESE suspension of spermatocytes in a case of azoospermia due to maturation arrest



consistently shows superiority of open micro-TESE [57–62]. Successful TESE for nonobstructive azoospermia (NOA) is a matter of finding those rare sperm in an ocean of other spermatogenic cells and somatic cells (Fig. 11.46).

## 11.10 The Round Spermatid Controversy

When we first described TESE for nonobstructive azoospermia (NOA), there was a flurry of confusion created by embryologists and gynecologists who knew nothing about spermatogenesis, looking for “round spermatids” to inject, and even fraudulently sometimes claiming success. What these mistaken IVF centers were injecting, thinking they were round spermatids, were in fact Sertoli cell nuclei with a prominent nucleolus that were mistaken for an acrosomal vesicle on Hoffman optics (Figs. 11.48 and 11.49). It is difficult to identify round spermatids in Hoffman optics but easy with

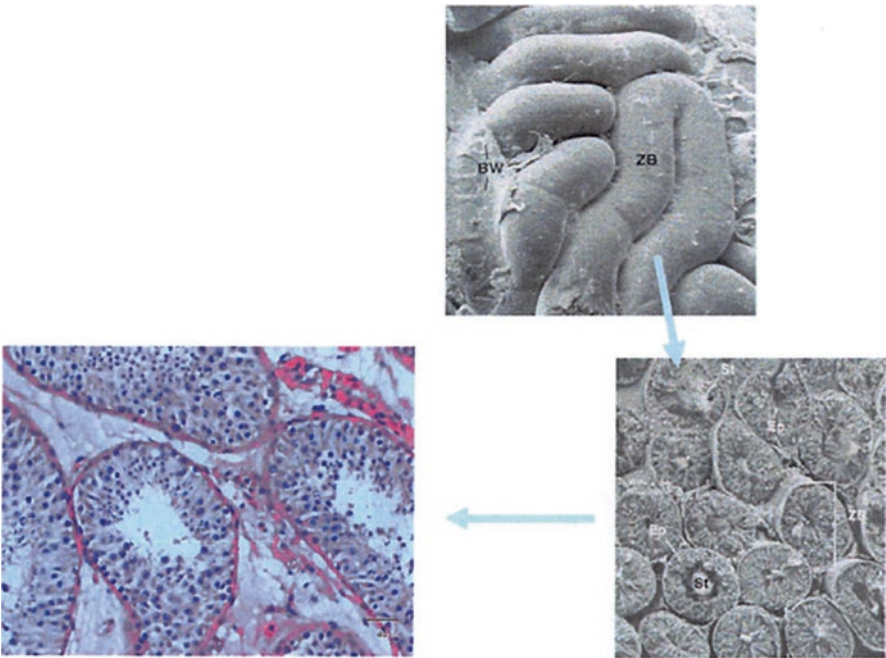
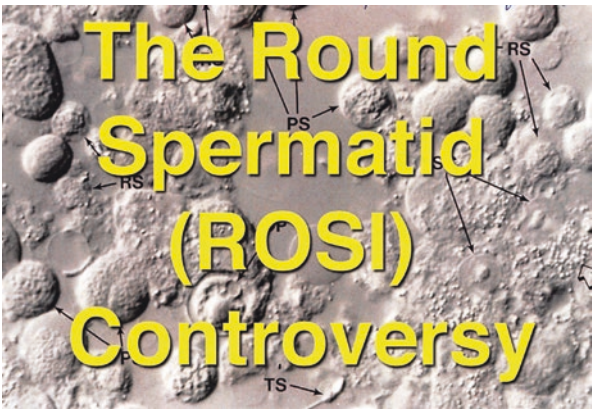


**Fig. 11.47** Testis-anatomic stem cell approach to TESE. This anatomy demonstrates that if there is any normal spermatogenesis stem cell in any tubule of the testis, sperm will be found in the periphery where the seminiferous tubule makes its U-turn at the periphery

**Fig. 11.48** The round spermatid (ROSI) controversy



phase contrast. Under phase contrast, the acrosomal vesicle actually “glows,” and it is easy to identify round spermatids (Figs. 11.50 and 11.51). Again and again whenever round spermatids were found, elongated sperm with tails were found. So the goal of TESE is not to erroneously try to find immature round spermatids but rather to find the tiny number of normal sperm that do not exist in sufficient quantity to spillover into the ejaculate. So in truth those round spermatid enthusiasts were doing

**Fig. 11.49** This is a Hoffman optics view of round cells that consist of red blood cells, pachytene spermatocytes, and where the arrows point, to Sertoli cell nuclei with prominent nucleoli. These have been confused with round spermatids, even though they are Sertoli cell nuclei, i.e., somatic cells

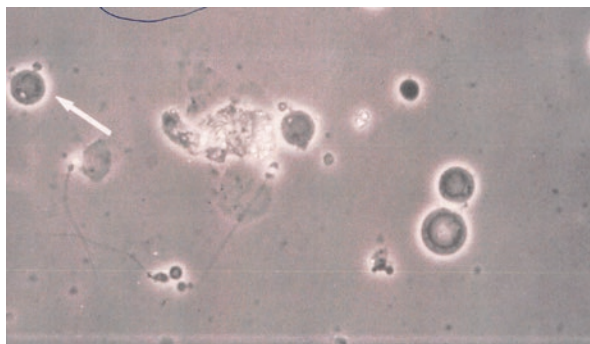
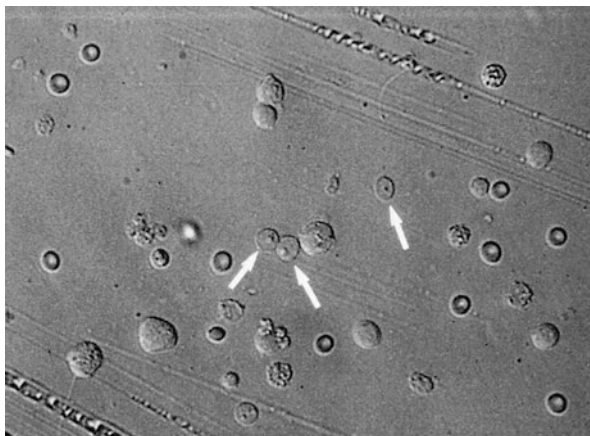


**Fig. 11.50** A phase contrast view of a TESE specimen with normal spermatid the arrows pointing to a typical round spermatid with its “glowing” acrosomal vesicle. The acrosomal vesicle is the absolute indication of an early round spermatid. Normally, if a round spermatid is found in a TESE specimen, there should also be mature elongated sperm. However the acrosomal vesicle is not always seen, even on phase contrast. It is never seen on Hoffman optics

somatic cell nuclear transfers. They even got cleavage (which of course can happen from any such injection). But many even claimed pregnancy and live baby, and this has been proven to be a fraudulent claim. So Dr. Larry Johnson and Dr. Andre Van Steirteghem and I investigated this and exposed this in *Fertility and Sterility*. We demonstrated that in the human, there are rarely round spermatids in the absence of elongated sperm with tails [63–65]. In fact a very famous doctor presented his demonstration at the HIFA in London and showed fraudulently these round spermatids “growing tails in culture” in an NOA case, and I had to counter this by demonstrating that this was just a photoshop re-creation. Nonetheless in some cases there may be round spermatid arrest, indicating ROSI should be performed [66].

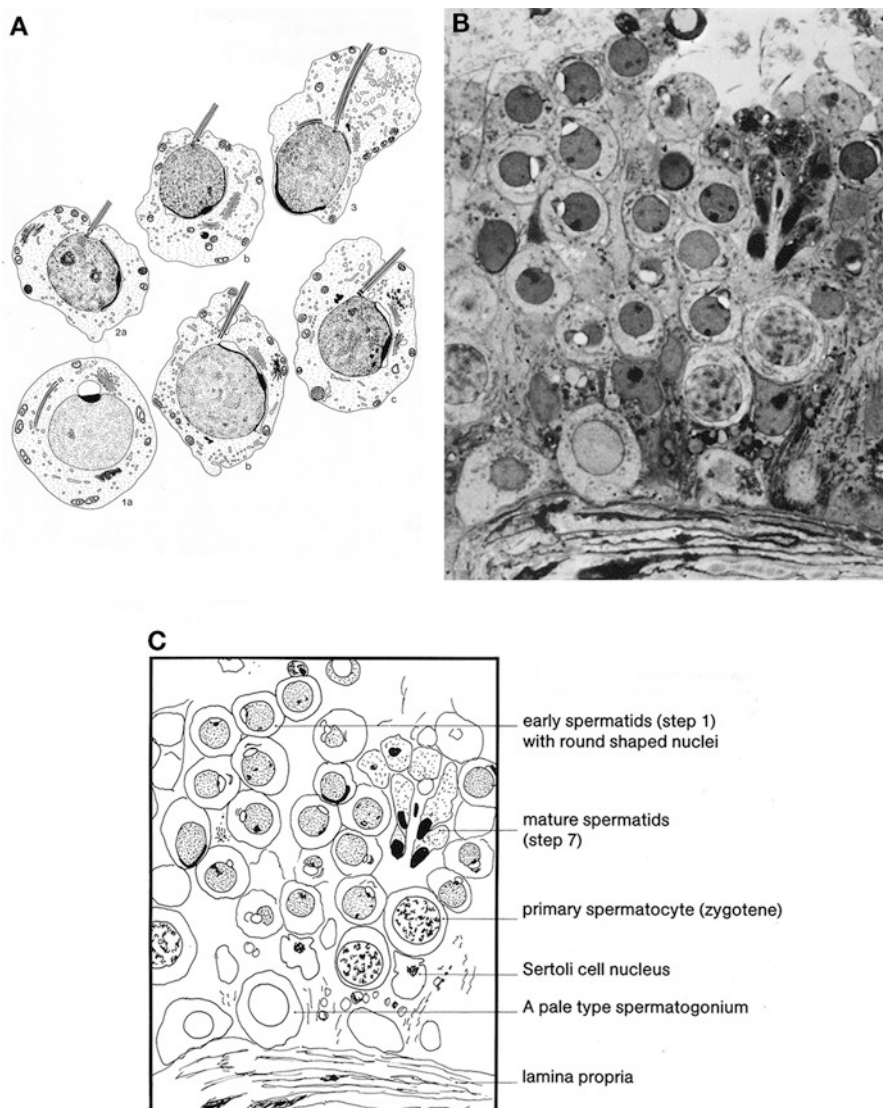


**Fig. 11.51** (a) Drawings of the stages of spermiogenesis after the second meiotic division has occurred. Before the formation of the tail, the round spermatid can always be recognized by the prominent acrosomal vesicle (1A). As the acrosomal vesicle recedes, the tail begins to form. (b) Electron micrograph of a section of human spermatogenesis demonstrating pale “type A” spermatogonia, Sertoli cell nuclei, pachytene spermatocytes, early round spermatids with acrosomal vesicle, and mature spermatids with an oval, dark staining head. (c) Diagrammatic depiction of 13B with labeling of the specific cells involved in spermatogenesis (from Holstein AF, Roosen-Runge ED, eds. *Atlas of Human Spermatogenesis*. Berlin: Grosse Verlag, 1981). (d) Tauaka et al. 2015 various types of spermatogenic cells seen after enzymatic dissociation of seminiferous tubules. Spermatogonia (red arrows and red arrow heads), primary spermatocytes (blue arrow heads), round spermatids (yellow arrow heads), and Sertoli cells (green arrow heads).

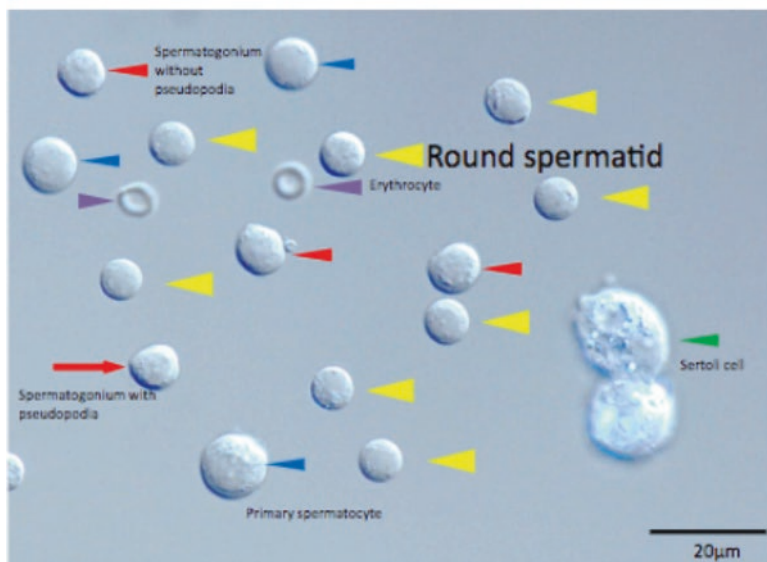
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Fig. 11.51 (continued)

### 11.11 Micro-TESE: What Is the Best Method?

The initial approach to TESE for nonobstructive azoospermia was crude, often involving numerous extensive biopsies from multiple areas of the testis until sperm were located. Legitimate concerns were apparent, including the following: (1) How do you counsel the couple to be prepared for IVF and ICSI (with all that it entails for the woman) when there is only a 55–60% chance that you will find any sperm? (2) Can you prognosticate which patients will have sperm successfully retrieved and which will not, so you may better advise who should and should not go through this procedure? (3) With such severely compromised testes, how do we assure the couple that they can undergo multiple repeat procedures with successful sperm retrievals in future cycles? and (4) Is it possible to simply freeze the unused sperm derived from a TESE procedure without diminishing the results and thereby avoid the necessity of having to time the woman's stimulation cycle to the man's sperm retrieval?

Whereas it is clear that good results can be obtained with frozen-thawed testicular sperm for cases of obstructive azoospermia, sperm retrieved from the testicle in nonobstructive azoospermia cannot be quite as reliably frozen and thawed with a result equivalent to that of fresh. Therefore, two major goals of ours were to determine (1) whether a previous diagnostic biopsy or any other test could predict the success or failure of testicle sperm extraction and (2) whether a technique for TESE could be used that would be relatively painless and would not compromise future attempts at fresh sperm retrieval. A small previous diagnostic testis biopsy is predictive of the likelihood of finding sperm in a TESE procedure in 85% of patients, but in 15% of patients, a small previous diagnostic testis biopsy was not predictive [58–60]. Our solution to this dilemma is a microsurgical approach to TESE (micro-

surgical TESE). However, we must warn that there are some microsurgical approaches called “microdissection” that are very damaging and not as reliable or safe as our stem cell-specific lobule micro approach.

In nonobstructive azoospermia, occasional mature spermatids are noted in the testis biopsy of men who might have been thought to have had no spermatogenesis (Fig. 11.33). At least three mature spermatids per tubule must be present in the testis biopsy for any spermatozoa to reach the ejaculate. More than 50% of patients with azoospermia with germinal failure thus have some minute foci of spermatogenesis, which are not of sufficient quantity to transport spermatozoa into the ejaculate. When spermatogenesis exceeds three mature spermatids per tubule, the patient has sperm “spillover” into the ejaculate and then has severe oligospermia rather than azoospermia.

Extensive multiple biopsies from every area of the testis are often performed in an effort to find sufficient sperm for TESE [58, 59]. This can result in a great deal of testicular damage and may even limit “successful” patients to only one attempt [58, 59]. Some try to limit damage by using needle rather than open biopsy to obtain sperm for ICSI [60]. However, control studies have shown that for difficult cases of nonobstructive azoospermia in which spermatogenesis is meager, needle biopsy is much less likely to find the rare foci of spermatogenesis for ICSI than is open biopsy [61, 62]. Furthermore as we have demonstrated in earlier sections of this chapter, penetrating to the center of the testis can cause both seminiferous tubule obstruction and vascular damage.

We studied the distribution of spermatogenesis in men with azoospermia and have outlined a microsurgical approach to TESE that minimizes tissue loss and pain and makes TESE easily repeatable for an indefinite number of cycles. Knowledge of the distribution of spermatogenesis and use of microsurgical technique help to prevent testicular damage and postoperative pain, making multiple repeat TESE procedures safe and reliable [23, 24]. By “microsurgical TESE,” I do not mean “microdissection” which attempts to remove only small amounts of tissue but in truth destroys a great deal of tissue by diffusely and indeed massively interfering with vascular supply.

There is a lot of unnecessary confusion about testicular sperm, mature spermatids, and round spermatids. For example, the tail of the sperm is seldom seen on histology, and only the thicker sperm head shows up in thin sections. It is usually only the oval head that is observed in histologic sections. Mature spermatids at TESE are no different in appearance than sperm. Furthermore, the solution to cases in which there are no sperm to be seen on TESE is seldom to look for “round spermatids” [63, 64]. We seldom see round spermatids in the human in the absence of mature spermatids, which at TESE are what just usually appear to be sperm (Figs. 11.51) [63–65]. However in some cases, there may be arrest at the round spermatid stage. So it is important to be able to recognize round spermatids (Fig. 11.51d) [66].

## 11.12 Best Method for Micro-TESE

All microsurgical procedures are performed using local anesthesia only. This involves both cord block and local infiltration of the incision line in the scrotum. The procedure is truly painless. In fact now that we use a combination of ropivacaine,

morphine, and Toradol in the local anesthetic mixture, there is no pain even for several days afterward. It is truly a virtually painless procedure for the man. The tunica vaginalis is opened and the testicle exteriorized. The operating microscope is then used with  $\times 16$  to  $\times 40$  magnification. Sometimes with Sertoli cell only, tubular dilation can be observed in a sea of tubules that are very thin and “stringy” so that just a tiny microscopic removal of single dilated tubules can be used to retrieve sperm. However, the larger looking tubules often also have no sperm, and an endless search can take place trying to find sperm by only picking at a few larger tubules and still finding nothing. Furthermore, in maturation arrest, all the tubules are normal size just like with normal spermatogenesis. So a better approach is needed to sample all of the testis. I call this the spermatogenic stem cell anatomic lobule approach.

Large strips of tissue (no greater than the total amount of peripheral tissue that would have been removed in the conventional “blind” TESE technique) can be excised if necessary, with no damage to blood supply or pressure atrophy. But it is crucial not to “dissect” into the substance of the testicle, as this causes devascularization and fibrosis. It is crucial to stay on the periphery of the testis, and all anatomic lobules must be sampled peripherally. If there is a single one niche of spermatogenic stem cells in that lobule, then sperm will be found peripherally. So it is all an issue of where any rare spermatogenic stem cells are located in that lobule because of the U-shape loop of the seminiferous tubule in that anatomic lobule. Therefore all anatomic lobules can be harvested peripherally.

The tunica albuginea is closed with 9-0 nylon interrupted sutures after meticulous hemostasis with micro-bipolar forceps (Figs. 11.52 and 11.53). This prevents any increase in intratesticular pressure, resulting in minimal pain and no subsequent atrophy. Using this approach TESE for nonobstructive azoospermia, up to 60% have sperm recovered. Even in those microsurgical procedures in which relatively larger amounts of tissue have to be removed, minimal damage is incurred because blood supply is not interrupted, all removed tissue is peripheral, microscopic bleeders are meticulously coagulated, tunica albuginea is not encroached on because of the closure with 9-0 nylon interrupted stitches, and consequently, there is no increase in intratesticular pressure (Figs. 11.52–11.59). A longitudinal incision at the dome of

**Fig. 11.52** Microsurgical closure of the tunica albuginea of the testes after a microsurgical TESE procedure results in no increase in intratesticular pressure and subsequently no loss of testicular function



**Fig. 11.53** A single, large, testicular incision for a TESE procedure using the operating microscope results in minimal to no testicular damage, minimal to no postoperative pain, and an ability to analyze each specific seminiferous tubule for the presence of spermatogenesis

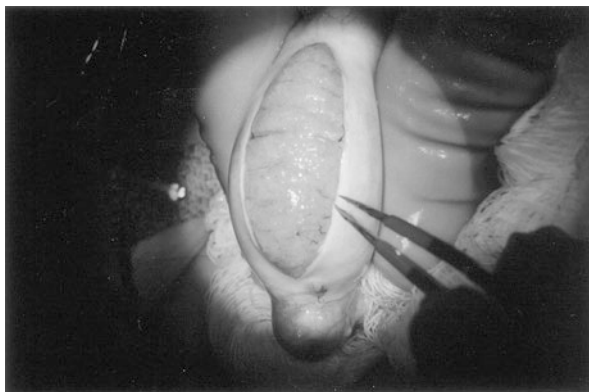


**Fig. 11.54** TESE for NOA: sample every anatomic lobule. If there is a single niche anywhere in any anatomic lobule, then sperm will be found peripherally in the U-shaped curve of the seminiferous tubule at the periphery

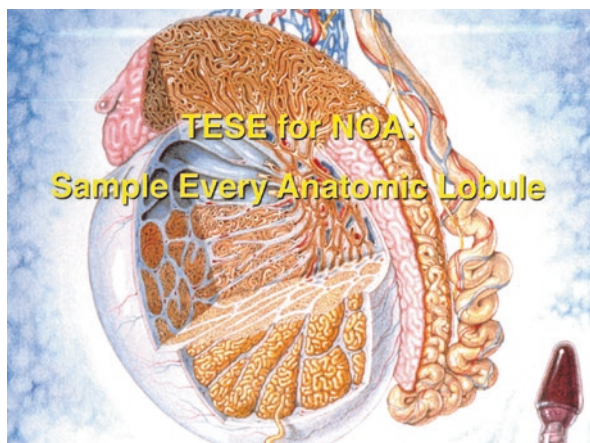
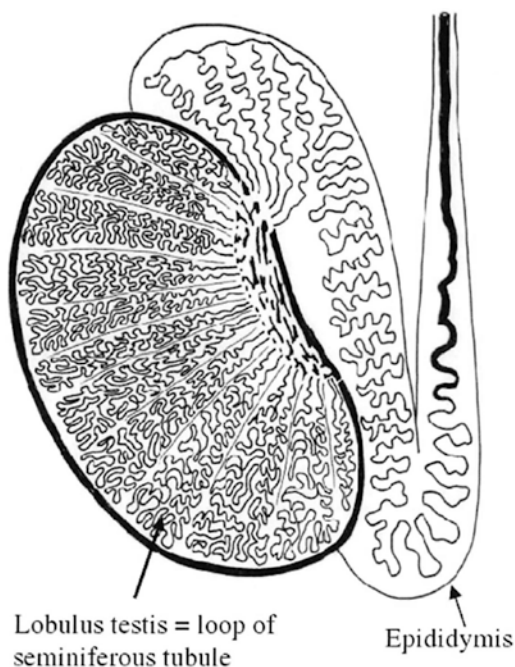


**Fig. 11.55** This diagram shows how all the seminiferous tubules loop toward the periphery



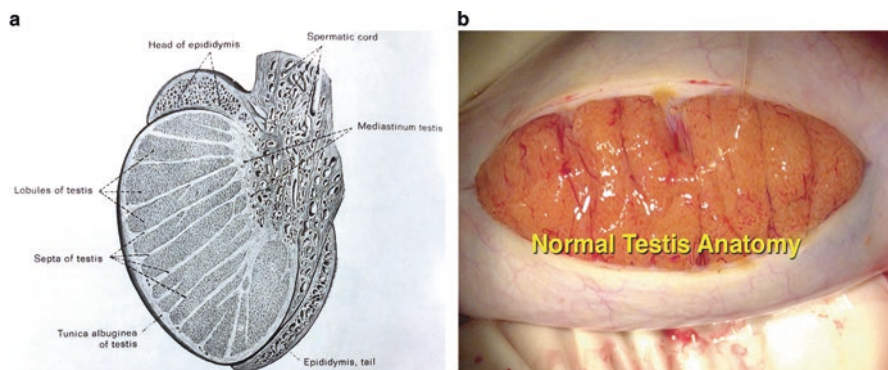
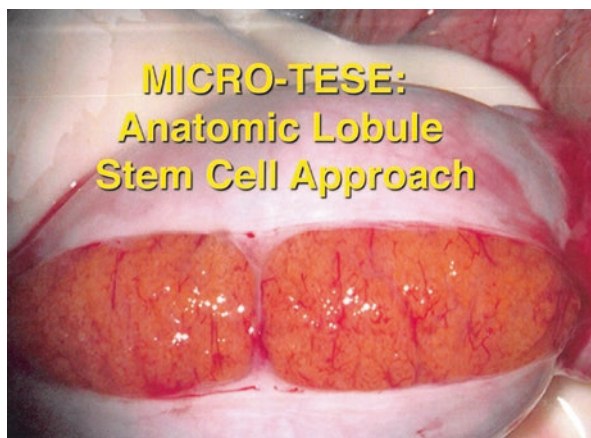


**Fig. 11.56** Anatomical lobules. This side section view of the testis demonstrates how you can sample every anatomic lobule by just staying on the periphery. (a) A longitudinal incision in tunica albuginea allows maximum exposure of the periphery of all anatomic lobules, with no damage to the blood supply

**Fig. 11.57** Micro-TESE: anatomic lobule stem cell approach. You can see the septa separating each anatomic lobule. Stay only on the periphery, and there will be no harm to the testis



the tunica albuginea of the testes allows full exposure of all tubules with minimal risk of damage. This approach results in no testicular damage and minimal pain.

Our direct mapping provides evidence for a diffuse rather than regional distribution of spermatogenesis in nonobstructive azoospermia [23, 25, 26]. Furthermore, the variation in sparseness of spermatogenesis verified by observation of contiguous strips of testicular tissue explains why a single random biopsy may or may not yield sperm and why with nonobstructive azoospermia, removal of small amounts of tissue blindly with a needle has a low success rate.

The formidable testicular deterioration that has been observed with overly aggressive TESE procedures such as “microdissection” is caused by either direct

**Fig. 11.58** Microsurgical closure 9-0 nylon. By closing the large tunica albuginea incision microsurgical after perfect hemostasis, there will be no intratesticular pressure that otherwise could cause pain and pressure atrophy. (a) After hemostasis with micro-bipolar forceps and pulsatile irrigation with heparinized saline, the tunica albuginea is closed with a 9-0 nylon interrupted sutures. (b) Completed closure of tunica albugenia, this approach results in no testis damage from the TESE procedure

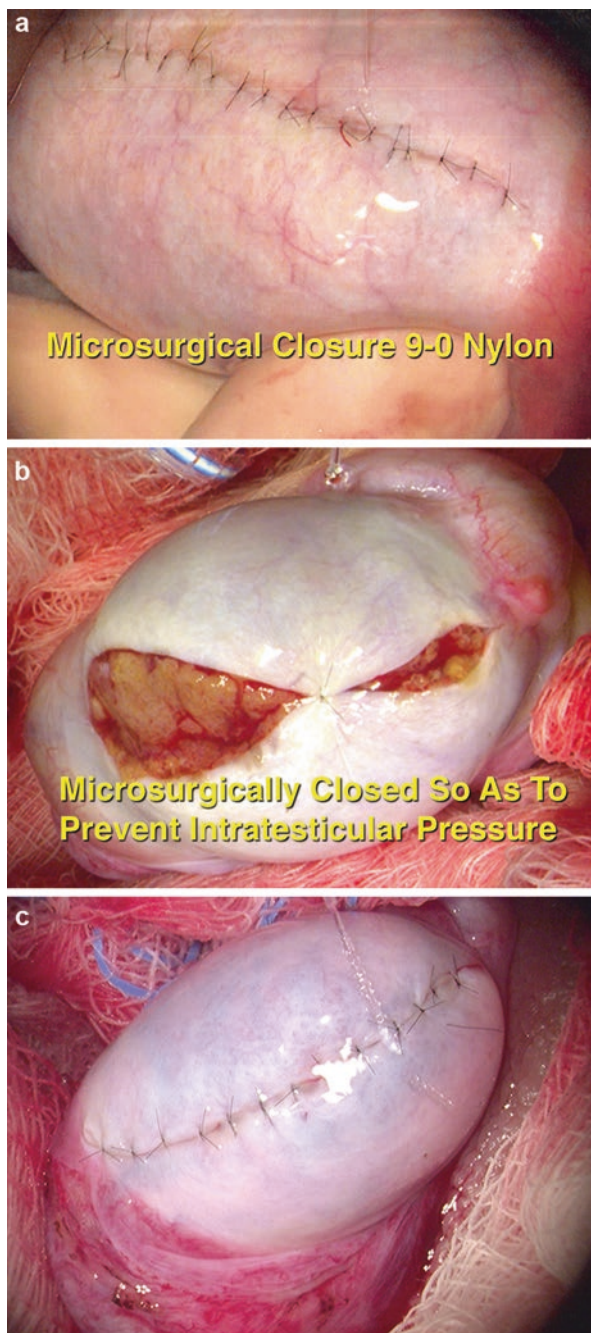
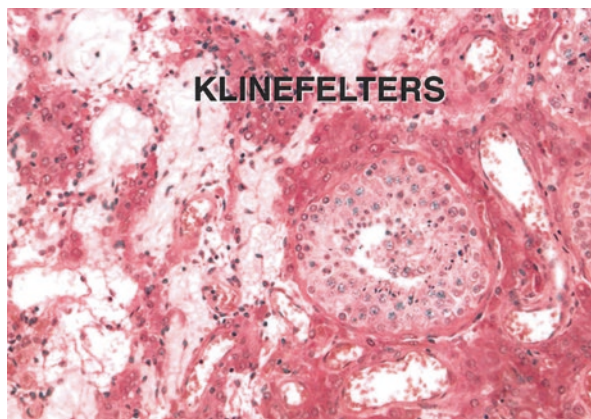


**Fig. 11.59** No adhesions 6 months post-op. This is the same testis as in Fig. 11.58. Note that there are no adhesions or other damage



**Fig. 11.60** Klinefelter TESE with small focus of a single tubule with normal spermatogenesis



interference with microvascular supply of the seminiferous tubules or by increased intratesticular pressure caused by minor amounts of bleeding within the enclosed tunica albuginea. The tunica albuginea is a nonflexible enclosure. A small degree of intratesticular bleeding causes a noticeable increase in intratesticular pressure. This can be readily observed by anybody performing conventional, multiple testicle biopsy samplings for TESE. Furthermore, the closure of open biopsies with the usual non-microsurgical suture, particularly in a running rather than interrupted fashion, with conventional TESE, further compromises the intratesticular volume and thereby adds to the increased pressure.

It must be noted that the recently popularized “microdissection” technique is ironically very destructive despite being microsurgical. The reason is that digging

deep into the testis is inevitably destructive and does not give a greater sperm recovery rate than staying on the periphery but sampling the periphery of every anatomic lobule.

Even men with the most tiny testicles, like with XXY Klinefelter or XO-XY male Turner, will often have a minute focus of a tubule with normal spermatogenesis. Such a tubule in completely abnormal testes occurs because these sex chromosomal abnormalities are mosaics with rare normal XY chromosomes even if the peripheral karyotype does not appear to be mosaic (Figs. 11.60–11.65). It should be noted that contrary to expectations, the majority of men with XXY Klinefelter or XO-XY male Turner have some focus of spermatogenesis and are amenable to micro-TESE. But these testes are so small that it is critical to do no harm.

**Fig. 11.61** Testis of a Klinefelter case. Doing TESE for such a tiny Klinefelter testis there is no normal architecture and abundant yellow tissue showing Leydig cells



**Fig. 11.62** Closure with 9-nylon interrupters of a tiny Klinefelter testis. A delicate perfect closure with 9-0 nylon interrupted is essential in such tiny testes to preclude the slightest damage

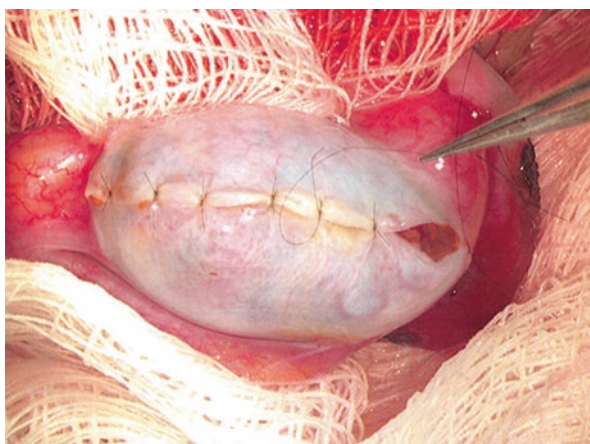


**Fig. 11.63** Tiny Klinefelter testis held in the surgeon's fingertips, sperm were found, and he now has two babies

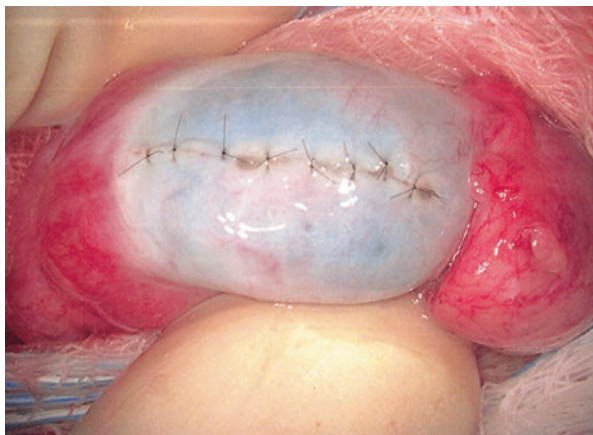


**Fig. 11.64** XY-XO mosaic Turner. These TESE cases in male tuners are very similar in appearance to XXY Klinefelter, and usually there is a small focus on a normal tubule with normal spermatogenesis

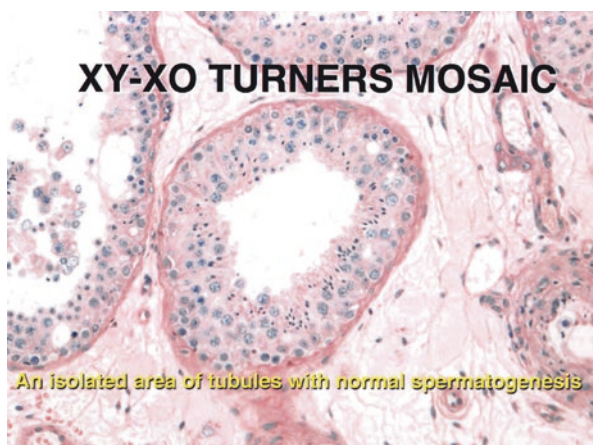
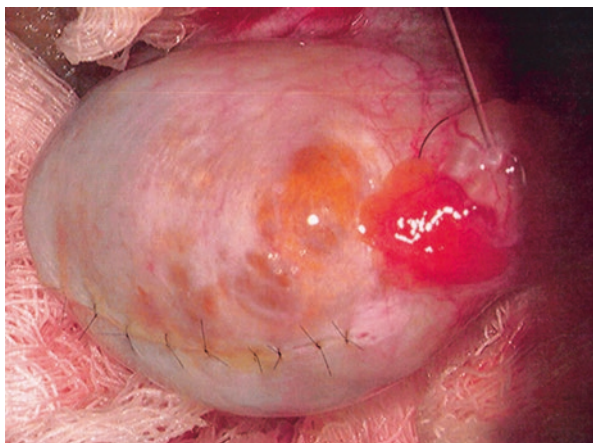


**Fig. 11.65** Microsurgical closure of XO/XY male Turner TESE where sperm were found, and now he has two babies. A very delicate 9-0 nylon closure of the tunica on this male Turner testis



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# Chapter 12

## Karyotyping of Infertile Males and Their ICSI Offspring



### 12.1 Karyotyping

A massive summary of karyotyping results in newborn populations, reviewed by Van Assche, was published to determine to what extent chromosomal abnormalities could be responsible for the diagnosis of azoospermia. It revealed an incidence of balanced translocations in a normal newborn population of 0.25%. A similar review of 7876 men with infertility undergoing karyotyping revealed an incidence of balanced translocations of 1.3%, more than four times that found in normal newborns [1–3]. When the analysis is restricted to men with oligospermia (i.e., less than 20 million per mL), some type of autosomal chromosome anomaly, either balanced Robertsonian translocations, balanced reciprocal translocations, balanced inversions, or extra markers, is found in 3% of patients. In azoospermic men, the incidence of such translocations was less than in patients with severe oligospermia but still greater than 1%. Sex chromosomal anomalies, such as Klinefelter's syndrome, were found in more than 5% of azoospermic men and in 1.6% of oligospermic men. It is known that Klinefelter's (XXY) occurs in 1/1000 (0.1%) of newborns. If 0.5% of men have azoospermia, we would expect 5% of azoospermic men to have XXY Klinefelter's.

The cytogenetic and pediatric follow-up of the first 1987 children born as a result of ICSI has been meticulously studied and reported by the originators of ICSI in the Dutch-speaking Free University [138, 139]. In 1082 karyotypes of ICSI pregnancies, nine (0.83%) had sex chromosomal abnormalities, including 45 X (Turner), 47 XXY (Klinefelter's), 47 XXX, and mosaics of 47 XXX, as well as 47 XYY. This is a very low frequency but nonetheless is four times greater than the expected frequency of sex chromosomal abnormalities in a newborn population (0.19%). Four (0.36%) of these 1082 children had de novo balanced translocations or inversions (Table 12.1) [2, 3]. These children were apparently normal, but this incidence of de novo balanced translocations is five times greater than what would be anticipated in a normal newborn population (0.07%). It is felt that this is not due to the ICSI

**Table 12.1** Chromosome abnormalities of ICSI offspring in comparison to control populations (Silber et al. 2012)

| Type of chromosome abnormality | ICSI offspring (1586) | Spontaneous pregnancy (56,952) | Spontaneous pregnancy (34,910) | Amniocentesis females >35 years of age (52,965) |
|--------------------------------|-----------------------|--------------------------------|--------------------------------|-------------------------------------------------|
| De novo                        | (25) 1.58%            | 0.45%                          |                                | 0.87%                                           |
| Sex chromosome                 | (10) .63%             | 0.19%                          | 0.23%                          | 0.27%                                           |
| Autosomal                      | (15) .95%             | 0.26%                          | 0.61%                          | 0.60%                                           |
| Numerical                      | (8) .50%              | 0.14%                          |                                | 0.33%                                           |
| Translocations (de novo)       | (7) .44%              | 0.11%                          |                                | 0.19%                                           |
| Transmitted                    | (22) 1.39%            | 0.47%                          |                                | 0.33%                                           |
| Balanced translocations        | (21) 1.32%            | 0.45%                          |                                | 0.27%                                           |
| Unbalanced translocations      | (1) 0.06%             | 0.023%                         |                                | 0.07%                                           |
| Total                          | (47) 2.96%            | 0.92%                          | 0.84%                          |                                                 |

procedure but rather to the nature of the severe oligospermic spermatozoa. Finally, there were ten cases of translocations inherited from the infertile male (0.92%), and although nine of these ten were balanced translocations in normal newborns, one (0.09%) was an unbalanced translocation that was terminated. This incidence of cytogenetically recognizable chromosomal abnormalities in the offspring of patients undergoing ICSI is extremely low but much greater than what would be anticipated in the normal newborn population.

Because approximately 2% of oligospermic men have chromosomal translocations (compared with a control population of 0.25%), it is not surprising that 0.9% of offspring resulting from ICSI would inherit such a translocation from their father. It is somewhat reassuring that only 10% of those inherited translocations were unbalanced and 90% were balanced. The pregnancy was terminated in one unbalanced transmission of a paternal chromosomal translocation, and this represents an incidence of only 0.1% of offspring resulting from ICSI. However, the other nine translocations that were transmitted in a balanced manner, with “normal” offspring, are likely to potentially have the same infertility defect as their father (0.83%).

Thus, of the first 1082 offspring resulting from ICSI and undergoing meticulous prenatal diagnosis and karyotyping, we can anticipate that almost 2% (based strictly on cytogenetic studies) will be infertile or sterile, which is more than fivefold of what would be anticipated in a normal newborn population. However, the karyotype study of these offspring is more reassuring than alarming. The incidence of congenital abnormality in children resulting from ICSI (2.3%) is no greater than in every normal population studied [2, 3]. Even in the reported ICSI offspring of Klinefelter’s syndrome, patients have normal chromosomes. There is no greater incidence of autosomal aneuploidy than what is predictable from maternal age. Sex chromosome aneuploidy (0.83%) is not a high incidence, although it is clearly greater than normal. Thus, the evidence based on cytogenetic and pediatric follow-up of offspring

resulting from ICSI is reassuring, despite the possible occurrence of infertility and sex chromosomal disorders in a very tiny percentage of patients.

## 12.2 Klinefelter's Syndrome

Klinefelter's syndrome (47,XXY) is found in a frequency in the general population of 1:1000 males. There is a very wide phenotypic spectrum, but all males with Klinefelter's syndrome will demonstrate testis deficit as both the spermatogenic and androgenic capabilities of the testes are dysfunctional. It is a wide phenotypic spectrum most likely because of mosaicism, just as in Turner. If virilization is delayed or absent (the most severe cases) at the expected time of puberty (the "classical" presentation of Klinefelter's syndrome), referral may be made to a pediatric endocrinologist where the diagnosis will be discovered and testosterone therapy initiated. Alternatively, teenage boys who are virilizing properly but who have small testes found on physical examination may be diagnosed for that reason and have some spermatogenesis present.

However, most Klinefelter's men are discovered only at the time of infertility evaluation (or with their mother's prenatal diagnosis). They would have virilized adequately during the time of puberty and escaped detection and are perfectly normal aside from the infertility. Typically libido and erectile capability are normal in these men. Therefore, although it is commonly thought that all Klinefelter's males will have a eunuchoid body habitus, it is not true. Their phenotype appearance depends on testosterone production, and they have no other discernible phenotypic abnormalities.

The vast majority of non-mosaic Klinefelter's males will be azoospermic. All will show marked elevation of FSH and LH, reflecting compensatory pituitary secretion due to impaired spermatogenesis and testosterone production. Gynecomastia may be present reflecting a relative increase in the estradiol/testosterone ratio. Because often they have normal mosaic XY cells that are not found with routine karyotyping, they can make rare but entirely normal sperm. Turner syndrome girls with X-O karyotype are not always girls missing an X chromosome but rather are "boys" missing a Y chromosome. Although there was a hypothetical fear that the Klinefelter's ICSI offspring could exhibit numerical chromosomal anomalies themselves, there have been no aneuploidy offspring reported in the literature. That is because a few foci of 46,XY spermatogonia may be present in these patients, reflecting gonosomal mosaicism. Therefore, 47,XXY men can achieve biologic paternity and normal offspring with the help of ICSI. There also can be Turner syndrome males who have an XO\_XY karyotype. They are short, but normal otherwise, and will be found (like XXY men) to have some normal sperm in their testes during TESE.

Although sex chromosomal abnormalities can account for 7–15% of azoospermia, and autosomal anomalies another 1–2%, most commonly, the karyotype of azoospermia is normal. However, molecular study of the x and y chromosomes

reveal that observable numerical karyotype chromosome abnormalities are only the tip of the iceberg. The sperm count even in oligospermia is most likely genetically determined. But one has to go beyond karyotyping to detect most genetic etiologies of male infertility. We will discuss that in the next chapter [1–4].

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## Chapter 13

# Donor Sperm



In the past, if the husband had no sperm in his ejaculate, the only solution was for the wife to have artificial insemination with donor sperm. Since we have developed the ICSI technique, there are now very few couples who would need to consider donor sperm, because the vast majority of previously hopeless cases of male infertility can now be treated successfully with ICSI. In most cases in which ICSI is not successful for dealing with male infertility, it is because of the wife's eggs, not the sperm. However, there are still a small number of cases in which no sperm at all can be found with TESE-ICSI or in which the sperm are completely abnormal. Therefore, the need for donor sperm banks may have dwindled, but some couples will still have this need. The purpose of this chapter is to explain the process of using donor sperm for couples for whom there is no alternative.

In a sense, using donor sperm is really no different from adoption. It is simply a matter of adopting sperm. You're adopting the baby at a much earlier stage—prior to conception. Using the sperm of a well-selected anonymous donor is the most realistic and sensible solution for the few couples who simply have no sperm at all, even in the testes. The use of donor sperm or donor eggs (or even donor sperm and donor eggs) has tremendous advantages over classic adoption for parental bonding and child development, not to mention less cost.

### 13.1 Is It My Baby?

There has always been a heated debate about where a child's personality and abilities come from, its environment or its genes. After counseling many hundreds of couples who have used donor sperm and having seen the results over the last 30 years, as well as having studied rather extensively the early childhood development research coming from the Harvard and the Ypsilanti early childhood projects, I have some definite views that could be of benefit to couples who are facing this issue of whether to use donor sperm (or donor eggs). It is obviously preferable emotionally

to do whatever is possible to allow patients to have their own genetic baby using the husband's sperm. However, if such a solution is not at hand, the following observations should be seriously considered.

The divorce rate among couples who choose to elect donor sperm is less than 1%, even though the general divorce rate in our population is over 50%. This is not because the decision to have a baby by donor insemination in some way holds the marriage together. Rather, couples who wind up choosing this route have a solid relationship and a good basis of communicating with each other. Couples who have any flaws or weaknesses in their ability to communicate on tough issues usually won't choose donor insemination. Couples who do use donor insemination are a select group that have an extraordinarily solid marriage. They can deal with a potentially divisive issue and come to a common understanding that makes them both happy. So if a couple decides to use donor sperm, it is a very good sign that the marriage is going to remain solid.

Couples will frequently ask how the baby will do when its genes come half from the mother and half from some stranger. Very often they will say, "Well, at least if we go this route, as opposed to adoption, the baby will be one half ours." This outlook is a strong reason *not* to have donor insemination. My observation has been that when the husband and the wife both accept the baby as being 100% theirs, and take the view that the genetic contribution is of no significance, then the father-infant bonding is completely normal.

Although there is a controversial view held in some circles that a child's personality, intelligence, and athletic ability are basically genetically transmitted and that there is only a partial contribution from parental rearing, evidence from most donor sperm and donor egg couples, and from early childhood education projects, strongly refutes this commonly held genetic bias. In truth, the child's personality, intelligence, and even athletic skill (though not size, hair color, eye color, or body build) are overwhelmingly related to how he or she was raised in the first several years of life.

Children in the first year and a half of life are, in many respects, like parrots. They learn by copying what they see around them. A personality tends to emerge more clearly as a sense of individual identity around the age of a year and a half, when language skills first become readily apparent. The way in which a nongenetic offspring mimics his or her rearing father, regardless of genetic origin, is so striking that these parents are sometimes shocked and do double takes when a friendly neighbor (who knows nothing about the child's genetic origin) comments admiringly on how the child is the spitting image of his father. In couples who elect donor insemination, most of the time the father's bonding with the infant is no different than if it were his own "genetic" child.

The interesting thing is that the courts take a similar view in ruling on cases where a husband might allege that his wife got pregnant by having an affair with another man and that the child his wife bears is not really his. The courts define the father as the person who is living with the woman and are not terribly concerned about whose sperm fertilized the mother. (Of course, the courts have a different agenda in that their major concern is establishing a responsible father to handle

financial arrangements for the child's future care.) Still, it is of interest that the official legal view does not contradict what the latest studies on early child development demonstrate.

It is true that we have all witnessed how a 2-year-old's personality, intelligence, and skill level are quite predictive in many cases of how that child will eventually turn out as an adult, and this observation leads many to conclude unquestioningly that "it's just all genetic." That viewpoint is enhanced even further among natural parents (who have not required any infertility treatment) who are in one way or another unhappy with how their child has turned out. Even though it may be "their own genetic child," they blame the result on "genes," assuming that the child just had the bad luck of getting the parents' worst genes, because it takes away any possibility that they might have to blame themselves and their rearing efforts. There can be tremendous attractiveness in being able to allow genetics to shoulder the responsibility for how a child turns out. These erroneous "genetic" arguments can raise fear among couples who are considering donor sperm.

It is clear to me that in every case I have studied where parents have been unhappy with how their children turned out, the child's problems can be traced to the parents' dysfunction as parents and not to any genetic predetermination. When the final human genome sequence papers came out in *Science* and in *Nature*, the molecular geneticists who actually sequenced the entire human genome warned against two myths: determinism and reductionism. It was clear to them that who a person becomes is *not* in his or her genes.

Nonetheless, couples electing to use donor sperm prefer to make sure that the sperm comes from the healthiest, most intelligent possible source, and they might want to match up hair color, skin color, eye color, body build, etc. A good donor sperm program will pay close attention to these selective characteristics so that the couple may get as close a physical match to the husband as they'd like.

## 13.2 How to Select the Donor

Artificial insemination was first successfully used in women by the famous physician John Hunter in England in the eighteenth century. In 1890, Dr. Robert Dickinson of New York was the first to use donor sperm for women whose husbands had untreatable infertility. This early use of artificial insemination with donor sperm was carried out in great secrecy. However, it became so popular by 1990 that more than 30,000 babies were being born every year in the United States as a result of it. Patients are accepting it when there is no other solution, and most of the psychological, social, and legal fears about it have disappeared.

In 1964, Georgia was the first state to issue legislation that guaranteed that a child conceived in this manner would be considered legitimate. Oklahoma passed a similar law in 1967, as did Kansas in 1968, and all other states followed suit. Even prior to these legislative decrees, common law provided some protection for the legitimacy of such children. Unless it is proved that the husband had no access to the

wife, any child born of her is considered to be his by the law. Whether or not the husband is the true father, he is the legal father of any child born to his wife while they are living together.

From a medical point of view, artificial insemination is extraordinarily simple. A sperm specimen obtained from the donor is drawn up into a syringe, and then either simply squirted into the vagina near the cervix or washed and placed with a catheter into the uterus. Since the sperm are only capable of fertilization for about 48 h in the female reproductive tract, and since the egg is only capable of being fertilized within 8 h of ovulation, the insemination must be timed appropriately just before ovulation. However, the pregnancy rate per cycle with donor sperm is much higher per attempt if it is used with IVF (60%) rather than with simple insemination (12%).

There are very strong reasons to use ICSI-IVF for donor sperm cases, instead of just insemination or IUI. Firstly the timing with IUI to ovulation is inherently flawed because the egg is so briefly fertile after ovulation. Secondly, just one single specimen is all that is needed for all the children the couple might want. Thirdly, the time till pregnancy is shorter and less emotionally trying. Fourthly, it is less stressful to the relationship to have washed individual sperm injected sucrose capsule into the wife's eggs, than to have after semen's squirmed into her vagina.

All donors have to be checked for the presence of hepatitis, AIDS, or venereal disease. Because of the possible 6-month incubation time of the AIDS virus, frozen sperm is virtually always used today (as opposed to 10 years ago), because it allows the donor to be observed for 6 months after the collection of the sperm and have a repeat blood test for AIDS. Only if his blood test for AIDS is still negative 6 months after donating the sperm is his sperm used for donor insemination. Furthermore, the donor is screened for any history of genetic disease in his family. Even a history of diseases that are only partially genetically transmitted, such as diabetes, would generally rule him out as a sperm donor. The specimen is also carefully cultured for bacteria, such as gonorrhea or chlamydia, and the remainder of the sample is prepared for freezing and storage.

It has always been thought best for the sperm donor to be anonymous because problems could arise if the genetic father is known to the couple. With a greater openness in our society, and a better understanding of the relative lack of significance of genetic contribution as opposed to rearing, some physicians (though not the majority) are becoming more open to allowing selected couples to use sperm from donors they know. However, the vast majority of couples prefer anonymous sperm from a well-respected donor sperm bank rather than use a known sperm donor.

It is generally preferable to obtain donor sperm from a reputable sperm bank located in a region completely different from your own. Because of the requirement that anonymous donors' sperm be frozen in order to make certain that the donor doesn't become AIDS positive over the ensuing 6 months, before the sperm is used, and because of the meticulous detail that couples using donor insemination have a right to expect regarding the characteristics of the donor, many physicians are turning more and more to formally operated and carefully regulated frozen sperm banks. One of the very best sperm banks in the United States is the California Cryobank. It

makes shipments of sperm all over the world and is presently the largest sperm bank in North America. It provides a detailed pedigree of every single donor, including race, hair color, eye color, skin color, body build, religious preference, nationality, degree of education, and even hobbies. In truth, all that really makes a difference is that there is no history of genetic disease; that the donor doesn't have hepatitis, AIDS, or venereal disease; and that he is a reasonable match of inherited appearance. The other issues, such as level of education achieved, hobbies, and interests, are simply a way of reassuring future recipients who might have lingering doubts about the possibility of any broad genetic transference of intellectual ability or personality.

There are so many cases of short parents having tall children that are genetically their own, of dark-haired parents having a redheaded child, that all of us are aware of the importance of recessive genes expressing themselves unpredictably in children that are genetically our own. In fact, even if there is a perfect matching of the donor to the recipient, the child may have a very different appearance because of these recessive genes.

The sperm bank should also make sure that no particular donor is overused. Population scientists have made it very clear that if a particular donor were to be used in more than ten different couples around the United States, there would be an ever-so-slight risk of a future unknown first-cousin marriage if the offspring of any of these families were to ever meet and get married. The temptation for a poorly organized sperm bank would be to use a particular donor over and over again and thereby avoid all of the expense related to the selection of new donors. Despite the increased cost, it is important that the sperm bank resist any such overuse of its donors, and that is why only the most reputable sources for sperm should be used.

There was a great deal of publicity in the early 1980s associated with the founding of the Nobel Prize Sperm Bank. This bank was started in the San Diego area by a very old, former Nobel Prize winner (who has subsequently died) who received his prize for inventing the transistor. It was his firm belief that intelligence was genetically transmitted and that the future of our society depended upon sperm from these most intelligent men being used for donor insemination. His sperm bank was never really taken very seriously and never caught on. As one of my patients put it: "If genes do have anything to do with superintelligence and winning Nobel Prizes (which I don't believe they do), then we should be using the sperm from Nobel Prize winners' fathers, not from the Nobel winners themselves. By and large, the children of Nobel Prize winners are not any more distinguished than children born from parents with less illustrious minds."

The brain of modern man is really no different from the brain of Cro-Magnon man, who lived 40,000 years ago in caves and drew crude paintings on the wall. The human brain has been able to take us as far as we have come not because of inborn abilities, but rather because of its remarkable flexibility and capacity to learn. If I were born 40,000 years ago in a Cro-Magnon cave, I would not be a sophisticated microsurgeon or in vitro fertilization specialist. Nor would I have developed the complex language abilities that have allowed me to write this book. My brain would have developed in other directions appropriate to allowing me to figure out how to

survive in an entirely different, more primitive world. If a genetically Cro-Magnon child were to be born today and reared by parents who encouraged the spark of curiosity, enthusiasm, and intellectual challenge, he or she would be just as likely to win a Nobel Prize as the child of anyone else born today.

### 13.3 Frozen Sperm and Sperm Banks

All people dream from time to time about the possibility of immortality. Science-fiction novelists frequently toy with the idea of human beings being placed in a deep freeze just prior to the moment of death, to be revived perhaps 200 years later, at which time science may have better treatments for illnesses and a way of prolonging life indefinitely. Life is, in a sense, a series of chemical events proceeding irreversibly toward death, and these chemical events cannot take place at  $-196^{\circ}\text{C}$ . Thus, if an organism could be “safely” placed in a deep freeze, it could be preserved until a future century and revived with subsequent warming.

Of course, freezing large animals would kill them immediately because of damage created by crystallization of water within their cells during the freezing process. However, it has been known since 1776 that human sperm are remarkably resistant to the damaging effects of freezing (because there is so little water in them). In that year, an Italian scientist exposed spermatozoa to freezing temperatures and noted that, after warming, some of them regained their motility. It was speculated then that frozen semen might be used not only in breeding the finest farm animals but also for saving the sperm of a man going off to war so that his wife might have a child from him even though he had already died on the battlefield. Although these crude, early studies established that sperm could survive freezing and thawing, the sperm were so terribly damaged that there was no possibility of practical application.

But in 1949, British scientists discovered, completely by accident, that if a relatively common chemical, glycerol, is added to the semen before it is frozen, the majority of the sperm survive freezing and thawing uneventfully. The researchers who made this discovery were so surprised to find live, healthy sperm in large concentrations after thawing that they had to go back to their laboratory shelf to find out which of the chemicals accidentally added to their sperm suspension was the one that protected the sperm against freezing. They finally discovered that it was glycerol. It took very little time after their remarkable discovery for frozen sperm banks to find acceptance in the field of cattle breeding, and today, the vast majority of calves born in the world are the result of artificial insemination from frozen bull semen. In 1953, 4 years later, it was demonstrated that frozen and thawed human sperm could result in pregnancy and the delivery of normal babies. The first human sperm bank was established the next year.

With the advent of the ICSI technique, no matter how poorly sperm might thaw out after the freeze, they can be used quite successfully unless there is absolutely zero motility whatsoever, which is quite unusual. Therefore, although sperm of the majority of men do not freeze well enough for a subsequent pregnancy to occur with

simple insemination, the sperm of almost all men will freeze adequately for subsequent pregnancy with ICSI.

Sperm freeze better than most other cells because there is so little cellular water content. The sperm head is basically an extremely compact, dense arrangement of DNA with much less water content than any other cell. Therefore, there is very little intracellular ice crystal formation to damage it. Nonetheless, even sperm require some sort of cryoprotectant, in this case glycerol, whose function is to pull water out of the cell and to get inside it to act as a sort of antifreeze, preventing ice formation from any water still remaining inside. Subsequently, there was further improvement in cryoprotection by adding test yolk buffer to the glycerol and diluting the sperm 50% instead of 10% in the cryoprotectant.

There is no increased risk of birth abnormalities from frozen sperm. Whatever harm may come to sperm from freezing, either in the sperm's structure or ability to fertilize, there does not appear to be any increased risk of defective children. Extensive experience using both cattle and humans has now documented that frozen sperm from sperm banks is safe. Literally hundreds of thousands of normal pregnancies and births in humans using donor sperm have been reported in the scientific literature.

Perhaps the most exciting benefit for sperm banking in the modern era is that because of the development of the ICSI technique we can freeze the sperm of anyone who is about to undergo a vasectomy, chemotherapy, or radiation for cancer or a sperm aspiration procedure and who doesn't want to have multiple surgeries for future attempts at pregnancy. For all these conditions, we can freeze the man's sperm now, no matter how poor its quality, and offer a high chance of pregnancy once this sperm is thawed, if it used in conjunction with ICSI. Freezing of sperm has been attempted in the past with men who were about to undergo cancer chemotherapy, but it wasn't used very frequently because the chance for pregnancy from any one insemination would be very low. To have any kind of assurance, the patient might have to collect 20 or 30 samples of sperm to freeze prior to undergoing cancer treatment, and this would be too long a delay for his cancer treatment. Now, with ICSI, it is simply a matter of having one single sperm sample frozen and saved, and then the man can have whatever treatment he needs that may subsequently make him sterile, without the fear that he will not be able to have his own genetic child someday in the future.

### **13.4 Insemination with Donor Sperm or ICSI with Donor Sperm**

Not all infertility specialists agree, but I believe that if donor sperm is used, it should be used in conjunction with ICSI, not just insemination. There are several reasons for this. First, IVF with donor sperm gives a 60% pregnancy rate (or more) per cycle, while simple donor sperm insemination gives only a 12% pregnancy rate per cycle. You are thus five times more likely to get pregnant in any given donor sperm

cycle using IVF. So in the long run, IVF is actually more cost-effective than artificial insemination. Second, the emotional impact of going through multiple unsuccessful donor sperm inseminations month after month can be daunting. If you don't get pregnant in one of the early donor sperm insemination cycles, you may even run out of that donor's frozen sperm sample and then have to go through the trouble of selecting yet another donor. With IVF, you are more likely to get pregnant earlier, and with ICSI, you need never run out of that particular donor's sperm.

Most couples would prefer to have the same genetic origin for each of their children. With ICSI, just one single frozen specimen will be enough for you to have all subsequent children from the same donor. For each ICSI procedure, we just scrape a tiny chip off of the frozen pellet, leaving almost all of the rest of the sperm still frozen. One single donor sample can be used for hundreds of cycles. You do not have to worry that your original donor has disappeared or refuses to donate or that the sperm bank might have exhausted all of that donor's deposits. Most patients confronted with the choice of insemination or ICSI with donor sperm agree fully that ICSI is the better choice.

# Chapter 14

## Cryopreservation of Sperm



### 14.1 Introduction

The basic principles for cryopreservation of sperm are not different from cryopreservation of embryos, eggs, and ovarian tissue. However freezing with sperm is much easier because there is such a minute amount of water in this small, otherwise highly specialized gamete. In fact very little advance has been made in sperm freezing research, because the results seem to be so adequate with the simplest of methods [1–22]. However I will describe, in addition to routine sperm cryopreservation, a simple new method for freezing individual sperm for difficult cases of TESE.

Slow cooling results in preferential freezing on the outside of the cell where the osmolality is lower. This draws water out of the sperm making the inside even more hyper-osmolar. Thus as the temperature cools gradually and ice forms preferentially on outside of the cell, that pulls water out of the cell, and then cryoprotectants enter the cell and lower the freezing point inside the sperm cell even further. Eventually the inside of the sperm actually vitrifies when the temperature is low enough and the osmolality is high enough in this extremely low-volume cell. This slow cooling need not be so precise as it is with using a controlled rate freezing machine (like for slow freeze of embryos). In fact one can simply have a vial containing sperm mixed with cryoprotectants over an open liquid nitrogen dewar, vapor freeze it, and then plunge it into the tank. Before holding over liquid nitrogen vapor, it can be first put in an ordinary refrigerator at 4 °C. It is amazingly simple. Despite the crudeness and lack of graduality of such sperm freezing and thawing protocols, the effectiveness of this vapor slow freeze approach for sperm cryopreservation is very good.

## 14.2 Methodology Details

For sperm freezing and simple testicular tissue freezing in sophisticated studies documented by Brinster, Sadri-Ardekani, Shinohara, and Van Pelt with sperm and spermatogonial stem cells (SSC'S), the best results were remarkably obtained with just this simple vapor freeze. A media containing test yolk buffer provided by Irvine Scientific (TYP) with glycerol and gentamycin is the usual favorite. It contains 20% egg yolk USDA certified and 12% glycerol. The steps are as follows:

1. The sperm simply liquefies at room temperature.
2. Use a thawed 5 m vial of medium.
3. Add liquefied sperm sample drop by drop very slowly over 30 s until there is a 50:50 ratio of semen or sperm to media, and mix slowly by slowly very thoroughly. A failure to do this mixing slowly, and drop by drop, will result in complete failure.
4. For this manually assisted vapor freeze method, we recommend the mixture should be placed in the refrigerator (2–5 °C) for an hour before putting over the liquid nitrogen vapor.
5. Then after an hour, plunge the vial into liquid nitrogen at –196 °C for storage.

We have sent difficult samples to complex research programs all over the world with this simple approach of sperm freezing, and indeed testicular tissue freezing, and remarkably we are told this works well with no need for any further improvement. So this simple sperm freeze protocol using glycerol and egg yolk, with vapor phase, freeze followed by plunging, is quite adequate for sperm and for testis tissue. We are benefited by the tiny water content of sperm and by the very small size of the sperm cell. Glycerol is a reasonable cryoprotectant for sperm, but the TEST yolk gives better results by stabilizing the membrane.

## 14.3 Single Sperm Cryopreservation

For freezing single sperm, or just a few sperm derived from TESE with non-obstructive azoospermia, one can purchase a small platform with tiny microwells and put just 1  $\mu$ L of sperm freezing solution in each tiny well (Figs. 14.1 and 14.2). Slip that tiny platform into the ICSI dish and pick the individual sperm from the ICSI dish using the injection pipette. Then drop each sperm into the one microliter droplet of cryoprotectant solution. Then plunge that whole tiny platform into liquid nitrogen directly after holding in vapor. With this approach, even if only a few sperm are found in an ejaculated or in a TESE specimen, they can be successfully cryopreserved, just as readily as a normal semen sample from a fertile man with conventional freezing.



**Fig. 14.1** Sperm boat device Mix a 1 to 1 solution of the 10% SPS with HEPES and Quinn's Sperm Freezing Media (contains glycerol) Sperm boat device needs  $\leq 1 \mu\text{L}$  per drop of freezing media mix Cover droplets with enough mineral oil to fill moat With a sharpie, circle droplets Pick up sperm from patient's ICSI dish Load an ICSI needle with  $\sim 25$  viable motile sperm per droplet Do not immobilize sperm Locate droplet and expel the # of sperm retrieved into the droplet Put the sperm loaded boat device into a cryovial with a rounded bottom Hold device in vapor for a 60–90 s using forceps Drop device into the liquid nitrogen When thawing, remove the device from LN<sub>2</sub> Open the cryovial and remove the sperm boat with forceps Allow the sperm boat to come to room temperature; oil and droplets will be opaque at first When clear and transparent, check the droplet for motile sperm Add pentox if needed



**Fig. 14.2** Sperm VD device (tray like) Mix a 1 to 1 solution of the 10% SPS with HEPES and Quinn's Sperm Freezing Media (contains glycerol) Sperm VD device needs  $\leq 1 \mu\text{L}$  per drop of freezing media mix in each circle Place the sperm VD device into an ICSI dish; located parallel to PVP drop Device is thin and will easily be covered with the mineral oil Pick up  $\sim 25$  viable, motile sperm from the PVP drop Do not immobilize sperm Locate the droplet ICSI needle will enter between circle opening Expel the # of sperm retrieved into the droplet Put the sperm loaded VD device into a cryovial with a rounded bottom Hold device in vapor for a 60–90 s using forceps Drop device into the liquid nitrogen When thawing, remove the device from LN<sub>2</sub> Open the cryovial and remove the sperm VD device with forceps Place directly into an ICSI dish, parallel with the PVP drop Droplets will quickly turn clear; check the droplet for motile sperm Add pentox if needed

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## Chapter 15

# Varicocele



There is probably no subject that has been more controversial in the area of male infertility than varicocele. Most non-urologist infertility specialists in the world are skeptical of the role of varicocele or varicocelectomy in the treatment of male infertility, especially since the advent of ICSI [1]. However many urologists strongly recommend varicocelectomy, and now in 2017, there is another push to reestablish this surgery for infertile couples with male factor infertility. The September 2017 issue of Fertility and Sterility was dedicated to varicocelectomy with all positive, and no negative, reviews [2]. It has been suggested that in addition to infertility, varicocele has a negative impact on Leydig cell function, testosterone level, and overall “male health,” and that varicocelectomy will raise testosterone levels and improve “overall male health” [3–5]. It has also been suggested that varicocelectomy improves the results of ART (assisted reproductive technology) even though most early papers on ICSI showed no relation of sperm parameters to success or failure [6–9]. However, another equally large and very similar cohort study showed no difference in success with ICSI in men with varicocele who underwent varicocelectomy than in men who did not [10]. Interestingly, the study which showed improvement was only when there were younger female partners, and not older female partners. To confuse things more, a meta-analysis of these studies concluded no improvement in pregnancy rate with varicocelectomy performed before ART but there was improvement in birth rate [6]. With azoospermia TESE cases there was only a “strong trend” toward improvement after varicocelectomy [6]. Because of this enthusiastic resurgence of interest in varicocelectomy, I would like to once again review previous literature to objectively evaluate whether it is appropriate to perform varicocelectomy for male infertility in the era of ICSI.

There have been several, credible studies in the past which show no effect of varicocelectomy on fertility [11–14]. However newer studies are showing an effect

on varicocele. There were also in the past a few “controlled” studies that favor varicolectomy, but they have been disregarded because of patient selection bias and protocol deviations [10, 11]. The weight of evidence in the past has been against varicolectomy. For example, reports that semen parameters are improved by varicolectomy were troubled by uncontrolled observations and the failure to take into account the extreme variability of semen analysis and its regression toward the mean [15–20]. Many controlled studies have demonstrated that, because of this variability, men with an initially low sperm count tend later to have higher sperm counts in the absence of any treatment whatsoever, because of regression toward the mean.

Randomized control trials (RCTs) should eliminate this effect on sperm count of regression toward the mean. But these RCTs are very scarce, often possibly because urologists who have this interest might naturally be biased. Therefore, it will be useful to reevaluate whether past studies on varicocele, sperm count, and fertility might have been flawed or not.

It is commonly known that a left varicocele occurs in 15–20% of all men. In fact we found a large varicocele in 17% of fertile men coming for vasectomy reversals who clearly were fertile before their vasectomy [21]. It has been claimed, however, that in infertile men, 35% or more have a varicocele [2, 22]. However the incidence of an easily discoverable and palpable, visible varicocele is in fact similar with infertile and fertile men [23, 24]. The problem with such claims of high varicocele incidence is that for infertile males (or more precisely the male partner of an infertile couple alleged to have some abnormality in semen analysis parameters), there is an intense effort by the examining urologist in such cases to find a varicocele. So for infertile males, there is a bias toward finding a varicocele that does not exist in a routine normal population physical exam. In fact, for infertile men there has been an intense hunt for varicocele ever since the astonishing case report of Tulloch in 1955 alleging that an azoospermic man developed a high sperm count and fertility after varicolectomy [25].

Varicolectomy is not without complications, such as hydrocele or even testicular atrophy. Microsurgery for varicocele, which I originally introduced, has reduced these complications dramatically. Nonetheless, these occasional complications of varicolectomy have been known for >20 years. Of course, microsurgical approach to varicolectomy should avoid such complications [26–28]. Nonetheless, the occasional serious risk of varicolectomy cannot be disregarded. The more common risk for postoperative hydrocele (5%) is obviously just a nuisance and not as serious as devascularization.

Semen analyses are often highly variable, and spontaneous pregnancies without treatment are so common that there is much skepticism about many treatments for male infertility [12, 15–20, 29–35]. No treatment of male infertility is without the risk of delaying more effective treatment until the wife is older. So we should be cognizant of the pitfalls of trying to evaluate either pregnancy results or sperm count results in patients undergoing varicolectomy. The same critique applies even to recent papers suggesting that varicolectomy can reduce the need for ART [36, 37].

## 15.1 Lack of Effect of Varicocele on Pregnancy Rate Following Vasovasostomy

In 1989, we reported a 10-year follow-up of men undergoing vasovasostomy (who had spermatozoa in the vas fluid without secondary epididymal blowouts) and their long-term results [21]. Even many years later, their fertility was not affected by the varicocele. A decade later, essentially the same question was addressed when varicocelectomy was performed simultaneously with vasovasostomy in vasectomy reversal patients who had varicocele, but varicocelectomy was not carried out in the other vasectomy reversal patients who had a varicocele. There was no statistically significant difference between the two groups.

## 15.2 Evidence-Based Practice of Medicine

In 1995, Nieschlag proposed a basic axiom that needs to be followed in male infertility treatment: “Therapeutic interventions in male infertility should be based on properly controlled clinical trials” [13]. Several reports on spontaneous pregnancy rates with no treatment in couples with severe male factor justify Nieschlag’s axiom. In 1993, Hargreave reported on patients with severe oligozoospermia, high serum FSH concentrations, and varicocele whose wives became pregnant after an initial infertility consultation without any treatment of the male [14]. A total of 33% of men in this category had a varicocele and did not have time to undergo varicocelectomy before their wife became pregnant. The point of his study was that, with alarmingly low sperm counts, fertile women can become pregnant without any treatment of the male, verifying concepts that have been clear for many years [32–35].

To understand the importance of a controlled study in evaluating the validity of varicocelectomy, one has only to look at the spontaneous conception rates in the wives of men with various low sperm counts. Baker and Burger in 1986, reported life-table pregnancy rates over 3 years in couples with varying categories of semen parameters compared to control groups [19]. Although low sperm counts resulted in lower pregnancy rates, a substantial percentage of couples achieved pregnancy spontaneously despite severe oligoasthenozoospermia [38]. In 1983 Schoysman reported an extensive 12-year experience following 1291 oligozoospermic men who underwent no improvement in semen parameters [35]. These studies demonstrated the difficulty of interpreting whether any treatment of the male with oligozoospermia, e.g., varicocelectomy, has any discernible effect on the pregnancy rate.

It is easy to become enthusiastic about any treatment of male infertility that is performed without adequate controls, even crypt-azoospermia. We all have seen men who are initially azoospermic, who will eventually, in subsequent semen analyses, have spermatozoa in the ejaculate without any treatment [18]. In fact in the early days of ICSI, this was commonly called “cryptazoospermia” [1, 30]. Without

a control group to compare with, one should not be surprised to see a spontaneous pregnancy rate of 9–23% without any treatment of the male partner with severe sperm defects, particularly if the couple has had a short period of infertility and/or if the wife is young [30, 31].

## 15.3 Varicolectomy and Sperm Count

MacLeod and Gold, as far back as 1951 [39], first demonstrated that sperm concentration and motility tend to increase with repeated testing in oligozoospermic and asthenozoospermic men despite no treatment. This was a peculiar mathematical quirk related to the highly variable nature of the sperm count. This means that, without any treatment whatsoever, if you continue to get sperm counts and semen analyses longitudinally on men who initially have low sperm counts and poor motility, the low sperm count and the poor motility will routinely tend to increase with repeated tests and no treatment. Baker et al. were the first to clearly and mathematically explain this phenomenon of “regression toward the mean” [14, 18, 20]. “Regression toward the mean” has profound implications for all clinical trials. Whenever there is a highly variable measurement, if patients have a controlled period followed by a treatment period, there is likely to be a significant improvement even if the treatment is ineffective. Baker et al. observed the same phenomenon that McLeod and Gold had observed 30 years earlier, that sperm concentration and motility increased progressively in their study of day-to-day variability of semen analyses in infertile men [38]. Sperm motility increased equally on both active drug and on placebo treatment in a double-blind controlled trial of erythromycin for asthenozoospermia [16]. Clearly, erythromycin had no impact whatsoever on either sperm count or sperm motility. However, in this double-blind controlled study, it was obvious that the sperm motility increased in an equal manner in patients that were on erythromycin and patients that were on placebo. “In a similar fashion, sperm motility increased in men with varicoceles whether or not they had testicular vein ligations performed” [12]. No matter what the treatment, whether erythromycin or watchful waiting, clomiphene citrate or varicolectomy, an initially low sperm count (because of intrinsic variability) will gravitate higher because of “regression toward the mean.”

Baker and Kovacs also concluded in 1985 that although “a group of subjects selected for low results will on average have higher results on re-measurement”, conversely a group of men with high sperm counts will on average have lower results after any treatment [18]. Repeated tests after treatment of oligospermia will generally show an increase which has nothing to do with biology but is simply a mathematical event that has to occur. As Baker and Kovacs showed, therefore, a low sperm count will generally improve, with or without any treatment. Similarly, a very high sperm count will generally become lower with or without any treatment. Thus, whenever uncontrolled varicolectomy studies mention an

improvement in motility, or sperm count, this is what one often would expect to find with no treatment whatsoever when you are beginning with oligozoospermic couples [18, 19].

One might, without proper control studies, be very enthusiastic about varicocelectomy in a practice involving younger couples and less enthusiastic in a practice involving older ones. We discovered a similar confounding phenomenon in the treatment of obstructive azoospermia with sperm retrieval and ICSI. The only factor that significantly affected the variation in pregnancy rate in couples undergoing ICSI with retrieved spermatozoa was the age of the wife [38, 40]. Thus, it seems that in any kind of infertility treatment for male factor, regardless of sperm count, and whether for varicocele or obstructive azoospermia, the most important confounding factor, aside from duration of infertility, is the age and ovarian reserve of the wife.

As long ago as 1978, Rodriguez-Rigau et al. reported a large group of patients which was not prospective and not randomized, but was controlled, some of whom underwent varicocelectomy and others who did not [41]. Rodriguez-Rigau et al. noted a slightly increased percentage motility in patients undergoing varicocelectomy. However, there was no difference in pregnancy rate among those who had varicocelectomy versus those who did not. Furthermore, there was no relation of improvement in postoperative sperm count to pregnancy rate. Those patients who conceived after varicocelectomy had a mean sperm count of  $28 \times 10^6$  spermatozoa/ml, and those who did not conceive had a mean sperm count of  $26 \times 10^6$  spermatozoa/mL. Of patients with sperm counts of  $>10 \times 10^6$  spermatozoa/ml, those who conceived had a mean sperm count of  $40 \times 10^6$  spermatozoa/mL and those who did not conceive had a mean sperm count of  $48 \times 10^6$  spermatozoa/mL.

## 15.4 Does Varicocele Cause a Progressive Decline in Fertility?

As far back as 1968, Uehling studied the fertility of 440 married men in the military coming in for routine physical examination, with and without varicoceles. Of this group, 138 had no children (31.4%) and 302 did have children (68.6%). To break it down further, of the 75 men with a varicocele, 69% had children, and of the 227 men without a varicocele, 68% had children. Thus, there was no difference in fatherhood of those young married military recruits who had varicocele versus those who did not have varicocele. The presence or absence of a varicocele in these young men had no influence on whether or not their wives were able to get pregnant [23]. At least in young men, varicocele seemed to have no negative impact on fertility.

So what is the prevalence of varicocele in a group of otherwise healthy young men? Thomason et al. in a similar study of military recruits, in 1979, concluded, "It is apparent that the prevalence of varicoceles in young men occurs with significant frequency and does not interfere with the fertility in all individuals" [24]. It was found that 30.7% of all recruits had a left varicocele (14% were small, and 16.7% were moderate or large), and 29.4% of recruits who had fathered children also had

a varicocele (15% were moderate or large). This is similar to the frequency of large or moderate left varicocele in older vasectomy reversal patients [21]. They concluded, “the prevalence of a left-sided varicocele occurs with such frequency among a group of healthy men that one would question the association of a varicocele and poor semen quality.” Furthermore, we have observed no difference in fertility after vasovasostomy in older men with or without varicocele [21]. Nonetheless many adults with varicocele do have atrophied left testicle causing some concern that the varicocele could be the cause.

It is therefore likely that there will be a widespread resurgence in varicocelectomy, but it is this author’s view that varicocelectomy is not indicated for infertility treatment.

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## Chapter 16

# Kallman's Syndrome and Hypopituitarism



Idiopathic congenital hypogonadotropic hypogonadism (CHH) is a rare reproductive disorder that is primarily caused by a gonadotropin-releasing hormone (GnRH) deficiency but with significant genetic heterogeneity. Clinically, this disorder is characterized by abnormally low plasma levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in conjunction with very low concentrations of circulating testosterone, and of course azoospermia. In approximately 50% of cases, CHH patients also suffer from a reduced or deficient sense of smell (hyposmia or anosmia, respectively), which is then termed as Kallmann syndrome (KS) [1–3].

KS was first recognized in 1856 by Maestre de San Juan [1] who observed patients with defective olfactory structures and a microphallus. A few years later, Kallmann et al. [2] identified the hereditary nature of this condition. In the 1950s, De Morsier and Gauthier [4] further described the partial or complete absence of the olfactory bulb (OB) and its axons in multiple hypogonadal males. Hypopituitarism can also be caused by brain injury or removal of a pituitary tumor. All these causes of hypopituitarism are rare, but their treatment is interesting in understanding the hormonal control of spermatogenesis.

In a large study at NIH by Sherins et al., azoospermic men with Kallman's and hypogonadotropic hypopituitarism were treated with gonadotropins [5–8]. They all had very small testes and were eunuchoid, with undetectable FSH, LH, and testosterone levels. First he puts them on HCG 2500 units three times per week for up to 1 year. They all developed normal testosterone levels and androgenization, but they remained azoospermic. Testis biopsy revealed recovery of all stages of spermatogenesis up to the pachytene spermatocyte but no maturation beyond that. In other words, with HCG replacement alone, the spermatogonia began to enter meiosis but were arrested before MI could be completed.

When they then were administered FSH, meiosis was completed, and normal spermatogenesis was achieved, with sperm now appearing in the ejaculate. However,

the count never got very high. Interestingly, despite low sperm count, these men were fertile and impregnated their young wives readily. This demonstrated the flaw in equating low sperm count with male infertility.

Then if the patient stays on the HCG but discontinues the FSH, he continues to be fertile with no decrease in spermatogenesis despite no FSH. Thus, once FSH is added to the HCG and the maturation arrest and meiotic arrest are unlocked, the FSH can be discontinued, and spermatogenesis can be maintained with HCG alone.

However, if HCG is also discontinued, then restarting the HCG will once again only stimulate spermatogenesis up to the pachytene spermatocyte and arrest in late meiosis once more. Furthermore in attempts to treat the hypopituitarism with only FSH, and no HCG, there may be a few spermatozoa develop, but the patient will not become fertile until HCG is added. Thus, HCG alone is not adequate, and FSH alone is not adequate.

These findings were duplicated in a different set of very rare cases, called facetiously "the fertile eunuch" syndrome. Men with normal FSH levels, but no LH production, do have sperm production, but it is poor quality and very sparse. When you give them HCG, the spermatogenesis becomes completely normal, and then they truly are fertile. So HCG can initiate spermatogenesis but does not complete it. FSH can initiate the completion of spermatogenesis but is not sufficient for qualitatively or qualitatively normal spermatogenesis.

Therefore to treat hypopituitarism or Kallman's syndrome requires both FSH and HCG. Either alone is not adequate. The usual dose is 75 units of FSH three times per week along with 2500 units of HCG three times per week. This may require some adjustments based on monitoring of hormone levels. It may take 4 months to 9 months to see sperm finally in the ejaculate. This treatment protocol is prolonged and expensive. So once there is sperm in the ejaculate, it is best to freeze for ICSI (see later part of this section) and switch over to simple testosterone replacement or just stay on HCG and discontinue the FSH; almost all men can have successful results.

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## **Part III**

# **New Frontiers**

## Chapter 17

# The Genetic Control of Male Infertility and Understanding the Y Chromosome



The Y chromosome has been a very rich area for beginning the study of the genetic control of spermatogenesis. The Y chromosome contains 60 multicopy genes composed of 9 different gene families concentrated in regions of multiple repeat sequences called amplicons arranged in mirror images called palindromes. It contains many testis-specific spermatogenesis genes. This very complicated pattern is susceptible to deletions caused by homologous recombination with itself and can explain the presence of small numbers of sperm in otherwise azoospermic men. It is also the beginning of understanding the genetic control of spermatogenesis.

Almost concurrently with the development of intracytoplasmic sperm injection (ICSI) and testicular sperm extraction (TESE) for azoospermia in 1993, we began to study the genetic causes of male infertility via the first mapping of the Y chromosome in azoospermic males and in fertile control male populations, eventually leading to complete sequencing of the Y chromosome. This led to an understanding of why tiny amounts of sperm are often found in the testis of azoospermic men previously thought to be making no sperm [1–8]. Subsequently, much of the research into the genetics of male infertility has been focused on aberrations of the Y chromosome. This specialized male chromosome, the Y chromosome, contains many genes that are involved in spermatogenesis, concentrated in a peculiar pattern of nucleotide repeats and mirror image inversions called amplicons and palindromes. Deletions that involve these regions of the Y are found in 15% of severely infertile males and have been shown to be transmitted to male offspring via ICSI, presumably causing fertility problems in these children later in life [5, 9, 10].

However, the sequence of the Y also gives us a perspective about other male infertility genes that are most likely widespread throughout the genome and which also could transmit infertility to future generations of ICSI offspring. More importantly perhaps, understanding the Y chromosome helps us to comprehend why men who are seemingly azoospermic often have some residual tiny amount of spermatogenesis that can be used for successful TESE-ICSI [11–13]. Many genes on the Y chromosome required for spermatogenesis have multiple copies and even “backup” homologues elsewhere in the genome, such as *DAZL* and *CDYL* [6, 14, 15]. Another

broader scientific benefit is that it has amplified our understanding of sex determination in general and has even helped to explain the origin of Turner syndrome [16]. Indeed, studies of male infertility and the Y chromosome have gone so far as showing why XO female patients with Turner syndrome are often caused not by the loss of an X chromosome in the female embryo, but rather by a loss of the Y chromosome in a male embryo, and why XO women are always mosaics, even if their karyotype does not show it. This also explains why in XXY Klinefelter's males there is often a tiny amount of spermatogenesis somewhere in their tiny testes. They too are mosaic.

## 17.1 The Y Chromosome and ICSI

Until 1992, there were no good treatment options for infertile couples when the male had severely impaired spermatogenesis. In fact, there are still no clinical therapies to correct deficient spermatogenesis [17–30]. Since the introduction of TESE and ICSI by us and the Brussels Dutch-Speaking Free University in 1992, however, there has been a revolution in our thinking about male infertility [31, 32]. Infertile couples with the most severe cases of male infertility, even with almost 100% abnormal morphology and/or only rare spermatozoa in the ejaculate, can now have pregnancy and delivery rates not apparently different from conventional IVF with normal sperm [13, 33–35].

In 1993, we first introduced microsurgical epididymal sperm aspiration (MESA) in conjunction with ICSI for the treatment of obstructive azoospermia [3, 12, 36–38]. A few months later, TESE was also found to be effective for the majority of cases of nonobstructive azoospermia as well [3, 12, 13, 38, 39]. The reason is that 60% of azoospermic men with presumably no sperm production actually do have a minute amount of sperm production in the testis that is not quantitatively sufficient to spill over into the ejaculate, but which is adequate for ICSI [11, 13, 27, 39–46]. Thus, even men with extremely low sperm counts, or with spermatogenesis so deficient in quantity that no sperm at all can reach the ejaculate, could now have children with the use of TESE-ICSI [3, 10, 47].

It is with these cases of nonobstructive azoospermia and severe oligozoospermia that the greatest concern has been registered for the fertility of future generations. If severe oligozoospermia or azoospermia is of genetic origin, then ICSI might conceivably create a potential problem of proliferation of male infertility in future generations [48]. In fact, if one-half of all azoospermic men were to undergo ICSI, the incidence of male infertility would double within seven generations [49]. Our patients generally are not concerned about this reality, because they figure, if they can get pregnant with ICSI, then their children can also.

Karyotyping reveals that autosomal structural chromosomal abnormalities, such as translocations and inversions, are found in about 1% of azoospermic men, but the most common karyotyping chromosomal abnormalities in azoospermic men involve the sex chromosomes, such as in Klinefelter's syndrome [47, XXY] [50–64]. In the

majority of these cases, surprisingly, we can usually find a few rare sperm adequate for fertilization using ICSI [44, 65–67]. The reason for this presence of normal X and Y sperm in the testes of men with Klinefelter's (XXY) is that they are truly mosaics in the testis if not in the peripheral blood. The incidence of Klinefelter's in the population of azoospermic men is controversial, ranging from 5% to 15%. Thus Y chromosome deletions (15%), and Klinefelter's, and translocations add up to 20–30% of cases of nonobstructive azoospermia.

Suspicion of involvement of the Y chromosome in male infertility actually arose originally from cytogenetic evidence reported more than 40 years ago [68]. This karyotyping study showed grossly obvious terminal Y chromosome deletions in a very small percentage (0.5%) of azoospermic men who were otherwise phenotypically normal. It was then postulated that the Y chromosome contained a so-called *azoospermia factor* (AZF) gene. During the middle 1990s, we showed that the long arm of the Y chromosome contains not one but many distinct deletion intervals and at least 60 genes belonging to 9 gene families whose exclusive functions are in spermatogenesis [1–4, 6–8, 69, 70]. Interestingly, several of these multicopy spermatogenesis genes on the Y chromosome have “backup” homologues elsewhere in the genome, like *DAZZL* on chromosome 3 and *CDYL* on chromosome 6. This is the reason (backup autosomal genes) that in men with azoospermia from such deletions, we can still find a few sperm on TESE sufficient for ICSI.

In fact, the deletion frequency of one or more of these regions on the Y chromosome in men with azoospermia or severe oligozoospermia is 15% (Fig. 17.1) [1, 3, 70]. After our initial report, most laboratories throughout the world now routinely report on these submicroscopic deletions of the Y chromosome in azoospermic and severely oligozoospermic men [25, 69, 71–101]. In fact, deletion screening of the Y chromosome is now considered standard practice for severely oligozoospermic and azoospermic patients undergoing assisted reproduction in most countries in the world. But in view of the autosomal backup knowledge of these genes, it is probable that a thorough search of the human exome will reveal many more diverse genes that specifically control spermatogenesis. They would have to be very specific for spermatogenesis or germ cell development, as are these deletions, since these azoospermic men are otherwise quite healthy.

## 17.2 AZFa

The *AZF<sub>a</sub>* region differs from the *AZF<sub>b</sub>* and *AZF<sub>c</sub>* regions because of its nonrepetitive structure and its low deletion frequency (Fig. 17.2). *AZF<sub>a</sub>* deletions are very uncommon and only a few rare patients have been described [69, 79, 87, 102–105]. However, studies of this region are highly useful in understanding the genetic basis of male infertility. The *AZF<sub>a</sub>* region spans 800 kilobases (kb) and contains two functional single-copy genes: *USP9Y* and *DBY* [104, 105]. This mutation represented the first and only case of a point mutation rather than a large microdeletion causing a single gene defect on the Y chromosome associated with spermatogenic

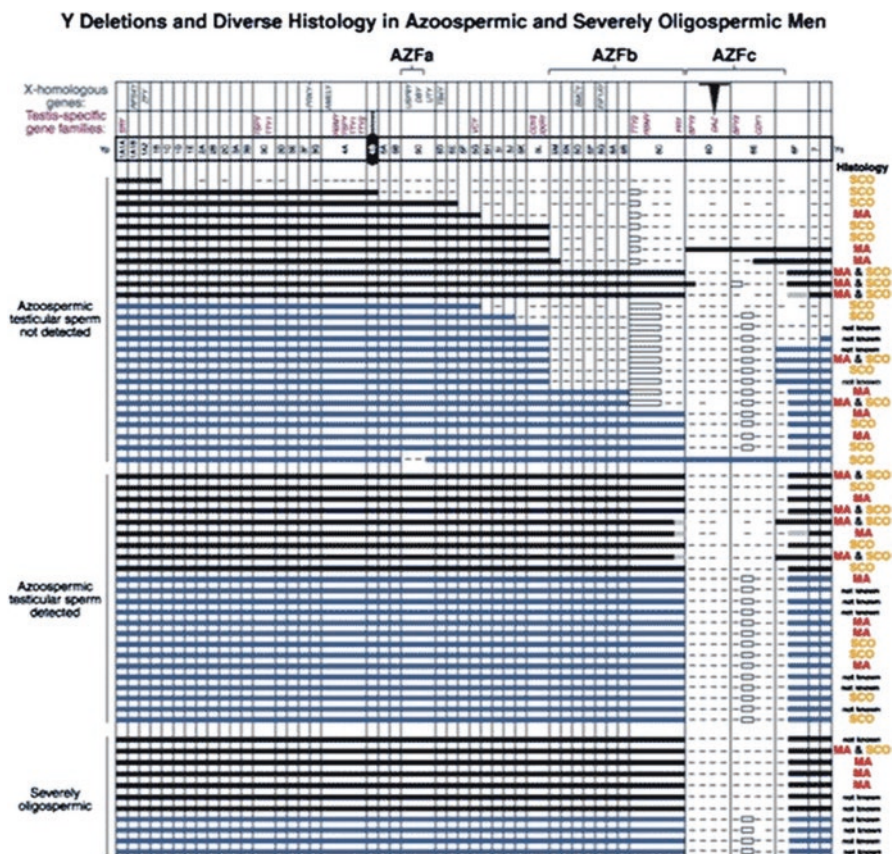


**Fig. 17.1** Y deletions and diverse histology in azoospermia and severely oligospermic men

failure [104]. The lack of sequence repeats which characterize the rest of the Y chromosome made this particular region of the Y amenable to such a point mutation search very early in the study of the Y. Because almost all other spermatogenesis genes on the Y chromosome, such as those in *AZFb* and *AZFc*, are multicopy, searching for point mutations in these genes is very problematic. It is also questionable whether point mutations in a single gene of a gene family will give rise to a severe infertility phenotype, because the remaining intact copies of the gene could potentially compensate for the loss of function of the mutated gene. This is an important lesson for why spermatogenic defects come in varying degrees of severity and why even in azoospermic men there are usually still a tiny number of healthy sperm “hiding” in the testes [4, 5, 8, 10].

The *AZFa* region provides a good model for the interaction and overlapping functions of multiple genes and sheds light on the polygenic nature of the genetic control of spermatogenesis. When the entire *AZFa* region is deleted, taking out both *DBY* and *USP9Y*, the spermatogenic defect is severe and the patient is always

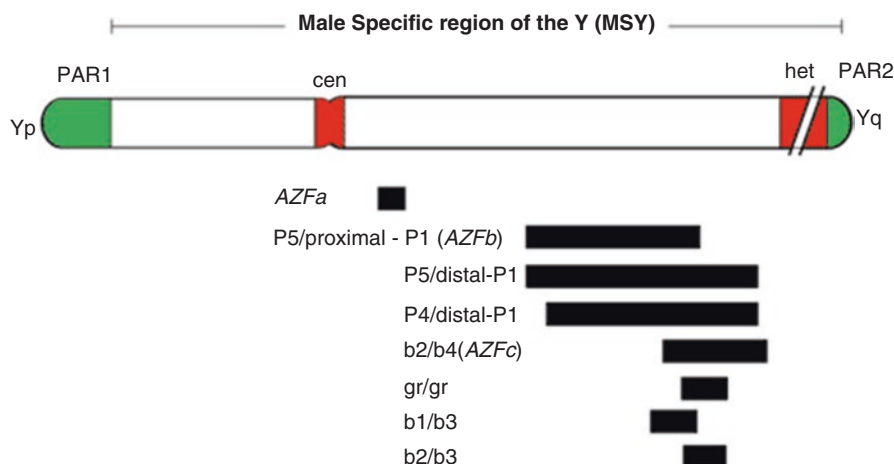


**Fig. 17.2** Simplified diagram of the main areas for Y chromosome microdeletions, including some that are less frequently studied

azoospermic. In contrast, when only one gene is affected, such as loss of *USP9Y* function owing to a specific point mutation, this results in a less severe phenotype of maturation arrest with a few pachytene spermatocytes developing into a few mature sperm in some seminiferous tubules. Thus, the loss of *DBY* (the only other gene in the *AZF*a region) likely exacerbates the spermatogenic consequences of the loss of *USP9Y*.

### 17.3 AZFb

Deletions of the *AZF*b region are somewhat more common than deletions of the *AZF*a region but are still found in a very small percentage of azoospermic men [69, 95, 106–108]. Interestingly, all men with deletions of *AZF*b described to date are azoospermic and show complete absence of spermatozoa in the testis [14, 69, 71, 81, 106–111]. Therefore, similarly to *AZF*a deletions, there are no reports on transmission of an *AZF*b deletion to ICSI offspring. Interestingly, these azoospermic men, despite missing a huge section of nucleotide repeats, suffer from maturation arrest (at the pachytene stage) rather than Sertoli cell only. Many functionally active genes are clustered within the *AZF*b region [81, 106, 112, 113]. Because of the presence of multiple sequence repeats within this region, in contrast to *AZF*a, efforts to define the *AZF*b region precisely had been hampered, but now the exact content and extent of the *AZF*b region has been elucidated, and the entire Y chromosome has been fully sequenced [8, 106].

In fact, the region frequently referred to simplistically as *AZF*b overlaps *AZF*c and has a very large number of repeat copies of genes and pseudogenes (*RBM*Y,

*PRY*, *TTY*) arranged in a complex of palindromes (inverted repeat sequences). That is why we technically object to this commonly used terminology of “*AZFb*.” We prefer the more specific palindromic terminology. Deletions in this region of the Y chromosome are extensive and are much less common than in *AZFc*, and they are caused by weak “breakpoints” in the center of its inverted repeats. This is a less common deletion mechanism than that which plagues the *AZFc* region.

## 17.4 *AZFc*

The most commonly deleted and best-studied region on the Y chromosome is the *AZFc* region. Deletion of the *AZFc* region is found in 12% of azoospermic men and in 6% of severely oligozoospermic men [1–3, 70]. The complete nucleotide sequence of the *AZFc* region reveals an extraordinary structure and genetic composition. The region is constructed from very large areas of absolute sequence identity repeats called amplicons, which are arranged in direct repeats, inverted repeats, or palindromes. The *AZFc* region spans 3.5 Mb (huge but not quite as huge as *AZFb* with its length of 8 Mb) and contains 7 separate families of genes with a total of 19 genes that are all exclusively expressed in the testis (Fig. 17.3). This so-called microdeletion, *AZFc*, is actually a huge section of DNA, but not huge enough to show up with karyotyping. Interestingly, absence of this large 3.5-Mb *AZFc* chunk of the Y chromosome seems to have no other deleterious effects except upon spermatogenesis, exemplifying the remarkably specialized function of this region of the Y chromosome [2]. These genes only affect spermatogenesis and nothing else.

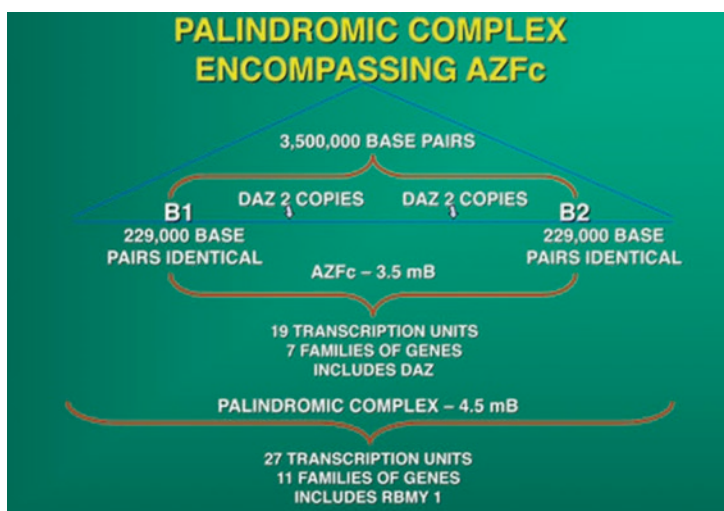


**Fig. 17.3** Diagram of DNA sequence boundaries of *AZFc* deletion and the mechanism of *AZFc* deletion

## 17.5 The DAZ Gene Family

The *DAZ* gene family, which is one of the seven gene families located within *AZF<sub>c</sub>*, was one of the first spermatogenesis genes identified on the human Y chromosome and comes in four almost identical copies [1, 6]. As it turns out, *DAZ* or its autologous homologues have other enormous, and evolutionarily, ancient roles in licensing primordial germ cells to become oogonia or spermatogonia. The human *DAZ* genes were shown to be transcribed specifically in spermatogonia and in early primary spermatocytes [114]. Interestingly, homologues of *DAZ* in other species were also shown to be involved in the control of spermatogenesis, supporting an essential and ancient role of this gene in male fertility in humans as well as almost all other animals. Homologues of *DAZ* have been found in *Drosophila* (termed *boule*), in mice (termed *Dazl*), in frogs (termed *Xdazl*), and even in worms (termed *daz-1*) [115–120]. Therefore, *DAZ* is the most ancient and well-conserved spermatogenesis gene. In contrast to its presence in humans, the *DAZ* gene in these other species is single copy and located on an autosome rather than on the Y chromosome. In the human, *DAZ* is present on the Y chromosome in four near-identical repeat copies (99.9% homology) arranged in two clusters with two genes in each cluster [2, 7].

The human, and most animals of course as well, also retains an autosomal homologue of *DAZ* called *DAZL*, which is located on chromosome 3 in humans [6]. During evolution, some time after the split of Old and New World monkeys 30 million years ago, the *DAZL* gene was transposed to the Y chromosome like a simple translocation event. Once autosomal *DAZL* was transposed from chromosome 3 to Y, it was amplified and pruned until it became the modern-day human *DAZ* gene family of four copies on the Y and still two autosomal copies on chromosome 3. In fact there is yet another *DAZ* family homologue in humans, termed *BOULE*, that resembles the fly homologue *boule*, even more closely than *DAZ* or *DAZL* [121]. The exact interaction and possible functional overlap between these three members of this interesting gene family holds the clue to the peculiar finding of a few surviving sperm in the testes of azoospermic men because of genetic “backup.” In fact, >80% of azoospermic men with an *AZF<sub>c</sub>* deletion have some sperm in the testis retrievable with TESE [4, 9, 10].

*DAZL* actually is a key universal germ cell development gene for both males and females, and is needed in order for PGCs (primordial germ cells) to be able to develop from the embryo’s earliest epiblast differentiating cells (see Part 1) [122]. When the early PGCs are specified from a corner of the epiblast, they begin to migrate toward the dorsal spine where eventually they will enter the gonadal ridge (the early testis in the male and the early ovary in the female). However they cannot truly become PGCs and develop into germ cells (spermatogonial stem cells or oogonia) unless they are first “licensed” to do so by *DAZL*. Of course retinoic acid in the gonadal ridge upregulates *stra 8*, which initiates meiosis in the female and is blocked by Sertoli cells in the male fetus. However, neither in the male or female can the epiblast stem cells function as PGCs and become germ cells unless they are allowed to do so by *DAZL*.

Now to get back to spermatogenesis and the infertile male, the *DAZL* gene on chromosome 3 was the original human *DAZ* gene, which jumped over to the Y chromosome 30 million years ago and resulted in our modern Y chromosome *DAZ* gene. The Y chromosome, with its lack of recombination during meiosis, represents a safe harbor for the proliferation of genes that are beneficial to the male but detrimental to the female. This is how the Y chromosome, over evolutionary time, tends to accumulate genes from throughout the entire rest of the genome that control spermatogenesis. The Y chromosome in mammals, and other sex-determining chromosomes such as the W chromosome in birds, evolved differently over and over again in different genera, always with totally different genes and nucleotide sequences, but always with the same general theme: a degradation of its X homologues owing to failure of recombination and at the same time an accumulation and amplification of multiple copies of male-specific spermatogenesis genes from all over the genome. It is this multiplicity of gene copies (most in fact originating from autosomal homologues), all of which contribute quantitatively to total sperm production that explains the minute amount of sperm we can find at TESE in azoospermic men, because of “rescue” from the continued presence of at least one of these gene copies (such as *DAZL* on chromosome 3) even though most copies are missing or deleted.

Deletions of the entire *AZFc* region result in loss of all four *DAZ* copies. Recent reports, however, indicate that deletions involving not all of the *DAZ* genes are also found in infertile men, but these smaller deletions are found in men with only mild oligozoospermia, indicating a possible gene dosage effect, i.e., men with a deletion of only two *DAZ* genes are less affected than men with a deletion of all four copies [123–127]. These are called GR-GR deletions, that is, half of *AZFc* is deleted. There are also less studied *AZFc* deletions, such as B1-B3 and B2-B3. These data illustrate that infertility is a complex multigenic disorder and that disruption of different genes or disruption of some genes of a gene family can result in different degrees of spermatogenic failure.

## 17.6 Mechanism of De Novo Y Chromosome Deletions

It is strange that men with nonobstructive azoospermia, or severe oligospermia, rarely have any other genetic or health problems. In the *Drosophila* literature, this is referred to as “pure sterile.” Research into deletions involving *AZFa* and *AZFc* has provided interesting data on the mechanism of deletions, which sheds light on the inexorable decline in spermatogenesis in humans and in other species which have no “sperm competition.” Deletions on the Y chromosome are caused by “illegitimate” homologous recombination between highly similar and identical sequences which are found on the Y in great abundance (Fig. 17.3). For example, homologous recombination between two identical sequence stretches results in dropout of the intervening *AZFa* or *AZFc* region [105, 128, 129]. Because the sequences of identical nucleotide repeats are shorter in *AZFa* than those in *AZFc*, *AZFa* deletions are very rare, whereas *AZFc* deletions are common.

For *AZF<sub>c</sub>* the substrates for homologous recombination are two repeats that are >99.9% identical and as long as 229 kb in length [2]. The frequency with which these deletions occur seems to correspond to the length of the stretch of homology. Therefore, deletions of *AZF<sub>c</sub>*, caused by homologous recombination between 229-kb repeats, are far more common than deletions of *AZF<sub>a</sub>*, which are caused by repeats of only 10 kb in length. The very repetitive nature of the Y chromosome seems to be the cause of its instability over an evolutionary time frame, as well as in our current infertile male patient population, but it is also the method that the Y has adopted (albeit inefficient) for its survival, called “gene conversion” [130]. Gene conversion simply means that since most of the Y cannot recombine with its homologous partner, the X, like sequences just anneal with each other. This is less efficient DNA “repair” than meiotic recombination, but it is enough to allow the otherwise deteriorating Y to survive quite well with amplification into multiple copies of its male-specific genes.

What is fascinating is how we survived at all, as our X-homologous genes on the Y chromosome deteriorated because of failure of meiosis with its nonrecombining mate [131, 132]. In fact, this deterioration of X-homologous Y genes was the whole reason for the evolution of “X inactivation,” to put males and females on an equal footing despite different gene dosages. As the X-homologous genes on the Y chromosome degenerated, a mechanism had to evolve for the species to survive. The X genes had to develop increased expression, so the male could survive, and they had also had to allow half of them to “unactivate” (X-inactivation), so the female could survive.

## 17.7 Evolution of the Human Y Chromosome

What makes the Y chromosome, with its confounding repeats, polymorphisms, and degenerating regions, such an interesting object of study for male infertility? The answer lies in the evolutionary history of the X and Y chromosomes (Fig. 17.4). Over the course of the past 240–320 million years of mammalian evolution, the X and Y chromosomes have evolved from what was originally a pair of ordinary autosomes [14, 131–138]. During that evolution, just as most of the ancestral X genes were decaying on the Y because of the lack of meiotic recombination, genes that control spermatogenesis arrived on the Y from autosomes. Once on the Y, these formerly autosomal genes amplified into multiple copies and achieved greater prominence through the process called “gene conversion” [6, 130, 139]. Spermatogenesis genes that arrived on the Y, but came originally from autosomes, include the *DAZ* (from autosome 3) and *CDY* (from autosome 6) genes which are among the seven gene families located in *AZF<sub>c</sub>* [6, 15]. Other spermatogenesis genes on the Y, such as *RBMY*, have persisted in their original position as on the X [140–143]. The ancestral gene that remained on the X chromosome (*RBMY*) retained its widespread cellular functions, whereas *RBMY*, which persisted on the receding Y chromosome, evolved a male-specific function in spermatogenesis [142, 144–146]. Male-benefit genes have thus arrived and accumulated on the evolving Y

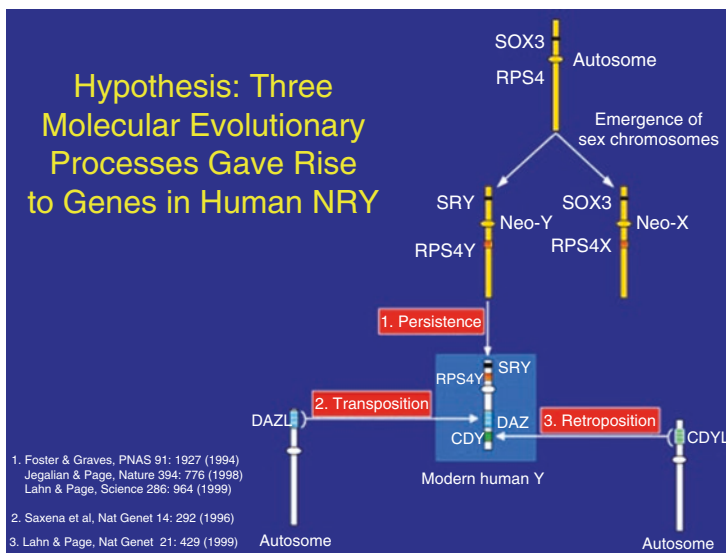


**Fig. 17.4** Evolution of Y chromosome from what was originally a pair of ordinary autosomes 300,000,000 years ago

chromosome over many millions of years via three mechanisms: (1) persistence of genes on the ancestral X that evolved a male-specific function (*RBM* to *RBMY*), (2) retroposition from an autosome via reverse transcription (*CDL* to *CDY*), and (3) transposition from an autosome via translocation (*DAZL* to *DAZ*).

This evolution of the modern X and Y chromosomes was initiated by the emergence of a male sex-determining gene (now known as *SRY*) on what was originally an ordinary pair of autosomes [15, 137, 138, 142]. Genes associated with the non-recombining *SRY* region that were specifically beneficial for male function or antagonistic to female function flourished on the evolving Y chromosome despite the deterioration of more generalized genes and despite absence of the DNA repair benefits of meiosis [146–151].

The next question that the evolution of the X and Y chromosomes presents, which is crucial for understanding male infertility and ICSI, is how this degraded Y chromosome survives at all. The lack of recombination of the male-determining Y chromosome has led to complete deterioration and loss of most of its original 1438 genes (the number of genes on its corresponding X) and an accumulation of only 60 genes (only 9 gene families) which are male specific and are located in areas of sequence identity that promotes further deletions. So how does the Y survive at all, and why do we humans retain any spermatogenesis at all?

The answer is “gene conversion” [130]. It answers the question of how these ampliconic repeats and palindromic inversions occur. When autosomes recombine during meiosis, DNA is exchanged in a way that the accumulated mutational errors of life get corrected in the germ cells via this DNA exchange. In a sense, the autosomes “have sex” with each other (homologous recombination). This correctional

meiosis cannot occur with the nonrecombining region of the Y chromosome. Instead, the Y chromosome “has sex” with itself. That is, the like-sequence repeats of the Y chromosome “recombine,” in a sense, with each other, an “illegitimate” homologous recombination. This “gene conversion” creates and repairs the multiple copies and inverted DNA sequence repeats that characterize the Y and indeed all sex-determining chromosomes. So if there is a missing or deleted copy of a spermatogenesis gene, there are other backup copies that can still rescue spermatogenesis to some degree.

Throughout our whole study of Y deletions, the most confusing deletion was the so-called “terminal deletion,” shown in the top six cases of Fig. 17.1. With molecular analysis, they appeared with initial mapping not to be interstitial deletions, but rather “isodicentric,” meaning that the Y chromosome did not just come to an end point proximally, but rather was doubled over on itself with like copies annealed to each other. This is caused by a kind of “gene conversion” that went awry. Thus, “gene conversion” as a mechanism for maintenance and repair of the Y chromosome has more limited value than the standard meiosis that the X chromosome in the ovary and the autosomes enjoy. In fact, gene conversion, and the resulting isodicentric Y chromosome of 12% of Y-deleted men (about 1.5% of azoospermic men), is the cause also of Y chromosome dropout leading to XO Turner women. That is, centromeres that are far apart from each other in an isodicentric Y cause the Y to disappear completely resulting in XO Turner syndrome. So the appearance on karyotyping of a terminal deletion of the Y is in truth an isodicentric Y resulting from an incorrect gene conversion mechanism.

## 17.8 The Y Chromosome and Spermatogenesis in Humans and in Apes and “Sperm Competition”

Comparing spermatogenesis in humans, chimpanzees, and gorillas has always been fascinating [52, 150]. Chimpanzees, which weigh only about 100 pounds, have enormous 8-cm-diameter round (not oval) testes with sperm counts of over 1 billion/mL. Yet gorillas, which weigh as much as 600 pounds or more, have tiny testes, have very poor spermatogenesis, and, in the sparse literature on gorilla testicular histology, in the majority of cases, have what appear to be Sertoli cells only [152]. Humans, the closest living relatives to chimpanzees and gorillas, fall somewhere in between.

Most intriguing is to compare the human Y chromosome to the chimpanzee Y chromosome, both of which have been fully and accurately sequenced [8, 153]. Unfortunately, the gorilla Y has not yet been sequenced. Some interesting differences are noted between the human and chimpanzee Y chromosomes [153]. The chimpanzee Y chromosome has many more amplicons and palindromes than the human, but it nonetheless has much fewer ampliconic genes (25 compared with 60). So the increased sperm production of chimpanzees cannot be explained by the presence of more testis-specific genes on their Y chromosome. But interestingly, the

chimpanzee Y is missing a gene (*PRY*) that is present on *AZFc* in two copies in the human. You cannot attribute the vast superiority of chimpanzee spermatogenesis to having more copies of Y chromosome spermatogenesis genes. But the *PRY* gene, which humans have and chimpanzees do not have, could speculatively be a suppressor of spermatogenesis. Thus, a deletion of *PRY* in the chimpanzee could speculatively be a reason for its huge amount of spermatogenesis. Furthermore, it is fascinating that this gene has also been found to be present in only one copy in rare humans who have incredibly high sperm counts, approaching half a billion [154]. Thus, comparing the superfertile chimpanzee Y chromosome to its less fertile human cousin can help us to better understand the genetic control of spermatogenesis in our infertile male patients. Ultimately it is the promiscuous mating pattern of the chimpanzees which fosters tremendous “sperm competition” that allowed them to have a tenfold improvement in spermatogenesis over humans.

## 17.9 Other Male Infertility Genes Not Discernible on the Y Chromosome

Although most research into the genetics of male infertility has focused on the Y chromosome, many other spermatogenesis genes are surely present throughout the human genome. These genes must be rare variants and thus will require huge population studies of azoospermic men to elucidate. Recent studies and earlier speculations indicate that the Y chromosome is not the only chromosome that accumulates genes that benefit spermatogenesis over an evolutionary time span [134, 149, 155, 156]. Its counterpart, the X chromosome, also seems to be an ideal locus for spermatogenesis genes. Like the Y chromosome, the X chromosome is present as a single copy in the heterogametic XY male. Of course, in the egg, i.e., in the female, the X chromosome undergoes normal meiosis and repair with its paired X mate. However, the X chromosome, like the Y chromosome, does not get to undergo normal meiotic repair in the testis. So it cannot escape completely some of the problems that the Y chromosome faces. Therefore, the X chromosome also has large ampliconic regions (12% in the mouse and 2% in the human) [157]. The X chromosome spends 1/3 of its evolutionary life in the testis, not the ovary. Furthermore, any genetic alteration on the X would have an immediate impact in the male, because there is no other X chromosome to compensate or counteract the effect. Therefore, a recessive gene mutation on the X that is beneficial to the male would preferentially be allocated to the X chromosome as opposed to an autosome (155). The X chromosome (which is only repaired at meiosis in the egg, not in the testis) has large ampliconic regions, similarly to the Y chromosome, which have not yet been completely characterized and which might have a rich complement of spermatogenesis genes.

In fact, reverse-transcription PCR subtraction studies of spermatogonia in mice have demonstrated that a large fraction of genes that are expressed exclusively in premeiotic male germ cells are indeed X chromosomal in origin [156]. Eleven of the

36 genes expressed specifically in mouse spermatogonia were found exclusively on the X chromosome. Because the X chromosome is well conserved in all mammals (much more conserved than any of our other chromosomes), it seems very likely that evolution has also conferred on the human X chromosome ironically a large portion of the burden for spermatogenesis.

## 17.10 Transmission of Y Deletions to ICSI Offspring

Once it had been shown that many cases of male infertility were caused by deletions on the Y chromosome, concern was registered immediately about the possibility of transmitting these deletions (or other genetic causes of male infertility) to offspring via what was the relatively new technique of ICSI, first introduced in 1992 [5, 6, 49]. It seems likely that these offspring if they carry the same genetic defect as their father on their Y chromosome, they will be found to be infertile just as their fathers were.

Microdeletions on the long arm of the Y chromosome do not appear to adversely affect the fertilization or pregnancy that results in either severely oligozoospermic or azoospermic men, from whom sperm was successfully retrieved by TESE [3, 5, 9, 10]. Concern had been registered that ICSI results might be poorer in Y-deleted men, but this has not been the case [10, 101]. Thus, men with Y deletions have the same chance of obtaining offspring via ICSI as non-Y-deleted males undergoing ICSI. Most genes involved in these Y deletions are expressed specifically in the testis during spermatogenesis but do not seem to be essential for fertilization or embryogenesis. The only problem for obtaining a pregnancy is the low number of sperm available in these patients, and this is circumvented by the ICSI procedure.

There has been some concern expressed about a possible widening of the *AZF<sub>c</sub>* deletion when it is transmitted to the next generation, but in our centers all of the male offspring from Y-deleted men have had the same Y deletion as their infertile father without any expansion [5, 9, 126, 158]. In fact, there was no cogent reason to expect there would be any expansion, considering the mechanism of these Y deletions.

So we also examined the Y chromosome of fathers, brothers, and paternal uncles of the infertile men for Y deletions and fertility. In all of the infertile Y-deleted men, the deletions were shown to be completely new, i.e., the fertile fathers of the infertile Y-deleted patients had no Y deletion, the deletion first appearing in the infertile sons. However, all male offspring of these infertile Y-deleted men derived from ICSI procedures had the same Y deletion of their father transmitted to them without any change (Fig. 17.5) [9, 158]. So there is no evidence that the male offspring will be any worse off than his father. Therefore, most couples with male infertility who have a baby are not very troubled by the possibility that their son might have a similar problem (Fig. 17.6) [159–161].

It can therefore be assumed that most Y chromosome deletions arise during spermatogenesis in the fertile testis of the infertile man's fertile father, and not dur-



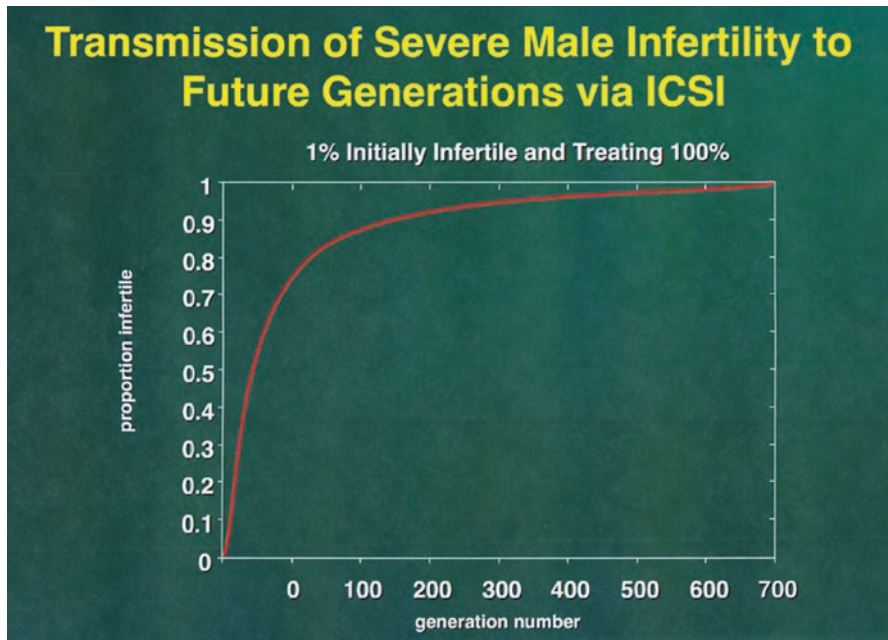


**Fig. 17.6** Transmission of severe male infertility to future generations via ICSI

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# Chapter 18

## Spermatogenic Stem Cell Cryopreservation and Transplantation



### 18.1 Brinster's Original Studies

It was first shown by Ralph Brinster that spermatogenic stem cells derived from testis tissue of normal mice can be transferred via the rete testis into mice with no spermatogenesis, and they will gradually populate stem cell niches all along the seminiferous tubules of large areas of the previously sterile recipient mouse testis and result in normal spermatogenesis and ultimately in normal offspring (Fig. 18.1) [1, 2]. Subsequently spermatogenic stem cells from a variety of species were successfully transplanted into SCID mice. The closer the phylum of the donor spermatogenic stem cells to the sterilized recipient mouse, the farther along the spermatogenesis of the transplanted germ cells would develop (Fig. 18.2). For example, rat stem cells would develop mature sperm in the SCID mouse but at the rate of rat spermatogenesis rather than mouse spermatogenesis. For more distant phyla, the final stages of spermatogenesis are not seen although earlier stages are well supported [3–8]. Frozen spermatogenic stem cells did just as well as fresh [9].

In fact, it has been very difficult to find markers for positively defining adequately human spermatogonial stem cells. They have to exist, obviously, but we cannot identify them except for this Brinster model of spermatogonial stem cell (SSC) transplantation in SCID mice. Even though human (or primate) SSCs injected into SCID mice cannot progress to complete spermatogenesis, they will “colonize” the seminiferous tubules of SCID mice, and that is the most reliable assay for them. In fact, testis tissue from any animal, and cryopreserved, can restore spermatogenesis to that animal after it has been sterilized by chemotherapy, because of the SSCs in that testis tissue.

Among the SSCs which are located in the dark spermatogonia, the SSCs are the least active cells and undergo the fewest mitoses (which reduces the mutation rate). The undifferentiated progenitor cells (dark A spermatogonia) that differentiate into pale A

## Animal models - transplantation

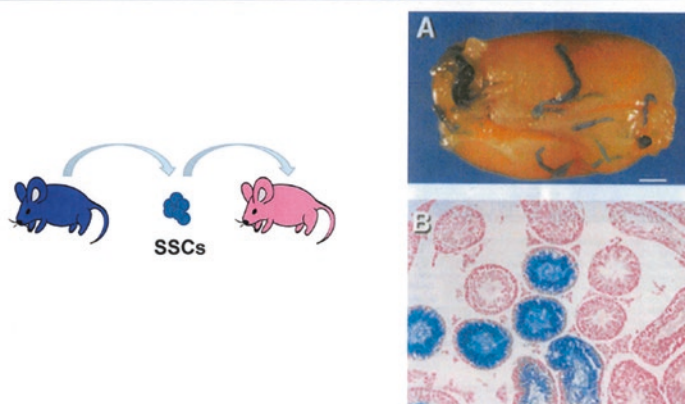


**Fig. 18.1** Animal models of SSC transplantation

spermatogonia divide less rapidly than pale spermatogonia or type B spermatogonia. Once you get past the SSC stage, all spermatogonia are committed to becoming sperm. The progenitor cells are not stem cells and cannot self-renew only SSC's do that. The only way to identify these SSCs has been with the Brinster transplant procedure. However, it will soon be possible to do so with chip transcriptome sequencing.

## 18.2 Clinical Applications for Cancer Patients and Also to Improve Sperm Count

In his groundbreaking spermatogonial stem cell transplant work, Brinster's lab sets the stage for a potential clinical application for preserving fertility in prepubertal male cancer patients and also sets the stage for a method finally of raising the sperm count of azoospermic or severely oligospermic men [10, 11]. Most of the human clinical progress in this field comes from Hooman Sadri-Ardekani.

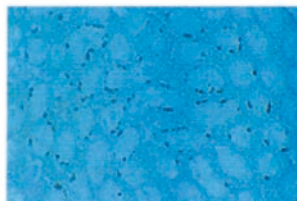
Over the last three decades, effective cancer treatments have improved the survival rates for many types of cancer. In children, the survival rate for all cancers combined improved from 58% to 80% (Fig. 18.3a, b) [12]. Currently it is estimated that 1 in 250 young adults between 20 and 29 years of age is a long-term survivor of childhood cancer [13, 14]. The adverse side effects of their cancer treatment include gonadal failure and sterility, and great efforts are now being made to find a way to prevent or modify these complications of cancer therapy [15]. For postpubertal boys or men, it is simple just to cryopreserve mature sperm found in ejaculate [16]. For girls or women, ovarian cortical strips can be cryopreserved [17]. But for prepubescent boys, the only way to preserve their future fertility is through testis biopsy,

**a**

## Animal models - transplantation

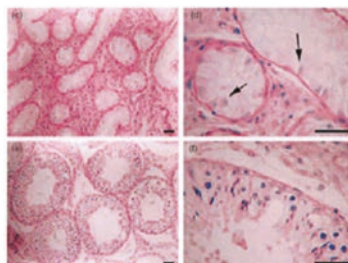
### Xenotransplantation:

Rat to mouse (Cloutier et al., Nature 1996)  
 Hamster to mouse (Ogawa et al., Biol Reprod 1999)  
 Rabbit/dog to mouse (Dobrinski et al., Biol Reprod 1999)  
 Baboon to mouse (Nagano et al., Biol Reprod 2001)  
 Bull to mouse (Izadyar et al., Reproduction 2002) →  
 Human to mouse (Nagano et al., Fert Steril 2002)



### Autotransplantation:

Mouse to mouse  
 Bull to bull (Izadyar et al., Reproduction 2003) →  
 Goat to goat (Honaramooz et al., Mol Reprod Dev 2003)  
 Ram to ram (Ridriguez-sosa et al., Theriogenology 2006)  
 Dog to dog (Kim et al., Reproduction 2008)

**b**

## In vitro culture of Spermatogonial Stem Cells

### • Successful animal models:

- Mouse (Nagano et al, 1998)
- Mouse-Long term (Kanatsu-Shinohara et al, 2003)
- Other species:
  - Rat (Hamra et al, 2005)
  - Bovine (Aponte et al, 2008)
  - Hamster (Kanatsu-Shinohara et al, 2008)
  - Dog (Kim et al, 2008)

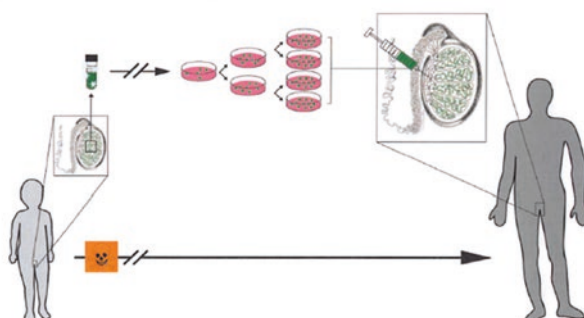


### • Human Spermatogonial Stem cells culture ?

**Fig. 18.2** (a) Summary of species in which SSC transplantation has been studied. (b) Summary of species in which SSC transplantation has been studied

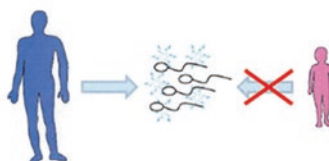
**a**

## Theoretical approach

**b**

## Clinical problem

- Sperm cryopreservation not possible in children



- Increased success in pediatric oncology
  - ~80% survives childhood cancer
  - 1:250 young adults is childhood cancer survivor

**Fig. 18.3** (a, b) Clinical application for prepubertal male cancer patients

spermatogonial stem cell culture and amplification, cryopreservation of spermatogonial stem cells, and their subsequent transfer back to the patient when he is an adult (Figs. 18.2, 18.3, 18.4, and 18.5a) [18–24].

As explained in Part 1 of this book, when the male goes through cancer chemotherapy or radiation, the more rapidly dividing or metabolizing cells are the first to die. It is the more slowly dividing cells (like Sertoli cells or Leydig cells) that clearly survive. For germ cells, it is the SSCs that divide the slowest and are the most resistant to the damage caused by chemotherapy. Clinically and histologically these are a small portion of the population of dark type A spermatogonia. It is from the dark type A spermatogonia that spermatogenesis (if it recovers) will derive from. For that reason it can take years sometimes for recovery of spermatogenesis in these patients,

## Xenotransplantation human SSCs

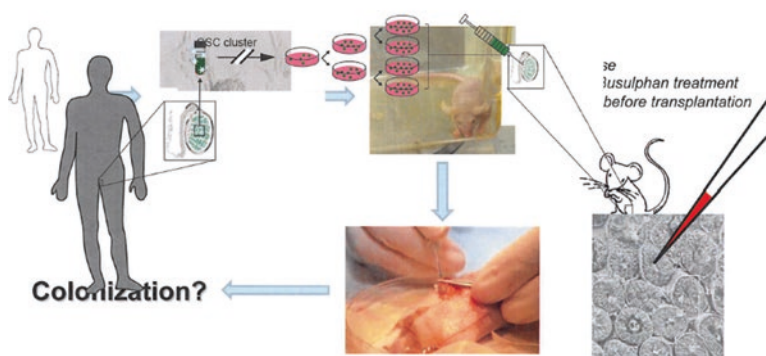


**Fig. 18.4** Xenotransplantation human SSCs

because the SSCs that are the most resistant cells to damage are of course the most slowly metabolizing and slow to reproduce themselves.

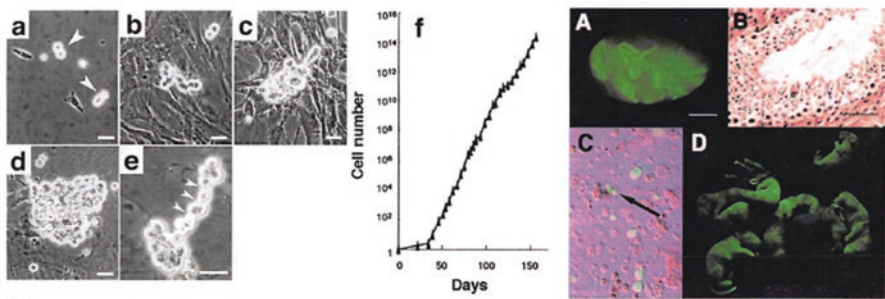
As mentioned in the first section of this book, the SSCs are very sparse, and they are imbedded below the basement membrane. Most of the spermatogonia are connected to each other as paired or aligned cells, (basically “connected to each other”) as they propagate and form spermatocytes and then mature sperm. Once the connected spermatogonia develop from the single SSCs, they do most of the propagation as they differentiate. The SSCs are single and attached to tissue. Thus when you dissociate testis tissue for a TESE procedure, if you do it in the usual way you retrieve sperm, you may “throw away” the precious SSCs. You must keep the dissected tissue because that is where the SSCs reside, and you won’t see them (because we have no good markers) other than transplanting all of the cells into the recipient to see the seminiferous tubules “colonize” (Fig. 18.1).

Studies led by Sadri-Ardekani et al. have shown that long-term culture of spermatogenic stem cells prior to transplanting them via efferent ducts or rete testis to a sterile recipient allows their amplification in number so as to get a better ultimate result (Fig. 18.5) [24, 25]. Since these stem cells represent a tiny 0.03% (less than 1/3000) of germ cells and only 1.25% of spermatogonia, in vitro culture and amplification of cryopreserved spermatogenic stem cells will enhance this clinical application of preserving fertility for prepubertal male cancer patients and possibly of converting very oligospermic or azospermic males to normospermia. Interestingly, leukemic cells which contaminate these spermatogenic stem cells will die in multiple transfers, while the spermatogenic stem cells survive. Thus a pure culture of safe spermatogenic stem cells can be transplanted back with Brinster’s method and restore fertility to the male prepubertal cancer patient when he becomes an adult.

**a**

# Animal models - culture

- Essential for future clinical application
  - Only limited number of SSCs from prepuberal testis



**b**

# In vitro propagation

| Human sample             | Culture days (passage number) | Number of injected cells( $10^5$ ) | Number of colonies / $10^5$ cells | Dilution factor | Human SSCs fold increase |
|--------------------------|-------------------------------|------------------------------------|-----------------------------------|-----------------|--------------------------|
| Testicular cells culture |                               |                                    |                                   |                 |                          |
| URO0021                  | 28(2)                         | 2.55                               | 2                                 | 133             | 53 ← 19 day s            |
| URO0021                  | 47(5)                         | 3.1                                | 0.8                               |                 |                          |
| GSCs subculture          |                               |                                    |                                   |                 |                          |
| URO0021(C21)             | 77(7)                         | 2                                  | 1.25                              | 8,870           | 18,450 ← 64 day s        |
| URO0021(C21)             | 84(8)                         | 0.5                                | 5                                 |                 |                          |
| URO0021(C21)             | 141(12)                       | 1.9                                | 2.6                               |                 |                          |

**Fig. 18.5** (a) Animal models for SSC transplantation in male cancer patients. (b) Table showing amplification of SSCs in serial culture to increase spermatogenesis

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## Chapter 19

# Making Sperm from Skin Cells



However, there is even more of a future possibility for actually restoring the fertility to prepubertal male cancer patients when they are adults, via IPS cells (induced pluripotent stem cells) (Figs. 19.1 and 19.2). IPS cells can be derived from skin cells and then can be differentiated into primordial germ cells (PGC-like cells) or PGCLCs and then into spermatogenic stem cells (SSCs) when they are transferred to the testis. This is not science fiction. We are doing this daily in the lab in mice and soon will be able to do this in humans. These cells derived from IPS cells (that are derived from skin cells) can be transplanted into the sterile testis, and there they colonize the seminiferous tubules and make normal spermatogenic stem cells (SSCs) that will then colonize the testis with spermatogonia. Spermatogonial stem cell (SSC) culture with transplantation and conversion of skin cells to sperm will be the most fruitful areas of future research in male infertility, aside from molecular characterization of spermatogenesis genes.

However, there are a few provisos to be worked out before this amazing and proven technology in mice can be applied to humans. Firstly, if ES cells (embryonic stem cells) or IPS cells (induced pluripotent stem cells) are injected directly into the testis (or ovary) without being at least 95% converted to PGC-like cells, they will just form a tumor. Fertilized eggs are “totipotent” in that they can form normal embryonic and adult structure. But ES cells are only “pluripotent” in that although they can form every kind of tissue, they cannot form an organized structure of cells like the heart or lungs or an organism. So firstly, after making IPS cells from skin or fibroblasts, these stem cells must be differentiated in culture into PGC-like cells (PGCLCs), i.e., into primordial germ cells. This is fairly easy to do and has even been done successfully in humans, as well as obviously in mice.

IPS cells can be made readily by incubating somatic cells (like the skin) with just four known genes (which can be readily purchased commercially). One can use just three genes, but all four originally described genes are best: KLF4, SOX2, OCT4, and C-MYC. That is all it takes to make stem cells (IPS) from the skin. You can then maintain those IPS cells (like ES or embryonic stem cells) in just three genes: LIF, FGF-i, and GSK2B-i.

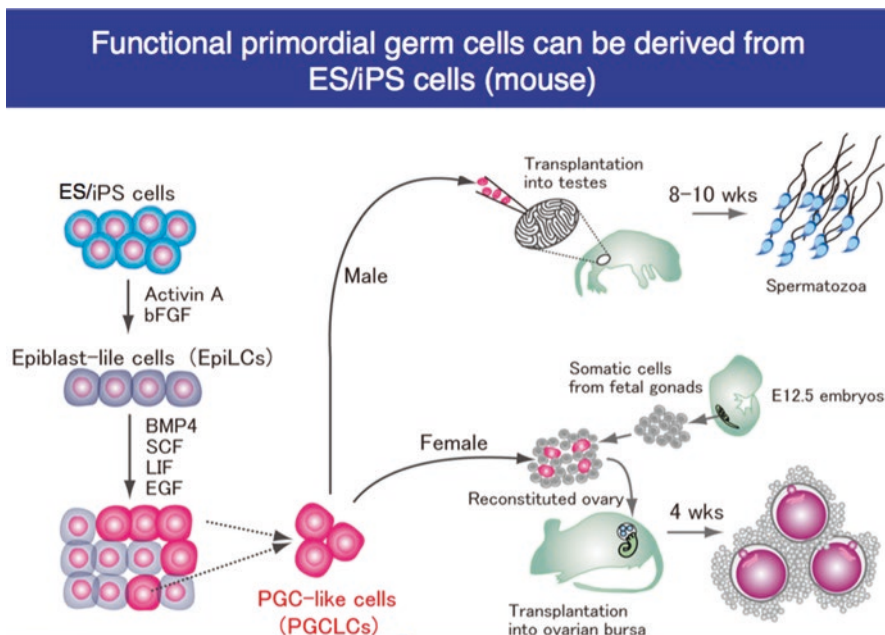


**Fig. 19.1** Derivation of artificial gametes from iPS cells in mouse. Note that ES cells (embryonic stem cells derived from inner cell mass (ICM) of the blastocyst) are essentially the same as iPS cells (derived from skin by incubation in 4 pluripotency genes). They can recapitulate in vitro exactly what happens in vivo. They can be cultured into epiblast cells, and from them into PGCs (primordial germ cells). The PGCs will create normal spermatogenesis when transferred into neonatal testis, by becoming SSCs (spermatogonial stem cells)

Next, you incubate those IPS cells in three genes (again all commercially available) to make epiblast-like cells. Note that primordial germ cells (PGCs) arise and are “specified” from a corner of the epiblast just at the beginning of gastrulation. So you need epiblast-like cells (EpiLCs) in order then to make PGCs (Fig. 19.2). The three genes required to make epi-L cells are activin A, bFGF, and KSR. Then the epi-L (epiblast) cells are incubated with five genes, and behold, you have in vitro PGCs (primordial germ cells), or, i.e., PGCL cells. The five commercially available genes needed to do this are BMP4, SCF, LIF, EGF, and BMP8a. So with this simple in vitro culture, you now have primordial germ cells that can be made into functionally normal spermatogonia, or even oocytes (Fig. 19.2a–c)

It normally will require 3–4 weeks in culture for skin cells to transform into IPS (stem cells). Then it requires 2 days for the IPS cells to convert in culture to epiblast-like cells and 6 days in culture for the epi-like cells to become PGCL cells.

The PGC-like cells can become sperm or oocytes in the following ways: If they are injected into fetal testis, they become spermatogonial stem cells (SSCs), and then when this fetus becomes an adult, its testis will make normal sperm. Alternatively, if the PGC-like cells are injected into fetal ovaries, they become normal oogonia and oocytes. These sperm and eggs in mouse IVF make normal

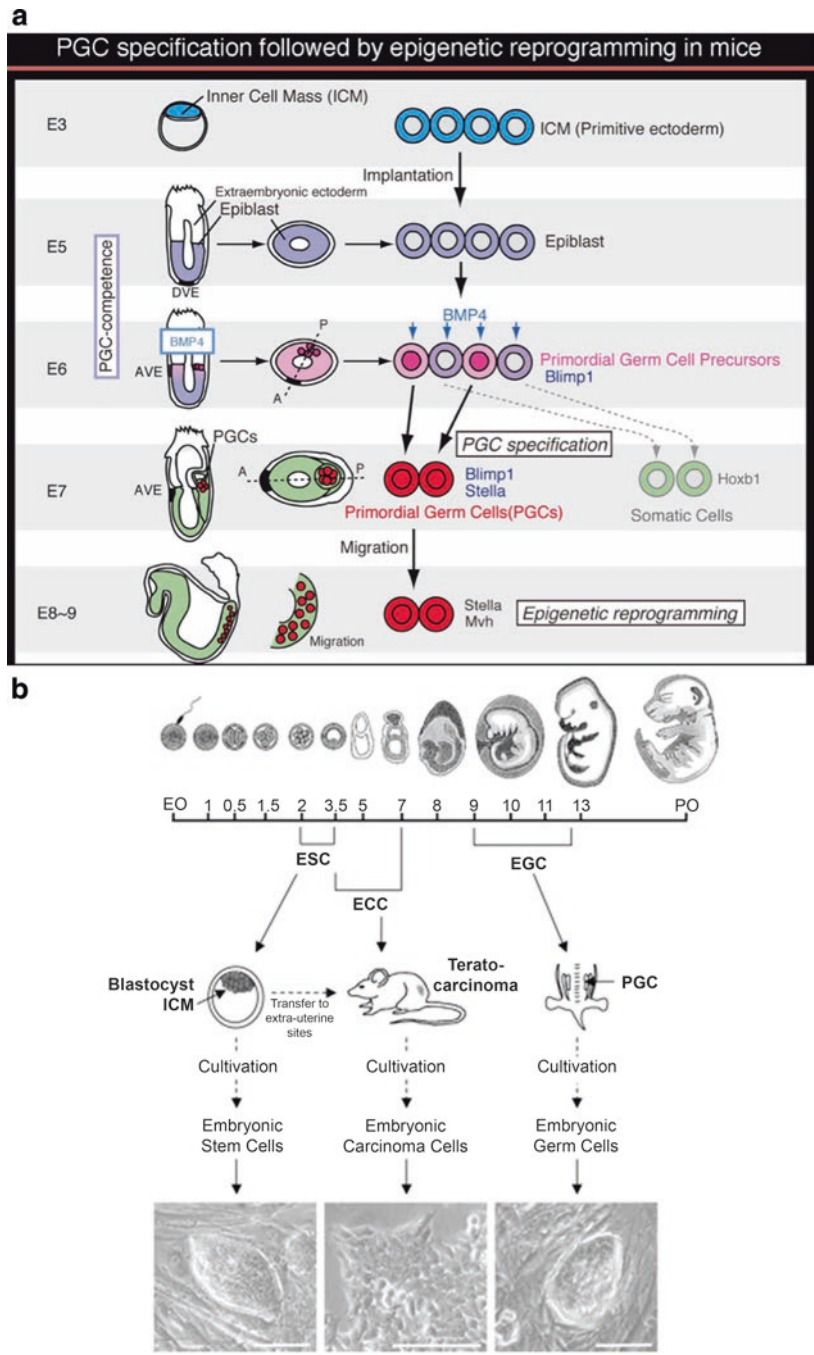


**Fig. 19.2** IPS cells or ES cells can be differentiated into epiblast cells and then PGC-like cells. Then the PGC-like cells can be differentiated into spermatogenic stem cells (SSCs) or even into oocytes. (a) When embryonic stem cells (ES) or induced pluripotent cells (iPS) are transferred to

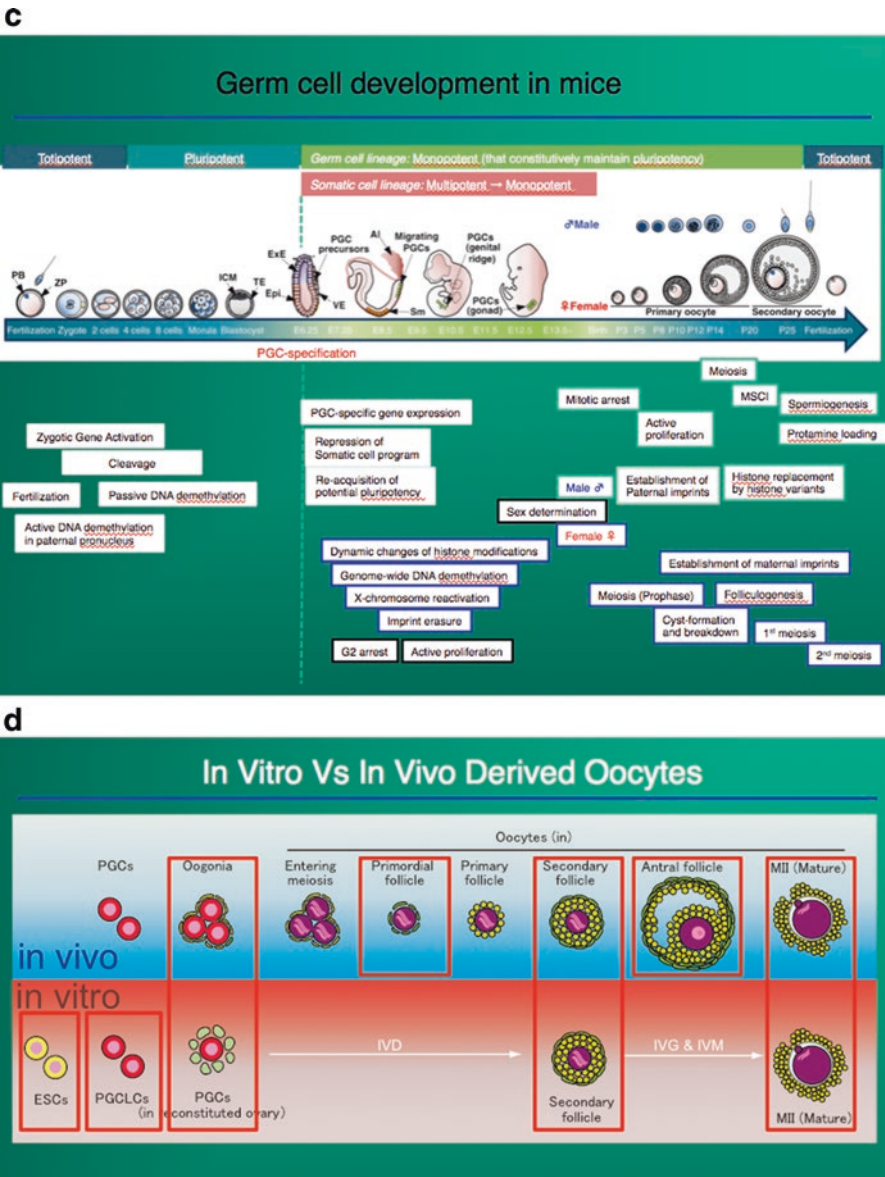


**Fig. 19.2** (continued) the testis before becoming PGCs they will produce a cancer. **(b)** A summary diagram of the steps of in vivo development of PGCs in the early embryo. In the female ovary these PGCs become eggs. In the male testis, fetal PGCs become SSCs. **(c)** Comparison graphic of in vivo generation of oocytes from PGCs to in vitro generation of oocytes from ES cells to PBCs. **(d)** Listing of steps in culture of skin cells to IPS cells to epiblast cells to PGCs, which can become oocytes or sperm

e

## Outline Of Making An Oocyte In Vitro

- Primordial follicles are “locked” by Fox 3 and other factors. Ovarian tissue cannot be cultured to oocyte as easily as skin cells.
- Skin cells to IPS cells: 3-4 weeks.
- IPS cells to epiblast cells: 2 days.
- Epiblast like cells to PGCLC cells 6 days.
- PGCLC with granulosa cells: an aggregate for 3 weeks.
- FSH culture: after dissociation for 11 days.
- HCG culture plus FSH: one day. Do this earlier than later.

Fig. 19.2 (continued)

offspring, and these normal offspring will grow up to be fertile and in turn have more normal offspring naturally.

So now the issue is what if these PGCL cells are injected into adult testes or ovaries rather than fetal or neonatal, before converting them to SSCs? They will just die. They need the fetal or neonatal gonad, just like in real life: when the fetal primordial germ cells (PGCs) migrate from the epiblast to the gonadal ridge, they differentiate into spermatogonial stem cells (SSCs) in the seminiferous tubules (if it is a fetal testis) and then into spermatogonia. If it is a fetal ovary, the migrating PGCs become oogonia and oocytes. That is what happens normally to PGCs in vivo in the fetus. But how can we make sperm (or eggs) from PGCs in the adult? When PGCs are injected into an adult testis, or ovary they just die. They have to be made into SSCs in a fetal or neonatal testis. Then SSCs survive and make sperm in the adult testis.

For making sperm from PGCs in the adult, you have to make SSCs (spermatogonial stem cells) in vitro, either with culture in the appropriate genes, which will have to include DAZL and VASA and others, or you would have to incubate in fetal testis. This issue is trickier than making oocytes in vitro. To make oocytes in vitro from PGCL cells, you just incubate in fetal granulosa cells for 3 weeks, and fetal granulosa-like cells can undoubtedly be made via a different pathway from IPS cells. The reason making sperm is so much more difficult than making oocytes is

that the PGCs need to be surrounded by these fetal or neonatal somatic cells. The Sertoli cells in the seminiferous tubule are supported by Leydig cells. It is easier to make oocytes in vitro with unstructured fetal granulosa cells in the female than to make SSCs in vitro with a structure like the seminiferous tubule. But no doubt the plasticity of IPS cells and PGC cells in vitro should eventually lead to making sperm and eggs from the skin.

## Chapter 20

# Identification of Spermatogonial Stem Cells and Genetic Control of All Stages of Spermatogenesis



An ability to identify SSCs by their transcriptome analysis would greatly benefit future treatment of male infertility. If you could determine the RNA expression of every stage of spermatogenesis, that would be a more reliable identification of cell type by gene expression than just histology. It would mean the elucidation of the total genetics of spermatogenesis. The identification of spermatogonial stem cells (SSCs) in particular in the human would help us better understand the origin of testicular cancers such as teratoma, choriocarcinoma, embryonal carcinoma, and seminoma, which arise in the testis from germ cells [1–5]. It is known that primordial germ cells (PGCs) arise from the fetal epiblast at the time of gastrulation and migrate eventually to the gonadal ridge to become spermatogonial stem cells (SSCs) in the male gonad. Any pluripotent stem cells that have not fully differentiated into PGCs when they enter the fetal testis could become a testis cancer. In fact cancers of the adult testis are composed of differentiated cells which represent all basic somatic cell types, much like what ES cells develop into if not suppressed in culture or in a niche. So the origin of testis cancer is most likely embryonic stem cells rests that entered the testis before fully converting to PGCs.

PGCLCs (primordial germ cell-like cells), though differentiated, still retain expression of stem cell genes like Oct4, SOX 2, and KLF 4, even though they are not functionally pluripotent and therefore not in danger of teratoma formation. They are locked into non-functionality by the genes DAZL and VASA. Without this final “locking,” some PGCs could still possibly result in teratoma, as they still have ES cell pluripotent potential. It is possible that teratomas in testis and in ovary could result from PGCs which still have some residual pluripotent ES cell expression not completely silenced by DAZL and VASA. There is also an apoptosis “security system” that is indeed necessary to protect against ES rests developing into teratoma. In fact one could speculate that ovarian teratomas are benign, and testis teratomas are malignant because the adult testis retains stem cell potential and the ovary does not. With the complete single-cell transcriptome of human SSCs and later spermatogenic cells, it will be easier to study this hypothesis of the origin of testis cancer in adult humans.

An interesting clinical use for the complete human spermatogenic transcriptome analysis is to clear up the diagnostic confusion in the pathology literature about testis histology in azoospermic men, i.e., “hypospermatogenesis,” “Sertoli cell only,” and “maturation arrest” [1–3]. Now that it is known that 60% of azoospermic men have some quantitatively minuscule amount of sperm production in their testes (enough in fact for successful ICSI), the non-quantitative frankly erroneous pathologic diagnoses for spermatogenic defects are no longer tolerable [4–6]. The single-cell spermatogenic transcriptome will allow a much more accurate depiction of spermatogenic deficiency, which may even help predict which azoospermic men will be found to have sperm in a TESE procedure (testicular sperm extraction). The reason is that the most reliable and safe microsurgical technique for this is to sample the periphery of every anatomic lobule, because if there is just one spermatogonial stem cell (SSC) in that lobule, the surgeon will find sperm at its periphery sufficient for successful ICSI.

Perhaps the most exciting clinical application of a spermatogenic transcriptome resource is in predicting the recovery of spermatogenesis after chemotherapy or radiation for cancer [6–8]. It may sometimes be years before the azoospermia induced by cancer treatment recovers. The only way to predict this would be an accurate determination of SSC survival. The human SSC has been elusive up till now, with no reliable markers, and the only reliable assay has been transplantation to the SCID mouse. With a transcriptome determination of SSC survival, recovery of spermatogenesis in cancer patients might finally be predictable.

Another application for fertility preservation in prepubertal boys with leukemia is to culture spermatogonial cells from testis biopsy before cancer treatment and culture SSCs along with the leukemia cells. In multiple passages, the leukemia cells should die before the SSCs. So SSC transcriptome analysis would help to determine if all the remaining cells are SSCs rather than leukemia. Thus, it should be possible to simply now (today) freeze the testis biopsy of prepubertal boys with cancer and when they are adults (years from now) transplant back their SSCs and thereby restore their fertility. Thus the treatment of male infertility has advanced scientifically by leaps and bounds from the days of invalidated empirical treatments that did not work. It is the “fundamentals” (i.e., the science) “of male infertility” that will bring the field of andrology forward into this exciting “brave new world.”

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