Sijo J. Parekattil
Sandro C. Esteves
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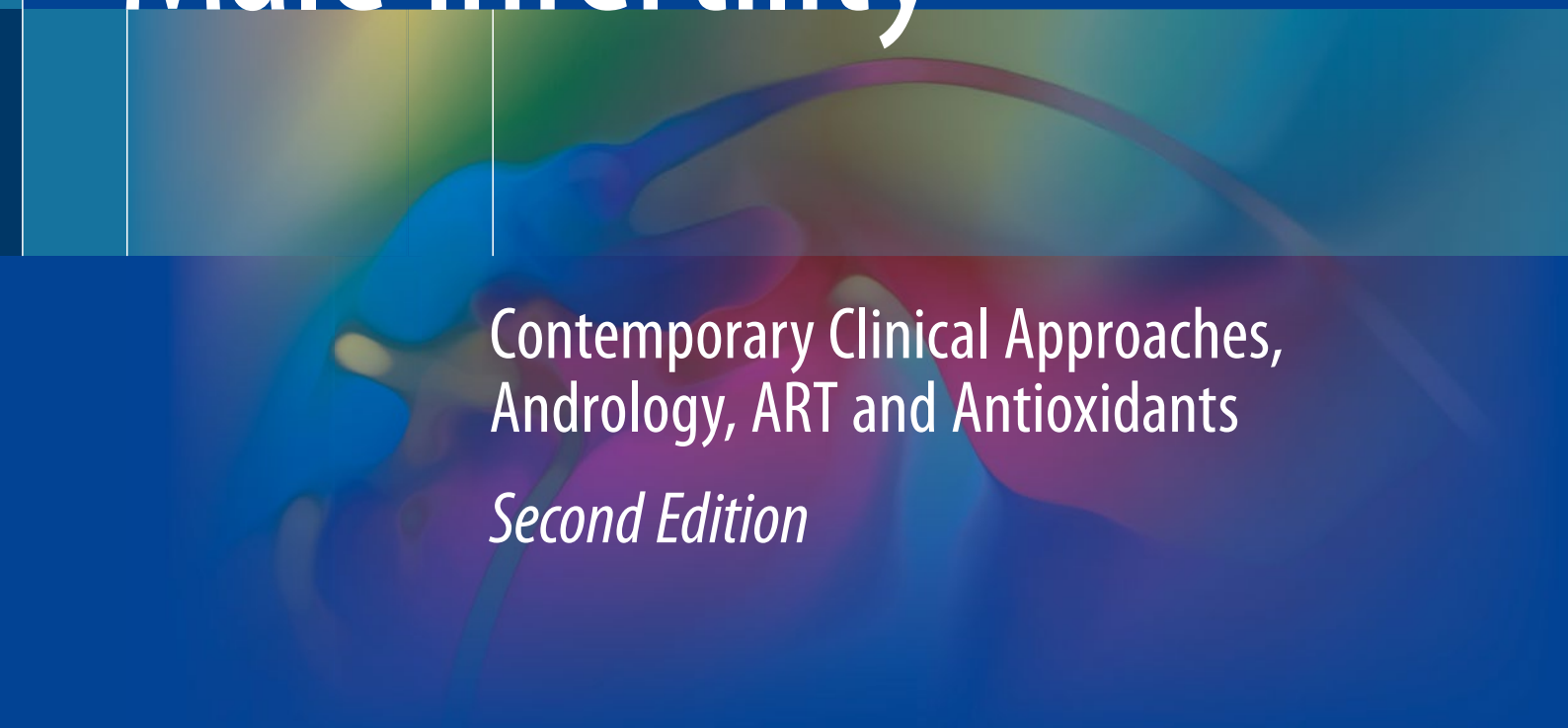
Editors

Male Infertility

Contemporary Clinical Approaches, Andrology, ART and Antioxidants

Second Edition



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This work is dedicated to my wife and sons, who have been ever patient and considerate in allowing me to pursue my academic interests. It is also dedicated to my mentors who believed in me and gave me the opportunity to grow. It is also dedicated to our patients who place their faith in our hands and who allow us the honor and privilege to continue to learn and better the art of medicine for a better tomorrow.

–Sijo J. Parekattil

To my father Waldemar Esteves (late) for instilling the virtues of integrity, perseverance, and enthusiasm. To my wife, Fabiola, my sons, Alexandre and Catarina, for their devotion, love, and support. To Prof. Nelson Rodrigues Netto Jr. (late) (UNICAMP), Prof. Anthony Thomas (late) (Cleveland Clinic), Prof. Sidney Glina (ABC University), and Prof. Ashok Agarwal (Cleveland Clinic) for their guidance and support and for being giants who take their apprentices by their hands and teach them how to climb; once on their shoulders they show us what to see, helping us to see better and further.

–Sandro C. Esteves

To my father Professor RC Agarwal (late) for instilling the virtues of honesty, dedication, and hard work. To my wonderful wife, Meenu, sons, Rishi and Neil-Yogi, for their unconditional love and support. To Prof. Kevin Loughlin (Harvard Medical School), Prof. Anthony Thomas (late) (Cleveland Clinic), and Prof. Edmund Sabanegh (Cleveland Clinic) for their friendship, guidance, and support and for making an indelible positive impression on my life. To my associates at work, large number of researchers and students, and most importantly the patients who placed their trust in our work.

–Ashok Agarwal

Foreword

I have been eagerly awaiting the release of the second edition of this text, *Male Infertility: Contemporary Clinical Approaches, Andrology, ART and Antioxidants*, which is edited by three luminaries in the field of reproduction, Sijo J. Parekattil, MD; Sandro C. Esteves, MD; and Ashok Agarwal, PhD. The first edition of this text served as an invaluable resource to those of us involved in the clinical care of couples encountering reproductive challenges and also to those of us investigating the pathophysiology underlying disordered fertility. In the same vein, this second edition builds upon the important principles shared in that first edition but also includes new and important insights that have been subsequently reported in both the basic science and clinical literature.

The editors have done a masterful job of assembling a panel of expert authors who are also fantastic writers. Each chapter is rich with important information, yet at the same time very pleasant to read. Additionally, while each chapter is a "stand-alone" piece of academic work, collectively, the chapters build upon one another to provide the reader with a truly 360-degree, state-of-the-art understanding of andrology, ART, and antioxidants.

In an era where the clinical and research focus in reproductive medicine often pivots solely to assisted reproductive techniques, this text does a wonderful job of comprehensively and holistically examining andrology, assisted reproductive techniques, and the role of antioxidants. The "winners" in all of this are the clinicians who will use this book to enhance the integrated and informed care that they are providing their patients, as well as the patients themselves, who are on the receiving end of this optimized care. The editors are to be congratulated for passionately assembling this important group of academic manuscripts. There is no doubt that countless couples will benefit from the collective knowledge and inspiration that this text imparts within reproductive clinicians and scientists alike.

Chicago, IL, USA — Robert E. Brannigan, MD

Preface

The practice of male infertility has dramatically evolved over the past few decades. This has been achieved after the realization of the important contribution the male counterpart holds in the couple's infertility. It is also supported by a number of breakthroughs in the diagnosis and treatment of this medical condition. The field of male infertility truly illustrates the need for a multispecialty approach to the effective diagnosis and management of such conditions. From the initial referral possibly from a reproductive endocrinologist or gynecologist to the male infertility urologist or andrologist, a multidisciplinary team including doctors, nurses, embryologists, technicians, researchers, and alternative medicine specialists needs to work as a cohesive unit to provide our patients with the most effective and highest quality care.

Due to the success of the first edition of this book in 2012, we decided to work on the second edition in 2018 with the goal to bring out a medical textbook that can serve as the most reliable source of accurate information on various aspects of the rapidly developing field of male infertility. This book was an attempt to gather experts from each of these fields and present an integrated management approach with detailed descriptions of topics ranging from the initial clinical diagnosis, management, new treatment options, and scientific rational for the various approaches. The book initially focuses on the clinical aspects of male infertility and then dives into the use of antioxidants as adjunctive therapy and the current state of affairs in antioxidant research. In this second edition, which includes 72 chapters in total across 7 parts, over 20 new chapters were added to enhance the book's appeal by including the most recent advances brought to the male infertility arena. Additionally, this edition has new features including video clips illustrating some of the most fascinating male infertility treatment modalities and a dedicated new part on current guidelines on male infertility and antioxidants to enlighten readers on how to most optimally manage male infertility clinical scenarios. The 129 contributing authors hail from leading institutions in 18 countries across 6 continents in an attempt to capture a wide range of techniques and approaches. New to this edition are dedicated sections within each chapter covering key points and review criteria, carefully bookended by introductory and concluding content that sets the stage for and sums up the state of the art in male infertility. We are hoping that this text may serve as a reference guide for specialists across the male infertility team to further enhance dialogue, discussion, and refinement in our multidisciplinary approach.

We would like to thank the authors for their contributions and our families for their patience in allowing us to put together this project. We wish to acknowledge the Glickman Urological & Kidney Institute at the Cleveland Clinic Foundation, the PUR Clinic with University of Central Florida, and Androfert Fertility Center for institutional support for this endeavor as well. We are grateful to Kristopher Spring, senior editor, for his support and advice, and to Michael D. Sova, developmental editor, for his tireless efforts in reviewing and editing each of the manuscripts. We hope that this book will provide a comprehensive, consolidated reference for a broad range of topics in male infertility.

Clermont, FL, USA — Sijo J. Parekattil
Campinas, Brazil — Sandro C. Esteves
Cleveland, OH, USA — Ashok Agarwal

About the Editors

Sijo J. Parekattil, MD is Co-director of PUR Clinic (Personalized Urology & Robotics) at South Lake Hospital and Orlando Health and Associate Professor of Urology at the University of Central Florida (UCF). He was an Electrical Engineer (University of Michigan) prior to his medical training and thus has interests in surgical techniques incorporating technology, robotics, and microsurgery. He completed his urology residency training at Albany Medical Center and then went on to complete dual fellowship training from the Cleveland Clinic Foundation, Cleveland, in Laparoscopy/Robotic Surgery and Microsurgery/Male Infertility.

Dr. Parekattil has received numerous awards and published several articles in the field of robotic microsurgery. He is an editor of a few textbooks on Male Infertility and a surgical textbook on Robotic Microsurgery. He is a pioneer in robotic microsurgery in urology. He has developed novel treatments for chronic groin and testicular pain. He is one of the founding board members of the Robotic Assisted Microsurgical and Endoscopic Society (RAMSES) and is on the foundation board for Florida Polytechnic University, Lakeland, FL.

Sandro C. Esteves is Medical and Scientific Director of ANDROFERT—Andrology and Human Reproduction Clinic—a referral Fertility Center for Male Reproduction in Brazil.

He earned his MD in 1990 from the University of Campinas (UNICAMP), Brazil, where he did residency training in General Surgery and Urology. He completed his training in the United States (1995–1996) as a Research Fellow at the Cleveland Clinic's Center for Reproductive Medicine. He was awarded a Master's degree in Surgery in 1998, at UNICAMP, and a PhD in Medicine (Urology) in 2001, at the Federal University of São Paulo (UNIFESP). Dr. Esteves is a board-certified Urologist by the Brazilian Society of Urology, and a certified-ART Center Director and IVF Consultant by the Brazilian Society of Assisted Reproduction.

Dr. Esteves is a Collaborating Professor in the Department of Surgery (Division of Urology) at the University of Campinas (Brazil) and Honorary Professor of Reproductive Endocrinology at the Faculty of Health, Aarhus University, Denmark. He is also Research Collaborator at the Cleveland Clinic's Center for Reproductive Medicine (USA). His major contributions in the

field of reproductive medicine have been in the area of male infertility and microsurgery, reproductive endocrinology, assisted reproductive technology, cleanroom technology, and quality management. His center, ANDROFERT, was one of the first centers, both in Brazil and worldwide, to introduce quality management and IVF cleanroom technology, and it is certified according to ISO 9001:2015 and ISO 14644-1 standards.

Prof. Esteves has published approximately 300 peer-reviewed scientific papers, authored over 80 book chapters, and presented over 150 papers at both national and international scientific meetings. His current Hirsch index is 46 (Google Scholar) while his citation count is more than 6000. Sandro is also a Faculty Member of F1000Prime in the area of reproductive endocrinology and infertility since 2016 and is a co-founder of Group POSEIDON (**P**atient-**O**riented **S**trategies **E**ncompassing **I**ndividualize**D** **O**ocyte **N**umber) (www.groupposeidon.com). He is also a member of the Society for Translational Medicine's male infertility cooperative group.

Dr. Esteves has served as an editor of eight textbooks related to male infertility, reproductive medicine, and assisted reproductive technology. He is also the guest editor of six special issues in scientific journals on topics related to reproductive medicine. He currently serves on the editorial board of several journals and is Associate Editor of *International Brazilian Journal of Urology* and *Frontiers in Endocrinology* (Reproduction).

Dr. Esteves is a much-requested international lecturer and gives approximately 50 lectures annually. He has been invited as guest speaker in over 40 countries. He is the recipient of the "Alumni of the Year" Award from the Cleveland Clinic Center for Reproductive Medicine, and consecutive Star Awards from the American Society for Reproductive Medicine for the last 6 years.

Ashok Agarwal, PhD, HCLD(ABB), ELD(ACE) is the Head of Andrology Center and Director of Research at the American Center for Reproductive Medicine since 1993. He holds these positions at The Cleveland Clinic Foundation, where he is Professor of Surgery (Urology) at the Lerner College of Medicine of Case Western Reserve University. Ashok was trained in Male Infertility and Andrology at the Brigham and Women's Hospital and Harvard Medical School and later worked as Assistant Professor of Urology at Harvard from 1988 to 1992. Ashok has over 26 years of experience in directing busy male infertility diagnostic facilities and fertility preservation services. He is very well published with over 730 scientific papers and reviews in peer-reviewed scientific journals and is ranked in Scopus as the #1 author in the world in the fields of Male Infertility/Andrology and Human Assisted Reproduction, based on the number of peer-reviewed publications, citation scores (Scopus: 32,001; Google Scholar: 67,039), and h-index (Scopus: 96; Google Scholar: 125). He is currently an editor of 39 medical text books/manuals related to male

infertility, ART, fertility preservation, DNA damage, and antioxidants and active in basic and clinical research. His laboratory has trained over 1,000 scientists, clinicians, graduate, and undergraduate students from the United States and more than 55 countries. His current research interests include proteomics of male infertility and the molecular markers of oxidative stress and DNA integrity in the pathophysiology of male reproduction.

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Part I

Male Infertility Diagnosis and Management

Causes of Male Infertility

1

Graham Luke Machen and Jay I. Sandlow

Key Points

- The majority of the causes of male infertility may be either preventable or treatable.
- Pre-testicular causes of male infertility exert their negative effect via imbalances in the hormonal milieu of sperm production. Sexual function is also negatively impacted with effects seen at the level of erectile function, ejaculatory function, and sexual desire.
- Varicocele is a common modifiable cause of male infertility and negatively affects global testicular function through oxidative stress.
- Severe oligospermia or azoospermia requires genetic screening, given their high associated prevalence of Klinefelter's syndrome, karyotypic abnormalities, and microdeletion of the Y chromosome.
- Many testicular causes of male infertility (radiation, toxins, environmental factors, genital tract inflammation, varicocele, testicular hyperthermia) lead directly to sperm DNA damage.

1.1 Introduction

Of all sexually active couples, 12%–15% are infertile, of which a male component can be identified 50% of the time either in isolation or in combination with a female factor [1, 2]. Previous research in a US male fertility clinic analyzing 1430 patients identified causes of infertility from most to least common: varicocele, idiopathic, obstruction, female factor, cryptorchidism, immunologic, ejaculatory dysfunction, testicular failure, drug effects/radiation, endocrinology, and all others [3]. However, despite recent technologic and diagnostic advances, idiopathic infertility remains a common diagnosis, with approximately 25% of patients not having an identifiable cause of infertility [4, 5]. Regardless, many recognizable causes of male infertility are treatable or preventable; thus, a keen understanding of these conditions remains paramount. This chapter will provide an overview of the causes of male infertility, divided into pre-testicular, testicular, and post-testicular etiologies.

1.2 Causes of Male Infertility

1.2.1 Pre-testicular

1.2.1.1 Hypogonadotropic Hypogonadism

Hypogonadotropic hypogonadism (low or absent gonadotropins) affects fertility at multiple levels. Sperm production is deleteriously affected by a lack of testosterone (from low LH) and a lack of a stimulatory effect of FSH on the Sertoli/germ cell complex. Hypogonadism may also manifest itself through effects on sexual function, including sexual desire as well as erectile and ejaculatory dysfunction. There are many etiologies of hypogonadotropic hypogonadism. The most common include elevated prolactin, medications, illicit drugs, and pituitary damage. Idiopathic hypogonadotropic hypogonadism, including Kallmann syndrome, is another, albeit rare, cause of hypogonadotropic hypogonadism.

1.2.1.2 Elevated Prolactin

Elevated prolactin may cause hypogonadism by suppressing the release of GnRH. Symptoms of hypogonadism, especially erectile dysfunction and loss of libido, are the most common presenting symptoms in males with hyperprolactinemia, though galactorrhea and gynecomastia may also be evident [6].

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Elevated prolactin may be secondary to various etiologies. The most common of these is a prolactinoma, which typically arises from the pituitary gland. Because prolactinomas in men are more likely to manifest through mass effect, visual disturbances and headaches may be present [7]. Normal prolactin levels range from 2 to 23 ng/mL. In the setting of prolactinomas, the prolactin level frequently corresponds to tumor size. Specifically, levels typically range from 50 to 300 ng/mL in microadenomas but may be as high as 5000 ng/mL in patients with macroadenomas (greater than 1 cm in greatest dimension) [8, 9].

There are other significant causes for hyperprolactinemia as well. Prolactin elevation may be associated with common medical conditions, such as renal failure, hypothyroidism, and cirrhosis. Prolactin levels may also be elevated in certain systemic diseases such as systemic lupus erythematosus, rheumatoid arthritis, celiac disease, and systemic sclerosis. Many drugs elevate prolactin levels, especially those which block the effects of dopamine, such as antipsychotics [10].

1.2.1.3 Pharmacologic

Various medications may cause hypogonadotropic hypogonadism. Estrogens and progestins may cause a decrease in testosterone levels via negative feedback to the hypothalamic–pituitary–gonadal (HPG) axis. Marijuana is known to decrease testosterone levels by working on the endocannabinoid receptors present at multiple levels of the hypothalamic–pituitary axis [11]. Both ethanol and cannabinoids suppress GnRH secretion at the level of the hypothalamus. Endocannabinoid receptors have been found in the pituitary and so may also affect the hypothalamic–pituitary axis at that level as well [12].

LHRH agonists and antagonists are used for the treatment of prostate cancer, precocious puberty, and gender reassignment surgeries. These medications produce a tonically stimulated state which, unlike the physiologic circadian rhythmicity of normal LHRH stimulation, acts to decrease LH and FSH secretion.

Opioids may also produce profound hypogonadism; these medications bind to mu-receptors in the hypothalamus inhibiting the production and release of GnRH [13]. Of note, opioids may also directly inhibit spermatogenesis in the testis, and chronic opioid users have been shown to have decreased sperm concentration, motility, and increased DNA fragmentation [14, 15].

Another increasingly common medication resulting in infertility is exogenous testosterone replacement for hypogonadism. Testosterone downregulates the HPG axis, leading to a decrease in spermatogenesis [16]. Despite this established effect on fertility, many physicians, including urologists, still prescribe testosterone to hypogonadal men with infertility; in fact a recent survey found that 25% of urologists prescribed testosterone to men in this specific patient cohort [17].

1.2.1.4 Idiopathic Hypogonadotropic Hypogonadism and Kallmann Syndrome

Idiopathic hypogonadotropic hypogonadism (IHH) is a rare disorder caused by defects in gonadotropin-releasing hormone, including its action and/or release, in the setting of an otherwise normal HPG axis [18]. While the age and severity of presentation may vary, most commonly patients present during adolescence with failure to go through puberty. Other presenting symptoms may include anosmia, cleft palate, and hearing loss [19]. In the presence of anosmia, IHH is referred to as Kallmann syndrome, which affects 1 in 8000–10,000 males [20, 21]. The etiology is likely genetic, and multiple genes have been identified as playing a role in pathogenesis including *KAL1* [22, 23]. These most commonly manifest through the same mechanism whereby GnRH-secreting neurons fail to migrate to the hypothalamus. Lack of these neurons in the hypothalamus results in a lack of GnRH secretion and thus hypogonadism.

1.2.1.5 Testicular

Varicocele

A varicocele is a dilation of the pampiniform plexus likely caused by the absence or incompetence of the venous valves of the internal spermatic vein. Varicoceles have long been associated with infertility. The first written description is attributed to Celsius who noticed the association between the varicocele and testicular atrophy [24]. In the 1800s, surgical correction was seen to improve semen quality. It is the most common surgically correctable cause of male infertility. Roughly 12% of all men have a varicocele, but this number jumps to 35%–40% in men with infertility [25, 26].

Varicoceles may affect multiple semen parameters, including total sperm count, sperm motility, and sperm morphology [27, 28]. There are many theories about the underlying pathophysiology of a varicocele, with heat, renal metabolites, and hormonal abnormalities all playing a role. However, most agree that disruption of the countercurrent heat exchange mechanism in the testis, causing hyperthermia, is the most likely mechanism. Numerous studies point to impaired sperm production and a decrease in semen quality when scrotal temperatures are elevated [29–34]. One study showed that men with scrotal skin temperatures above 35 °C for >75% of the day had sperm concentrations of 33 million/mL as compared with densities of 92 million/mL in men with scrotal skin temperatures greater than 35 °C for <50% of the day [33].

The mechanism by which heat causes decreased sperm counts is poorly understood, but one hypothesis is that increased temperature could increase the metabolic rate of testicular and epididymal sperm. Regardless, men with varicoceles demonstrate increased reactive oxygen species and decreased antioxidant capacity, which may alter sperm DNA and can affect sperm maturation [35–37]. Furthermore, data has suggested that larger varicoceles may be associated with more profound oxidative stress [38, 39].

A somewhat controversial area with regard to the management of varicoceles is associated with hormonal abnormalities. It has been hypothesized that the pathophysiology associated with spermatogenesis may be applicable to Leydig cells and testosterone synthesis as well [40, 41]. Accumulating data supports this hypothesis of global testicular dysfunction, and by repair of the varicocele, testosterone may improve [42, 43]. In fact, a meta-analysis in 2012 found a mean improvement in testosterone of 97.5 ng/dL [44]. However, the evidence is somewhat mixed as to the degree of improvement, with several recent prospective studies demonstrating more modest testosterone increases [45–47]. Thus, the clinical implications of varicoceles and their effects on hypogonadism remain somewhat controversial.

Cryptorchidism

Cryptorchidism is well known to affect fertility. The severity of its effect on fertility is directly proportional to the severity of the cryptorchidism, with bilateral cryptorchidism having more severe effects than unilateral and with higher testes having worse function than lower testes [48–51].

Similarly, orchidopexy has been shown to improve fertility, with the best results obtained with fixation at a young age, especially prior to 1 year of age [52]. Fixation after age 10 may not improve fertility, or may improve it only modestly, suggesting that permanent and progressive damage is done to the testis while in an abnormal position, which is supported by histologic studies [53, 54]. Actual paternity rates in men who underwent orchidopexy for unilateral cryptorchidism are 89%, slightly less than the non-cryptorchid group, which had a 94% paternity rate. Bilaterally cryptorchid men post-orchidopexy had markedly lower paternity rates, at 62% [50, 51].

The pathophysiology of cryptorchidism is complex, with heat likely playing a partial but significant role [55, 56]. A number of other factors are likely to come into play, including the underlying genetics, hormonal milieu, and environmental exposures which originally led to the cryptorchidism [57–59].

Testicular Cancer

Testicular cancer is strongly associated with infertility. There are multiple ways in which testicular cancer can contribute to reduced fertility. Both testicular cancer and impaired spermatogenesis may be related in their etiology of embryologic testicular dysgenesis. The testicular dysgenesis syndrome is a spectrum of disease that may involve cryptorchidism, hypospadias, decreased spermatogenesis, and testis cancer. In this syndrome, it is thought that all of these share an origin of abnormal fetal testis development. As a result of this developmental anomaly, any number of these manifestations may be present in a boy [60]. Testicular tumors may also directly contribute to infertility by secreting hormones, which can downregulate sperm production in the contralateral testis [61–64]. This is uncommon but has been seen with Leydig and Sertoli cell tumors as well as seminomas. Tumors may directly disrupt spermatogenesis by mass effect, by alterations of the endocrine cascade, or by the effects of the inflammatory reaction to the tumor [65].

At presentation, roughly 10% of men will be azoospermic, and roughly 50% will be oligospermic. While orchiectomy will result in a rebound in semen parameters in roughly 90% of these men [66], adjuvant treatments with surgery, chemotherapy, or radiation can further decrease fertility.

1.2.1.6 Ionizing Radiation

Excellent data on the effects of ionizing radiation is available from two similar studies, which are unlikely to be repeated. Researchers in these studies prospectively irradiated the testes of prisoners with single or multiple doses of radiation up to 600 cGy [67, 68]. Sperm counts were followed, and serial testicular biopsies were done. These studies showed that sperm counts declined when testes were irradiated and that decline was dose dependent. At low doses of ~7.5 cGy, a mild decline of sperm counts was seen, and this decline increased to severe oligospermia by 30–40 cGy and azoospermia by 78 cGy. The time to recovery was also seen to be dose dependent, with those receiving 20 cGy beginning to have a recovery of sperm counts by 6 months, those with 100 cGy at 7 months, 200 cGy at 11 months, and 600 cGy at 24 months. The percentage of men achieving a complete recovery and time to achieve a complete recovery also declined with increasing radiation doses.

Decline to the nadir of sperm counts was seen at roughly 64 days, corresponding roughly to the time required for sperm cell production from spermatogonia. More rapid declines were seen with higher radiation doses, indicating increased damage to the more highly differentiated cells undergoing spermatogenesis. Biopsy results from these studies showed that spermatogonia numbers nadired at much lower levels with higher doses of radiation and that these nadirs took longer to achieve than those which had received lower doses of radiation.

These studies provide excellent information into the biology of the effects of radiation on spermatogenesis on the

healthy young testis. Clinically, however, the effects seen are often more pronounced given the setting of the radiation, namely, cancer patients undergoing radiotherapy. Fractionated radiation has been shown to be more damaging than single-dose radiation [69]. One report showed that fractionated radiation with a total dose of 200 cGy may cause permanent azoospermia [70].

1.2.1.7 Chemotherapy

Chemotherapy typically targets rapidly dividing cells and thus has profound effects on the germinal epithelium. As such, the expected outcome of acute chemotherapy is a decline in spermatogenesis, and this has been well documented since the late 1940s [71]. The mechanism by which chemotherapeutics decrease fertility and the rates of recovery is both drug and dose dependent [72–75]. Further, it should be noted that it is impossible to predict recovery of spermatogenesis following chemotherapy.

Alkylating agents, such as cyclophosphamide and cisplatin, are perhaps the most cytotoxic classes of medications, possessing the greatest risk of prolonged azoospermia [76]. Decreases in semen parameters typically occur within 1–2 months of beginning treatment, and azoospermia can be seen after 2 months. Of those men who do become azoospermic, the probability of recovery is the highest within 2 years and rarely occurs after 5 years [77].

Furthermore, while not as toxic as the aforementioned chemotherapeutics, certain drugs, such as anthracyclines and microtubule inhibitors, can have an additive effect on gonadotoxicity when paired with higher risk agents. Other chemotherapeutics such as topoisomerase inhibitors and metabolic inhibitors (methotrexate and 5-fluorouracil) are toxic to differentiating spermatogonia, but less so to testicular germ cells. This frequently leads to only transient decreases in sperm counts [77].

1.2.1.8 Genetic Azoospermia/Oligospermia

It is estimated that 2%–8% of infertile men have an underlying genetic abnormality, with this number rising to 15% in azoospermic men [78]. Although the majority of male infertility does not have an identifiable genetic cause, two potential etiologies are Y chromosome microdeletions and karyotypic abnormalities. The two most common karyotypic abnormalities are Klinefelter's (47,XXY) and chromosomal translocations.

Y chromosome microdeletions occur in approximately 10% of men with azoospermia or severe oligospermia [79]. Research has focused on the azoospermia factor (AZF) region on the long arm of the Y chromosome at Yq11. This area itself contains three separate regions, AZFa, AZFb, and AZFc, and microdeletions of these areas lead to slightly different phenotypes [80]. Deletions in the AZFa and AZFb regions both cause azoospermia, but they differ histologically with AZFa deletions resulting in Sertoli cell-only syndrome and AZFb deletions causing an arrest of spermatogenesis at the primary spermatocyte stage [78]. AZFc deletions are the most common of the Y chromosome microdeletions and are found in 5%–7% of oligospermic men [80]. Unlike the AZFa/AZFb deletions, they do not uniformly result in azoospermia; rather, a spectrum of phenotypes are seen with partial deletions being found in normospermic men, from oligospermia to azoospermia in some full deletions [78]. In men undergoing micro-TESE sperm extraction with ACFc deletions, reported success rates have ranged from about 35% to 72% [81–84].

Classic and mosaic Klinefelter's are common karyotypic abnormalities found in infertile men. Klinefelter's has a prevalence of one in 660 males and is found in 15% of men with nonobstructive azoospermia; thus, it is the most common chromosomal cause of male infertility [85–88]. Klinefelter's affects fertility through two routes: direct effects on spermatogenesis and indirect hormonal effects on spermatogenesis [85, 89, 90]. These men uniformly present with very small testicles, increased gonadotropins, and hypogonadism. Interestingly, sex hormone levels are normal until puberty. During puberty, they may rise to low-normal levels, but plateau. By adulthood, serum testosterone levels are typically below normal. Histologic studies demonstrate gradual degeneration of the testes with development, with hyperplasia of poorly functioning Leydig cells [91].

As far as altered spermatogenesis is concerned, many Klinefelter's patients do produce sperm, with micro-TESE recovery rates ranging between 55% and 65% [84, 92]. Additionally, one report found 8% of men with non-mosaic Klinefelter's had sperm in their ejaculate [84]. However, the quantity of sperm produced is typically very low. Biopsy studies of Klinefelter's testes have demonstrated that spermatogenesis is halted pre-pachytene in the vast majority of aneuploid cells and that meiosis was seen mainly in cells with normal karyotypes [90].

Robertsonian translocations are a third significant genetic cause of infertility. They occur in 0.8% of infertile men, and this number rises to 1.6% in oligospermic men [93]. Phenotypes are highly variable given the possibilities of recombination [78].

1.2.1.9 Lifestyle Factors

Hyperthermia is considered to be a major contributor in the pathogenesis of infertility in men with varicocele and cryptorchidism. Subsequently, one may infer that other lifestyle factors, such as underwear type, heated car seats, or occupational exposures, could have a similar effect. However, these cases have not been manifest in existing studies. The role of underwear style in male infertility has

been investigated. One small study of 14 normospermic men, a tight polyester scrotal support, when worn day and night, was shown to make all azoospermic at a mean time of 140 days. After removal of the scrotal support, all men regained function at a mean time of 157 days [94]. However, normal underwear (i.e., boxer or brief style) has not been shown to exert a significant influence on semen parameters [95]. Other types of heat exposure, such as occupational heat exposure in a group of welders, have been shown to decrease semen quality [96]. Sedentary posture, heated car seats, and sauna and hot tub use are all lifestyle factors that increase scrotal temperature as well and may contribute to a decline in fertility [97].

Cell phones have been implicated as possibly playing a role in decreasing male fertility, and several studies show that there may be some basis for this theory. One observational study assessed semen parameters and cell phone usage in 361 men who presented to an infertility clinic. Approximately 60% of the men in this study had greater than 2 h of cell phone use per day, with 30% using their cell phones for more than 4 h per day. They found that sperm counts, motility, viability, and morphology all worsened with increasing cell phone use [98]. The mechanism by which cell phones affect semen parameters has not yet been elucidated, but one hypothesis is that cell phone-generated electromagnetic radiation (CPEMR) alters mitochondrial function and acts to increase reactive oxygen species. This is somewhat corroborated by one study which looked at the effects of CPEMR on semen parameters and found increased levels of reactive oxygen species with decreased viability and motility in the sperm exposed to CPEMR [99].

Tobacco use has been implicated in the pathogenesis of numerous cancers and medical diseases. While the use of tobacco significantly impacts female fertility, its impact on male fertility is less clear. Semen parameters, including sperm density, motility, and morphology, have all been shown to be worsened with tobacco use [100–103]. However, a significant reduction in fertility has not yet been proven.

Another increasingly common condition negatively effecting fertility is obesity. A 2006 study of over 20,000 families found that increasing body mass index was associated with worsening male fertility [104]. This finding has been corroborated by further studies demonstrating worse semen parameters, increased DNA fragmentation, and possibly genetic alterations in obese men [105–108]. The mechanism for this is multifaceted. Obese men are at in increased risk of hypogonadism via both decreased secretion and response to LH and increased peripheral aromatization of testosterone to estradiol [109, 110]. Furthermore, obese men are at an increased risk of developing cardiovascular disease and diabetes, which may exacerbate these negative effects [111].

1.2.1.10 Testicular Injury

Injury to the testicle can be sustained either directly or indirectly. Direct trauma to the testis is typically managed by debridement of devitalized seminiferous tubules and closure of the tunica albuginea [112]. The resultant loss of volume of seminiferous tubules and possible obstruction from scarring is one possible cause of decreased fertility. Reports on testicular salvage after bilateral trauma indicated that preserved volume of testis is the key to preserving fertility [112–117].

Indirect damage to the testis may be sustained by exposure to infection or inflammation of the testis. While rare in developed nations, the classic infectious agent causing infertility is mumps. The mechanism by which mumps causes orchitis is via pressure atrophy. Infection of the testis with the mumps virus causes inflammation and swelling, which is limited by the tunica albuginea; this in turn leads to atrophy [118]. Approximately 1.5% of postpubertal males with mumps may become infertile as a result of the illness [119].

Other bacterial and viral pathogens may also cause infertility at the testicular level, most commonly; this is the result of spread of infection from the epididymis [120, 121]. The mechanism for infertility in these cases may be persistent inflammation which suppresses testicular function or obstruction secondary to resultant sclerosis.

1.2.1.11 Primary Ciliary Dyskinesia

Ultrastructural defects that affect sperm motility are described under the grouping of primary ciliary dyskinesia (PCD). PCD is a rare and heterogeneous genetic disease which affects one in 20,000–60,000 [122]. Many components of cilia and flagella are affected, though the defect is found in the dynein in over 80% of cases [123]. The key clinical finding is chronic respiratory infections leading to bronchiectasis. When situs inversus is present in addition to the other components, it is termed Kartagener's syndrome. Male infertility secondary to sperm dysmotility is related to the dysfunction of the flagellate tail of the sperm. It is a common finding, though not universal, given the phenotypic heterogeneity.

1.2.1.12 Antisperm Antibodies

In the normal male, sperm reside in an immunoprivileged site. The blood–testis barrier prevents proteins from the sperm from interacting with the immune system and setting up an immune reaction against them. Trauma, infection, and inflammation all may disrupt this barrier and result in immunity against the germinal epithelium and spermatozoa.

Antisperm antibodies (ASA) are very common, with 8%–17% of men and 1%–22% women in infertile couples

testing positive for serum ASA [124, 125]. As expected, ASA are heterogeneous in their binding sites and, as such, have wide ranging effects on sperm function. Some ASA will not significantly affect fertility, and 0.9%–2.5% of fertile men will test positive for serum ASA [126, 127]. ASA targeted against proteins on the head region are more likely to affect zona binding and sperm penetration, whereas ASA targeted against the tails of spermatozoa are more likely to decrease motility and cervical mucus penetration and cause sperm agglutination [128]. While ASA clearly may affect fertility in some cases, serum ASA positivity is not a strong predictor of infertility.

1.2.2 Post-testicular

1.2.2.1 Absence of the Vas Deferens

Congenital bilateral absence of the vas deferens (CBAVD) is a condition strongly related to cystic fibrosis (CF), and the same genetic mutation of the CFTR is typically responsible for both disease processes [129]. While men with CF do not necessarily have CBAVD, most men with CBAVD do have a CFTR mutation [130–132]. The pathophysiology of CBAVD thus clearly involves altered chloride transport in the majority of cases, and, similar to the respiratory and pancreatic sequelae seen with CF, there is evidence that the genital abnormalities and pathology seen are a progressive disease. Namely, intentionally aborted CF fetuses demonstrate normal vas deferens, albeit with secretions filling their lumens. This suggests that the mechanism for CBAVD is atresia, and not aplasia, when a CFTR mutation is present [133]. One interesting sequela of this case is that renal agenesis is not associated with CBAVD [134].

Congenital unilateral absence of the vas deferens (CUAVD) is a different entity altogether [135]. While there remains a significant rate of CFTR mutations in men with CUAVD, especially when the obstructive azoospermia is present [129], the majority of CUAVD is the result of an embryologic Wolffian duct aberrancy [136]. As such, renal agenesis is often seen with CUAVD, though CUAVD is not always seen in men with unilateral renal agenesis, as there are many other embryologic missteps that may occur to result in renal agenesis. While there is only a 20% rate of CUAVD seen in those with a unilateral renal agenesis, there is a 79% rate of unilateral renal agenesis seen in men with CUAVD. Since CUAVD not associated with a CFTR mutation is usually a unilateral and isolated phenomenon, fertility is often preserved.

1.2.2.2 Young's Syndrome

Young's syndrome is a rare disorder which presents clinically as obstructive azoospermia and chronic sinopulmonary infections [137]. Thus, it can be difficult to differentiate clinically from cystic fibrosis variants and primary ciliary dyskinesia. Indeed, definitive diagnosis of Young's syndrome requires negative CFTR genetic testing and investigation of ciliary ultrastructure to rule out primary ciliary dyskinesia [138]. Normal spermatogenesis is seen, and the obstructive azoospermia is due to inspissated secretions in the vas deferens.

The etiology of Young's syndrome is unclear with childhood mercury exposure having been postulated to play a role in the past [139]. Interestingly, the incidence of Young's syndrome has plummeted from estimates of one in 500 in the 1980s down to case reports and articles which question the existence of Young's syndrome today [140]. The observation that the reduced incidence over the last 50 years coincides with a decrease in mercury use and poisoning is tempered by the fact that our knowledge of genetics has rapidly advanced. Thus, the decreased incidence of Young's syndrome is more likely due to the increased correct genetic diagnosis of CF spectrum disease.

1.2.2.3 EjDO/Seminal Vesicle Dysfunction

Ejaculatory duct obstruction is a common etiology of male infertility, occurring in 1%–5% of men presenting with infertility [141]. There are many causes of ejaculatory duct obstruction, including cystic fibrosis spectrum disease, Wolffian or Mullerian origin cysts, calcifications, tuberculosis and other GU infections, calculi, and urinary tract instrumentation [142, 143]. Additionally, chronic ejaculatory duct obstruction may affect the seminal vesicle in a manner analogous to the effect of bladder outlet obstruction on the bladder. Namely, with longstanding obstruction, the seminal vesicles may lose contractility, and resolution of the anatomical obstruction may not improve seminal vesicle emptying during ejaculation.

1.2.2.4 Nerve Injury

Nervous injury affecting ejaculation may occur at many levels and have a diverse etiology ranging from spinal cord injury to neural damage during retroperitoneal or pelvic surgery to neuropathy from systemic diseases. Ejaculatory dysfunction is present in 90% of spinal cord injury patients [144]. The type and severity of ejaculatory dysfunction are dependent on the level and extent of the injury. Higher cord lesions, typically at T10 or above, often result in an intact reflex arc which allows for penile vibratory stimulation to induce ejaculation. Men with sacral lesions or lesions of the efferent parasympathetic nerves are typically not responsive to penile vibratory stimulation and may require endorectal electrical stimulation to induce ejaculation [140, 145]. These men may additionally have trouble initiating erections, as injuries at this level can interrupt the arc for reflex erections [146].

While sperm concentration in men with spinal cord injuries characteristically remains normal, many have low motility and viability [147, 148]. The exact reason for this phenomenon remains unclear, but is possibly a result of decreased mobility, voiding dysfunction, accessory gland dysfunction, and/or inflammatory changes [146, 149]. Of note, an interesting physical finding seen in 25%–50% of men with spinal cord injuries likely related to seminal vesicle dysfunction is brown semen [150]. The exact reason for this phenomenon is unclear; brown coloration is not derived from heme and is not related to semen stasis per se.

Retroperitoneal lymph node dissection (RPLND) for testicular cancer resulted in a high rate of ejaculatory dysfunction until the development of methods to spare the sympathetic nerve fibers. Both emission and bladder neck contraction are mediated by the sympathetic nervous system, and damage to the sympathetic chain and the hypogastric plexus overlying the great vessels results in a high degree of ejaculatory dysfunction. In the past, RPLND was associated with a 55%–60% chance of ejaculatory dysfunction [151, 152]. Modified templates have helped to reduce the rates of retrograde ejaculation, with one study demonstrating an 82% rate of antegrade ejaculation with a modified unilateral template [153]. Another study using a modified bilateral template demonstrated an 88% rate of preservation of antegrade ejaculation [154].

Nerve-sparing RPLND, developed in the late 1980s, has reduced the incidence of retrograde ejaculation even further to 0%–7% [155, 156]. Nerve-sparing RPLND may also be done after chemotherapy, though only 136 of 341 men qualified for this method as compared with standard RPLND in one series [157]. Rates of ejaculatory dysfunction were also higher at 21%.

1.2.2.5 Medications

Medications affecting ejaculation do so by altering adrenergic signaling. This scenario is most clearly seen with alpha-1 antagonists. Tamsulosin and silodosin, especially, are known to cause ejaculatory dysfunction [158, 159]. Previously, this case was thought to be retrograde ejaculation. Recent studies have shown that the ejaculatory dysfunction induced by alpha-1 antagonists is actually a failure of emission [160, 161].

Antipsychotics have long been associated with sexual dysfunction, including ejaculatory dysfunction. Antipsychotics have effects on many different neurotransmitters including dopamine, norepinephrine, acetylcholine, and serotonin. Predictably, altered ejaculatory function with antipsychotics use correlates with anti-adrenergic actions of the antipsychotics [162]. Even atypical antipsychotics such as risperidone may affect ejaculation [163, 164].

1.2.2.6 Coital

Abnormal coital practices may play a role in infertility when they interfere with semen deposition in the vagina or affect their timing with the female reproductive cycle. Similarly, erectile dysfunction and penile abnormalities such as hypospadias and chordee may interfere with semen deposition and thus may play a role in infertility.

Lubricants are commonly used by infertile couples, and many vaginal lubricants have been shown to negatively affect fertility. Many synthetic lubricants not only affect sperm motility but have also been shown to increase the DNA fragmentation index. In one study, FemGlide, Replens, and Astroglide all affected sperm motility, and FemGlide and K-Y jelly increased DNA fragmentation. One lubricant that has not been shown to have a significant impact on sperm motility or DNA fragmentation is Pre-Seed [165]. Another study showed similar findings with decreased motility in sperm exposed to K-Y jelly and Touch. Nonviability was seen in sperm exposed to Replens and Astroglide which was comparable to the nonviability seen when sperm were exposed to the spermicide nonoxylnol-9 [166]. In this study, canola oil was not found to affect sperm motility or viability. Yet another study showed that K-Y jelly, saliva, and olive oil all reduced sperm motility, while baby oil did not significantly affect motility [167].

1.3 Conclusion

The etiologies of male infertility are robust and varied. It must be remembered that the majority of the causes of male infertility are either preventable or treatable. This fact, along with a growing body of evidence suggesting infertility may have implications for overall health, highlights the importance of an evaluation for all male partners of couples struggling to conceive. While idiopathic infertility still comprises a large portion of the men evaluated for problems with reproduction, this percentage will likely continue to decrease in the future with scientific advancements, particularly in the field of genetics. It is crucial that reproductive urologists have an understanding of the causes of infertility in men so as to provide the optimal management of couples struggling to conceive (see Table 1.1).

1.4 Review Criteria

MEDLINE was searched for articles published in English from January 1980 to November 2018. Abstracts and papers were reviewed with a focus on the etiologies of male infertility. In addition, the reference sections of these articles, along with relevant chapters of infertility textbooks, were consulted.

Table 1.1 Summary table

Pre-testicular		Pathophysiology
	Hypogonadotropic Hypogonadism	Lack of stimulation of Sertoli/germ cell complex
	Elevated prolactin	Suppression of GnRH
	Pharmacologic	Downregulation of HPG axis
	Idiopathic hypogonadotropic hypogonadism	Defective action or release of GnRH
Testicular	Varicocele	Testicular hyperthermia resulting from a disruption of the countercurrent heat mechanism
	Cryptorchidism	Testicular hyperthermia; possible role of genetic and/or environmental factors
	Testicular cancer	Embryologic testicular dysgenesis, mass effect, inflammation, alteration of endocrine cascade
	Radiation	Direct cytotoxic effect on spermatogenesis
	Chemotherapy	Damage to testicular germ cells, fibrosis of testicular interstitial tissues
	Genetic	YCMD – Sertoli cell only syndrome or arrest in primary spermatocyte stage (AZFa or AZFb deletion), quantitative spermatogenesis impairment (AZFc deletion) Klinefelter's – direct effect on spermatogenesis and indirect hormonal effect on spermatogenesis
	Lifestyle	Testicular hyperthermia, increased DNA fragmentation, and/or genetic alterations
	Injury	Loss of seminiferous tubules, scarring leading to obstruction
	Primary ciliary dyskinesia	Dysfunction of the flagellate tail of the sperm
	Antisperm antibodies	Impairment of sperm motility and/or penetration
Post-testicular	Absence of vas deferens	CFTR mutation resulting in vasal or seminal vesicle atresia; embryological Wolffian duct aberrancy
	Young's syndrome	Inspissated secretions in the vas deferens
	Ejaculator duct obstruction	Obstruction of ejaculatory ducts and/or resultant loss of seminal vesicle contractility
	Nerve injury	Ejaculatory dysfunction
	Medications	Alteration of adrenergic signaling leading to impaired ejaculation
	Coital	Interference with semen deposition in vagina; lubricants may impair semen parameters

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Notable Suggested Readings from the Last 5 Years

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Epidemiologic Considerations in Male Infertility

2

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Key Points

- There is a 15% lifetime incidence of infertility among couples in Western countries; only 3%–5% of couples are sterile.
- If the timing of sexual intercourse relative to ovulation is deliberately controlled, then the monthly probability of pregnancy may approach 40%; however, the human average for monthly fecundity is 20%.
- The absence of a gold-standard test for male infertility is a fundamental problem, preventing an accurate understanding of its epidemiology and of the predictive value of tests employed for its diagnosis.
- Sperm concentration correlates with the probability of conception up to a density of 40 million/mL, but there is no additional likelihood of pregnancy at higher sperm densities.
- Purported declines in semen quality during the twentieth century may be an artifact of bias from geographical differences in sperm counts.
- Economic analyses suggest that pathology-directed treatment of male infertility is generally more cost-effective than proceeding directly to ART.

2.1 Introduction

This chapter primarily aims to discuss the epidemiologic relationship between infertility and male reproductive potential. It will focus in detail on the accuracy and diagnostic value of semen studies. Controversial reports of declining sperm counts during the twenty-first century will also be addressed, followed by a review of trends in health-care resource utilization and cost analysis models pertinent to the management of male infertility.

Antioxidant therapy for male infertility has an epidemiologic basis in the understanding that reactive oxygen species (ROS) contribute to sperm damage and are present in higher levels in the semen of infertile men [1–4]. However, given the increasing availability and use of assisted reproductive technology (ART), it may be surprising to encounter an epidemiologic approach to male infertility in the modern era [5]. The relative ease of surgical sperm retrieval in cases of azoospermia and severe oligozoospermia has seemingly rendered comprehensive evaluation and treatment of infertile men less relevant. Even though such an evaluation may identify one or more modifiable risk factors for infertility (as well as potentially serious underlying or coexisting illnesses and genetic abnormalities), the outcome of pathology-directed treatment may take longer to realize.

However, risk and uncertainty are also attendant to the use of ART. Cost-effectiveness studies have demonstrated that a straight-to-ART approach is less efficient than pathology-directed treatment in many situations involving male infertility. Therefore, renewed emphasis is being placed on the paradigm of intervention to correct modifiable male risk factors [6].

2.2 Epidemiology of Infertility

It is important to distinguish reproductive potential, or capacity, from actual reproductive performance, or outcomes [7, 8]. Infertility is ultimately defined by a reproductive *outcome* (i.e., childlessness), but "male infertility" is a diagnosis of relative impairment in male reproductive *potential*. Before discussing male infertility in more detail, we will review the epidemiology of infertility in general.

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2.2.1 Incidence and Prevalence of Infertility in Developed Countries

Childlessness within long-standing marriage has been observed to occur in roughly 10% of couples in developed countries [9]. This figure represents a measure of reproductive outcomes, but it does not exclude couples who remain voluntarily childless or do not have regular intercourse during the fertile phase of the female's menstrual cycle.

The World Health Organization (WHO) has defined infertility by "the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse," which captures the concept of impaired reproductive potential [10]. By this criterion, the lifetime *incidence* of unwanted infertility is approximately 15% among couples in Western countries [11]. Two-thirds of these cases are primary, that is, in couples who have never previously conceived, while one-third are secondary [9].

It is not uncommon to see infertility defined by other durations of time, such as 2 or 5 years. Also, while the outcome of interest in the WHO's definition is conception, others prefer to regard infertility as the absence of live birth [7, 8]. This distinction is important, because 10–25% of recognized pregnancies end in miscarriage [12].

The annual *prevalence* of infertility among married US women aged 15–44 years was 6.7% in 2015, down from 11.2% in 1965, 8.5% in 1982, to 7.4% in 2002 [13, 14]. This trend may reflect improvements in reproductive potential, perhaps through better awareness of ideal timing for intercourse, which may be attributable to at-home ovulation tests. However, other factors could also be involved, such as more couples utilizing infertility treatment prior to reaching the 12-month threshold necessary for inclusion in the rate's numerator. Declining marriage rates among lower socioeconomic classes may also be disproportionately removing women at greater risk of infertility from the denominator [13].

A popular misconception is that infertility is synonymous with, or virtually approximates, sterility [8]. In fact, only 3%–5% of couples are sterile [9, 15].

2.2.2 Infertility in the Developing World

In 2002, the WHO estimated that 16%–30% of married women in developing countries were experiencing infertility of at least 5 years duration, with the vast majority being secondary [16]. A more recent study, focused on child-seeking women, lowered these estimates to 9%–20% (Table 2.1) [17]. Interestingly, this study found little difference in the prevalence of infertility between high-income countries and low- or middle-income countries of certain regions. Sexually transmitted infections and postpartum complications have been identified as key factors contributing to secondary infertility in the developing world [18].

Table 2.1 Prevalence of infertility of 5-year duration among child-seeking women in high-income countries and low- or middle-income countries of various regions, 2010

	Primary or secondary infertility (%)	Primary infertility (%)	Secondary infertility (%)
High-income countries	9.9	1.8	8.1
Latin America/ Caribbean	8.8	1.5	7.3
Middle East/North Africa	9.1	2.6	6.5
Sub-Saharan Africa	11.8	2.0	9.8
East Asia/Pacific	12.4	1.6	10.8[a]
South Asia	13.7	2.4	11.3
Central/Eastern Europe and Central Asia	20.2	2.3	17.9

Adapted from Mascarenhas et al. [17]. With permission from Creative Commons

[a]Estimate excludes China

The rate of childlessness in developing countries among sexually experienced women who are beyond childbearing age is approximately 3% [16]. This figure is comparable to the estimated prevalence of sterility in Western societies.

High cost represents an important barrier to the accessibility of most infertility treatment, and specifically ART, in the developing world [18]. Therefore, further identification of avoidable gonadotoxins, as well as clarification of the role of less expensive therapies, would be especially welcome there [19].

2.2.3 Reproduction—a Matter of Chance: The Natural History of Infertility

Reproduction has been described as "a matter of chance depending on the subtle balance between success or failure of complex, mostly poorly understood, sequential processes that may lead to a pregnancy and eventually to the birth of a healthy child" [15]. Failure of a couple to reproduce is a unique medical problem in that it occurs *between* rather than *within* individuals [7].

Individuals' reproductive potential is a continuous, as opposed to dichotomous, variable. It reflects the influence of many factors, including age. Reproductive potential declines in members of both sexes over 30 years old, but female age has the most profound effect on the likelihood of conception [20, 21].

Since a couple's reproductive capacity is the composite of its individual members' reproductive potentials, it, too, is a continuous variable. Impairment of a male's reproductive

potential may be compensated for—or compounded—by that of his female partner and vice versa. This concept is illustrated by the fact that artificial insemination with donor semen is more often successful in partners of azoospermic men than in partners of men with oligozoospermia [22].

Couples' monthly, or cycle-wise, likelihood of conception falls along a spectrum of probability. This is referred to as fecundability or monthly fecundity rate (MFR). The average MFR for human couples having regular, unprotected intercourse is approximately 20%, and the overall distribution of human MFRs is believed to range from 0% to 60% [23, 24]. The variable likelihood of pregnancy at 6, 12, 24, and 60 months has been calculated for couples with different MFRs (Table 2.2). Based on these values, a hypothetical model has been constructed of the proportion of couples with varying degrees of reproductive potential (MFR) among residual nonpregnant couples after specified durations of infertility (Table 2.3) [25].

Table 2.2 demonstrates that couples with average fecundability have a better than 90% chance of conceiving within 12 months. However, 30% of couples who do not conceive within 12 months are still of average reproductive potential (see Table 2.3). Population-based studies have found that couples who are infertile after 12 months retain a roughly 50% likelihood of achieving unassisted pregnancy by 24 months [26]. Thereafter, the odds of conception decline precipitously, as illustrated in Fig. 2.1. The proportion of couples that are sterile increases with the duration of infertility.

An obvious but nevertheless critically important factor in determining reproductive potential is the timing of sexual intercourse relative to ovulation (Fig. 2.2) [27]. In a cohort of 340 German couples who received natural family planning education intended to improve their timing of intercourse, the monthly probability of achieving pregnancy averaged 38%, markedly higher than the average human MFR of 20% [28].

2.3 Diagnostic Accuracy and Utility of Semen Studies

Male infertility is diagnosed in approximately 50% of couples presenting for evaluation of infertility [11]. Basic semen analysis remains the most widely utilized laboratory study for this purpose. However, the limitations of semen analysis should be clearly understood.

The parameters usually assessed by semen analysis include ejaculate volume, sperm concentration (density), sperm motility, and sperm morphology. From the ejaculate volume, concentration, and percentage of motile sperm, the total sperm count and total motile count are calculated. Semen pH, viscosity, white blood cell concentration, and the degree of sperm agglutination may also be reported [29].

More specialized studies that are undertaken on a case-by-case basis include evaluation of sperm viability, antisperm antibodies, and functional assays, such as assessment

Table 2.2 Cumulative spontaneous pregnancy rates of couples in five hypothetical categories of reproductive potential

		Cumulative pregnancy rate (%) after period of months			
	MFR (%)	6 months	12 months	24 months	60 months
Highest fecundability	60	100	–	–	
Average fecundability	20	74	93	100	
Below-average fecundability	5	26	46	71	95
Severely impaired fecundability	1	6	11	21	45
Sterile	0	0	0	0	0

Reprinted from Evers [22]. With permission from Elsevier
MFR monthly fecundity rate; cumulative pregnancy rate = $1-(1 - \text{MFR})^{\#\text{ of months}}$

Table 2.3 Hypothetical model of the proportion of couples with varying degrees of reproductive potential in the residual population, dependent on the duration of infertility

		Composition of residual nonpregnant couples (%) after period of months				
	MFR (%)	0 months	6 months	12 months	24 months	60 months
Highest fecundability	60	3	–	–	–	–
Average fecundability	20	79	58	30	–	–
Below-average fecundability	5	10	21	30	30	8
Severely impaired fecundability	1	5	13	24	40	44
Sterile	0	3	8	16	30	48

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MFR monthly fecundity rate

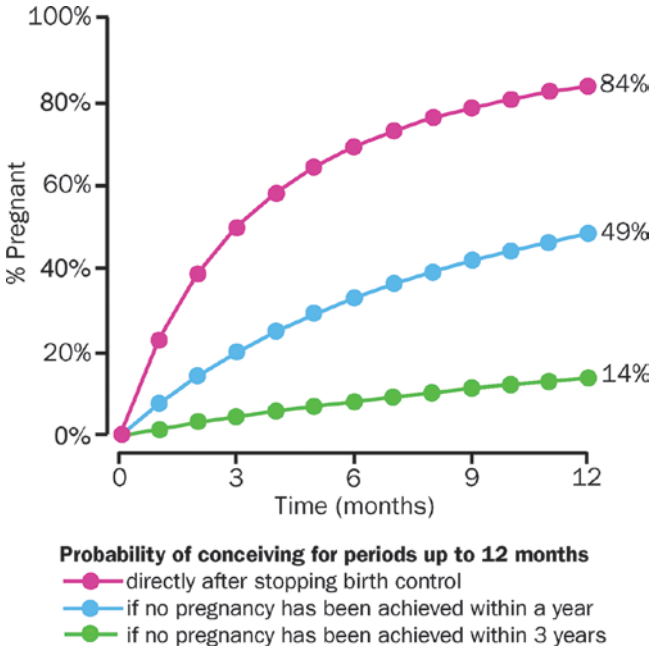

Fig. 2.1 Cumulative probability of conception in couples having unprotected intercourse. (Reprinted from the Velde et al. [15]. With permission from Elsevier)

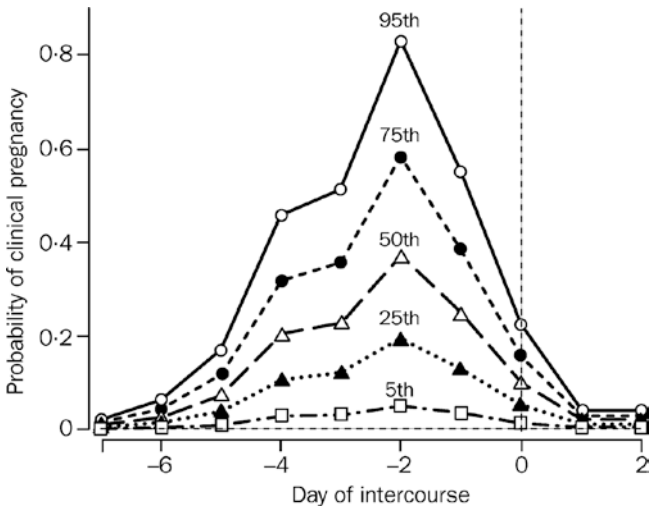

Fig. 2.2 Probability of clinical pregnancy after intercourse on a particular day relative to ovulation (day 0) for couples at specified percentiles of the population distribution of reproductive potential. (Adapted from Dunson et al. [27]. With permission from Oxford University Press)

of sperm–cervical mucus interaction, capacitation, and sperm penetration of a zona-free hamster oocyte [30]. Relatively recent developments have included the introduction of tests for seminal ROS levels and sperm DNA fragmentation [31, 32].

Preliminary studies have also suggested that sperm epigenetics may have a future role in the evaluation of male infertility. Gene H19 is a common target for study and was associated with infertile men by a factor of 9.9 in one meta-analysis [33]. Epigenetic research remains exploratory and is limited by the wide number of possible gene targets for study.

Table 2.4 WHO reference values for analysis of semen parameters

	1992	1999	2010
Ejaculate volume (mL)	≥2.0	≥2.0	≥1.5
Sperm concentration (10^6/mL)	≥20	≥20	≥15
Total sperm number (10^6/ejaculate)	≥40	≥40	≥39
Sperm motility (% motile)	≥50 (a + b)[a]	≥50 (a + b)	≥40 (a + b + c)
Sperm morphology (% normal)	≥30	≥14[b]	≥4
Sperm viability/vitality (% live)	≥75	≥75	≥58
White blood cells (10^6/mL)	<1.0	<1.0	<1.0

Reprinted from Menkveld [34]. With permission from Asian Journal of Andrology

[a]Motility is graded as follows: a = rapid progressive motility (>25 μm/s); b = slow/sluggish progressive motility (5–25 μm/s); c = nonprogressive motility; d = immotility

[b]Kruger (Tygerberg) strict criteria were adopted by the WHO in 1999

2.3.1 Relationship Between Semen Parameters and Male Infertility

Semen analysis definitively confirms male factor infertility in men who are found to have semen characteristics at the negative extremes, that is, azoospermia, nonmotile sperm, or the severest cases of teratozoospermia. However, only a small fraction of men who present for evaluation of infertility have such findings [11]. The present section explores the relationship between male infertility and the entire spectrum of semen quality.

Since 1980, the WHO has published reference values for human semen parameters. These values have been changed periodically (Table 2.4) [34]. As of 2010, they represent the fifth percentile in the distribution of semen parameters from a population of men with proven fertility [35]. They provide no information regarding the distribution of semen parameters in men who are infertile.

Although the WHO criteria are commonly used as thresholds for designation of male infertility, the diagnostic picture in a clinical setting is considerably more complex. This scenario is because of substantial overlap between the distributions of semen characteristics in empirically fertile men and those with infertility whose female partners have had a normal fertility evaluation (Fig. 2.3).

Basic performance measures of a test such as semen analysis include calculation of its sensitivity and specificity. These concepts are illustrated in Tables 2.5 and 2.6. If a sperm concentration below 15 million/mL is the criterion for a "positive" result (i.e., diagnosis of male infertility), then men who are

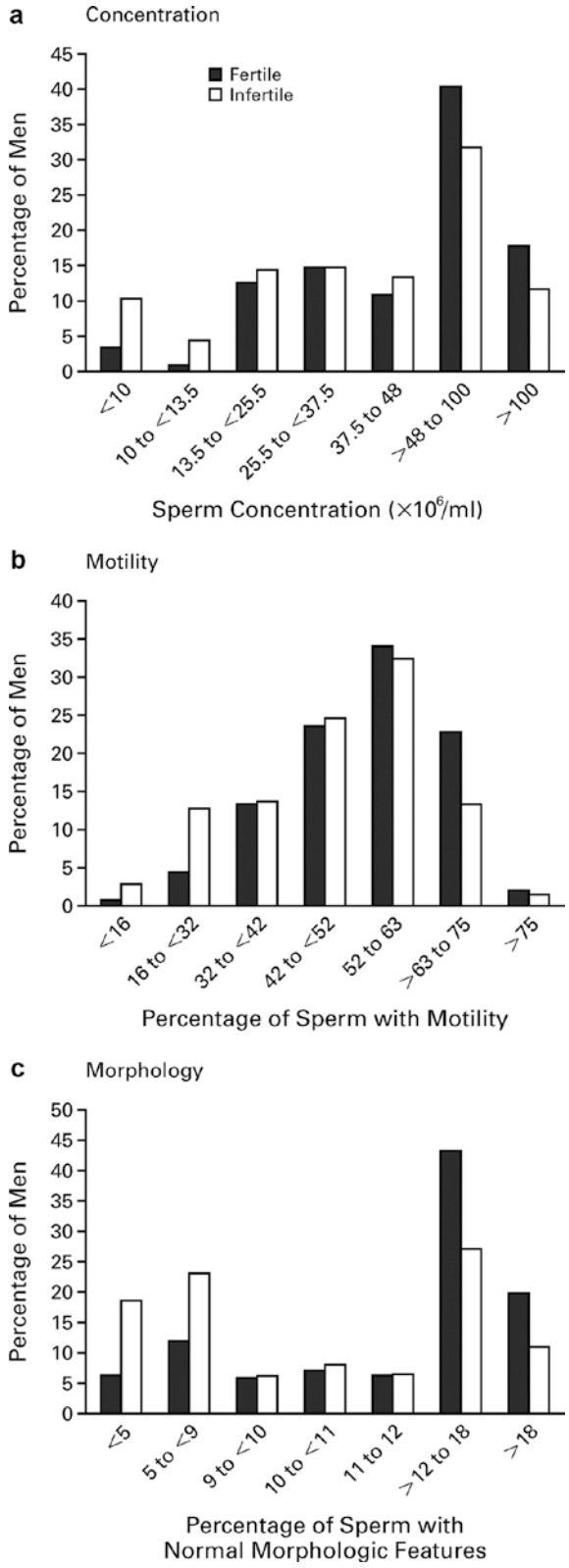

Fig. 2.3 Frequency histograms depicting the percentage of men from fertile (*shaded bars*) and infertile (*unshaded bars*) couples with sperm concentration (**a**) sperm motility (**b**) and sperm morphology (**c**) within specified ranges. Data were obtained from 696 fertile men and 765 men from infertile couples whose female partners had an unrevealing fertility evaluation. (Reprinted from Guzick et al. [36]. Copyright 2001, with permission from Massachusetts Medical Society. All rights reserved)

Table 2.5 2 × 2 Table depicting actual fertility status vs. test results using the 2010 WHO reference value for sperm concentration as a threshold for diagnosis of male infertility, in a hypothetical population of 200 men presenting for evaluation of infertility

		Actual fertility status	
		Infertile	Fertile
Test results	Abnormal	15 (TP)	5 (FP)
	Normal	85 (FN)	95 (TN)

TP true positives, *FP* false positives, *FN* false negatives, *TN* true negatives

Table 2.6 Accuracy metrics based on Table 2.5, using the 2010 WHO reference value for sperm concentration (15 million/mL) as a threshold for diagnosis of male infertility

Accuracy metric	Formula for calculation	Result (%)
Sensitivity	TP/(TP + FN)	15
Specificity	TN/(TN + FP)	95
Positive predictive value	TP/(TP + FP)	75
Negative predictive value	TN/(TN + FN)	53
Classification accuracy	(TP + TN)/N	55

TP true positives, *FP* false positives, *FN* false negatives, *TN* true negatives, *N* = total number

actually fertile will be correctly classified 95% of the time. In other words, the threshold of 15 million/mL has a specificity of 95%. However, 85% of infertile men also have sperm concentrations above 15 million/mL; thus, the sensitivity of the test at this threshold is only 15% [36]. Many men with impaired reproductive potential will not be recognized as such.

If we assume that 50% of couples presenting for an evaluation of infertility have a contributing male factor, then the predictive value of a positive result (i.e., its likelihood of being correct) would be 75%. The predictive value of a negative, or normal, result would be only 53% (see Table 2.6).

Of course, diagnostic thresholds can be changed. If they are increased, sensitivity improves, but there is a reciprocal decline in specificity, and vice versa. One way to assess the diagnostic accuracy of a test across all thresholds is by a receiver operating characteristic (ROC) curve [28, 34]. ROC curves are constructed by plotting the probability of detecting true positives (sensitivity) against that of detecting false positives (1 − specificity) at each threshold.

The total area under the ROC curve (AUC) for a particular test represents its overall discriminatory capability. A perfect test has an AUC of 1.0, while a test is useless if the likelihood of a true positive matches that of a false positive at every threshold, resulting in the no-discrimination line from (0, 0) to (1, 1) and an AUC of 0.5. The amount by which a test's ROC curve diverges from this line—and by extension, to which its AUC exceeds 0.5—is the degree to which it is diagnostically helpful. An AUC that exceeds 0.9 is considered excellent, while an AUC of less than 0.7 is poor.

Figure 2.4 represents an example of an ROC curve generated from MacLeod's data on sperm concentration in fertile and infertile men [37]. Its AUC is only 0.59, indicating that the overall accuracy of sperm concentration for diagnosis of

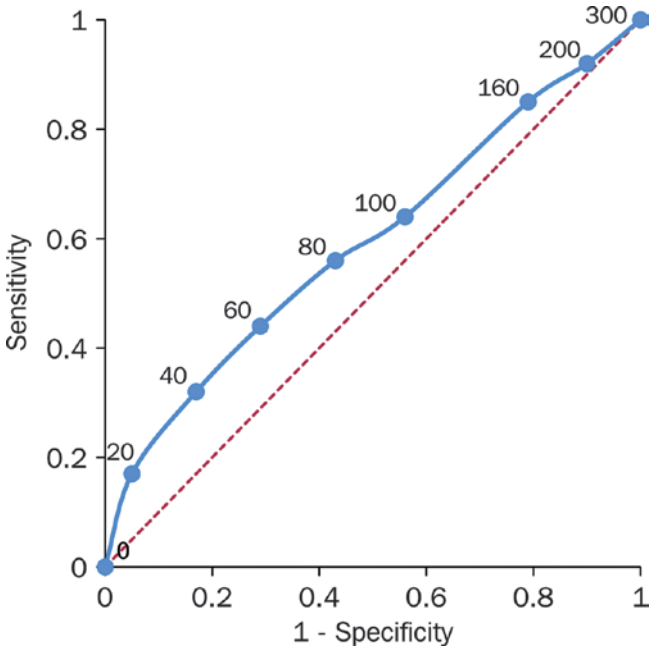

Fig. 2.4 Receiver operating characteristic (ROC) curve for diagnosis of male infertility at specified thresholds of sperm concentration in millions/mL (data from [37]). The no-discrimination line is shown in *red*. (Modified from Niederberger [38]. With permission from Elsevier)

male infertility just narrowly exceeds that of random chance. In Guzick and colleagues' series, which was depicted in Fig. 2.3, the AUC for sperm density, motility, and morphology were 0.60, 0.59, and 0.66, respectively [38].

The ROC curve alone does not provide information regarding the likelihood that a specific patient's positive or negative test result is correct. This probability is dependent both on test performance and the prevalence of disease in the relevant population. As previously illustrated, a diagnosis of male infertility based on sperm concentration below 15 million/mL may be correct 75% of the time in the population of males from infertile couples. False-positive results for infertility are relatively rare in this population. By comparison, if semen analysis were performed on men in the general population (e.g., to assess sperm donors with no prior reproductive history), its positive predictive value would be considerably less, on account of a much lower prevalence of male infertility.

Other studies have reported AUCs for sperm density, motility, and morphology, as described in Table 2.7 [39–43]. In general, motility and morphology demonstrated greater discriminatory capability than sperm concentration. Although the authors of these studies reported "optimal thresholds" for discrimination of fertile and infertile men, some of these thresholds have been criticized for having an unacceptably low positive predictive value in the setting of an infertile population [44].

Guzick and colleagues took a different approach to selecting diagnostic thresholds, using classification and regression tree (CART) analysis to determine *two* thresholds for each parameter that define the upper and lower boundaries of an indeterminate range lying between the fertile and infertile ranges (see Table 2.7). Unfortunately, a large number of men presenting for infertility evaluation fall into the indeterminate range, leaving unanswered the question of whether they warrant intervention for modifiable risk factors. The odds of male infertility multiply if more than one semen parameter is within the infertile range [36], but only 1% of men fail to meet all five WHO reference criteria for ejaculate volume, total sperm number, sperm concentration, motility, and morphology [45].

Table 2.7 Reported areas under the ROC curve for various seminal characteristics

	Population	AUC density	AUC motility	AUC morphology (strict)
MacLeod [37][a]	1000 fertile; 800 infertile[b] (ages NR)	0.59	NR	NR
Guzick et al. [36][a]	696 fertile (avg age, 33.5 ± 5.0); 765 infertile (avg age, 34.7 ± 4.9)	0.60 (13.5, 48)	0.59 (32, 63)	0.66 (9, 11)
Ombelet et al. [42]	144 fertile; 143 infertile (ages NR)	0.69 (34)	0.61 (45)	0.78 (10)
Gunalp et al. [39]	61 fertile (avg age, 29.9); 62 infertile (avg age, 31.3)	0.56 (34)	0.71 (42)	0.70 (12)
Menkveld et al. [41]	107 fertile (avg age, 33.8 ± 4.3); 103 infertile[b] (avg age, 33.7 ± 3.9)	NR	0.79 (45)	0.78 (4)
Jedrzejczak et al. [40]	113 fertile (avg age, 31 ± 4.7); 109 infertile (avg age, 32.2 ± 4.1)	0.80 (45–50)	0.91 (24)	0.82 (11)
Sripada et al. [43][b]	488 fertile (avg age, 30.6 ± 4.8); 938 infertile[b] (avg age, 32 ± 5.4)	NR	0.55 (30)	0.57 (9.5)

Based on data from Ref. [38]

AUC area under the ROC curve, *NR* not reported, ages of study populations are reported as mean ± SD, values in parentheses are the "optimal thresholds" identified by the respective studies for discrimination of fertile from infertile men—see text for additional details

[a]For data from these studies, the ROC curve was plotted and AUC was reported by Niederberger [38]

[b]These reports did not specify female partners as having had a negative fertility evaluation

2.3.2 Do Semen Parameters Prospectively Predict Fertility and Assisted Reproductive Technique Outcomes?

Only a handful of studies have attempted to prospectively identify variables associated with male reproductive poten-

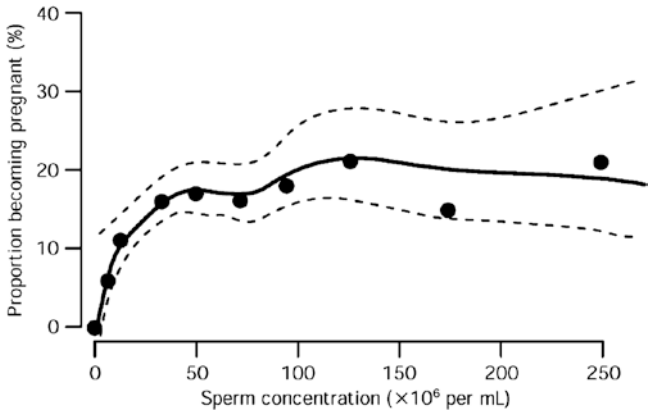

Fig. 2.5 Probability of pregnancy per menstrual cycle relative to sperm concentration. (Reprinted from Bonde et al. [47]. with permission of Elsevier)

tial. One such project included 200 couples, some of whom had had prior pregnancies [46]. After discontinuing contraception, the couples were followed for up to 12 months. Around 78% conceived during the study period, and both sperm motility and morphology were significantly associated with fertility. The difference in sperm concentration between fertile and infertile couples remained statistically insignificant.

Bonde and colleagues investigated 430 Danish couples between 20 and 35 years old who had never previously been or tried to become pregnant, following them for up to six menstrual cycles after discontinuation of contraception [47]. Around 60% of couples became pregnant. The probability of conception increased up to a threshold sperm concentration of 40 million/mL, but there was no additional likelihood of pregnancy at higher sperm densities (Fig. 2.5). This finding has led some reproductive specialists to argue that the appropriate threshold of sperm concentration for diagnosis of male infertility should be 40 instead of 15 million/mL [48]. A change of this sort, however, would also increase the number of false-positive diagnoses, prompting unnecessary evaluation and treatment.

Leushuis and colleagues have published an incisive review of prediction models in reproductive medicine, including several that use one or more semen characteristics to predict conception by infertile couples [49]. One such model, which has been externally validated in a population excluding men with total motile sperm counts of less than three million, takes account of sperm motility and the characteristics of the female partner and the duration of the couple's infertility; it is available online at www.freya.nl/probability.php [50]. Another model utilizes inputs of sperm concentration, motility, morphology, and hypoosmotic swelling to assess the likelihood of pregnancy, with a reported accuracy greater than 85% [40]. Of note, however, the AUCs for each variable in this study substantially exceeded those published in the other reports described in Table 2.7, and the predictive model has yet to be validated.

Sperm quality also affects ART outcomes, at least to some degree. Several investigations have demonstrated a positive correlation between semen characteristics, including ROS levels, and in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI) success rates; however, there was no apparent association with clinical pregnancy rates [51, 52]. Studies of intrauterine insemination (IUI), by contrast, have shown a correlation between successful outcomes and sperm concentration, motility, and morphology [44, 53–55].

2.3.3 Novel Assays for Diagnosis of Male Infertility

Andrologists often lament the lack of more accurate studies for diagnosis of male infertility. Several tests are currently in various stages of development, including genomic, epigenomic, proteomic, glycomic, lipidomic, and metabolomic analyses [56, 57]. Testing for seminal ROS levels was found to have an AUC of 0.82 in a study of 105 patients [58]. Further evaluation in a larger cohort of patients is necessary.

Sperm DNA fragmentation, or damage, may also have diagnostic value. The etiology of DNA fragmentation appears to be multifactorial, including accumulated defects during spermiogenesis, exposure to oxidative stress, exogenous toxicity (e.g., smoking), and elevated temperature (e.g., secondary to a varicocele) [59].

The degree of DNA fragmentation may have implications for ART success. Evaluation of 1633 IVF and ICSI cycles found the likelihood of a live birth after IVF to be significantly lower at higher levels of fragmentation. Using the sperm chromatin structure assay (SCSA) and DNA fragmentation index (DFI), the odds ratio of live birth after IVF was 0.61 (95% confidence interval 0.38–0.97, p = 0.04) when DFI was >20%. This decrease is believed to be due to impaired fertilization, since DNA fragmentation was not associated with a lower birth rate after ICSI [60].

However, questions remain as to the diagnostic accuracy and predictive value of different DNA fragmentation assays. Cissen and colleagues performed a meta-analysis of various techniques for assessment of DNA fragmentation, finding that only terminal nick-end labeling was able to predict IVF/ICSI outcomes [61].

2.4 Are Sperm Counts Declining?

One of the most controversial issues in reproductive medicine during the past 30 years has been a purported decline in semen quality during the twentieth century. In 1992, a widely publicized meta-analysis of 61 studies by Carlsen and

colleagues appeared to demonstrate a worldwide decrease in average sperm concentration from 113 million/mL in 1940 to 66 million/mL in 1990 [62]. This report was echoed by additional publications, raising the question of whether exposure to environmental toxins, such as estrogenic compounds like diethylstilboestrol (DES), was adversely affecting testicular function [63, 64].

Important methodological shortcomings have since been identified in Carlsen and colleagues' analysis and in the quality of many studies that were included in their review [65, 66]. Geographical differences were found to be the source of much of the variance in sperm density [67]. All studies included in the review from before 1970 were performed in the USA; however, because US studies generally reported higher sperm concentrations than those conducted elsewhere, the review was biased toward an apparent decline in sperm concentration by inclusion of international studies post-1970.

In subsequent investigations, Fisch and colleagues found no evidence of a decline in sperm density in the USA, although they did identify substantial differences between the average sperm counts of men from different states [68, 69]. Fisch and colleagues also concluded that worldwide studies did not reveal a global decline in semen quality, although more limited, locoregional trends could not be excluded [65].

More recently, Levine and colleagues published a large meta-regression analysis of 185 worldwide studies (including almost 43,000 men) that reported on sperm concentration and total sperm count between 1973 and 2011 [70]. They found a 52% decrease in sperm concentration and a 59% decrease in total sperm count among Western countries (North America, Europe, Australia, and New Zealand) but no significant decline of either parameter in non-Western countries. This decline has not been uniformly reported, though. A key study of nearly 5000 Danish men found a slight increase in sperm concentration, total sperm count, and total normal morphology from 1996–2000 to 2006–2010 [71].

2.5 Health-Care Resource Utilization for Male Infertility

Medical intervention for male infertility may take the form of outpatient care, surgical procedures, and ART. The Urologic Diseases in America (UDA) Project, which published its first report in 2007, has facilitated a better understanding of the scope of health-care resource utilization for male infertility in the USA.

2.5.1 Office Visits and Ambulatory Surgery Cases

According to data from the National Ambulatory Medical Care Survey (NAMCS), which are summarized in the UDA Project's report, the average number of physician office visits for male infertility in the United States exceeded 150,000 annually between 1992 and 2000, with little variability [72]. However, there was a 29% decline in hospital outpatient visits and 23% decline in outpatient visits for male infertility from 2002 to 2006 [73]. The reasons for this trend are unclear but may include preferential use of ART.

Age-wise, the greatest utilization of health-care services for male infertility between 2002 and 2006 was among men 35–39 years old. Varicocele was the most commonly identified diagnostic code, accounting for 53% of office visits and 67% of ambulatory surgeries.

Other data from the NAMCS demonstrate substantial regional variation in resource utilization for male infertility. Men living in the Northeast United States had a rate of ambulatory surgery visits associated with a diagnosis of infertility of 227 per 100,000, while those in the Midwest, South, and West had rates of just 110.9, 128.9, and 119.2 per 100,000, respectively. The decline in outpatient visits was disproportionately experienced in the Northeast and West, decreasing by 36% and 32%, respectively, from 2002 to 2006. Visits in the South and Midwest decreased by 17%. The explanation for this variability is probably multifactorial, reflecting a combination of patient demand and availability of services.

2.5.2 Assisted Reproductive Technology

About 231,936 ART cycles were performed in the United States in 2015, an increase from 142,435 in 2007 and 81,438 in 1998 [74]. A total of 60,778 live births resulted from these cycles, representing a cumulative success rate of 26.2%. Approximately 33% of ART cycles were associated with a diagnosis of male factor infertility, and 17% were linked to a combination of male and female factors. The overall likelihood of a live birth per ART cycle was 34.4%.

The percentage of ART procedures involving the use of ICSI has steadily increased, from 71% in 2006 to 79% in 2015 [74]. Although ICSI was originally developed specifically to overcome severe oligozoospermia or azoospermia, nearly 70% of ART cycles now utilize ICSI even when no male factor is identified [74].

2.5.3 Cost of Treatment for Male Infertility

The overall economic burden of health care for male infertility is difficult to estimate precisely. While expenditures for office and ambulatory surgery visits were reported by the NAMCS to be $17 million in 2000, this figure does not account for IVF/ICSI or out-of-pocket expenditures [75]. If the assumed cost per IVF cycle is $15,715, then expenditures on ART alone for male infertility exceeded $1.8B in 2015,

given the percentage of ART procedures involving a diagnosis of male factor infertility [74, 76] .

On an individual basis, the average annual expenditures on health care for a privately insured male with infertility was $11,437 from 2003 to 2006, compared with $6942 for men without infertility. The difference in expenditures increases with patient age, from $2249 for men aged 18–34 to $8127 for men aged 55–64, likely due to increased utilization of more costly IVF technology [73].

There are significant disparities between countries in the cost of utilizing ART. In 2009, the average cost of a standard IVF cycle in the United States was $12,513, compared with $8500 in Canada, $6534 in the United Kingdom, and $3956 in Japan [77].

2.6 Cost Analysis Models for Management of Male Infertility

When a male risk factor for infertility is identified, the couple is often faced with the choice of using ART or having pathology-directed treatment. Evaluating the economic efficiency of these alternatives is the domain of cost analysis.

Cost analysis is only meaningful with respect to treatments that have previously been demonstrated to be effective. Our intention in this chapter is not to review the studies that have established the effectiveness of the interventions discussed here—or, in some cases, the controversies surrounding them. Rather, our focus is limited to a brief introduction to cost analysis studies as they pertain to male infertility.

Two types of cost analysis are utilized in reproductive medicine: cost-minimization analysis and cost-effectiveness analysis. The first, cost-minimization analysis, is also known as cost identification. It involves assessment (and comparison) of the costs associated with particular treatments. Direct and/or indirect costs may be taken into account—direct costs being health-care expenditures and indirect costs being "downstream" burdens such as transportation expenses, lost wages, etc. In well-conducted economic analyses, future costs should be appropriately discounted to present values by a factor of 3%–5% per year.

When the outcomes of alternative interventions are not equivalent, cost-effectiveness analysis is useful to compare them, as it involves not only identifying the costs that accrue but also expressing them relative to the probability of a particular result (e.g., dollars per pregnancy or live birth). Decision analysis, such as Markov modeling, is the most common technique employed for this purpose in the field of male infertility. Details of relevant methodology are covered in *Methods for the Economic Evaluation of Health Care Programmes* [78].

Comparison of cost-effectiveness has been applied to several management options that may be encountered in male reproductive medicine, including varicocele treatment vs. immediate ART (with or without surgical sperm retrieval), vasectomy reversal vs. ART, and hormonal therapy vs. ART for hypogonadotropic hypogonadism. Pathology-directed intervention has almost invariably been found to be more cost-effective than a straight-to-ART approach [79, 80]. One exception is when a varicocele is present in the setting of nonobstructive azoospermia; in this situation, microsurgical testicular sperm extraction (TESE) for ICSI is more cost-effective than varicocelectomy [81].

Every cost-effectiveness analysis in the arena of male infertility is sensitive to assumptions about treatment costs, success and complication rates, and the subsequent management of couples for whom first-line intervention is unsuccessful [82, 83]. Precise characterization of the clinical scenario(s) is very important and should be considered when determining the generalizability of results. For instance, the age of the female partner has a significant influence on the relative cost-effectiveness of vasectomy reversal and ART [75].

2.7 Conclusion

This chapter examined a number of issues that are commonly misunderstood and/or misrepresented with respect to the epidemiology of infertility, in general, and male infertility, in particular. First, infertility should not be confused with sterility—or even below-average fecundability. Approximately 10% of couples with average reproductive potential will not conceive within 12 months of unprotected intercourse and are therefore designated as infertile. Such couples may comprise a substantial proportion of those who present for evaluation of infertility (see Table 2.3). Their odds of spontaneous, unassisted conception remain high.

From a clinical standpoint, it would be ideal to accurately identify men with below-average reproductive potential. However, while semen analysis is the most common test employed for this purpose, its utility is limited by substantial overlap between the distributions of semen characteristics in empirically fertile and infertile men. Using relatively low diagnostic thresholds, such as the 2010 WHO reference values, carries the advantage of high specificity and perhaps a relatively decent positive predictive value in the setting of an infertility clinic; unfortunately, the negative predictive value only narrowly exceeds that of a coin flip.

The diagnostic inaccuracy of semen analysis is a fundamental problem for several additional reasons. From an epidemiologic perspective, the absence of a gold-standard test for male infertility means that we have, at best, an uncertain grasp of its actual prevalence, let alone its association with putative risk factors, such as varicocele, cryptorchidism, sexually transmitted infections, etc. Moreover, if the true prevalence of male infertility is unclear then so, too, is the predictive value of tests employed for its diagnosis.

Finally, there is the question of how to counsel infertile men whose semen parameters exceed diagnostic thresholds. Given the poor predictive value of these "negative" results, should we advise consideration of further evaluation and treatment for modifiable risk factors identified in these individuals? An affirmative response to this query carries the risk of unnecessary treatment and costs, while the alternative may deprive some couples of an improvement in reproductive potential that facilitates natural conception, allows the use of IUI instead of IVF, or increases the odds of successful IVF/ICSI [84]. Well-designed studies to address this question are necessary.

2.8 Review Criteria

An extensive search was performed to identify studies regarding the epidemiology, diagnosis, and treatment of male infertility. Search engines such as MEDLINE (Ovid), PubMed, and Google Scholar were used. For the second edition, emphasis was placed on studies published between 2010 and 2018. Key search terms included male infertility, incidence, epidemiology, sperm count, semen parameters, prediction, cost analysis, DNA fragmentation, IVF, ART, and ICSI. Data were abstracted from publications by the Centers for Disease Control and Prevention (CDC), including the National Ambulatory Medical Care Survey and the CDC's ART National Summary Report, as well as the Urologic Diseases in America report issued by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). Articles published in languages other than English were not considered. Websites and book-chapter citations provide conceptual content only.

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Laboratory Evidence for Male Infertility

3

Neel Parekh and Ashok Agarwal

Key Points

- The WHO manual is currently in its fifth edition and updated the reference values. The lower reference values provide a broader range of "normal" parameters.
- Azoospermia is defined as the complete absence of sperm after examination of a centrifuged pellet on two separate occasions obtained more than 2 weeks apart.
- Healthy sperm DNA is bound to protamine and tightly packed to protect from stress and breakage during transport through the female reproductive tract.
- The TUNEL assay identifies "nicks" or free ends of DNA by incorporating fluorescent nucleotides into the site of damage. It is both highly specific and associated with a high positive predictive value to quantify SDF.
- An AZFa or AZFb Y-microdeletion has rarely successful sperm retrieval rates. However, sperm retrieval rates are as high as 80% in patients with an isolated AZFc deletion.

3.1 Introduction

The semen analysis has been the primary biomarker to evaluate male infertility and is a direct reflection of the male's fertility and overall health in the preceding 74 days [1]. Semen analyses first began with the advent of microscopy in 1678, when van Leeuwenhoek first described spermatozoa or "semen animals" in the ejaculate [2]. However, it was not until 1951 that the integral role of semen in reproduction became evident. MacLeod's landmark study compared semen parameters of 1000 men who conceived naturally and 800 men who were presumed infertile and paved the way for modern semen analyses [3]. MacLeod plotted the semen parameters on histograms, and the plots were divided into quartiles for each of the two groups of men. He believed that men in the lowest quartile would be infertile while men above this threshold were likely fertile. The data was instrumental in developing reference ranges for semen parameters used in the first edition of the World Health Organization (WHO) manual in 1980 [4]. Since then, advances in assisted reproductive technologies (ART) have shown considerably more options for subfertile and infertile men to achieve fecundity. Such changes have necessitated a more comprehensive and methodological assessment of infertile men. The aim of this chapter will be to discuss modern laboratory exams and techniques available to successfully evaluate and treat male factor infertility.

3.2 WHO Guidelines for Assessment of Semen Specimen

Since 1980, the WHO has endeavored to standardize how laboratories analyze and report semen parameters globally. The WHO manual is currently in its fifth edition which was published in 2010 [5]. The fifth edition aims to provide evidence-based reference ranges based off of populational studies. The new reference ranges are significantly lower than the previous manuals (Table 3.1) [5, 6]. Cooper et al. analyzed semen samples from more than 4000 men from 14 different countries on four continents, and reference values were calculated from men who naturally conceived with their partner within 12 months [7]. After the data was examined, the 95th percentile for semen volume, concentration, motility, vitality, and morphology was formulated, and the

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fifth percentile was set as the lower reference limit. There are, however, several limitations to this study including significant population biases. Specifically, the majority of the cohort was from Europe, and only 10% of the study population was from the southern hemisphere. Furthermore, the data is derived solely from a fertile cohort and was not compared to an infertile one; therefore, a "cutoff" between infertile and fertile men cannot be determined [8]. As a result of the lower reference values, men who would have been categorized as having abnormal parameters using the old reference values are now being classified as having normal parameters. Murray and colleagues determined that roughly 15% of men who had one or more aberrant parameters using the fourth edition values were categorized as having all parameters within the normal fifth edition reference ranges [9]. The fifth edition manual provides clinicians a table which reviews where individual semen parameters lie within the 2.5th to 97.5th percentiles for men who naturally conceived with their partner within 12 months (Table 3.2) [5, 7]. This information can be informative for men when reviewing semen analysis parameters in the office.

Table 3.1 Evaluation of fourth and fifth editions of the WHO manual semen analysis reference values

Parameter (units)	Fourth edition reference value	Fifth edition lower reference limit fifth centile (95% CI)
Semen volume (ml)	2.0	1.5 (1.4–1.7)
Total sperm number (10^6/ejaculate)	40	39 (33–46)
Sperm concentration (10^6/ml)	20	15 (12–16)
Total motility (%)	50	40 (38–42)
Progressive motility (%)	25	32 (31–34)
Sperm morphology normal forms (%)	Not given but suggests possibly 15	4 (3–4)
% Vitality (live spermatozoa)	50	58 (55–63)
Peroxidase-positive leukocytes (10^6/ml)	<1.0	<1.0

3.3 Laboratory Evaluation of Male Factor Infertility

3.3.1 Basic Semen Analysis

The process of natural conception is intricate, culminating in fusion of a healthy sperm to healthy ovum. Semen analyses provide valuable information for clinicians and patients but is only a surrogate for male fertility and does not guarantee paternity. It is unclear why some men with "normal" semen analyses suffer from infertility and those with "abnormal" semen analyses may remain fertile [8]. Reproductive potential is also strongly influenced by female factors in many couples and should be assessed when appropriate. To appropriately assess for male factor infertility, a thorough history and physical exam should be performed. Ideally, two semen analyses should be obtained and performed one month apart. Clinicians should be able to appropriately counsel male patients on the protocol for proper semen collection and ultimately interpret the results [9].

3.3.1.1 Collection

It is generally recommended that a semen specimen be collected at least 3 months after a febrile illness or stressful life event. The WHO recommends providing a semen specimen after 2–7 days abstinence [5]. However, the optimal abstinence interval may be 2–4 days, as studies have shown a relative improvement in total sperm concentration and motility after 4 days [10]. A longer abstinence interval has been associated increased sperm DNA damage [11]. Preferably, the specimen should be obtained in a sterile cup via masturbation either at home or in the laboratory. However, sexual intercourse using a special collection condom that is not detrimental to sperm may also be utilized. Lubricants should be avoided as they may alter sperm motility [12]. The semen specimen should be evaluated within an hour of collection and stored at room or body temperature until then. The Clinical Laboratory Improvement Amendments (CLIA) has specific guidelines by which laboratories should observe to ensure quality and accurate semen analysis results [13]. Once the semen liquefies, typically within 20–60 minutes, a

Table 3.2 Review of semen analysis percentiles for men whose partners had a time to conception of ≤12 months

Percentiles									
Parameters	2.5th	5th	10th	25th	50th	75th	90th	95th	97.5th
Semen volume (ml)	1.2	1.5	2.0	2.7	3.7	4.8	6.0	6.8	7.6
Total sperm (10^6/ejaculate)	23	39	69	142	255	422	647	802	928
Sperm concentration (10^6/ml)	9	15	22	41	73	116	169	213	259
Total motility (%)	34	40	45	53	61	69	75	78	81
Progressive motility (%)	28	32	39	47	55	62	69	72	75
Sperm morphology normal forms (%)	3	4	5.5	9	15	24.5	36	44	48

macroscopic assessment of the semen sample is performed which includes volume, pH, color, and viscosity. Subsequently, microscopic examination is utilized to calculate the concentration, motility, morphology, and vitality [14].

3.3.1.2 Volume

Over 70% of the semen volume is provided by the seminal vesicles, and current WHO criteria for normal semen volume is >1.5 ml [5]. A low semen volume following an appropriate abstinence interval and complete collection may indicate partial retrograde ejaculation, ejaculatory duct obstruction (EDO), congenital absence of the vas deferens, or severe hypogonadism. A high semen volume (>4 ml) may be seen in instances of prolonged sexual abstinence.

3.3.1.3 Liquefaction and Viscosity

Fibrinolysin is a proteolytic enzyme that is secreted by the prostate and facilitates liquefaction of semen from the coagulum state (~30–60 minutes). A defect in liquefaction may be indicative of an EDO or inadequate secretion of fibrinolysin by the prostate. Semen viscosity is related to the fluid nature of the specimen and can be determined by utilizing a 1.5 mm pipette to drop a semen specimen into a cup and examining the length of the thread formed. A thread length >2 cm is considered abnormally viscous. There is a concern that increased viscosity impairs sperm motility and therefore fertility, but this is controversial [8]. "Non-liquefaction" and "hyperviscosity" are two distinct conditions that are commonly interchanged inappropriately. "Non-liquefaction" is a result of the semen remaining in the coagulum state. "Hyperviscosity" is when the semen specimen pours thick as opposed to in drops. This is associated with hypofunction of the prostate or seminal vesicles, infection, or high levels of leukocytospermia [15]. Trypsin can be used to treat hyperviscous semen specimens prior to additional testing.

3.3.1.4 pH [normal > 7.2]

Measurement of pH is primarily determined by the balance of alkaline seminal vesicle fluid and the acidic prostatic secretions. The normal range of semen pH typically lies between 7.2 and 8.0. An acidic pH is associated with obstruction or seminal vesicle hypoplasia seen in men with congenital absence of the vas deferens [12, 13]. An alkaline pH > 8.0 may be associated with an underlying infection [8, 12].

3.3.1.5 Concentration

Normal sperm concentration is defined as >15 million sperm/mL and is determined after careful light microscopic examination of the wet preparation [5]. Typically, a minimum of 200 spermatozoa are counted utilizing counting chambers within a grid pattern for accurate assessment (Fig. 3.1). The concentration is then calculated and reported per milliliter. Alvarez et al. demonstrated that compared to other sperm parameters (count, morphology, motility, and volume), sperm concentration has the highest intra-observer variation [16]. Oligozoospermia is defined as <15 million sperm/mL, but incomplete collection or short abstinence interval should be ruled out prior to making this diagnosis. Azoospermia is defined as the complete absence of sperm after examination of a centrifuged pellet on two separate occasions obtained more than 2 weeks apart. EDO, ejaculatory dysfunction, and abnormal spermatogenesis are potential causes of azoospermia.

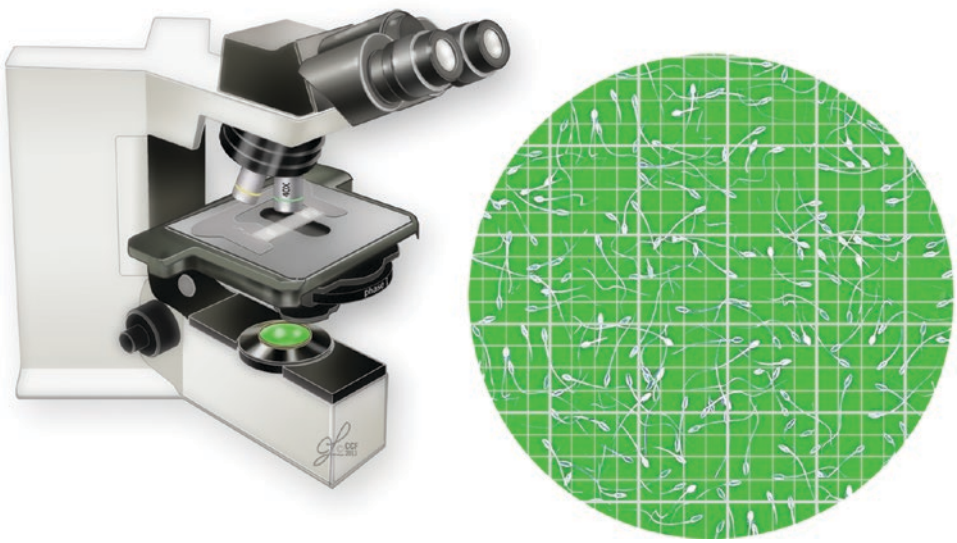

Fig. 3.1 Measurement of sperm concentration and motility by a fixed counting chamber and a phase-contrast microscope. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2013–2019. All Rights Reserved)

Recently, studies have revealed that the total motile sperm count (volume × concentration × % motility) is more predictive of infertility compared to concentration, motility, and volume alone [17, 18]. Compared to infertile men with normal semen parameters, those with total motile sperm count (TMSC) <1 million experienced 83% fewer natural pregnancies, while those with 10–20 million had 55% fewer pregnancies [18]. Surprisingly, among men with TMSC 0–1 million, 23% were able to achieve a natural pregnancy within 3 years, further highlighting the predictive and diagnostic limitations of semen analyses [18]. TMSC was also recently shown to be a better predictor than total sperm count for intracytoplasmic sperm injection (ICSI) outcomes compared with WHO values [19]. Clinically, TMSC facilitates decision-making when counseling couples on appropriate treatment options, including assisted reproductive technologies.

Computer-assisted semen analysis (CASA) is an automated method of determining sperm parameters which offers several advantages over manual semen analyses. Specifically, the basic parameters evaluated manually by an operator (concentration, motility, and morphology) can be measured more precisely by CASA. CASA also provides useful kinematic information. However, CASA systems suffer from other challenges such as costly equipment, more complicated procedure, and poorer performance with low sperm concentrations [20].

3.3.1.6 Motility

Sperm motility is determined by assessing sperm for signs of movement and is a critical indicator of the functionality of spermatozoa. It should be characterized immediately following liquefaction to avoid temperature changes and dehydration. The WHO considers normal total motility to be >40% and is dependent on the ability of the sperm to traverse the epididymis and successfully mature [5]. A motility of <5%–10% can be attributed to ultrastructural defects in the reproductive tract. The previously utilized WHO subclassification of progressive motility into fast and slow has fallen out of favor due to high variability and inaccuracy among laboratory technicians. Currently, motility is simply divided into (1) progressive, space gaining motion; (2) nonprogressive, motion in place or in small circles; and (3) nonmotile or immotile [6].

Asthenozoospermia (sperm motility <40%) can also be a result of a poor specimen collection that may have been exposed to a rubber condom, lubricants, or spermicides. Sporadic clumps of agglutinated sperm are typically not concerning. However, clumping of sperm seen >10%–15% of the time is associated with antisperm antibodies (ASAs). ASAs may impair sperm motility and elicit an unusual "shaking pattern" that can impede transit of sperm through the cervical mucous. Lengthy abstinence intervals, infection, partial EDO, ultrastructural defects, and varicocele have all been implicated as potential causes of asthenozoospermia [12, 16].

3.3.1.7 Morphology

Over time, there have been significant shifts in the classification of sperm morphology which is a source of controversy. Normal morphology reference values have shifted from ≥80.5% in the WHO first edition to ≥4% normal forms in the WHO fifth edition [5, 21]. Stricter approaches and lack of standardization in technician training may have contributed to these changes [21, 22]. To appropriately classify morphology, the sperm should routinely be analyzed in a fixed, air dried, and stained semen smear. Either Shorr, Papanicolaou, or Diff-Quik smear stains are approved by the WHO. This process alone has been implicated in affecting morphologic appearance [23, 24]. Normal sperm are typically comprised of a smooth oval head with a distinct acrosomal region covering 40%–70% of the sperm head. Normal morphology dimensions have been defined as a sperm head of 3–5 μm in length and 2–3 μm in width. Furthermore, the sperm should be devoid of head, midpiece, or tail defects [25].

- *Head defects* include micro- or megalocephalic heads, tapered head, missing acrosome (globozoospermia), heads with irregular forms, and bicephalic or multicephalic heads.
- *Midpiece defects* consist of abnormal or no tail insertions. Distended, thin, bent, or elongated midpieces are abnormal.
- *Tail defects* include coiling (associated with osmotic stress), multiple, short, 90° bending, or broken tails.

Sperm morphology male infertility–laboratory evaluation is reported as the percentage of atypical forms present in the ejaculate and classified according to the WHO criteria or the Kruger's strict criteria. Teratozoospermia is defined as <4% normal morphology by the WHO method. Early studies demonstrated that it is most likely the morphologically normal appearing spermatozoa that are best able to navigate the female reproductive tract and fertilize the egg [26, 27]. More recent data suggests that abnormal morphology should not deter intrauterine insemination (IUI) or require couples to proceed immediately to in vitro male infertility–laboratory evaluation: fertilization/intracytoplasmic sperm injection (IVF/ICSI) [28–30]. The American Urological Association (AUA) recommends against utilizing isolated abnormalities in strict morphology to counsel couples on treatment decisions [31].

3.3.1.8 Agglutination

Microscopic examination may identify sperm agglutination or clumping and is characterized by sperm adhering to one another without other cells or debris. Sperm agglutination may be indictive of ASA. However, if there is clumping of

sperm, cells, and debris, it is most likely a result of aggregation. Aggregates typically only consist of dead sperm while agglutination from ASA consists of a proportion of motile sperm. Clinicians should be aware that a small amount of agglutination may be unremarkable [6]. However, when significant agglutination is present, additional testing with ASA testing and/or semen cultures is recommended [12, 31].

3.3.1.9 Leukocytospermia

Leukocytospermia is defined as >1 × 10^6/ml WBCs in the semen and is commonly associated with idiopathic male infertility [32]. It is important to avoid classifying immature spermatozoa – spermatids and spermatocytes, which appear as round cells – as seminal leukocytes. The primary sources of leukocytes are from the prostate, seminal vesicles, vas deferens, and epididymis. They are unlikely to arise from the testis, secondary to the blood–testis barrier [33]. If there are >5 round cells per high-powered field (HPF), further investigation is recommended. Immunocytochemistry is the gold standard, but due to cost and difficulty standardizing the monoclonal antibodies, it is not commonly performed by most laboratories. Peroxidase staining or the Endtz test is a reliable substitute to properly identify leukocytes and distinguish them from immature spermatozoa [34].

Although leukocytospermia can be indicative of an infectious process (male accessory gland infection), recent investigation has determined that WBCs can exist in the absence of infection or immune response [35]. Furthermore, leukocytospermia has been implicated in negatively affecting sperm functionality through the formation of reactive oxygen species (ROS) [35, 36]. Sharma and colleagues have shown that ROS levels are increased, even with leukocytospermia levels <0.2 million/mL, suggesting leukocyte levels lower than the WHO "cutoff" are detrimental [37].

Athayde et al. demonstrated that men with a normal Endtz test had a 24% chance of natural conception, while men with leukocytospermia levels <1 million/mL lowered natural conception rates to 16% on 12-month follow-up [38]. Unfortunately, identifying leukocytospermia is not standard practice in many andrology laboratories or must be ordered separately from the basic semen analysis. Routine urine and semen cultures should be performed if leukocytospermia is identified. Current therapies to treat leukocytospermia include anti-inflammatory medications, antioxidants, and antihistamines. Antibiotics are used to treat concurrent clinical infections.

3.4 Retrograde or Post-Ejaculatory Urinalysis (PEUA)

Men with retrograde ejaculation (semen is redirected into the urinary bladder), incomplete collection, EDO, congenital bilateral absence of the vas deferens (CBAVD), hypogonadism, or lack of emission may present with low-volume ejaculate on semen analysis. A PEAU is a routine laboratory test used to differentiate retrograde ejaculation from the aforementioned conditions. The AUA Best Practice Statement recommends that clinicians perform PEUA in patients with ejaculate volume <1.0 ml and in the absence of CBAVD or hypogonadism [31]. PEUA is performed by microscopically assessing the pellet at ×400 magnification after approximately 10 minutes of centrifugation at a minimum of 300 *g*. In patients diagnosed with aspermia or azoospermia, identification of any sperm on PEUA is indictive of retrograde ejaculation. However, in patients with low volume oligozoospermia, "significant numbers" of sperm should be identified on PEUA in order to diagnose retrograde ejaculation. There is no expert consensus on "significant numbers" of sperm in PEUA.

3.5 Reactive Oxygen Species (ROS) Testing

There is mounting evidence that elevated ROS levels play an independent role in the etiology of male infertility [39, 40]. ROS are produced by leukocytes and abnormal spermatozoa and are a natural byproduct of metabolic pathways. Small quantities of ROS are actually required to ensure normal spermatogenesis, successful capacitation, and the acrosome reaction [40, 41]. An overabundance of ROS levels compared with antioxidants, however, may result in oxidative stress (OS) which has been observed to impair spermatogenesis as well as sperm kinetics [41]. Mitochondria have been identified as an essential component of seminal ROS production, which is primarily mediated through the formation of superoxide in the electron transport chain [42]. Oxidative stress has been shown to negatively affect sperm physiology and function through several pathways: increased rates of DNA fragmentation [43], decrease in sperm motility [44], and diminished quality of sperm [45]. Elevated seminal ROS levels have been identified in 25%–80% of infertile men, but levels are significantly lower in the fertile population, and it remains unclear if this scenario is predominately a correlative or a causative relationship with infertility [46, 47]. Clinically, measurement of seminal oxidative stress can identify men where oxidative stress is associated with infertility and identifies a patient who may benefit from antioxidant supplementation [46].

The ideal method of testing for ROS is controversial, likely due to a lack of standardization among laboratories regarding reference values, equipment, and technique. Currently, there are a variety of direct and indirect ROS testing modalities utilized to determine seminal OS. However, the most reliable and accurate modality has been luminol-based chemiluminescence assay. This technique accurately measures both intracellular and extracellular ROS. Luminol

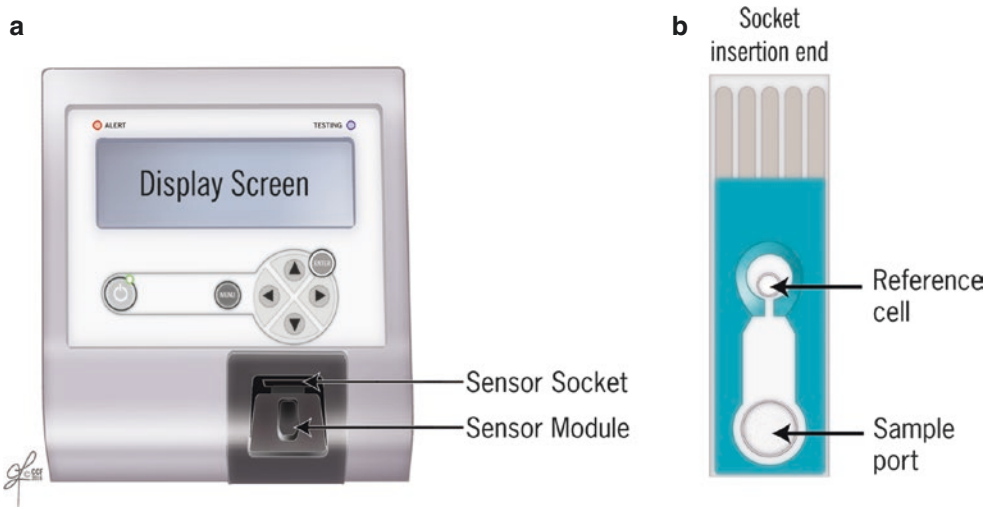

Fig. 3.2 Measurement of ORP using (**a**) MiOXSYS analyzer and the (**b**) sensor. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2013–2019. All Rights Reserved)

is also able to react with various forms of ROS, including hydrogen peroxide, hydroxyl radical, and superoxide anion. An adequate semen specimen is required to perform this test (>1 × 10^6/ml) and analyzed within 1 h of collection. Luminol adheres to the free radicals to produce a light signal that the luminometer converts to an electrical signal. The quantity of free radicals produced is measured as relative light units (RLU)/s 10^6 sperm. Agarwal and colleagues [48] determined that in healthy controls with normal semen parameters, the physiologic ROS level is 102.2 RLU/s/10^6. Men above this threshold value can be considered to be oxidative stress positive and should be counseled appropriately.

The latest advancement in laboratory diagnostics is the Male Infertility Oxidative System (MiOXSYS) (Fig. 3.2). It allows for an easy and accurate measurement of ROS through the oxidation-reduction potential (ORP). ORP, or redox balance, measures for the homeostasis between reductants and oxidants in a variety of biologic specimens. Several studies have validated the reproducibility and reliability of the MiOXSYS in measuring the ORP levels in semen specimens of patients evaluated for male infertility [49, 50]. The MiOXSYS has several advantages over standard chemiluminescence assays which include ease of use, smaller sample volume (30 μL vs. 400 μL), and more forgiving measurement protocol (up to 120 minutes after specimen is produced) [50].

While ROS measurement is not routinely recommended on initial evaluation of male infertility, it is a clinically useful tool to further evaluate patients with varicocele, lifestyle factors (i.e., smoking), and idiopathic infertility [12, 31].

3.6 Acrosome Reaction Testing

The acrosome reaction is an essential step of successful fertilization. The acrosome is a modified Golgi apparatus that encompasses 40%–70% of the sperm head. The primary function of the acrosome reaction is to release the lytic enzymes acrosin and hyaluronidase to digest the zona pellucida which ultimately allows for spermatozoa fusion with the oocyte. Acrosome reaction testing is not routinely performed in many laboratories, but may be considered when substantial irregularities of head morphology exist or the setting of recurrent IVF failure. Acrosomal status can be examined through triple staining with transmission electron microscopy, optic microscopy, flow cytometry, and fluorescently labeled lectins [51–55].

3.7 Antisperm Antibody (ASA) Testing

A number of urologic conditions including testicular cancer, torsion, and traumatic or surgical disruption of the testis can violate the blood–testis barrier [56]. Breakdown of the blood–testis barrier may predispose men to an immune response to sperm in the form of ASA. ASA are deemed an important factor impairing pregnancy rates and are seen in approximately 10% of infertile men as opposed to 2% of fertile men [57, 58]. Extensive sperm agglutination is a result of ASA which can impair the process of fertilization by inhibiting sperm penetration of cervical mucous and preventing fusion of the sperm and oocyte. Sperm concentration and motility have also been shown to be lower with the presence of ASA [12, 56].

The mixed antiglobulin reaction (MAR) and immunobead test are qualitative laboratory tests used to detect IgA and IgM sperm antibodies. If ≥50% of motile sperm are bound by antibodies, then these tests are considered abnormal [5]. The clinical utility of ASA testing is varied, but in men with isolated asthenospermia and normal concentration or with significant sperm agglutination, ASA testing should be considered [12, 31].

3.8 Sperm Viability Testing

Sperm viability testing or a vitality assay is a laboratory test utilized to facilitate identification of living sperm in the setting of low motility (<25%). In ejaculated specimens with low motility or nonmotile surgically derived sperm to be used for IVF/ICSI, it is necessary to differentiate between necrozoospermia (dead sperm) and viable immotile sperm (asthenozoospermia). This is done by evaluating the integrity of the sperm cell membrane which should be intact in living sperm. The fifth edition WHO reference parameter for vitality is ≥58% [5]. Cell membrane integrity can be evaluated through the hypoosmotic swelling test (HOS test) and the dye exclusion test.

The dye exclusion test is dependent on the intact cell membrane of a live sperm resisting absorption of specific dyes. Eosin Y and nigrosin are two of the most commonly utilized dyes. Nigrosin is particularly useful as it will stain the background dark and provide good contrast for sperm assessment. Eosin Y will stain sperm heads red or dark pink if they are dead. It will stain live sperm heads white or light pink. The HOS test is dependent on the ability of the live sperm (intact cell membrane) to swell in hypotonic solutions. The HOS test is favored over the dye exclusion test due to the ability of quickly assessing for necrozoospermia without damaging viable sperm [31]. Therefore, sperm used for a HOS test can still be used for subsequent IVF/ICSI [58, 59].

3.9 Advanced Semen Testing

3.9.1 Sperm DNA Fragmentation (SDF)

Evaluation of sperm DNA fragmentation has evolved into an essential tool for the overall assessment of male infertility. Sperm DNA is bound to protamine and tightly packed to protect from stress and breakage during transport through the female reproductive tract. The cause of DNA damage is likely multifactorial (i.e., smoking, drug use, environmental exposure, malignancy, chemotherapy, and varicocele) [60]. Elevated SDF has been identified in roughly 8% of subfertile men with normal semen parameters [61]. The integrity of sperm DNA has been shown to affect couple's fecundity through its impact on successful fertilization, healthy embryo development, implantation, and pregnancy [62].

Several sperm DNA integrity assays have been introduced to evaluate the degree of SDF. In general, the assays were developed to facilitate clinicians in implementing early and effective management plans for specific clinical scenarios. These scenarios include couples with recurrent pregnancy loss, patients with idiopathic infertility, ART failure, and varicocelectomy candidates. Commonly utilized methods for evaluating SDF are summarized in Table 3.3.

Table 3.3 Methods of evaluating sperm DNA damage

Assay	Measures	Method
Sperm chromatin structure assay (SCSA)	Susceptibility of sperm DNA to denaturation by heat or low pH	Flow cytometry based
Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)	Single- and double-stranded DNA breaks	Fluorescence microscopy or flow cytometry based
Single-cell gel electrophoresis assay (Comet)	Single- and double-stranded DNA breaks Altered bases	Objective and quantitative, fluorescence microscopy
Sperm chromatin dispersion (SCD)	Absence of sperm DNA damage - Halo	Flow cytometry based

3.9.2 Sperm Chromatin Structure Assay (SCSA)

The SCSA is dependent on the principle that heat or acid will denature sperm with an abnormal chromatin structure. Specifically, SCSA is a measure of the susceptibility of sperm DNA to acid or heat denaturation utilizing flow cytometry [63]. The metachromatic shift from green to red fluorescence is measured using flow cytometry. The percentage of spermatozoa with red fluorescence (red–red + green fluorescence) is expressed as DNA fragmentation index (DFI). The clinical threshold for DFI is <30%. Couples with >30% DFI have lower conception rates with natural intercourse and ART. It is a very sensitive assay and has been reported as an excellent tool to detect dose–response relationship in men exposed to environmental toxicants [64]. SCSA can evaluate over 10,000 cells rapidly and has a well-standardized protocol, thus minimizing interlaboratory variations. The major drawbacks include expensive instrumentation (flow cytometry) and skilled technicians.

3.9.3 Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL)

The TUNEL assay identifies "nicks" or free ends of DNA by incorporating fluorescent nucleotides into the site of damage [65]. Single- and double-stranded DNA damage can be examined by either fluorescence microscopy or flow cytometry. Advantages of the TUNEL assay include proven reliability, accuracy, and ease of use with low interlaboratory discrepancies [66]. Furthermore, by utilizing a threshold of 16.8% for SDF, the TUNEL assay is both highly specific and associated with a high positive predictive value and will aid in its clinical utility [67].

3.9.4 Single-Cell Gel Electrophoresis Assay (Comet)

The comet assay provides an objective and quantitative measure of the degree of DNA damage per sperm [68]. Comet is able to detect not only single- and double-strand breaks but also abasic sites. The sperm are subject to electrophoresis at high pH which results in structures resembling comets under fluorescence microscopy. The number of DNA breaks is quantified by the intensity of the comet tail relative to the head. An advantage of this assay is that it requires only 5000 sperm to evaluate SDF and is particularly useful in cases of severe oligozoospermia [69].

3.9.5 Sperm Chromatin Dispersion (SCD)

The SCD test or Halosperm test is unique in that it is a measure of the absence of SDF. The SCD test produces sperm nucleoids with a central core and a peripheral halo of dispersed DNA loops. Sperm with nonfragmented DNA will demonstrate the characteristic halo, but sperm with DNA fragmentation will produce very small or no halo on fluorescent microscopy. While the SCD test is easy to use, high interlaboratory variations has impeded widespread standardized usage [12].

3.9.6 Endocrine Evaluation

Approximately 3% of infertile men will have a hormonal imbalance as a basis of their subfertility [70]. The 2011 AUA Best Practice Statement recommends endocrinologic assessment in men with (1) abnormal semen analysis (sperm concentration < 10 million/ml), (2) diminished sexual function, or (3) clinical findings of a small firm testis or altered masculine features. The hypothalamus secretes gonadotropin-releasing hormone (GnRH) which traverses the hypophyseal portal system to the anterior pituitary and stimulates the release of FSH and LH. These gonadotropins exert their effect on the testicle in a pulsatile fashion. Under normal conditions, LH stimulates the testicular Leydig cells to produce testosterone. Testosterone provides negative feedback onto the hypothalamus by inhibiting GnRH release. FSH stimulates the testicular Sertoli cells to promote spermatogenesis and secrete inhibin B and activin. Inhibin B causes negative feedback by inhibiting the release of FSH from the anterior pituitary, whereas activin stimulates the release of FSH from the anterior pituitary. The relationship between these hormones can help determine an underlying cause of subfertility.

At a minimum, the initial endocrinologic workup should include a measurement of serum follicle-stimulating hormone (FSH) and morning serum testosterone levels. Morning collection of blood for hormone testing is recommended due to a normal physiologic decrease in testosterone levels throughout the day. If the testosterone level is low, then a repeat measurement of total and free testosterone should be obtained. In addition, measuring serum prolactin and luteinizing hormone (LH) is also advised.

While normal serum FSH levels do not guarantee active spermatogenesis, an elevated or "high normal" FSH level is indicative of an abnormality in spermatogenesis such as hypergonadotropic hypogonadism (primary testicular failure). Causes of primary testicular failure include Klinefelter's syndrome, Noonan syndrome, and poorly functioning testes (i.e., cryptorchidism, atrophy, or torsion). Obstructive azoospermia typically presents with normal testosterone and FSH/LH levels. When serum testosterone levels are low and gonadotropins are also either low or "inappropriately normal," hypogonadotropic hypogonadism (secondary testicular failure) may be the cause. Elevated prolactin levels, hemochromatosis, opioid use, pituitary/hypothalamic damage, or genetic conditions (i.e., Kallman's syndrome) are associated with hypogonadotropic hypogonadism.

If hypogonadotropic hypogonadism is diagnosed or symptoms suggest prolactinoma, then obtain a serum prolactin. Due to significant physiologic variability, serum prolactin levels should be repeated if initially abnormal. Slight elevations of serum prolactin (<50 ng/ml) can be associated with stress, kidney disease, certain medications, or be idiopathic in nature. However, if the serum prolactin remains abnormally elevated or is extremely high, then magnetic resonance imaging (MRI) should be performed to assess for a pituitary tumor.

Obese men have increased activity of the hormone aromatase, an enzyme found in adipose that converts testosterone to estrogen. An overabundance of estrogen is associated with diminished libido, gynecomastia erectile dysfunction, and hypogonadism. Evaluation of estrogen should be performed in obese infertile men or those with symptoms suggesting hyperestrogenism.

3.9.7 Genetic Evaluation

Genetic testing is a key component of the male infertility evaluation. It provides necessary information to establish causes of infertility, identify clinically significant medical comorbid conditions, and assess the likelihood of success of certain fertility treatment options (i.e., microdissection testicular sperm extraction or varicocele repair). Furthermore, it allows the clinician to counsel partners on the potential risk of transmitting genetic conditions to future progeny. The most common genetic causes of male infertility are cystic fibrosis transmembrane conductance regulator (CFTR) gene mutations, Y-chromosome microdeletions, and chromosomal

Table 3.4 Frequency of karyotype abnormalities in infertile men

Azoospermia	10%–15%
Oligozoospermia	5%
Normal	<1%
All infertile men	*7%*

anomalies. The CFTR gene is located on chromosome 7 and is associated with cystic fibrosis (CF), CBAVD, and unilateral absences of the vas. Almost all men with CF have a CFTR mutation and CBAVD. However, not all CFTR mutations are currently detected, and it is best to assume that men with CBAVD have a CFTR mutation and may be carriers of CF. It is thus imperative to assess the female partner for CFTR carrier status prior to proceeding with ART.

Karyotype evaluation and Y-chromosome microdeletions are frequently utilized genetic tests to evaluate patients with severe oligozoospermia (<5 million/ml) or nonobstructive azoospermia (NOA). A numeric or structural chromosomal abnormality is identified in roughly 7% of infertile men, and the degree of infertility inversely correlates with the presence of chromosomal abnormalities (see Table 3.4). Klinefelter's syndrome is the most common chromosomal abnormality in infertile men and accounts for over 60% of chromosomal abnormalities identified in workup of infertility. Couples should be counseled regarding the potential for paternal transmission of chromosomal abnormalities as it can result in miscarriage, birth defects, and other genetic syndromes.

Y-chromosome microdeletions are identified in roughly 10%–15% of males with severe oligozoospermia or NOA [71, 72]. Typically, microdeletions occur in the AZF regions (AZFa, AZFb, and AZFc) on the long arm of the Y-chromosome (the site of several genes required for normal spermatogenesis). A deletion within the Y-chromosome does not necessarily result in infertility, but sperm retrieval attempts are rarely successful with an AZFa or AZFb deletion. However, sperm retrieval rates are as high as 80% in patients with an isolated AZFc deletion. It is unclear if there are any medical conditions associated with Y-chromosome microdeletions, but all of the male progeny will inherit the abnormality, and appropriate genetic counseling is recommended.

3.9.8 Conclusion

The laboratory evaluation of male infertility extends far beyond a basic semen analysis. The clinician is tasked in identifying and treating reversible conditions that may improve the male's fertility or even potentially detect harmful underlying conditions. The fertility evaluation must be complemented by a thorough history and physical examination with an understanding of genetic and endocrinological conditions which cause male infertility. Advances in sperm function tests have been developed to provide earlier and more individualized treatment for couples [73, 74]. Ongoing investigation in the field of proteomics will continue to aid in the development of more successful treatment options and provide more answers for couples experiencing infertility.

3.10 Review Criteria

We extensively searched Google Scholar, PubMed, Medline, Clinical Key, and Science Direct for articles focusing on semen analyses, male infertility, advanced sperm testing, hormonal, and genetic assessment. We began our literature search in September 2018 and completed it by November 2018. The following key words were utilized in our search: "semen analysis," "sperm DNA fragmentation," "oxidative stress," "WHO manual," "TUNEL," "oxidative-reduction potential," "computer-assisted semen analysis." We reviewed only English language articles. Tables were created with assistance from Microsoft Excel.

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Imaging Modalities in the Management of Male Infertility

4

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Key Points

- Testicular MR spectroscopy is promising for the assessment of metabolic integrity and differentiation between normal and markedly decreased spermatogenesis testicles, with further clinical studies necessary to assess its full diagnostic and therapeutic capability.
- Microsurgical technique remains the gold-standard procedure for the varicocele repair, but the concomitant use of intraoperative Doppler should be seriously considered as a tool to improve surgical outcome and safety.
- Microsurgical organ-sparing resection with micro-TESE and tissue cryopreservation may be considered for patients presenting with azoospermia and incidental testicular lesions, especially for those with solitary testicles and bilateral tumors.
- Annual ultrasound follow-up is recommended for testicular microlithiasis, when accompanied by other potentially premalignant features. Patients with testicular dysgenesis syndrome features, such as infertility, may be in higher risk of intratubular germ cell neoplasia.

4.1 Introduction

Infertility affects approximately 15% of couples desiring conception, and male infertility underlies almost half of the cases. Assisted reproductive technology (ART) is increasingly being used to overcome multiple sperm deficiencies and because of its effectiveness it has been suggested by some to represent the treatment for all cases of male factor infertility regardless of etiology. Although the use of these technologies may allow infertile couples to achieve pregnancy rapidly, associated higher cost, potential safety issues, and the fear of transferring the unnecessary burden of invasive treatment on healthy female partners weigh down this treatment option heavily.

Diagnostic imaging techniques may be indicated as part of the complete male fertility evaluation. Reproductive therapy can be instituted only after completion of a thorough evaluation that begins with a detailed history and physical examination. Due to the introduction and enhancement of newer imaging modalities, reliable adjuncts to clinical examination can be obtained to diagnose a variety of causes of male infertility including varicocele, epididymal blockage, testicular microlithiasis (TM), seminal vesicle (SV) agenesis, and ejaculatory obstruction. Imaging plays a key role in the evaluation of the hypospermia or azoospermic man. It can detect correctable abnormalities, which can lead to a successful conception. It can also reveal potentially life-threatening disorders in the course of an infertility evaluation such as testicular tumors. The goal of this article is to provide the reader with a foundation for a comprehensive evaluation of the male partner and emerging technologies that can improve the treatment of correctable causes of male infertility.

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4.2 Testicular Tissue Imaging for Guided Sperm Retrieval

4.2.1 Doppler Duplex Flow Imaging

Testis biopsy with cryopreservation of sperm is a procedure performed in men with possible nonobstructive azoospermia (NOA), and at times, in men with a previous vasectomy (who do not want a reversal) and men with spinal cord injuries (who fail electro-ejaculation or vibratory ejaculation). Recent studies have illustrated that it is likely to find active spermatogenesis in areas with good blood supply within the testicle.

Nowroozi et al. [1] also performed a prospective study in 130 patients with azoospermia, both NOA and obstructive azoospermia (OA). OA patients had previous history of vasectomy and failed vasovasostomy or vasoepididymostomy (21 out of 24 patients), 4 patients had obstruction due to recurrent UTI, 1 patient had a known congenital bilateral absence of vas deferens, and 1 patient had a prostatic cyst with failed surgical treatment. All patients underwent ultrasonography with Doppler before surgery, with location and number of vessels recorded and testicular artery resistive index (RI) registered. Testicular parenchyma was mapped in upper, middle, and lower segments and according to visible vessels at Doppler: grade 1 – no visible vessels, grade 2 – 1 to 3 visible vessels, and grade 3 – more than 3 visible vessels.

All patients with OA had successful fine needle aspiration. Only 4 patients with NOA had successful aspiration, and all of them had grade 3 perfusion. A micro-TESE was performed for all other patients with NOA.

Patients with OA revealed a significantly higher intratesticular perfusion than NOA, and there was a correlation between visible vessels on Doppler and probability of sperm retrieval success. Among patients with grade 3 testicular perfusion, 60% required less than 3 biopsies, while 82% of the patients with grade 1 perfusion required more than 3 biopsies. Differences were statistically significant. No acceptable cutoff value of FSH or testicular artery RI through ROC curve analysis was able to allow discrimination between NOA or OA with these isolated parameters. Doppler-guided sperm retrieval would be suitable for increasing probability of success and decreasing the number of required biopsies in patients with NOA.

Evaluation of handheld Doppler phase shift flow mapping of the testicle at the time of biopsy appears to have promise in detecting areas of spermatogenesis. However, further data analysis on more patients has now shown that the predictive value was not as high as we initially had expected, and so there remains more work that has to be done in this area before conclusions can be made about the use of this technology for this application.

4.2.2 MRI Spectral Imaging

Patients with idiopathic oligozoospermia or azoospermia, especially those with normal serum gonadotrophins and physical examination, always present a diagnostic dilemma. Both situations can represent a ductal obstruction or a testicular failure, but they have completely different prognoses. Testicular functions are currently evaluated in rather indirect ways, by seminal parameters and hormonal assays. Histological analysis, which involves obtaining specimens by biopsies or surgical explorations, can directly evaluate testicular tissue. However, it cannot be widely used in clinical situations due to possible damage to testicular functions and its invasive character.

Eliveld et al. [2] performed a recent meta-analysis of 15 studies regarding TESE-induced hypogonadism. The authors highlight transient, although significantly decreased, total testosterone. Patients usually recover to baseline levels from 18 to 26 months. Unfortunately, in terms of hypogonadism symptoms, available data is limited.

Nevertheless, heterogeneity of testicular tissue also limits broad indications for diagnostic biopsies, because aleatory tissue samples may not reflect the total parenchyma. Chances of sperm retrieval in a following procedure may only be estimated. Ideally, noninvasive techniques for testicular functions in vivo would allow better evaluation without inconvenience of procedures and potentially guide treatments [3].

Ultrasound is the initial radiological method that is used to evaluate the testis. However, the increased availability of magnetic resonance imaging (MRI) has allowed this noninvasive diagnostic tool to further evaluate testicular function. This technique was already used in a number of experimental studies on testis [3, 4]. On humans, there are few magnetic resonance spectroscopy (MRS) reports; one describes its application on a patient with testicular non-Hodgkin's lymphoma to monitor response to irradiation [5], and the other reveals the in vivo tissue characterization of the testis in patients with carcinoma in situ [6]. Also, differentiation between normal healthy testes and those with markedly decreased spermatogenesis presenting with oligozoospermia or azoospermia in whom spermatogenesis is completely absent was achieved [7, 8].

MRI spectroscopy is a noninvasive technique for obtaining metabolic information from living tissue based upon differences in the ratio of peaks of lipid and choline levels [9]. These metabolites may be used to evaluate the state of fertility and to investigate ischemia–reperfusion disorders.

More recently, in vivo hydrogen MR spectroscopy using stimulated echo acquisition mode measurements was performed with a short echo time, improving the detection of signals from low-molecular-weight metabolites including glutamate, choline, creatinine, and glycine not only in the

normal state but also in diseased conditions such as ischemia [10]. In addition to these metabolites, a lactate signal could be observed in the ischemic testis. The presence of a lactate signal in the H spectra could be utilized to distinguish between normal and ischemic testes [10].

Other recent studies describe MRS for spermatogenesis identification. Aaronson et al. [11] observed significantly lower concentrations of phosphocholine in patients with Sertoli-cell only samples in caparison with fertile patients. Phosphocoline is enrolled in membrane synthesis and may be a marker for active cell proliferation.

Storey et al. [12] performed a study with a quantitative ^{1}H-MRS at 3T, a proton spectroscopy, to compare testicular metabolite concentrations between infertile men and fertile controls. The most prominent peak of spectra was the choline compounds, in accordance with previous studies. Researches also observed lower concentrations of creatine and myo-inositol. Creatine and phosphocreatine are involved in cellular energy metabolism, crucial in high energy-demanding cells such as spermatozoa. Myo-inositol is related to sperm motility. Heterogeneity and size of the cohort limits direct clinical application before proper validation. Nevertheless, it represents a potential shift toward non-invasive techniques. Patients with higher metabolites concentration would represent the best candidates for surgical sperm retrieval.

MR spectroscopy is a sensitive tool for assessment of testicular metabolic integrity and differentiation of normal testicles from those with markedly decreased spermatogenesis. MRS may improve sperm retrieval rates by better identifying isolated foci of spermatogenesis during testicular sperm retrieval in men presenting with nonobstructive azoospermia. MR spectroscopy of the testis might be a promising new modality that warrants further clinical studies to assess its diagnostic and therapeutic capability.

4.2.3 Testicular Artery Mapping During Varicocelectomy

Current data supports the statement that varicocele repair does indeed have a beneficial effect in reversing the harmful effects of varicocele upon testicular function in selected patients by improving seminal parameters in the majority of controlled studies [13]. A diversity of open surgical techniques has been used to repair this condition, including retroperitoneal, inguinal, and subinguinal. Recently, open microsurgical inguinal or subinguinal varicocelectomy techniques have been shown to result in higher spontaneous pregnancy rates and fewer recurrences and postoperative complications than conventional varicocelectomy techniques in infertile men [14]. The subinguinal approach has the same principles as the inguinal approach but is performed through an incision below the external inguinal obviating to the need to open the aponeurosis of the external oblique causing less postoperative pain.

The microsurgical subinguinal varicocelectomy is the preferred approach for most experts. On the one hand, the use of an operating microscope allows the preservation of the testicular artery and lymphatics vessels, resulting in lower recurrence rates and hydrocele after the procedure [15]. On the other hand, the spermatic cord at the subinguinal level has a greater number of internal spermatic veins and an increased likelihood of encountering multiple spermatic arteries [16]. Previous studies reported that multiple spermatic arteries are identified in approximately 40% of the spermatic cords during microsurgical varicocelectomy at the subinguinal level [16, 17]. The recognition of the main spermatic artery can be confirmed by visualization of clear pulsatile movement and/or evidence of antegrade, pulsatile blood flow with gentle lifting, and partial occlusion of the vessel. However, the identification of tiny secondary arteries is not at all times apparent, and a sterile intraoperative probe attached to a 9.3-MHz VTI surgical Doppler and a disposable probe flow detector (Vascular Technology Inc., USA) (Fig. 4.1) have been used at this point [18]. Consequently, it is possible that an inadvertent unrecognized ligation of a small internal spermatic artery occurs more frequently than reported [19]. Following are some of the reasons that could explain how the injury occurs. First, the size of the arteries may be so small that

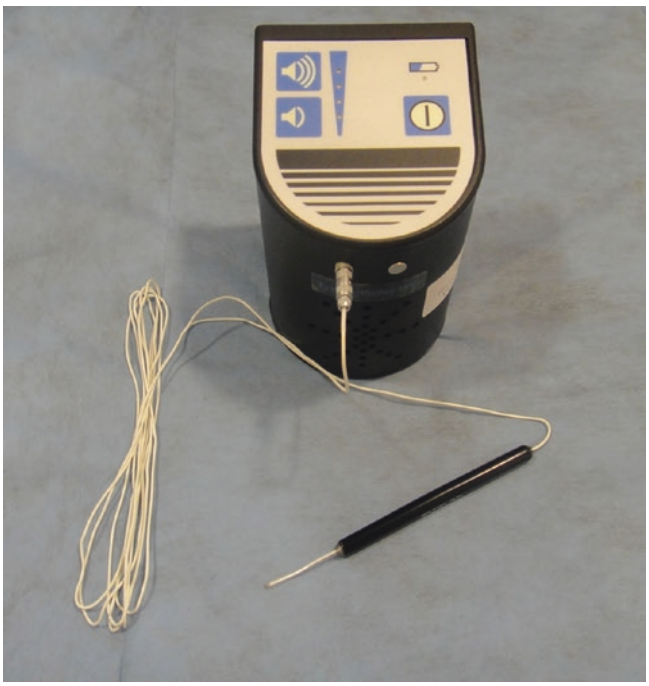

Fig. 4.1 VTI surgical Doppler 9.3 MHz and a disposable probe flow detector (Vascular Technology Int., USA)

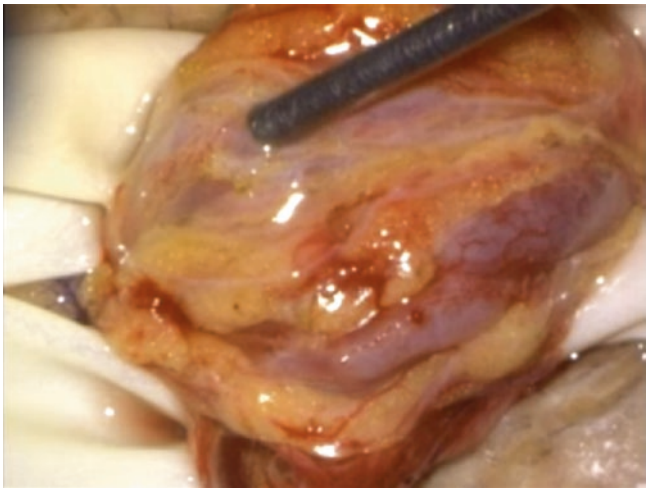

Fig. 4.2 Using a vascular Doppler probe to preserve all testicular arterial branches during varicocele repair

Table 4.1 Intraoperative evaluation of internal spermatic veins ligated, number of lymphatic spared, and arteries preserved and injured in 377 spermatic cord dissections during microsurgical subinguinal varicocele repair with and without vascular Doppler

Variable	With Doppler (no. spermatic cords = 225)	Without Doppler (no. spermatic cords = 152)	*P* value
Number of veins ligated[a]	8.0 (3.1)	7.3 (2.8)	0.02
Number of arteries preserved[a]	1.6 (0.6)	1.3 (0.5)	<0.01
Number of arteries injured[b]	0	2 (1.1%)	0.06
Number of lymphatics spared[a]	2.2 (1.2)	2.0 (1.5)	0.21
Operative time unilateral repair (min)[a]	52.8 ± 17.8	53.0 ± 36.7	0.98
Operative time bilateral repair (min)[a]	101.0 ± 16.2	101.9 ± 16.3	0.37

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[a]Values are mean and SD. Compared using student's unpaired *t* test.
[b]Data presented as number (percentage) of patients. Compared using chi-square test. $P < 0.05$ was considered statistically significant.

the pulsation is difficult to identify. Second, aggressive manipulation of the vessels during dissection can lead to spasm, making it difficult to identify arterial pulsation. Third, the arteries tend to be in close proximity to or buried under complex branches of veins [19]. In all these situations, the use of vascular Doppler may help to preserve the arterial branches (Fig. 4.2). Even though there is no agreement about the necessity to preserve all testicular arterial branches during varicocele surgery [20, 21], not doing so might be responsible for suboptimal improvement in seminal parameters in some cases [22].

A recent study showed that concomitant use of intraoperative vascular Doppler during subinguinal varicocelectomy allows a higher number of arterial branches to be identified and therefore preserved [23]. Data concerning surgery using the Doppler vascular device and without it show that a solitary artery is identified in 45.5% and 69.5% of cords, respectively; 2 arteries are identified in 43.5% and 28.5%, respectively; and 3 or more arteries are identified in 11% and 2%, respectively. Also, the authors reported that a higher number of internal spermatic veins were ligated when Doppler was used (Table 4.1). The use of intraoperative Doppler gives the surgeon more confidence during dissection of a dense complex of adherent veins surrounding the artery present in 95% of cases when the subinguinal approach is used [16]. Accidental artery ligation documented by a pulsatile twitching of the ligated vessel stump under magnification is less common when Doppler is applied [23].

The clinical implication of these findings can be supported by recent studies showing that the total number of veins ligated was significantly positive correlated with improvements in total sperm motility and sperm concentration [24, 25]. These results suggest that ligating a larger number of veins should decrease reflux, which in turn would lead to diminished insult to spermatogenesis.

More recently, Guo et al. [26] conducted a prospective randomized study comparing outcomes for microscopic subinguinal varicocelectomy with and without the use of Doppler ultrasound. A total of 172 patients were strictly selected and randomized in two groups, that is, simple microscopic varicocelectomy and intraoperative vascular Doppler ultrasound-assisted microscopic varicocelectomy, and each group was designated to a single surgeon. Procedures were performed in the same hospital and with the same technique, by surgeons trained in microsurgery and male infertility. Groups were different only in the systematic use of intraoperative Doppler to identify all vessels before ligation in the Doppler-assisted group. The Doppler-assisted group had a statistically higher number of internal spermatic veins ligated and higher number of internal spermatic arteries preserved during surgery. Authors attribute the reduction of operative time to rapid and precise identification of vessels with the use of intraoperative Doppler. No testicular atrophy was described. Results showed improvements in semen parameters, from the third month of follow-up, and were durable at 6 and 12 months, with significantly higher sperm motility in the Doppler-assisted group. Probably due to sample size limitation, pregnancy rates were not different between the two groups compared in the study.

4.3 Management of Testicular Lesions in Infertile Patients

4.3.1 Organ-Sparing Microsurgical Resection of Testicular Tumors in Infertile Patients

Epidemiologic studies have given attention to a worldwide possible increase in testicular cancer incidence in the last two decades, particularly in industrialized, developed countries [27]. One of the possible explanations for this augmented detection of testicular lesions is the widespread use of ultrasound as a screening method in all fields of medical practice, including scrotal ultrasonography in urology [28, 29].

In patients presenting with bilateral tumors (Fig. 4.3) or tumors in a solitary testis, the gold-standard procedure is to perform a radical orchiectomy which leads to permanent sterility, lifelong dependence on androgen replacement therapy, and psychological problems of castration at a young age [30]. As a result, organ-sparing surgery has been reported as a safe procedure in selected patients, especially for infertile men desiring to preserve their fertility [31–33]. The German Testicular Cancer Study Group established a guideline for organ-sparing surgery for testis tumors that includes cold ischemia during spermatic cord clamping, restriction of the procedure for organ-confined tumors of less than 20 mm that do not infiltrate the rete testis, performance of multiple biopsies of the tumor bed, and application of adjuvant local radiotherapy to eradicate carcinoma in situ and avoid local recurrence [33].

Testicular germ cell tumors (TGCTs) are the most common type of malignancy during the reproductive age [34, 35]. However, as the majority of incidental testicular nonpalpable lesions with negative markers diagnosed in scrotal ultrasonography performed during andrology investigation show a benign histology, surgical approach must be as conservative as possible for the testicular parenchyma [36]. In such cases, the most important step is the confirmation that frozen section analysis is an oncologically useful method for assessing small incidental testicular tumors when performed by an experienced pathologist [37]. The high degree of oncological efficiency achieved by the frozen section analysis during resection of testicular masses supports organ-sparing approaches that reduce the chances of facing difficult decisions intraoperatively [38].

Due to the advances of microsurgical techniques, partial orchiectomy was appointed as first-line therapy in infertile men even for nonpalpable small testicular lesions [31, 39, 40]. Also, infertile patient presenting with azoospermia and incidental testicular lesions now can experience the chance to father their own genetic offspring [37]. The combination of organ-sparing surgery, microdissection for TESE, cryopreservation, and assisted reproductive technologies represents a powerful tool to preserve fertility even for azoospermic men [41]. The complete procedure was meticulously described by Hallak et al. as follows [39]: During the procedure, the testis can be delivered through the inguinal incision, respecting principles for oncological procedures to avoid any potential spillage of tumor cells. The vas deferens must be carefully isolated from the spermatic cord and blood circulation interrupted by a delicate vascular clamp placed across the spermatic cord (Fig. 4.4). Slugged ice may be used to prevent warm ischemia, and a temperature probe inserted far from the tumor location controls the temperature at 12–15 °C (Figs. 4.5 and 4.6). A linear ultrasound transducer at 15 MHz guides real-time intraoperative placement of a 30-gauge 10-cm-long stereotaxic hook-shaped needle (Guiding-Marker System; Hakko, Tokyo, Japan) adjacent to the tumor to guide microsurgical resection (Figs. 4.7 and 4.8). Using a surgical

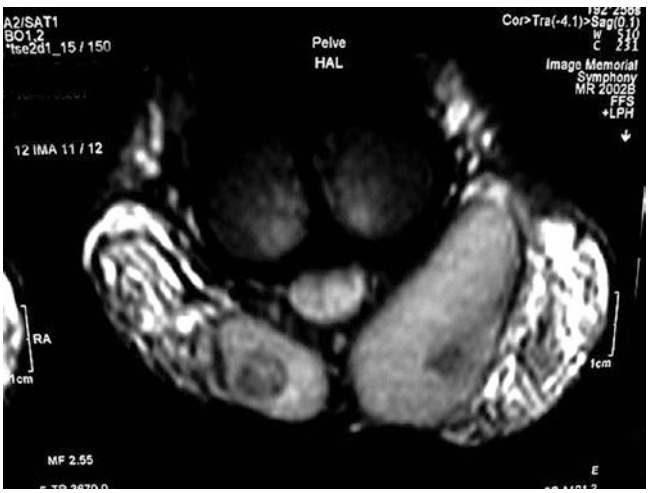

Fig. 4.3 Magnetic resonance showing a solid bilateral testicular lesion

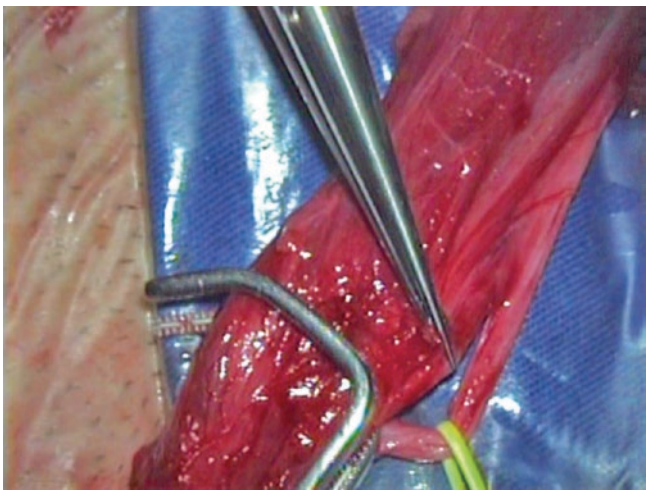

Fig. 4.4 Blood circulation was interrupted by a delicate vascular clamp placed across the spermatic cord after the vas deferens was carefully isolated

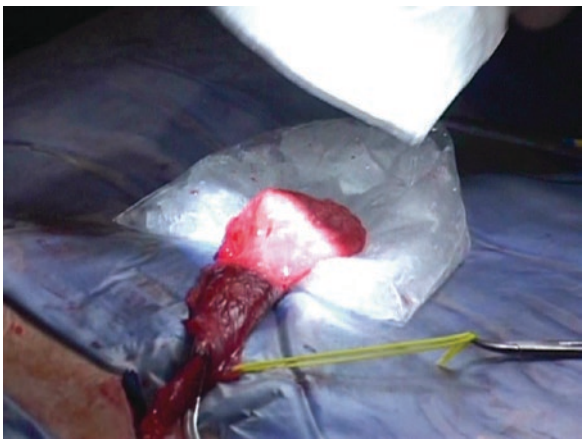

Fig. 4.5 Slugged ice wrapped the testicle, thus preventing from warm ischemia

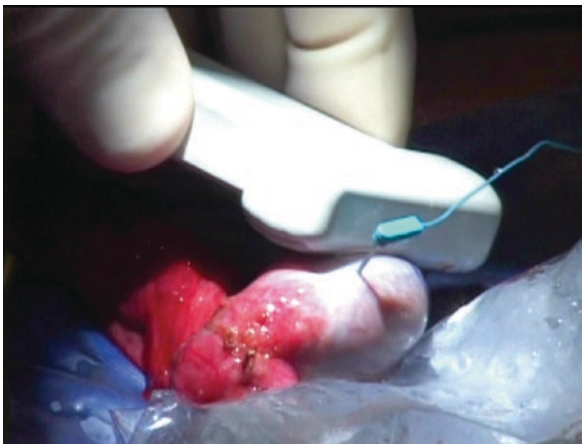

Fig. 4.6 A temperature probe was inserted far from the tumor location in the upper pole of the testicle

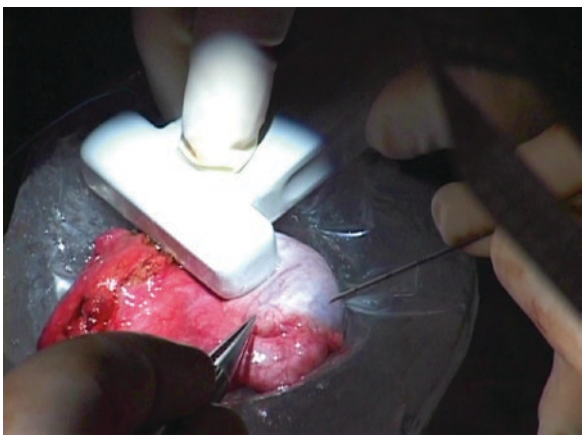

Fig. 4.7 Linear ultrasound transducer at 15-MHz guides real-time intraoperative placement of the stereotaxic hook-shaped needle adjacent to the nodule. The 30-gauge stereotaxic hook-shaped needle that permits the hook to be completely contained in and pass through the needle lumen. The introducing needle can thus be optimally positioned prior to engaging the hook. The hook is ejected and released from the needle tip reforming to the hook and anchoring into the tissue adjacent to the tumor

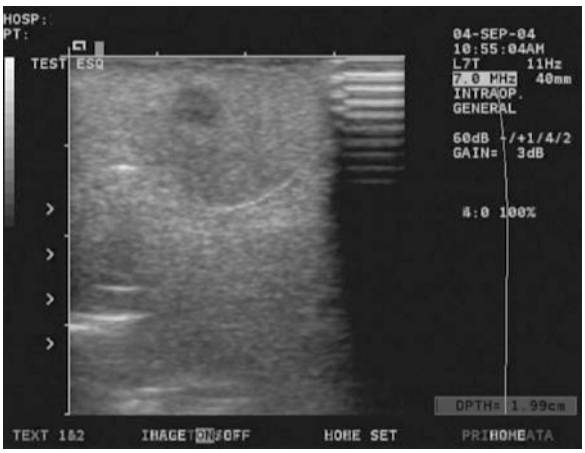

Fig. 4.8 Intraoperative ultrasound image showing a nonpalpable hypoechoic intratesticular lesion to guide real-time needle placement

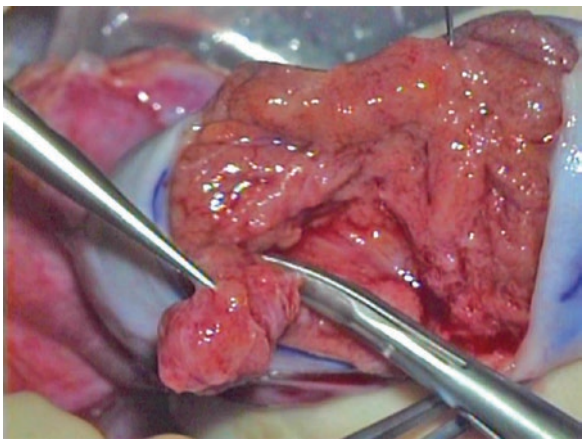

Fig. 4.9 After the tunica albuginea is incised in an avascular region using an operating microscope, dissection respecting testicular lobules and arteries is conducted. Seminiferous tubules were separated carefully by blunt dissection following the needle until the lesion that is excised using micro-instruments is found, leaving 2–3-mm borders as safe margins around the nodule

operating microscope, the tumor can be gently dissected and removed along with the adjoining parenchymal tissue (Fig. 4.9). Frozen section studies must be performed, and if malignancy is confirmed, then biopsies of the tumor cavity margins and the remaining parenchyma must be obtained to ensure the absence of residual tumors. After biopsies are sent for frozen section, the testicular parenchyma must be meticulously microdissected for identification of functioning seminiferous tubules, as reported by Schlegel [41]. After excision of selected enlarged and opaque tubules, viable spermatozoa can be retrieved for cryopreservation in 80% of cases [39]. This procedure integrates modern skills accumulated in the field of male infertility, combining knowledge in testicular vascular anatomy, oncology, microsurgery, organ preservation, tissue preparation, and sperm cryopreservation.

Recently, De Stefani et al. [42] presented a retrospective series of 20 patients submitted to microsurgical testis-sparing surgery for small testicular masses. The authors report the procedures using a similar technique of sperm retrieval for infertility, as previously described, also with the use of a guided needle for tumor identification and frozen section examination. Using the infertility approach to the testis, researchers were able to preserve testicular parenchyma and avoid areas of increased vascularity. Intraoperative ultrasound was used for smaller lesions difficult to locate even with microscopic enhancement. No postoperative clinical hypogonadism was identified, although the series was retrospective. Authors emphasize the importance of an experienced pathologist and a relatively high-volume center for adequate approach.

The possibility of intracytoplasmic sperm injection (ICSI) with cryopreserved testicular sperm has given infertile azoospermic men the chance to have their own genetic offspring [43]. As previously highlighted, it is very important to keep in mind that this approach is only appropriate in centers experienced in managing testicular cancer for patients who want to preserve fertility.

4.3.2 Testicular Microlithiasis

Testicular microlithiasis (TM) is an entity of unknown etiology that results in the formation of intratubular calcifications. TM is detected in 0.6% of adult males with clinical indications for scrotal ultrasonography [44]. Recently, microlithiasis, although uncommon, has been considered a feature of the testicular dysgenesis syndrome. This poorly defined entity is based on the assumption of a testicular dysgenesis during fetal development [45]. A range of testicular and male reproductive problems may arise or be associated with microlithiasis, such as Klinefelter's syndrome, cryptorchidism, varicocele, testicular atrophy, torsion, tumors, and infertility [46–49]. The strength of the association of microlitiasis with any of these entities has been difficult to precisely determine. In terms of infertility, the exact mechanism by which microlithiasis affects spermatogenesis is unknown [49]. Scrotal ultrasound is diagnostic, and typically, there are small echogenic foci (1–3 mm).

The clinical significance of TM is debatable because inconsistencies exist in the literature. Therefore, there are conflicting recommendations regarding the appropriate interval and duration of subsequent surveillance of patients with testicular microlithiasis, or even if there is a definitive association with TM and cancer [50]. Nevertheless, it is a general consensus that patients with microlithiasis and testicular dysgenesis syndrome features, such as infertility, may be in higher risk of intratubular germ cell neoplasia of unclassified type (ITGCNU) [45]. Therefore, follow-up at this time should be dictated based on risk factors for developing testis cancer more than on the presence of TM [46]. Casteren et al. suggested taking a testicular biopsy in a selected patient population with at least one additional risk factor for testicular germ cell tumors [51]. This would be the case for patients in particularly high risk of testicular germ cell tumor (TGCT): gonadal dysgenesis and cryptorchidism. For these patients, an early biopsy at age 18 would be beneficial for early diagnosis [45]. Finally, there is no definitive proof that TM by itself can cause infertility. Probably, decreased seminal function is not directly due to the TM but rather to an underlying testicular abnormality or associated condition such as cryptorchidism [52].

4.3.3 Seminal Vesicle and Ejaculatory Duct Imaging

Seminal vesicles and vas deferens are important and essential urogenital organs. They share close embryological relationship with the kidneys and ureters. Although both agenesia and hypoplasia are rare, unilateral seminal vesicle and vas deferens agenesis, as well as seminal vesicles cysts, may correlate with unilateral kidney agenesis, and bilateral seminal vesicle agenesis may correlate with cystic fibrosis diagnosis. On the one hand, congenital bilateral agenesis of vas deferens is usually associated with seminal vesical aplasia. Ejaculatory duct obstruction (EDO), on the other hand, usually associates with dilated seminal vesicles [53].

Ultrasonography, computed tomography, and magnetic resonance are imaging modalities that have been used to evaluate these structures. Knowledge of embryology and anatomy of these structures is essential for correct interpretation [54].

MRI has the advantage of allowing visualization of the intra-abdominal segment of the vas deferens. A tubular structure of low intensity in both T1 and T2 may be traced from the internal ring towards the ejaculatory duct topography, where a small portion of intraluminal fluid may be detected [55]. Seminal vesicles demonstrate low signal intensity on T1-weighted images and high signal on T2-weighted images, been identified as elongated structures with thin septa and fluid-filled.

Apart from identifying the presence and anatomy elucidation of seminal vesicles, MRI is also useful to identify other entities, such as congenitally acquired cysts (Müllerian cysts, paramedian prostatic – wolffian – cysts), vesiculitis, and primary and secondary neoplasms [54].

Chen et al. [56] performed a prospective study in 30 patients with agenesis or hypoplasia or seminal vesicles, submitted to both TRUS and MRI. Congenital defects of the seminal vesicles were diagnosed in 22/30 patients through both imaging methods, with a concordance rate of 73.3%. The authors suggest that an inconclusive TRUS should motivate an MRI study. In contrast, Chiang et al. [55] also performed a prospective

study with both CUAVD and CBAVD. Sensitivity and specificity values of TRUS compared with MRI were 0.62 and 0.25, respectively. The authors highlight the disagreements of study data with previous publications.

Ejaculatory duct obstruction is usually suspected in patients with low ejaculate volume (<1 ml) and acidic pH semen, when vas deferens is palpable bilaterally. The use of TRUS facilitates the diagnosis, when there is dilation of seminal vesicle (>1.5 cm internal section diameter), dilatation of vassal ampullae, or midline prostatic cysts [53].

Ejaculatory duct has also been studied with the use of MRI. For improvement of anatomy precision, an endorectal coil has been used. Although it allows useful images, MRI carries limitations regarding the presence of patient's hardware or devices. Nevertheless, evidence comparing MRI and TRUS for diagnosis of ejaculatory duct abnormalities shows controversial results [57]. Engin et al. [58], comparing both techniques, reached a similar conclusion of seminal vesicle imaging: TRUS is a cheaper and more accessible tool, with MRI probably more precisely indicated when initial TRUS is inconclusive.

Turek et al. presented a technique of transrectal vasodynamics (pressure-flow study of the seminal vesicle) to assess for partial ejaculatory duct obstruction (EDO). We have been exploring the use of a 3D transrectal ultrasound imaging and needle-targeting system to perform vasodynamics for the treatment of necrospermia with partial EDO (Target Scan Touch, Envisioneering, St. Louis, MO).

A patient with necrospermia (viability staining of sperm) and low ejaculatory volume (<0.5 cc) was evaluated for EDO by vasodynamics performed utilizing a 3D transrectal ultrasound needle guidance system (Target Scan™, Envisioneering) in January 2008. This system was utilized to access and maintain a flexible needle within the seminal vesicle (SV) during vasodynamics. The decision to perform transurethral (TUR) unroofing of the ejaculatory duct would be based on the pressure readings obtained during vasodynamics.

The patient had no visible left SV and a dilated right SV (>1.5 cm). The system provided an easy stable-guided platform to place and maintain a flexible needle within the right SV. A pressure reading of 41 cm H_2O was achieved with delayed emptying of the right SV (on simultaneous cystoscopic examination of the ejaculatory ducts within the urethra). Transurethral (TUR) unroofing of the right ejaculatory duct was performed, and the post-TUR SV pressure reading dropped to 31 cm H_2O. The right SV drained promptly post-TUR on cystoscopic examination. Postoperatively, the patient initially had retrograde ejaculation. At 1 year post-op, the patient now has an ejaculate volume of 1 cc with 70 million sperm/hpf with 64% motility.

The use of a 3D transrectal ultrasound needle guidance system to perform vasodynamics is feasible for the treatment of patients with necrospermia and partial EDO, and it enhances a surgeon's ease in performing vasodynamics. Further testing and evaluation are needed.

4.4 Conclusion

The objective of this chapter was to discuss the potential role of imaging modalities in the management of male infertility. The advanced technologies discussed may not only provide new treatment options but also reduce the number of couples without a definitive diagnosis of the cause of failure to conceive. There is no hesitation that all technical advances such as those explained in this chapter will drive the development of pioneering approaches to the management of the infertile male by andrologists.

On the one hand, imaging may help to diagnose reversible causes of infertility and treat them to achieve seminal improvement and pregnancy. On the other hand, the use of imaging in the field of infertility is no longer just for diagnosis but also as part of the arsenal that provides more precise surgery procedures as described above.

Collaborations with radiologists have provided a rich opportunity to explore and expand imaging techniques for intraoperative use during urologic surgery. Due to the continual improvement of a variety of imaging and tissue characterization modalities, surgeons will have an increasing number of tools at their disposal to improve intraoperative surgical decision-making. Only through the application of evidence-based assessment and evaluation, however, will there be a firm understanding of the true impact of these new technologies on the field of urology. However, further studies will be needed to confirm whether or not these techniques can evolve into widespread clinical practice.

4.5 Review Criteria

The current chapter is based on an electronic search using Pubmed/MEDLINE database and references of the identified articles performed between November 2018 and January 2019. The following key words were used on the search engines: "imaging in male fertility," "Doppler flow imaging," "ultrasound," "ejaculatory duct imaging," "testicular microlithiasis," and "MRI spectral imaging."

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Endocrinopathies

5

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Key Points

- Hypogonadotropic hypogonadism, characterized by deficient production of gonadotropins (FSH and LH), results in low testosterone levels and can arise due to either congenital or acquired causes.
- Males with Klinefelter syndrome have a 47, XXY karyotype and also commonly have hypergonadotropic hypogonadism with impaired or absent spermatogenesis.
- Androgen excess, which commonly arises through the use of prescription exogenous testosterone therapy or illicit use of anabolic steroids, suppresses intratesticular testosterone production which typically results in partial or complete suppression of spermatogenesis.
- Estrogen excess, which is commonly associated with obesity, results from the conversion of testosterone to estradiol by aromatase in adipose tissue and can lead to low testosterone levels.
- Clomiphene citrate and human chorionic gonadotropin (hCG) are both off-label therapies for testosterone deficiency that can support both intratesticular testosterone production and spermatogenesis.

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5.1 Introduction

Spermatogenesis depends on an intricate interplay of hormonal factors both centrally and in the testis. Centrally, the hypothalamus releases gonadotropin-releasing hormone (GnRH), which acts on the anterior pituitary to cause secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). At the level of the testis, FSH acts on Sertoli cells to induce the maturation process in spermatogonia. LH exerts its effect on Leydig cells, stimulating the production of testosterone. Effective spermatogenesis requires local testosterone concentrations to be much higher than serum concentrations. This intratesticular testosterone then acts indirectly to stimulate germ cell maturation through actions on Sertoli cells [1].

Although endocrinopathies only account for a small minority of cases of male infertility, about 1%–2% [2], the treatment of these conditions offers patients a strategy of directed therapy. Broad classification of endocrinopathies involves two main categories, that is, hormonal deficiency and hormonal excess, with specific hormonal abnormalities falling under each of the previously mentioned categorizations.

In this chapter, we provide an overview on diseases in which hormonal imbalance can negatively impact fertility. We discuss the etiology and clinical presentation for each disease. The standard diagnostic process and best management approach will be addressed as well, and future directions for research in this area will be explored.

5.2 Hormonal Deficiency

5.2.1 Hypogonadotropic Hypogonadism

As the name suggests, hypogonadotropic hypogonadism is a state of testosterone deficiency associated with subnormal levels of gonadotropins (FSH and LH). Etiologies of hypogonadotropic hypogonadism can be numerous and are divided into congenital and acquired causes.

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Kallmann syndrome is one identified congenital etiology of hypogonadotropic hypogonadism. Inherited in an X-linked recessive fashion, Kallmann syndrome can arise due to a variety of mutations, the most prevalent of which involves the KAL1 gene. Features include hypogonadism as well as anosmia, facial defects, renal agenesis, and neurologic abnormalities [3]. The hypogonadism and associated clinical sequelae (delayed puberty, infertility) result from a failure of migration of GnRH-secreting neurons. This failure of migration leads to the absence of GnRH secretion which in turn leads to the absence of LH and FSH secretion [2].

Hypogonadotropic hypogonadism can also be acquired, as in the case of pituitary insufficiency resulting from pituitary tumors, surgery, infarct, or infiltrative disease. Regardless of the myriad etiologies of hypogonadotropic hypogonadism, the underlying disturbance is low gonadotropin levels, and treatment can be affected through pharmacologic replacement.

Treating hypogonadotropic hypogonadism involves replacement of the deficient hormones through gonadotropin therapy. Agents used in this therapy include human chorionic gonadotropin (hCG), human menopausal gonadotropin (hMG), and recombinant follicle-stimulating hormone (rFSH). Human chorionic gonadotropin use stems from its properties as an LH analogue, acting at the Leydig cell to stimulate androgen secretion. Human menopausal gonadotropin is a product purified from the urine of postmenopausal women that contains both LH and FSH. Regimens of gonadotropin therapy for men with hypogonadotropic hypogonadism typically begin with hCG administration alone for 3–6 months. Dosages range from 1000 to 1500 USP units either IM or SC three times per week. Adequacy of therapy can be assessed by measuring serum testosterone levels, with the goal of achieving sustained normal levels. Although the pertinent goal for spermatogenesis is adequate intratesticular testosterone concentrations, this value is not normally assessed in gonadotropin replacement therapy. However, intratesticular testosterone levels show linear correlation with administered hCG dosage [4]. After titration to sustained normal testosterone levels, usually after 3–6 months of hCG monotherapy, therapy is initiated to replace FSH levels. One method of FSH replacement involves hMG given at doses of 75–150 IU IM/SC three times a week at a separate injection site. Alternatively, rFSH can be used at dosages of 150 IU SC three times a week [5]. Relative efficacy of hMG versus rFSH has been studied to some extent in women undergoing IVF, but comparisons in male patients are lacking. Replacing gonadotropins in this manner has shown promising results as more than 90% of treated males experience spermatogenesis [2]. The time to spermatogenesis can be quite variable, with the average response occurring in about 6–9 months. However, therapy may be required for up to 1–2 years before a response may occur, and some individuals unfortunately never respond to this modality [6]. An Australian study of 38 men with hypogonadotropic hypogonadism found that median time to first sperm in the ejaculate was 7.1 months, while median time to conception was 28.2 months [7].

While spermatogenesis occurs in a strong majority of patients, sperm concentrations achieved through gonadotropin therapy still sometimes fall below goal ranges (<20 million sperm/mL). Despite this, fertility outcomes with gonadotropin therapy are very good. In a study of 24 men with hypogonadotropic hypogonadism treated with gonadotropin therapy, 22 men achieved pregnancy, even though mean sperm concentration was 16.7 million sperm/mL [8]. A retrospective study of Japanese men found sperm production in 71% of men treated with hCG (3000 IU) and hMG (75IU), provided testicular size was greater than prepubertal sizes (>4 mL) [9]. A recently published Saudi Arabian paper studied 87 infertile men with hypogonadotropic hypogonadism treated with IM gonadotropins for a median of 26 months, with the primary outcome of fertility. Overall, 35 of the 87 patients (40%) were able to achieve pregnancy [10].

An important area of newer research focuses on determining predictors of response to gonadotropin therapy. The aforementioned long-term study in Japanese men found a correlation between testicular size pretreatment and response to gonadotropin therapy. Men with testicular size >4 mL had a 71% chance of responding to treatment, whereas men with testicular size <4 mL had only a 36% chance of responding to treatment [9]. In addition, the above Saudi Arabian study found that only pretreatment testicular size was predictive of conception. In particular, responders to treatment had a mean testicular pretreatment volume of 9.0 ± 3.6 mL, while the pretreatment testicular volume of nonresponders was only 5.7 ± 2.0 mL. Interestingly, there was no significant difference in conception rates between men with hypogonadotropic hypogonadism due to congenital or acquired etiologies [10]. Larger baseline testicular size has also been shown as an independent predictor of response time to gonadotropin therapy, and achieving summed testis volume >20 mL after treatment increased the odds of achieving both goal sperm parameters and pregnancy by at least twofold [7]. It is worth noting that the lower sperm concentrations found in these studies, while below traditional goals of infertility management, may allow for pregnancy with adjunctive use of assisted reproductive therapies such as intrauterine insemination or in vitro fertilization. Additionally, such medical treatment may allow increased efficacy of surgical sperm extraction.

Another method of treatment for men with hypogonadotropic hypogonadism involves the use of antiestrogen agents. These agents competitively bind to estrogen receptor sites in the hypothalamus. Normally, estradiol acts via negative feedback at this endocrine center to inhibit gonadotropin secretion.

By binding at these sites, antiestrogen agents block estradiol's feedback inhibition of the hypothalamus and thus increase the hypothalamic secretion of GnRH. The increased secretion of GnRH leads to increased pituitary secretion of gonadotropins, which thereby stimulates an increase in intratesticular testosterone production. The most commonly used agent in this class is clomiphene citrate, but similar agents include tamoxifen, raloxifene, and toremifene. These drugs have been previously studied in the setting of empiric therapy for idiopathic infertility with mixed results [6]. However, the directed use of clomiphene in patients with proven hypogonadotropic hypogonadism has shown to be useful in limited settings. An American study treated four men with hypogonadotropic hypogonadism with clomiphene citrate 50 mg three times a week and found improved testosterone levels and semen parameters in three of these patients. Subsequently, two of these three men achieved documented pregnancy [11]. Similar success at the biochemical level has also been described in case reports, although fertility was not a goal of these treatments [12, 13]. A recent retrospective study of 31 men compared clomiphene citrate with testosterone therapy. Serum testosterone levels were highest in the group injected with testosterone and lower for the groups that received topical testosterone and clomiphene treatment. Despite these findings, the ADAM questionnaire determined that level of satisfaction was similar in all groups [14]. Clomiphene treatment of male infertility can be associated with such side effects as visual disturbances, GI upset, weight gain, hypertension, and insomnia [6].

Although using testosterone therapy to improve spermatogenesis is not recommended, a significant number of providers still utilize this approach. A recent study found that around one fourth of urologists surveyed have prescribed testosterone in an attempt to enhance spermatogenesis [15]. However, exogenous testosterone actually has detrimental effects on spermatogenesis. Increased levels of exogenous testosterone result in negative feedback to the hypothalamus, leading to lower levels of GnRH, gonadotropin, and ultimately intratesticular testosterone. Previous research indicates that suppression of intratesticular testosterone to levels less than 20 ng/mL can substantially impair normal spermatogenesis [16].

It is worth noting that exogenous GnRH treatment represents another avenue of medical therapy for hypogonadotropic hypogonadism. Synthetic analogues of GnRH can be administered to stimulate secretion of gonadotropins. However, the short half-life of these agents combined with the necessary pulsatile release to recreate normal physiology requires a method of frequent administration, such as frequent injections, nasal sprays, or an implantable pump. These methods are obviously less convenient, and further, studies have not shown this treatment to have a strong benefit for hypogonadotropic hypogonadism [1].

5.2.2 Hypergonadotropic Hypogonadism

In hypergonadotropic hypogonadism, the main perturbation is an inadequate or absent function of the testes. Gonadotropins are appropriately elevated secondary to lack of negative feedback from estradiol, testosterone, and inhibin B from the testis. Without appropriate androgen secretion, spermatogenesis is impaired. These men also typically have significant testicular atrophy with fibrosis and markedly reduced germ cell number, also leading to abnormally low levels of spermatogenesis. Hypergonadotropic hypogonadism can occur as a result of genetic etiologies (e.g., Klinefelter syndrome) or from acquired conditions. Acquired etiologies of hypergonadotropic hypogonadism include destruction of normal gonadal tissue from chemotherapy or radiation, trauma, mumps orchitis, or androgen decline in the aging male. Men with hypergonadotropic hypogonadism not desiring fertility can be treated with exogenous testosterone therapy, but men trying to conceive should generally not be given exogenous testosterone. The treatment for men trying to conceive is less well characterized. Aromatase inhibitors have been suggested as treatment for men with Klinefelter syndrome [4]. A small cohort of patients with Klinefelter syndrome treated with aromatase inhibitors showed hormonal improvements with treatment, although the study did not comment on semen parameters in the Klinefelter subset. In particular, for this subset of patients, testolactone therapy was more efficacious with respect to hormonal levels than anastrozole [17].

It is important to mention the additional potential advantage in the setting of surgical sperm extraction after adjuvant medical therapy in men with Klinefelter syndrome. Surgical sperm extraction alone has resulted in successful retrieval in up to 50% of attempts [18]. Ramasamy et al. retrospectively studied 68 azoospermic men with Klinefelter syndrome. Of these 68 men, 56 were treated for low testosterone levels (<300 ng/dL) with a combination of medical therapies (aromatase inhibitors, hCG, clomiphene) before microdissection TESE. Of the 56 men receiving medical therapy before TESE, 28 received testolactone alone, 12 received testolactone and weekly hCG, 9 received anastrozole alone, 1 received anastrozole and hCG, and 4 received hCG alone. Three patients received clomiphene citrate. While there was no difference among specific agents in terms of successful sperm extraction, these medical regimens collectively resulted in improved sperm retrieval when patients responded to medical therapies with posttreatment testosterone >250 ng/dL. More specifically, successful sperm extraction was seen in 77% of men with posttreatment testosterone >250 ng/dL versus 55% of men with posttreatment testosterone <250 ng/dL [18].

Most commonly, androgen supplementation is initiated when luteinizing hormone levels rise above normal during puberty. Despite the paucity of data, studies in progress may

help provide the foundation for producing evidence-based guidelines on the timeline of androgen supplementation. A recent retrospective study found that infants with Klinefelter syndrome who received testosterone treatment had superior cognitive development at 3 and 6 years old, but the generalizability of these findings is questionable due to the lack of randomization and blinding [19]. To date, no randomized controlled trials of testosterone supplementation in Klinefelter syndrome children have been published, but a study is currently in progress at Children's Hospital Colorado. Another randomized controlled trial on Klinefelter syndrome in early adolescence is in the process of enrolling as well, with the purpose of evaluating the psychosocial impact of topical testosterone [20]. Despite the deficit in data, studies in progress may help provide the foundation for producing evidence-based guidelines on the timeline of androgen supplementation.

The rate of diagnosis of Klinefelter syndrome may increase substantially in the coming years as prenatal testing for the disease may be integrated into routine prenatal care in the near future. This change would lead to an estimated increase in rate of diagnosis by up to five times the current rate [21]. As a result, the higher rate of diagnosis may potentially drive the research community to allocate more energy and resources to studying patient-centered Klinefelter syndrome health and treatment outcomes.

5.2.3 Hypothyroidism

Thyroid hormones are essential in organ development and routine metabolism. However, there have been few studies evaluating hypothyroidism and male reproduction in humans. Substantial research has been performed on rats studying the relationship between hypothyroidism and male fertility. Rats with drug-induced hypothyroidism have been shown to have fewer and smaller seminiferous tubules, lighter testes, less testicular germ cells, and impaired sperm parameters in contrast to their control rat counterparts [22–24].

In humans, hypothyroidism has long been associated with diminished libido and erectile dysfunction [25]. Additionally, a recent study by Meeker et al. revealed a correlation between thyroxine (T4) level and sperm concentration, with higher T4 being correlated with better sperm concentrations [26]. Sperm concentration may not be the only parameter affected, as a study by Krassas et al. showed that men with hypothyroidism have a lower than normal percentage of sperm with normal morphology as well as impaired sperm motility and decreased ejaculate volume. Correcting the hypothyroidism resulted in 76% of the patients having a normal morphology [27]. Overall, there is a relative scarcity of data regarding hypothyroidism and semen parameters. Nonetheless, these studies do suggest a link between thyroid function and spermatogenesis.

5.3 Hormonal Excess

5.3.1 Androgen Excess

Within the hypothalamic–pituitary–testis axis, testosterone exerts negative feedback inhibition on the hypothalamic secretion of GnRH. This effect is indirect and thought to occur via aromatization of testosterone to estradiol. Acting in this manner, excess circulating testosterone can suppress this axis and cause inhibition of spermatogenesis.

Testosterone excess can result from exogenous testosterone administration or from endogenous production.

Therapeutic administration can inadvertently result in testosterone excess, but testosterone excess can also result from the illicit use of anabolic steroids. Regardless of the cause, exogenous androgens typically suppress gonadotropin secretion with resultant decreased levels of intratesticular testosterone and decreased spermatogenesis. Diagnosis is suggested by normal to high serum testosterone levels with suppressed gonadotropins. The first step in treating a male with suspected androgen excess is to remove the exogenous source. Return of spermatogenesis usually occurs within 4 months but in some instances can take up to 3 years [28, 29]. If sperm parameters do not improve adequately or are slow to improve, then some evidence suggests beneficial effects of gonadotropin therapy in improving intratesticular testosterone levels [29, 30]. If response to treatment remains suboptimal after a trial of gonadotropin therapy, then limited evidence suggests a possible use of clomiphene in reestablishing the hypothalamic–pituitary–testis axis [31].

Although abuse of anabolic androgenic steroids has not been a focus in mainstream medicine, a recent review has brought to light the toll this issue has had on the health of young men in America. Retrospective analysis of over 6000 patients revealed that steroid abuse was the etiology in over a third of the patients with profound hypogonadism, and perhaps even more worrisomely, about one fifth of men treated for symptomatic hypogonadism reported previous use of anabolic androgenic steroids [32]. Given the rise in incidence of steroid abuse, growing numbers of psychiatrists accept anabolic androgenic steroid dependence as a diagnostic entity [33]. Counseling patients with anabolic steroid-associated hypogonadism and understanding the patients' motivation for use is crucial for both preventing future use and recognizing other diseases the patient may have such as primary hypogonadism that the provider can safely medically manage.

Androgen excess can also result from endogenous androgen production. The most common endogenous source is congenital adrenal hyperplasia, although functional tumors (adrenal or testicular) and androgen insensitivity syndromes could also be responsible [2]. Although congenital adrenal hyperplasia is more commonly discussed in the context of female fertility, there have been multiple studies linking the

disease to decreased fertility in men [34, 35]. In one of these studies, only two thirds of men with congenital adrenal hyperplasia who attempted pregnancy achieved success [36]. In terms of treatment, numerous strategies have been studied and proven efficacious, including hCG with FSH, clomiphene citrate, and intracytoplasmic sperm injection [37–39].

5.3.2 Estrogen Excess

As mentioned earlier, testosterone's ability to inhibit GnRH secretion at the hypothalamus is mediated through conversion to estrogens. A primary excess of estrogens can act similarly to inhibit the hypothalamic–pituitary–testis axis and thus contribute to decreased fertility. While estrogens are produced in the testis along with testosterone, the main source of estrogens in males is peripheral aromatization of testosterone by the enzyme aromatase, found in adipose tissue. The rising prevalence of obesity in our society puts more men at risk for estrogen excess. In particular, the ratio of testosterone to estradiol (T:E2) appears to be an important measure of estrogen excess, with a goal ratio > 10:1 sought by many clinicians. Pavlovich et al. examined a cohort of infertile men and found significantly reduced T:E2 ratios in the infertile men compared with a fertile control group (6.9 vs. 14.5) [40].

Treatment of relative estrogen excess involves inhibitors of the aromatase enzyme. There are two main classes of aromatase inhibitors: steroidal agents (e.g., testolactone) and nonsteroidal agents (e.g., anastrozole). Both have shown utility in treatment of infertile men with low T:E2 ratios. The above Pavlovich study treated 63 men with male factor infertility and low T:E2 ratios with testolactone, 50–100 mg twice daily. Treatment was effective in improving both T:E2 ratio and sperm quality, as defined by concentrations and motility [40]. A more recent study by Raman and Schlegel treated 140 infertile men with abnormal T:E2 ratios with either testolactone (100–200 mg daily) or anastrozole (1 mg daily). Both treatment arms showed improvement in T:E2 ratio and improved sperm concentration and motility. Further, the study did not show any significant difference between the two classes of aromatase inhibitors in terms of hormonal profile or semen analysis, except in the setting of Klinefelter syndrome, where testolactone was superior in treating the abnormal T:E2 ratios [17]. These studies combined show a clear role for aromatase inhibitors in infertile men with abnormal T:E2 ratios. This treatment strategy may be of particular importance in obese patients [41].

5.3.3 Thyroid Excess

As was touched upon earlier, the role of thyroid hormones in spermatogenesis is not entirely clear. However, hyperthyroidism appears to adversely affect semen parameters. Abalovich et al. found that patients with hyperthyroidism have lower bioavailable testosterone, higher sex-hormone-binding globulin, and higher LH levels compared with controls [42]. Hyperthyroid patients were reported to have markedly impaired semen parameters, including low motility, low ejaculate volume, low sperm concentration, and abnormal morphology. The authors noted that 85% of the seminal abnormalities normalized on semen testing conducted 7–19 months after achievement of euthyroid status. A more recent study also found that hyperthyroidism can impair semen parameters [43]. The authors of this study reported that hyperthyroid patients had significantly lower sperm motility than controls. Motility was improved after euthyroid status was achieved with medical thyroid ablation. Just as with hypothyroidism, there is a scarcity of data regarding hyperthyroidism and spermatogenesis. However, the available studies seem to suggest that hyperthyroidism can adversely affect semen parameters.

5.3.4 Prolactin Excess

Hyperprolactinemia, an excess of the hormone prolactin, is another hormonal etiology of male infertility. The diagnosis is relatively straightforward, as hyperprolactinemia can be detected on routine serum testing, but determination of a particular etiology can be more challenging. Hyperprolactinemia can occur in the case of hypothyroidism, liver disease, stress, use of certain medications (i.e., phenothiazines, tricyclic antidepressants), and with functional pituitary adenomas (prolactinomas). Clinical suspicion must be high for excess prolactin, as the manifestations can range from asymptomatic in many patients to galactorrhea or hypoandrogenic states (i.e., low libido, erectile dysfunction) in affected patients. Patients with pituitary adenomas may also present with bilateral temporal visual field defects. This state, known as bitemporal hemianopsia, is the result of the close anatomic proximity of the pituitary gland to the optic chiasm. Growth of the pituitary tumor compresses the optic nerve, leading to visual field deficits.

Hyperprolactinemia can cause male infertility through its inhibitory effects on the hypothalamus. The high levels of prolactin suppress the secretion of GnRH from the hypothalamus, which subsequently impairs the release of gonadotropins, the production of testosterone, and spermatogenesis. The multiple effects on the hypothalamic–pituitary–testicular axis can result in a patient presenting with multiple problems such as decreased libido, inability to achieve erection, and abnormal semen parameters.

Once the diagnosis of hyperprolactinemia is made, the practitioner should obtain an MRI study focusing on the pituitary gland. If a prolactinoma is found, then it can be

characterized based on its size and appearance. The main differentiation is between microadenomas, lesions <10 mm, and macroadenomas, which are lesions >10 mm. If a prolactinoma is discovered, then medical therapy focuses on blocking the secretion of prolactin through the use of dopamine agonists. Examples of these agents include bromocriptine, cabergoline, pergolide, and quinagolide, with the most well-characterized agents being bromocriptine and cabergoline. These agonists make use of the natural inhibition of prolactin secretion by dopamine. This can actually cause regression of the tumor, although the process generally occurs over months. Possible side effects of dopamine agonists include nausea, vomiting, and postural hypotension. While inhibition of excess prolactin secretion prevents the disruption of the hypothalamic–pituitary axis, there have been few studies specifically elucidating the effects of these dopamine agonists on spermatogenesis and fertility. A 1974 study treated men with functional prolactinomas and hypogonadism with bromocriptine and found no increase in sperm motility [44]. However, more recently, DeRosa and colleagues compared bromocriptine and cabergoline in such patients. Both treatments showed overall improvements in sperm number, motility, rapid progression, and morphology within 6 months of therapy [45]. A subsequent study from the same institution compared seminal fluid parameters between men with prolactinomas and control men. After 24 months of treatment with cabergoline (initial dose 0.5 mg weekly, subsequently titrated to PRL levels), two-thirds of men showed restored gonadal function as compared against healthy control men [46].

Comparing cabergoline and bromocriptine, it appears that cabergoline is more efficacious at normalization of prolactin levels and regressing tumor burden [47]. Further, a higher percentage of patients show a clinical response to cabergoline when compared with bromocriptine. Finally, there is a higher overall rate of permanent remission and fewer side effects with cabergoline compared to bromocriptine [47]. Considering all of these findings, cabergoline is often the first therapy utilized in treating men with prolactinomas.

While treatment of prolactinomas with dopamine agonists can be effective in many cases, a significant percentage of men may still remain persistently hypogonadotropic. Recent research suggests that clomiphene citrate may be an effective treatment for these men. Ribiero and Abucham treated 14 persistently hypogonadal men with clomiphene (50 mg/day for 12 weeks) and noted both improved testosterone levels and sperm motility [48].

Ablative therapy for prolactinomas—in the form of radiation therapy or transsphenoidal resection—is also available. Ablative therapy is typically reserved for those who fail medical management. Ablative treatments remove the source of prolactin and thus the inhibition of GnRH secretion. Measurement of the patient's gonadotropin levels posttreatment remains important, as further intervention with exogenous gonadotropins may be necessary to optimize therapeutic benefit.

Medical treatments for male infertility have traditionally centered on empiric approaches to enhance spermatogenesis. Over the past two decades, improved insight has been gained into the pathophysiology of male infertility and the outcomes associated with empiric therapy. With this insight, more targeted and directed use of medical agents has been described. As such, "empiric therapy" is used with less frequency now than was the case 20 years ago. Many available medical therapies for infertile men are used to optimize the hormonal milieu and thus optimize spermatogenesis. This has indeed been the focus of this chapter. However, numerous other medical agents are used routinely to address other specific pathophysiologic conditions leading to male factor infertility. These agents include antimicrobial drugs, anti-inflammatory medications, and sympathetic agonists. Each of these classes of drugs has clear indications for use in specifically targeted subgroups of infertile men, and they are each described in other specific chapters of this text.

One important point clearly delineated in the literature over recent years is this: empiric medical therapy generally has limited utility and benefit in the treatment of infertile men. While randomized, double-blinded, placebo-controlled studies are costly in terms of time and money, they remain the proving ground for effective medical therapies. More than one agent has failed to pass this test in recent years, but this is a good thing. While the armamentarium of available medical agents for the treatment of male infertility is somewhat limited, this fact should push us to strive harder to gain enhanced insight into the pathophysiological mechanisms leading to decreased male reproductive potential. It is with this enhanced insight into the fundamental problems leading to male infertility that we will develop additional, effective medical therapies.

5.4 Conclusion

Male fertility relies on an intricate hormonal balance and thus a deficiency or excess in a number of hormones can result in impaired fertility. This imbalance can impact fertility by affecting the more central hypothalamic–pituitary axis or by causing dysregulation in the testis where LH and FSH act on Sertoli cells and Leydig cells, respectively, to stimulate maturation of spermatogonia. Despite the low percentage of infertility cases involving endocrinopathies, further research on better understanding the etiology of the mechanism behind infertility is essential as it may lead to the development of more tailored therapies for endocrinopathy-induced infertility. As the rates of metabolic diseases continue to rise

in this country, addressing the impact of hormonal imbalance on male fertility will play an increasingly important role in improving patient quality of life.

5.5 Review Criteria

A meticulous search of studies addressing the topic of endocrinopathies related to male infertility was performed using the search engines PubMed and MEDLINE. The start and end dates for these searches were November 2018 and December 2018, respectively. Manuscripts published in academic meeting proceedings were not included. The overall strategy for study identification and data extraction was based on the following key words: male infertility, spermatogenesis, endocrinopathy, hypogonadotropic hypogonadism, hypergonadotropic hypogonadism, hyperprolactinemia, hyperthyroidism, androgen excess, and estrogen excess.

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Oxidative Stress and Its Association with Male Infertility

6

Ashok Agarwal and Pallav Sengupta

Key Points

- ROS mediate some essential intracellular signaling cascades needed for normal male reproductive functions.
- Excess ROS production leads to redox imbalance and OS that cause oxidative damage to the sperm.
- ROS can affect male reproductive functions through lipid peroxidation, sperm DNA fragmentation, apoptosis of germ cells, and adversely affecting ART media.
- Laboratory assessment of ROS in male infertility includes both indirect methods via lipid peroxidation markers, TAC and seminal ORP, and direct method through chemiluminescence assay.

6.1 Introduction

Infertility is a major global medical and social concern, and male etiology contributes to half of the overall infertility [1]. The mechanisms, by which multivariate causative factors influence male fecundity, are not yet fully understood. The advances in research in the arena of male reproductive science could potentially recognize some of the most deleterious factors attributing to male factor infertility. These include physiological, environmental, as well as genetic and epigenetic factors. At the molecular level, most of these factors interfere with normal male reproductive functioning via the imbalance in reactive oxygen species (ROS) generation and antioxidant capacity, a condition of oxidative stress (OS) [2].

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ROS are the oxygen metabolism derivatives. They present as a set of toxic metabolites, while possessing some vital physiological role in normal male reproductive functions [3]. At normal physiological levels, ROS participate in essential intracellular signaling cascades to ensure proper reproductive functions such as capacitation and hyperactivation of sperms, and acrosome reaction (AR) [3, 4]. On the contrary, the imbalance between ROS and antioxidant levels causes a disparity in the redox balance, which has deleterious effects over the cellular micro- and macromolecules including the carbohydrate components, lipids, proteins, and nucleic acids [5]. Spermatozoa are very vulnerable to OS as they lack proper cell repair machineries and have inadequate antioxidant capacity [6]. These deficiencies in spermatozoa are due to their structural peculiarity possessing diminutive cytoplasmic content. Spermatozoa are highly susceptible to OS-induced lipid peroxidation (LPO) due to high abundance of PUFA in their cell membrane. As a result of LPO, spermatozoa lose their membrane permeability causing efflux of the much-needed ATPs, thereby impairing their own motility with disrupted flagellar movements [7, 8]. OS impairs the structural and functional aspects of sperm including its production, viability, and fertilizing potential [9].

This chapter provides a concise overview by discussing (a) the generation and sources of ROS in the male reproductive tract, (b) physiological role of ROS in male reproductive functions, (c) pathological role of ROS in causing male infertility, and (d) assessment as well as management of OS-induced male infertility. This chapter aims to aid in better understanding of association of ROS with male infertility from physiological and clinical perspectives.

6.2 Biochemistry of Oxidative Stress

Reactive oxygen species (ROS) are generated as a result of physiological cellular metabolism. Biological energy production mostly involves oxidative phosphorylation by the enzymatically regulated reaction of oxygen with other bio-

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molecules within the mitochondria [10]. In the course of these enzymatic reductions of molecular oxygen to produce energy, free radicals are formed [11]. A free radical refers to an oxygen molecule that has a single or several unpaired electron(s) in the molecular orbitals. Superoxide anion ($O_2^{\bullet-}$) radical, the primary ROS molecule, is produced by incorporation of an electron to the molecular O_2. This primary product, either by direct or indirect conversions, is then transformed to secondary ROS products, namely, peroxyl radical ($ROO^{\bullet-}$), hydroxyl radical ($OH^{\bullet-}$), or hydrogen peroxide (H_2O_2) [8]. Among these, H_2O_2 cannot be categorized as a free radical as it is devoid of any unpaired electron. Reactive species derived from nitrogen (reactive nitrogen species or RNS) also have vital physiological and pathological importance. RNS include nitrous oxide (N_2O), peroxynitrite ($ONOO^-$), peroxynitrous acid (HOONO), nitrogen dioxide (•NO2), and dinitrogen trioxides (N_2O_3) etc. [10, 12].

Spermatozoa generates ROS via two mechanisms: (a) in sperm plasma membrane, ROS is be produced by the nicotinamide adenine dinucleotide phosphate (NADP) oxidase system and/or (b) in sperm mitochondria, ROS can be generated via the NAD-dependent redox reaction. Spermatozoa are mitochondria-rich cells owing to their constant requirement of energy for their motility [13]. Increased number of dysfunctional spermatozoa triggers higher ROS production in semen which in turn affects sperm mitochondrial function and motility. As discussed earlier, the leading ROS in human spermatozoa is $O_2^{\bullet-}$ that reacts with itself through dismutation reactions to yield H_2O_2. In presence of transition metals (Fe^{2+} and Cu^{2+}), H_2O_2 and $O_2^{\bullet-}$ can generate the most reactive $OH^{\bullet-}$ via the Haber–Weiss reaction which can initiate a LPO cascade disrupting membrane fluidity and impairing sperm functions [14, 15].

6.3 Sources of ROS in Male Reproductive Tract

6.3.1 Endogenous Sources

6.3.1.1 Leukocytes

The significant percentage of semen specimens contain peroxidase-positive leukocytes (polymorphonuclear leukocytes, predominantly neutrophils, 50~60%) and macrophages (20~30%) that originate from prostate gland and seminal vesicles. Leukocytospermia is also a sperm-disrupting disorder characterized by the presence of $>1 \times 10^6$ WBC/mL of semen [16]. Any infection or inflammatory response triggers these cells to produce 100 times more ROS than normal production rate as a defense mechanism and also promote NADPH production through the hexose monophosphate (HMP) shunt [5]. ROS generation is one of the prime mechanisms of leukocytes-induced immune defense with leukocytes being quite potent OS-inducing cells [15]. In addition, the elevation of pro-inflammatory mediators (predominantly IL-6, IL-8, and TNFα) and reduction of antioxidants in inflammatory reactions can induce a respiratory burst resulting in OS (Fig. 6.1) [17].

6.3.1.2 Immature Spermatozoa

In normal conditions, the cytoplasm is extruded from the maturing spermatozoa to prepare itself for fertilization. Any disruption in spermiogenesis may result in retention of excess residual cytoplasm (ERC) around the mid-piece of the spermatozoon, as in immature teratozoospermic sperms (Fig. 6.1). These ERCs have considerable content of enzymes to regulate glucose metabolism, namely, the glucose-6-phosphate dehydrogenase (G6PD). They also mediate intracellular β-nicotinamide adenine dinucleotide phosphate (NADPH) production through the HMP shunt that is the major fuel for ROS production via NADPH oxidase located within the sperm membrane [18]. Thus, teratozoospermic sperms produce more ROS compared with morphologically normal sperm. The calcium-dependent NADPH oxidase (NOX 5) present in the sperm differs from those present in the leukocytes, as the sperm-specific NOX5 activity is not dependent upon protein kinase-C [19]. However, the correlation of overexpression of NOX5 in spermatozoa in OS-induced infertility remains under debate.

6.3.1.3 Infections, Autoimmune/Inflammatory Conditions

Genitourinary Tract Infection

Prostatitis may affect up to 50% of men worldwide, and 10% of them suffer from chronic prostatitis [20]. Bacteria that cause prostate infection are of urinary tract origin and/or transmitted sexually [21, 22]. Some important nonsexually transmitted bacteria are gram-negative bacteria (*E. coli*, *Proteus mirabilis*), Streptococci (*S. viridans* and *S. pyogenes*), atypical mycoplasma strains (*Ureaplasma urealyticum)*, and *Mycoplasma hominis* coagulase-negative Staphylococci (*S. epidermidis*, *S. haemolyticus*). These agents commonly produce acute inflammatory responses by promoting leukocyte influx into the male reproductive tract and, ultimately, triggering ROS production [13]. Viral infections by cytomegalovirus, herpes simplex virus (HSV), Epstein–Barr virus, and others can also initiate oxidative damage to spermatozoa. Herpes simplex DNA has been found in semen of 4–50% of infertile men [23], with a tenfold increase IgM antibodies toward HSV [24].

Systemic Infection

Several chronic systemic infections have been correlated with increased OS in the male genital system. Human immu-

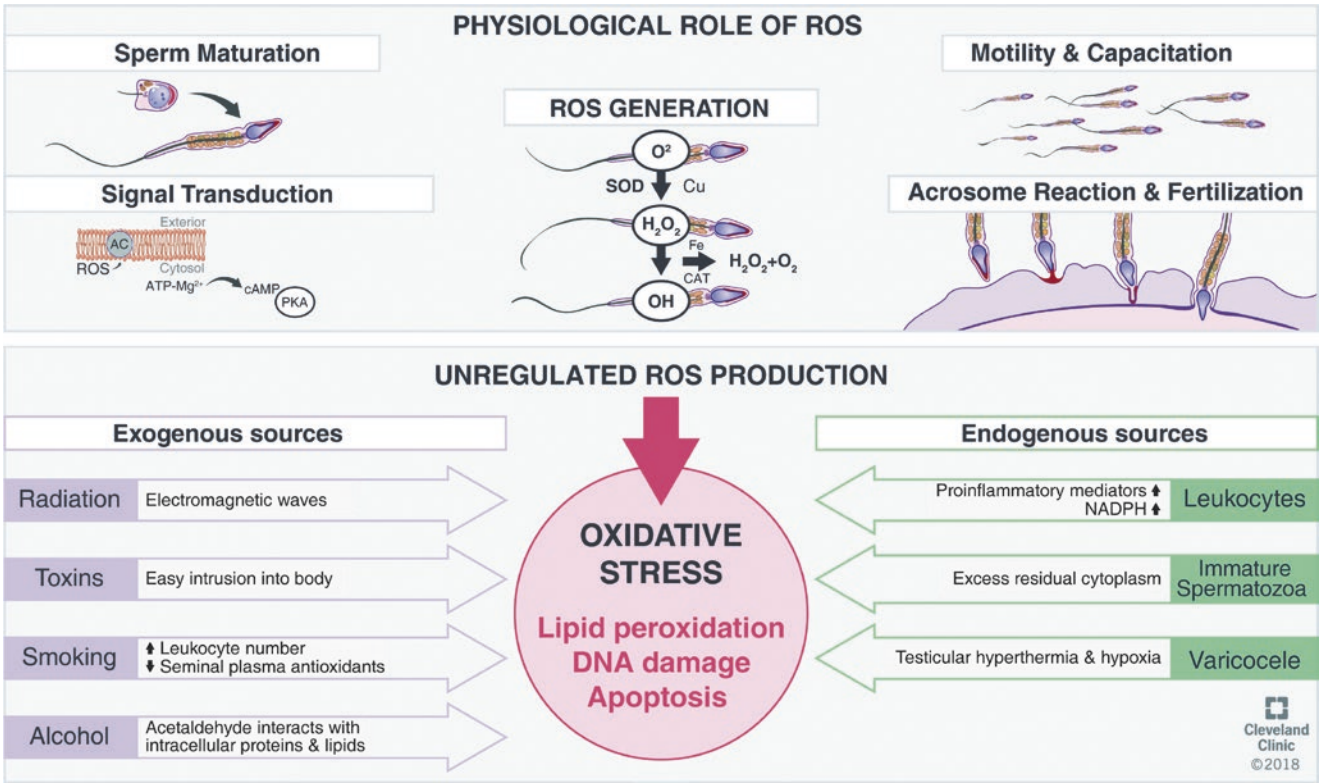

Fig. 6.1 Physiological roles of ROS and generation of excess ROS by exogenous and endogenous sources. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2017. All Rights Reserved)

nodeficiency virus (HIV) infection is associated with an elevation of leukocyte number in semen [25]. Hepatitis B and C infection have also been linked with significant hepatic and seminal OS [26, 27]. Chronic infections such as tuberculosis [28], leprosy [29], malaria [30], and Chagas disease [31] have all been connected with elevated degrees of systemic and male genital OS.

Autoimmune/Inflammatory Condition

Chronic nonbacterial (noninfective) prostatitis is an inflammatory condition of the prostate that has been linked with considerably elevated OS within semen [32]. It accounts for more than 90% of all cases and effects 10% of men [20]. The majority of the cases are reported to have adverse autoimmune responses to seminal or prostate antigens, leading to an elevation in seminal pro-inflammatory mediators and ROS-generating leukocytes [32, 33]. The specific mechanism inducing this response is unidentified, but a probable cause is polymorphism of the T_H-2 cytokine IL-10 [34]. Thus, reduction in IL-10 may shift immune responses to T_H-1 mechanism that induces T-lymphocytes against prostate antigens. The prime cytokines involved in this regulation are IFN-Ð, TNF-α, and IL-1b activating chemotaxis and activation of leukocytes and thereby inducing seminal OS [33]. OS, as discussed earlier, results in a significantly decreased semen quality and sperm membrane integrity [35]. OS also affects infertility after vasectomy reversal, due to disruption of blood–testis barrier. This causes loss of testicular immune privilege and initiation of immune responses against sperm [36], such as elevated seminal leukocytes, inflammatory cytokines, and generation of free radicals [36, 37].

6.3.1.4 Varicocele and Cryptorchidism

Varicocele, a pathogenic condition characterized by an abnormal venous dilation in the pampiniform plexus around the spermatic cord, is detected in about 40% of male partners of all infertile couples and is thought to be the leading cause of male factor infertility [38]. Many mechanisms have been proposed in the pathophysiology of varicocele. Testicular hyperthermia and hypoxia are the most commonly accepted theories resulting in OS-induced testicular dysfunction [38, 39] (Fig. 6.1). Recent studies have confirmed the presence of significantly higher OS parameters such as ROS and lipid peroxidation in semen samples from infertile patients with varicocele compared with normal fertile donors [38, 40]. The increase in varicocele-induced ROS generation is strongly correlated with a reduction in sperm DNA integrity [41]. The seminal ROS levels have been reported to be directly associated with the grade of varicocele [42].

Cryptorchidism is yet another cause of male factor infertility. It involves hypo-spermatogenesis with inability of gonocytes to mature to type-A spermatogonia [43]. Cryptorchidism-affected men may be surgically treated with orchidopexy in early stage of life still, but they will continue to possess an increased sperm ROS generation and DNA fragmentation [40].

Spermatic cord torsions are also associated with male infertility. It is suggested that OS-induced ischemia reperfusion injury results in damage to contralateral testis and with torsions. A prolonged ischemic phase follows either surgical or spontaneous restoration of testicular blood flow. This results in sudden influx of activated leukocytes into the testis [1, 40] that accelerates the generation of free radicals [6].

6.3.1.5 Other Chronic Diseases

Diabetes, one of the most prevalent chronic diseases, impairs male reproductive functions influencing both spermatogenesis and erectile processes. Diabetic men have shown higher sperm DNA fragmentation induced by OS compared with normal men [43]. Chronic inflammation and OS are also found in men with chronic kidney diseases [44]. Men with hemoglobinopathies, such as β-thalassemia, possess systemic OS [45] that can cause oxidative damage to sperm owing to iron overload by multiple blood transfusions [46]. Homocysteines can also lead to reproductive dysfunctions and OS by accumulated toxins [46]. Hyperhomocysteinemia generally occurs via suboptimal homocysteine re-methylation to methionine by methyl tetrahydrofolate reductase (MTHFR). This is caused by dietary folate deficiency or a single nucleotide polymorphism (SNP) in the MTHFR gene [47]. SNPs in the MTHFR gene are more commonly reported in the infertile men [48–50], rendering these men at elevated risk for homocysteine-induced OS.

6.3.2 Exogenous Sources

6.3.2.1 Radiation

Mobile phone radiation magnifies ROS generation in seminal plasma and causes deleterious effects on semen quality. It induces sperm DNA damage affecting sperm motility, count, and vitality [6, 51]. The radiofrequency electromagnetic waves interfere with the intracellular electron transfer along the membranes owing to cytosolically charged molecules, and thus interfering with the normal germ cell functions [52–54]. Radiofrequency waves affect male fertility through both thermal and nonthermal mechanisms (Fig. 6.1). Since testis depends mostly upon surface conduction for its temperature regulation, it is susceptible to damage by electromagnetic energy than any other organs who mainly depend on blood flow for temperature control. Radiation may elevate scrotal temperature, and even if it gets increased by 1 °C, normal spermatogenesis gets affected. The nonthermal effects of radiation include induction of oxidative stress or cell membrane potential alterations which disrupt germ cell proliferation and trigger apoptosis. Radiation may also cause sperm DNA fragmentation and epigenetic modifications. Chronic exposure to radiation severely disrupts steroidogenesis and brings about degeneration of Leydig cells [52–54].

6.3.2.2 Lifestyle Factors

Smoking disrupts the balance of ROS production with adequate antioxidant defense. Smoking has been reported to elevate concentrations of seminal leukocytes by 48% that accounts for the increase in seminal ROS by 107%. This leads to reduction in seminal antioxidants capacity and subsequent rise in 8-OHdG concentrations (a potent oxidative damage biomarker) [52]. Furthermore, smoking elevates cadmium and lead concentrations in blood and semen. This in turn increases ROS generation and damages sperm motility [52]. Germ cell apoptosis and DNA damage are generally identified in smokers, thus compromising male fecundity [55].

Alcohol is another inducing factor leading to uncontrolled seminal ROS generation and deterioration of antioxidant capacity. Acetaldehyde, produced as a by-product through ethanol metabolism, yields ROS via reaction with sperm cellular components. This significantly reduces the percentage of functional spermatozoa [56].

6.3.2.3 Toxins

Industrialization and domestic sophistications have led to the generation of a huge mass of environmental toxins and endocrine disruptors in an individual's immediate environment. These intrude into the body and may potentially induce excessive testicular ROS generation (Fig. 6.1). ROS thereby impairs morphology and functions of sperms. Exposure to environmental toxins leads to ROS-induced germ cell apoptosis [55]. Increased use of plastics have led to rise in exposure to phthalates which in addition to toxic metals such as lead, manganese, cadmium, chromium, and mercury afflict sperm quality, count, and spermatogenesis [52, 57].

6.4 Physiological Role of ROS

ROS, at physiological limits, is crucial for the quintessential phases of sperm development such as their maturation, capacitation, hyperactivation, and finally acrosome reaction (Fig. 6.1). The efficiency of ROS as signaling molecule attributes to its compatible small size, abundant generation in cells, and short half-life [58]. Redox modulation of cysteine residues is the key regulatory step by which ROS influence

the most of the signaling cascades. It is to be mentioned that activity of any specific enzyme is determined by the redox status of its thiol groups. ROS operates through activation of adenyl cyclase (AC), thus increasing intracellular cyclic AMP (cAMP) production. This turns on protein kinase A (PKA) that activates the downstream messengers specific to the spermatozoa maturational state [59].

6.4.1 Capacitation

Capacitation is the process of ultimate functional maturation of spermatozoa, which is essential to fertilize an ovum. Maturation of spermatozoa takes place mostly in the epididymis. It involves cell membrane restructuring, surface protein redistribution, and alterations in nuclear components and enzymes [3, 59]. ROS mediates the spermatozoa maturation by regulating the vital cell signaling cascades [59, 60]. Spermatozoon in human has compactly packed chromosomal DNA with smaller proteins than histone proteins. Chromatin stability is achieved through ROS-aided disulfide bonds between the cysteine residues of protamines. Thus, ROS helps in conferring chromatin stability and in preventing DNA damage. ROS also protects mitochondria from proteolytic dilapidation by participating in the development of "mitochondrial capsule" formed by disulfide bonded proteins [3, 61]. ROS facilitates spermatozoa capacitation by signaling cascades increasing intracellular cAMP levels. This activates protein kinase A followed by subsequent phosphorylation of MEK (extracellular signal regulated kinase)-likeproteins,sheathproteins,andthreonine-glutamate-tyrosine [18, 62]. This cellular pathway ultimately leads to spermatozoa capacitation, which is a prerequisite for acrosome reactions [52, 63].

6.4.2 Hyperactivation

Hyperactivation refers to a state of increased sperm motility with enhanced amplitude and asymmetric sperm flagellar movements. It confers a nonlinear movement of sperm with an increased sideward displacement of the sperm head [64]. This process just follows capacitation or can be considered as a later part of capacitation. It is required to determine proper sperm penetration into the zona pellucida for successful fertilization. The initiation of the process is mediated by calcium ion influx, which together with ROS activates adenylyl cyclase (AC), inducing cAMP production and activation of PKA. This triggers NADPH oxidase to further induce ROS generation [60]. PKA triggers protein tyrosine kinases (PTK) and phosphorylates the tyrosine residues present within the axonemal fibrous sheath and flagellar cytoskeleton. ROS induces the phosphorylation of tyrosine by influencing the activation of PTK and deactivation of phosphotyrosine phosphatase (PTPase) [3, 52].

6.4.3 Acrosome Reaction

The hyperactivated spermatozoon crosses the cumulus oophorous, to get attached to the zona pellucida of the oocyte. This is followed by a pore formation in the extracellular matrix of zona pellucida by spermatozoa exocytotic release of proteolytic enzymes [61]. These reactions enable sperm attachment and entry into ova and together is termed as acrosome reaction. These reactions are initiated by phosphorylation of tyrosine residues of membrane proteins that is aided by ROS-regulated cell signaling pathway. The primary pathway as discussed in the earlier sections involves Ca^{2+} influx, rise in intracellular cAMP, and PKA activation [3, 59].

6.4.4 Fertilization

ROS also assists in the process of fertilization by enhancing the membrane fluidity for sperm–oocyte fusion to occur [52]. ROS inhibits protein tyrosine phosphatase activity and thus prevents phospholipase A2 (PLA2) from getting deactivated. Hence, the activated PLA2 cleaves the secondary fatty acid present in the membrane to effectively enhance membrane fluidity [65].

6.5 Mechanisms of ROS-Mediated Male Infertility

6.5.1 Lipid Peroxidation

Sperm has substantially high lipid content in its plasma membrane. They are mostly present as PUFAs with unconjugated double bonds in methylene groups. This weakens the methyl bond between carbon and hydrogen. Thus, hydrogen is rendered vulnerable to oxidative attack. The uncontrolled rise in intracellular ROS initiates a progressive reaction cascade and triggers LPO [39, 66, 67]. LPO is a self-propagating autocatalytic reaction that destroys almost 60% of the membrane fatty acids affecting its fluidity. It also imparts nonspecific permeability of the membrane and disrupts membrane receptors and enzymes that severely impairs fertilization [52, 66, 68].

Oxidative damage once initiated follows steady propagation until it is terminated. "Initiation" is marked by isolation of hydrogen atoms from the carbon–carbon bonds, followed by generation of free radicals and lipid radicals. This reacts with oxygen to produce peroxyl radicals [66]. These peroxyl radicals further abstract hydrogen atom from the lipids

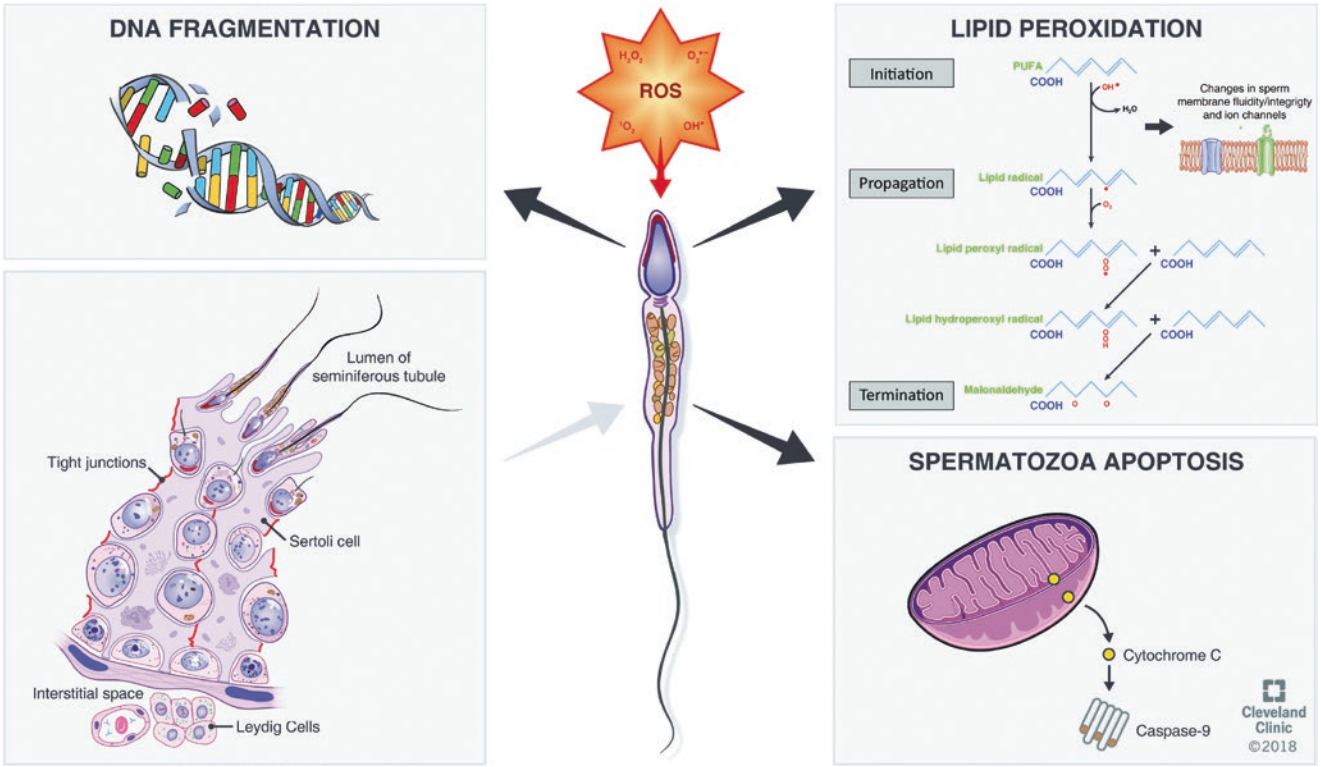

Fig. 6.2 Mechanisms of ROS-induced impairments in sperm functions. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2017. All Rights Reserved)

(action enhanced by the presence of metals such as copper and iron). This triggers the chain of autocatalytic reaction. The "propagation" of oxidative damage refers to continuation of the damaging reaction with the existing radicals reacting with succeeding lipids, giving rise to toxic aldehydes by hydroperoxide degradation (Fig. 6.2) [15, 18, 66]. Cytotoxic peroxyl and alkyl radicals are generated in a cyclical manner until a stable end product, malondialdehyde (MDA), is produced to terminate the reaction [66]. MDA thus bears importance as a marker to predict the level of peroxidative damage caused to spermatozoa. Another deleterious product of LPO is the hydrophilic 4-hydroxynonenal that severely impairs spermatozoa function affecting its proteomics and genomic constitution [52].

6.5.2 Sperm DNA Fragmentation

ROS adversely affects sperm nuclear DNA through sperm DNA fragmentation (SDF), inducing chromatin cross-linking, randomly modifying the base-pairing and chromosomal microdeletions (Fig. 6.2) [52, 66, 69]. ROS also aids adenine and pyridine nucleotides removal by initiating LPO [52, 69]. Numerous intrinsic and extrinsic factors are suggested in the etiopathogenesis of male SDF, including infection, varicocele, advanced male age, lifestyle factors, heat stress, environmental toxins, radiations, defective protamination, and sometimes even remain idiopathic [43, 70]. Several of the mentioned etiologies are mediated by uncontrolled ROS generation leading to increased SDF [71]. Testicular SDF has been characterized by abortive germ cell apoptosis [72] and defective sperm maturation [73]. As a consequence of imbalance between ROS production (via testicular hypoxia, reflux of metabolites, endocrine disruption, and scrotal hyperthermia) and protective antioxidant capacity, a state of OS is established. This induces elevated lipid peroxidation products in infertile men with pathological conditions that serve as etiology for SDF [74]. Moreover, treatment of such pathological state with antioxidants seems to be effective in concurrent decrease in both ROS [75] and SDF [76].

6.5.3 Apoptosis of Spermatozoa

ROS is capable of disrupting the inner and outer mitochondrial membranes releasing cytochrome C. This cytochrome C in turn activates the apoptotic caspases [52, 62]. ROS induces apoptosis of spermatozoa by elevating cytochrome C level, which have been reported by seminal plasma analysis in infertile men (Fig. 6.2). The increased cytochrome C level in seminal plasma is an indicator of severe sperm mitochondrial damage [6, 52]. Besides the defects in sperm chromatin compaction, numerous intrinsic and extrinsic factors have been reported in the etiopathogenesis of SDF, including

varicocele, infection, advanced male age, heat stress, lifestyle factors, environmental toxins, ionizing and nonionizing radiations, defective protamination, and, at times, idiopathic [43, 70]. Many of the above etiologies, but not all, are mediated by ROS leading to high SDF [71]. Abortive apoptosis [72] and defective maturation [73] theories have been proposed to correlate the role of intrinsic factors in testicular SDF. Moreover, evidences showed more DNA fragmentation in epididymal and ejaculated sperm than the testicular sperm, signifying the impact of extrinsic factors in most patients [77]. The presence of a large amount of polyunsaturated fatty acids (PUFA) in the plasma membrane makes sperm susceptible to ROS-induced damage [78].

The close relationship between ROS and SDF is also evident from the etiopathologies of all grades of clinical varicocele. The result of imbalance of ROS (produced by testicular hypoxia, scrotal hyperthermia, reflux of metabolites, and endocrine disruption) and protective antioxidant system was demonstrated by the higher level of ROS and lipid peroxidation products in infertile men with varicocele than infertile men without varicocele [74]. Moreover, treatment of varicocele is effective in decreasing both ROS [75] and SDF [76].

6.6 ROS in Assisted Reproduction

ROS has been shown to possess a significant role in fertilization. Oocyte metabolism produces ROS, but it actually lacks adequate protective antioxidant mechanisms [79], which results in OS. Moreover, preimplantation embryonic development needs a preferred modulation in cellular energetics [80]. Thus, the established OS serves as beneficial state where oxidative phosphorylation is needed as energy source for preimplantation embryo development. This oxidative phosphorylation dependency shifts to glycolysis to increase energy production [80, 81]. Oxygen may oxidatively phosphorylate ADP to ATP, which is utilized in folliculogenesis and oocyte maturation. In case of any alterations in these reactions using oxygen, it results in excess production of ROS which adversely affects embryo development [81].

Sources of ROS differ in case of conventional IVF and for ICSI [82]. The oocytes for ICSI are devoid of cumulus cells, rendering the oocyte and the injected spermatozoa as the only potential ROS sources in the culture media environment [81, 83]. While in IVF, ROS gets generated from the several oocytes per culture dish, a large mass of cumulus cells, and also from the inseminated spermatozoa. Moreover, unlike IVF, in ICSI there is no sperm–oocyte contact, thus limiting the scope of ROS production by faulty spermatozoa [59]. This explains why lesser the concentration of spermatozoa in the in vitro culture media, higher is the possibility of proper fertilization, implantation, and pregnancy [84]. ICSI utilizes a single spermatozoon at a time and therefore a better procedure to minimize OS.

In either IVF or ICSI, both the culture media and external environment influence the ultimate outcomes [8]. The procedures differ mostly in the fact that for ICSI there is shorter incubation time that reduces exposure to modulating external environmental factors [83, 85]. The predicted limit of the incubation time is 1–2 hours which yields better ART outcomes. In both IVF and ICSI, ROS is generated from the gametes, the embryo, or may arise indirectly from external factors, such as cumulus cells, leukocytes, etc. Since fertilization followed by embryo development in vivo takes place under low oxygen tension, a state of OS in the in vitro setting definitely accounts for high failure rate in ARTs. Hence, in order to improve ART outcomes, in vivo conditions should be emulated via minimizing exposure to the factors that promote generation of excess ROS [83, 86].

6.7 Measurement of OS

ROS-induced sperm damage is a major contributing male reproductive pathology (almost 30–80% of idiopathic male infertility) [87]. Assessment of ROS levels in the infertile men thereby is very crucial for its treatment and management. However, the difficulties in ROS assessment arise from the factors such as high cost, screening inconveniences, and scarcity of accepted specific analysis method. With the advent of research and technology, more than 30 different ROS measurement assays have emerged lately that can predict the level of OS in infertile men [52].

6.7.1 Assessment of Sperm OS from Routine Semen Analysis

Routine semen analysis (sperm count, morphology, and motility) provides diagnostic clues to the clinicians with asthenozoospermia being a major OS marker [88, 89]. Seminal plasma hyperviscosity is suggestive of increased MDA and reduced seminal antioxidant levels [88]. In addition, *Ureaplasma urealyticum* infection in the semen can also increase seminal viscosity and can be associated with high seminal ROS production [88]. The presence of round sperm cells may infer leukocytospermia which is again a potent source of ROS. However, it is needed to ascertain that the round cells are actually not immature spermatozoa. For this, several other tests are required, such as peroxidase test, seminal elastase measurement, or staining of CD45 (transmembrane glycoprotein) antibody. OS status is also presumed from disruption in sperm morphology and cytoplasmic droplets featuring anomalous spermatozoa. Poor sperm membrane integrity is diagnosed by hypoosmotic swelling test (HOST), which also associates with a state of OS [52].

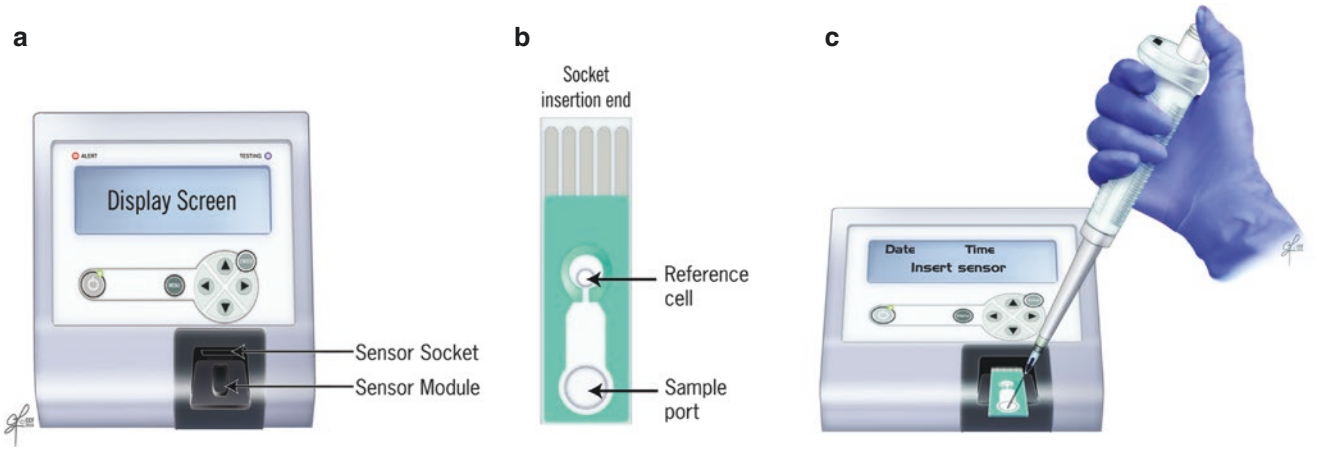

Fig. 6.3 Measurement of ORP by the MiOXSYS system. (**a**) MiOXSYS analyzer showing the socket and the sensor module, (**b**) sensor showing the reference cell and the sample port where the sample is loaded, and (**c**) loading of semen samples on the sample port of the sensor strip. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2017. All Rights Reserved)

6.7.2 Total Antioxidant Capacity (TAC)

Antioxidant capacity testing has been reported to potentially enhance prognostic information for infertile men. Luminol, a chemical exhibiting chemiluminescence when reacted with particular oxidizing agent, is being widely used to measure seminal total antioxidant capacity (TAC). It is quantified in contrast to a vitamin E analog "Trolox," a water-soluble tocopherol. The results signify ROS-TAC score and aid to infer the level of antioxidants produced as a combined efforts of all seminal constituents such as vitamins, lipids, and proteins [89].

6.7.3 Lipid Peroxidation Markers

Spermatozoa often carries accumulated lipid peroxides, produced as end-products. These metabolites, hydroxynonenal, MDA, 2-propenal (acrolein), and isoprostanes, can be used as OS indicators [66]. Thiobarbituric acid (TBA) assay is used for MDA measurement. The principles include combination of MDA with TBA to produce 1:2 adduct. This is a colored substance measured by fluorometry or spectrophotometry [52, 89].

6.7.4 Seminal Oxidation-Reduction Potential (ORP)

Oxidation-reduction potential (ORP) or redox potential measures the potential of electrons transfer between different chemical species [90, 91]. ORP helps to infer the association between oxidants and antioxidants and thereby suggest the level of OS. Recently, a novel method has been developed based on galvanostatic electron measure to assess OS changes in patients in trauma [92, 93].

Seminal ORP is efficiently assessed with the MiOXSYS System (AytuBioScience, USA) which is a standardized, reproducible, and reliable method. It provides greater application for OS analysis in research and clinical settings (Fig. 6.3) [94, 95].

6.7.5 Direct Laboratory Assessments of OS

Seminal ROS can be directly measured the chemiluminescence assays. The procedure involves a luminometer and a chemiluminescent probe such as luminal (5-amino-2,3, -dihydro-1,4-phthalazinedione). Luminol measures both extracellular and intracellular ROS. The free radicals contained in the semen sample produce a light signal reacting with luminal, which is converted by the luminometer to an electric signal (photon). Results are expressed as relative light units/s/10^6 sperm. The range of normal ROS levels in washed sperm suspensions is 0.10–1.03 units/s/10^6 [89].

6.8 Management of OS-Associated Male Infertility

Primary steps in OS management involve unveiling its underlying cause(s) followed by effective treatment.

6.8.1 Lifestyle Management Approach

Stress arises from both personal lifestyle adoptions and competitive professional load. The ill habits, such as substance

abuse, alcoholism, tobacco smoking and chewing, and an unbalanced diet are all major contributors to cause OS. Therefore, minimizing the avoidable lifestyle disorientations may help to prevent OS [52, 96].

Exposure to endocrine and reproductive disruptors which include pollutants, heavy metals, and other toxins greatly account for OS development. Increase in scrotal temperature via rigorous exercise, hot baths, driving for long hours, sedentary office works, etc., induce heat stress that create OS [39, 87, 97].

6.8.2 Vitamin and Antioxidant Supplementation

Antioxidant supplementation may eradicate excess ROS or decrease its production, to halt the damaging oxidative chain reactions. On the one hand, preventive antioxidants such as the metal chelators or binding proteins, for example, transferrin and lactoferrin, prevents OS by restricting ROS generation [98]. On the other hand, the scavenging antioxidants (vitamins C and E) combat OS by mitigating excess ROS [99].

Antioxidants can also be classified as enzymatic and nonenzymatic. Natural antioxidants are mostly enzymatic antioxidants that include superoxide dismutase (SOD), catalase, and glutathione reductase (GSH). Examples of nonenzymatic antioxidants include vitamins B, C, and E, carnitines, carotenoids, cysteines, pentoxifylline, taurine, hypotaurine, albumin, and some metals. They can also be obtained from certain foods and food supplements [52, 100, 101].

Available reports suggest antioxidant supplements are a beneficial treatment option for OS-induced male infertility that may reduce sperm DNA damage and improve semen parameters [102–107]. Specific antioxidants such as vitamin C, vitamin E, N-acetyl-cysteine, selenium, and zinc have been reported to be beneficial in treatment of OS-induced male infertility [108].

6.8.3 Surgery

Surgeries effectively correct the venous abnormality in varicocele patients and decrease seminal ROS, thus preventing further oxidative damage [87]. Surgical repair improves several sperm parameters and has been shown to improve pregnancy rates as well [52, 75]. It has been reported that inflammatory and OS markers such as MDA, H2O2, and nitric oxide (NO) are reduced while antioxidant levels improved following a successful varicocelectomy [109]. This scenario suggests that varicocelectomy decreases seminal ROS levels and increases TAC to restore male reproductive functions in varicocele patients [109, 110].

6.9 Conclusion

OS arises from disrupted balance between ROS production and their elimination. While at physiological levels, ROS contribute in maturation of sperm and normal sperm functions, and excessive seminal ROS induce OS that impairs sperm quality and functions. This deleterious chain of oxidative reactions leads to infertility. With proper assessment and management strategies, along with healthy life style practices, it is possible to improve male reproductive potential in OS-induced infertile men.

6.10 Review Criteria

An extensive literature search has been performed to find the relationship between oxidative stress and male infertility using search engines such as Science Direct, OVID, Google Scholar, PubMed, and MEDLINE. The overall strategy for study identification and data extraction was based on the following key words: “oxidative stress,” “reactive oxygen species,” “infertile men,” “infertility,” “semen parameters,” and “assisted reproduction” and the names of related oxidative-stress markers and specific ROS assessment methods. Articles published languages other than English were also considered. Data that were solely published in conference or meeting proceedings, websites, or books were not included. Websites and book-chapter citations provide conceptual content only.

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Oxidative Stress Measurement in Semen and Seminal Plasma

7

Rakesh Sharma and Ashok Agarwal

Key Points

- Abnormal spermatozoa and leukocytes are major producers of reactive oxygen species (ROS).
- Physiological levels of ROS have an important role in sperm function; pathological levels result in oxidative stress.
- Various direct and indirect methods are available to measure oxidative stress. Each method captures only a single dimension of oxidative stress from which the overall oxidative stress status in a given biological system is generalized. These methods fail to provide a complete picture of the true oxidative stress status environment. They only reflect the redox status at a single point.
- Current markers of oxidative stress are antiquated, time-consuming, and time-sensitive.
- Oxidation reduction potential (ORP) is a novel direct marker of oxidative stress. It provides the relative proportion of oxidants (ROS) and reductants (antioxidants) that are present in a given biological system at any given time. ORP can be used as an adjunct tool to basic semen analysis in all patients who present for infertility. It can provide valuable information about sperm function and the fertilizing ability of the man.

7.1 Introduction

Infertility is defined as the inability to achieve a clinical pregnancy after 12 months of unprotected sexual intercourse. While contributing to about 50% of cases overall, males are solely responsible for 20–30% of infertility cases [1]. Approximately 7% of men worldwide are infertile [2]. When encountering male infertility, routine semen analysis is the first step for laboratory evaluations. Almost 15% of infertile men have semen parameters that are within normal reference range [3] and categorized as idiopathic infertility cases. Thus, assessing a man's fertility not only depends on gross physical parameters of spermatozoa but also on their functional capability.

Male infertility is strongly correlated to excess reactive oxygen species (ROS) in human semen [3–6]. The delicate balance in the cellular environment is maintained by the presence of a scavenging system via enzymatic and nonenzymatic antioxidant pathways. Physiological levels of ROS are required for normal cellular metabolism, spermatogenesis, sperm maturation, capacitation, hyperactivation, acrosome reaction, and sperm–oocyte fusion [7]. However, when ROS threshold supersedes the balance between ROS and antioxidants cells undergo oxidative stress (OS), this may lead to cellular dysfunction via lipid peroxidation, changes in protein conformation, and DNA integrity (Fig. 7.1) [8]. Due to its negative effect on cells, OS is associated with numerous pathological conditions in addition to infertility [7, 9–14].

Defective spermatozoa and presence of activated leukocytes are the major sources of ROS in human semen [5]. OS is one of the most common etiologies affecting 30–80% of men with infertility [3, 15], especially those who have unexplained and idiopathic male infertility [16]. OS can also potentially have consequences at a systemic level by decreasing the amount of testosterone or LH [17].

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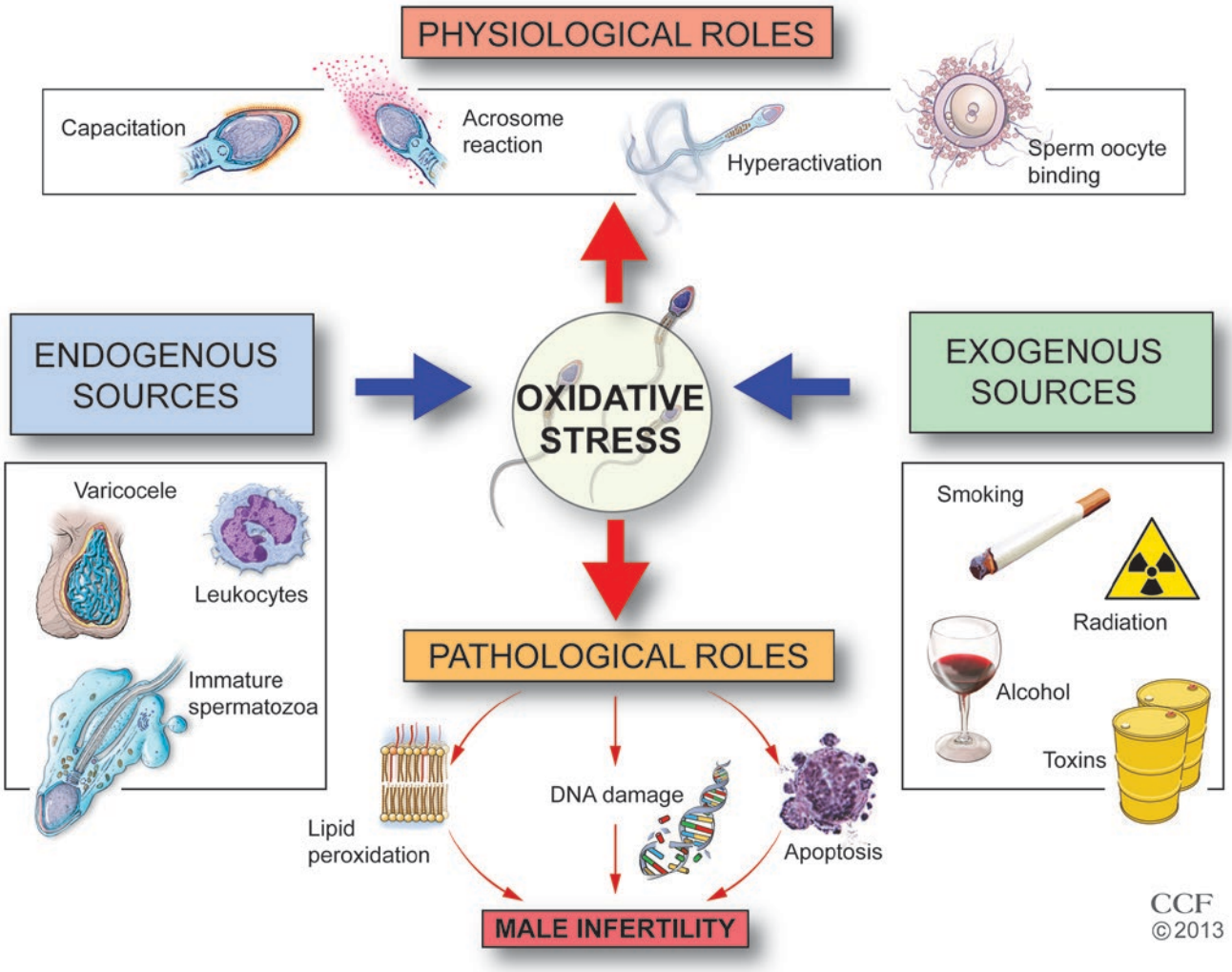


Fig. 7.1 Physiological and pathological effects of RODS and exogenous and endogenous sources of ROS resulting in oxidative stress and male infertility. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2013–2019. All Rights Reserved)

Increased OS is detrimental to sperm DNA and may compromise the paternal genomic contribution to the embryo [18]. Numerous studies have reported that sperm DNA fragmentation (SDF) is linked to reduced fertilization rate [19–23]. Several hypotheses have been proposed to understand the origin of sperm DNA fragmentation [19, 24, 25]. The first hypothesis describes endonuclease-mediated DNA cleavage, also called abortive apoptosis. This occurs when sperm with damaged DNA escape from normal programmed cell death [19]. The second hypothesis involves DNA strand breaks induced by oxidative stress [24]. The third hypothesis is characterized by an increase in torsional stress during spermiogenesis that can in turn increase the activity of endogenous endonucleases, which may stimulate DNA fragmentation [19]. Due to these characteristics, OS in male infertility can lead to serious health risks including cardiovascular and diabetes if not carefully controlled over time [26–29].

7.2 Seminal Reactive Oxygen Species and Antioxidants: Physiological and Pathological Roles

7.2.1 Physiological Roles

ROS are highly reactive free molecules or non-radicals that are generated from oxygen metabolism. Free radicals are molecules that contain at least one unpaired valence electron in their outer shell, making them highly reactive and short-lived [5]. Among all the ROS, superoxide anion ($\bullet O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\bullet OH$) are the most common examples. Reactive nitrogen species (RNS) are a subclass of ROS that contain nitrogen compound [30]. Examples of reactive nitrogen species are nitric oxide ($\bullet NO$), lipid peroxyl ($\bullet LOO^-$), and thiyl ($\bullet RS$) and peroxynitrite ($ONOO^-$). ROS/RNS are formed during

normal cellular and sperm mitochondrial metabolism and normal levels are essential to perform many physiological processes, including the activation of immune system [31]. They are also generated by sperm mitochondria during normal cellular respiration [32, 33] regulating different intercellular signaling pathways and facilitating normal maturation and fertilization in reproductive systems [16, 34–37].

7.2.2 Pathological ROS and Oxidative Stress

Contributing factors to OS include genito-urinary infections, varicocele, chronic inflammation (inflammatory bowel diseases) metabolic syndrome, cigarette smoking, alcohol abuse, recreational drug abuse, ionizing radiation, mobile phone use, psychological stress, strenuous exercise, spinal cord injury, and environmental pollution [3, 38–42]. Major exogenous sources include lifestyle factors: smoking, alcohol use, nutrition, obesity, stress, medication, environmental; pollutants, pesticides, xenoestrogens, cell phone radiation [27, 43–48]. Whereas primary contributors of endogenous ROS production include immature and morphologically abnormal spermatozoa with excess residual cytoplasm in the mid-piece of the flagellum [49] and infiltrating leukocytes (especially granulocytes) [4, 16, 50] (Fig. 7.1).

The number of leukocytes in seminal plasma increases with genital tract infection and/or inflammation (i.e., epididymitis, prostatitis). Peroxidase-positive leukocytes can produce approximately 1000 times more ROS than spermatozoa by enhancing nicotinamide-adenine dinucleotide phosphate (NADPH) production via the hexose monophosphate shunt [51]. These cells predominantly originate from the prostate and seminal vesicles [52]. A strong association is seen between the seminal ROS levels and presence of male accessory gland infections (MAGI), such as prostatitis, prostato-vesiculitis, and prostato-vesiculo-epididymitis [16].

7.2.3 Oxidative Stress and Male Infertility

High levels of ROS have shown a detrimental effect on parameters such as sperm concentration [53–58], motility [7, 56, 57], sperm morphology [7, 52, 55], as well as increased sperm DNA damage [53, 59], apoptosis [59, 60], and overall decrease in sperm function [6, 18]. Furthermore, oxidative stress disrupts the sperm membrane by altering its permeability, fluidity, and key functions. It also affects both natural and assisted reproduction outcomes, and low pregnancy rates have been reported in men with high ROS levels [61, 62].

Spermatozoa are prone to OS because their plasma membrane contains an unusually high percentage of polyunsaturated fatty acids (PUFAs), which are susceptible to lipid peroxidation [53]. Lipid peroxidation is characterized by the breakdown of PUFAs into lipid peroxides due to oxidative stress [63] as electrons from plasma membrane lipids are stripped away by ROS. This propagates a chain of redox reactions that eventually generates highly mutagenic and genotoxic electrophilic aldehydes such as malondialdehyde (MDA), 4-hydroxynenal (4-HNE), and acrolein [64]. Compared to free radicals, these aldehydic products are relatively stable and are able to move freely and react with molecules like DNA, proteins, and lipids.

Oxidative stress is responsible for single-strand breaks in DNA [52, 65]. Furthermore, apoptosis can also occur as a result of increased oxidative stress and can result in DNA fragmentation. Several studies show that infertile men have high number of single- or double-stranded sperm DNA fragmentation (SDF) [23, 66–71]. Several etiological factors have been implicated including cigarette smoking, irradiation, chemotherapy, leukocytospermia, varicocele, cancer, elevated levels of ROS, abnormalities during chromatin packaging, and advanced age that have demonstrated compromised sperm DNA quality [7, 18, 60, 65, 72–74]. Increased levels of ROS are associated with reduced fertility; impaired embryo development increased miscarriage or pregnancy loss [20–23, 75–80].

For these reasons, measuring the accurate levels of oxidative stress markers specifically ROS is important and can be offered as a diagnostic test or screening test for differentiating fertile population from infertile or men with unexplained/idiopathic infertility [81].

7.2.4 Types of Samples for Oxidative Stress Measurement

The types of semen samples that can be used to measure OS levels include unprocessed seminal ejaculates, simple wash and resuspend samples, and samples processed by swim-up or density gradient centrifugation [52]. An ejaculate comprises not only of spermatozoa but also of all other secretions from prostate and seminal vesicles and other accessory glands and cellular components such as round cells, leukocytes, and epithelial cells. Levels of ROS are reflective of the de novo status of the ROS in the sample. In a simple wash and resuspend sample, the seminal plasma is removed; however, the leukocytes, round cells, and debris remain in the sample. In samples prepared by swim-up the actively motile sperm are separated from the non-motile sperm and the debris, whereas in the density gradient separation, spermatozoa are separated on the basis of their densities and motility, which results in the separation of actively motile and morphologically normal sperm [52].

7.2.5 Markers of Oxidative Stress and their Assessment

Many individual markers have been used to reflect oxidative stress. The rationale for various laboratory techniques used to measure OS are a result of direct measurement of ROS or indirect measurements through oxidized products of ROS production. Direct laboratory techniques measure OS or free radicals such as ROS and reactive nitrogen species. Direct laboratory techniques include chemiluminescence, nitroblue tetrazolium (NBT), cytochrome C reduction test, fluorescein probe, electron spin. Indirect laboratory techniques measure lipid peroxidation, antioxidants, cofactors, or other end products secondary to ROS production. More specifically, indirect measurements could be an accumulative result of oxidized products resulting from sources of ROS such as the oxidized form of nicotinamide adenine dinucleotide (NADPH)-oxidase in the sperm, the reduced form of NAD (NADH)-dependent oxidoreductase in mitochondria, or leukocytospermia [82]. Indirect laboratory techniques include measurement of Endtz test, lipid peroxidation, chemokines, antioxidants/micronutrients/vitamins, ascorbate, total antioxidant capacity (TAC), or DNA damage (Table 7.1).

7.3 Common Methods to Measure Oxidative Stress

7.3.1 Nitroblue Tetrazolium Test

The nitroblue tetrazolium test, or the NBT test, is based on the detection of ROS produced by spermatozoa and leukocytes by using the compound nitroblue tetrazolium. NBT is a yellow water-soluble nitro-substituted aromatic tetrazolium compound that reacts with superoxide ions to form formazan derivative that can be monitored spectrophotometrically [83]. This test is based on the principle that when heterogeneous samples such as ejaculate are stained with NBT, it results in the formation of colored formazan due to reduction of NBT. This has been shown to correlate with impaired sperm function [83]. NBT is an electron acceptor that becomes reduced in the presence of free oxygen radicals to form a blue–black compound, formazan [84]. Spermatozoa containing formazan can be stained histochemically and scored under the microscope. Spermatozoa are scored as follows: formazan occupying 50% or less of the cytoplasm (+) and more than 50% of cytoplasm (++) [84]. Leukocytes are scored as: no detectable formazan (−), scattered or few formazan granules (+), intermediate density (++), and cells filled with formazan (+++). NBT reaction reflects the ROS generating activity in the cytoplasm of cells, and therefore it can help determine the cellular origin of ROS in semen [85]. The advantages of this method include that it is readily available, inexpensive, and has high sensitivity. It provides information about the differential contribution of leukocytes and abnormal spermatozoa in the production of ROS, i.e., the cellular origin of ROS in the sample [84]. The major limitation of this test is that presence of other cellular reductases may also reduce NBT. Furthermore, changes in the cellular content of various oxido-reductases may also alter the rates of NBT reduction [85, 86].

7.3.2 Chemiluminescence Assay

Chemiluminescence is one of the most commonly employed methods used to detect ROS in a semen sample [87–89]. The reaction causes emission of light, which is measured with a luminometer. The two major types of luminometer include the photon-counting luminometer and the direct current luminometer. The photon counting measures the individual photon, whereas the direct counting measures the current passing through a luminometer. These are measured as photons per minute or relative light units, respectively [7].

The basic principle of chemiluminescence is the measurement of emitted light due to a chemical reaction occurring between chemical reagents and the ROS generated. The following equation shows two reactants A and B in presence of an excited intermediate [◊] resulting in emission of light. The decay of this excited state [◊] to a lower energy level causes light emission.

$$[\mathbf{A}]+[\mathbf{B}]\rightarrow[\Diamond]\rightarrow[\text{Products}]+\text{light}$$

There are two major types of probes used in chemiluminescence which include luminol for global measurement of ROS and lucigenin for measurement of superoxide anion [82, 89, 90].

The reagents used for global measurement of ROS are the stock luminol probe (100 mM), the working luminol (5 mM), and the dimethylsulfoxide (DMSO) solution [81, 91]. The test is light sensitive and performed under subdued light. A luminometer is attached to a computer (Fig. 7.2). A total of 11 tubes are used including 3 blank tubes which contain only phosphate buffer saline (PBS), 3 negative controls which contain PBS + luminol (working solution), 2 tubes which have the patient sample and + luminol, and 3 positive controls which contain PBS + hydrogen peroxide (50 µL) + luminol (Fig. 7.3). The tubes are loaded into the luminometer (Berthold, Autolumat Plus LB 953), and a real-time plot of the ROS levels produced in each sample is visualized on the computer monitor and analyzed (Fig. 7.4) [91].

Table 7.1 Advantages and disadvantages of direct and indirect measurement of ROS in semen

Assays	Principle/Probe	Instrument	Type of sample	Advantages	Disadvantages
Direct methods					
Chemiluminescence	Luminol Lucigenin	Luminometer	Extracellular and intracellular Extracellular	1. Robust, high sensitivity, and specificity	1. Time-consuming, large and expensive equipment 2. Interfering variables 3. Requires high sample volume.
Nitro blue tetrazolium (NBT)	NBT reduced to formazan by superoxide ions	Spectrophotometer	Extracellular	1. Cost-effective, user friendly 2. Detect neutrophils at a concentration of 0.5×10^6/mL or higher	1. Subjective interpretation of a positive vs. negative neutrophils
Cytochrome C reduction test	Ferricytochrome C	Spectrophotometer	Extracellular	1. Quantify $O_2^{\bullet-}$ released during the respiratory burst of neutrophils or by isolated enzymes 2. Good for high level of ROS production	1. Relatively insensitive to detect NADPH oxidase activity, if enzymatic activity is low 2. Cannot detect intracellular $O_2^{\bullet-}$
Fluorescein probe	2′-7′-Dichlorodihydrofluorescein diacetate (DCFH-DA)	Flowcytometry	Spermatozoa		2′-7′-Dichlorodihydrofluorescein diacetate (DCFH-DA)
Oxidation induced fluorochrome probe	Dihydroethidium (DHE)	Confocal microscope	Spermatozoa		
Fluorescein isothiocyanate (FITC)-labeled lectins				1. Detect acrosome status	1. Difficult to distinguish true and false acrosomal reactions 2. Impossible to detect sperm viability and acrosomal status in one picture 3. Fluorescent signal fades in times
Electron spin resonance	Paramagnetic species	Electron spin resonance spectroscopy	Extracellular and intracellular	1. Broad usages such as observations of free radicals, analysis of free radical characteristics, quantitative analysis of free radicals, and kinetic analysis 2. Good for high level of ROS production	1. Limitation if a free radical reacts immediately with a molecule other than the spin-trapping agent 2. Inference factors such as possible neutralization

(continued)

Table 7.1 (continued)

Assays	Principle/Probe	Instrument	Type of sample	Advantages	Disadvantages
Indirect methods					
Myeloperoxidase or Endtz test	Peroxidase activity	Colorimeter	Spermatozoa	1. Clearly distinguishes WBCs especially ROS-producing granuloctyes from other immature germ cells in semen	1. Cannot be used to detect ROS generation by spermatozoa
Lipid peroxidation levels	Thiobarbituric acid-reactive substances	Colorimeter	Oxidized components in seminal plasma	1. Malondialdehyde is a colored substance that can be measured by fluorometry or spectrophotometry 2. Low sperm concentration of malondialdehyde can be measured through sensitive high-pressure liquid chromatography equipment or spectrofluorometric measurement of iron-based promoters	1. Not widely used in clinical practice at this time
Chemokines	Specific antibodies ELISA	ELISA reader	Seminal plasma	1. Produced as a result of ROS-induced inflammation	1. Requires a large amount of biological material (>0.5 L of culture supernatants).
Antioxidants, micronutrients, vitamins (vitamin E, vitamin C, ascorbate)	Column chromatography	HPLC	Seminal plasma	1. Cofactor of essential enzymatic reactions of ROS	1. Assess an end state occurring secondary to other unknown pathologic processes
Antioxidants- total antioxidant capacity (TAC)	2,2′ -azinodi-[3-ethylbenzthiazolinesulfonate] (ABTS)	Luminometer	Seminal plasma	1. Rapid colorimetric method 2. Total antioxidants in seminal plasma measured	1. Does not measure enzymatic antioxidants or individual antioxidants. 2. Requires expensive assay kit and microplate reader
DNA damage	TUNEL SCSA Comet SCD	Flow cytometer	Spermatozoa	1. Robust and sensitive method 2. Multiple methods are available to measure DNA fragmentation such as sperm chromatin structure assay (SCSA), sperm chromatin dispersion, TUNEL, comet, sperm chromatin dispersion assay, nuclear protein composition, sperm nuclear maturity test, and 8-OHdG.	1. Accessibility of the DNA (TUNEL) 2. Inter observer, intra observer + inter-assay and intra-assay variability. Lack of standardized reference (Comet, SCD).

Fig. 7.2 Autolumat 953 Plus Luminometer used in the measurement of ROS by chemiluminescence assay. Multiple tubes can be loaded simultaneously for measuring ROS. The luminometer can be connected with a computer and monitor. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2013–2019. All Rights Reserved)

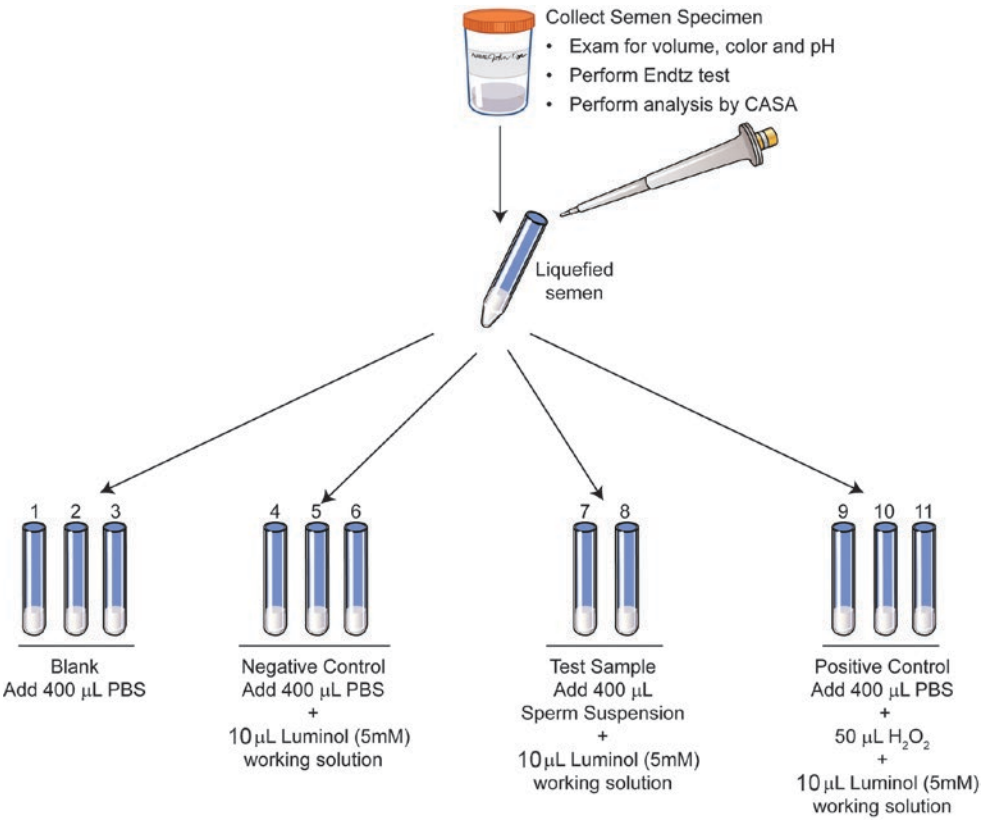


Fig. 7.3 Setup of the tubes for ROS measurement. A total of 11 tubes are labeled from S1-S11: Blank, negative control, test sample and positive control. Luminol is added to all tubes except the blank. Hydrogen peroxide is added only to the positive control. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2013–2019. All Rights Reserved)

7.3.3 Factors Affecting the ROS Measurement

Semen age, viscosity, repeated centrifugation, and the use of media containing albumin that can generate spurious signal spikes and sensitivity of luminol to pH changes are potential variables that influence ROS production. Other factors affecting ROS measurement are: (i) luminometer calibration; (ii) sensitivity and the dynamic range as well as the units used; (iii) concentration and the type of probe used; (iv) concentration and the volume of semen used; and (v) temperature of the instrument at the time of measurement.

This test effectively differentiates between fertile and infertile population using ROS cutoff values. ROS levels >102 RLU/sec/10^6 sperm are considered abnormal. At this cutoff, ROS sensitivity is 76.4%, specificity of 53.3% with a positive predictive value of 82.1% and a negative predictive

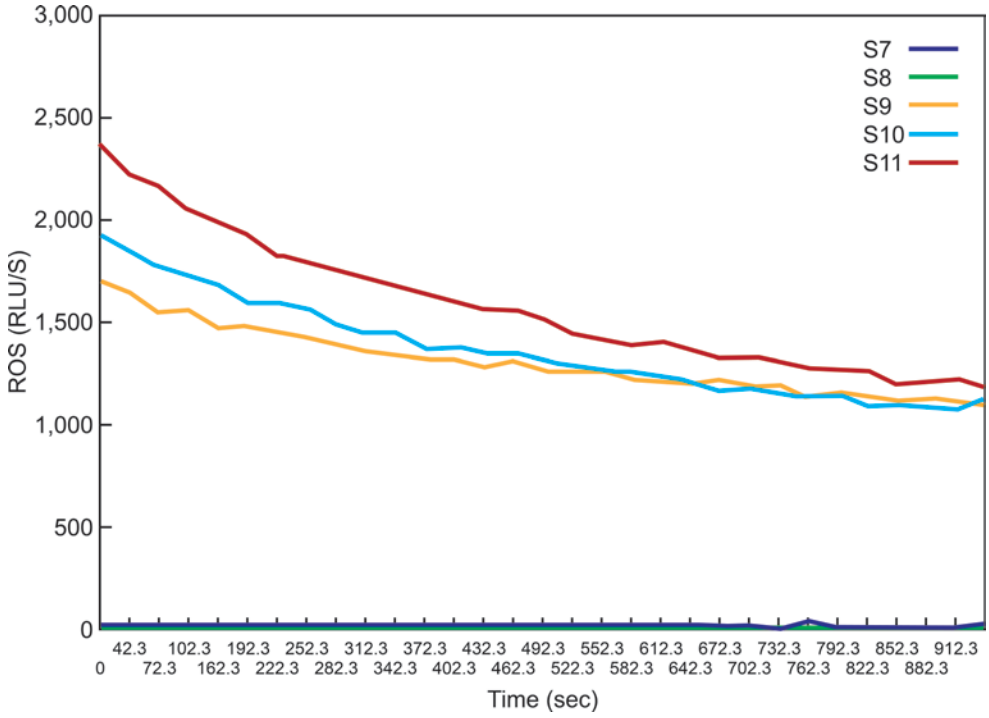


Fig. 7.4 A typical graph showing the ROS levels in the 11 tubes (S1–S11). As seen here, only the positive controls have significantly higher levels of ROS. Those producing low levels (Tubes S1–S8) of ROS are seen very close to the X axis. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2013–2019. All Rights Reserved)

value of 44.5% in differentiating infertile men from controls [81]. When the controls were strictly comprised of individuals who had established pregnancy, the cutoff was slightly lower at <93 RLU/sec/ × 10^6 sperm. The sensitivity increased to 93.8% indicating that the test can differentiate subjects that are fertile from those that are not. Levels of ROS >102 RLU/sec/ × 10^6 sperm must be considered pathological [7].

The major advantages of this approach include high specificity and sensitivity and its ability to measure global ROS, i.e., both intracellular as well as extracellular [81]. The major disadvantages are: (i) it cannot measure multiple markers simultaneously and (ii) highly time sensitive as the levels of ROS decline with the time after ejaculation due to short half-life of ROS.

7.3.4 Measurement of Intracellular ROS

Intracellular levels of ROS can be measured by flow cytometry using dihydrofluorescein diacetate (DCFH) to detect intracellular hydrogen peroxide radicals. This dye is oxidized to the highly fluorescent derivative dichlorofluorescein (DCF), which is detected by the use of a flow cytometer [11, 92, 93]. A counterstain dye for nucleic acid (propidium iodide) is used to exclude apoptotic spermatozoa [72]. Dihydroethidium (DE) can be used to detect intracellular levels of superoxide anions [11, 72, 92, 94]. The results are interpreted as percentage of fluorescent spermatozoa [72].

7.3.5 Measurement of DNA Fragmentation

Several tests have been introduced to measure the sperm DNA damage [7, 19, 20, 66, 95–103]. The methodological approaches by which sperm DNA damage is investigated in these tests are varied. Some tests measure abnormalities in sperm chromatin, whereas others measure direct DNA strand fragmentation. Among such tests, the most commonly used to measure DNA fragmentation are the sperm chromatin structure assay (SCSA) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), comet, and sperm chromatin dispersion assay [71, 73, 97, 99, 104–106]. The advantages and disadvantages of various tests of sperm DNA fragmentation are described in Table 7.2, whereas individual test details are described below.

7.3.6 Sperm Chromatin Structure Assay (SCSA)

Sperm Chromatin Structure Assay (SCSA) detects damaged sperm DNA using flow cytometry of acridine orange stained sperm. It is based on the susceptibility of DNA breaks to acid denaturation [66, 107, 108]. Low pH treatment opens the DNA strands at the sites of breaks. Staining by acridine orange is highly precise, repeatable, and comparable between fresh and frozen samples. The DNA damage is induced by exposing it to denaturing conditions. This

Table 7.2 Tests for measurement of DNA fragmentation

Assay	Advantages	Disadvantages
SCSA		
For single- and double-stranded DNAs 1. Mild acid treatment denatures DNA with single-strand or double-strand breaks 2. Acridine orange binds to DNA double-stranded DNA (nondenatured) fluoresces green, single-stranded DNA (denatured) fluoresces red 3. Flow cytometry counts 10,000 cells. 4. DNA fragmentation index (DFI)—the percentage of sperm with a ratio of red to (red + green) fluorescence greater than the main cell population	1. Direct and objective 2. Established clinical thresholds 3. Many cells rapidly examined 4. High repeatability 5. Fresh or frozen samples 6. Most published studies and is reproducible	1. Proprietary method 2. Not available in commercial kits 3. Expensive equipment 4. Acid-induced denaturation 5. Small variations in lab conditions affect results 6. Calculations involve qualitative decisions 7. Very few labs conduct this assay
COMET		
For single- and double- stranded DNA 1. Electrophoresis of single sperm cells 2. DNA fragments form tail 3. Intact DNA stays in head *Alkaline COMET* 1. Alkaline conditions, denatures all DNA 2. Identifies both single- and double-strand breaks *Neutral COMET* 1. Does not denature DNA 2. Identifies double breaks	1. Indirect assay, subjective 2. Poor repeatability 3. High sensitivity 4. Fresh samples only 5. Correlates with seminal parameters 6. Small number of cells required 7. Versatile (alkaline or neutral)	1. Variable protocols 2. Unclear thresholds 3. Not available in commercial kits 4. Time- and labor-intensive 5. Small number of cells assayed 6. Subjective 7. Lacks correlation with fertility 8. Requires special imaging software
SCD test		
1. Individual cells immersed in agarose 2. Denatured with acid then lysed 3. Normal sperm produce halo	1. Easy 2. Can use bright-field microscopy	1. Thresholds for SCD are not clearly established for men with unexplained infertility 2. Low-density nucleoids are faint and produce less contrasting images 3. Few studies have shown correlation between sperm DNA damage and ART outcome 4. Cannot discriminate the type of DNA fragmentation or quantify the amount of DNA damage at the spermatozoa level
TUNEL		
1. Adds labeled nucleotides to free DNA ends 2. Individual template 3. Labels single- and double-strand breaks 4. Measures percent cells with labeled DNA	1. Direct objective 2. Performed on few sperm (10,000) 3. High repeatability 4. Objective, high sensitivity (flow cytometry) 5. Fresh or frozen samples 6. Indicative of apoptosis 7. Correlates with semen parameters 8. Associated with fertility 9. Available in commercial kits	1. Thresholds not standardized 2. Variable assay protocols 3. Not designed specifically for spermatozoa 4. Need for special equipment (flow cytometer) 5. Template independent 6. Requires proper controls

SCSA sperm chromatin structure assay, *TUNEL* terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), *SCD* sperm chromatin dispersion

test utilizes the metachromatic properties of acridine orange to distinguish single-stranded/red fluorescence and double-stranded/native DNA/green fluorescence [66, 72, 103]. The results of this test are expressed as DNA fragmentation index (DFI) which is the ratio of percentage of sperms showing red fluorescence/total fluorescence (red + green) [11, 66]. The SCSA also measures sperm with high DNA stainability (%HDS) which is related to the nuclear histones retained in immature sperm and shown to be predictive of pregnancy failure [103]. The current clinical threshold is 25% DFI, which categorizes patients into a statistical probability of the following: (a) longer time to natural pregnancy, (b) low odds of IUI pregnancy, (c) more miscarriages, or (d) no pregnancy [103]. The test is precise and repeatable with an acceptable DNA fragmentation threshold which places a man at risk of infertility.

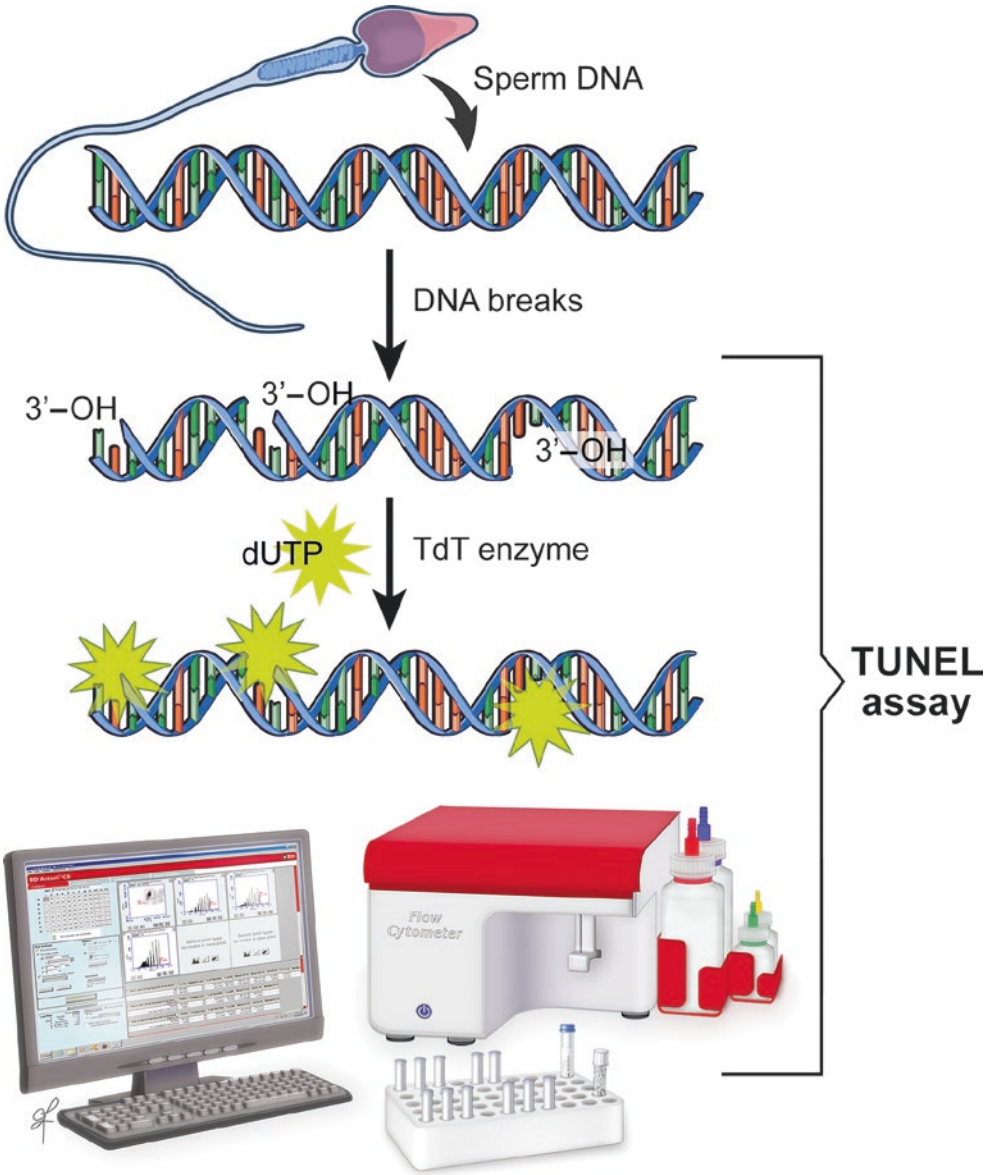


Fig. 7.5 Schematic of the DNA staining principal by the TUNEL assay. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2013–2019. All Rights Reserved)

7.3.7 Terminal Deoxynucleotidyl Transferees dUTP Nick End Labeling (TUNEL) Assay

The TUNEL assay utilizes a template-independent DNA polymerase called terminal deoxynucleotidyl transferase (TdT) that non-preferentially adds deoxyribonucleotides to 3′ hydroxyl (OH) single- and double-stranded DNA. Deoxyuridine triphosphate (dUTP) is the substrate that is added by the TdT enzyme to the free 3′-OH break-ends of DNA (Fig. 7.5) [23, 99, 109].

The more DNA strand breaks sites are present, the more labels are incorporated within a cell. This identifies the in situ DNA breaks. Here, the 3′ hydroxyl-free ends are labeled using a fluorescent label which on passing through a flow cytometer generates fluorescence, which is directly proportional to the number of strand breaks [99, 110]. DNA damage can be detected via TUNEL assay such as fluorescein isothiocyanate labeled dUTP system and Apoptosis detection kit. The reagents used in the assay are shown in Fig. 7.6. DNA fragmentation can be measured by a bench top flow cytometer. About 2.5 × 10^6 sperm are fixed and permeabilized with 3.57% paraformaldehyde. Staining is carried out for 1 h at 37 °C in the dark followed by rinsing and staining with propidium iodide. Both assay kit controls (positive and negative controls), as well as internal controls of known DNA fragmentation are included along with the test samples. TdT is omitted from the negative controls and positive controls are treated with hydrogen peroxide 2% at 50 °C for 1 h. According to the acquisition and alignment strategy, the percentage of DNA fragmentation in each sample is calculated.

Fig. 7.6 Staining reagents for the TUNEL staining using the Apodirect detection kit. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2013–2019. All Rights Reserved)

This test is highly sensitive and specific [99, 110]. It measures a definite end point and is considered to provide better prediction regarding the potential of embryo implantation [23, 70, 71, 79, 104, 111] .

Studies have reported DNA fragmentation measured by TUNEL assay to range from 12% to 36.5% at which no pregnancies were reported by microscopy and flow cytometry [99, 109, 112–118]. A cutoff value of 16.7% has been recently shown to have 91.6% specificity and positive predictive value of 91.4% [99]. The specificities can be further increased by including only men with established pregnancies as controls. The high specificity and positive predictive value is important particularly in cases of idiopathic and unexplained infertility.

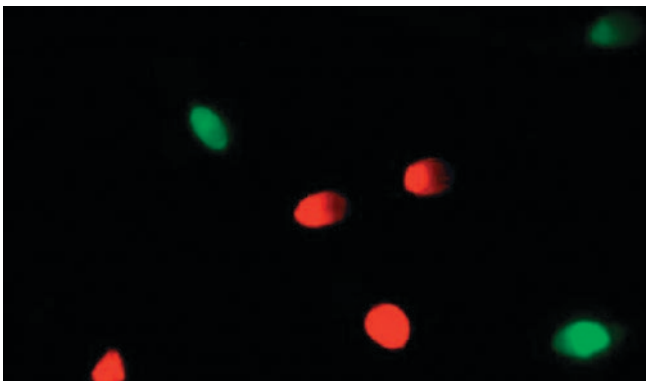


Fig. 7.7 Fluorescent staining showing intact (red) spermatozoa and spermatozoa with DNA fragmentation (green). (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2013–2019. All Rights Reserved)

7.3.8 Epifluorescence Using Acridine Orange Dye

Acridine orange is a nucleic-acid-specific, fluorescent, cationic dye. It interacts with DNA by intercalation and by electrostatic interaction with RNA or single-stranded DNA. Exposure to acid in a pre-incubation step denatures labile DNA sites with single- or double-stranded breaks, to which acridine orange intercalates leading to either red or green fluorescence, respectively, after excitation with ultraviolet light (Fig. 7.7). Sperm stained green indicate sperm with DNA fragmentation. This method is relatively rapid, simple, and inexpensive. The major disadvantage of this technique is the heterogeneous staining and color fading of the slides.

7.3.9 Comet Assay

The comet assay is a single gel electrophoresis method which measures the breaks in DNA [119]. Electrophoresis is used to mobilize DNA fragments that are produced from nucleoids after being depleted of proteins. The method is based on the general concept that DNA fragments resulting from preexisting DNA breaks have different mobility in the electrophoretic field depending on the relative size of the fragment. This generates morphological differences between nuclei containing fragmented DNA when examined under fluorescent microscopy. The resulting image represents a "comet" that consists of a head and a tail chromatin in the direction of the anode. The larger the size of the comet, the higher the level of DNA fragmentation [95, 119–122].

The assay can be performed in both neutral as well as alkaline environments. In neutral buffer, double-stranded DNA damage is measured, while in alkaline environment, DNA can be denatured; both single- (SS) and double-stranded (DS) DNA damage can be measured due to unwinding of the DNA strands [123, 124]. Under the influence of an electric field, broken DNA strands are separated (SS and DS) [122, 125–128]. Thus, sperm with more DNA breaks showing intense comet tails can be measured using the fluorescence microscope or cytometer [95, 124]. The comet's tail length and fluorescent intensity is directly proportional to the degree of DNA fragmentation [71, 122, 129].

7.3.10 Sperm Chromatin Dispersion (SCD) Assay

The sperm chromatin dispersion (SCD) assay uses the Halosperm kit to differentiate between non-fragmented from fragmented sperm DNA [95]. It is based on a controlled species-specific DNA denaturation to produce single-stranded DNA stretches from any DNA breaks, coupled with controlled DNA depletion [101, 130–133]. This test is usually used in laboratories without access to flow cytometry as it can be evaluated using bright field or fluorescence microscopy. The process involves (1) integration of sperm sample into an inert agarose microgel on pretreated slide, (2) controlled acid denaturation of DNA, and (3) controlled protein depletion.

Normal sperm produce halos of dispersed chromatin around a dense core. Contrary, fragmented DNA does not produce halos of dispersed chromatin [76, 132, 134]. The halos can further be classified according to their morphology, and the results can be expressed for each patient against the established cutoff criteria (Fig. 7.8). The results show a strong correlation when compared with indirect assessments of DNA damage such as the SCSA or the comet assay [95, 135].

7.4 Measurement of Total Antioxidant Capacity

The total antioxidant capacity (TAC) is a parameter that can be measured by evaluating the reducing ability of various antioxidants present in semen against an oxidative reagent, such as hydrogen peroxide, and measuring the effect on the substrate [136, 137]. Clear seminal plasma is used for the assay. The reaction can be measured with a spectrophotometer or colorimeter depending on the substrate. Most techniques employed to estimate TAC measure the low molecular weight, chain-breaking antioxidants and do not include the contribution of antioxidant enzymes (glutathione group of enzymes, catalase, and superoxide dismutase) and metal-binding proteins. TAC can be measured in two ways:

7.4.1 Colorimetric Analysis

The antioxidant assay is based on the principle that aqueous and lipid antioxidants in seminal plasma specimens inhibit the oxidation of the 2,20-Azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) to $ABTS^{+}$. Under the reaction conditions used, the antioxidants in seminal plasma cause suppression of the absorbance at 750 nm to a degree that is proportional to their concentration to produce a stable blue–green color [136–138]. The capacity of the antioxidants present in the sample to prevent ABTS oxidation is compared with that of standard Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid), a water-soluble tocopherol analogue.

Ten microliters of metmyoglobin and 150 μL of chromogen are added to all standard/sample wells. The reaction is initiated by adding 40 μL of hydrogen peroxide as quickly as possible. The plate is incubated for 5 min at room temperature on a horizontal shaker and absorbance is monitored at 750 nm using a microplate reader. The total antioxidant concentration of each sample can be calculated using the equation obtained from the linear regression of the standard curve by substituting the average absorbance values for each sample into the equation:

$$\text{Antioxidant}\left(\mu M\right) = \frac{\text{Unknown average absorbance} - \text{Y-intercept} \times \text{dilution} \times 1000}{\text{Slope}}$$

Results are reported as micromoles of Trolox equivalent. A diagnostic cutoff value of 1947 mM Trolox in seminal plasma distinguishes infertile patients from healthy men with a sensitivity of 59.5% and specificity of 63.0% [138].

7.4.2 ROS-TAC Score

There is a novel parameter derived from the ROS concentration and seminal TAC values called ROS-TAC score. It is superior than the individual ROS and TAC alone as it is capable of discriminating fertile from infertile men [139]. ROS and TAC values from controls are used to create a scale of these two variables using controls as reference point [14, 140, 141]. Both values are normalized for distribution after converting ROS to the log of ROS +1. Both log ROS +1 and TAC are

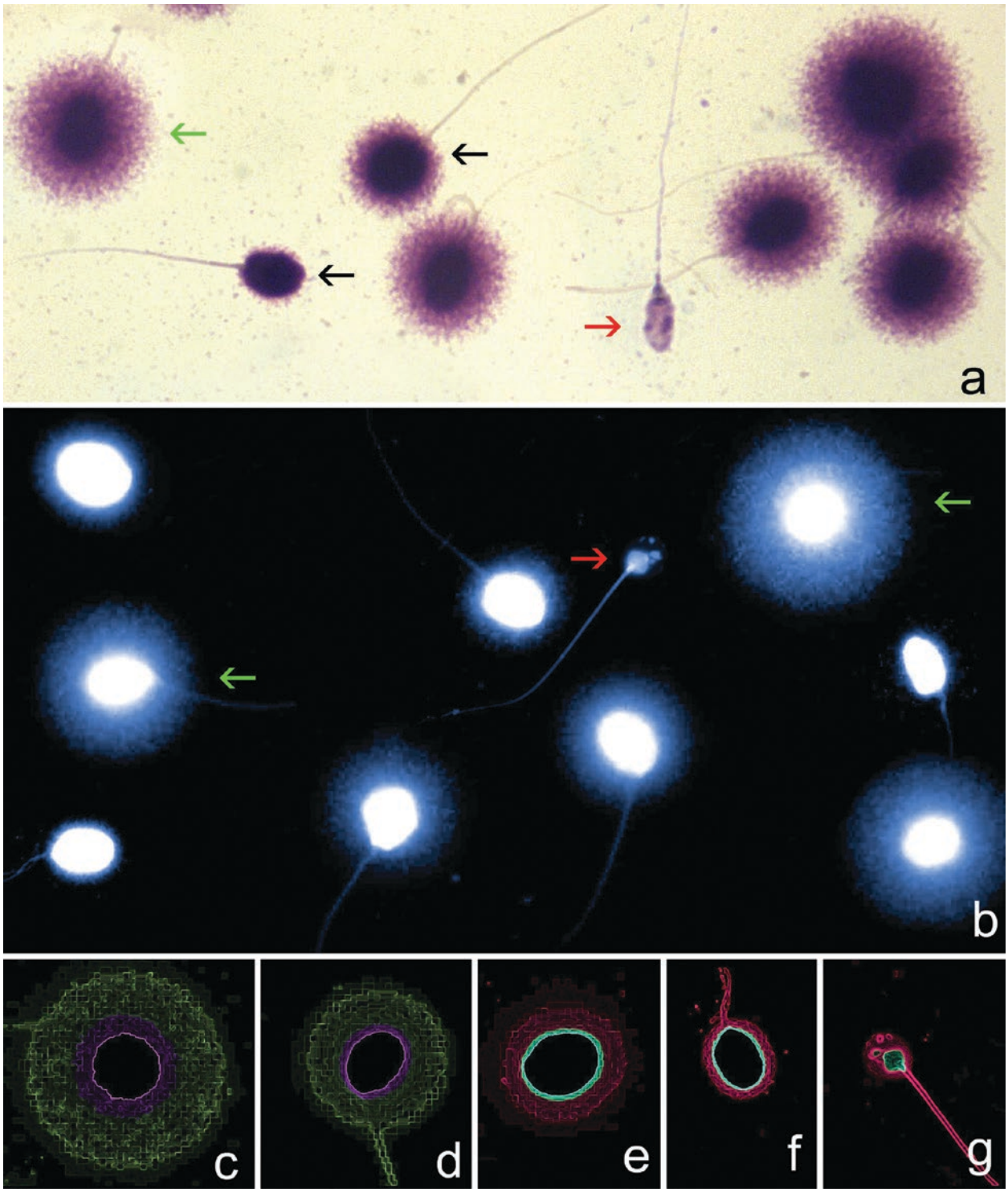


Fig. 7.8 Assessment of sperm DNA fragmentation using the sperm chromatin dispersion (SCD) test. Nucleoids from human spermatozoa obtained with the improved SCD procedure (Halosperm, Halotech DNA, SL, Madrid, Spain) under (**a**) bright field microscopy and Wright's stain (**b**) under fluorescence microscopy and DAPI staining. Green arrows target spermatozoa containing a normal DNA molecule. Red arrows target a highly fragmented spermatozoon (degraded sperm). (**c**–**g**) Electronic filtered images showing a series of nucleoids with different levels of sperm DNA damage. Nucleoids with highlighted core delineation in green correspond to (**c**) large (**d**) and medium halos of dispersed chromatin representing a normal DNA molecule. Nucleoids in red are spermatozoa containing fragmented DNA and are represented by (**e**) small or (**f**) no halos of dispersed chromatin and (**g**) degraded spermatozoa. Bright-field and fluorescence microphotographs were obtained using a motorized fluorescence microscope controlled with software for automatic scanning and image digitization (Leica Microsystems, Barcelona, Spain). The microscope was equipped with a Leica EL6000 metal halide fluorescence light source and Plan-Fluotar 60 × objectives with three independent filter blocks (DAPI-5060B; FITC- 3540B and TRITC-A; Semrock, Rechestern NY, United States). A charge coupled device (Leica DFC350 FX, Leica Microsystems, Barcelona, Spain) was used for image capture. (Courtesy of Prof. Jaime Gonsálvez, Madrid, Spain)

Table 7.3 Various techniques to measure lipid peroxidation

Technique	Principle	Advantage	Disadvantage
Thiobarbituric acid assay (TBARS)	MDA-TBA adduct detection by colorimetry or fluoroscopy	Simple but non-specific	Rigorous controls are required
Isoprostane	EIA/liquid chromatography-tandem mass spectrometry	Specificity, stable compound	Labor-intensive and high expense of equipment
HNE-his adduct ELISA	ELISA	Rapid, helps in quantification	Chances of cross reactivity

MDA malondialdehyde, *TBA* thiobarbituric acid, *HNE-His* hydroxynonenal histidine

standardized to Z scores (mean = 0, SD = 1) so that both will have the same variability. Standardized scores are calculated by subtracting the mean value of the controls from the individuals observed values and dividing by the standard deviation of the control population. The standardized values of ROS and TAC are analyzed with principal component analysis, which provides linear combinations or weighted sums that account for most variability among correlated variables [14]. Infertile men with male factor or idiopathic diagnoses were reported to show low ROS-TAC scores. Infertile men with higher ROS-TAC scores were able to initiate pregnancies compared to those who had low scores and had failed pregnancy [14].

7.5 Measurement of Lipid Peroxidation

Measurement of the end products of lipid peroxidation is a widely accepted marker of oxidative stress. Some of the common methods to measure lipid peroxidation are described below:

7.5.1 TBARS Assay

Malondialdehyde (MDA) is a reactive compound formed as one end product of lipid peroxidation [142]. The thiobarbituric acid (TBA) assay is one of the commonly used tests to assess MDA concentrations. The assay detects TBA-reactive substances (TBARS) via spectrophotometry, high performance liquid chromatography (HPLC), colorimetry, or spectrofluorescence [143]. In this reaction, which is carried out in acidic conditions and under a high temperature (90–100 °C), MDA and TBA react together to form an MDA–TBA adduct. This adduct formation is measured colorimetrically at 530–540 nm or fluorometrically at an excitation wavelength of 530 nm and an emission wavelength of 550 nm [144]. Lipid peroxidation in sperm is expressed as nmol MDA 10^{-7} sperm [145].

7.5.2 4-Hydroxynonenal-Histidine Adduct ELISA Assay

The 4-Hydroxynonenal-Histidine (HNE-his) adduct ELISA assay is a method to detect HNE bound to proteins using ELISA assay. This immunoassay has been developed for quantification and rapid detection of HNE-His protein adducts. 4-HNE can react with lysine, histidine, or cysteine residues in protein to form adducts. A 96-well ELISA titer plate is taken and protein samples (10 μg/mL) or BSA standards 0-200 μg/mL are adsorbed onto it. The HNE-protein adducts present in the standard or sample are probed with a primary antibody (anti-HNE-His antibody). This is followed by addition of a HRP-conjugated secondary antibody. Standard curves prepared from predetermined HNE-BSA standards are used to compare and quantify the HNE-protein adduct content in an unknown sample. Using a primary wavelength of 450 nm, the absorbance of each well is read on a microplate reader. Results are expressed as pmol/mg [146].

7.5.3 Isprostane (IsoP) Method

Another important biomarker of lipid peroxidation is 8-isoprostane (IsoP) measured as ng/mL [147]. It is a specific end product belonging to the family of eicosanoids derived from nonenzymatic peroxidation of polyunsaturated fatty acids [37]. The advantages of using the IsoP marker include the fact that it is not produced by enzymatic pathways like cyclooxygenase and lipoxygenase pathways of arachidonic acid, its stability and ease of quantification in seminal plasma [147]. The advantages and disadvantages of various lipid peroxidation methods are shown in Table 7.3.

7.6 Measurement of ROS-Induced Post-translational Modifications

Reactive oxygen species can modify proteins resulting in altered functions such as activation or inhibition of transcription factors, signal transducers, and enzymes [148, 149]. These result in alterations in structural and functional integrity of specific proteins. Most of the oxidants will react with several amino acids to yield multiple products. Three principal type of post-translational modification of proteins are induced by ROS and consist of S-glutathionylation (GSS-R), carbonylation, and nitrotyrosine modifications (Nitro-Y). Carbonylation, nitration, and thionylation of proteins are

regarded as the most common post-translational modifications leading to dysfunction of proteins [149–151].

Reactive carbonyls are produced by direct protein oxidation (oxidation on Trp, Lys, Arg, Pro, and Thr) with low- or high-molecular weight dicarbonyls (modifications of Lys, Arg, and Cys) generated during lipid peroxidation and glycoxidation, and oxidative degradation of Amadori products. Products of oxidation such as protein carbonyls are useful for detection and in estimation of ROS levels in a semen sample [150]. Protein carbonyls are chemically stable, more reliable, and a frequently used marker for protein oxidation [150, 152].

ELISA can measure both, nitrosylation and carbonylation. For detection or quantification of carbonyl modification of spermatozoa proteins, BSA standards or protein samples (10 µg/mL) are adsorbed onto a 96-well plate for 2 h at 37 °C. The protein carbonyls present in the sample or standard are derivatized to DNP hydrazone and probed with an anti-DNP antibody, followed by an HRP-conjugated secondary antibody. The protein carbonyl content in an unknown sample is determined by comparing with a standard curve prepared from a predetermined reduced and oxidized BSA standard.

Nitrotyrosine is formed by the reaction of peroxynitrite or donors of NO• with tyrosine residues. It can be produced by sperm cell by reaction of superoxide with NO•. The nitrotyrosine protein modification can result in alteration of protein function or structure. Higher amounts of nitrotyrosine were found in patients with impaired motility (asthenozoospermia) or spermatic cord blood in varicocele patients [150–155].

A competitive ELISA kit can be used for nitrotyrosine quantitation. In this assay, the unknown protein nitrotyrosine sample or nitrated BSA standards is first added to a nitrated BSA pre-absorbed EIA plate. After a brief incubation, an anti-nitrotyrosine antibody is added, followed by an HRP-conjugated secondary antibody. The protein nitrotyrosine content in an unknown sample is determined by comparing it with a standard curve prepared from predetermined nitrated BSA standard.

7.7 Measurement of ROS-Induced Protein Alterations: Proteomic Analysis

Proteomics and bioinformatics tools can be utilized to understand alterations in proteins as a result of exposure of spermatozoa to reactive oxygen species or oxidative stress. It also helps in demonstrating how post-translational modifications such as phosphorylation, proteolytic cleavages, glycosylation, and mutations can bring about changes in the physiological functions of spermatozoa. Global change occurs in the proteomic profiles of human spermatozoa and seminal plasma under oxidative stress conditions [12, 156, 157].

Differential regulation of protein expression in infertile patients has been reported with variations in ROS level as evidenced by global proteomic profiling [12]. Both, spermatozoa and seminal plasma proteome influence fertilization and implantation in infertile men with various levels of ROS or oxidative stress [12, 156–158]. Most commonly employed techniques to understand sperm-specific proteins include 2D polyacrylamide gel electrophoresis (2D-PAGE), differential in-gel electrophoresis (DIGE), and liquid chromatography-mass spectrometry, or LC-MS/MS. Global proteomic analysis involves analysis of pooled or individual test samples (either spermatozoa or seminal plasma from semen samples of infertile men exhibiting oxidative stress). Seminal proteome, proteins involved in biomolecule metabolism, protein folding and protein degradation are differentially modulated in infertile patients exposed to low, medium, and high ROS [12]. Similarly, in the seminal plasma proteome, pathways involved in post-translational modification of proteins, protein-folding (heat shock proteins, molecular chaperones), and developmental disorder are overexpressed in the high ROS group compared with fertile control group [155, 158].

7.8 Limitations of Current Oxidative Stress Markers

All markers of oxidative stress such as measurement of ROS by chemiluminescence assay [81, 87, 159], antioxidants by colorimetric assay, [11, 136, 160–162], lipids by measuring oxidized products by thiobarbituric acid assay or 4-hydroxynonenal (4-HNE) [144, 146], apoptotic markers such as annexin V [163, 164], DNA fragmentation by TUNEL, SCSA, comet or SCD, and oxidatively modified protein alterations using proteomic tools [12, 81, 158] described above are individual methods. Each method captures only a single dimension of oxidative stress from which the overall oxidative stress status in a given biological system is generalized. Therefore, these methods fail to provide a complete picture of the true oxidative stress status environment. They only reflect the redox status at a single point. Furthermore, these methods are antiquated, time-consuming, and time-sensitive, requiring large financial investments such as measurement of ROS by chemiluminescence or proteomics. It is therefore important to identify a test that includes all constituents of OS and helps in our understanding of the real-time redox status in a given semen sample and thereby improve the management of these patients. This is accomplished by the redox potential marker called oxidation reduction potential described below.

7.9 What Is Oxidation Reduction Potential?

Measuring oxidation reduction potential (ORP), or redox potential, is the latest addition in the attempt to identify a single direct marker of oxidative stress that has diagnostic value especially in male infertility. ORP, also known as redox balance, provides the relative proportion of oxidants (ROS) and reductants (antioxidants) that are present in a given biological system at any given time. It is a measure of the potential of electrons to move from one chemical species to another. In an attempt to quench the damaging effects of oxidants, antioxidants donate electrons thereby reducing the chances of oxidants to acquire electrons from adjoining cells and cause damage. This test was initially used to measure ORP in water treatment industry to determine if the oxidant activity was sufficiently high to kill bacteria and other microbes [165]. Later, it was used to measure ORP in biological fluids, such as blood plasma of patients with brain injury, trauma, and stroke [166–170]. ORP testing was introduced in the andrology setting as a reliable marker of OS in the human semen in 2016 [171, 172].

7.9.1 Measurement of Oxidation Reduction Potential Using MiOXSYS

The development of the *M*ale *i*nfertility *Ox*idative Stress *Sys*tem, or MiOXSYS, system and the measurement of ORP in semen samples represents an invaluable clinical tool that obviates the need for complicated oxidative stress assays. The MiOXSYS system is an instrument that overcomes the negative aspects of other more complicated and expensive methods to evaluate oxidative stress in semen samples.

ORP in human semen samples can be measured in real time by using the MiOXSYS system, a galvanostat-based technology [173]. The MiOXSYS system is designed to capture the transfer of electrons from reductants to oxidants in the sample and is based on the Nernst equation [174].

$$\mathrm{ORP} = \mathrm{E}^{\circ} - RT / nF \ \ln\left(\left[\mathrm{Red}\right] / \left[\mathrm{Ox}\right]\right)$$

where

E° = standard reduction potential
R = universal gas constant
T = absolute temperature
N = number of moles of electrons exchanged
F = Faradays constant
Red = concentration of reduced species
Ox = concentration of oxidized species

The MiOXSYS system is comprised of the MiOXSYS analyzer and the MiOXSYS sensor (Fig. 7.9a, b). The analyzer uses a constant negligible current and measures the passive exchange of electrons between the oxidants and reductants. This is also called the static or passive or current state of activity between the oxidants and antioxidants. Static ORP (hereafter referred as ORP) represents the actual redox balance in a given sample and is expressed as milliVolts (mV). A higher ORP is indicative of higher oxidative stress reflecting the imbalance in the redox system in favor of the oxidant activity. Monitoring ORP levels in semen may therefore help predict treatment efficacy in such patients. Higher ORP levels are indicative of the progression of infertility.

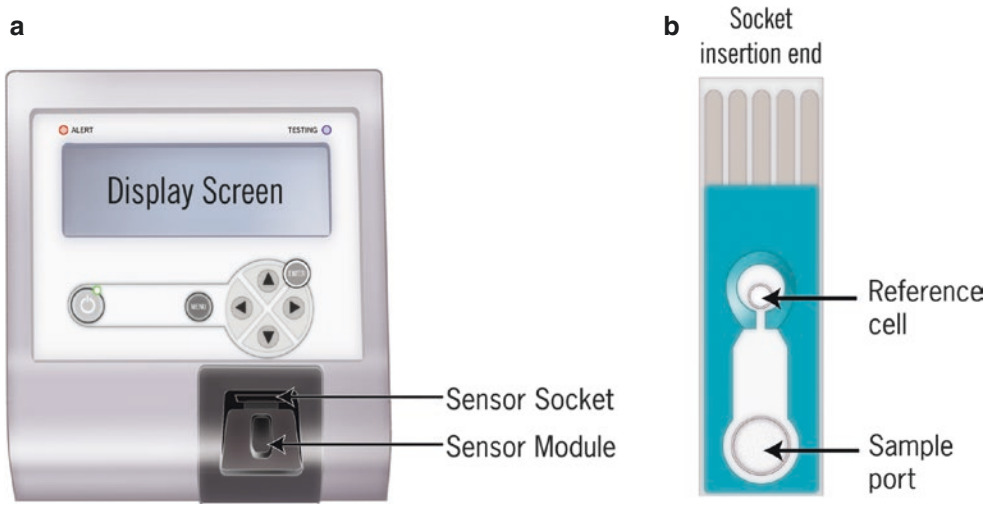


Fig. 7.9 Oxidation reduction potential can be measured by the MiOXSYS system comprised of (**a**) MiOXSYS Analyzer and (**b**) MiOXSYS Sensor. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2013–2019. All Rights Reserved)

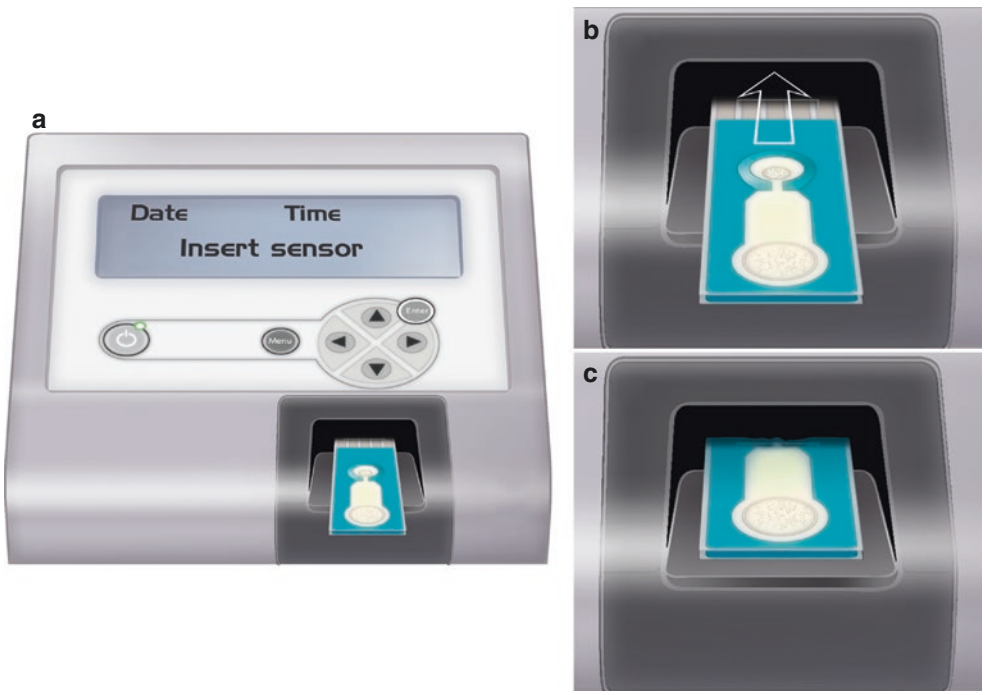
7.9.2 Protocol to Measure Oxidation Reduction Potential

To measure ORP in a given semen samples, a MiOXSYS sensor is placed on the MiOXSYS port with the sensor electrodes facing the analyzer (Fig. 7.10). Using a pipette, a small volume (~30 μL) of liquefied neat semen sample is added on the pre-inserted sensor (Figs. 7.11 and 7.12a–c). The results are normalized to express ORP in the sperm by dividing the ORP with the sperm concentration and expressed as milliVolts (mV)/10^6 sperm/mL ORP provides redox balance in real time, is less time consuming, is independent of sperm concentration, and requires less expertise than other laborious methods such as ROS [171, 172]. ORP has demonstrated excellent intra- and inter-observer reliability indicating that the measurements are highly reproducible and samples need not be tested in duplicate or triplicate [175].

Fig. 7.10 The MiOXSYS Analyzer is turned on and the sensor is gently placed on the sensor module. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2013–2019. All Rights Reserved)

Fig. 7.11 A 30 μL sample is carefully aspirated and loaded on the sensor sample port. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2013–2019. All Rights Reserved)

Fig. 7.12 (**a**–**c**) The sensor (**a**) after the sample is loaded and (**b**) gently inserted in the analyzer to make sure that (**c**) it is inserted all the way in the analyzer so that the electrodes make a connection with the analyzer. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2013–2019. All Rights Reserved)



7.9.3 Value of ORP in Fresh and Frozen Semen Samples and Seminal Plasma

The MiOXSYS system can measure ORP levels in semen and seminal plasma both in fresh and frozen samples [172, 176] and also not affected by the age of the semen [172]. Semen ORP levels were also correlated with those in the seminal plasma both at time 0 and at time 120 min. ORP levels in semen and seminal plasma showed strong negative correlation with semen parameters, especially concentration. Similar negative correlation was seen between ORP and sperm parameters at time 0 and 120 min. The fact that ORP levels in semen samples were stable and not affected by time up to 2 h makes the ORP technology easy to employ in a clinical setting [172]. ORP measurement can also be made in fresh and frozen semen samples [172].

7.9.4 Assessment of Semen Quality and Fertility Status Using Oxidation Reduction Potential

ORP levels measured in semen from male partners of infertile couples suggest that ORP levels are significantly lower in infertile men that have normal individual semen parameters [177].

Agarwal et al. examined 106 infertile and 51 healthy men who were categorized into normal and abnormal based on the semen parameters according to the 2010 WHO guidelines [178, 179]. A strong negative correlation was seen between ORP and semen parameters both in controls and patients combined especially sperm concentration ($r = -0.823$; $P < 0.001$); total sperm count ($r = -0.728$; $P < 0.001$); and motility ($r = -0.048$; $P < 0.001$) as well as between ORP and controls only for sperm concentration ($r = -0.688$; $P < 0.001$); total sperm count ($r = -0.540$, $P < 0.001$); and motility ($r = -0.255$; $P < 0.001$). Higher ORP levels were seen in patient group and they were inversely correlated with sperm concentration ($r = -0.846$; $P < 0.001$); total sperm count ($r = -0.765$; $P < 0.001$) motility ($r = -0.445$; $P < 0.001$); and morphology ($r = -0.226$; $P < 0.020$) [175].

In a study comprising of 293 infertile men and 15 fertile men, ORP was highly significantly ($P < 0.0001$) and negatively correlated with sperm concentration ($r = -0.840$); motility ($r = -0.429$); and weakly correlated with morphology ($r = -0.288$) [176]. Arafa et al. [180] examined 365 infertile men and 50 fertile men for semen parameters and ORP levels. These authors reported significantly higher ORP levels in infertile men with abnormal semen parameters (total sperm count, motility/morphology) compared with those of normal semen parameters. Furthermore, infertile men had higher ORP values (Mean ± SE; 5.00 ± 0.56 mV/10^6 sperm/mL) compared to fertile donors (1.26 ± 0.15) ($P < 0.001$). ORP was negatively correlated with sperm concentration ($r = -0.909$; $P < 0.001$); total sperm count ($r = -0.799$; $P < 0.001$); and motility ($r = -0.554$; $P < 0.001$) [180].

Furthermore, Agarwal and Wang examined 194 infertile men, including 28 who underwent repeated semen analysis and ORP measurement; they reported that patients with oligozoospermia appeared to have the highest ORP levels compared to those with asthenozoospermia or teratozoospermia [177]. These authors also reported that infertile men ($n = 42$) who had normal semen parameters had significantly lower ORP values [0.74 (0.41, 1.3)] compared to 152 infertile men who exhibited at least abnormal semen parameter [3.76 (1.19, 16.4); $P < 0.001$] [177]. Patients who had significantly poorer sperm parameters and higher ORP levels on the first evaluation showed significant improvement in sperm concentration and motility with a concomitant decrease in ORP after a repeat semen test was done 16.8 ± 7.7 weeks later [177].

In a recent study, Majzoub et al. examined semen parameters and ORP levels in 1168 infertile men and 100 fertile men [181]. They reported significantly poor sperm parameters in infertile men including count ($P < 0.001$); total motility ($P < 0.001$); total morphology ($P < 0.001$) compared to fertile men. Furthermore, the ORP levels were significantly higher in infertile men [mean ± standard error, (5.44 ± 0.34) compared with fertile men (1.18 ± 0.94) ($P < 0.001$). Similarly, when various cutoff values for normal morphology (0% to >4%) were examined, ORP levels were highest (13.12 ± 1.84 mV/10^6 sperm/mL) in samples with 0% normal morphology compared to those with >4% normal morphology (1.99 ± 0.26 mV/10^6 sperm/mL) [181].

7.9.5 Oxidative Reduction Potential: Establishing Reference Values

Higher ORP values indicate a higher state of oxidative stress. Data from ORP studies conducted in healthy men and in infertile men [172, 177] reinforce the impression that oxidative stress is related to poor semen quality [7, 13, 54, 61, 141, 182, 183].

In the study reporting the reference values of ORP for the first time in semen and seminal plasma [172], a cutoff of 1.48 mV/10^6 sperm/mL and 2.09 mV/10^6 sperm/mL in seminal plasma was reported (Fig. 7.13a, b). At these cutoffs, ORP was also able to distinguish semen quality based on good (≥40%) and poor (≤40%) motility in semen and seminal plasma (Fig. 7.13c, d).

In a later study comprising of 106 infertile men and 51 healthy men with normal semen parameters, a cutoff of 1.36 mV/10^6 sperm/mL was able to distinguish normal

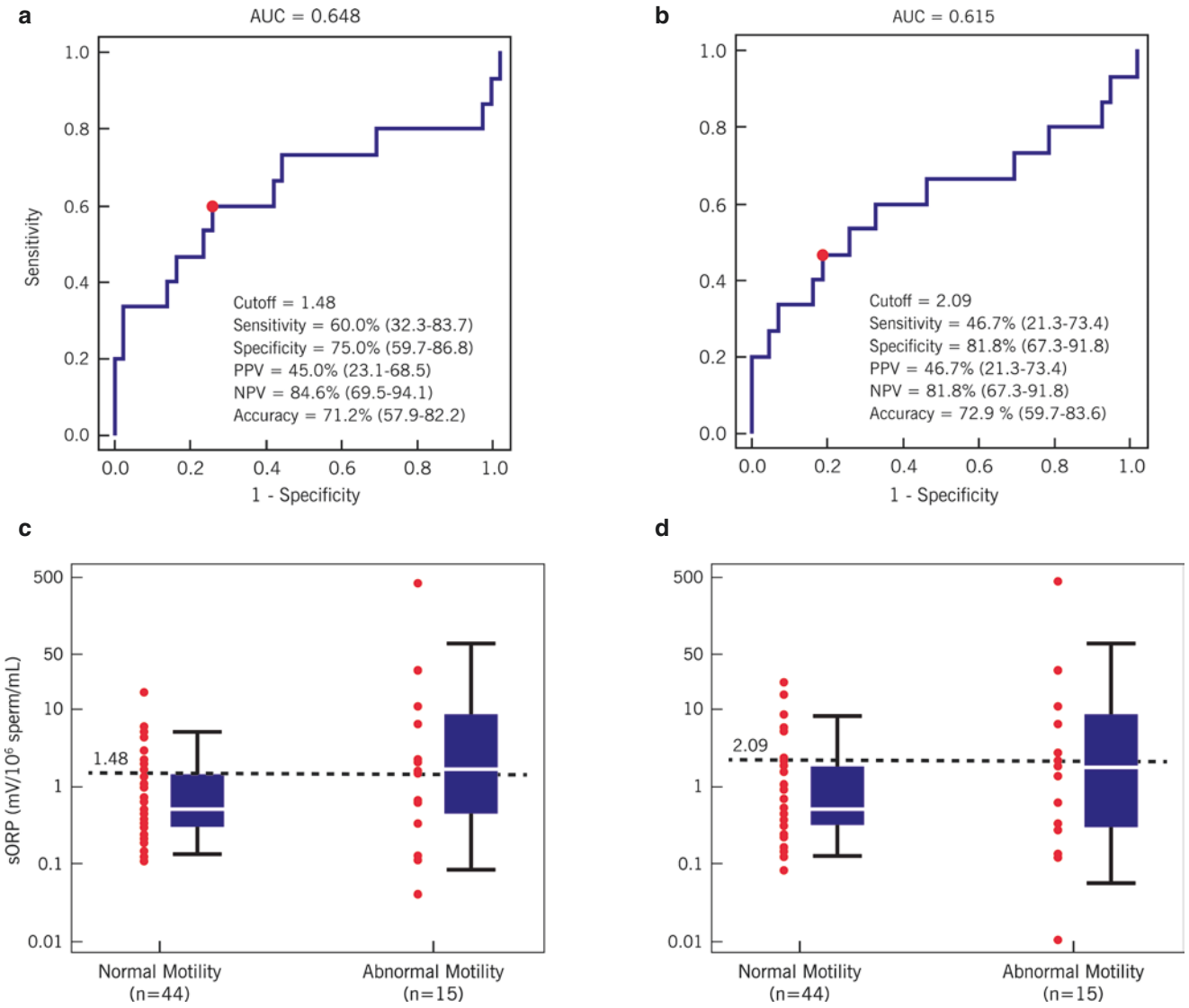


Fig. 7.13 ROC curve showing ORP cutoff in (**a**) semen and (**b**) seminal plasma. Distribution of ORP in subjects with normal and abnormal motility based on good (≥40%) and poor motility (≤40%) motility in (**c**) semen and (**d**) seminal plasma. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2013–2019. All Rights Reserved)

healthy men from infertile men with a sensitivity of 69.6% and specificity of 83.1% (Fig. 7.14) [175].

In a study by Agarwal et al. [176], ORP was measured in 293 patients and 15 controls and categorized according to WHO criteria as normo-, oligo-, astheno-, terato-, and oligoasthenoteratozoospermic. When subjects were grouped based on concentration, motility, morphology, or a combination of these, the area under curve for ROC curve, sensitivity and specificity, positive predictive value, and ORP cutoffs were significantly different. The ROC curves generated were able to differentiate between fertile controls and infertile men and other categories. The ORP cutoffs were also able to differentiate between subjects and any two semen parameters between various categories (Fig. 7.15).

In another study by [177], ORP at a cutoff of 1.57 (mV/10^6 sperm) was able to detect at least 1 abnormal sperm parameter with a sensitivity of 70.4% and specificity of 88.1% (Fig. 7.16a, b). Similarly, ORP at a cutoff of 2.59 (mV/10^6 sperm) had the highest predictive values in detecting oligozoospermia with 88% sensitivity and 91.2% specificity [177]. In another study by Majzoub et al. [181], comprising of 1168 infertile men and 100 men with confirmed fertility, using a cutoff of 4% normal morphology to determine the presence of teratozoospermia, Majzoub et al. showed that ORP cutoff of 1.73 mV/10^6 sperm/ mL had a sensitivity of 76%, specificity 72%, positive predictive value of 69.32%, and a negative predictive value of 78.6% with an accuracy of 73.9% [181].

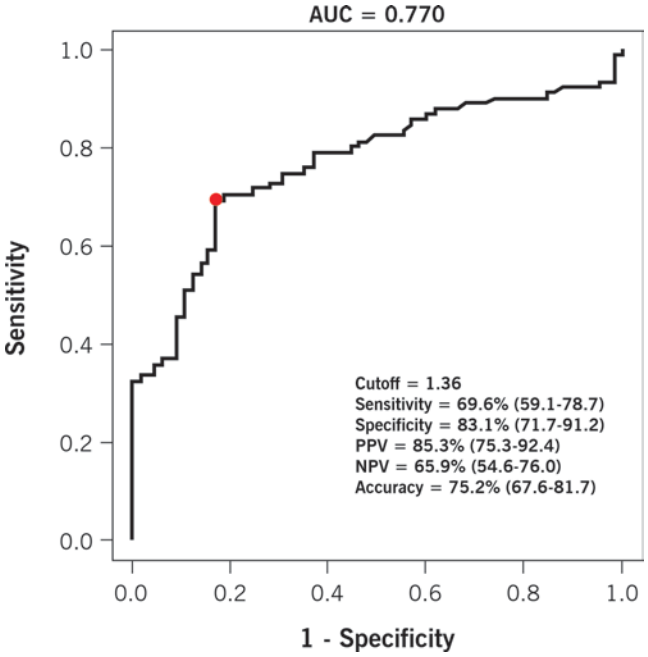


Fig. 7.14 Receiver operating curve showing a cutoff of 1.36 able to distinguish normal healthy men from infertile men. AUC area under the curve, NPV negative predictive value, PPV positive predictive value. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2013–2019. All Rights Reserved)

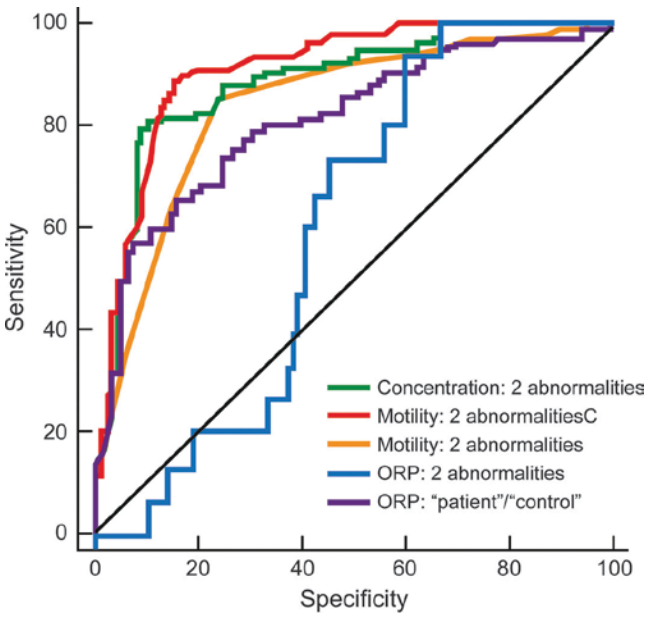


Fig. 7.15 Receiver operating curve showing sensitivity and specificity for sperm parameters with two abnormalities alone and ORP combined with two semen abnormalities as well as ORP in patients and controls. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2013–2019. All Rights Reserved)

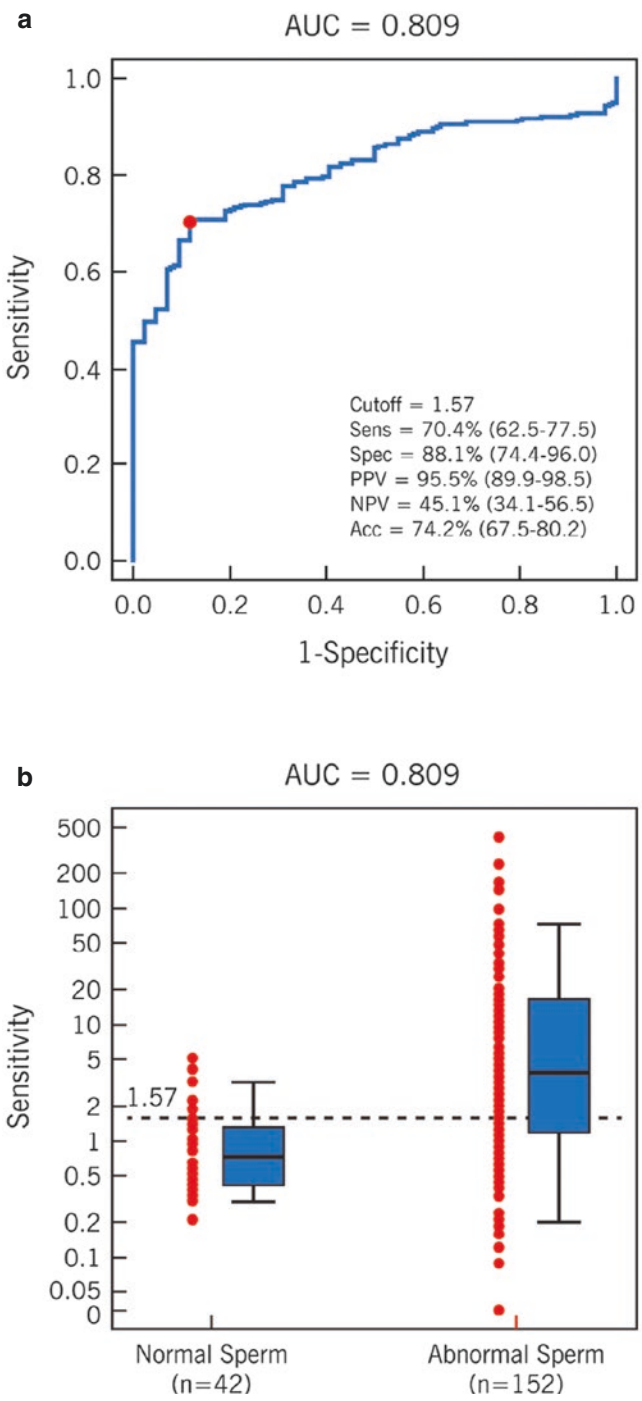


Fig. 7.16 Receiver operating characteristic curve of ORP (**a**) cutoff capable of detecting at least 1 abnormal sperm parameter. (**b**) Box-and-whisker plots showing the distributions of ORP cutoff between normal and abnormal sperm parameters. AUC area under the curve, NPV negative predictive value, PPV positive predictive value, Sens sensitivity, Spec specificity. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2013–2019. All Rights Reserved)

Arafa et al. [184] reported a cutoff of 1.38 mV/10^6 sperm/mL; sensitivity of 63.3%, specificity 87.8%, PPV 97.6% and NPV 23.2%, and accuracy of 66%. This cutoff was able to distinguish and identify semen samples with abnormal quality such as sperm motility, various morphological abnormalities, ORP, and sperm DNA fragmentation. The odds ratio that a semen sample with a sORP value greater than 1.38 mV/10^6/ml was abnormal in quality was 10.05 ($P < 0.001$) [184].

7.9.6 Update on Research on Oxidative Reduction Potential: Multicenter Findings

In a multicenter study [175] conducted in Cleveland Clinic, the United States, and the other in Doha, Qatar, semen analysis and ORP measurements were compared between the two laboratories. The U.S. dataset comprised of 194 patients and 51 fertile donors, while the Qatar dataset comprised of 400 patients and 50 fertile donors. In both individual datasets and combined datasets, infertile men had significantly lower sperm concentration, total and progressive motility and morphology. Furthermore, ORP levels were higher when compared to the fertile men ($P < 0.05$). The dataset from the United States identified ORP cutoff of 2.26 mV/10^6 sperm/mL and 1.42 mV/10^6 sperm/mL for the Doha data. The combined dataset showed a cutoff of 1.42 mV/10^6 sperm/mL in distinguishing infertile patients from healthy controls (Fig. 7.17a–c). The overall ROC characteristics with cutoff, sensitivity, specificity, area under curve, accuracy, positive predictive value, negative predictive value examined for all studies published on ORP is shown in Table 7.4. The findings show that although semen parameters showed significant differences between the two centers, ORP levels were consistent in both datasets individually or combined showing reproducibility and reliability of ORP [175].

7.9.7 Clinical Utility of Oxidation Reduction Potential in Male Infertility

Application of ORP measurement in clinical practice can benefit infertile men with varicocele, infection, inflammation, spinal cord injury, and severe oligozoospermia [178, 180, 181]. The ability to measure ORP in cryopreserved semen samples as a single marker for oxidants and the available antioxidant reserves is important as it can help in predicting the success of assisted reproductive techniques [185]. Combining sperm parameters with advanced tests, such as DNA fragmentation, can help recognize sperm with greater reproductive potential despite having abnormal morphology [181, 186]. This can further provide knowledge for developing novel sperm selection techniques that can be used during ART. ORP can also be used as a screening tool to identify patients who are more likely to benefit from various sophisticated tests and treatment [172, 181, 187].

7.9.8 Oxidation Reduction Potential: A Test Replacement or a Test in Conjunction with Semen Analysis?

All reported studies demonstrate that individual semen parameters are poor predictors of sperm infertility [188, 189]. It would be useful to use ORP in conjunction with sperm parameters to support the interpretation of standard semen analysis rather than as an independent marker. A significant overlap was reported in individual sperm parameters in infertile and fertile men [3]. Assessment of progressive motility is subjective, and its reliability as a fertility marker is questionable and both total and progressive motility were lower in proven fertile donors [190], suggesting a more complex relationship between motility and infertility.

ORP is a measure of oxidative stress, and oxidative stress affects sperm numbers, motility, and morphology [7, 54, 183, 191]; ORP is not influenced by semen age or BMI [184]. Changes in ORP reflect site-specific changes in oxidative stress as the testis is an immune privileged site [183, 192]. Therefore, elevation in oxidative stress at the site of spermatogenesis and maturation is more likely to translate into changes in semen and sperm quality. These changes may not be reflected in systemic alterations. Therefore, by focusing on the ability to measure small site-specific alterations in semen, ORP can help focus on the relationship between oxidative stress, semen quality, and male infertility.

7.9.9 Clinical Relevance of Combination of OS Markers and Other Sperm Function Tests

We have highlighted several recent studies that have measured ORP in relevance to sperm functional parameters. The findings point out the relationship between oxidative stress and semen parameters, specifically sperm count, motility, and morphology. Using receiver-operating characteristic curves, various ORP cutoffs have been reported that can help distinguish infertile from healthy men. Negative association of ORP with SDF has also been reported in high SDF groups (4.03 ± 0.61 mV/10^6 sperm/mL) versus low SDF groups (2.14 ± 0.14 mV/10^6 sperm/mL, respectively, $p < 0.001$) [180, 184]. A five-fold higher ORP was seen in the semen of infertile men when compared with the fertile controls ($P < 0.0001$). Head defects were significantly higher amongst infertile patients (54%) than fertile controls (48%)

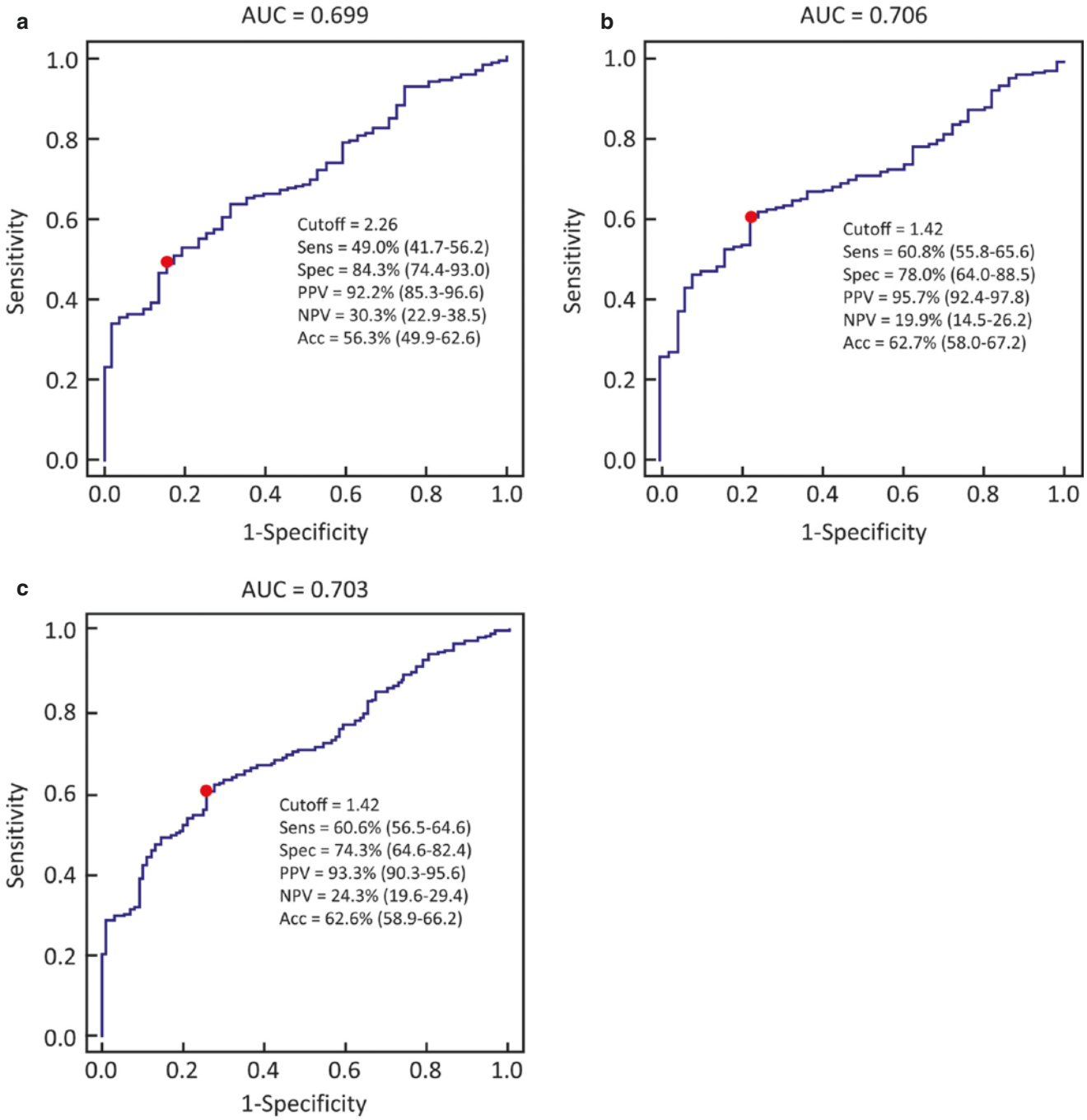


Fig. 7.17 Receiver operating characteristic of the oxidation-reduction potential in distinguishing infertile patients from healthy controls in (**a**) Cleveland Clinic, U.S. dataset, (**b**) Doha, Qatar dataset, and (**c**) combined dataset. AUC area under the curve, NPV negative predictive value, PPV positive predictive value, Sens sensitivity, Spec specificity, and Acc accuracy. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2013–2019. All Rights Reserved)

($P < 0.001$). Majzoub et al. [187] also reported the positive association of ORP with abnormal morphology, especially abnormal sperm heads ($P < 0.001$) [180, 181, 184]. Ayaz et al. [193] reported higher pregnancy rates in patients with low ORP (<1.36 mV/10^6 sperm/mL) than in those in a high ORP group (>1.36 mV/10^6 sperm/mL) ($p = 0.006$) [193]. In ART monitoring, ORP allows for better preparation and selection of sperm as it detects not only oxidative stress associated damage but also provides important clues on prognosis and an individual's clinical health. Higher ORP levels have also been reported in infertile men with varicocele that exhibited poor sperm parameters ($P < 0.001$) [175, 184,

Table 7.4 Review of studies examining the receiver operation characteristic curves to identify the ORP criteria that best predicts the normal and abnormal semen parameters as well as differentiates normal healthy controls from male factor infertility patients

No.	Reference	Study population	Cutoff	Sensitivity% (95% CI)	Specificity% (95% CI)	PPV% (95% CI)	NPV% (95% CI)	Accuracy% (95% CI)	AUC
1	Agarwal et al. [172]	Controls (n = 26); infertile men (n = 33)							
		Semen	1.48	60.0% (32.3–83.7)	75.0% (59.7–86.8)	45.0% (23.1–68.5)	84.6% (69.5–94.1)	71.2% (57.9–82.2)	0.648
		Seminal plasma	2.09	46.7% (21.3–73.4)	81.8% (67.3–91.8)	46.7% (21.3–73.4)	81.8% (67.3–91.8)	72.9% (59.7–83.6)	0.615
2	Agarwal et al. [171]	Control; n = 51 (proven fertile; n = 15 and unproven fertility; n = 36); Infertile men (n = 106; varicocele: n = 36; idiopathic: n = 13; unclassified: n = 55)	1.36	69.6% (59.1–78.7)	83.1% (71.7–92.4)	85.3% (75.3–92.4)	65.9% (54.6–76.0)	75.2% (67.6–81.7)	0.770
3	Agarwal and Wang [177]	Healthy control (n = 49); infertile men (n = 194) Categorized into three groups:							
		Oligozoospermic (n = 92)	2.59	88.0% (79.6–93.9)	91.2% (83.9–95.9)	90.0% (81.9–95.3)	89.4% (81.9–94.6)	89.7% (84.5–93.6)	0.754
		Asthenozoospermic (n = 102)	6.08	52.0% (41.8–62.0)	91.2% (83.4–96.1)	86.9% (75.8–94.2)	62.9% (54.0–76.8)	70.5% (63.5–76.8)	0.751
		Teratozoospermic (n = 95)	1.57	65.3% (54.8–74.7)	62.0% (50.4–72.7)	67.4% (56.8–76.8)	59.8% (48.3–70.4)	63.8% (56.2–70.9)	0.693
		Detecting at least one sperm parameter (n = 152)	1.57	70.4% (62.5–77.5)	88.1% (74.4–96.0)	95.5% (89.9–98.5)	45.1% (34.1–56.5)	74.2% (67.5–80.2)	0.809
4	Arafa et al. [180]	Fertile (n = 50); infertile (n = 365) Differentiate normal from abnormal semen quality	1.38	63.35%	87.8%	97.6%	23.2%	66%	–
		Fertile (n = 50); infertile (n = 365) Differentiate fertile from infertile samples	1.41	57.3% (52.0–62.4)	78.0% (64.0–88.5)	95.0% (91.2–97.5)	20.0% (14.6–26.3)	60%	–
5	Majzoub et al. [181]	Fertile (n = 100) Infertile (n = 1168) Differentiating normal (≥4%) from abnormal sperm morphology (≤4%)	1.73	76%	72%	69.2%	78.6%	73.9%	0/804
6	Agarwal et al. [175]	U.S. dataset Fertile control (n = 50); infertile men;(n = 194)	2.26	49.0% (41.7–56.2)	84.3% (74.4–93.0)	92.2% (85.3–96.6)	30.3% (22.9–38.5)	56.3% (49.9–62.6)	0.699
		Qatar dataset fertile control (n = 51); infertile men;(n = 400)	1.42	60.8% (55.8–65.6)	78.0% (64.0–88.5)	95.7% (92.4–97.8)	19.9% (14.5–26.2)	62.7% (58.0–67.2)	0.706
		Combined U.S. and Qatar dataset Fertile (n = 101); infertile men (n = 594)	1.42	60.6% (56.5–64.6)	74.3% (64.6–82.4)	93.3% (90.3–95.6)	24.3% (19.6–29.4)	62.6% (58.9–66.2)	0.703
7	Agarwal et al. [176]	Fertile controls (n = 15) Infertile men (n = 293) Able to differentiate control vs. patients	>2.63	40.4%	93.3%	99.2%	7.4%	–	0.596
		Oligozoospermic vs. non-oligozoospermic	>2.63	81.5%	92.7%	89.1%	87.2%	–	0.919
		Asthenozoospermic vs. non-asthenozoospermic	>5.2	46.9%	86.3%	75.3%	64.7%	–	0.685
		Teratozoospermic vs. non-teratozoospermic	>1.4	65.7%	65.3%	64.3%	66.7%	–	0.655
		Oligoasthenoteratozoospermic vs. non-oligoasthenoteratozoospermic	>5.3	69.0%	87.4%	58.8%	91.5%	–	0.822
		Subjects with any two semen abnormalities	>2.7	64.6%	83.9%	75.7%	75.4%	–	0.803

PPV positive predictive value, *NPV* negative predictive value, *AUC* area under curve

194]. When compared to infertile men with idiopathic infertility, varicocele patients had significantly lower sperm concentration, fewer morphologically normal sperm, and higher ORP levels ($P < 0.005$) [194]. Lastly, genitourinary infections are a major contributor of oxidative stress-induced male infertility. Sikka et al. [195] proposed monitoring of ORP as an indicator of oxidative stress in leukocytospermia as ORP levels were also shown to be similar to certain active inflammation biomarkers such as Toll-like receptor-4 and cyclooxygenase [195].

7.10 Future Directions

The current literature is convincing to recommend the use of ORP as an adjunct tool to basic semen analysis in all patients who present for infertility, as ORP can provide valuable information about sperm function and the fertilizing ability of the man. ORP can also be used as an important ancillary tool for male infertility evaluation and assessment of a patient's initial oxidative stress status. Additional studies, however, from other centers and increasing use of ORP in the evaluation of basic semen analysis will further help establish the clinical utility of ORP in the infertility evaluation. This can be beneficial in guiding clinicians with appropriate therapeutic interventions. The utility of ORP in monitoring patients before and after varicocelectomy, idiopathic infertility and after life style modifications needs to be investigated. Future studies should also aim to examine the value of ORP in longitudinal studies to monitor the progress in patients with specific clinical conditions.

7.11 Conclusions

Oxidative stress is recognized as an important factor in male infertility. Accurate assessment is therefore critical in the laboratory evaluation of male infertility attributed to oxidative stress. In this chapter, we have described various techniques that can be used to measure reactive oxygen species or their end-products using simple techniques such as the nitroblue tetrazolium test to ROS measurement by chemiluminescence assay, measuring antioxidants or end products of oxidative stress such as lipid peroxidation, DNA damage, or protein modifications. We have highlighted the value of the newer tools such as proteomics in identifying oxidatively modified proteins and validating these proteins. The ultimate goal is to identify potential markers of oxidative stress to assist in elucidating the underlying mechanism of oxidative stress-related sperm dysfunction that ultimately results in male infertility and assists the clinicians in the management of these patients.

We described novel approach (ORP analysis by MiOXSYS system) to measure oxidative stress in semen and seminal plasma supported by contemporary literature evidence. ORP provides a comprehensive measure of oxidative stress with a high sensitivity and specificity. Measuring ORP using a portable MiOXSYS system can assess seminal oxidative stress quickly and in small volume samples both in fresh and frozen semen and seminal plasma. It offers the wider application of oxidative stress measurements both in clinical and research settings. MiOXSYS system provides a novel diagnostic method that may be used in conjunction with routine semen analysis. It is a valuable tool to identify patients who will potentially benefit from treatment. It is an advanced and independent test of semen quality that can fit in the algorithm of the male infertility workup and help the clinicians in the management of their patients. Appropriate treatment strategies can be recommended in patients presenting with high oxidative stress.

7.12 Review Criteria

An extensive search of studies examining the relationship between oxidative stress and male infertility was performed using search engines such as Google Scholar and PubMed. The start and end dates for these searches were September 1996 and September 2018, respectively. The overall strategy for study identification and data extraction was based on the following key words: "male infertility", "oxidative stress", "reactive oxygen species", "infertile men", "antioxidants", "DNA fragmentation", "oxidation reduction potential", and "pregnancy rate" as well as the names of specific oxidative-stress markers. Articles published in languages other than English were excluded. Data published in conference or meeting proceedings, websites, or books were also included.

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Sperm Chromatin Integrity Tests and Indications

8

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Key Points

- Assessment of sperm chromatin integrity is important. It provides useful information in cases of male idiopathic infertility, post-chemo/radio therapy, and in couples pursuing assisted reproduction.
- Pathologically increased sperm DNA fragmentation (SDF) is a paternal-derived cause of repeated ART failures.
- Assessment of SDF is important for evaluating semen samples prior to their use in assisted reproduction particularly in men with multiple sperm defects.
- There are multiple assays that can be used to evaluate sperm chromatin and SDF.
- It is important to evaluate the real cause of DNA damage and provide proper therapeutic strategies.
- Several studies have correlated sperm DNA fragmentation with pregnancy outcome in IVF.
- The predictive value of SDF testing in regards to IVF/ICSI outcomes is controversial.
- There is a strong recommendation that sperm DNA fragmentation be included in the evaluation of infertile men.

8.1 Introduction

Laboratory-based semen analysis is the cornerstone assessment for male factor infertility [1]. However, impaired semen parameter cannot predict the fertility [2]. As a result, this laboratory test cannot differentiate fertile from infertile men. Earlier studies have shown that male infertility did not only include abnormal conventional semen parameters but also showed a correlation between compromised efficiency of DNA integrity and reduced fertility potential [3]. Human sperm DNA is a complex configuration which is very susceptible to damage resulting in defective chromatin structure and quality leading to infertility. One of the main causes of sperm DNA damage is reactive oxygen species (ROS) [4]. ROS are known to induce lipid peroxidation of the plasma membrane of the sperm which is the primary cause of sperm DNA fragmentation (SDF). High levels of SDF have been shown to have a negative impact on semen parameters and reproductive potential. This abnormality cannot be detected in routine semen analysis.

Many tests have been developed to measure SDF. The most commonly used SDF tests are the terminal deoxynucleotidyl transferase deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) assay, sperm chromatin structure assay (SCSA), and sperm chromatin dispersion (SCD) assay [5]. A panel composed of fertility experts worldwide extensively studied the utility of sperm DNA fragmentation test as part of the evaluation of infertile men [6]. Their practice recommendations are based on clinical scenarios, and recommend SDF testing for men with varicocele (high grade varicocele with normal semen parameters or low grade varicocele with abnormal semen parameters), unexplained infertility, recurrent pregnancy loss, recurrent intrauterine insemination (IUI) failures, exposure to environmental pollutants, drugs, radiation, smoking, febrile illness, varicocele, advanced age, and obesity can increase SDF fertilization (IVF) and intracytoplasmic sperm injection (ICSI) failures and men with lifestyle risk factors such as exposure to environmental pollutants, drugs, radiation, smoking, febrile

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illness, varicocele, advanced age, and obesity can increase SDF smoking [6]. Despite the impact of SDF testing to evaluate infertile men, many reproductive societies such as the American Society for Reproductive Medicine (ASRM), European Association of Urology (EAU), American Urological Association (AUA), and National Institute of Clinical Excellence (NICE) do not recommend its use as part of the routine assessment of male infertility. Good methodological studies should be conducted to determine chromatin packaging defects as well as the clinical utility of SDF testing in the evaluation of infertile men. In this chapter, we describe the sperm chromatin structure, factors associated with abnormal chromatin structure, and discuss the methods available to analyze sperm DNA chromatin and DNA integrity.

8.2 Limitation of Semen Analysis

Routine semen analysis continues to be the cornerstone of laboratory evaluation of male infertility. However, its ability to diagnose male fertility is limited. Semen analysis provides important information about spermatogenesis, sperm production, formation of spermatozoa, and the individual components of semen and their function; however, there is a significant overlap in the semen parameters such as concentration, motility, and morphology between fertile and infertile [7]. Although sperm motility, concentration, and morphology are parameters that collectively provide important information about the efficiency of spermatogenesis, it is important to look for tests beyond traditional semen analysis [8].

The latest edition of the WHO 2010 laboratory manual provides statistically derived 5th centile lower reference limits. The traditional semen analysis has limited ability to identify the underlying alterations in the molecular and cellular mechanisms that play a critical role in fertilization and fertility. Standard measurements of semen parameters do not provide information about the subtle sperm defects that can affect fertility. The head must contain DNA that has been correctly assembled during spermatogenesis and disassembled during spermiogenesis and finally correctly reassembled to partner with the female DNA to ultimately form the new genome. Similarly, the midpiece must contain mitochondria to provide energy, and the flagellum must be present to transfer the energy to provide the motility. Thus, the outcome can help alleviate the emotional and financial burden to the patient [9, 10]. The introduction of improved automated semen analyzers, smart phone semen testing, microfluidics, and proteomics are all platforms that may potentially provide useful information [11–14], but the technology is still far away from predicting the correct diagnosis with 100% accuracy.

Therefore, there is a need to combine other diagnostic tools to better discriminate infertile men from fertile men, predict pregnancy outcome in female partners, and calculate the risks associated with adverse reproductive events. Perhaps the candidate test with the greatest potential for inclusion is DNA integrity assessment.

8.3 Sperm DNA Integrity

Sperm chromatin abnormalities have been extensively studied as a cause of male infertility. With the increasing use of assisted reproductive technologies (ART), especially ICSI, attention has focused on the genomic integrity of the male gamete and concerns about the transmission of damaged DNA. The ability of the oocyte to repair DNA damage is limited, and excessive DNA damage raises concerns about potential chromosomal abnormalities, congenital malformations, and developmental abnormalities in ICSI-born children [15–18]. Disturbances in the male genomic content are negatively associated with fertility potential of spermatozoa, both in vivo and in vitro [19–27]. Sperm DNA damage is indicative of male subfertility regardless of the normal semen parameters [28, 29]. High sperm DNA fragmentation can compromise embryo quality and result in pregnancy loss following ART [23]. With increasing reports in the literature regarding the association of sperm DNA fragmentation with pregnancy outcome, there is a strong recommendation for inclusion of sperm DNA fragmentation in the evaluation of male infertility [30].

Sperm chromatin is different from that of somatic cells in terms of the chromatin packaging [31, 32]. The histones are replaced by protamines, which make the sperm nuclei highly compacted [33]. While in the mice, 95% of nucleoproteins are comprised of protamines which confers 40% less nuclear volume than that of normal somatic nuclei [34], human sperm nuclei retains about 15% of the histones and contains considerably fewer protamines (85%) when compared to the sperm nuclei of other species such as bull, stallion, hamster, and mouse [35, 36]. The distinct mammalian sperm chromatin packaging called toroids is comprised of 50–60 kb that are cross-linked by disulfide bonds [37, 38]. This condensed, insoluble, and highly organized structure confers genetic integrity during transport of the paternal genome through the male and female reproductive tracts, ensuring proper fusion of the male and female genome and correctly expressing the genetic information in the developing embryo [30, 39, 40].

In contrast to other species which contain only one type of protamine (P1), human and mice spermatozoa contain a second protamine called P2 which is deficient in cysteine residues [31]. P2 diminishes the disulphide cross-linking which is responsible for stable packaging and makes the sperm nuclei less compacted and more susceptible to DNA strand

breaks [41]. Furthermore, the alterations in P1/P2 ratio and absence of P2 are associated with male fertility problems [42–46]. Distinct differences in P1/P2 ratio are reported between fertile and infertile men and P1/P2 ratio has been shown to correlate with sperm DNA fragmentation [47]. Alterations in P1/P2 ratio have been shown to be a good indicator of disturbances in spermatogenesis and lead to male infertility [48].

8.4 Indications and Importance of Sperm DNA Integrity in Male Infertility

Evaluation of sperm chromatin integrity can be challenging; it is difficult to link it with the known physiological mechanisms. Furthermore, its role in clinical practice especially ART is still controversial [49–51]. Evaluating sperm chromatin structure is complex and different methods are necessary to assess this structure. Several confounding factors such as lack of standardized protocols, validated reference ranges, and the assay principles of various sperm DNA testing assays complicate the interpretation of the results. Not all DNA damage is lethal and the oocyte has the ability to repair sperm DNA damage. Although a sperm with damaged DNA can fertilize an egg, it can result in compromised embryonic growth, miscarriage, or childhood deformities [52–56]. Numerous studies have demonstrated significant differences in sperm DNA damage levels between infertile and fertile men [49, 57–61]. Several etiological factors have been implicated in damaging sperm DNA including environmental and lifestyle factors, varicocele, male accessory gland infections, advanced paternal age, and systemic diseases [5, 52, 62–66].

Varicocele has been shown to have an adverse effect on SDF [67]. Higher SDF has been shown in men with varicocele compared to fertile men [68]. Another study reported higher SDF in infertile men with varicocele compared to infertile men without varicocele [69]. Similarly, the same study reported higher SDF rates in men with varicocele and no history of infertility compared with fertile men without varicocele [69]. In a study by Esteves et al., using SCD, varicocele was identified with 94% accuracy based on the rates of degraded sperm determined by the proportion of degraded sperm in the population of spermatozoa with fragmented DNA which was eight-fold higher in men with varicocele than in donors [62]. SDF is also increased in the presence of oxidative stress in these men [70–72]. Varicocelectomy has been shown to be beneficial in select cases [73]. Varicocele repair has been shown to lower SDF and increase the chances of a natural pregnancy [67]. A meta-analysis comprised of six studies that evaluated the effect of varicocelectomy on SDF and found an overall reduction in SDF with a mean difference of −3.37% (95% CI: −4.09 to −2.65; $p < 0.00001$) [68]. Another study evaluated 92 patients with clinical varicocele and examined semen parameters and SDF before and after subinguinal microsurgical varicocele repair [74]. Significant improvement in SDF was reported from a preoperative mean of 42.6–20.5% post-operative ($p < 0.001$). Similarly, Smit et al. [75] evaluated 49 men with varicocele, oligozoospermia, and primary infertility. Following varicocele repair, SDF was significantly reduced post-operatively, and higher pregnancy rates through natural conception with ART were reported. Other reasons for testing DNA fragmentation are recurrent pregnancy loss, unexplained infertility, ART, and cancer patients undergoing chemotherapy or radiotherapy [6].

8.5 Clinical Relevance of DNA Integrity with ART Outcomes

Sperm chromatin integrity is important for effective transmission of genetic information. Abnormal sperm chromatin affects both natural fertility and ART outcomes [70, 73, 76–80]. High SDF as determined by SCSA was shown to result in natural pregnancy failure with high odds ratio of 7.01 (95% CI: 3.68–13.36) [81]. High SDF was also shown to result in increased time to pregnancy in first-time couples without any history of infertility [82, 83]. Both, SCD and TUNEL were able to predict natural pregnancy with over 80% sensitivity and specificity [84, 85]. High levels of SDF have also been associated with poor IUI outcomes [86, 87]. A significant adverse effect of high SDF on clinical pregnancy in both IVF and ICSI was also demonstrated in a meta-analysis comprised of 8068 treatment cycles [86]. Another meta-analysis comprising of 2969 couples demonstrated a 2.2 fold risk of miscarriage when semen specimens with high SDF were used for ICSI (95% CI: 1.54–03.03; $p < 0.00001$) [88]. Similarly, another meta-analysis study comprising of pooled data from 14 studies, abnormally high SDF was associated with higher miscarriage rates in ICSI cycles (OR: 2.68; 95CVI: 1.40–5.14; $p = 0.003$) [89]. Furthermore, SDF was higher in couples experiencing recurrent pregnancy loss compared to fertile controls (18.8% ± 7.0% vs 12.8% ±5.3%; $p < -0.9001$) [90]. All these studies elucidate that higher SDF plays a role in both natural pregnancy and in ART outcomes.

8.6 Contemporary Sperm Chromatin Integrity Tests

Different methods such as aniline blue staining, acridine orange staining, SCSA, 8-hydroxy-2-deoxyguanosine (8-OHdG) assay, Comet assay, sperm chromatin dispersion (SCD) assay and TUNEL assay are available to evaluate sperm chromatin and DNA integrity. These are described below.

8.6.1 Aniline Blue Staining

The acidic dye aniline blue has a strong affinity for binding with loose or decondensed proteins due to residual histones. Aniline blue does not measure SDF. It does not even measure DNA features. It is only a measure for chromatin condensation. Aniline blue staining differentiates lysine-rich histones and arginine/cysteine-rich protamine nuclei. Immature sperm nuclei are rich in lysine-rich histones and sperm are stained blue, whereas protamines are rich arginine and cysteine and therefore do not pick up the stain, thus remain unstained. Therefore, this technique is able to differentiate immature and mature sperm in ejaculated spermatozoa [91].

The technique involves air-dried smears of ejaculated or washed spermatozoa fixed in 3% glutaraldehyde in phosphate buffered saline (PBS) for 30 min. The fixed slides are stained in acidic (pH 3.5) 5% aqueous aniline blue solution for 5 min. A total of 200 spermatozoa are counted under bright field microscopy. Percentage sperm stained with aniline blue is counted and should not exceed 25% [92]. Immature, decondensed sperm nuclei are stained light blue (Fig. 8.1a–c). A modified method combines aniline blue counterstained with 0.5% Eosin Y for 1 min [93].

The advantage of this technique is that it is simple and inexpensive and requires only a simple bright field microscope. The disadvantage is that the staining is not homogenous. A high percentage of sperm with nuclear instability is reported with aniline blue staining in patients with varicocele, idiopathic infertility, and unilateral cryptorchidism [94]. While its correlation with other sperm parameters is controversial, aniline blue-stained spermatozoa showed normal sperm count, motility, and morphology [95, 96]. While immature sperm chromatin may or may not correlate with abnormal morphology patterns in asthenozoospermic samples [91, 97], chromatin condensation observed by aniline blue staining was reported to be a good predictor of IVF outcome [93, 94]. The aniline blue test could be considered as one of the complimentary tests of semen analysis for assessment of male infertility [96, 98].

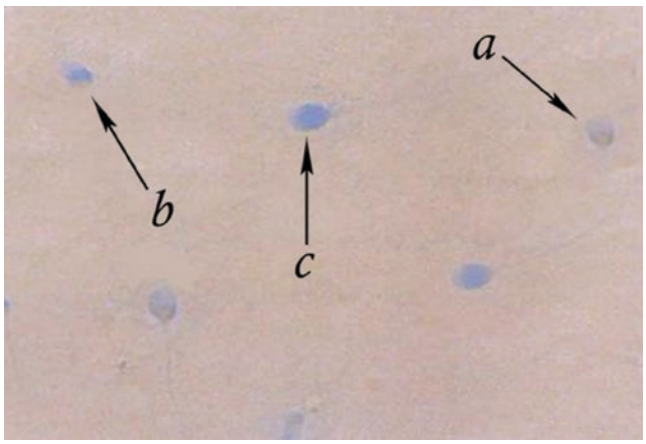

Fig. 8.1 Aniline blue (AB) staining of sperm chromatin in a man with spontaneous recurrent abortion showing (**a**) normal spermatozoa versus (**b**) spermatozoa with moderate level of remaining histones and (**c**) spermatozoa with extensive levels of histones. (Reprinted from Kazerooni et al. [96]. With permission from Springer Nature)

8.6.2 Toluidine Blue Staining

Toluidine blue is a basic thiazine metachromatic dye that binds selectively with acidic components of the tissue. It has a high affinity to bind with the phosphate residues present in sperm DNA of immature nuclei [6, 99, 100]. Air-dried sperm smears are fixed in ethanol-acetone (1:1) at 4°C for 30 min and hydrolyzed in 0.1 N HCl at 4°C for 5 min, followed by rinsing thrice in distilled water. Smears are stained for 5 min with Toluidine blue (0.05%) prepared in 30% citrate phosphates or McIlvain buffer, pH 3.5. Permanent preparations are prepared after dehydrating in tertiary butanol twice for 3 min each followed by xylene twice for 3 min each. Staining is observed using light microscopy. Normal sperm heads stain light blue, whereas sperm heads with abnormal DNA stain purple–violet due to the metachromatic shift [101, 102]. It is therefore a sensitive probe for DNA structure and packaging. Toluidine blue staining has demonstrated application in assessment of male fertility potential with 95% specificity and a sensitivity of 42% when the threshold is set at 45% [103, 104]. It is a simple and inexpensive stain that has been used for morphological assessment by light microscopy. It has also been shown to correlate with other advanced tests of sperm DNA fragmentation, i.e., SCSA and TUNEL [102, 105].

8.6.3 CMA 3 Assay

Chromomycin A3 (CMA3) is an indirect assay to measure protamine deficiency in sperm DNA. It is only a measure for chromatin condensation. Poor chromatin packaging is revealed by CMA3 a guanine-cytosine-specific fluorochrome which competes with protamine for binding to the minor grooves of DNA [106]. Spermatozoa exhibiting low protamination show high CMA3 binding [107]. The technique involves air drying of methanol-glacial acetic acid (3:1) fixed sperm at 4°C for 20 min. The slides are treated for 20 min with CMA3 solution prepared in McIvlain's buffer supplemented with 10 mmol/l $MgCl_2$. Slides are rinsed in buffer and mounted with PBS-glycerol (1:1 vol./vol.) and kept overnight at 4°C.

Staining is observed under fluorescence microscope and 200 spermatozoa are counted on each slide. CMA3-positive spermatozoa indicative of poor protamination stain bright

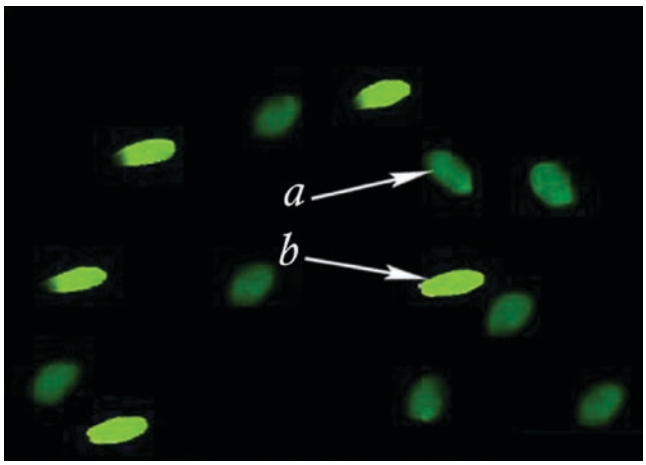

Fig. 8.2 Chromomycin A3 (CMA3) staining of sperm chromatin in a man with (**a**) spontaneous recurrent abortion showing normal spermatozoa versus (**b**) spermatozoa with protamine deficiency. (Reprinted from Kazerooni et al. [96]. With permission from Springer Nature)

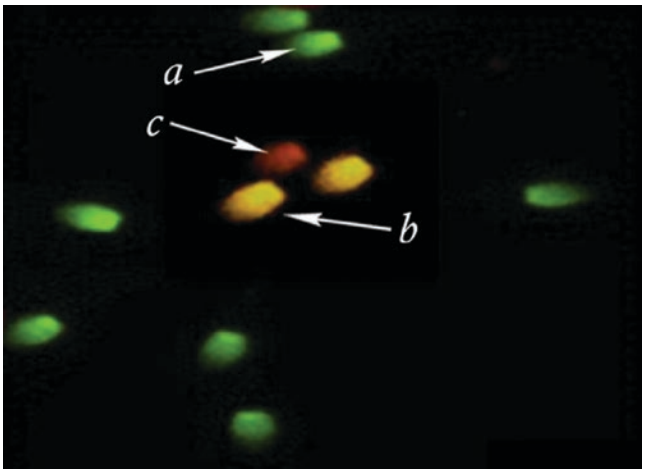

Fig. 8.3 Acridine orange (AO) staining of sperm chromatin of a man with spontaneous recurrent abortion showing (**a**) normal, (**b**) moderately denaturated, and (**c**) completely denaturated spermatozoa. (Reprinted from Kazerooni et al. [96]. With permission from Springer Nature)

yellow or bright green, whereas CMA3-negative spermatozoa (high protamination) stain faint yellow or dull green [96, 108, 109]. (Figure 8.2a–b). CMA3 staining has demonstrated a sensitivity of 73% and a specificity of 75% and reported to be a good discriminator of IVF success [110, 111]. CMA3 results have shown an inverse correlation with sperm concentration, motility especially morphology. Men with poor sperm morphology exhibited a greater degree of protamine deficiency and DNA damage [112, 113]. Furthermore, significantly higher CMA3 staining has been demonstrated in globozoospermic patients [114]. The sensitivity and specificity of CMA3 staining is strongly correlated with that of aniline blue staining [108]. However, this assay is limited due to inter-observer subjectivity.

8.6.4 Acridine Orange

Acridine orange is a flurochrome dye used to measure the susceptibility of the sperm nuclear DNA to acid-induced denaturation. It intercalates into double-stranded DNA as a monomer and binds to a single-stranded DNA as aggregate. Acridine orange fluoresces green when bound to native DNA and green the relaxed acridine orange fluoresces red in denatured DNA [115, 116]. Acridine orange is used both in fluorescence microscopy and in flow cytometry. For fluorescence microscopy, thick semen smears are fixed in Carnoy's fixative (methanol: acetic acid; 1:3) for 2 hours and stained in acridine orange for 5 min. After rinsing in deionized water, about 200 spermatozoa are examined for the intact (green) and damaged DNA (yellow–orange to red) [96] (Figure 8.3a-c). The DNA fragmentation index is calculated by measuring the ratio of yellow to red/green + yellow to red florescence [115].

For flow cytometry, 25–100 μL of sample containing about 1×10^6 spermatozoa are suspended in ice cold PBS at pH 7.4 and centrifuged at 600 × g for 5 min. The pellet is resuspended in ice-cold TNE (0.01 mmol/l of tris-HCl, 0.15 mol/l NaCl, and 1 mmol/l EDTA, pH 7.4) and centrifuged at 600 × g for 5 min. After removing the supernatant, the pellet is again resuspended in TNE containing 10% glycerol and fixed in 70% ethanol for 30 min. The fixed samples are treated with triton-X 100 solution (0.15 mol/l NaCl and 0.08 N HCl; pH 1.2) for 30 seconds. Acridine orange (6 μg/mL, 37 mmol/l citric acid, 126 mmol/l Na_2HPO_4, 1 mmol/L disodium EDTA and 0.15 mol/l NaCl, pH 6.0) is added. A minimum of 5000 cells are examined by flow cytometery using a light source with excitation at 488 nm light source. The ratio of acridine bound to intact double-stranded DNA fluorescing green at 513–530-nm and acridine orange bound to damaged single-stranded DNA fluorescing red is calculated (630 nm) [117].

Acridine orange positive cells are more likely to have structural abnormalities compared to acridine-orange-negative spermatozoa [118]. In infertile men, acridine orange technique demonstrated significantly higher DNA damage in infertile men compared to controls in men with varicocele. This was significantly reduced after varicocele repair demonstrating the clinical utility of this technique [104, 119]. The acridine orange assay shows low intra-assay variability, and the technique is highly reproducible. A strong positive correlation has been demonstrated between acridine orange assay and other techniques that are used to measure single-stranded DNA, such as TUNEL assay [120]. The cutoff to differentiate between fertile and infertile men varies between 20% and 50% [109, 121, 122]. Single-stranded DNA staining has also shown to negatively correlate with the classical IVF and lower pregnancy rates [116, 122–125].

8.6.5 Sperm Chromatin Structure Assay

The sperm chromatin structure assay (SCSA) measures in situ DNA susceptibility to the acid-induced conformational helix-coil transition by acridine orange (AO) fluorescence staining. The extent of conformational transition in situ following acid or heat treatment is determined by measuring the metachromatic shift of AO fluorescence from green (native DNA) to red (denatured or relaxed DNA) (Fig. 8.4). The SCSA acid method is much easier to use than the heat treatment. DNA damage that is SCSA-defined is manifested by the DNA Fragmentation Index (DFI) [121].

An aliquot of unprocessed semen (about 13–70 μl) is diluted to a concentration of 1–2 × 10^6 sperm/ml with TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, and 1 mM EDTA, pH 7.4). This cell suspension is treated with an acid detergent solution (pH = 1.2) containing 0.1% Triton X-100, 0.15 mol/l NaCl, and 0.08 N HCl for 30 seconds, and then stained with 6 mg/l purified AO in a phosphate-citrate buffer, pH 6.0. The stained sample is placed into the flow cytometer sample chamber [121]. The extent of DNA fragmentation is measured as % of sperm with fragmented DNA termed as DNA fragmentation Index, or DFI. It is the ratio of red fluorescence to total (red + green fluorescence). Native (double-stranded) or intact spermatozoa fluoresce green and fragmented DNA (single-stranded DNA) fluoresce red (Fig. 8.4). Since the SCSA is more constant over prolonged periods of time than routine World Health Organization (WHO) semen parameters, it may be used effectively in epidemiological studies of male infertility [126]. No significant male age-related increase in DFI was demonstrated [127]. SCSA has clearly established clinical thresholds for utility in the human infertility clinic [128]. In clinical applications, the SCSA parameters can distinguish fertile and infertile men. In addition, these parameters are also able to classify men according to the level of *in vivo* fertility, i.e., high fertility (pregnancy initiated in less than 3 months), moderate fertility (pregnancy initiated within 4–12 months), and no proven fertility (no pregnancy by 12 months). Furthermore, a DFI threshold of <30% can identify samples compatible with in vivo pregnancy (<30%) [25, 86, 129–131].

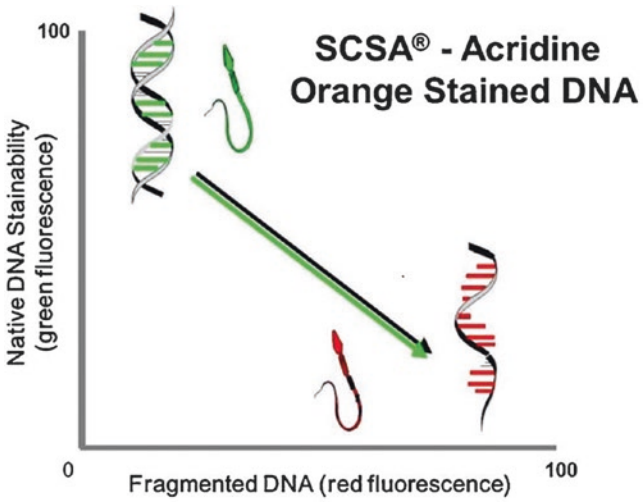

Fig. 8.4 Schematic of SCSA test showing fluorescence color shift from normal double-stranded (Normal or Native DNA) (green) to fragmented single-stranded DNA (red). (Reprinted from Evenson [221]. With permission from Elsevier)

SCSA can predict the various outcomes of ART. However, this is true only for neat semen [132] including the fertilization and implantation rates [22, 86, 133, 134], but this finding was not supported by a study by Lin et al. [135] who reported increased abortion rate in the high DFI (>27%) group. Reports suggest that DFI can be used as an independent predictor of fertility in couples undergoing IUI [22], but an association between SCSA results and IVF and ICSI outcomes are not strong enough [136]. It is also proposed that all infertile men should be tested with SCSA as a supplement to the standard semen analysis [137]. Recent data suggests that ICSI should be the method of choice when DFI exceeds 30% [86].

The SCSA accurately estimates the percentage of DNA-damaged sperm and has a cut-off point (30% DFI) to differentiate between fertile and infertile samples [22, 129]. However, it requires the presence of expensive instrumentation (flow cytometer) and highly skilled technicians. SCSA DFI shows significant association with TUNEL assay results when Spearman's rank correlation was used, however, regression and concordance correlation results showed that these methods are not comparable. SCSA rather measures DNA damage in terms of susceptibility to DNA denaturation, while TUNEL measures "real" DNA damage [138].

8.6.6 Measurement of 8-Hydroxy-2-Deoxyguanosine (8-OHdG)

This assay measures levels of 8-hydroxy-2-deoxyguanosine (8-OHdG), which is a byproduct of oxidative DNA damage in spermatozoa. It is the most commonly studied biomarker for oxidative DNA damage. Among various oxidative DNA adducts, 8-OHdG is representative of oxidative DNA damage owning to its high specificity, potent mutagenicity, and relative abundance in DNA [139].

The technique involves three steps: in step 1, DNA extraction is performed with chloroform-isoamyl alcohol (12:1 v/v) after the sperm cells are washed with sperm wash buffer (10 mmol/l Tris-HCl, 10 mmol/l EDTA, 1 mol/l NaCl, pH 7.0) and lysed at 55 °C for 1 hour with 0.9% SDS, 0.5 mg/ml proteinase *K*, and 0.04 mol/l dithiothreitol (DTT). After ribonuclease A treatment to remove RNA residue, the

extracted DNA is dissolved in 10 mmol/l Tris-HCl (pH 7.0) for DNA digestion. Step II involves the enzymatic DNA digestion that is performed with three enzymes: DNAase I, nuclease P1, and alkaline phosphatase. The final solution is dried under reduced temperature and pressure and is redissolved in distilled and deionized water for HPLC. The third step uses HPLC analysis and consists of a pump, a partisphere 5 C18 column, an electrochemical detector, an ultraviolet detector, an autosampler, and an integrator. The mobile phase consists of 20 mmol/l $NH4H_2PO_4$, 1 mmol/l EDTA, and 4% methanol (pH 4.7). The calibration curves for 8-OHdG are established with standard 8-OHdG, and the results are expressed as 8-OHdG/10^4 dG [97]. 8-oxoG can also be determined using a specific fluorescent probe (8-oxoG) from the OxiDNA assay kit.

8-OHdG provides the most direct evidence suggesting that oxidative sperm DNA damage is involved in male infertility, based on the finding that levels of 8-OHdG in sperm are significantly higher in infertile patients than in fertile controls and have an inverse relationship with sperm concentration [97]. 8-OHdG formation and DNA fragmentation assessed by TUNEL are highly correlated with each other [140]. 8-OHdG levels also are highly correlated with the disruption of chromatin remodeling [141]. Levels of 8-OHdG in sperm DNA have been reported to be increased in smokers, and they inversely correlate with the intake and seminal plasma concentration of vitamin C. It is demonstrated that infertile patients with varicocele have increased 8-OHdG expression in the testis which is associated with deficient spermatogenesis [142]. If not repaired, 8-OHdG modifications in DNA are mutagenic and may cause embryo loss, fetal malformations, or childhood cancer. Moreover, this modification could be a marker of OS in sperm, which may have negative effects on sperm function [143, 144].

8.6.7 Comet Assay

The comet assay or the single-cell gel electrophoresis is based on the principle of permeabilization and electrophoretic migration of cleaved fragments of DNA. In the neutral comet assay, the migration of the double-stranded DNA loops from a damaged cell comes in the form of a tail unwinding from the relaxed supercoiled nucleus. This unwinding is proportional to the damage that the cell is subjected to. This gives rise to the characteristic appearance of a comet with the tail when seen under fluorescence microscope using DNA stains.

This assay was later modified by Singh et al. [145] using alkaline electrophoresis buffers to expose the alkali-labile sites on the DNA and increase to the sensitivity of the assay to detect both single- and double-stranded DNA. The single- and double-stranded DNA breaks can also be evaluated by the modified two-tailed comet assay [146, 147]. Poor quality chromatin is closely associated with, and highly indicative of, some fertility problems. Many methodologies to assess DNA fragmentation in spermatozoa are available, but they are all unable to differentiate between single-stranded DNA breaks (SSB) and double-stranded DNA breaks (DSB) in the same sperm cell.

The two-tailed Comet assay (2 T-Comet) protocol overcomes this limitation. A modification of the original Comet assay was developed for the simultaneous evaluation of DNA SSB and DSB in human spermatozoa. In this assay, the DNA damage is quantified by measuring the displacement between the genetic material of the nucleus or the comet head and the resulting tail. The tail length is used as an index of the damage. In addition, the "tail moment" is a product of tail length and the intensity or the fraction of the total DNA in the tails. It can also be defined as similar to the torsional moment of the tail [148].

The comet assay is a simple, versatile, sensitive, and rapid assay and has demonstrated some correlation with other assays such as SCSA and TUNEL [149]. The assay requires expertise in result interpretation as it is based on the fluorescence microscopy. The 2 T-Comet assay is a fast, sensitive, and reliable procedure for the quantification and characterization of DNA damage in spermatozoa [150] (Figs. 8.5a–b and 8.6a–c).

The comet assay has been used to evaluate the DNA damage after cryopreservation [151]. It has been used to predict embryo development after IVF and ICSI, especially in couples with unexplained infertility [152, 153]. Although the clinical thresholds have been established for diagnosing infertility and predicting IVF outcome [154–157], not all studies have demonstrated such an association [158].

8.6.8 Sperm Chromatin Dispersion Test (Halosperm Assay)

The sperm chromatin dispersion (SCD) test produces sperm nucleoids consisting of a central or core and peripheral halo caused by release of DNA loops, signifying the absence of DNA fragmentation. When sperm are treated with an acid solution prior to lysis buffer, a complete absence or a minimal halo is produced in spermatozoa with fragmented DNA. A distinct halo is seen in spermatozoa with intact DNA integrity [159]. When spermatozoa with non-fragmented DNA are immersed in an agarose matrix and directly exposed to lysing solutions, the resulting deproteinized nuclei (nucleoids) show extended halos of DNA dispersion, which can be observed either by bright field microscopy or fluorescent microscopy. The presence of DNA breaks promotes the expansion of the halo of the nucleoid [80, 160–165].

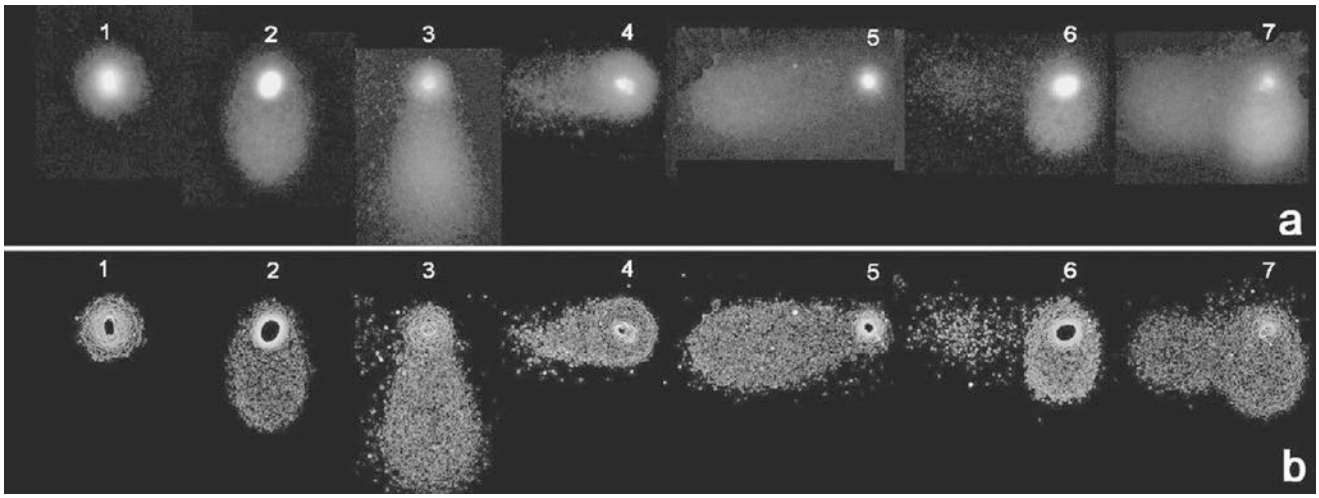

Fig. 8.5 Two-tailed (2 T) comet assay protocol detects seven comet types: (1) undamaged; (2) low level of single-stranded DNA breaks (SSB); (3) high level of SSB; (4) low level of double-stranded DNA breaks (DSB); (5) high level of DSB; (6) low level of SSB and low level of DSB; (7) high level of SSB and high level of DSB. Scale bar: 10 μm. (Reprinted from Enciso et al. [150]. With permission from Elsevier)

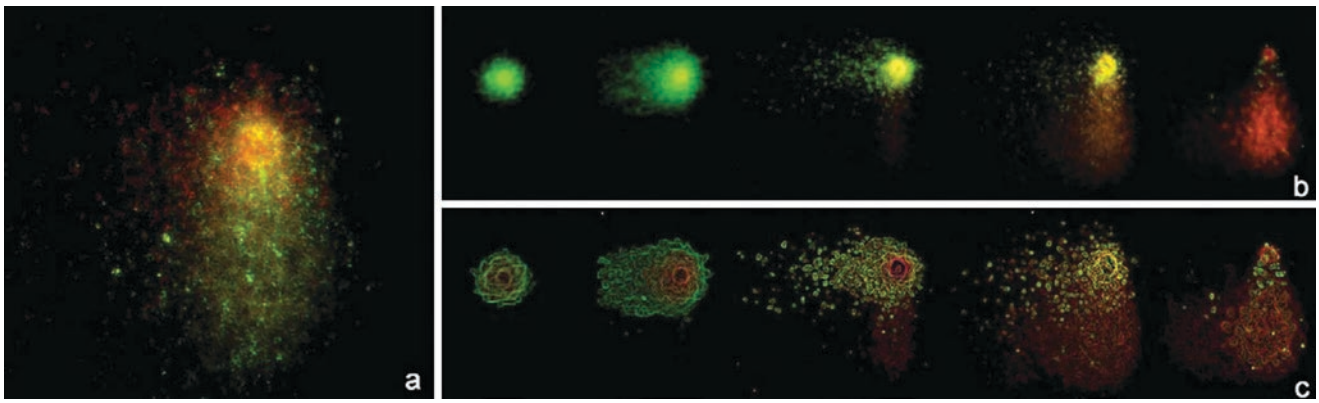

Fig. 8.6 Characterization of single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) by fluorescence in-situ hybridization (FISH) and acridine orange staining. (**a**) ssDNA detection by FISH with a digoxigenin-labeled whole human genome probe and fluorescein isothiocyanate-detected (green) on two-tailed (2 T)-Comets obtained from human spermatozoa, counterstained with propidium iodide (red). (**b**) 2 T-Comet types found in a normal semen sample first electrophoresed under neutral conditions (from right to left; X-axis) and then 90° electrophoresed under alkaline conditions (from upper to lower; Y-axis), stained with 0.5 × 10–4 mol/l acridine orange. (**c**) Same images after application of a common electronic filter. (Reprinted from Enciso et al. [150]. With permission from Elsevier)

In this assay, aliquots of sperm at a concentration of 5–10 million/ml are prepared by diluting in PBS. The samples are mixed with 1% low-melting-point aqueous agarose (to obtain a 0.7% final agarose concentration) at 37 °C. Aliquots of 50 μl of the mixture are pipetted onto a glass slide precoated with 0.65% standard agarose dried at 80 °C, covered with a coverslip, and left to solidify at 4 °C for 4 min. The coverslips are then carefully removed, and the slides are immediately immersed horizontally in a tray of freshly prepared acid denaturation solution (0.08 N HCl) for 7 min at 22 °C in the dark, which generates restricted single-stranded DNA (ssDNA) motifs from DNA breaks. Denaturation is then stopped, and the proteins are removed by transferring the slides to a tray with neutralizing and lysing solution 1 (0.4 mol/l Tris, 0.8 mol/l DTT, 1% SDS, and 50 mmol/l EDTA, pH 7.5) for 10 min at room temperature. The slides are then incubated in neutralizing and lysing solution 2 (0.4 mol/l Tris, 2 mol/l NaCl, and 1% SDS, pH 7.5) for 5 min at room temperature. The slides are thoroughly washed in Tris-borate EDTA buffer (0.09 mol/l Tris-borate and 0.002 mol/l EDTA, pH 7.5) for 2 min, dehydrated in sequential 70%, 90%, and 100% ethanol baths (2 min each), and air-dried. For bright-field microscopy in the improved SCD test (Halosperm® kit), slides were horizontally covered with a mix of Wright's staining solution (Figs. 8.7a–i and 8.8). Cells can also be stained with DAPI (4′,6-diamidino-2-phenylindole) (2 μg/ml) for fluorescence microscopy [109, 159].

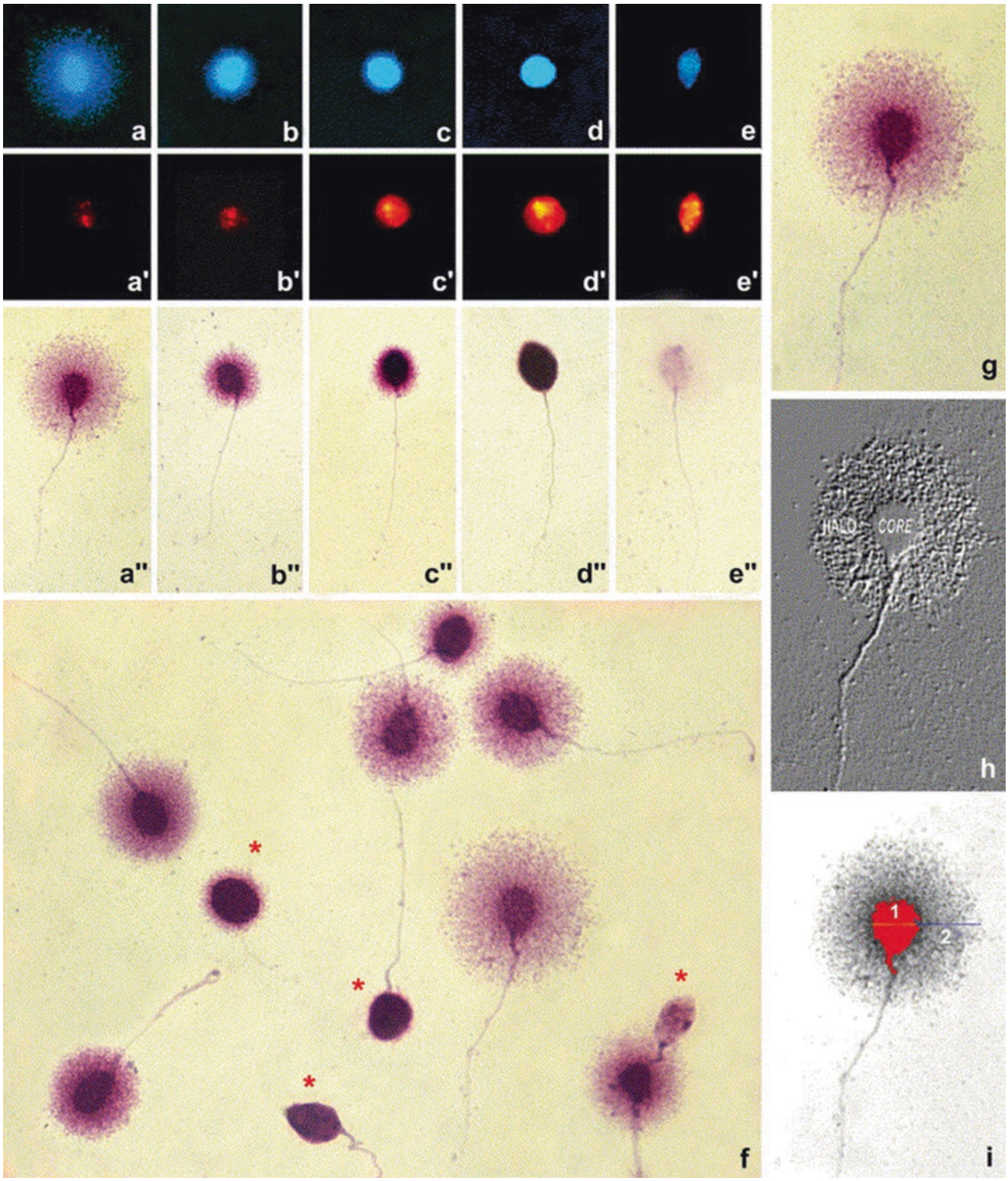

Fig. 8.7 Nucleoids from human sperm cells obtained with the improved SCD procedure. (**a**–**e**) DAPI staining for fluorescence microscopy. (**a′–e′**) Sequential DBD-FISH with a whole genome probe to demonstrate DNA breakage. (**a″–e″**) Wright staining for bright-field microscopy. (**a**, **a′**, **a″**) Nucleoids with big halo of DNA dispersion. (**b**, **b′**, **b″**) Nucleoids with medium-sized halo. (**c**, **c′**, **c″**) Nucleoids with small halo size. (**d**, **d′**, **d″**) Nucleoids without halo. (**e**, **e′**, **e″**) Nucleoids without halo and degraded. According to the DBD-FISH signal, those nucleoids with small halo, without halo, and without halo and degraded contain fragmented DNA. (**f**) Microscopic field visualized after Wright staining. Those sperm cells with fragmented DNA are indicated by an asterisk. (**g–i**) Besides the preservation of the tails, the improved SCD protocol allows for a better chromatin staining, obtaining highly contrasting images for bright-field microscopy (**g**), where the core and the periphery of the halo are well delimitated (**h**). (**i**) The estimation of the halo size was established by comparison of the halo width (2) with the minor diameter of the core (1). (Reprinted from Fernández et al. [222]. With permission from Elsevier)

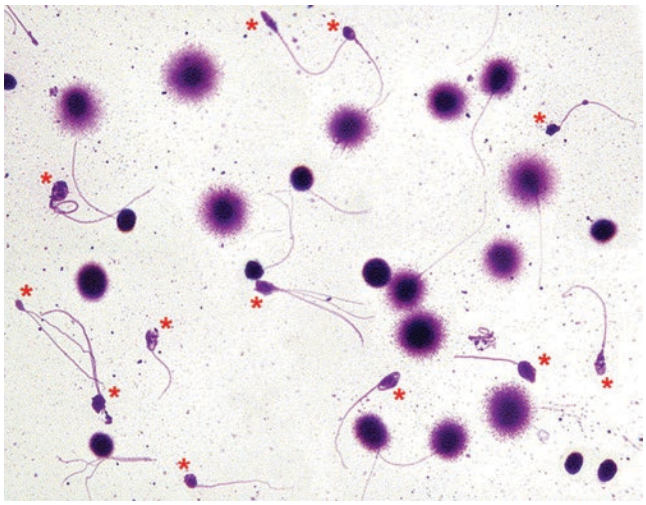

Fig. 8.8 The SCD test in an individual with varicocele. The frequency of sperm cells without halo and degraded (asterisks) is high, reflective of a very high degree of nuclear damage. (Reprinted from Fernández et al. [222]. With permission from Elsevier)

The SCD test is simple, fast, and reproducible with comparable results to those of the SCSA [162, 164] and TUNEL [166]. The currently available protocol is suitable for bright field microscopy as it significantly reduces equipment cost. The test is successfully used in clinical studies to detect sperm DNA damage [167] and can be simultaneously combined with the FISH (SCD-FISH) assay for detection of aneuploidy in sperm cells [168]. This is the only test allowing sperm DNA fragmentation and chromosomal aneuploidy by FISH in the same cell. Oxidative DNA damage can also be simultaneously determined in the same sperm cell by combining SCD and incubation with an 8-oxoguanine DNA probe [169]. A commercially available Halosperm kit is available to perform this test [170]. Reports suggest that sperm DNA fragmentation as reported by the SCD test is negatively correlated with fertilization rates and embryo quality in IVF/ICSI but not with clinical pregnancy rates or births [162, 171].

8.6.9 Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

This single step staining method labels DNA breaks with FITC-dUTP followed by flow cytometric analysis. TUNEL utilizes a template-independent DNA polymerase called Terminal Deoxynucleotidyl Transferase (TdT), which non-preferentially adds deoxyribonucleotides to 3′ hydroxyl (OH) single- and double-stranded DNA. Deoxyuridine triphosphate (dUTP) is the substrate that is added by the TdT enzyme to the free 3'-OH break-ends of DNA [172–174]. DNA fragmentation can be quantified with conventional or the bench top flow cytometry [172] (Fig. 8.9).

TUNEL utilizes a template-independent DNA polymerase called terminal deoxynucleotidyl transferase (TdT) that non-preferentially adds deoxyribonucleotides to 3′ hydroxyl (OH) single and double-stranded DNA. Deoxyuridine triphosphate (dUTP) is the substrate that is added by the TdT enzyme to the free 3'-OH break-ends of DNA. To assess the DNA fragmentation by TUNEL, an APO-DIRECT Kit (BD Pharmingen, CA) is used. It contains the reaction buffer, TdT, FITC-dUTP, and propidium iodide/ RNase stain. The assay kit also contains negative and the positive controls, which are not sperm cells. About 2.5×10^6 sperm are fixed with 3.7% paraformaldehyde for a minimum of 30 min at 4 °C. The sample is centrifuged at 300 × g for 7 min.

Paraformaldehyde is removed by centrifuging the samples at 300 × g for 7 min. Supernatants are discarded and the pellets resuspended with 1 mL of ice-cold ethanol (70% vol./vol.). The tubes are kept at −20 °C for at least 30 min. To create negative sperm controls, the enzyme terminal transferase is omitted from the reaction mixture. To create positive sperm controls, the samples are pretreated with 2% (v/v) hydrogen peroxide and incubation for 1 h at 50 °C. A 50 µL of the stain is added and incubated for 1 h. Following two washes with 1 mL of the "Rinse buffer," PI /RNase stain is added and incubated for 30 min. For flow cytometry, the laser excitation is provided at 2 wavelengths of 488 nm supplied by a solid blue laser at 20 mW and 640 nm powered by 14.7 mW diode red laser. Green fluorescence (480–530 nm) is measured in the FL-1 channel and red fluorescence (640 nm) in the FL-2 channel. The percentage of positive cells (TUNEL-positive) is calculated on a 1023-channel scale from the flow cytometer software (Fig. 8.10a–e). The analysis is conducted with a similar strategy on both the C6 and the C6 Plus flow cytometer, and dot plots are generated by the BD Accuri software (BD Biosciences, Ann Arbor, MI, United States) [173]. TUNEL results of SDF obtained from a standard (C6) flow cytometer have been compared with a newer version of the same instrument (C6 Plus), and the cut-off, sensitivity, specificity without calibration (adjustment), and after adjustment have been examined. Using identical sperm preparation, matched acquisition settings, the performance of two flow cytometers and two observers has been examined to assess the strength of agreement of the results [175]. After adjustment of the settings, overall concordance was high and the two cytometers showed 100% positive and negative predictive value with 100% area under the curve. The overall correlation coefficient observed between C6 and C6 plus was highly significant ($p < 0.0001$; $r = 0.992$; 95% CI 0.982–0.997). After adjustment, the two cytometers showed very high precision of 98% and accuracy of >99%. The inter-observer agreement on C6 flow cytometer for the two observers was 0.801 ± 0.062 and 0.746 ± 0.044 for C6 Plus. A strong agreement was demonstrated between the samples tested on the two flow cytometers after calibration and established the robustness of both instruments [175].

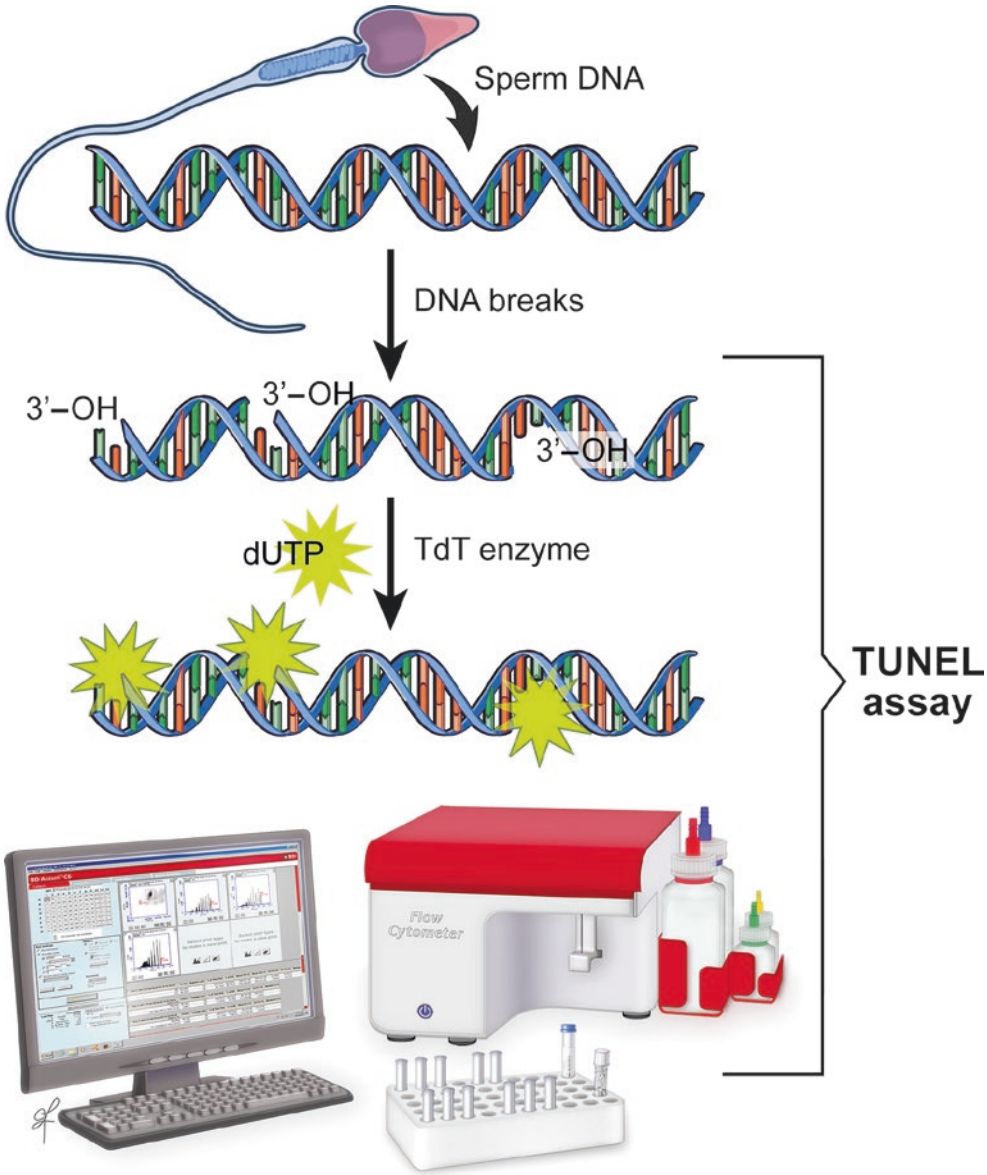

Fig. 8.9 Schematics of the TUNEL assay using the Apo-Direct kit and bench flow cytometer. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2015–2019. All Rights Reserved)

The standard TUNEL assay can be improved to become more sensitive to DNA fragmentation by incubating sperm cells in 2 mm dithiothreitol (DTT) solution for 45 min prior to fixation with formaldehyde. This modified version of the TUNEL assay was shown to significantly enhance its sensitivity. Mitchell et al. modified the TUNEL methodology by incubating spermatozoa for 30 min at 37 °C with LIVE / DEAD Fixable Dead Cell Stain (far red) (Molecular Probes, Eugene, OR, United States). The cells were then washed three times with culture medium before incubation with DTT allowing both DNA integrity and vitality to be simultaneously assessed [176].

The TUNEL assay has been widely used in male infertility research related to sperm DNA fragmentation. A negative correlation was found between the percentage of DNA-fragmented sperm and motility, morphology, and concentration in the ejaculate [120, 177]. It also appears to be potentially useful as a predictor for IUI pregnancy rates, IVF embryo cleavage rates, and ICSI fertilization rates [19]. In addition, it provides an explanation for recurrent pregnancy loss [149, 177–179]. A predictive threshold of 19.2% has been shown significant differentiation between fertile and infertile men with a sensitivity of 64.9% and a specificity of 100% [57, 149]. This is higher than that demonstrated for IUI procedures (12%) [179]. A very high specificity (91.6%) and positive predictive value (90%) was reported at a cutoff point of 16.8% (Fig. 8.11a, b). The high specificity of the TUNEL assay is helpful in correctly identifying infertile patients who do not have sperm DNA fragmentation as a contributory factor [172, 180]. Due to its high positive predictive value, the assay is able to confirm that a man who tests positive is likely to be infertile due to elevated sperm

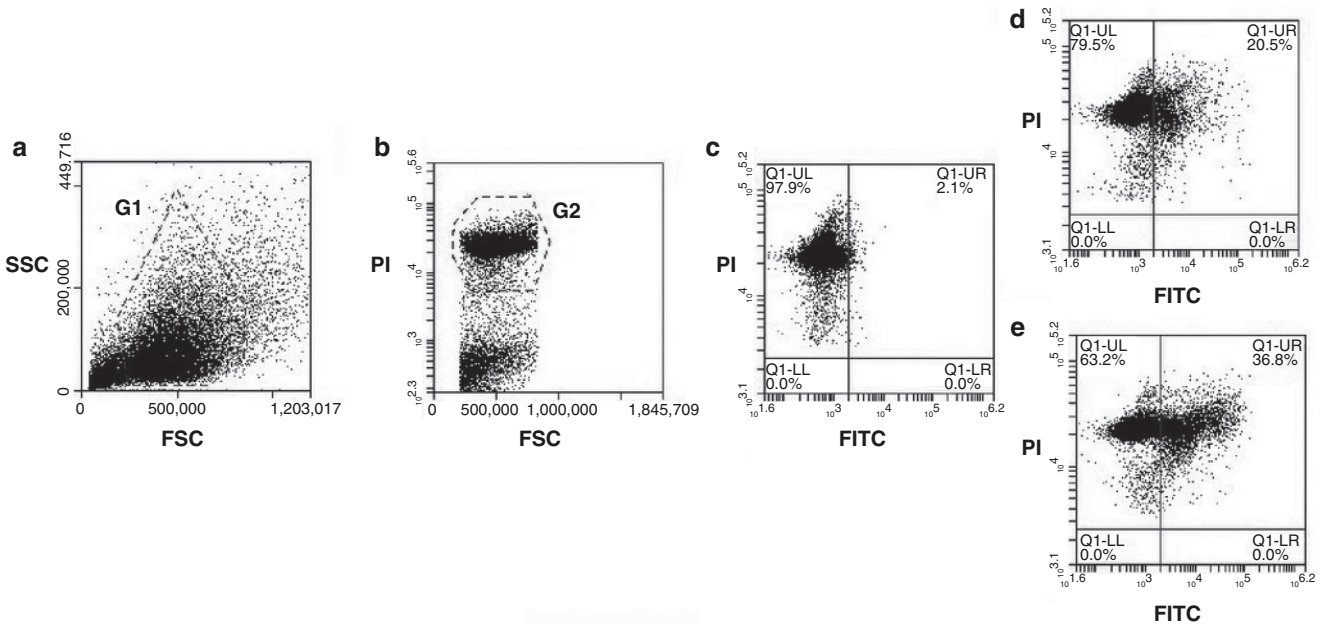

Fig. 8.10 Example of Accuri C6 Workspace and gating strategy used in both laboratories for TUNEL data analysis. (**a**) FSC/SSC plot showing the gate used for spermatozoa selection (G1). (**b**) PI/FSC plot with gating for PI positivity (G2). (**c**) PI/FITC plot of negative control sample (TdT enzyme omitted). (**d**) PI/FITC plot of standard sample. (**e**) PI/FITC plot of positive control sample. *FSC* forward scatter, *SSC* side scatter, *PI* propidium iodide fluorescence, *FITC* fluorescein isothiocyanate fluorescence, *Q1-UL* upper left quadrant, *Q1-UR* upper right quadrant, *Q1-LL* lower left quadrant, *Q1-LR* lower right quadrant

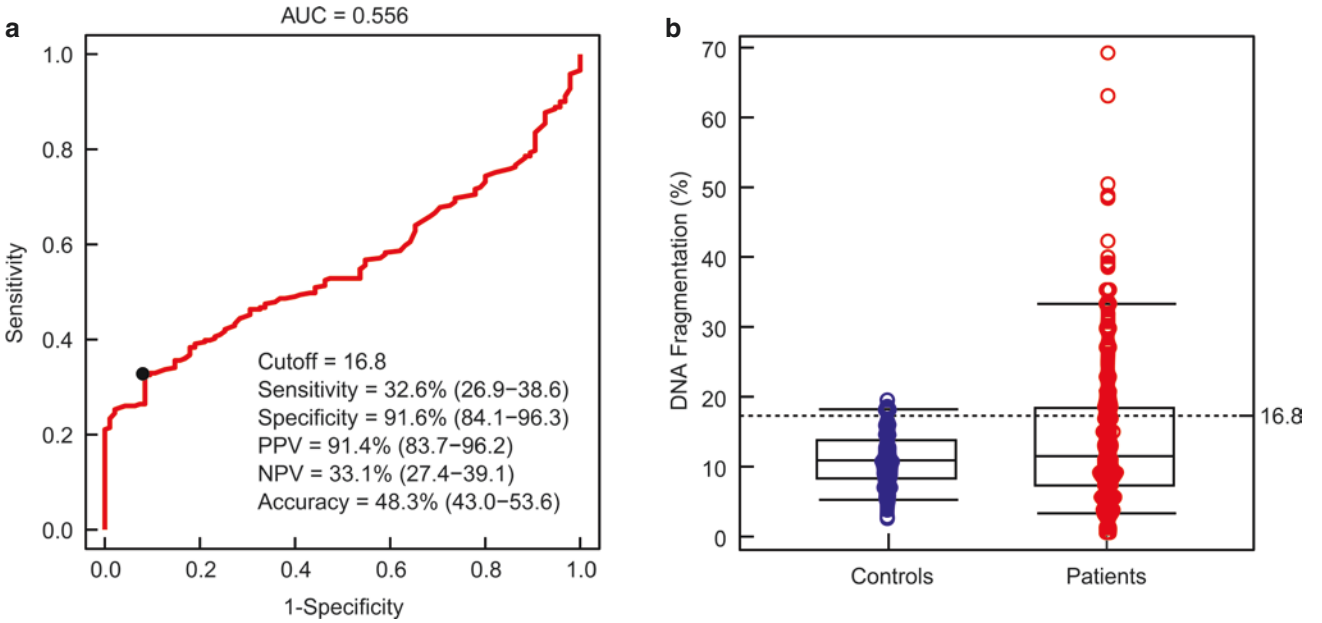

Fig. 8.11 Receiver operator characteristic (ROC) curve showing (**a**) TUNEL cutoff and the area under the curve. Values within the parentheses represent the 95% confidence interval and (**b**) Distribution of TUNEL values between controls and infertile men. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2015–2019. All Rights Reserved)

DNA fragmentation. A similar specificity (91%) of TUNEL was reported [181]. The calculated cutoff would be ideal as any value above this threshold will be strongly associated with infertility.

The TUNEL assay is relatively expensive and time- and labor-consuming. Also, a number of factors can significantly affect assay results including the type and concentration of fixative, fixed sample storage time, the fluorochrome used to

label DNA breaks, and the method used to analyze flow cytometric data [182]. The flow cytometric method of assessment is generally more accurate and reliable than fluorescent microscopy, but it is also more sophisticated and expensive and it presents limitations in the accuracy and reproducibility of the measures of sperm DNA fragmentation [182]. Fairly good quality control parameters with minimal inter- and intra-observer variation (<8%) have been demonstrated for the fluorescent TUNEL assay using the bench top flow cytometer [180, 183].

8.7 Limitations of Current Protocols

One of the criticisms on SDF testing is lack of standardization [184]. While it is true that some of the methods for SDF suffer from high inter-laboratory variation, other tests have been studied extensively to standardize the methods. To achieve this, it is recommended that the testing for SDF should be done in a facility equipped with appropriate instrumentation, qualified technicians, and with both internal and external quality control measures [185]. For example, in a blinded study, two experienced observers carried out inter- and intra-observer variation of TUNEL assay using bench top flow cytometer [183]. The mean TUNEL assay measurement from one observer was confined to the average measurement of the two observers combined with an absolute difference of 1.73% and percent difference of 6.68% in 80% of cases. On the other hand, one TUNEL assay measurement from one observer was likewise limited to the average measurement of the two observers with an absolute difference of 3% in 90% of the cases, while the percentage difference was 9.68% in 80% of the cases. In another study, standardization of the method on SDF testing was assessed in two reference laboratories [183]. No significant differences were detected between the duplicate results. The mean difference between the duplicate readings within each testing centers was 0.5%, while the correlation showed exemplary results ($r = 0.75–0.95$).

There are three categories in which SDF can be measured directly or indirectly [184]. These include the tests that measure the degree of compaction of the chromatin, tests that measure the DNA breaks before and after DNA denaturation and tests that measure SDF by inserting DNA probes or modified nucleotides at the site of damage. Due to these categories, the results measured from one SDF testing do not imperatively correlate with those obtained by the other tests [186]. In a prospective comparative experimental study on 20 sub-fertile men with unexplained infertility, SCD test measured significantly higher SDF compared to TUNEL assay (20.6 ± 14.0% vs 11.5 ± 7.3%, $p < 0.05$) [165]. However, these two tests were poorly correlated ($r = 0.29$) due to the fact that they implemented two distinct methods of SDF detection [165].

As it stands, there are still no clear cutoff values for SDF testing. Sharma et al. [172] provided a detailed protocol and quality control steps on SDF measurement using the TUNEL assay on 95 semen specimens from controls and 261 from infertile men. The positive predictive value was 91.4%, while the negative predictive value was 33.1%. Based on these findings, men with elevated SDF levels are most likely to present a challenge in their reproductive potential. Wewak et al. [84] determined the sperm deoxyribonucleic acid fragmentation index (DFI) of 26.1% as a cutoff point to differentiate infertile men and fertile men using the SCD assay. At this threshold, the prevalence ratio of 2.84 demonstrated the onset of male infertility. In another test using SCD, Lopez et al. [28] reported that the predictive cutoff value of 25.5% DFI could distinguish between successful and unsuccessful IVF or ICSI outcome from 152 infertile couples. SCSA was used to examine the relationship of DNA fragmentation with ART outcomes [59]. The chance of pregnancy in IUI was significantly higher if the value of DFI was 27%. On the other hand, IVF and ICSI outcomes were non-significant in groups with DFI less than 27%. In another study examining the effect of sperm DFI on pregnancy outcome and pregnancy loss in 531 couples after autologous ICSI ($n = 416$), donor egg procedure ($n = 39$), and IUI ($n = 76$), a cutoff of DFI 27% correlated with reproductive outcomes [187].

The current SDF testing methods cannot identify the nature of DNA damage and the location of DNA breaks [188]. Not all SDF tests measure the same damage. Aniline blue and toluidine blue determine the degree of chromatin decondensation while TUNEL, SCD, and SCSA measure DNA breaks [184]. Unlike the semen sample used in ART, SDF measurement is carried out using unprocessed semen [189]. Bungum et al. [132] assessed the effect of SCSA measurement in semen sample from 510 ART cycles prepared by density gradient centrifugation (DGC). No significant difference was seen in clinical pregnancy outcomes between SCSA performed in a neat semen compared to post DGC. In another prospective, observational study comprising of 44 non-azoospermic infertile men and nine fertile men, DFI measured by SCSA increased significantly in infertile men following DGC (25% vs. 15%, $p < 0.01$) [190]. This may reflect a potential deleterious effect of sperm processing on the integrity of sperm DNA. Similar results were demonstrated in 223 couples undergoing IVF where SDF measurements were analyzed by SCSA after swim-up preparation [191]. Although an abnormal DFI (> 27%) had a lower healthy embryo rate (13.2% vs 27.5%, $p < 0.05$), there was no noticeable significant differences in fertilization rate, clinical pregnancy rate, and delivery rate. Zini et al. [192] compared the effects of the two sperm processing methods on sperm DNA integrity in 22 semen samples collected from

non-azoospermic infertile men. SDF was significantly higher in Percoll-treated spermatozoa compared to samples processed by swim up (10.1% vs 4.8%, $p < 0.0001$). Mean sperm motility was significantly improved with the two methods ($p < 0.005$) [192].

8.8 Controversies of Sperm DNA Fragmentation

Varicocelectomy is recommended in men with a palpable varicocele and abnormal semen parameters [1]. In recent guidelines proposed by Agarwal et al. [193], elevated SDF testing levels may significantly shift the way fertility specialists are discussing varicocelectomy in men with normal semen parameters. Varicocele repair can be considered in infertile men with high grade varicoceles and normal conventional semen parameters or low grade varicoceles with abnormal conventional semen parameters. Multiple studies have determined that elevated SDF can be observed in men with varicoceles even in those with normal conventional semen parameters [63, 194]. Significant reduction in SDF is observed after varicocelectomy [195]. In a prospective study of 60 men with varicocele and abnormal semen parameters, DFI was significantly improved 3–6 months after varicocelectomy (from 29.49% to 18.78%, $p < 0.001$) [196]. There was a concomitant reduction in ROS ($p < 0.001$) and significant increase in total non-enzymatic antioxidant capacity (TAC) ($p < 0.001$) post varicocelectomy. In another prospective study of 72 infertile men with varicocele and oligozoospermia, a significant reduction in DFI was observed after varicocelectomy (from 34.5% to 28.2%, $p = 0.024$) [197]. Also, other semen parameters such as total sperm count, sperm concentration, percent progressive motility, percent normal morphology were also significantly improved. In recent study of 157 semen samples with varicocele, elevated sperm DFI measured by SCSA of more than 30% was significantly associated with poor motility and viability ($p < 0.01$). Likewise, abnormal sperm concentration was observed in men with high DFI (24.5% vs. 33.5%, $p < 0.05$) [198]. SDF testing can help physicians prognosticate infertile men with varicocele. Despite the improvement of SDF after varicocelectomy, there are a number of confounding variables [199]. Further research is warranted to understand fully the exact mechanism(s) of SDF in infertile men with varicocele.

Limited data is available to recommend the benefit of screening for SDF in unexplained infertility, recurrent pregnancy loss, and IUI failure [184]. Couples with unexplained infertility usually demonstrate DNA damage. Vandekerckhove et al. [200] examined the incidence of SDF in couples with previously unexplained infertility who underwent IUI. Using SCD test with a DFI of 20%, the incidence of SDF was 42.9%.

There is still an existing controversy regarding the predictive value of SDF testing the outcomes of IVF and ICSI procedures. SDF alone cannot predict the pregnancy outcomes due to a wide list of factors influencing the ART outcomes [184]. Although the negative impact of elevated SDF on the reproductive outcome in conventional IVF is well studied, its poor association with ICSI is not clearly demonstrated [6]. Lin et al. [135] investigated the relationship of SDF on the outcomes of IVF and ICSI. In 233 couples undergoing ART (IVF = 137; ICSI =86), no significant differences were observed in fertilization rate, good embryo rate and pregnancy rate between the two procedures even in low to high DFI (>27%). On the other hand, total and progressive sperm motility was negatively correlated with high DFI [201, 202]. The quality of post-implantation embryo and spontaneous abortion should be a concern with high DFI affecting the pregnancy outcomes [88, 203]. Cissen et al. [204] performed a systematic review and meta-analysis of 30 extractable data on SDF and clinical outcomes of medically assisted reproduction. TUNEL assay, SCD test, and Comet assay showed no predictive values between IVF and ICSI outcomes. Due to the significant limitations of the evidence available, further research is needed to determine the effect of SDF in reproductive outcome after medically assisted reproduction. In contrast to the recent systematic review and meta-analysis, Simon et al. [205] provided sufficient evidence that SDF has negative impact on reproductive outcome following ART procedures. SDF significantly affects the clinical pregnancy after IVF (OR = 1.65; 95% CI: 1.34–2.04; $p < 0.0001$) and ICSI (OR = 1.31; 95% CI: 1.08–1.59; $p = 0.0068$).

Numerous factors can affect the reproductive abilities not only of men but also of women. This is the reason why both partners in an infertile couple should be evaluated at the same time during the assessment of their fertility status. The role of female factors should be addressed in the management of men with SDF [206]. Jin et al. [207] investigated the impact of SDF in 2865 women with different ovarian reserves who underwent ART procedures. Live-birth rate and implantation rate were significantly reduced in women with reduced ovarian reserves when DFI is above 27.3%. In contrast, women with normal ovarian reserves did not demonstrate significance in clinical pregnancy, live-birth rate, and implantation rate with the same values of DFI. Carlini et al. [90] conducted a study on the male factor in 114 Italian infertile men whose wives had recurrent pregnancy loss following natural conception. SDF values were significantly higher in infertile men compared to the controls (18.8% vs 12.8%, $p < 0.001$).

Based on the results of SDF testing, clinicians may advise the infertile men to take measures to decrease SDF. Yet, there is a lack of evidence to demonstrate that lifestyle changes will improve SDF in men with borderline abnormal or normal conventional semen analysis [184]. Improvement in

dietary patterns was able to reduce the DFI ($p = 0.05$) in 336 men with normal sperm concentration or slight oligozoospermia. In addition, higher sperm concentration and higher level of serum testosterone were observed. The use of antioxidants has also shown a beneficial impact on basic semen parameters, advanced function, outcomes of ART procedures, and live-birth rate [208].

In a placebo-controlled, double-blind, randomized study of 77 infertile men with DFI more than 25%, there was no statistically significant difference observed in those men taking antioxidants for 6 months. Although sperm concentration was significantly improved after the treatment period of 3 months ($p = 0.028$) and 6 months ($p = 0.053$) [209]. Intake of antioxidants (vitamins with zinc and selenium) resulted in significant reduction of SDF (−19.1%, $p < 0.0004$) after 90 days of treatment. In another study, Greco et al. [210] evaluated 38 men with DFI more than 15% and whose wives had one failed ICSI attempt. After 2 months of treatment with antioxidants (vitamin C 1 g/day, vitamin E 1 g/day), significant improvement in clinical pregnancy (48.2% vs 6.9%) and implantation rates (19.6% vs 2.2%) were observed. Despite these findings, further studies with appropriate methodological designs are warranted to determine the benefit of antioxidant therapy to resolve SDF. Despite these findings, antioxidant therapy is a reasonable option for the management of infertile men [211].

8.9 Common Laboratory Protocols for Measuring DNA Fragmentation by TUNEL and Flow Cytometry (Direct and Indirect Methods)

Sperm DNA integrity is fundamental in sustaining the reproductive potential of a man. This is relevant because of the popularity of ART procedures, which bypass natural selection barriers unlike in the natural process, which only selects sperm with intact DNA to undergo fertilization. The endonucleases which are activated during programmed cell death degrade the sperm chromatin initially into fragments (30 kb) and then into much smaller fragments (50 kb) [180]. DNA fragments being produced during this process can be detected by TUNEL assay. Flow cytometry and fluorescent microscopy can identify the DNA strand breaks using TUNEL assay [28, 212].

The TUNEL assay is one of the most promising methods to measure SDF. This measures both single and double DNA strand breaks. It directly quantifies DNA damage while other tests assess the DNA integrity indirectly by examining its susceptibility to acid or alkaline denaturation. It is detected by flow cytometry making it a robust evaluation for SDF [173, 183, 213]. The modified dUTP can be labeled either directly with fluorescein-dUTP or indirectly through labeled antibodies or streptavidin. Flow cytometry is used in quantification with TUNEL assay which make rapid evaluation of more than 10,000 spermatozoa per sample. This is combined with staining of the nucleus with propidium iodide (PI) to factor out apoptotic bodies in the semen sample which allows additional diagnostic power in two distinct intensities of the spermatozoa. Spermatozoa with normal DNA integrity will only demonstrate background staining. On the other hand, those fragmented DNA with multiple chromatin 3'OH ends will fluoresce brightly [117].

The protocols must be standardized and validated before it can be used in clinical practice. Sharma et al. [180] reported an earlier detailed standardization of the TUNEL assay with less than 10% on both inter- and intra-observer variability and inter assay variability. With a sensitivity of 64.9% and specificity of 100%, a threshold value of 19.25% can differentiate infertile men with SDF from fertile men. In a more recent study on the standardization of TUNEL assay, the cut-off value of 16.8% showed a specificity of 91.6% and sensitivity of 32.6 with a positive predictive value of 91.4% and a negative predictive value of 33.1%. At this threshold, abnormal SDF level are strongly indicative that SDF may be the cause of reproductive incapacity of a man [172].

8.10 Challenges Using Indirect Assays to Measure DNA Integrity by TUNEL Assay

Ribeiro et al. [214] compared the efficacy between the indirect antibody-based labeling system (BrdUTP/fluorescein-anti-BrdUTP) and the direct labeling system (fluorescein-dUTP). Both labelling systems showed similar staining functions in live spermatozoa. When compared to direct labeling system, the TUNEL indirect labeling system underestimates the SDF with the differences ranging from 19.2% to 85.3% ($p < 0.05$). These differences were more pronounced in semen samples with total motility less than 40% or weak propidium iodide stained spermatozoa (PI dimmer spermatozoa) higher than 14%. In addition, a major difference was observed when dead spermatozoa were stained (40.1% vs 65.7%, $p < 0.05$). There was a correlation between the number of immotile spermatozoa and the intensity of difference between the two labeling systems. Indirect TUNEL labeled to lesser extent the PI dimmer population. Compared to direct labeling, indirect labeling only stained a small number from the PI – dimmer population (90.6% vs 17.9%). Overall, only 30–100% of the total number of spermatozoa stained with direct TUNEL labeling system was stained with indirect method. In this study, the most probable reason in the different staining efficiency of both the labeling system was due to the steric hindrance of the antibody during its binding to the BrdUTP. In addition, condensed chromatin

architecture in dead spermatozoa contributed to the staining differences.

In earlier studies, measurement of TUNEL assay was highly correlated with sperm vitality [176, 215]. There was a significant difference on the staining efficiency of the dead population of indirect labeling compared to direct labeling (40.1% vs 65.7%, $p < 0.05$). On the other hand, no significant difference was found on staining the number of live spermatozoa using both TUNEL labeling methods 8.9% vs 8.3%, $p > 0.05$) [214]. TUNEL assay should be standardized in all laboratory centers performing this specialized test. This will help clinicians to further assess these infertile men whose treatment options may highly depend in the results of SDF testing.

8.11 Common Direct Methods to Measure DNA Integrity

Apart from the TUNEL assay, the comet assay is likewise a direct method to measure SDF. This quantifies the amount of DNA damage per spermatozoon. The degree of SDF is proportional to the intensity and length of the DNA fragments that stream out of the sperm head. This represents the amount of migrated DNA which looks like a comet with a tail when viewed under the fluorescent microscope and DNA stains. This assay is beneficial for men with severe oligozoospermia as low as 5000 sperm [216]. Comet assay can detect alterations in DNA bases aside from its identification of the usual DNA strand breaks. However, this is not a rapid test, as it demands highly specialized personnel to run the assay.

8.12 Current Challenges in Sperm Chromatin Integrity Tests

Well-known professional specialty societies do not recommend the routine use of SDF testing in the evaluation of men presenting with problematic reproductive potential [217]. This is due to unavailability of good quality methodological studies in support of the application of this laboratory test in the clinical scenarios. On the other hand, there is a wide list of evidences including systematic reviews and meta-analyses demonstrating the beneficial impact of SDF testing to reproductive outcome. Despite these findings, different professional societies still do not recommend the routine use of SDF testing in the evaluation of infertile men [1, 217, 218]. In a strengths-weaknesses-opportunities-threats (SWOT) analysis by Esteves et al. on the clinical utility of SDF testing, 51 out of 58 fertility expert participants were supportive of the recommendation set by the clinical practice guideline. Thirty participants expressed their concerns regarding the technical weak points of the SDF testing [184].

Cost of the SDF testing is one of the drawbacks of requesting this procedure. It was reported that SDF testing costs roughly $170.4 ± 122.9 (range $0–450). In a survey, 46.9% of clinicians expressed their unwillingness to utilize these test due to cost to the patients [219]. Majzoub et al. [219] developed a questionnaire to survey fertility specialists on major aspects of SDF testing. Forty-nine invited scholars from 19 different countries mostly composed of urologists (44.9%) completed the questionnaires. The majority (79.6%) of the participants commonly requested SDF testing as part of the evaluation of infertile men. TUNEL (30.6%) and SCSA (30.6%) were frequently utilized to analyze SDF. DFI of 30% was used as a threshold value by 61.2% of the participants. The most common indication to request for SDF testing was recurrent conventional IVF failure or pregnancy loss following conventional IVF (91.8%). The least common reason was to assess patients with low grade varicocele and subnormal semen parameters (46.9%). Cost (46.9%) and lack of validation (36.7%) were major factors for not requesting for SDF testing.

8.13 Future Direction

With support from the Society for Translational Medicine in endorsing the clinical practice guidelines for SDF testing in the evaluation of infertile men, other specialty societies may re-review their recommendations on the potential benefit of this specialized test [193]. There should be an expanded list of indications on who will benefit from this testing and not only be limited to practice recommendations. Good methodological studies should be conducted to determine the positive impact on the clinical utility of SDF testing in the evaluation of infertile men [220]. With more research, a solid evidence-based foundation for future utilization of SDF testing will benefit those men with reduced reproductive potentials as this might be their only hope to father a child.

8.14 Conclusion

In this chapter, we have emphasized the importance of assessing sperm chromatin integrity. Results of sperm assessment provide useful information in cases of male idiopathic infertility and in couples pursuing assisted reproduction. There are multiple assays that can be used to evaluate sperm chromatin. Each assay has advantages and limitations. Choosing the right assay is important and depends on factors such as equipment cost, availability of an andrology laboratory, and the presence of experienced technicians. It is important to determine the underlying cause of DNA damage and provide proper therapeutic treatment before attempting ART to minimize downstream potential side effects of offspring.

It is important to design methods for selecting sperm with undamaged DNA in ART, especially in ICSI. Assessment of sperm DNA damage can be a potential tool for evaluating semen samples prior to their use in assisted reproduction. It allows for the chance to select spermatozoa with intact DNA or with the least amount of DNA damage for use in assisted conception. It provides better diagnostic and prognostic capabilities than standard sperm parameters for assessing male fertility potential. There is a strong data supporting the inclusion of sperm DNA fragmentation in the evaluation of the infertile male.

8.15 Review Criteria

An extensive search of studies examining the relationship between sperm chromatin and sperm DNA fragmentation and male infertility and ART outcome was performed using search engines such as Google Scholar and PubMed. The start and end dates for these searches were September 1996 and September 2018, respectively. The overall strategy for study identification and data extraction was based on the following key words: "male infertility," "sperm chromatin," "reactive oxygen species," "infertile men," "DNA fragmentation," "marks of sperm chromatin assessment," "direct and indirect markers of sperm DNA fragmentation," "sperm DNA fragmentation and male infertility," and "sperm DNA fragmentation and ART failure." Articles published in languages other than English were excluded. Data published in conference or meeting proceedings, websites, or books was also excluded.

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Proteomic and Metabolomic Fingerprinting in Male Infertility

9

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Key Points

- Semen proteomics is able to provide valuable information about the patho-physiological state of the spermatozoa.
- Integration of proteomics as a component of male infertility diagnosis in the clinical lab can help explain the underlying molecular etiology associated with specific infertility conditions.
- Metabolomics strategies could potentially be used to differentiate between fertile males and infertile patients or between two different infertility-related disorders.
- Male infertility-related disorders may each have specific metabolic profiles which could contribute toward the noninvasive diagnosis of infertility in males.
- Specific proteins or metabolites identified using the omics strategies may serve as potential biomarkers of a specific male infertility disorder and in the development of new therapeutic modalities.

9.1 Introduction

In the current scenario, infertility is a major concern among couples in the reproductive age group with a global prevalence of 9%. Infertility in 27% of these couples is due to both male and female factors while 38% is contributed by the female alone and 20% is attributed to the male factor. The remaining 15% of infertility problems are idiopathic [1]. An overall 50% of cases are contributed by male factor infertility [2]. Male infertility is a multifactorial disorder and it is evaluated based on the results of semen analysis tests. Conventional semen analysis is considered as the corner stone for the diagnosis of male infertility. It provides information about semen parameters such as sperm concentration, motility, morphology, and vitality. The World Health Organization (WHO) has provided well-established reference values for semen parameters to distinguish fertile men from infertile men [3]. Additionally, advanced tests are carried out along with basic semen analysis to determine the levels of oxidative stress and sperm DNA damage, which are a major cause of fertilization failure or male infertility [4, 5]. However, there are still other underlying mechanisms at a subcellular level of the spermatozoa that cannot be explained merely from the results of a conventional semen analysis.

On the other hand, omics studies are able to explain the molecular mechanisms underpinning male infertility using different approaches. The four main branches of omics include genomics, transcriptomics, proteomics, and metabolomics (Fig. 9.1). Among these omic studies, proteomics and metabolomics are being widely used in the field of male infertility. Semen proteomics and metabolomics are undertaken to understand the cellular pathways and metabolic pathways associated with normal gametogenesis and the role of proteins and metabolites in the fertilization process. Furthermore, proteomics and metabolomics analysis, with bioinformatic tools and metabolomic analysis along with chemometrics, serves as a promising tool in the identification of potential diagnostic and therapeutic biomarkers for the management of male infertility.

In recent years, the availability of advanced proteomic and metabolomic tools has increased the knowledge and understanding of the causes of male infertility. This chapter provides a brief overview of advanced proteomic and metabolomic techniques used in studies involving the sperm and seminal plasma of infertile males. It highlights the general steps involved in these omics approaches, including bioinformatic analysis of the proteomic data. Furthermore, pro-

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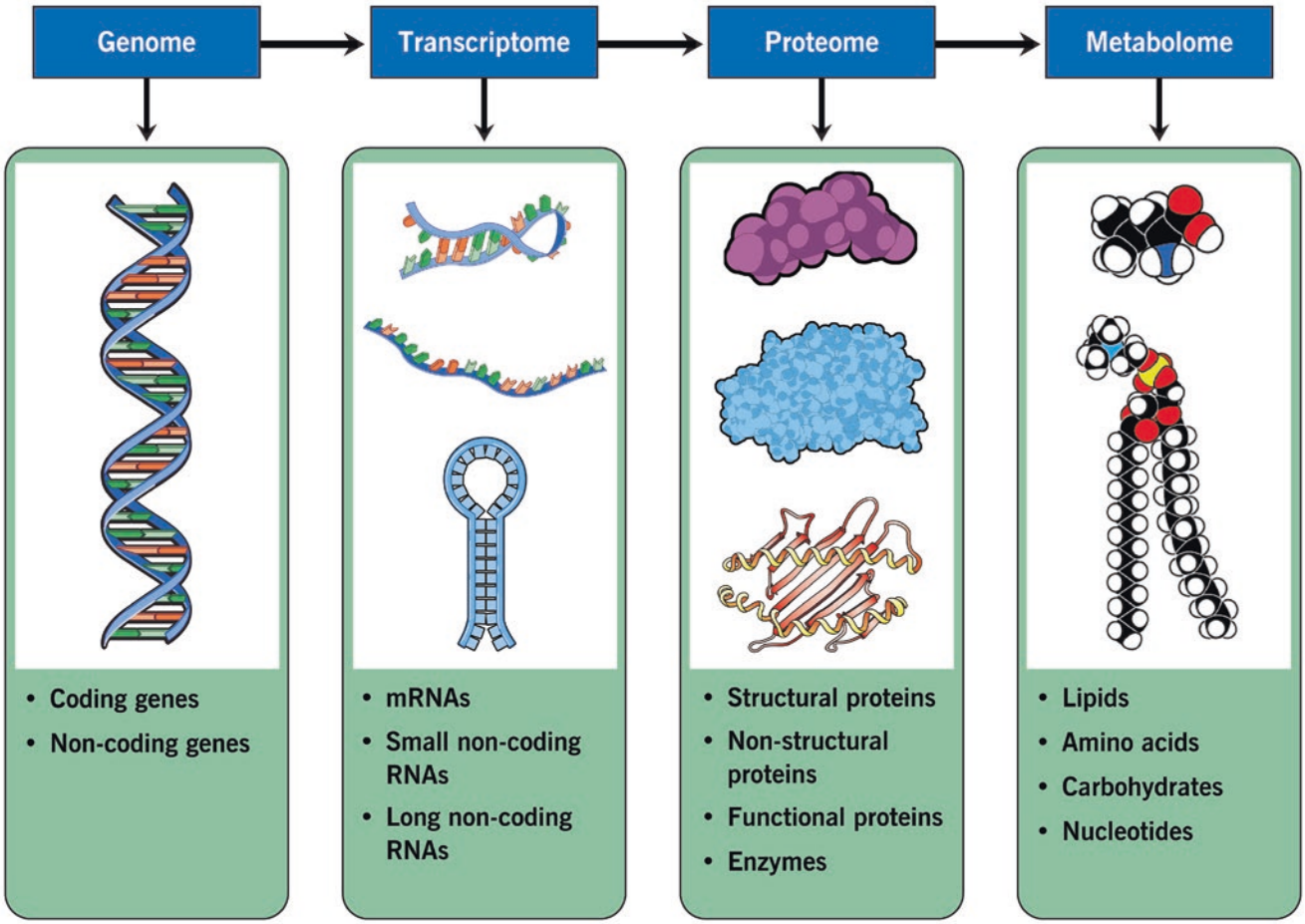

Fig. 9.1 Different classes of biomolecules detected in the spermatozoa using different omics techniques. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2018–2019. All Rights Reserved)

teomic- and metabolomic-based studies in sperm and seminal plasma are discussed in detail, along with the potential role of biomarkers in the prognosis and diagnosis of male infertility.

9.2 Proteomics in Male Infertility

Proteomics is defined as the complete profiling of proteins extracted from a tissue or cell. The most commonly used proteomic approaches, such as shotgun or bottom-up, can identify more than 1000 proteins in a short period of time. Semen is considered as the biological fluid used for the diagnosis of male infertility. Cellular component of semen is made up of sperm (5%) and seminal plasma (95%). Sperm are transcriptionally and translationally inert gametes and rely on proteins for their functional activity. Currently, proteomics is being widely used in the field of male infertility to study the protein profiles in spermatozoa and seminal plasma [6–8]. High throughput platforms such as matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and mass spectrometry coupled with liquid chromatography (LC-MS/MS) are used to profile a maximum number of proteins in the sperm and seminal plasma [9].

Advanced proteomic tools were used to identify more than 6000 sperm proteins. Using the data mining approach, 30 proteomic studies were analyzed and a total of 6198 proteins were identified in the spermatozoa [10]. A similar approach was used by Jodar et al., to identify 2064 proteins in the seminal plasma [7]. Any alteration in the sperm or seminal plasma proteome may have an adverse effect on the normal physiological function of the spermatozoa. Several proteomic studies have identified alterations in the sperm and seminal plasma proteome associated with male infertility conditions such as varicocele [11–16], idiopathic infertility [17–19], unexplained infertility [20–23], elevated oxidative stress [24–26], and testicular cancer [27, 28]. Moreover, the key proteins associated with vital sperm functions such as capacitation, hyperactivation, acrosome reaction, and fertilization process, are identified as potential noninvasive biomarkers to differentiate infertile men from normal healthy fertile men.

9.2.1 General Approach to Proteomics

Different proteomic techniques were used in the detection and identification of sperm proteins. The conventional approach includes the use of a two-dimensional (2D) gel electrophoresis of the extracted sperm or seminal plasma proteins. The proteins present in the sample are separated out based on the isoelectric focusing property and molecular weight of the peptides. Martinez-Heredia et al. identified a total of 98 distinct proteins in the human spermatozoa using 2D gel electrophoresis coupled with MALDI-TOF technique. The majority of these proteins were mainly involved in energy production, protein synthesis, and transcription process [29]. A modified version of the 2D gel electrophoresis technique, known as difference gel electrophoresis (DIGE), is used to identify differentially expressed proteins (DEPs) with a minimum error of <10% [30]. Based on the intensity of the different staining dyes (Cy3 and Cy5), the expression of the DEPs are determined on the same gel using automated image analysis software.

Conventional proteomic techniques have several limitations, such as decreased sensitivity, detection of a fewer number of proteins in a given sample, and the missing out in the detection of less abundant proteins. Investigators were able to overcome these limitations by using sophisticated and complex instruments such as MALDI-TOF and LC-MS/MS. These instruments can detect the maximum number of proteins even in samples that are of lesser concentration. Using the in-gel digestion-based LC-MS/MS approach, Johnston et al. identified 1760 sperm proteins and also reported the abundance of 26S proteasome complex [31]. Later on, several other studies also employed the LC-MS/MS-based proteomic profiling of spermatozoa in men with infertility disorders [22, 28, 32–34].

9.2.2 Assessment of Sperm and Seminal Plasma: Methods and Tools, Analysis, Bioinformatics

Proteomic analysis starts with the extraction of proteins either from the spermatozoa or seminal plasma. Seminal plasma are rich in proteins and are readily available for proteomic experiments without undergoing any purification process. However, prior to the extraction of proteins, sperm are subjected to several purification and processing steps. First, sperm are separated from the seminal plasma by the centrifugation technique. Apart from the sperm, semen also contains other cells such as round cells and immature germ cells. The round cells include both spermatogenic as well as non-spermatogenic cells. Investigators proposed that the use of sperm with round cells may contaminate the sperm proteome. Therefore, the density gradient centrifugation step was recommended and performed to isolate a pure fraction of the spermatozoa for proteomic analysis [35–39]. Recently, Paneer Selvam et al., conducted two proteomic studies to understand the role of contamination by round cell proteins in the proteome of sperm and their effect on biological pathways associated with sperm function [40, 41]. The presence of round cell proteins were masked by the sperm proteome, and the influence of non-spermatogenic round cell proteins was found to be very negligible or insignificant [40]. Moreover, the presence of these round cells and leukocyte proteins failed to show any effect on the molecular pathways associated with sperm function [41] (Fig. 9.2).

The isolated sperm are routinely washed for a minimum of three to four times with phosphate buffer saline to remove the remnants of seminal plasma. The sperm pellet, free from any contamination, is mixed with radioimmunoprecipitation assay (RIPA) buffer and left overnight. This results in the complete lysis of spermatozoa. Sonication of the spermatozoa suspended in an isotonic medium is also carried out to extract the sperm proteins. Extracted sperm proteins are checked for their purity and concentration, and then subjected to one-dimensional SDS-PAGE. Proteins separated by electrophoresis are subjected to in-gel digestion using trypsin. Digested proteins and peptides are eluted and injected into the mass spectrometry (MS) system. MS detects the peptides and proteins with an unbiased approach [42]. The proteins are identified with a very low false discovery rate based on their mass/charge ratio (m/z). To identify the post-translational modification such as acetylation, methylation, and phosphorylation in the sperm proteome, enrichment protocols are recommended. In addition, MS coupled with high performance liquid chromatography (HPLC) can simplify the detection of the complex proteins. Other techniques such as MALDI-TOF and SELDI-TOF (surface-enhanced laser desorption/ionization time-of-flight) are also successfully used to detect the sperm proteins [43, 44].

Initially, the complete scan of peptides detected by the MS is compared with the global database consisting of previously annotated and sequenced proteins. Computational software such as SEQUEST, Mascot and X! Tandem operating with different algorithms displays the complete list of proteins [45]. Furthermore, the proteins are categorized as DEPs based on spectral counts and abundance of each protein. These DEPs are used in the downstream bioinformatic analysis to understand the role of proteins in the molecular pathways [46]. Gene ontology (GO) analysis provides additional information such as localization and distribution of the proteins. Freely available bioinformatic tools such as STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) are used to understand the interaction between proteins [47]. In addition, commercially available sophisticated software such as Ingenuity Pathway Analysis (IPA) and Metacore™ are used to obtain a complete picture of the

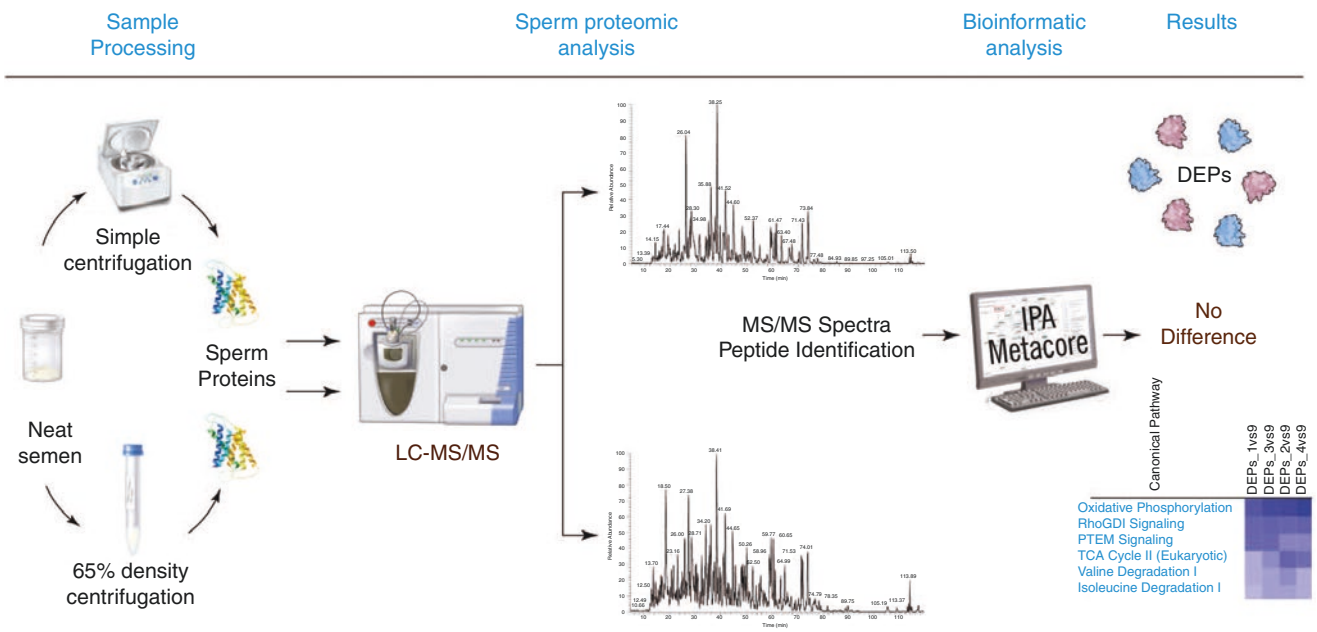

Fig. 9.2 LC-MS/MS analysis of sperm demonstrates the use of neat semen samples in proteomic/bioinformatic analysis. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2018–2019. All Rights Reserved)

interactions between the proteins and the transcriptional factors regulating their expression [47].

9.2.3 Sperm Proteomics

Any pathological state will alter the homeostasis and have a direct effect on the proteome of the tissue or cell. Changes in the proteome content of the spermatozoa may have deleterious effects on the fertilizing ability of spermatozoa. Several studies have identified the changes in the expression of the proteins associated with male infertility.

Proteomic profiling of sperm was performed on asthenozoospermic infertile men [48]. A total of 667 sperm proteins were identified in the asthenozoospermic men. These proteins were found to affect cellular pathways such as glycolysis, gluconeogenesis, and axoneme activation and nucleosome assembly [48]. Cao et al., demonstrated that proteins such as cytochrome c oxidase subunit 6B (COX6B), outer dense fiber 2 (ODF), and tubulin beta 2B (TUBB2B) involved in sperm motility were differentially expressed [32]. Similarly in another study on asthenozoospermic samples, Siva et al., identified that the proteins related to energy and metabolism, movement, and organization and protein turnover, folding, and stress response were significantly altered in their expression levels [49]. In addition to the proteins associated with sperm motility, the other protein components of proteasome complex were also differentially expressed [50, 51]. Moreover, phosphoproteome analysis of the asthenozoospermic samples showed that the dysregulation of these group of proteins were associated with cytoskeleton, fibrous sheath, and energy metabolism [52].

Globozoospermia is an abnormality of the spermatozoa that is associated with male infertility. Proteomic analysis of spermatozoa associated with globozoospermia revealed a total of 35 DEPs that play a vital role in spermatogenesis, cell skeleton, metabolism, and spermatozoa motility [53]. Also, perinuclear theca (PT) proteins were underexpressed and these were involved in acrosomal biogenesis, thus affecting the acrosome function in globozoospermic patients [54].

Proteomic analysis of sperm samples has identified the underlying changes or molecular pathology associated with varicocele condition. LC-MS/MS analysis of the sperm proteins in unilateral varicocele patients showed overexpression of 114 proteins and underexpression of 97 proteins. These DEPs were involved in sperm maturation, motility, capacitation, acrosome reaction, and fertilization [11]. Bioinformatics analysis demonstrated that small molecule biochemistry and post-translation modification proteins pathways were affected in infertile men with unilateral varicocele [11]. Agarwal et al. conducted a prospective study using the sperm samples from bilateral varicocele patients. A total of 73 DEPs were identified and the majority of these DEPs were involved in the regulation of functions such as metabolic processes, stress responses, and oxidoreductase activity. The remaining proteins were involved in sperm functions such as capacitation, motility, and sperm-zona binding [34]. Another proteomic study by the same researchers showed that the dysregulation of the mitochondrial proteins is a cause of male infertility in varicocele patients [16, 34]. The key DEPs

identified as biomarkers in different varicocele studies are presented in Table 9.1.

Testicular cancer has a deleterious effect on semen parameters and the fertilization potential of spermatozoa. The American Cancer Society estimated that there will be 9310 new cases and 400 deaths related to testicular cancer [55]. Several proteomic studies have been conducted for the diagnosis of testicular cancer [56–58]. These studies, however, did not examine the proteome of spermatozoa from testicular cancer patients prior to cancer treatment. Recently, Dias et al., profiled the sperm proteome in men with non-seminoma testicular cancer (NSTC) using the LC-MS/MS platform. They had identified a total of 189 DEPs in their study. Among the DEPs identified, NADH:Ubiquinone Oxidoreductase Core Subunit S1 (NDUFS1), ubiquinol-cytochrome C reductase core protein 2 (UQCRC2), and the testis-specific sodium/potassium-transporting ATPase subunit alpha-4 (ATP1A4) were proposed as the potential biomarker for NSTC patients. Furthermore, mitochondrial dysfunction is identified as the primary cause for the decrease in sperm concentration and motility [28].

9.2.4 Seminal Plasma Proteomics

Apart from sperm proteins, seminal plasma proteins are essential for sperm protection, maturation, and fertilization process. Male infertility conditions such as azoospermia, oligoasthenozoospermia (OAT), and varicocele showed alteration in the seminal plasma proteins [59].

Azoospermia may be either obstructive or non-obstructive. The seminal plasma proteome of azoospermic subjects revealed extracellular matrix protein 1 (ECM1) as a biomarker to differentiate obstructive azoospermia (OA) from non-obstructive azoospermia (NOA) and a differential expression of testis-expressed protein 101 (TEX101) in distinct NOA subtypes [60, 61]. A proteomic study by Yamakawa et al., proposed a total of 4 and 1 biomarker for NOA and

Table 9.1 Key differentially expressed proteins (DEPs) identified in various clinical conditions associated with male infertility

Condition	Sample	Method	DEPs	Reference
Varicocele	Spermatozoa	1D PAGE LC-MS	TEKT3, TCP11	Agarwal et al. (2016) [13]
	Spermatozoa	1D PAGE LC-MS	PKAR1A, AK7, CCT6B, HSPA2, ODF2	Agarwal et al. (2016) [34]
	Spermatozoa	1D PAGE LC-MS/MS	GSTM3, SPANXB1, PARK7, PSMA8, DLD, SEMG1, SEMG2	Agarwal et al. (2015) [11]
	Spermatozoa	LC-MS/MS	LETM1, EFHC, MIC60, PGAM5, ISOC2,TOM22, NDFSU1, UQCRC2, COX5B, ATPase1A4, HSPA2, SPA17, APOA1	Samanta et al. (2018) [16]
	Seminal plasma	2D- LC-MS/MS	ZA2G, KCRB, ALBU, NPC2, FINC, PIP, SEMG1, SEMG2, KLK3, TRFL, PPAP, ANXA3, CATB, EP3B, PTGDS, SODE, A1AT, ASAH1, CALM, CRIS1	Fariello et al. (2012) [64]
	Seminal plasma	2D- LC-MS/MS	IBP-3, SMG1, BRE1B, NPC2, IDH, E3-beta	Zylbersztejn et al. (2013) [65]
Testicular cancer	Spermatozoa	1D PAGE MS	NDUFS1, UQCRC2, ATP1A4, ACR, ANXA 2	Dias et al. (2018) [28]
Azoospermia	Seminal plasma		ECM1, TEX101	Drabovich et al. (2013) [60]
	Seminal plasma	2D DIGE LC-MS/MS	STAB2, CP135, GNRP, PIP, NPC2	Yamakawa et al. (2017) [62]
Asthenozoospermia	Spermatozoa and seminal plasma	UPLC-MS	PLXNB2, POTEKP, NIN, PHF3, DYNLL1, PROCA1, FASCIN-3; LRRC37B, PLC	Saraswat et al. (2017) [48]
	Spermatozoa	2D PAGE MALDI MS/MS	TPIS, GKP2, OXCT1, TUBB2C, TEKT1, PSMA3, HSPA2	Siva et al. (2010) [49]
	Spermatozoa	UPLC-MS(E)	GRP78, HSP70–2, TUBA4A, TUBA3C, TUBA8, ODF1, AKAP3, AKAP4, GAPDHS, ROPN1B, SPANXB, CLU, PIP, ATP5B	Parte et al. (2012) [52]
Oligoasthenozoospermia	Seminal plasma	1D PAGE/LC-MS/MS	AACT, TBCB, ALDR	Herwig et al. (2013) [19]
	Seminal plasma	2D PAGE LC-MS/MS	NPC2, M2BP, LCN1, PIP	Giacomini et al. (2015) [33]
	Seminal plasma	2D chromatography LC-MALDI	LTF, PIP, ECM1, HE1, PTGDS, CD177, PSA	Liu et al. (2018) [63]
Globozoospermia	Spermatozoa	2D DIGE MALDI-TOF/TOF MS	SAMP1, ODF2, SPANXa/d, TUBA2, TPI1, PIP	Liao et al. (2009) [53]

OA, respectively. The NPC2 protein was suggested as a potential biomarker for OA patients [62].

OAT is a semen abnormality condition associated with male infertility. Proteomic analysis of seminal plasma exposed a total of 2489 proteins in subjects with OAT [19]. Twenty-four proteins, primarily involved in metabolism and inflammation, defense, and stress responses, were highly expressed in idiopathic OAT (iOAT). A comparative proteomic analysis of oligoasthenozoospermic and normozoospermic seminal plasma revealed epididymal secretory protein E1 (NPC2) and galectin-3-binding protein (M2BP) to be underexpressed, while lipocalin-1 and a form of prolactin-inducible protein to be overexpressed in iOAT [33]. A recent comparative proteomic analysis identified DEPs involved in multiple biological functions such as binding activity (lactotransferrin, LTF; Prolactin-induced protein, PIP; extracellular matrix protein 1, ECM1), transporter activity (human epididymis-specific *protein* 1, HE1; Prostaglandin D_2 synthase, PTGDS), immune activity (CD177), and hydrolase activity (prostate-specific antigen) were differentially expressed in the seminal plasma of OAT subjects [63].

Seminal plasma proteins were also studied in varicocele patients. The first report on seminal plasma proteins identified 95 DEPs in cigarette smoking, adult varicocele patients. Seminal plasma proteins involved in sperm maturation and sperm–oocyte fusion were dysregulated in these varicocele patients [64]. Whereas, the expression of proteins associated with sperm motility and capacitation were altered in seminal plasma of adolescents with varicocele [65]. A study by Belardin et al. reported that insulin-like growth factor-binding protein 7 (IGFBP7) and deoxyribonuclease-1 (DNASE1) were involved in the regulation of apoptosis as seminal plasma biomarkers in adolescents with varicocele [15]. The seminal plasma proteomic signature also varied from before and after varicocelectomy. Cellular pathways such as oxidative stress and protein stabilization were enriched in patients after varicocelectomy. Proteins related to homeostasis function such as DJ-1, S100-A9, SOD, ANXA1, G3P, and MDH were upregulated and proteins associated with oxidative stress (such as NELFE) were downregulated after varicocelectomy [65].

Proteomic studies identifying DEPs associated with various male infertility conditions are listed in Table 9.1.

9.3 Metabolomics in Male Infertility

Metabolomics is the latest of the omics technologies that has been gaining traction in male infertility research over the last decade. Metabolomics involves the unbiased identification and quantification of all low molecular weight metabolites (< 1 kDa) within a biological system. The resulting metabolome consists of a complete set of these metabolites, which include secondary metabolites as well as hormones and other signaling molecules present in a biological sample [66]. Metabolomics may be applied on various biological samples, including that of bodily fluids (e.g. urine, blood plasma or serum, seminal fluid, follicular, or endometrial fluid) and various tissues in the body [67, 68]. As intracellular metabolites are in a state of dynamic balance with the metabolites in the biological fluids that perfuse these cells, the composition of biological fluids could therefore provide useful insights into the present metabolic state of the body [69].

Moreover, the study of the metabolome is comparatively less complex, provides more real-time information, and could give direct impact of a certain condition/stimuli on the body. For example, while genes and mRNA transcripts may run up to hundreds of thousands in numbers, and proteins to millions, the downstream products of metabolism (i.e., metabolites) only amount to a few thousand within the human metabolome [44]. These metabolites resemble the current phenotypic state of the cell more closely than the transcriptome and proteome do. This is because following gene expression, post-transcriptional and post-translational modifications take place, and these changes are relatively augmented in the metabolome compared to the transcriptome or proteome [70]. As such, metabolomic profiling of a particular biofluid or tissue could thus reveal the current health status of the individual [71].

9.3.1 General Approach to Metabolomics

There are several approaches that could be utilized when applying the metabolomics strategy to biological samples. Metabolomic fingerprinting provides a high-throughput, global and rapid biochemical analysis, which serves as a screening tool to differentiate between samples from healthy controls and that of diseased patients [66]. Changes detected in patient samples from that of normal samples are then correlated with the severity status of the disease or used to assess how an intervention is faring. On the other hand, metabolomic profiling involves the identification and quantification of a selected number of pre-defined metabolites that are involved in a particular metabolic pathway [66], while untargeted metabolic profiling is commonly done as a comparative analysis between the control and treatment groups. Both metabolomic fingerprinting and profiling have been applied to studies dealing with infertile males. Yet another approach is metabonomics, which deals with the quantitative analysis of metabolites in response to either disease or therapeutic treatment or to genetic modification. No matter the approach utilized, all metabolomics strategies include the identification and quantitation of metabolites [72].

The detection of metabolites in human metabolomics studies may be approached as either targeted or untargeted (global) (reviewed in Agin et al. [73]). Studies that employ the targeted approach have a specific hypothesis which warrants the investigation of certain biochemical pathways. Therefore, pre-determined metabolite-specific signals and analytical standards are employed to quantify the concentrations of a specific number of known metabolites in a precise and accurate manner. On the other hand, the global approach aims to measure and compare as many signals as possible without knowing the nature and identity of the metabolites beforehand. Thus, complex datasets are generated along with metabolites that are yet to be characterized. Global metabolomics studies provide only qualitative and semi-quantitative data, however, these studies help identify unknown metabolites, new pathways and generate hypotheses [74–77]. The study of the metabolome is multi-disciplinary and involves disciplines such as analytical chemistry, chemometrics, and biology. Analytical chemistry is useful during sample preparation, generation of metabolic profiles, and elucidation of metabolic structure. Chemometrics is required to extract the most pertinent information from the large datasets generated, for example, in studies using the metabolic fingerprinting strategy. Biology is necessary for understanding the observations, underlying mechanisms of action, and metabolomics pathways of interest [78].

9.3.2 Analysis of the Metabolome

Analysis of the metabolome is generally performed in four main stages: (1) collection of samples (sampling, quenching, and storage), (2) preparation of samples (extraction of metabolites, dilution, and clean up), (3) acquisition of data, and (4) analysis of data to generate a metabolic profile [78, 79].

Samples used in studies pertaining to male infertility include testicular tissue, seminal plasma, spermatozoa, blood serum and plasma, and urine (Table 9.2). The sample chosen for a particular study is influenced by the type of sample that can provide the most information for the intended study, its feasibility, as well as ease of collection [80]. Moreover, each of these biofluids could provide different types of information. For example, urine has the highest amount of water-soluble molecules, whereas blood composition is less variable than urine [81].

Following sample collection, the sample has to be stabilized so that it represents the actual metabolome composition at the time of sample collection. This is done via a metabolism-quenching step in order to halt further metabolic reactions that could either generate or degrade metabolites. Samples are snap frozen in liquid nitrogen for this purpose and stored at −80 °C [82]. Next, the sample is prepared according to protocol. For example, solid matrices undergo an extraction step to transfer the metabolome compound into a liquid phase, whereas low volatile analytes that will be analyzed via gas chromatography (GC) will undergo a derivatization reaction step (e.g., alkylation, acylation, silylation) to increase its volatility and reduce its polarity [83, 84].

Analysis of the metabolome can be approached through several techniques including nuclear magnetic resonance spectroscopy (NMR), Fourier transform infrared spectroscopy (FTIR), near-infrared (NIR), Raman spectroscopy, liquid chromatography, or gas chromatography coupled with mass spectrometry (LC-MS or GC-MS, respectively). Each of these techniques has its own advantages and shortcomings that have been previously reviewed elsewhere [66, 68]. The data acquired are processed using software tools and analyzed using suitable statistical techniques to segregate fewer, relevant metabolites (i.e., potential biomarkers/differential metabolites) that could possibly differentiate the compared sub-groups. Experiments that follow would then focus on the potential biomarker metabolites and explanations of the changes observed [78].

9.3.3 Sperm Metabolomics

Back in 2009, Huser's group had used the micro-Raman spectroscopy technique on individual sperm cells of healthy males to examine if the Raman spectra of sperm chromatin packed within the heads of normal or abnormally shaped sperm cells showed any correlation with its protein content and DNA conformation [85]. While the efficiency of DNA packaging and relative protein content per cell differed between the morphologically normal and abnormal sperm, the study also highlighted the significant variation that existed in protein content and DNA packaging within sperm cells with normally shaped heads [85].

Some years later, Paiva and colleagues were the first to obtain a comprehensive metabolomic profile of mature human spermatozoa through the use of two complementary untargeted metabolomics strategies: proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy and GC-MS in normozoospermic and asthenozoospermic samples [86] (Table 9.2). The NMR and GC-MS techniques identified 42 and 27 endogenous metabolites, respectively, with an overlap of four metabolites. The bulk of the identified metabolites belonged to the super classes of amino acids, peptides, analogues, organic acids, and lipids [86]. NMR and MS strategies provide complementary results that may be applied toward completing the metabolome of the mature human sperm cell.

Using the proton magnetic resonance spectroscopy (^{1}H-MRS) technique, Reynolds et al. determined the molecular composition of a live human spermatozoon from healthy vol-

Table 9.2 Metabolomics studies on male fertility/infertility

Study population	Human body fluid/tissue	Analytical technique	Main study outcomes	Reference
Spermatogenic failure; obstructive azoospermia; oligoasthenozoospermic infertility	Seminal plasma	^{1}H-NMR spectroscopy	^{1}H-NMR spectroscopy could potentially be used to differentiate OA from NOA Peak areas for GPC, citrate and lactate in seminal plasma were smaller in azoospermic men Peak area ratios for citrate:lactate and GPC:lactate were altered in control vs. (i) spermatogenic failure, (ii) OA Peak intensity ratio for GPE:GPC was different between spermatogenic failure and OA subjects	Hamamah et al. (1993) [89]
Non-obstructive azoospermia	Seminal plasma	GC–MS	Proposed a novel diagnostic method for NOA patients using the TIC data of metabolites in seminal plasma 36 metabolites were identified as potential discriminatory biomarkers for different groups in NOA	Gilany et al. (2017) [91]
Non-obstructive azoospermia	Seminal plasma	Raman spectroscopy	The seminal plasma metabolome could be used to detect spermatogenesis in NOA patients This metabolomics technique can be applied to detect major metabolomic alterations in seminal plasma of NOA from TESE(+) vs. TESE(−) cases TESE(−) patients had severe oxidative imbalance compared to TESE(+)	Gilany et al. (2018) [92]
Non-obstructive azoospermia (maturation arrest or Sertoli-cell only); normozoospermia (vasectomy reversal)	Testicular tissue	^{1}H-NMR spectroscopy	Testicular concentrations of phosphocholine and taurine were significantly different between normal, and SCO tissue Phosphocholine concentrations were significantly higher in testes with spermatogenesis ^{1}H-NMR spectroscopy could be used to discern a distinct metabolic signature for spermatogenesis, thus contributing to the non-invasive diagnosis of sperm in men with NOA	Aaronson et al. (2010) [96]
Non-obstructive azoospermia and obstructive azoospermia	Testicular tissue	Raman spectroscopy; GC/TOF-MS	Raman spectroscopy could be used to discriminate between samples of NOA and OA testicular tissue Raman spectroscopy could be used to non-invasively differentiate seminiferous tubules with complete and incomplete spermatogenesis. Levels of 12 metabolites were altered between NOA and OA testes (increased: cis-Phytol, glutamine; decreased: S-carboxymethylcysteine, fructose, arachidonic acid, etc.)	Liu et al. (2014) [97]
Non-obstructive azoospermia	Blood serum	HPLC-MS/MS	Serum metabolomic profile could be used to differentiate NOA patients from fertile males 24 metabolites were identified as potential markers, many of which were involved in energy production, oxidative stress, and cell apoptosis in spermatogenesis Metabolic pathways of glycometabolism, lipid metabolism, and amino acid metabolism were disrupted in NOA males Serum metabolic disorders may contribute to the etiology of NOA	Zhang et al. (2017) [115]
Oligozoospermia; normozoospermic infertility	Seminal plasma	^{1}H-NMR spectroscopy	^{1}H-NMR spectroscopy could potentially be used to differentiate normozoospermia from oligozoospermia 10 biomarkers were detected Alanine, citrate, GPC, tyrosine, phenylalanine could be used to screen for MI	Gupta et al. (2011) [102]

Table 9.2 (continued)

Study population	Human body fluid/tissue	Analytical technique	Main study outcomes	Reference
Oligozoospermia	Urine	LC/QTOF–MS	Oligozoospermic infertile men could be differentiated from fertile controls based on altered urinary metabolomic profiles Oligozoospermia seems to be associated with energy consumption and antioxidant defenses in spermatogenesis The combined pattern of acetylcarnitine, carnitine C3:1, and aspartic acid provided moderate diagnostic power for oligozoospermic infertility	Zhang et al. (2014) [95]
Asthenozoospermia	Seminal plasma	Raman spectroscopy	Metabolomic fingerprinting could be used to discriminate between asthenozoospermic and normozoospermic males	Gilany et al. (2014) [90]
Asthenozoospermia	Seminal plasma	^{1}H-NMR spectroscopy	Nineteen metabolites were either up- or down-regulated Asthenozoospermia-related changes of metabolites in lipid, phospholipid (choline), cholesterol, nucleoside, Krebs cycle, and energy metabolic pathways were detected Oxysterols (5α-cholesterol and 7-ketocholesterol) were raised in seminal plasma of patients with asthenozoospermia Oxidative stress is an underlying mechanism in the development of asthenozoospermia	Zhang et al. (2015) [93]
Asthenozoospermia	Seminal plasma	GC–MS	Of the 25 metabolites identified, 7 were significantly different in asthenozoospermic samples compared to controls Levels of oleic acid and palmitic acid were higher in seminal plasma of asthenozoospermic men, which may signify a metabolic disorder of the sperm membrane Deficiency of valine in seminal plasma of asthenozoospermic males may contribute to poor sperm motility	Tang et al. (2017) [116]
Asthenozoospermia	Spermatozoa	^{1}H-NMR spectroscopy and/or GC-TOF/MS	69 metabolites were identified in human sperm extracts: (i) 42 using NMR, (ii) 27 using GC-MS, (iii) 4 by both techniques (carnitine, L-threonine, gamma-aminobutyric acid, oxoglutaric acid) The majority of metabolites identified belonged to the amino acids, peptides, and analogues super class	Paiva et al. (2015) [86]
Idiopathic asthenozoospermia	Spermatozoa	GC-MS	33 metabolites were identified in spermatozoa 27 metabolites (e.g., 3-phosphoglycerate, lactic acid, glutamic acid, tryptophan, leucine, cysteine, guanosine, cytidine) were decreased in IAS group compared to controls 6 metabolites (zymosterol, dithioerythritol, orotic acid, 2-deoxyerythritol, benzoic acid, ethanolamine) were increased in IAS group compared to controls Pathways for nucleoside, amino acid and energy metabolism, and the Krebs cycle were either up- or downregulated) in IAS	Zhao et al. (2018) [88]
Idiopathic infertility, oligozoospermia, asthenozoospermia, teratozoospermia and azoospermia	Seminal plasma	NMR spectroscopy	Seminal plasma metabolomic profile of infertile males differed significantly from that of fertile males The metabolomic profile of idiopathic infertile men were clearly segregated from that of (i) fertile controls and (ii) other infertile groups The biomarker profile of idiopathic infertile patients differed from that of the other groups due to either up- or down-regulation of lysine, fructose, arginine, tyrosine, citrate, and proline	Jayaraman et al. (2014) [109]

(continued)

Table 9.2 (continued)

Study population	Human body fluid/tissue	Analytical technique	Main study outcomes	Reference
Unexplained male infertility	Seminal plasma	Raman spectroscopy	Metabolomic fingerprinting could be used as (i) a screening tool to diagnose male infertility and (ii) to study oxidative stress Men with unexplained infertility have (i) an imbalance of oxidative stress, (ii) increased biomarkers of oxidative stress, and (iii) absence of a functional antioxidant	Jafarzadeh et al. (2015) [94]
Unexplained male infertility	Seminal plasma	GC–MS	153 metabolites were identified in the seminal plasma of UMI subjects, of which 44 metabolites were differentially expressed in UMI compared to fertile controls Tryptophan, phenylalanine, glycine, serine, threonine, isoleucine, proline, and valine were decreased significantly; while urea and glutamine were increased significantly in UMI seminal plasma The major metabolic signature of UMI seminal plasma is the increased catabolism of various amino acids 4-Hydroxyphenylacetic acid is a key metabolite in differentiating between UMI and controls, and its seminal plasma concentration related positively to sperm counts	Qiao et al. (2017) [108]
Idiopathic normozoospermic infertility	Urine	LC/MicrOTOF–Q II MS	The urinary metabolomic profile could be used to differentiate normozoospermic infertile males from fertile controls 37 potential biomarkers were identified with functional roles in energy production, antioxidation, and hormone regulation in spermatogenesis A combination of the top 5 negative biomarkers (i.e. xanthosine, leukotriene E4, methoxytryptophan, 3-hydroxypalmitoylcarnitine, and aspartate) had the best diagnostic ability for detecting normozoospermic infertility	Zhang et al. (2014) [95]
Spinal cord injury-derived infertility	Seminal plasma	MALDI-TOF MS	85 ions were differentially present in seminal plasma of fertile and infertile men The lipids identified were mostly glycerolipids and were related to pathways for biosynthesis of CTP, UTP, and GTP Other metabolic pathways involved were sterol biosynthesis, arachidonic acid metabolism, and response to (i) hydrogen peroxide, (ii) steroid hormone, and (iii) vitamin Men with SCI-related infertility are likely to have altered signal transduction	Da Silva et al. (2011) [117]
Kidney yang deficiency syndrome-associated infertility (erectile dysfunction/premature ejaculation, weak ejaculation/orgasm disorder, oligozoospermia, asthenozoospermia)	Seminal plasma	LC/QTOF–MS	41 metabolites were differentially present in the seminal plasma of fertile men and infertile males with KYDS 7 metabolites were related to the 5 potential metabolic pathways Changes in metabolic pathways of biosynthesis and metabolism of aromatic amino acids, citric acid cycle, and sphingolipid metabolism may contribute to the development of KYDS-associated infertility	Chen et al. (2015) [118]

Table 9.2 (continued)

Study population	Human body fluid/tissue	Analytical technique	Main study outcomes	Reference
Kidney yang deficiency syndrome-associated infertility (erectile dysfunction/premature ejaculation, oligozoospermia, asthenozoospermia, azoospermia, teratozoospermia)	Blood plasma	GC–MS	Metabolomics profiles of infertile males with KYDS were identified 10 potential biomarkers (e.g., 1,5-anhydroglucitol, hydroxyvaleric acid) and 6 metabolic pathways (galactose glucitol, phenylalanine, glutamic acid, L-isoleucine, ornithine, lysine) could be used to discriminate between infertile males with KYDS and healthy controls Infertility in males with KYDS is probably associated with energy consumption and antioxidant defenses	Zheng et al. (2017) [119]
Erectile dysfunction; demen abnormalities	Blood plasma	GC–MS	Males with ED and semen abnormalities could be differentiated from fertile controls based on altered plasma metabolomic profiles 1,5-anhydro-sorbitol and α-hydroxyisovaleric acid are potential biomarkers to differentiate between infertile and fertile males Lactate, glutamate, and cholesterol could be used to discern between subjects with ED or semen abnormalities	Zhou et al. (2016) [120]
Young Danish men presenting with different sperm concentrations: (i) low (>0–20 × 10^6/mL), (ii) intermediate (45–75 × 10^6/mL), or (iii) high (>100 × 10^6/mL)	Blood serum	LC-MS	The serum metabolic profiles differed significantly between males presenting with different sperm concentrations Metabolites identified were mainly amino acids and carboxylic acids Peptides related to protein complement C3f (involved in innate immunity) were down regulated in men with low sperm concentrations and may serve as potential markers of fertility	Courant et al. (2013) [78]

unteers [87]. Sperm were obtained from fresh ejaculates using simple centrifugation or density gradient centrifuge (DGC) with either one or two washes. The DGC yielded sperm populations of 40% or 80%, which was then used to obtain the ^{1}H-MRS spectra. The lactate, lipid, and choline/glycerophosphocholine (GPC) peaks differed significantly between the ^{1}H spectrum of 40% and 80% sperm populations with greater differences evident in the 40% sperm population, which was more likely to possess greater structural defects [87].

Zhao and colleagues were the first to perform an untargeted metabolomics study utilizing GC-MS spectroscopy to compare the metabolic profile of spermatozoa from idiopathic asthenozoospermic and normozoospermic males [88] (Table 9.2). Results of this exploratory study showed dysregulation in amino acid and nucleotide metabolism pathways as well as disruption of glycolysis, Krebs cycle, and energy metabolism in patients with idiopathic asthenozoospermia compared to that of healthy males. Not only were the levels of amino acids (e.g., cysteine, leucine, tryptophan, and glutamic acid) found to be downregulated, guanosine and cytidine levels were also significantly lower in asthenozoospermic patients [88].

9.3.4 Seminal Plasma Metabolomics

A biochemical exploratory study of human seminal plasma in infertile males has been reported as far back as 25 years ago. Utilizing the ^{1}H-NMR method, the study measured the peak areas for glycerylphosphorylcholine (GPC), glycerylphosphorylethanolamine (GPE), citrate, and lactate in human seminal plasma to determine if these metabolites could act as biomarkers to differentiate between azoospermic and normozoospermic males [89] (Table 9.2). The study showed that azoospermic patients had significantly smaller peak areas for GPC, citrate, and lactate in seminal plasma compared to controls, while patients with spermatogenic failure and obstructive azoospermia differed significantly in their GPE to GPC peak intensity ratio [89].

Gilany et al. applied Raman spectroscopy in combination with chemometrics to differentiate between the seminal plasma metabolomic profile of asthenozoospermic and normozoospermic patients [90] (Table 9.2). Based on the Raman spectra obtained from the two groups, the researchers came up with a diagquadratic model that was able to predict between normal and asthenozoospermic samples with a validity of 83% [90]. Gilany's group went on to

examine the seminal plasma metabolome in patients with NOA using two different strategies: untargeted metabolomic profiling using GC-MS and advanced chemometrics [91] as well as metabolic fingerprinting via the Raman spectroscopy approach [92] (Table 9.2). Commonly, males with NOA undergo the invasive testicular sperm extraction (TESE) procedure for detection of sperm in their testes. However, in the untargeted metabolic profiling study, the researchers proposed the use of multivariate models as a new noninvasive diagnostic method to differentiate between fertile and NOA men who were either TESE positive or TESE negative [91]. Their 2018 study also proposed the use of the seminal plasma metabolome as a noninvasive method to detect spermatogenesis in NOA males who were either TESE positive or TESE negative. They also found that patients who were TESE negative had higher reactive oxygen species (ROS) levels compared to TESE positive males [92].

Similarly, Zhang's group conducted an untargeted metabolomic profiling study of seminal plasma using the H^1-NMR spectroscopy approach in asthenozoospermic males. They found that these patients had raised levels of oxysterols (i.e., 5α-cholesterol, 7-ketocholesterol), which suggests that oxidative stress is an underlying mechanism in their asthenozoospermic condition [93]. The role of oxidative stress in unexplained male infertility was also evident when Jafarzadeh and colleagues reported an increase in the –CH functional group (an oxidative stress biomarker) coupled with the absence of the –SH group (a functional antioxidant) that was detected in the Raman spectra of seminal plasma metabolome of these men [94] (Table 9.2).

9.3.5 Urine Metabolomics

In other studies, urine samples have been used to conduct a differential diagnosis between healthy men and diseased patients. For example, the urinary metabolome of normozoospermic infertile patients has been used to discern between normozoospermic infertile men and fertile controls [95] (Table 9.2). The group proposed that the potential negative changes in the citric acid cycle and hormonal activity during spermatogenesis, as well as oxidative stress are among the underlying events that could lead up to normozoospermic infertility [95].

In another study, Zhang's group showed that the urinary metabolomic profile of oligozoospermic patients (sperm concentration <20 M/mL) differed significantly from that of normozoospermic males [95] (Table 9.2). A stronger risk of oligozoospermia seemed to be indicated when biomarkers related to sperm concentration and amplitude of lateral head displacement was altered. Moreover oligozoospermia, a common indication in most male subfertility cases, was potentially associated with disruptions in fatty acid metabolism and antioxidant defenses in spermatogenesis [95].

9.3.6 Testicular Tissue Metabolomics

Metabolomics studies on testicular tissue have proposed noninvasive methods to diagnose either the presence of spermatogenesis in NOA men [96] or to differentiate between seminiferous tubules with complete or incomplete spermatogenesis cycles [97] (Table 9.2). In the latter, testicular tissue from azoospermic males (maturation arrest or Sertoli cell only) was snap frozen and subjected to ^{1}H-MRS. This method is potentially an alternative to testis biopsy as a diagnostic test to detect normal or abnormal spermatogenesis in azoospermic males [96]. In the former study, Raman spectroscopy was used to scan the seminiferous tubules within fresh testicular tissues retrieved from OA and NOA patients with a sensitivity of 90% and specificity of 85.71% in order to discern between seminiferous tubules that had complete spermatogenesis cycles and those that did not [97].

9.4 Potential Biomarkers of Male Infertility

By definition, a biomarker is identified as a characteristic biological marker that represents a condition, event, or process that can be quantitatively assessed, measured, and studied [98]. The biomarker should be highly sensitive, specific, and ideally be easily accessible in order to minimize the need for invasive and often inconvenient tests in the infertile male. In addition, such a biological molecule should facilitate a more detailed and precise classification of the infertile male [99]. Research studies that are evolving in the areas of genomics, proteomics, and metabolomics could potentially lead to the development of novel male infertility biomarkers [100].

The most commonly used biomarker to gauge the male fertility potential is semen analysis, which despite providing critical fundamental information, is highly variable and thereby a poor predictor of fertility [101]. In fact, some infertile males present with normal semen parameters in spite of their poor fertility potential [102]. As semen analysis alone is clearly inadequate to diagnose the infertile male, proteomic and metabolomic technologies are fast becoming potentially vital tools in identifying appropriate biomarkers for use in diagnosis, prognosis, and treatment of male infertility [103, 104]. Moreover, advances in bioinformatics and analytical technologies in the omics have helped further develop protein and metabolite profiling as a useful tool in biomarker discovery [59, 105].

Seminal fluid is made up mainly of seminal plasma and only a small volume of spermatozoa. Seminal plasma has a varied molecular composition and contains a high concentration of tissue-specific proteins which acts as an abundant source of potential biomarkers in the assessment of male fertility [61, 100]. Proteins such as the testis-specific TKTL1 (transketolase-like protein 1), LDHC (lactate dehydrogenase C), and PGK2 (phosphoglycerate kinase 2) seem to be able to serve as a biomarker to distinguish semen from fertile and infertile men [106]. TEX101, a cell membrane protein expressed specifically by testicular germ cells, is among the most promising biomarkers of male infertility [107]. TEX101 (testis-expressed protein 101) could act as a biomarker to predict TESE outcome and to differentiate between Sertoli cell-only syndrome and the other NOA subtypes (maturation arrest, hypospermatogenesis). In addition, the epididymis-expressed protein ECM1 (extracellular matrix protein 1) appears to be able to differentiate between NOA and OA [60]. Other examples of DEPs that could serve as potential biomarkers in the infertile male are shown in Table 9.1.

Metabolomics too shows great promise as a useful tool in disease diagnosis among infertile males [108]. Metabonomic profiling has been proposed as a tool to detect idiopathic infertility, as lysine concentration in the seminal plasma detected using ^{1}H-NMR was found to give a good indication of idiopathic infertility [109]. Qiao's group reported that patients with unexplained male infertility have increased catabolism of several amino acids that could impact male reproduction. Using a GC-MS-based metabolite profiling platform, the study had identified 4-hydroxyphenylacetic acid as a significant metabolite in seminal plasma that could help differentiate between males with unexplained infertility from those who were healthy [108]. Among asthenozoospermic males, deficiency in valine along with high levels of oleic and palmitic acids detected via GM-MS in their seminal plasma may serve as potential biomarkers of asthenozoospermia [108]. While 37 potential biomarkers were identified when comparing the urinary metabolome of infertile normozoospermic and fertile men, it seemed that the best diagnostic ability for the detection of normozoospermic infertility was obtained when a combination of the top five negative biomarkers (i.e., xanthosine, leukotriene E4, methoxytryptophan, 3-hydroxypalmitoylcarnitine, and aspartate) was applied [95].

9.5 Current Challenges and Future Outlook

Proteomics and metabolomics studies in the infertile male have to date identified a number of putative biomarkers for a variety of conditions related to male infertility, which can be used to distinguish between healthy fertile men and patients with a specific infertility disorder [6, 68]. Biomarkers that are identified using an omics approach may be associated with disease pathogenesis and could therefore offer novel therapeutic targets for the management of disease [110]. It is hoped that these omics studies would eventually pave the way for the development of biomarker molecules or panels of natural fertility in the diagnosis and treatment of male infertility [100]. In fact, a combination of biomarkers has the potential to deliver a higher predicative power than would a single biomarker [95, 111].

While differential proteomic and metabolomics studies have identified an extensive set of sperm proteins and metabolites that are present either in varied quantity or state in infertile males, further research is required before it can actually be utilized in a clinical setting [7, 68, 112]. An inherent challenge from all the datasets obtained is in developing clinically relevant biomarkers [112]. Discovery of potential biomarkers must be followed by analytical validation and evaluation of clinical utility, before it can actually be utilized clinically [113]. Despite the increase in the number of studies and the rapid advances in the strategies and analytics for biomarker discovery, translation of these biomarkers from bench to bedside remains at a slow pace [114]. However, as technology and analytics of omics studies improves, it is hoped that the cost of performing wider-scale omics studies also becomes concurrently more affordable in order to facilitate greater advancements in biological knowledge of human fertility.

9.6 Conclusion

There are a multitude of causes underlying male infertility which remain undiscovered. Greater understanding of the molecular and genetic mechanisms underpinning the male fertility potential is needed to determine possible intervention strategies for the management of the subfertile male. In addition to the advanced tests that aid in the determination of oxidative stress and DNA fragmentation in infertile patients, biomarker discovery promises of a viable alternative for the noninvasive diagnosis of male infertility-related pathologies. Proteomics and metabolomics strategies are rapidly growing analytical tools that complement each other in the goal to discover the biomarkers of infertility in the male. These biomarkers may aid in the evaluation of the male fertility potential, and to differentiate between the various etiologies of infertility, and perhaps even help predict successful outcomes of assisted reproduction technologies. Outcomes of studies utilizing omics approaches and bioinformatics would eventually yield in greater understanding of the spermatogenesis process, sperm function, as well as the events that follow fertilization. Awareness of the molecular and genetic basis of male infertility would immensely aid the clinician in the management of the infertile male.

9.7 Review Criteria

An extensive search of studies examining the relationship between proteomics and male infertility along with metabolomics and male infertility was performed using search engines such as PubMed, MEDLINE, OVID, Science Direct, and Google Scholar. The start and end dates for these searches were July 2018 and Dec 2018, respectively. The overall strategy for study identification and data extraction was based on the following key words: "omics", "proteomics", "proteins", "metabolomics", "metabolites", "male infertility", "infertility", "spermatozoa", "spermatogenesis", "seminal plasma", "semen", "seminal fluid", "urine", "serum", "blood", "testicular tissue", "biomarkers", "bioinformatics". Articles published in languages other than English were also considered, provided the abstract was in English. Data that were solely published in conference or meeting proceedings, websites, or books were not included.

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Epigenetics and Male Infertility

10

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Key Points

- Epigenetics, the study of changes in DNA expression without alterations in the DNA sequence, is now thought to underlie much of human disease, including infertility.
- All four known epigenetic mechanisms, DNA methylation, histone modification and imprinting, non-coding RNAs, and chromatin remodeling, are thought to be active in spermatogenesis.
- Sperm epigenetics has the potential to provide a root cause for much of what is now termed "unexplained" infertility as well as oligospermia and sperm reproductive competence.
- Epigenetic changes that occur in sperm with paternal age appear to be non-random and may involve increased risk of neurodevelopmental diseases in offspring.
- As sperm epigenetic patterns are heritable, their significance to future generations extends to far more than simply infertility and includes familial and *de novo* disease transmission

10.1 Introduction

10.1.1 Definition of Epigenetics

Epigenetics is the study of heritable changes in gene expression that do not alter the underlying DNA sequence. By altering the way in which genes are read, changes in phenotype can occur without changes in genotype. Although every cell in an individual is genotypically identical, epigenetically, each cell's epigenetic signature is distinct, thus facilitating organ-specific differentiation. That is why a nose is not an eye and vice versa, despite having identical copies of DNA. Epigenetic changes are both natural and common occurrences and are influenced by age, environment, lifestyle, and illness. Epigenetic modifications underlie both normal development and also pathologic diseases such as cancer and autoimmunity (Table 10.1) [17].

10.1.2 History of Epigenetics

The term "epigenetics" was first coined by Waddington in 1942 from his work with *Drosophila* fruit flies [18]. He used the word epigenetics to describe the molecular process whereby environmental stress resulted in genetic "assimilation" of phenotypic characteristics. Although conceptually distilled by Waddington, the idea that the environment can influence genetics, that nurture can alter nature, is actually much older with origins ascribed to the eighteenth-century French naturalist Jean Baptiste Lamarck. His concept of "soft inheritance" preceded Darwin's evolutionary theory by 50 years. Whereas Darwin pictured evolution as occurring in rather large, generational "steps," Lamarck had earlier proposed that offspring inherit smaller, environmentally induced changes acquired by parents over their lives. In essence, Lamarck outlined a pathway for evolution that involved passing along traits that were gained from simply living and surviving or the "inheritance of acquired characteristics," an apt description of what we now call epigenetics. Ironically, while Lamarck has historically been considered the one who "got it wrong" in describing the mechanics of evolution, we now believe that certain inheritance patterns are best described by Lamarck's theory.

Our knowledge of epigenetics has exploded over the last quarter century. As outlined in Table 10.1, we now know that epigenetics underlies much of normal cell and tissue function as well as cancer biology, autoimmunity, psychiatric disorders, and intellectual disorders [1, 19]. However, its role as

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Table 10.1 Diseases and disorders in which epigenetic mechanisms have been proposed

Description	Reference
Cancer	
Colorectal	Feinberg and Vogelstein [1]
Breast	Pasculli et al. [2]
Pancreas	Sato and Goggins [3]
Prostate	Ngollo et al. [4]
Intellectual disability	
ATR-X,	Schenkel et al. [5]
Fragile X	Kraan et al. [6]
Rett syndrome	Kubota et al. [7]
Beckwith–Weidman syndrome	Soejima and Higashimoto [8]
Prader–Willi syndrome	Butler [9]
Angelman syndrome	Lalande and Calciano, 2007 [10]
Neurodegenerative	
Schizophrenia	Akbarian [11]
Bipolar disease	Ludwig and Dwivedi [12]
Autism	Loke et al. [13]
Alzheimers	Sanchez-Mut and Gräff [14]
Immunity	
Systemic lupus erythematosus	Xiao and Zuo [15]
Rheumatoid arthritis	Ai et al. [16]

a cause or consequence of infertility is only beginning to be understood. There is a strong sense in the field, though, that epigenetics is critically important to normal human fertility. Factors that complicate the study of epigenetic infertility are the largely continuous nature of the variables involved, the fact that epigenetics can change with age, a serious lack of a defined "normal" cell signatures and the wide variety of epigenetics marks and measures that exist.

10.2 Epigenetics Mechanisms

Four general types of epigenetic modification have been described. All of these are thought to be active in sperm.

10.2.1 DNA Methylation

DNA methylation is one of the oldest and best-characterized epigenetic mechanisms, first described in 1969 [20]. DNA methylation refers to the addition of a methyl (CH_3) group to the DNA strand, typically to a carbon atom of a cytosine ring. It fixes genes in the "off" position and is important for cellular processes like embryonic development, X-chromosome inactivation, genomic imprinting, gene suppression, carcinogenesis, and chromosome stability. Abnormal DNA methylation has been linked to several human diseases including lupus, cancer, muscular dystrophy, and congenital defects [19]. As an example, cancer cell genomes tend to show overall hypomethylation (i.e., are activated) relative to healthy cells which partly explains their malignant behavior.

10.2.2 Chromatin Remodeling

Chromatin is term used to describe the DNA and its associated proteins that are packed within the nucleus of cells. DNA forms chromatin when it is tightly condensed and wrapped around nuclear proteins called histones. The DNA–histone complex is called a nucleosome. When packed tightly in a nucleosome, DNA is relatively inaccessible to transcription factors and therefore unavailable for transcription. In this state, the DNA is called "heterochromatin." When more loosely packed, and accessible for transcription, it is called "euchromatin."

10.2.3 Histone Modification

Epigenetic modifications to histone proteins, also termed histone modification, commonly occur through methylation, phosphorylation, acetylation, ubiquitylation, and sumoylation. These modifications can alter gene expression by grossly or slightly modifying histone structure and are known to underlie biological processes such as transcriptional activation, chromosome packaging, and repair of DNA damage. This process provides another modifiable layer of gene regulation with the potential for heritability.

10.2.4 Non-coding RNA

A non-coding RNA is a functional RNA that is transcribed from DNA but not translated into protein. Non-coding RNAs thought to have epigenetic functions include microRNA (miRNA,) short-interfering RNA (siRNA), piwi-interacting RNA (piRNA), and long-non-coding RNA (lncRNA). In general, non-coding RNAs regulate gene expression at both the transcriptional and post-transcriptional levels and are known to play a role in heterochromatin formation, chromatin and histone modification, DNA methylation targeting, and gene silencing.

10.2.5 Genomic Imprinting

Genomic imprinting is an epigenetic process that involves DNA methylation and histone methylation within the germline (sperm or egg cells) of an organism. After fertilization, these marks are maintained in the early embryo despite the extensive epigenetic reprogramming that takes place early in development. This generates regions in the genome that have DNA methylation present on one parental allele but absent on the other allele. Imprinted areas are then maintained through mitotic cell divisions in the somatic cells of the individual during its lifetime in a parent-of-origin-specific manner. The precise number of genes known to be imprinted is

debated with some studies claiming to have identified over 1000 imprinted genes [21]. The lack of congruence in the data is largely a result of different tissues and species being screened. What is known is that inappropriate imprinting of certain genes has been implicated in several diseases to date, including defective spermatogenesis.

10.3 Sperm Epigenetics

The sperm epigenetic program is uniquely customized to meet the needs of this highly specialized cell. Sperm chromatin structure is one of the most complex structures in the eukaryotic genome, for good reason. Sperm must transport its genome through the male and female reproductive tracts, which necessitates a chromatin structure that is between six and twenty times more dense and robust than somatic cell nucleosome-bound DNA [22, 23]. The extreme compaction of the sperm head is also thought to enhance sperm motility and to protect the DNA from damage in a cellular environment that lacks robust DNA repair abilities [24]. To achieve this uniquely compact chromatin structure, canonical histones are first replaced with transition proteins. Subsequently, two forms of protamines (P1 and P2) take the place of transition proteins in DNA compaction in humans. This process of protamination essentially "blocks" the DNA from any epigenetic change or gene transcription, which make sense given the need to preserve the sperm genome during transport through both the male and female reproductive tract. In fact, the ratio of P1:P2 is tightly regulated at 1:1 in mature sperm and aberrations in this ratio have been correlated with infertility and poor egg fertilization [25–28].

Even more interesting is the fact that the replacement of sperm histones with protamines is typically incomplete, with between 5% and 15% of chromatin remaining histone-nucleosome-bound. Furthermore, the incomplete replacement appears not to reflect random inefficiency but rather a purposeful and programmatic process occurring in deliberate locations [29, 30]. As such, it is thought that histone retention allows for epigenetic modification of genes important for the embryo, including developmental gene promoters, microRNAs, and imprinted loci [29]. These recent findings now suggest that the sperm epigenome, previously considered silent and inaccessible, is actually critical for regulation of early embryo development [31].

10.3.1 Current Technology Used to Evaluate Sperm Epigenetics

The evaluation of sperm DNA methylation profiles is typically based on bisulfite conversion of extracted sperm DNA. This is most commonly assessed in three ways: using arrays, whole genome bisulfite sequencing, and targeted bisulfite sequencing. Among the most popular techniques to screen DNA methylation signatures in humans is the 850K (EPIC) methylation array (Illumina, San Diego, CA, USA). This array assesses the amount of methylation, or lack thereof, at over 850,000 CpGs and reports these methylation signatures as intensity values. Since intensity values reflect a sperm population average, the value effectively represents a "fraction methylation" at each CpG site. Informatic analysis of these data typically includes regional assessments (such as "sliding window" analyses) and point data analyses (assessment of a single genomic site of DNA methylation). This relatively simple format allows for rapid and reliable screening of most known, well-annotated gene promoters, CpG islands, multiple enhancers, and gene body methylation sites with impressive single base pair resolution. The most comprehensive assessment of DNA methylation comes in the form of whole genome bisulfite sequencing. This technology is quite reliable and can cover the entire genome, but has drawbacks including a high cost per sample and the potential loss of sensitivity to identify small methylation changes. One innovative variation on this technology is termed reduced representation bisulfite sequencing (RRBS) that provides similarly high-quality data but with more targeted coverage and at a lower cost. In addition, RRBS can be tailored to cover specific genomic regions of interest depending on the research goals. In addition to the assessment of DNA methylation, newer technologies show great promise in the assessment of sperm RNAs. Because sperm are transcriptionally quiescent, evaluating RNA can be difficult due to very low transcript numbers. However, RNA sequencing methods developed and modified from somatic cell protocols can effectively be used to assess sperm RNAs. These technologies including DropSeq (McCarroll Lab, Harvard Medical School) and the 10× Genomics (San Francisco, CA) platform have shown excellent performance in the evaluation of somatic cell RNAs. Hopefully, they will soon allow for the assessment of single sperm RNA as well.

Sperm chromatin, protamines, and histone modifications have been investigated using several techniques, including simple staining. More advanced techniques including ATAC-Seq or ChIP-Seq allow for the determination of not only the amount of an individual histone present but also the precise genomic location.

10.3.2 Value of Sperm Epigenetics to the Male Infertility Evaluation

The well-recognized inability of the standard semen analysis to predict male reproductive potential [32] needs no emphasis. The fact is that a semen analysis can inform us regarding a potential fertility problem but does not constitute a formal diagnosis. Its ability to predict pregnancy outcomes or to guide clinical decisions is limited. The wide variability in

quality between ejaculates further complicates the potential of the semen analysis to predict "fertility." On the contrary, an understanding of the sperm epigenome has the ability to not only improve the prediction of fertility but also to provide clues to the root cause of the underlying spermatogenetic disorder. Add to this the fact that DNA methylation signatures in mature sperm remain remarkably stable throughout spermatogenesis provides a foundation for a more reliable and relevant diagnostic test for sperm. Limitations in the evaluation of sperm epigenetics include data contamination with somatic cells, which is technically possible to overcome, and the fact the infertility is inherently a couple phenomenon, which makes isolation of male and female factors difficult in most cases.

10.3.3 Sperm Epigenetic–Fertility Phenotypes

The increasing ability of technology to reliably and relatively inexpensively screen the epigenome with high resolution has helped our understanding of the relationship between the sperm epigenome and fertility phenotypes. For example, RNA sequencing has allowed for the assessment of non-coding RNAs, miRNAs, and mRNAs in sperm [33, 34]. While we know that overall RNA content is very low in sperm and that much of the RNA appears to be "remnant" leftovers from spermatogenesis, there appear to be forms of RNA present that may play a role not only in sperm development but also in embryogenesis [34–37]. To date, published research has correlated sperm RNA content to the following fertility phenotypes: decreased IVF success rates [34] and decreased IUI success rates [38]. Similarly, there appear to be signatures in mature sperm methylation patterns that predict the likelihood that an individual will need IVF to conceive or if less invasive therapeutic interventions may be effective [39].

10.3.3.1 Abnormal Semen Analysis

The earliest research on the relationship between epigenetics and semen parameters focused on imprinted loci and measured methylation of sequences in one or only a few genes [40] Marques et al., (2004) examined the *H19* imprinted locus of men with various sperm concentrations and observed abnormal methylation in 0.13% of normozoospermic men, 17% of those with moderate oligospermia, and in 30% of men with severe oligozoospermia. In a study of men with teratozoospermia or abnormal sperm morphology, 11 of 19 patients displayed a loss of methylation at either *IGF2* or both *IGF2* and *H19* genomic sites [40]. Moreover, several studies have confirmed that the abnormal methylation patterns occurring in men with low sperm counts occur at both paternal (hypomethylated) and maternal (hypermethylated) genomic sites [40–43]. Kobayashi et al. (2007) examined the methylation status of seven imprinted genes in the sperm DNA of infertile men and found that when *both* maternal and paternal DNA was abnormally methylated, the finding of severe oligospermia was more common. Thus, abnormal methylation patterns associated with several imprinted genes of both maternal and paternal origin appear to correlate with low sperm concentration and abnormal sperm morphology. At this time, it is unclear whether abnormal DNA methylation among imprinted genes arises from de novo methylation or improper erasure of pre-existing methylation, although the latter seems to be a simpler mechanism [44].

Subsequently, as measures of DNA methylation improved with the advent of methylation arrays, the possibility of examining hundreds or thousands of different methylation markers across the genome was now realizable. In the first study to use a more extensive array of methylation measures, elevated methylation was found at numerous sequences in the DNA of poor-quality sperm from infertile men [44]. The high-throughput analysis addressed hundreds of DNA methylation targets and revealed significant correlations between methylation levels in 35 gene sequences and sperm concentration, motility or morphology. In four gene sequences, *NTF3, MT1A, PAX8, and PLAGL1*, there were striking correlations between methylation levels and abnormalities involving all three semen parameters. Notably, this study was the first to demonstrate that methylation abnormalities in non-imprinted genes are also associated with abnormal semen parameters.

10.3.3.2 Unexplained Infertility

Analyses of DNA methylation patterns in sperm have also identified candidate genetic loci associated with decreased fecundity. In a paired analysis of semen samples from men who had conceived within 2 months of attempting and men unable to achieve a pregnancy within 12 months, two genomic regions were identified as having significantly different methylation patterns between the cohorts [45]. Interestingly, there were no differences in semen volume, sperm concentration or morphology on routine semen analysis testing between the groups. The two sites in which methylation was associated with reduced fecundity are closely related genes that are known to be expressed in sperm: *HSPA1L* and *HSPA1B*. These observations suggest that abnormal epigenetic patterns in sperm might be linked to sperm function, egg fertilization, or embryo development in addition to their previously described association with semen parameters.

A more recent study expanded on the notion that sperm epigenetic patterns correlate with natural fertility and IVF success. Aston et al. [39] studied whether genome-wide sperm DNA methylation patterns can be used to predict male fertility and IVF success. As illustrated in Fig. 10.1, semen samples from a control group of $n = 54$ men with

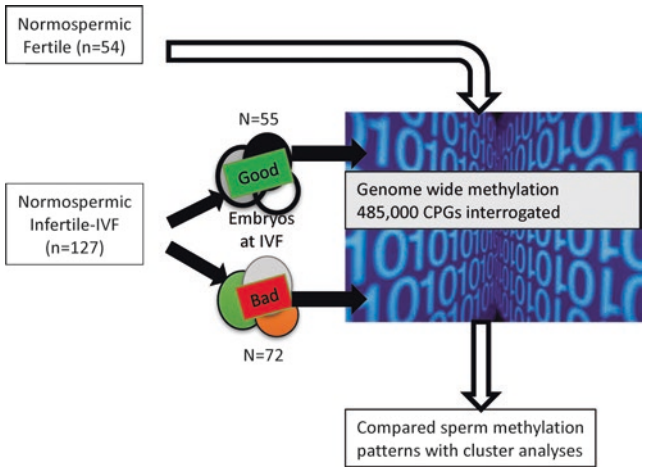

Fig. 10.1 Schematic of study design of genome-wide sperm DNA methylation patterns to predict male fertility and IVF success. Semen samples from a control group ($n = 54$ men) with normal semen quality and proven fertility were compared to infertile men ($n = 127$) with normal semen parameters with none-to-mild female factor infertility undergoing IVF. Genome-wide sperm DNA methylation analysis was performed to measure methylation at >485,000 sites across the genome

normal semen quality and proven fertility were compared to $n = 127$ infertile men with normal semen parameters whose partners were judged to have none to mild female factor infertility and were undergoing IVF. The infertile men were further divided into two groups: men whose partners produced high quality embryos at IVF along with many confirmed pregnancies ($n = 55$ men) and those whose partners produced generally poor-quality embryos ($n = 72$ men) with far fewer pregnancies. Genome-wide sperm DNA methylation analysis was performed to measure methylation at >485,000 sites across the genome. Notably, the sperm DNA methylation patterns were observed to be very stable across semen samples from each individual and maintained consistent difference in methylation patterns across individuals. They observed specific sperm methylation patterns that were highly predictive of fertility status, and somewhat predictive of IVF embryo quality. Predictive models generated based on cluster analysis were capable of correctly classifying male fertility status (fertile or infertile) with 82% sensitivity and 99% positive predictive value. In addition, modeling of the cluster analysis of sperm methylation patterns from infertile couples generating poor quality embryos achieved a positive predictive value of 94%. Finally, a comparison of sperm methylomes of fertile men vs. infertile men revealed >8500 CpGs that had differed significantly. When studying the specific genes with discrepant methylation, several gene classes were involved, including cellular adhesion, cellular morphogenesis and differentiation, and imprinted genes. This study was the first to use large array-based examination of sperm DNA methylation patterns and the first to build predictive models of fertility status using sperm methylation data. It also served as the basis for a commercially available mail in, sperm-based test of male fertility potential (Episona Seed® Assay) that was marketed in the United States from 2016 until 2018 and discontinued due to high testing costs.

10.3.3.3 Embryo Development and Miscarriage

If the sperm epigenome truly influences IVF success, might it act by altering embryo development and effecting miscarriage rates? A study by Denomme et al. [46] has provided early evidence to support the concept that the integrity of the sperm methylome correlates to embryo competence. The study involved comparing the blastocyst methylomes and transcriptomes of 128 couples undergoing IVF for male factor issues characterized by oligoasthenozoospermia to that of 72 surplus banked blastocysts derived from non-male factor patients. Sperm methylomes were not examined. Importantly, all blastocyst were biopsy-euploid to eliminate the influence of maternal or paternal chromosomal disorders on embryo development and pregnancy rates. Although the clinical pregnancy rates were similar after euploid embryo transfer in both male factor and non-male factor embryo transfers, the subsequent miscarriage rate was seven times higher in male factor cases (14.7% vs 2.2%, $p < 0.05$). In addition, there were significant differences in the embryonic methylomes (at 1111 Cpgs) and transcriptome (in 469 transcripts) analyses of embryos between the two cohorts. While the data do not show clear proof of inherited epigenetic dysregulation in blastocysts derived from severe male factor sperm, it does suggest an epigenetic consequence of male factor infertility on embryogenesis and miscarriage rates. The basis for a relationship between sperm DNA methylation patterns and IVF outcomes including miscarriages has now been realized and merits further study.

10.3.4 Sperm Epigenetics and Paternal Age

The relationship between advanced maternal age and pregnancy outcomes is undisputed [47]. Since the advent of epigenetics research, there is now increasing concern that paternal age is associated with non-random alterations in the sperm epigenome that may have implications not only for paternal fertility potential but also on offspring health. Several epigenetic alterations in sperm, particularly DNA methylation defects, have recently been correlated with advanced paternal age [48]. Sperm appear to accumulate hundreds of DNA methylation defects with paternal age that are localized to specific genomic sites, such as CpG regions [48–50]. Strikingly, many of these are found in regulatory or promoter regions and govern neurological, psychiatric, and behavioral disorders, including schizophrenia, bipolar disease, autism, and mood disorders [48–50].

A recent study analyzed age-associated sperm DNA methylation patterns in sperm [48, 51]. In addition to characterizing the type and magnitude of DNA methylation changes, the analysis examined if any specific genomic regions were consistently affected with age. As illustrated in Fig. 10.2, semen samples from men with known fertility were examined at two points in their lives: When they were "younger" (mean age 37.7 years) and "older" (mean age 50.3 years). Global methylation patterns were determined by pyrosequencing, and high-level CpG level array analysis and targeted bisulfite sequencing were performed. Overall, there was a significant global hypermethylation in sperm with paternal age along with localized regions of hypomethylation, which contrasts sharply with patterns of DNA methylation found in somatic tissues with age (i.e., global hypomethylation and localized hypermethylation) [52]. The authors calculated that the average fractional methylation change in sperm was 0.3% per year in hypermethylated regions and 0.28% in hypomethylated regions, both of which appear much higher than the 0.15% annual change in DNA methylation estimated to occur in somatic cells with age [52].

Equally or more intriguing were the study findings that consistently linked altered regions of sperm DNA methylation to genes associated with specific diseases (Fig. 10.1). In a 2014 study, Jenkins et al. found that the genomic loci exhibiting age-associated hyper- or hypomethylation appeared to be enriched at genes associated with bipolar disorder and schizophrenia. This finding suggests that sperm DNA methylation changes observed with paternal age are not randomly distributed within the genome, but could occur more frequently in neurodevelopmental gene sets. This observation is particularly striking when taken in the context of the increased incidence of neuropsychiatric disorders seen in the offspring of older fathers.

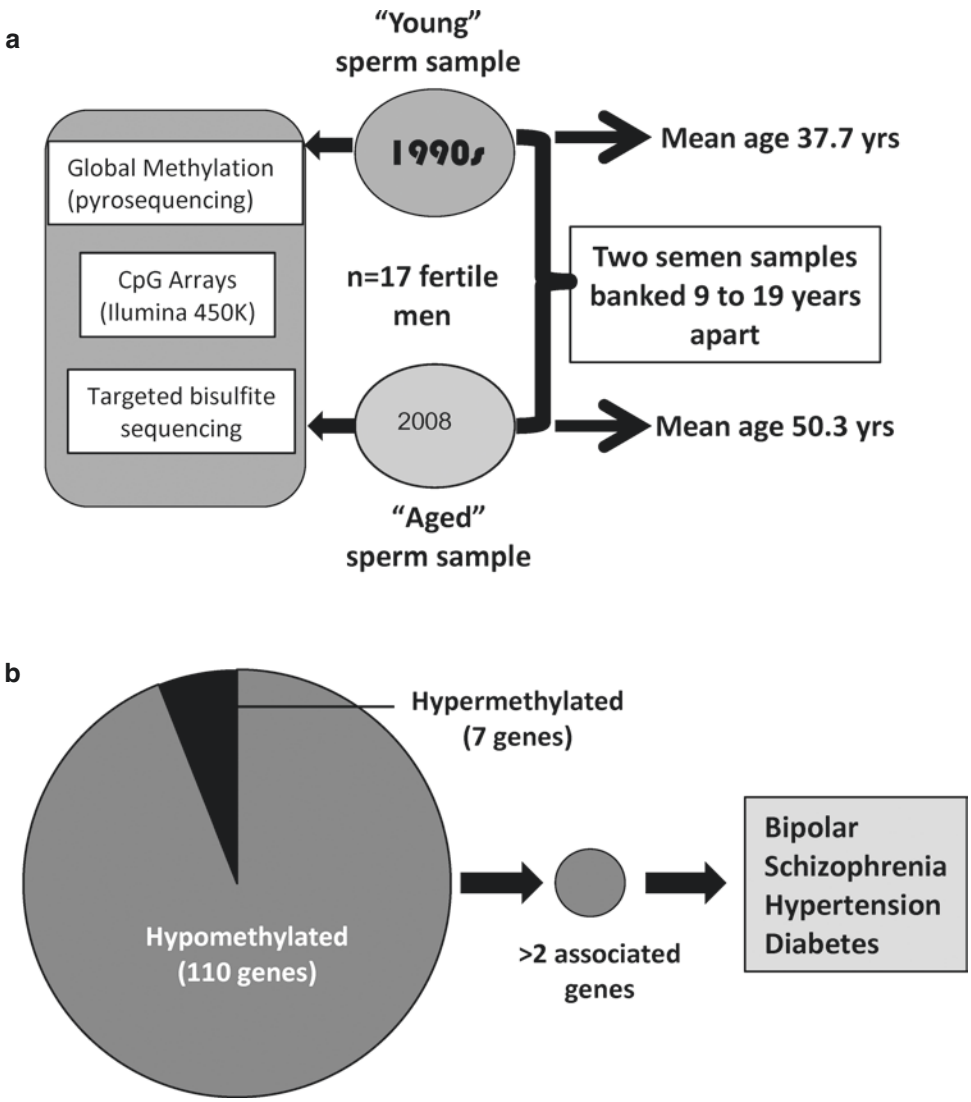

Fig. 10.2 Study of human sperm DNA methylation with age. (**a**) Schematic of study design and epigenetic investigations on sperm. The average difference in subject age between sperm samples was 12.6 years. (**b**) Schematic of study findings. Among the diseased associations with paternal age-related DNA methylation changes, only bipolar disorder reached statistical significance. (Reprinted from Yatsenko and Turek [59]. with permission from Springer Nature)

10.3.5 Lifestyle and Environmental Influences on Sperm Epigenetics

Not only paternal age but nutritional status (obesity) and physical activity levels have also been linked with dynamic epigenetic changes in human sperm [53, 54]. Although potential scientific confounders abound in the examination of environmental influences on sperm epigenetics, including the timing and type of environmental stimulus, the methylation methodology and choice of genomic sites, the type of bioinformatic analysis, somatic cell contamination and the source, purification and fractionation of sperm [55], studies to date are highly suggestive that lifestyle factors significantly modulate the epigenetic health of sperm.

10.3.6 The Hereditability of Sperm Epigenetics

The inheritance of epigenetic alterations in sperm is a plausible way to explain how phenotypic plasticity is transmitted across generations without involving formal genetic mutations [55]. It also lends a molecular mechanism to the mode of inheritance of acquired characteristics postulated by Lamarck more than 200 years ago. Animal models of paternal inheritance have shown that parental dietary factors can affect the metabolism of offspring through epigenetic inheritance [56, 57]. There is also burgeoning evidence from human epidemiological studies that the lifestyle of one generation can modify the risk of chronic disease in offspring through what is now termed "parental effects"[58]. Such modifications have to be transmitted through either sperm or eggs. Currently, the best evidence is that much of human epigenetic inheritance is paternal in nature [57], but this claim may be premature because the investigation of the oocyte epigenome (at least in humans) is ethically and technically far more challenging than studying the sperm epigenome.

10.4 Conclusion

The modern study of epigenetics is based on an old idea that recently found a molecular basis. Sperm epigenetics is a rapidly evolving field that is extremely pertinent to normal and aberrant human reproduction. Abnormal sperm epigenetic profiles appear to correlate not only with semen analysis parameters but also with reproductive competence as defined by embryo quality and miscarriage rates. Sperm epigenetic profiles also change with paternal age and are influenced by paternal lifestyle choices. As sperm epigenetic patterns are uniquely heritable, its significance takes center stage in the study of transgenerational transmission of disease to offspring.

10.5 Review Criteria

In order of importance, randomized controlled trials, scientific studies, meta-analyses, case-controlled cohort studies, and published reviews from 1942 to 2018 were used in this work. Articles published in languages other than English were considered. Data from conference or meeting proceedings, websites, or books were not included.

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Genetic Aspects of Male Infertility

11

David Prokai and Orhan Bukulmez

Key Points

- There are a myriad of genes involved in the development and maturation of the male reproductive systems, and perturbations in any of these pathways may manifest in male infertility.
- Chromosomal abnormalities are more common in infertile men; thus, a standard karyotype is recommended in men with severe oligospermia. Additionally, Y chromosome microdeletion testing may be informative regarding the possibility of successful sperm retrieval via microsurgical extraction techniques.
- Cystic fibrosis transmembrane conductance regulator (CFTR) gene mutation testing is recommended in cases of CBAVD in addition to testing for the intended partner as well. Commercial CFTR tests may miss novel mutations in these patients.
- Male infertility is associated with increased risk of testicular germ cell tumors.
- Advances into the genetic origin of male infertility are evolving at a rapid pace. Mitochondrial DNA mutations, epigenetic alterations, copy number variants, and artificial gamete production are emerging areas of interest in male infertility but have not yet reached the level of clinical practice.

11.1 Introduction

Despite advances in our knowledge regarding the causes of male factor infertility, most cases still remain idiopathic [1]. As the genes and genomic regulation involved in male genital tract development, gonadal development, and spermatogenesis become better understood and delineated, new insights into male reproductive health will be gained. It has been estimated that genetic abnormalities may account for 15–30% of male factor infertility [1]. As genetic causes of infertility are clinically significant not just for the patient themselves but potentially for their future children, it is essential to appraise the current information on genetic basis of male reproductive system disorders and to be aware of the future developments in the field.

11.2 Genomic Regulation of Male Sexual Development

As it is essential for the evolutionary success of an organism to be able to reproduce, male sexual development is under very precise and often redundant genetic control. Perturbations in any of the sequences governing the development and maturation of the male gonad can have profound effects on the ultimate reproductive potential of the patient. From the fourth to the sixth week of gestation, gender-specific development relies on the migration of primitive germ cells along the yolk sac to an area consisting mainly of mesenchyme and underlying cells of mesonephric origin known as the gonadal ridges. Male developmental pathways depend on the presence and correct function of a male-determining gene located on the Y chromosome, aptly named *Sry* (sex determining region Y). The principal protein product of this gene acts on a specific set of gonadal ridge cells to stimulate differentiation into Sertoli cells, which are the cells that interact with and nurture the germ cells. Sertoli cells play a role in orchestrating the differentiation of other cell types required for testis formation such as germ cells and steroid hormone producing cells [2]. Importantly, Sertoli cells are the source of anti-Müllerian hormone (AMH), which is responsible for Müllerian duct regression. In the absence of *Sry* function, the developmental pathway proceeds to female differentiation, although in reality, sexual

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development is more complex involving multiple networks of molecular signals and the fragility of these pathways are reflected to the fact that disorders of sexual development are among the most common birth defects. These disorders can range from hypospadias to complete sexual ambiguity and sex reversal, which are often associated with infertility.

11.2.1 Testicular Development

Early formation of indifferent genital ridges is a requirement before testicular development. Studies in mice have shown that several transcription factor genes are required for this process. These genes include empty spiracles homologue 2 (Emx2), GATA-binding protein-4 (GATA4), LIM homeobox protein 9 (Lhx9), steroidogenic factor-1 (SF-1/NR5A1), dosage-sensitive sex reversal, adrenal hypoplasia critical region, on X chromosome, gene 1 (DAX-1/Nr0b1), and Wilms' tumor 1 (WT-1) [3–8]. WT-1 and SF-1 are crucial for formation of genital ridges in humans. Furthermore, they are both important in sex-specific gonadal development [9, 10]. DAX-1 levels and expression thresholds on the other hand are important for male versus female functional development [8].

Timely expression of *Sry* is essential for testicular development [11]. Spatially dynamic *Sry* expression begins in waves within the gonadal ridge, peaks for a short time, and then declines as demonstrated in mice [12, 13]. The mechanism of this specific and tight regulation of *Sry* regulation remains elusive. Decreased expression of *Sry* was noted in the presence of splice variant mutants of WT-1, GATA4, Friend of GATA (FOG2), and the insulin receptor family [2, 14]. Delayed expression of *Sry* has been implicated in XY sex reversals, unilateral or bilateral ovotestes, and delayed testis formation [13, 15]. It is thought that *Sry* expression must reach a certain threshold within a specific temporal window of competence in the precursors of supporting cells for proper testicular development. *Sry* encodes for a nuclear high-mobility group (HMG) domain protein that binds and bends DNA.

Downstream of *Sry*, other factors such as SRY-box containing gene 9 (SOX9), SOX8, DAX-1, and fibroblast growth factor 9 (FGF9) have important roles in Sertoli cell differentiation and function [16]. SOX9 is considered an early acting and essential component of male development pathway.

Peritubular myoid cells of the testis are necessary for testicular cord development and integrity. Differentiation of these cells correlates with Sertoli cell specific secretion of desert hedgehog (DHH). DHH receptor patched (PTC) is expressed on peritubular myoid cells and Leydig cells, and it has been demonstrated that null mutation of DHH in mice leads to impaired differentiation of peritubular myoid cells and Leydig cells leading to feminized males [16, 17]. DHH mutations in humans lead to partial or pure XY gonadal dysgenesis accompanied by impaired cord formation and decreased testosterone levels [17, 18].

Fetal Leydig cell development is essential for male sexual differentiation. The candidate genes important in Leydig cell differentiation include aristaless-related homeobox gene (ARX), α-thalassemia/mental retardation syndrome, X-linked (ATRX) and platelet-derived growth factors (PDGFs), and their receptor PDGFRA [19, 20].

Primordial germ cells migrate from their origin at the posterior part of the embryo through hindgut to populate genital ridges where they interact with somatic cells to form primitive sex cords. Germ-cell migration is facilitated by interferon-induced transmembrane proteins 1 and 3 (IFITM1 and IFITM3) [21]. Stromal cell-derived factor 1 (SDF1) and its receptor CXCR4 function in the colonization of genital ridges [22]. New genes of interests continue to emerge in the literature regarding testicular development, and only time will tell if they rise to the level of clinical significance.

11.2.2 Testicular Descent

Testicular descent occurs in two phases. The transabdominal phase of testicular descent happens between 8 and 15 weeks of gestation in humans and is controlled by insulin-like 3 (INSL3) hormone produced by Leydig cells, acting via its receptor LGR8 (also known as GREAT) [23]. The inguinoscrotal phase is usually completed by the 35th week of gestation and is facilitated by the neurotransmitter calcitonin gene-related peptide (CGRP or CALCA) released by genitofemoral nerve under the influence of androgens. Mutations in genes involved with androgen signaling and those that encode transcription factors homeobox A10 (HOXA10), HOXA11, and developmentally and sexually retarded with transient immune abnormalities (DESRT) lead to second stage arrest in testicular descent [24].

11.2.3 Spermatogenesis

More than 2300 genes have been estimated to play a role in spermatogenesis [25]. For example, the number of sperm-specific membrane proteins alone has been estimated to be greater than 200 [26]. One study showed that of 1652 genes whose expression increased with onset of meiosis, 351 of them were expressed only in the male germline [27], and there are many genes involved in DNA condensation, sperm maturation, adhesion, and motility as well. In patients with azoospermia, deleted regions on Yq have attracted clinical attention. Deletions in the azoospermia (DAZ) gene on Y chromosome belongs to a family of three members: DAZ, BOULE, DAZ-like. Their proteins contain a highly conserved RNA-binding motif [28]. DAZ proteins bind to RNAs and may be involved in posttranscriptional regulation of

mRNA expression [29]. The DAZ gene family is expressed exclusively in germ cells.

Many forms of partial deletions occur on Y chromosome in some male infertility cases [30]. These infertility related deletions are not considered to be inherited [31], and most infertile males do not show any mutations or deletions on the Yq. The majority of genes involved with spermatogenesis are located on autosomal chromosomes [32, 33]. For instance, mutations in protamine (PRM) and transition protein (TPN) genes involved with histone to protamine replacement which are located on autosomes have been found in 1/200–1/300 of male infertility cases in Japan [33].

As shown in mice, the X chromosome is enriched for spermatogenic genes functioning both in pre-meiotic and post-meiotic germ cells [34]. In humans, the examples of genes on the X chromosome important in spermatogenesis include structural maintenance of chromosomes 1A (component of meiotic cohesion complex, SMC1A) on Xp11.22-p11.21 and testis expressed 11 (binding protein expressed only in male germ cells, TEX11) on Xq13.1. The X chromosome seems to play more important role in the pre-meiotic stages of mammalian spermatogenesis.

11.2.4 Male Genital Tract Development

Full differentiation of the Wolffian (mesonephric) ducts (WDs) leads to the mature male genital tract. Urogenital sinus contributes to the genital tract by developing prostate. In XY embryos, Müllerian ducts degenerate in an active process facilitated by anti-Müllerian hormone (AMH) which is secreted by Sertoli cells. AMH binds to its receptor AMHR2 on the surface of Müllerian duct mesenchymal cells inducing secretion of matrix metalloproteinase 2 (MMP2), leading to apoptosis of Müllerian duct epithelial cells [35]. Failure of this process in humans results in persistent Müllerian duct syndrome (PMDS), an autosomal recessive condition, which can lead to male infertility [36, 37].

Wolffian ducts differentiate under the vital influence of high local concentrations of testosterone into the epididymis, vas deferens, and seminal vesicles [38]. Mice lacking androgen receptor (AR) show agenesis of the Wolffian duct structures. Bone morphogenetic protein 4 (BMP4), BMP7, BMP8, HOXA10, and HOXA11 genes play important roles in epididymis development. Additionally, fibroblast growth factor 10 (FGF10) and growth and differentiation factor 7 (GDF7) are essential in proper development of seminal vesicles [2, 33].

11.2.5 Male External Genitalia Development

Phenotypically male external genitalia largely depends on the expression of 5α-reductase in the genital tubercle mesenchyme converting testosterone to 5α-dihydrotestosterone (DHT) which is the most potent ligand for AR. Mutations of 5α-reductase result in abnormalities in male external genitalia and prostate development.

Complete androgen insensitivity syndrome due to X-linked AR gene mutation can lead to a full external female phenotype, but partial forms may present with various phenotypes ranging from ambiguous genitalia to male infertility. Other mediators important in male external genitalia include cell surface molecules like ephrins and their receptors (Ephs), Wnts, FGFs, BMPs, Noggin, and Hox genes [2, 39].

Urethral fusion defects can lead to hypospadias. HOXA13 and HOXD13 gene mutations reported in hand-foot-genital syndrome suggest that these genes are important in pathogenesis of hypospadias [40].

11.3 Genetic Defects Associated with Male Infertility

11.3.1 Numerical and Structural Chromosomal Abnormalities

Infertile men have an eight- to tenfold higher prevalence of chromosomal abnormalities than fertile men [41]. Chromosomal abnormalities can be detected in about 5% of infertile men and rise to approximately 15% in azoospermic men [1]. In a review of studies involving 9766 azoospermic and severely oligospermic men, sex and autosomal chromosomal anomalies were found in 4.2% and 1.5% of infertile men, as compared with 0.14% and 0.25%, respectively, in control population [42]. It should also be noted that 0.37% of sperm donors with normal sperm parameters have been reported to have chromosomal translocations [43].

Aneuploidy is the most common error resulting from chromosomal anomalies in infertile men [44]. Although many autosomal and sex chromosomes can be involved, the most common are Klinefelter syndrome, XYY syndrome, XX male syndrome, mixed gonadal dysgenesis, autosomal translocations, and Y chromosome microdeletions. Men with non-obstructive azoospermia present with the highest incidence of aneuploidy of up to 13.7% (predominantly numerical or structural defects) [45]. In men with oligospermia, a 4.6% prevalence of autosomal translocations and inversions was reported [46]. Sex chromosomal aneuploidy may account for approximately two-thirds of chromosomal abnormalities observed in infertile men [47].

11.4 Klinefelter Syndrome

Klinefelter syndrome is seen in about 1 in 500 male live deliveries and is the most common known genetic cause of azoospermia accounting for up to 14% of all cases [1]. It

results from X chromosomal aneuploidy in which 90% of cases carry an extra X chromosome (47, XXY) and 10% are mosaics as 47XXY/46XY. In about half of the Klinefelter syndrome cases, the extra X chromosome is paternally derived. The classic triad associated with the syndrome includes small and firm testes, azoospermia, and gynecomastia. It is also associated with eunuchoid body habitus with increased height, low intelligence quotation scores, varicosities, obesity, diabetes, increased incidence of extragonadal germ cell tumors, leukemia, and breast cancer. There is high phenotypic variation and many patients may not demonstrate these classic findings. The only invariant finding of non-mosaic form is that of small testes volume of 2–4 ml. The laboratory findings include severe oligospermia or azoospermia and low testosterone levels with increased LH and FSH. Testicular histopathology is consistent with seminiferous tubular sclerosis and hyalinization and sometimes Sertoli cell only. In some cases, remarkable small islands of spermatogenesis can be observed creating opportunity to obtain testicular sperm [48, 49].

Mosaic forms of the syndrome are associated with spontaneous fertility. Testicular sperm extraction (TESE) with intracytoplasmic sperm injection (ICSI) has been successful in achieving successful pregnancies in non-mosaic forms. The success with TESE in those cases ranges from 27% to 69% [49, 50]. Interestingly 80–100% of mature sperm obtained from 47XXY patients show normal haploid sex chromosome with either X or Y [51, 52]. This may be either due to somatic-germline mosaicism or abnormal germ cells simply do not develop due to meiotic arrest. Nevertheless, the rates of aneuploid sperm, although low in absolute terms, are increased in men with Klinefelter syndrome as is the prevalence of aneuploid embryos (both sex and autosomal) making genetic and preimplantation genetic testing (PGT) counseling an important component of management [53–55].

11.5 XYY Syndrome

This syndrome is seen in 1/1000 live male births. Phenotypic characteristics include increased height, decreased intelligence, higher risk of some malignancies like leukemia, and aggressive or anti-social behavior [1]. XYY syndrome is associated with severe oligospermia or azoospermia with elevated FSH but normal testosterone and LH levels. Testis biopsies were found consistent with maturation arrest or Sertoli cell only. Similar to Klinefelter syndrome, the majority of the sperm obtained from these patients show normal haploid sex chromosomes, but higher rates of both sex and autosomal chromosomal imbalances have been reported in 47, XYY men [56, 57].

11.6 XX Male Syndrome

Its main characteristics are gynecomastia at puberty and azoospermia. It is less frequent than Klinefelter or XYY syndromes with a frequency of 1 in 20,000 live male births. Patients usually show elevated FSH and LH levels with low testosterone. Testicular histology shows absent spermatogenesis with hyalinization of seminiferous tubules, fibrosis, and Leydig cell clumping [1]. It is thought that the translocation of *Sry* to X chromosome results in testes development; however, there is no spermatogenesis since Yq is totally lacking [58]. Since there is no spermatogenesis, any surgical or medical treatment will not be successful for fertility purposes. These patients may require testosterone treatment for hypogonadism.

11.7 Mixed Gonadal Dysgenesis

This is another rare condition with male or female phenotype usually with a unilateral testis and a contralateral streak gonad. Patients may have ambiguous genitalia and abdominal testes showing Sertoli cell only. The gonads are predisposed to malignant germ cell tumors and need to be removed prior to puberty. The karyotype may be 45X/46XY or 46XY. The mutations of Sry have not been detected in the majority of cases with some suspect genes downstream to *Sry* [1].

11.8 Translocations and Inversions

Translocations or inversions of autosomal chromosomes can be detected in 1 in 600 to 1 in 1000 live deliveries. Exchanges between chromosomes may interrupt important genes at the break point or may interfere with normal chromosomal pairing during meiosis. Robertsonian translocations involving chromosomes 13, 14, 15, 21, and 22 and reciprocal translocations are at least eightfold more common in infertile men [1].

Robertsonian translocations occur when two acrocentric chromosomes fuse with loss of the short arm material; hence, the chromosome number will be 45. They are the most common chromosomal abnormalities in humans seen in 0.1% of newborns. Most commonly they involve chromosomes 13;14 and 14;21 (Fig. 11.1). Robertsonian translocations may be seen in 1.5% of oligospermic and 0.2% of azoospermic men [1, 59, 60]. Furthermore, carriers of Robertsonian translocations are at risk of pregnancies with miscarriages or birth defects. Interestingly, within some families, fertility is unaffected despite the same apparent translocation of t(13;14)(q10;q10) [43]. Of those sperm produced, most have a normal balanced chromosomal comple-

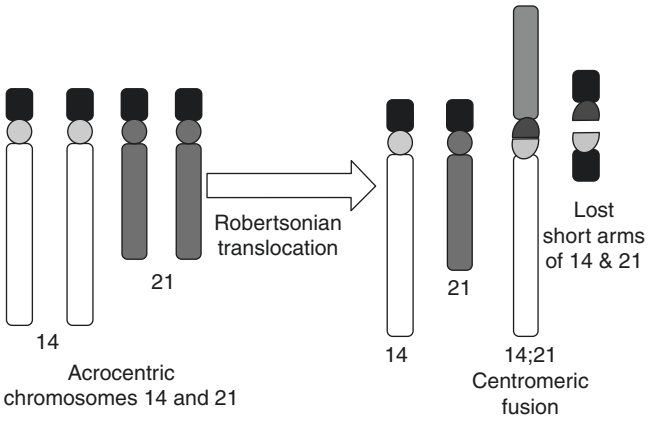

Fig. 11.1 A Robertsonian translocation involves centromeric fusion of the long arms of the acrocentric chromosomes, while the short arms are lost

ment, but an unbalanced karyotype can be present in 4–40% of sperm [61, 62]. Therefore, there is increased risk of trisomies or uniparental disomy. Since the carriers of Robertsonian translocations may pass translocation of imbalanced chromosomal abnormalities to the offspring, genetic counseling and PGT is recommended if sperm from ejaculate or testis is used for ICSI.

Reciprocal translocations are due to the exchange of material between autosomes or between the X or Y chromosome and an autosome and occur in 0.7% of severely oligospermic or azoospermic men [63]. The chromosomal number is normal, and the chromosomes and the break points may be unique to that particular family involved. Depending on the chromosomal material lost, the phenotype may vary. When the sperm is produced, more than 50% are chromosomally unbalanced [61, 64], making genetic counseling even more important.

Chromosomal inversions may involve the centromere (pericentric) or a peripheral segment of the chromosome (paracentric). Although many inversions may be clinically harmless, they may have pathological implications according to the chromosome and the site involved. For example, inversions of chromosome 9 are more frequently observed in infertile men. Due to formation of abnormal loops during chromosomal pairing, chromosomal unbalance can occur, which can effect spermatogenesis or the resulting embryo [61, 65].

11.9 Y Chromosome

The human Y chromosome is 60 megabases (Mb) in length with the least number of genes but the highest copy number of the repetitive sequences as compared to autosomal chromosomes [66]. Approximately 104 coding genes encode about 48 proteins. Among these proteins, 16 proteins have been discovered in the azoospermia factor (AZF) region [67]. The much smaller pseudoautosomal regions (PAR1 2.6 Mb and PAR2 320 bp) (Fig. 11.2), which pair with X chromosome during meiosis, are located at both ends of the Y chromosome. The region outside of the PARs, previously known as the non-recombining region of Y chromosome (NRY), is now called the male specific Y (MSY), which comprises 95% of the chromosome's length. It has also been shown that the MSY may also be somewhat involved in X-Y crossing over during male meiosis while the MSY is flanked on both sides by PARs [68]. But again, the vast majority of the Y chromosome, including the MSY, is less recombining and transmitted as a single block from generation to generation with functional variants and neutral polymorphisms being linked [67].

The MSY is made up of a combination of three classes of gene-rich euchromatic (X-transposed, X-degenerate, and ampliconic) and heterochromatic sequences (Fig. 11.2). The MSY encodes about 27 proteins, and within the MSY, the X-transposed sequences which only encode for two genes (3.4 Mb) are 99% identical to the DNA sequences in Xq21 [67]. X-degenerate sequences are surviving relics of ancient autosomes and encode 16 proteins of the

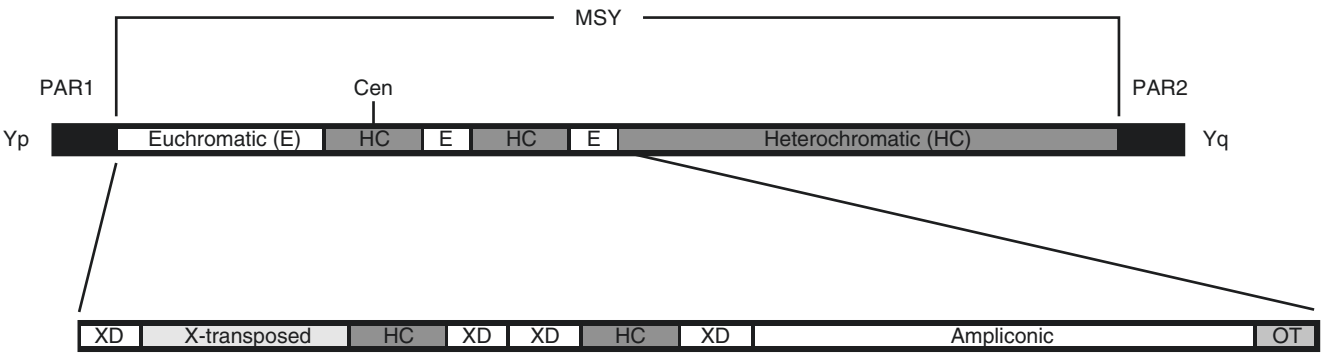

Fig. 11.2 Y chromosome: MSY, Yq, Yp, centromere (Cen), pseudoautosomal (PAR1 and PAR2), and heterochromatic (HC) regions followed by the enlarged view of euchromatic (E) region extending from boundary between PAR1 and Yp to the heterochromatic region of Yq. XD X-degenerate, OT Other. (Reprinted from Li et al. [67]. With permission from Elsevier)

MSY. Amplicons which encode nine proteins are sequence of nucleotides that are nearly identical large repeats reading in the same (direct) or opposite (inverted) directions. Genes in ampliconic segments may be replicated by recombination between the repetitive sequences. Most Y chromosome genes expressed in the testes are in the ampliconic regions. The Yp (Yp11) and the proximal part of the Yq (Yq11 subdivided into Yq11.1, 11.21, 11.22, 11.23) consist of euchromatin, while the distal part of Yq is made up of heterochromatin which is one-half to two-thirds of the Yq (Yq12) [68, 69] (Fig. 11.1). Loci identified in Y chromosome are thought to be involved in the production and differentiation of the sperm since microdeletions of these loci are associated with severe oligospermia or non-obstructive azoospermia. Accordingly seven deletion intervals were described both on Yp and Yq [70].

Male infertility affects one in 20 men, and primary spermatogenic failure accounts for about 50% of the cases [70]. Yq microdeletions were detected in 5–15% of males with spermatogenic failure. More specifically, these deletions occur in 6–8% of severely azoospermic men and in 3–15% of azoospermic men [70]. These deletions include the total Yq12 heterochromatin block and the part of Yq at Yq11.23. Consequently, it was suggested that at least one genetic Y factor essential for spermatogenesis is located in the distal Yq11 called as azoospermia factor (AZF). Of note, about 6% of severe oligospermia cases occur with deletions outside the AZF region [69].

11.10 AZF Region

The AZF region on Yq is the most thoroughly studied male fertility locus in humans [29]. The AZF region is further divided into three regions defined as AZFa, AZFb, and AZFc [71] (Fig. 11.2). While the AZFa region is truly separate and distinct, the AZFb and AZFc regions actually overlap one another and are simply different stretches of Yq within one much longer, encompassing expanse [28]. It is thought that AZF microdeletions result from intrachromosomal recombination events between homologous repetitive sequence blocks in Yq11 [28]. As there is no counterpart in the genome for mitotic pairing and meiotic recombination of the MSY, this repetitive palindromic sequence structure might have evolved to protect the long-term genetic integrity of Y chromosome by allowing MSY to pair with and to repair itself. However, on rare occasions, this non-allelic homologous recombination may go wrong when two spatially separate ampliconic regions permanently stick together during Y chromosome replication, resulting in loss of all chromosomal material in the intervening portion. In some cases, this may occur due to the deficiency of enzymes necessary for DNA repair. P8-P1 represents eight palindromes ordered nearest to farthest from the centromere within the euchromatic region of Yq. P5-P1 region is statistically more prone to non-allelic homologous recombination due to its unique molecular structure [72].

11.10.1 AZFa

AZFa microdeletions are responsible for azoospermia in 1% of males with non-obstructive azoospermia. The AZFa region is not palindromic, is 792 kb in length, and is located in proximal Yq. AZFa region's candidate genes include USP9Y (ubiquitin-specific protease 9, Y chromosome), or DFFRY (Drosophila fat facets-related Y), DBY (DEAD box on the Y), and UTY (ubiquitous TPR motif on the Y) [68, 71, 73–75]. In AZFa deletions, the most frequent presentation is Sertoli cell-only syndrome (SCO). Two subtypes of SCO have been described. In SCO I cases, there are no germ cells in the seminiferous tubules. In SCO II, associated with partial AZFa deletions, some germ cells with incomplete differentiation and maturation and degeneration can be seen.

11.10.2 AZFb

AZFb microdeletions are 6.2 Mb long and begin in the P5 palindrome and end in the proximal portion of P1, hence the name P5/proximal P1 microdeletion. The so-called AZFb/AZFc microdeletion is also named as P5/distal P1 microdeletion since it also starts in P5 but spans a larger area of 7.7 Mb and ends in distal P1. AZFb or AZFb/AZFc microdeletions are observed in 1–2% of males with non-obstructive azoospermia. AZFb candidate genes include EIF1AY (eukaryotic translation-initiation factor 1A, Y isoforms) and RBMY (RNA binding motif on the Y). For the former, no deletion specifically removing EIF1AY has been reported. Complete AZFb deletions are associated with maturation arrest at the primary spermatocyte or spermatid stages.

11.10.3 AZFc

The AZFc region stretches from the distal portion of the P3 palindrome to the distal portion of P1 and is 3.5 Mb in length (Fig. 11.3). Initially it was believed that the AZFb and AZFc were non-overlapping areas, but subsequent studies demonstrated that both the AZFb and AZFb/AZFc regions overlap the AZFc region. AZFc microdeletions (3.5 Mb) are also called b2/b4 (Fig. 11.3) since it occurs when non-allelic homologous recombination happens between the b2 and b4 amplicons in P3-P1 with loss of all intervening material [29]. AZFc microdeletions are the most common microdeletions found in men with non-obstructive azoospermia, occurring

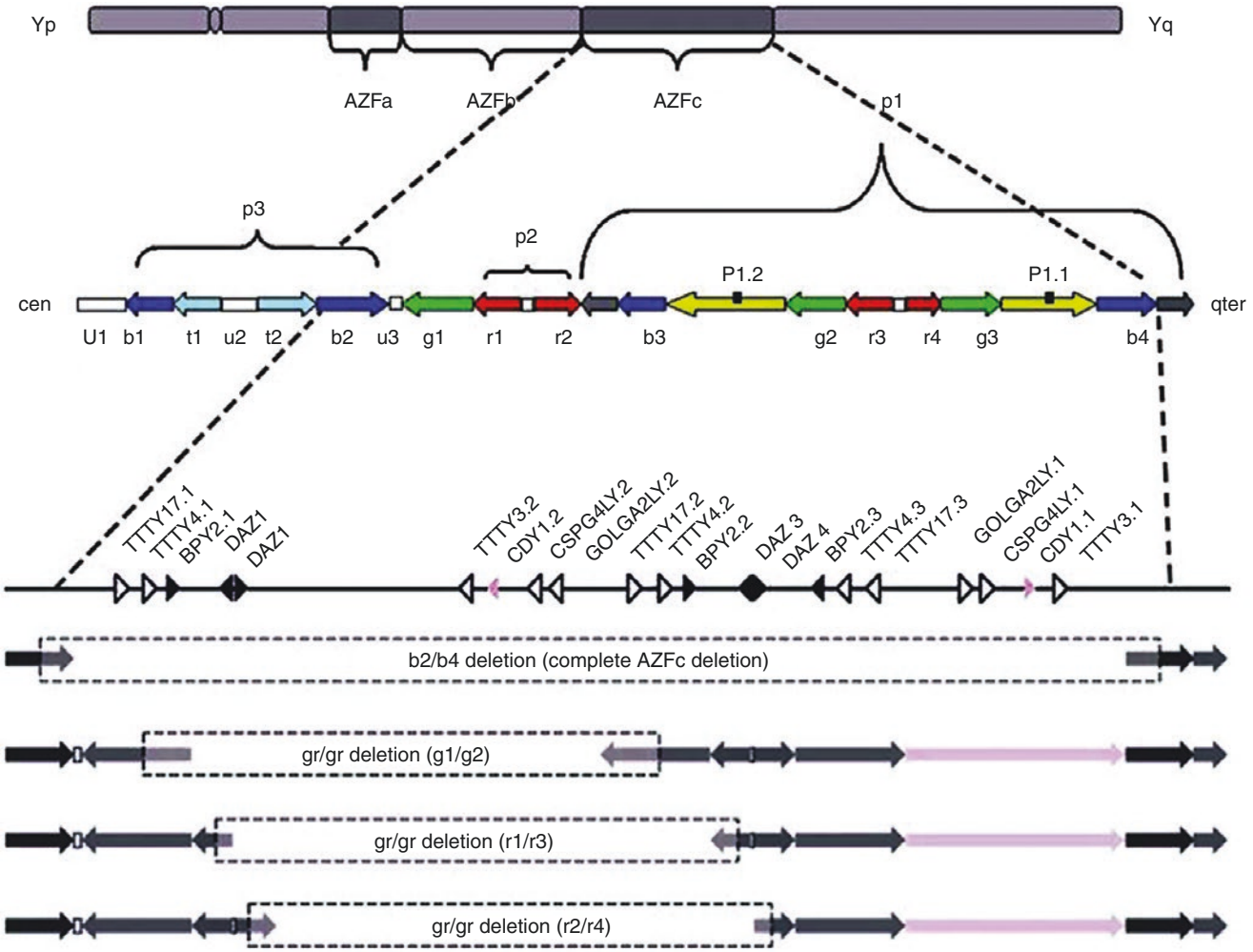

Fig. 11.3 AZFc is comprised of distinct families of nearly identical amplicons. Amplicons b, g, u, r, and t have 4, 3, 3, 4, and 2 repeats, respectively, in the haplotype. Palindromes are indicated as P1, P2, P3. Repetitive sequences are either direct repeats or inverted repeats based on the direction of the arrows. AZFc contains 12 families of transcription units which are all expressed in testis such as BPY2, DAZ, CDY1, and others. AZFc microdeletion subtypes include complete deletion b2/b4, partial deletion g1/g2, r1/r3, and r2/r4. (Reprinted from Li et al. [67]. With permission from Elsevier)

in up to 13% of cases with azoospermia and 6% of men with severe oligospermia. The so-called "Deleted in azoospermia" (DAZ) cluster is the primary candidate gene in the AZFc region. Several other genes in addition to DAZ were mapped in this region including CDY1 (chromodomain Y 1), BPY2 (basic protein Y2), PRY (PTA-BL related Y), and TTY2 (testis transcript Y 2). AZFc deletions are associated with a wide range of conditions from azoospermia to mild to severe oligospermia. This is reflected in testicular histology being consistent with hypospermatogenesis or SCO II in which there is a better chance of finding focal areas of spermatogenesis.

Among all cases with Yq microdeletions, deletions involving DAZ seem to be the most frequent one. Some reports suggest that DAZ deletions may be encountered in up to 13% of cases of male infertility [28]. DAZ was thought to be acquired by Y chromosome from an autosomal homologue DAZL (DAZ-Like) on chromosome 3p24 which shows a single DAZ repeat. The DAZ gene cluster on Y chromosome consists of seven copies of DAZ of which four copies are located relatively close together within a deletion interval on Yq11. DAZ encodes an RNA-binding protein exclusively expressed in early germ cells and is thought to be responsible for activation of silent mRNAs during pre-meiosis stages. It was reported that AZFc may not be critical for meiotic recombination, whereas absence of AZFc regions results in extension of the zygotene stage and reduction of chromosomal condensation [69]. Although most deletions involve all four DAZ genes, an absence of only two is also associated with defective spermatogenesis [28].

Some other partial microdeletions detected in AZFc region such as b2/b3, b1/b3, and gr/gr do not seem to have

any clinical significance, although race specific various phenotypic outcomes were reported [76, 77]. Notably, microdeletions of the AZFc may present with spermatogenic failure in Dutch, Spanish, Chinese, and Italians. However, the presence of AZFc deletion in healthy French, Germans, and Han Chinese questions its importance in male infertility [78, 79]. A partial AZFc microdeletion such as gr/gr can be passed from father to son, but again its clinical significance is still debated [80]. Whereas frequent deletion of DAZ gene cluster in male infertility cases suggests its importance in spermatogenesis, the variable penetration of AZFc deletions in general suggests some redundancies in its function. Perhaps the microdeletion is not an independent event but is compensated by the activation of other genes by gene replication or dosage compensation [67].

11.11 Yq Microdeletions in Clinical Practice

In infertile men with non-obstructive azoospermia or severe oligospermia, it is critical that the patients know the results of Y chromosomal microdeletion analysis before testicular sperm extraction (TESE) and ICSI. Briefly, complete AZFa, AZFb, and AZFb/AZFc microdeletions predict that TESE will be unsuccessful since sperm will not be found and presently there is no available treatment [1]. Men with Y chromosomal microdeletions rarely have a sperm density above five million per milliliter. Although a vast majority of AZFc microdeletions are de novo which means that the father of the patient is not affected, rare cases of natural transmission were also reported [28].

As noted above, it is thought that finding of ejaculated sperm in complete AZFa and AZFb deletions is highly unusual [30]. Combined deletions of two or more regions that include AZFb are associated with SCO or maturation arrest histology. Patients with AZFc deletions have the best prognosis for finding testicular sperm during TESE. Many reports suggest that 50% to 60% of azoospermic AZFc-deleted men will have testicular sperm enough for ICSI [30]. In patients with complete AZFa or AZFb microdeletions, the probability of finding sperm during TESE attempts is extremely low if not impossible. Successful TESE is often not possible in cases with deletions involving one or more regions that include AZFa or AZFb as well.

Small studies are not clear regarding the outcome of ICSI when testicular sperm is used in men with AZF deletions. Some have reported normal fertilization rates but poorer embryo quality as compared to those without AZF deletion and other demonstrated comparable fertilization and pregnancy rates [1]. There is also evidence that men harboring AZFc microdeletions may show time-dependent decline in sperm production. Therefore, counseling patients for sperm cryopreservation for future use is essential.

Men with AZF deletions who conceive via assisted reproduction are likely to pass on the Yq deletion to male offspring [81]. However, the children conceived by testicular sperm seem to be somatically healthy, and their AZFc deletions have not been shown to be altered, although it is expected that male offspring will suffer from similar deficiencies of spermatogenesis.

Since microdeletions cannot be detected by conventional cytogenetic methods, Yq analysis is performed on peripheral blood lymphocytes via polymerase chain reaction (PCR) where various center specific primers were used to amplify sequence tagged sites (STSs) of DNA, which makes evaluation of data challenging. By PCR amplification of STSs, which are specific to each of the AZF regions under review, deletions are identified by the absence of one or more ampliconic products.

11.12 Other Y Chromosome Conditions

Massive palindromes in the human Y chromosome harbor mirror-image gene pairs essential for spermatogenesis. These gene pairs have been maintained by intrapalindromic arm-to-arm recombination. Isodicentric Y (idicY) chromosomes may be formed by homologous crossing over between the opposing arms of palindromes in sister chromatids. This event is usually associated in mosaic event with 45X cell line giving a karyotype of 45X/46XidicY. These patients retain two Yps and two SRYs although the latter may not be functional due to mitotic instability leading to a female phenotype. In cases with male phenotype, the end result would be azoospermia possibly due to Yq segment loss at break points [82]. It remains elusive if successful TESE and ICSI with PGD can be performed in these cases.

The short arm of the Y chromosome also harbors genes related to spermatogenesis. TSPY gene is one of these genes with copies on Yq as well [83]. A study of copy number variation of TSPY demonstrated that more copies were found in infertile men [84].

11.13 X Chromosome

Many X chromosome genes influence male infertility. From rodent studies, it was suggested that the X chromosome may play an important role in pre-meiotic stages of mammalian spermatogenesis [34]. Deletions, translocations, and inversions of X chromosome may result in severe infertility and azoospermia [85–87]. For example, paracentric inversions involving Xq12–25 or deletion of a portion of Xp may result in a phenotype consistent with Klinefelter syndrome. Several X-linked gene mutations were reported in infertile men with oligospermia or azoospermia.

The androgen receptor (AR) gene is located on Xq11–12. Knockout mice studies have suggested that AR signaling in Sertoli cells plays an important role in meiosis I during spermatogenesis [88]. Lack of AR in Leydig cells may lead to spermatogenic arrest at the round spermatid stage [89]. The functional AR in germ cells however was not found to be essential in spermatogenesis [90]. Therefore, androgens control spermatogenesis, but germ cells themselves do not express a functional AR. Androgen regulation is thought to be mediated by Sertoli and peritubular myoid cells [91]. While complete AR gene mutations are associated with androgen insensitivity syndrome with a female phenotype, incomplete forms of AR gene mutations were detected more frequently in infertile men [92].

X-linked spinal and bulbar muscular atrophy or Kennedy's disease is caused by expansion of a CAG repeat in the first exon of AR gene. The CAG repeat encodes a polyglutamine tract in AR protein. The greater the expansion of the CAG repeat, the greater the polyglutamine repeat expansion and the earlier the disease onset and more severe the disease manifestations [93]. Glutamine repeat motifs in the first exon of AR gene (polyQ region) are polymorphic in the general population numbering 10 to 36 repeats. In Kennedy's disease, the polyQ region is expanded between 40 and 62 repeats [93].

The CAG repeat expansion mutations in the AR gene do not affect sexual differentiation. The repeat expansion likely causes a toxic accumulation of mutated AR in nuclei and cytoplasm of motor neurons, resulting in their degeneration and loss [94]. An important point regarding this issue relates to patients with complete androgen insensitivity syndrome. Like patients with Kennedy's disease, there is no androgen receptor function, but there are no neurologic issues in patients with androgen insensitivity as there are no issues with toxic damage to neurons. Patients present with amyotrophic, proximal, or distal weakness and wasting of the facial, bulbar (dysphagia, dysarthria), and limb muscles, occasionally sensory disturbances, and endocrinologic disturbances, such as androgen resistance, gynecomastia, elevated testosterone, and reduced fertility due to defects in spermatogenesis and testicular atrophy. The onset of neurological symptoms is between the ages of 30 and 50 years. In parallel to those observations, CAG repeat polymorphism has been investigated as a possible cause for male infertility [95]. However, it is still controversial if longer or shorter CAG repeats are associated with higher or lower sperm quality [96–98].

The X-linked form of Kallmann syndrome is related to the deletions in KAL-1 gene located in the short arm of X chromosome (Xp22.32). This gene codes for a cell adhesion protein, anosmin-1, which is involved in the migration of gonadotropin-releasing hormone (GnRH) neurons during embryonic development. The condition is associated with hypogonadotropic hypogonadism with sexual infantilism due to the deficiency of GnRH, anosmia or hyposmia due to the absence or hypoplasia of olfactory bulbs and tracts, cognitive and ocular abnormalities, and even with midfacial clefts and renal agenesis [99]. While X-linked KAL-1 mutations are responsible from 30% to 70% of the condition, the remaining cases are related to the deletions in fibroblast growth factor receptor 1 (FGFR1) gene on chromosome 8 which shows autosomal dominant inheritance [100]. With treatment, favorable reproductive outcomes can be attained in addition to maturation of secondary sex characteristics.

11.13.1 Congenital Bilateral Absence of the Vas Deferens (CBAVD) and Cystic Fibrosis

CBAVD is estimated to occur in 1/1000 to 1/10,000 and may be encountered in 1–2% of cases with male infertility. It is detected in 9.6% of cases with obstructive azoospermia [101], and it is thought to result from abnormal development of WD although it is not clear if absence of vas deferens is always congenital [101]. Absence of distal WD derivatives may be related to the early obstruction of these ducts by viscous secretions rather than an embryonic developmental defect. Approximately 80% of CBAVD cases are caused by mutations on both alleles of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. CBAVD is usually associated with absence of the body and tail of the epididymis, vas deferens, and seminal vesicles, but the head of the epididymis is intact (Table 11.1).

Given the fact that almost all CF male patients are infertile due to CBAVD, it was investigated if CFTR was also involved in infertility because of CBAVD alone. In an earlier small study, it was reported that 41% of azoospermic men with CBAVD were found to be heterozygous for F508del CFTR mutation as compared to the population risk of 2.8% [102]. Later, R117H mutations were also found at a higher

Table 11.1 Clinical detection of CFTR mutation-related CBAVD

Azoospermia
Low seminal fluid volume (<2.0 ml)
Biochemical features of the semen: pH <7.2, absent or decreased fructose, and α1–4 glucosidase (markers of properly functioning seminal vesicles and epididymis, respectively)
Absence of palpable vas deferens
On transrectal ultrasound: absence of the intra-abdominal tract of the vas deferens, globus major, and different degrees of hypoplasia of the seminal vesicles
Normal plasma follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone levels
Normal karyotype
Normal testicular size

Based on data from Ref. [108]

frequency in CBAVD cases [103]. In a more recent large study, analysis of 7420 alleles of CFTR gene showed that a CFTR mutation can be identified in 78.9% of patients with CBAVD. In French men with CBAVD, about 71% of the CBAVD patients had a mutation on both CFTR genes and about 16% had a mutation on one CFTR gene, and the remaining 13% of CBAVD patients had no mutation [104].

In 20% of CBAVD patients, the absence of vas deferens is associated with renal malformations [105]. Although a minority of CBAVD patients may have a mild lung disease or a positive sweat test, most CBAVD patients do not have lung disease. It is possible that a mild mutant of CFTR protein with partial chloride channel activity can sustain a normal non-diseased phenotype except for proper functioning and maintenance of vas deferens after its development [106].

The CFTR gene spans about 190 kb and contains 27 exons (chromosome 7q31). The CFTR protein is a glycosylated transmembrane protein, which functions as a chloride channel. In the CFTR gene, currently, 2061 sequence variations have been reported in populations with various geographic locations and ethnicities (http://www.genet.sickkids.on.ca/cftr/StatisticsPage.html) [107]. These are found in both CF and related phenotypes called CFTR-related disorders (CFTR-RD). These are clinical disorders with CFTR dysfunction where the diagnosis of CF cannot be established. These entities include CBAVD, disseminated bronchiectasis, chronic pancreatitis, and chronic rhinosinusitis [108]. As CF is inherited in a recessive manner, CF will develop when deleterious mutations are found on both CFTR alleles. If the mutation is only on one allele, the individual is a CF carrier. One in 2500 newborns has CF and 1 in 25 Caucasians is a CF carrier [106] (Table 11.2).

More than 1200 CF causing CFTR mutations have been identified. A CF patient may carry two identical or different mutations, with the latter condition being called a compound heterozygote for two CFTR mutations. Most mutations are point mutations and the distribution of these mutations varies according to ethnicity. The most common mutation F508del is seen in 70% of Northern European populations, but it is seen in lower frequencies in Southern Europeans [106]. About 1–5% of mutations remain undetermined in CF patients and even more in patients with atypical presentations as undetected mutations may lie within the introns or regulatory regions, which are not routinely analyzed. Additionally, the frequency of undetected mutations increases from Northern to Southern European populations [108]. Besides F508del, other mutations exist in most populations, each reaching frequencies of about 1–2% such as G5542X, G551D, R553X, W1282X, and N1303K. Therefore, in most populations, these mutations and some ethnic specific mutations comprise 85% to 95% of all CFTR mutations. The remaining mutations are rare and sometimes can be found in a single family or population. Depending on the effect at protein level which predicts the severity of the clinical condition, CF mutations are divided in several arbitrary classes [106]. One large study reported that CF patients had two severe mutations (88%) or one severe and one mild/variable mutation (12%), whereas CBAVD men had either a severe and a mild/variable (88%) mutation or two mild/variable (12%) mutations [104].

Most commercial genetic tests for CFTR mutations screen only for the most frequent CF-causing mutations and not the milder mutations, therefore resulting in a CFTR mutation detection rate of only about 60% in CBAVD patients [104]. The most common CFTR mutation presenting with a mild phenotype found in CBAVD patients is the 5T polymorphism (variant) [101].

The 5T splicing variant of the intron 8 acceptor splice site is not considered a CF-causing mutation, but it may be associated with CFTR-RD [108]. At the polypyrimidine tract of intron 8 acceptor splice site, the variants are named according to the number of thymidines as 5T, 7T, and 9T. The lower the number, the lower the efficiency of exon 9 splicing. The extent of splicing is further related to the number of adjacent TG repeats; the higher the number of TGs, the lower the efficiency of splicing. Patients with (TG)13/5T in *trans* with a CF-causing mutation may have mild CF. R117H CFTR-RD mutation can be found in *cis* with 5T or 7T. R117H/5T is considered a mild CF mutation, but R117H/7T is considered as a CFTR-RD mutation. If R117H/5T is trans with a F508del severe CF mutation, the signs of CF may be present. The presence of R117H/7T in the same configuration has been shown to be symptom free [109].

When 5T is found in compound heterozygosity with a severe CFTR mutation, or even with another 5T, CBAVD can be observed. However, not all men who are compound heterozygous for a severe CFTR mutation and 5T develop CBAVD, such as fathers of some CF children [109].Therefore 5T polymorphism is a mutation with partial penetrance. Again, the R117H mutation can either result in CF or CBAVD by being associated by either 5T or 7T allele [109]. Its association with 7T allele may result in CBAVD, and

Table 11.2 Cystic fibrosis detection and carrier rates before and after testing[a]

Ethnic group	Detection rate	Carrier rate before testing	Carrier risk after negative test result
Ashkenazi Jewish	94%	1/24	1/400
Non-Hispanic Caucasian	88%	1/25	1/208
Hispanic American	72%	1/46	1/164
African American	65%	1/65	1/186
Asian American	49%	1/94	1/184

[a]Modified from ACOG Committee Opinion No.691 [110]

R117H/5T may result in CF. F508del mutation is found at a higher frequency in CF patients as compared with those with CBAVD, while R117H is more frequently observed in patients with CBAVD.

Initial CF screening guidelines included 25 pan-ethnic mutations that were present in at least 0.1% of patients with CF. The commercially automated methodology uses PCR with allele-specific oligonucleotide primers [110]. Some screening panels may identify 5T, 7T, and 9T variants, although they are not offered in routine CF carrier screening. As detailed above, the presence of 5T allele may decrease mRNA stability affecting exon 9 [65]. Since CF may occur when 5T is on the same chromosome (*cis*) with R117H missense mutation along with a CFTR mutation on the other chromosome, reflex 5T testing is done if R117H is detected in the screening panel. Since males with 5T allele on both chromosomes are at increased risk for CBAVD, 5T testing should be ordered in CBAVD cases. CBAVD patients with two 5T variants and female with an R117H mutation with 5T variant in *cis* position need genetic counseling to discuss the risk of having an offspring with CF. It should always be remembered that the primary goal of universal CF screening test is to detect CF and not CBAVD, with a reasonable sensitivity. Therefore, at times, complete analysis of CFTR gene by DNA sequencing may be necessary in patients with CF and in patients with CBAVD who tested negative with commercial CFTR screening test results.

Spermatozoa of CBAVD patients used in an ICSI program may transmit CFTR mutation to the offspring. Most CBAVD patients may carry a severe CF-causing CFTR mutation with 50% chance of transmitting that mutation. If the carrier risk of a Caucasian female is 1/25 (0.04), with herself having a 50% chance of transmitting the gene to the offspring, the risk of having a child with CF would be 1 in 100 ($0.5 \times 0.04 \times 0.5 = 0.01$) as compared with a risk of 1/2500 in general population. Therefore, partner testing with genetic counseling is of the utmost importance. Since the commercial genetic tests have about 90% of sensitivity (Table 11.1) when no mutation is found in the test, the partner still has a risk of 1/250 being a carrier of an untested mutation; therefore, the CBAVD couple may still run a risk of 1/1000 of having a child with CF [106].

11.13.2 Genes Involved in Meiotic Recombination

Although the incidence of chromosome abnormalities is about 10 times higher in infertile males than in the general population, most infertile men have normal karyotype. However, these patients may show an increased incidence of aneuploid sperm and diploid sperm in their ejaculate or in sperm obtained from testes [111, 112]. It has also been shown that the risk of aneuploidy or diploidy in sperm correlates with decreasing numbers of sperm and total progressive motility [113]. Many times, meiotic disturbances are the culprits in these cases.

Meiotic recombination in germ cell occurs in prophase of meiosis and involves the induction of double-strand DNA breaks, the pairing of parental homologous chromosomes, followed by the repair of double-strand breaks using the intact homologous chromosome as a template. Several studies have suggested significantly lower rates of meiotic recombination and impaired synapsis in infertile men [114–117]. Faulty meiotic recombination can also cause fertility problems especially if the meiotic errors cannot be corrected (i.e., as in the case of meiotic checkpoint molecules activating apoptotic pathways leading to testicular failure). Furthermore, it was estimated that 5–10% of cases of non-obstructive azoospermia may be due to meiotic arrest [117]. There are many genes involved in meiotic recombination, investigation of which has been mostly relevant to etiology of many cancers [118].

11.13.3 Gene Mutations Associated with Sperm Functional Defects

Primary ciliary dyskinesia presents with immotile but viable sperm along with varying degrees of respiratory tract dysfunction, situs inversus totalis (Kartagener's syndrome), and hydrocephalus. Its frequency is 1 in 20,000 to 60,000 live births [119]. Most of the genetically characterized primary ciliary dyskinesia variants exhibit mutations in the genes dynein, axonemal, heavy chain 5 [DNAH5, 5p15.2], dynein, axonemal, heavy chain 11 [DNAH11, 7p21], dynein, axonemal, intermediate chain 1 [DNAI1, 9p13.3], and dynein, axonemal, intermediate chain 2 [DNAI2,17q25] that encode axonemal dynein-arm components responsible for ciliary beat generation and sperm-specific thioredoxin domain containing 3 [TXNDC3, 7p14.1] encoding a thioredoxin [120]. Some cases may have mutations in retinitis pigmentosa GTPase regulator (RPGR) gene on Xp11.4 which is also associated with retinitis pigmentosa [121]. The patient initially may present with severe asthenospermia, and in these cases, other clinical signs of primary ciliary dyskinesia should be sought. The majority of patients have sperm in the ejaculate, and ICSI has been successfully used in those cases [120].

11.13.4 Copy Number Variations (CNVs)

CNVs are pieces of 1 kb or longer DNA segments that vary in number between individuals, which are considered as submicroscopic duplications and/or deletions of the genome

[31]. CNVs can be detectable by higher-resolution genome-wide microarray comparative genomic hybridization assays and can be further confirmed by PCR-based methods. The complexity about CNVs lies on the facts that their presence can cause overt disease and a predisposition to a disease or may have no effects at all. In general, CNVs may affect up to 20% of the human genome [65]. CNVs have been investigated in many medical disorders; however, the data on male infertility are scarce and more investigation is still needed.

11.14 Mitochondrial Genetics

Mitochondrial DNA is a double-stranded circular DNA molecule coding for 2 rRNAs, 22 tRNAs, and 13 polypeptides essential for respiratory enzyme complexes involved in oxidative phosphorylation [122]. Mitochondrial DNA has no introns, and it mutates at 10 to 20 times higher rates than nuclear DNA due to its unique structure and replication system [123]. Mid-piece of mammalian sperm contains about 80 mitochondria with a single copy of DNA in each organelle. Spermatozoa are dependent on mitochondria for energy needed for rapid progressive motility. Mitochondrial DNA mutations caused by the oxidative damage induced by reactive oxygen species or free radicals may lead to male infertility [124]. In general, about 85% of sperm samples may contain various mitochondrial DNA deletions, which may partly explain the age-related decline in fertility in males.

The presence of multiple mitochondrial mutations has been reported to be associated with oligoasthenoteratospermia [125, 126].The key nuclear enzyme involved in the elongation and repair of mitochondrial DNA strands is DNA polymerase gamma (POLG). The catalytic subunit of POLG is encoded by POLG gene on chromosome 15q24 which includes a CAG repeat region [127]. POLG mutations are associated with mutations in mitochondrial genome, which subsequently affects ATP production and sperm function. Expanded CAG repeats in the region of POLG gene are also associated with several neuromuscular disorders, which may be associated with male factor infertility as well. Many of these disorders like Huntington's disease are transmitted in an autosomal dominant fashion and can show genetic anticipation.

11.15 Epigenetic Alterations

Epigenetics refers to the alterations of the gene expression without any change in DNA nucleotide sequence. Epigenetic mechanisms are associated with the way in which the genome is packed, thus affecting the ability of genes to be activated. It is involved mostly with regulation of transcription or translation.

The most established epigenetic mechanism that is heritable through the germline is DNA methylation. This is a post-replicative modification in which a methyl group is covalently added to CpG (cytosine-guanine) dinucleotide residues of DNA by DNA methyltransferases [128]. Other well-known epigenetic mechanisms include chromatin condensation and histone modifications. The regions of chromatin can be transiently condensed or uncondensed leading to variation in gene expression through transcriptional suppressors, functional RNAs, or interaction with various proteins [129]. Histones are subjected to modifications like phosphorylation, acetylation, methylation, ubiquitination, carbonylation, and such affecting gene expression [130]. Small non-coding RNAs like micro (mi) RNAs or Piwi-interacting (pi) RNAs are additional epigenetic mechanisms acting through transcriptional or translational regulation [131, 132].

Reprogramming of methylation patterns in mammals occurs usually after fertilization (pre-implantation stage) and during fetal development of the germline (gametogenesis) especially during germline differentiation [133]. Allelic differences in methylation which is characteristic of imprinted genes are also delineated during the germ cell line establishment [134]. Imprinted genes conserve their methylation patterns through generations. Therefore, if methylation changes are induced in imprinted genes or new methylation sites are established during germ cell differentiation or after fertilization, heritable factors can either diminish or persist affecting the ultimate phenotype in the offspring [135]. Most endocrine disruptors or environmental factors do not promote DNA sequence mutation but induce modifications of DNA without altering nucleotide composition, i.e., epigenetic changes [128, 136].

Imprinting abnormalities associated with Angelman, Prader-Willi, Beckwith-Wiedemann, and Silver-Russell syndromes have been associated with assisted reproductive technologies (ART), whereas correlations have been found to be weak. It seems these imprinting syndromes may be associated with infertility factors associated with pre-existing methylation aberrations rather than ART itself [137]. Favoring this assumption, it has been reported that epigenetic abnormalities are common in sperm of men with severe oligospermia [138, 139]. Rodent studies also revealed that perturbation of DNA methyltransferases or DNA methylation in male germ cells can affect fertility and sperm function [140]. Abnormal sperm chromatin packaging may have a role in proper establishment of methylation patterns, and abnormal protamine 1 and protamine 2 ratios (P1:P2 equals roughly 1 in fertile men), which were detected in infertile men [141–143], may lead to changes in imprinted genes [144]. Many studies have implicated altered P1:P2 ratios (both greater than and less than 1) with negative impacts on embryo quality, development, and overall IVF outcomes [44]. It is proposed that the erasure and resetting of DNA methylation that

takes place in primordial germ cells is an important stage to prevent DNA methylation defects; however, the effectors and modulators of these steps need further investigation before the applicability of this information to clinical practice [145].

11.16 Malignancy Risks Associated with Genetic Perturbations in Infertile Men

Multiple studies have suggested an association between male infertility and testicular germ cell tumor which is the most common malignancy in men aged 15–35 years [118, 146]. Infertility probably precedes the development of occult testicular cancer, and this association suggests common genetic and environmental factors in both infertility and testicular germ cell tumors. Increased risk of these cancers has been associated with factors related to genetics and epigenetics which include cryptorchidism, chromosome 12 aneuploidy, DNA mismatch repair gene defects, Y chromosome instability, and stem cell dysregulation via abnormal RNA interference [146–148]. Cryptorchidism itself may be associated with mutations in HOXA10, INSL3 and INSL3 receptor LGR8/GREAT, AR, estrogen receptor (ER)α, and SF-1 gene mutations [149]. While elucidation of all these factors and pathways is required, all men evaluated for infertility need to be adequately assessed and screened for testicular tumors.

11.17 Male Genetic Testing in Clinical Practice

Many potential genetic causes of both spermatogenic failure and obstructive azoospermia have been demonstrated, but despite much progress in animal data [150], the validation of the proposed genetic tests has been slow [151]. Therefore, only a limited number of tests are currently recommended in the evaluation of infertile men. Arbitrarily defining severe oligospermia as sperm concentration less than five million/ml and azoospermia as the sperm density below the detection limit, infertile men with those two conditions are recommended to undergo genetic testing.

Men with congenital unilateral or bilateral absence of vas deferens should be tested for a CFTR mutation, which also includes 5T variants. Almost all men with clinical CF have CBAVD. At least two-thirds of men with CBAVD have mutations of the CFTR gene. However, failure to identify a CFTR mutation by commercial tests in a man with CBAVD does not rule out a mutation, since they may still harbor a mutation undetectable with the currently recommended screening panel. It has even been recommended that patients with CBAVD should be assumed to have some sort of CFTR mutation [47]. Although most men with CBAVD have normal spermatogenesis, coexisting spermatogenesis defects should always be ruled out before harvesting sperm for ICSI [47]. Furthermore, since about 25% of men with unilateral absence of vas deferens and 10% of men with CBAVD may have unilateral renal agenesis, an abdominal ultrasound is also required [152].

A karyotype is recommended for infertile men with persistent or severe oligospermia (<10 million depending on the male phenotype or <5 million per ml for all) or non-obstructive azoospermia [61] (Fig. 11.3). Yq deletions are more frequent in azoospermia cases than men with severe oligospermia. Nevertheless, routine Yq microdeletion STS-PCR testing is required before testicular sperm harvesting or before ART in men with non-obstructive azoospermia or severe oligospermia. Chromosomal abnormalities may result in impaired testicular function, while Y chromosome microdeletions result in isolated spermatogenic failure.

At present, testing sperm for aneuploidies or inversions in men with abnormal sperm analysis or men with abnormal karyotype is not recommended since the exact data set establishing threshold levels for the percentage of sperm with abnormal karyotype to assist in clinical and PGT decision making is lacking [65]. Currently, routine assessment of CNVs and epigenetic assessment of infertile men or their sperm do not find any support in the clinical practice.

11.18 Gene Therapy for Male Infertility

Around 15% of the male infertility patients are azoospermic, and normal sperm production with obstructive cause accounts for 40% of these cases. In the remaining 60% of the cases with spermatogenesis defects, approximately half of them may have low levels of sperm, which may be obtained through TESE techniques to be used for ICSI [131, 153]. Only for cases without any viable sperm in the testes, gene therapy may be considered.

There are many challenges to gene therapy for male infertility. Karyotypic abnormalities like Klinefelter syndrome and Y chromosome deletions involve additions or deletions of large amounts of DNA. Currently, there is no technology to manipulate large amounts of DNA for gene therapy. Furthermore, although we mentioned some genetic causes of male infertility in this article, many men with severe infertility do not have any identifiable genetic defects even if the genetic cause is likely. Knowledge of the exact gene(s) involved is essential to proceeding with any gene therapy [154, 155].

Another obstacle in gene therapy for male infertility may involve both somatic (Sertoli, Leydig cells) and germ cells needing more complex approaches. Serious ethical and safety concerns exist regarding inducing genetic alterations

in the germline, and currently, germline genetic therapy is prohibited [156]. In somatic cells, using viral vectors to integrate wild type of gene carries the risk of insertional mutagenesis or carcinogenesis since technology does not allow selected site insertion [157]. With the introduction of CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/Cas9 nuclease) system, genome editing can now more efficient and more precise. This has opened the door to possible safe germline genome editing in the future, though ethical questions still remain [158].

Other suggested approaches in dealing with gene therapy obstacles include the use of embryonic stem cells, transplantation, and the production of artificial gametes [159–162]. Enucleated oocyte can be combined with patient's somatic cell nucleus, the nucleus can be reprogrammed, and the oocyte is stimulated to become a blastocyst since it contains diploid chromosomes. Then stem cells can be differentiated into germ cell lineage, and these cells can be transplanted back to patient's seminiferous tubules, or further development of germ cells can be achieved in vitro to be used for ICSI. In animals, artificial oocytes and artificial sperm have been created from germline stems cells, induced pluripotent stems cells, and embryonic stem cells [163]. These artificial gametes have been combined to produce viable animal offspring. In humans, various routes of creating "artificial sperm" have been proposed [164]. In males with spermatogonial maturation arrest, in vivo or in vitro induction of maturation of these arrested cells and subsequent reimplantation may allow for the re-establishment of ongoing sperm production [164]. For pre-pubertal patients or those facing gonadotoxic therapies, the harvesting of testicular tissue followed by in vitro maturation and later self-transplantation offers a path to comprehensive fertility preservation. Lastly, somatic cells could be induced into pluripotent stem cells followed by differentiation into mature sperm cells which could allow for reproduction in men with a complete absence of sperm cells. As always, safety and ethical concerns about these procedures remain.

11.19 Conclusion

The continued refinements of approaches such as microarray profiling, comparative genomic hybridization, and mutagenesis screening will open up new avenues in the efforts to understand genetic origins of male infertility [165–168]. As always, new technologies provide a vast amount of data with plenty of background "noise" at times. Therefore, the results from advanced genomic, proteomic, and metabolomic techniques should be confirmed by PCR, Western blot, flow cytometry, mass spectroscopy and chromatography, and protein function assays. These approaches will lead to better preconception counseling and more directed approaches for PGT. Future directions should involve continued research into artificial gamete creation as well as the identification of single gene defects associated with male infertility which may be amenable to gene editing.

11.20 Review Criteria

An extensive search of studies examining genetic aspects of male infertility was performed using search engines such as ScienceDirect, Ovid, Google Scholar, PubMed, and MEDLINE. The start and end dates for these searches were November 2018 and February 2019, respectively. The overall strategy for study identification and data extraction was based on the following keywords: "male infertility," "genetics of male infertility," "genomic regulation of male development," "testicular development," "spermatogenesis," "Y chromosome," "AZF deletions," "genetic testing for male infertility," "CBAVD and male infertility," "copy number variations," "mitochondrial genetics and male infertility," "epigenetics and male infertility," "malignancy risk and male infertility," and "future treatment of male infertility." Articles published in languages other than English were not considered. Data that were solely published in conference or meeting proceedings, websites, or books were not included.

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Surgical Treatment for Male Infertility

12

Ricardo Miyaoka and Sandro C. Esteves

Key Points

- Varicocele treatment is recommended for infertile men with clinically palpable varicocele and abnormal semen parameters or sperm functional tests. Overall, open microsurgical inguinal or subinguinal techniques are considered the best treatment modalities due to the higher pregnancy rates, fewer recurrences, and fewer postoperative complications than laparoscopic, radiologic embolization and macroscopic inguinal or retroperitoneal varicocelectomy techniques.
- There are no absolute predictive factors for successful varicocele repair. However, existing evidence does not support the recommendation for treating infertile men with subclinical varicocele.
- Surgical repair of varicocele improves semen parameters and functional markers of oxidative stress and DNA integrity. The chance for either natural or assisted conception is increased after repair of clinical varicoceles.
- Recovery of spermatogenesis can be achieved after repair of clinical varicocele in selected infertile men with non-obstructive azoospermia. Testicular histopathology is predictive of success, and men with maturation arrest and hypospermatogenesis are more likely to ejaculate motile spermatozoa after surgery. Also, the chance of retrieving testicular sperm for ICSI is optimized in non-obstructed azoospermic men with treated clinical varicocele.
- Men with obstructive azoospermia may father children either by surgical correction of the obstruction, which may allow the couple to conceive naturally, or retrieval of sperm directly from the epididymis or testis, followed by intracytoplasmic sperm injection.
- Best results with reconstructive surgery of the male reproductive tract are achieved by surgeons who have necessary microsurgical training and continuous hands-on experience with the procedures. Ideally, procedures should be performed by surgeons who can perform both vasovasostomy and vasoepididymostomy since, in many cases, the need of the latter cannot be anticipated.
- In experienced hands, microsurgical vasectomy reversal is highly successful. Sperm return to the ejaculate after surgery in 70–95% of patients, and 30–75% of couples can be expected to achieve unassisted pregnancy. Patency and pregnancy after microsurgical vasectomy reversal are inversely related to the interval of obstruction since vasectomy. Other factors that affect success rates include the intraoperative appearance of vasal fluid, the presence or absence of sperm in the vasal fluid and their quality, the length of the remaining segment adjacent to the epididymis, the age of the female partner, and the experience of the microsurgeon.
- Vasoepididymostomy should be performed only by those having the requisite training and experience in reproductive microsurgery. When treatment requires an elaborate reconstruction of the male reproductive tract, cryopreservation of retrieved sperm should be considered because surgery may not be successful.
- Ejaculatory duct obstruction is a potentially treatable cause of male infertility. Transurethral resection of the ejaculatory ducts (TURED) is the

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treatment of choice, but minimally invasive vesiculoscopy has been applied with promising results. After TURED, sperm return to the ejaculate in approximately 50% to 75% of men, and approximately 20% of couples achieve pregnancy. However, results are highly variable and depend on the etiology (acquired or congenital) and type (partial or complete) of obstruction. Complications of TURED occur in approximately 20% of men, including hematuria, hematospermia, urinary tract infection, epididymitis, and a watery ejaculate due to reflux of urine.

Table 12.1 Distribution of diagnostic categories in a group of infertile men who attended a male infertility clinic

Category	N	Percentage
Varicocele	629	26.4
Infectious	72	3.0
Hormonal	54	2.3
Ejaculatory dysfunction	28	1.2
Systemic diseases	11	0.4
Idiopathic	289	12.1
Immunologic	54	2.3
Obstruction	359	15.1
Cancer	11	0.5
Cryptorchidism	342	14.3
Genetic	189	7.9
Testicular failure	345	14.5
TOTAL	2383	100.0

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12.1 Introduction

Infertility complaint is common in the urologic office [1]. According to recent estimates, at least 30 million men worldwide are infertile with the highest rates in Africa and Eastern Europe [2]. Thus, regardless of having formal training in male infertility, urologists should be able to diagnose, counsel, provide treatment, or refer the affected patient for assisted reproductive technology (ART). Urologists with formal training in male infertility may also join the multiprofessional reproductive team in an assisted reproduction unit, being responsible for the above-cited tasks as well as for surgical sperm retrieval whenever needed.

In a group of 2385 infertile couples attending our tertiary center for male reproduction, we identified potentially correctable surgical conditions in 48.4% of the male partners [3] (Table 12.1). In our series, clinical varicocele and obstructive azoospermia (OA) were the most common conditions that could be treated by surgical interventions. Notably, about 1/3 of the patients evaluated in the series mentioned above had azoospermia. While reconstructive surgery might be possible in about 30% of the azoospermic men, the majority would require the use of sperm retrieval techniques once enrolled in ART. Thus, the surgical management can be offered to over 50% of the male infertility population seen in daily practice.

At least two breakthroughs occurred in the area of male infertility concerning treatment. One was the development of microsurgery which increased success rates for reconstruction of the reproductive tract. The second was the development of intracytoplasmic sperm injection (ICSI) and the demonstration that spermatozoa retrieved from either the epididymis or the testis were capable of fertilization and pregnancy [4, 5]. As a result of the latter, several sperm retrieval methods were developed to collect either epididymal or testicular sperm, or both, for ICSI. Microsurgery was also incorporated into this armamentarium, either for harvesting sperm from the epididymis in men with obstructive azoospermia (OA) or from the seminiferous tubules in those with non-obstructive azoospermia (NOA) [4, 6].

This chapter focuses on the most common surgical treatments to overcome male infertility. It includes reparative and reconstructive interventions for the male reproductive system. Sperm retrieval techniques are out of the scope of this chapter as they will be addressed later in Chap. 50.

12.2 Surgical Treatment

12.2.1 Varicocele Repair

12.2.1.1 Indications

About 15% of the general male population and up to 35% of the male population with infertility complaints have a varicocele [1]. The etiology of varicocele is likely to be multifactorial. Several theories tried to explain the impact of varicoceles on testicular function, but none of them fully elucidates the variable effect of varicocele on human spermatogenesis and male fertility [7–9]. Recently, several investigators have highlighted the critical role of reactive oxygen species and oxidative stress in the pathophysiology of varicocele-related infertility. Detailed reviews on varicocele pathophysiology can be found elsewhere [10–12].

According to the ASRM Practice Committee report [13], varicocele treatment should be considered in male partners of infertile couples when the following conditions are met: (1) varicocele is palpable on physical exam in standing position in a warm room; (2) the couple has known infertility; (3) the female partner has normal fertility or a potentially treatable cause of infertility (and is willing to treat it) and time to conception is not a concern; and (4) at least one seminal parameter or functional sperm test is altered [13]. According

to the ASRM, varicocele repair is not recommended in cases of isolated teratozoospermia [13] as improvement is modest in this situation [14]. The European Association of Urology (EAU) has somewhat similar recommendations. The EAU states that varicocelectomy is indicated in the presence of a clinically palpable varicocele, oligozoospermia, infertility duration of more than 2 years, and otherwise unexplained infertility in the couple [15].

Other possible indications for varicocele repair include the following: (i) men presenting with palpable varicocele and altered seminal parameters desiring future fertility but not attempting to have children [16]; (ii) young men with semen parameters within normal ranges but at increased risk of ipsilateral testicular dysfunction, such as coincident varicocele and hypotrophic testis; (iii) varicocele-associated pain; (iv) men with large varicoceles and symptomatic testosterone deficiency [15]; (v) prevention or reversal of testicular hypotrophy in adolescent males; (vi) infertile men with palpable varicoceles, semen parameters within normal ranges, and high sperm DNA fragmentation [17–19]; and (vii) infertile men with palpable varicoceles and non-obstructive azoospermia [20].

The recommendations for varicocele treatment relate to the reported association between varicocele and infertility [21], as well as with both reduced semen parameters and testicular size [22]. Furthermore, surgical treatment of clinical varicoceles can improve semen quality and increases the likelihood of pregnancy [23–25]. Despite that, it is still unclear why most men with varicocele retain fertility and why treatment does not always improve the fertility status [11, 26, 27].

12.2.1.2 Preoperative Planning

Patient Evaluation

Treatment of varicocele in the context of infertility aims at restoring or improving the testicular function. A detailed medical history must be taken, and the prognostic factors identified. Physical examination with the patient standing in a warm room is the preferred diagnostic method. Varicoceles identified by this method are termed "clinical" and are graded according to size. Large varicoceles (grade III) are varicose veins seen through the scrotal skin. Moderate (grade II) and small-sized varicoceles (grade I) are dilated veins palpable without and with the aid of the Valsalva maneuver, respectively [28]. In the presence of a bilateral palpable varicocele, it is recommended to perform surgery on both sides at the same operative time [29].

Physical examination may be inconclusive or equivocal in cases of low-grade varicocele and men with a history of previous scrotal surgery, concomitant hydroceles, or obesity. Imaging studies may be useful when the physical examination is inconclusive. The presence of retrograde blood flow to the pampiniform plexus in men with non-palpable varicoceles, identified by other diagnostic modalities such as venography, Doppler ultrasonography, scintigraphy, or thermography, might indicate the presence of subclinical varicocele [30, 31]. The role of subclinical varicocele as a cause of male infertility remains debatable, and current evidence does not support the recommendation for treating infertile men with only subclinical varicocele [32–34].

Notwithstanding, recent evidence suggests that in patients with left clinical varicocele and right subclinical varicocele, bilateral varicocelectomy is superior to unilateral varicocelectomy [35]. In a 2018 randomized controlled trial involving 358 infertile men with varicocele, the bilateral group showed significantly higher improvements than the unilateral group in sperm concentration, normal sperm morphology, and progressive motility. Moreover, the pregnancy rate was statistically higher in the bilateral group after the surgery (42.5% vs. 26.0%, bilateral vs. unilateral group).

A preoperative hormonal profile including serum levels of follicle-stimulating hormone (FSH) and testosterone might add prognostic information. Testicular volume should be assessed using a measurement instrument such as the Prader orchidometer or a pachymeter. At least two semen analyses must be obtained and evaluated according to the World Health Organization (WHO) guidelines [15, 36, 37]. It seems that infertile men either with higher preoperative semen parameters or undergoing varicocele repair for large varicoceles are more likely to show postoperative semen parameters improvement [38]. On the other hand, reduced preoperative testicular volume, elevated serum FSH levels, diminished testosterone concentrations, and subclinical varicocele are negative predictors for fertility improvement after surgery [32, 39–44].

Men with clinical varicoceles presenting with azoospermia might be candidates for varicocele repair. In such cases, the genetic evaluation including Giemsa karyotyping and polymerase chain Yq microdeletion screening for AZFa, AZFb, and AZFc regions is recommended. The reason is varicocelectomy in azoospermic men with genetic abnormalities is doubtful and should be carefully balanced. In azoospermic men with varicocele, testis biopsy (open or percutaneous) might be informative since testicular histology results seem to be the only valid prognostic factor for sperm return to the ejaculate among treated men [36, 45]. Caution should also be applied to recommend varicocele repair in men with hypotrophic testes, history of cryptorchidism, testicular trauma, orchitis, and systemic or hormonal dysfunction since varicocele in such cases might be coincidental rather than contributory to infertility [45]. Any recommendation for varicocele repair should be established rapport with the patient. Moreover, as for all surgical reconstructive procedures, the evaluation of the female partner's reproductive potential is recommended before any intervention, and the alternatives to varicocele repair should be fully discussed.

12.2.1.3 Operative Aspects

Anesthesia

Varicocele repair may be carried out using local, regional, or general anesthesia, according to surgeons and patient's preferences. In our Clinic, we routinely perform microsurgical subinguinal varicocele repair on an outpatient basis using intravenous anesthesia with propofol delivered by an infusion pump, associated with the blockage of the spermatic cord using 10 mL of a 2% lidocaine hydrochloride [36].

Techniques

Varicoceles are surgically treated either by open (with or without magnification) or laparoscopic approaches. Regardless of the technique, the ultimate goal is the occlusion of the dilated veins of the pampiniform plexus. The high retroperitoneal and laparoscopic approaches aim for the ligation of the internal spermatic vein, whereas the inguinal and subinguinal methods enable the ligation of the internal and external spermatic and cremasteric veins that contribute to the varicocele.

Retroperitoneal Techniques High retroperitoneal open varicocele repair involves incision medial to the anterior superior iliac spine at the level of the internal inguinal ring (Fig. 12.1). The external oblique muscle is split, the internal oblique muscle is retracted, and the peritoneum is teased away. Exposure of the internal spermatic artery and vein is carried out retroperitoneally near the ureter. At this level, only one or two internal spermatic veins are present, but the internal spermatic artery might not be easy to identify. The veins are ligated near to the point of drainage into the left renal vein. Neither the parallel inguinal and retroperitoneal collateral veins that usually exit the testis and bypass the retroperitoneal area of ligation nor can the cremasteric veins be identified in the retroperitoneal approach. Some evidence indicates that these collaterals cause the high recurrence rate seen in high retroperitoneal varicocelectomy [46]. The surgical approach on the right side may be more difficult because the right gonadal vein drains in the inferior vena cava.

A laparoscopic varicocelectomy is a retroperitoneal approach using high magnification. The spermatic artery and the lymphatics are easily identified and spared; collateral veins can also be clipped or coagulated. However, external spermatic veins, the second cause of varicocele recurrence, cannot be treated, leading to a recurrence rate of approximately 5% [47]. Furthermore, it has been argued that laparoscopy varicocele repair is more invasive, costly, and associated with higher complication rates than open procedures [47–49]. A discussion about laparoscopic varicocelectomy is out of the scope of this chapter, but more details can be found elsewhere [50].

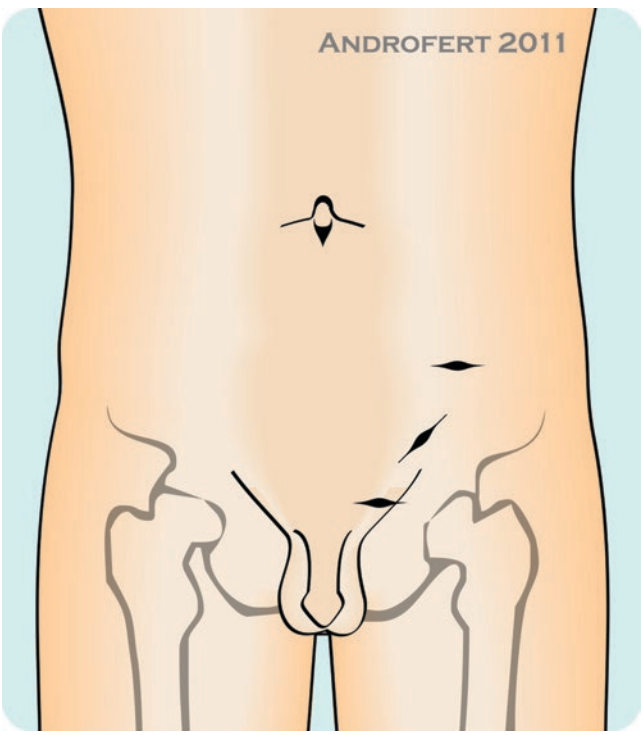

Fig. 12.1 Incision sites commonly used for subinguinal, inguinal, and retroperitoneal varicocele repair. (Reprinted with permission, ANDROFERT© 2011. All Rights Reserved)

Inguinal and Subinguinal Techniques The classic approach to the inguinal varicocelectomy involves a 5- to 10-cm incision over the inguinal canal, an opening of the external oblique aponeurosis, and isolation of the spermatic cord (Fig. 12.1). The internal spermatic veins are dissected and ligated. An attempt is made to identify and spare the testicular artery and the lymphatics. External spermatic veins running parallel to the spermatic cord or perforating the floor of the inguinal canal should be identified and ligated. Although internal and external spermatic veins can be identified macroscopically, the use of magnification facilitates identification and preservation of internal spermatic artery and lymphatics, which may prevent testicular atrophy and hydrocele formation, respectively [51].

Microsurgical varicocelectomy can be performed either by an inguinal or subinguinal approach. The main advantage of the subinguinal over the inguinal approach is that the former obviates the need to open the aponeurosis of the external oblique, which usually results in more postoperative pain and a longer time before the patient return to work. In our practice, varicoceles are treated using a testicular artery and lymphatic-sparing subinguinal microsurgical repair (Marmar's technique) [24, 36, 50]. Briefly, we first make a 2.5-cm skin incision below the external inguinal ring (Figs. 12.2a). Then, we dissect the subcutaneous tissue until the spermatic cord is exposed. The cord is elevated with a Babcock clamp, and the

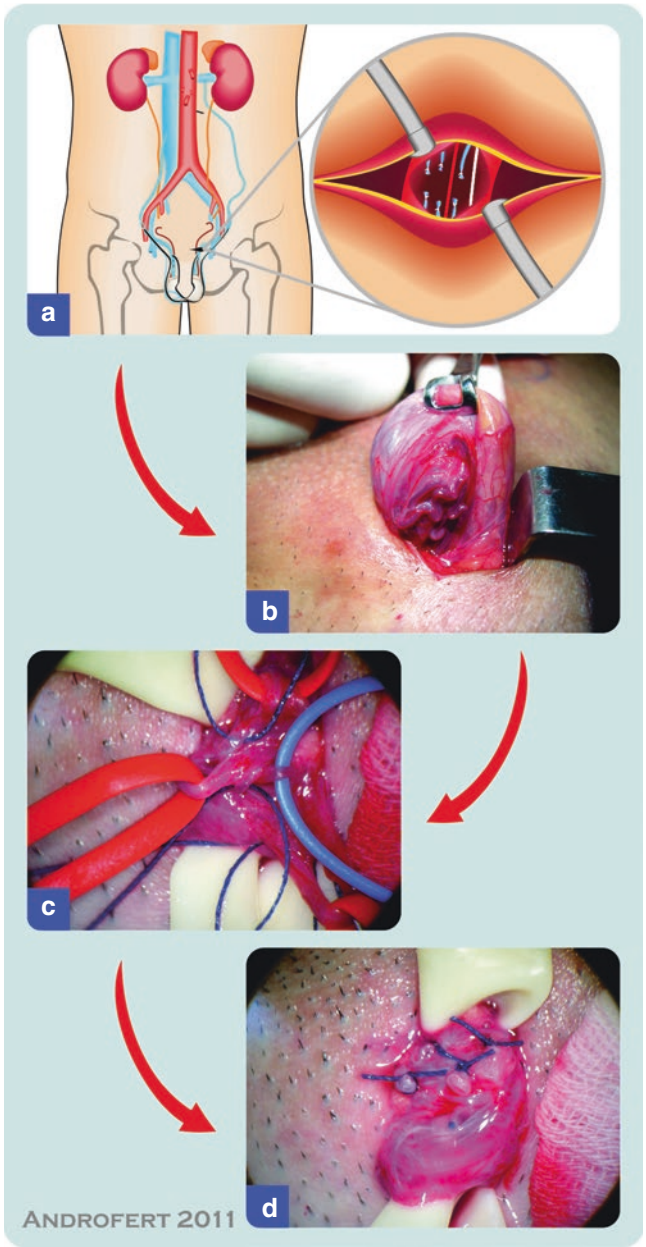

Fig. 12.2 Microsurgical subinguinal varicocele repair. (**a**) In the subinguinal approach, a transverse incision is made just below the level of the external inguinal ring. (**b**) The cord is easily identified and secured, and dilated cremasteric veins are identified by elevating the spermatic cord with a Babcock clamp. (**c**) Testicular artery (blue vessel loop), lymphatics (blue cotton suture), and dilated varicose veins (red vessel loops) are demonstrated. (**d**) Final surgical aspect of varicose veins transected and ligated with non-absorbable sutures. (Reprinted with permission, ANDROFERT© 2011. All Rights Reserved)

posterior cremasteric veins are ligated and transected (Fig. 12.2b). We place a Penrose drain behind the cord without tension. Then, the cremasteric fascia is opened to expose the cord structures, and the dissection proceeds using the operating microscope with magnification ranging from 6× to 16×. We ligate and transect the dilated cremasteric veins within the fascia. The lymphatics and arteries are visually identified and preserved (Fig. 12.2c). Identification of the spermatic artery(ies) can be confirmed by visualization of clear pulsatile movement and/or evidence of antegrade, pulsatile blood flow after gentle lifting and partial occlusion of the vessel. Although papaverine hydrochloride solution might be used on the cord structures to increase the arterial beat, we prefer to use the intraoperative microvascular Doppler (20 MHz) ultrasound to identify and spare small secondary arteries. All dilated veins of the spermatic cord are identified, isolated and tagged with vessel loops, then ligated using non-absorbable sutures, and transected (Fig. 12.2d). We only ligate vasal veins if they exceed 2 mm in diameter and do not use sclerotic agents to occlude small veins.

This subinguinal technique may also be carried out using the da Vinci TM (Intuitive Surgical) robot assistance, but results are still preliminary. In a study involving 258 procedures, persistent varicocele could be detected by Doppler examination in 9.6% of patients at 3 months postoperatively [52]. Improvement in semen parameters occurred only for sperm concentration with a median increase of 37.3% (p <0.03). The treatment did not affect motility and morphology. In this study, the authors did not assess ICSI outcomes, pregnancy rates, and cost-effectiveness [52]. Detailed information about robotic-assisted varicocelectomy is provided in Chap. 21.

Current evidence supported by prospective studies shows that subinguinal microsurgical varicocele repair offers better results regarding fewer complications and lower recurrence rates than the other techniques described above [15, 53].

12.2.1.4 Postoperative Care

We recommend the use of local dressing and scrotal supporter for 48–72 hours and 1 week, respectively. Also, we always recommend scrotal ice packing to decrease the local edema for the first 48 postoperative hours. We counsel our patients to restrain from physical activity and sexual intercourse for 2–3 weeks. Oral analgesics usually suffice to control postoperative pain. Postoperative follow-up aims to evaluate improvement in semen parameters, complications, and natural or assisted conception. Semen analysis should be performed every 3 months until the semen parameters stabilize, or pregnancy occurs.

Semen Improvement Results

Varicocelectomy studies report significant improvements in one or more semen parameters in approximately 65% of men [54]. The mean time for semen improvement and natural pregnancy after surgery is approximately 5 and 7 months, respectively [25, 55].

A 2007 study analyzed seminal improvement following varicocele correction using a new meta-analytic method. The authors reviewed 17 studies where patients with at least one

abnormal seminal parameter and unilateral or bilateral clinically detected varicocele had undergone high ligation or inguinal microsurgical varicocelectomy correction [10]. After microsurgical repair, the combined analysis demonstrated an improvement in sperm count of 9.7 M/mL (95% CI 7.3–12.1, p <0.00001) and 9.9% (95% CI 4.9–14.9, p = 0.0001) in motility. After high ligation, sperm count increased by 12.0 M/mL (95% CI 5.7–18.3, p = 0.0002) and motility increased by 11.7% (95% CI 4.3–19.1, p = 0.002). Morphology in both techniques showed an improvement of 3.2% (95% CI 0.7–5.6, p = 0.001) [10]. The comparisons were made against baseline values.

Along the same lines, Samplaski et al. evaluated the sperm count improvement rate following varicocele repair in men with oligozoospermia. The authors reported an improvement in about 60% of the treated individuals. The baseline total motile sperm count (TMSC), which was below five million, increased to >5 million, thus allowing the use of intrauterine insemination (IUI) or even natural pregnancy [56].

In a 2017 prospective study, 92 subjects with either primary (n = 57) or secondary (n = 35) infertility and varicocele were compared according to semen parameters after microsurgical repair. Baseline parameters were not significantly different between groups. The mean semen parameters improved significantly in both groups and did not differ between primary and secondary infertility patients [57].

Abdel-Meguid et al. conducted a prospective RCT to compare microsurgical varicocele repair versus no intervention. The results at 1-year follow-up did show significant improvement in sperm count, motility, and morphology, both in a within-arm analysis for treatment group and also against the control group [58]. Another study by Bryniarski et al. compared the improvement in semen parameters after varicocele repair with either microsurgery or laparoscopic approaches. Both groups showed improvement in all parameters at 1 year follow-up [59]. Nasr et al. comparing microsurgical varicocele repair and percutaneous embolization at 1-year of follow-up also showed improvement of sperm count and motility. In their study, there was no significant difference between the procedures regarding sperm quality [60]. Tables 12.2 and 12.3 synthetize published RCTs and meta-analyses on varicocelectomy and its effect on spontaneous pregnancy rates, respectively [61–71].

Another study by Baazeem et al. that corroborated these findings [53] showed not only improvement in sperm count and total and progressive motility and ultramorphology but

Table 12.2 Randomized controlled trials of varicocelectomy and its effect on natural pregnancy rates

First author and reference	Year	Population	Interventions and follow-up	Treated	Controls	*P* values
Madgar [60]	1995	45 infertile men with abnormal semen parameters and clinical varicocele (any grade); female partners with no demonstrable infertility factor	High ligation of the internal spermatic vein and observation; 3 years	PR[c]: 60.0%	PR[c]: 10.0%	0.001
Yamamoto [61]	1996	85 infertile men with subclinical left varicocele	High ligation of the internal spermatic vein and observation; 2–5 years	PR[c]: 6.7%	PR[c]: 10.0%	0.96
Nieschlag [62]	1998	125 infertile men with at least one abnormal semen parameter[a] and left clinical varicocele (any grade)	Embolization or surgical repair and counseling; 12 months	PR[c]: 29.0%	PR[c]: 25.4%	NS
Unal [63]	2001	42 men with subclinical varicocele; all couples with primary infertility	Surgical vein ligation and clomiphene citrate; 15 months	PR[c]: 12.5%	PR[c]: 6.7%	0.589
Krause [64]	2002	67 infertile men with at least one abnormal semen parameter[a] and clinical varicocele (any grade)	Antegrade of retrograde sclerotherapy and observation; 12 months	CPR[d]: 30%	CPR[d]: 16.2%	0.189
Abdel-Meguid [58]	2011	145 men with at least one abnormal semen parameter[b] and clinical varicocele (any grade); all couples with primary infertility	Subinguinal microsurgical varicocelectomy and observation; 12 months	PR[c]: 32.9%	PR[c]: 13.9%	0.010
Mansour Ghanaie [65]	2012	136 men with normal semen parameters[b] and clinical varicocele (any grade); all couples with secondary infertility (recurrent pregnancy loss)	Loupe-assisted inguinal varicocelectomy and observation; 12 months	CPR [1]: 44.1%; miscarriage [2]: 13.3%; LBR [3]: 38.2%	CPR [1]: 19.0%; miscarriage [2]: 69.2%; LBR [3]: 5.9%	0.003 [1]; 0.002 [2]; 0.003 [3]

CPR clinical pregnancy rate, *LBR* live birth rate, *NR* not reported, *NS* not significant
[a]According to the WHO 1992 criteria
[b]According to the WHO 1999 criteria
[c]Delivery and miscarriage rates not reported
[d]Based on as-treated groups

Table 12.3 Meta-analyses of varicocelectomy and its effect on natural pregnancy rates

Author, year, and reference	Population	Included studies	Interventions	Natural pregnancy after varicocelectomy	Remarks
Evers, 2008 [38]	607 couples and male partners with varicocele	8 RCTs reporting pregnancy rates as an outcome measure	Surgical ligation or radiological embolization of the internal spermatic vein and untreated groups	Observed odds ratio (OR) of the eight studies was 1.10 (95% CI 0.73 to 1.68), indicating no benefit of varicocele treatment over expectant management in subfertile couples in whom varicocele is the only abnormal finding	Two trials involving clinical varicocele included some men with normal semen analysis. Three studies specifically addressed only men with subclinical varicoceles
Ficarra, 2006 [39]	Infertile men with abnormal results on semen analyses and a palpable varicocele. Group 1: 120 infertile men with varicocele subjected to surgical repair; Group 2: 117 men with untreated varicocele	3 RCTs reporting pregnancy rates as an outcome measure	Surgical ligation or radiological embolization of the internal spermatic vein and untreated groups	Significant increase in pregnancy rate (PR) in patients who underwent varicocele treatment (36.4%) compared with the control group (20%) ($p = 0.009$)	This study is a revaluation of the Cochrane meta-analysis (Evers et al., 2008) excluding men with normal semen analysis and subclinical varicocele
Marmar, 2007 [40]	Infertile men with abnormal results on semen analyses and a palpable varicocele. Group 1: 396 men subjected to varicocele repair; Group 2: 174 infertile men with untreated varicocele	2 RCTs and 3 observational studies	Surgical varicocelectomy compared with no or medical treatment for palpable varicocele and at least one abnormal semen parameter	Significant increase in pregnancy rate (PR) in patients who underwent varicocele treatment (33.3%) compared with the control group (15%), OR of natural pregnancy after surgical varicocelectomy, compared with no or medical treatment was 2.87 (95% confidence interval [CI], 1.33–6.20) with use of REM and 2.63 (95% CI 1.60–4.33) with use of FEM	The number needed to treat was 5.7 (95% CI 4.4–9.5)
Baazeem, 2011 [12]	380 infertile men with oligozoospermia and a palpable varicocele	4 RCTs reporting on pregnancy outcome after repair of clinical varicoceles in oligozoospermic men	Surgical ligation and untreated groups	Using the random-effects model, the combined OR was 2.23 (95% confidence interval [CI], 0.86–5.78; $p = 0.091$), indicating that varicocelectomy is moderately superior to observation, but the effect is not statistically significant	22, 17, and 5 prospective studies reporting on sperm concentration, total motility, and progressive motility, respectively, before and after repair of clinical varicocele were evaluated. The REM indicated improvement in sperm concentration by 12.32 million sperm per milliliter (95% CI, 9.45–15.19; $p < 0.0001$) and by 10.86% (95% CI 7.07–14.65; $p < 0.0001$) and 9.69% (95% CI 4.86–14.52; $p = 0.003$) total and progressive motility, respectively
Kroese, 2012 [41]	894 couples and male partners with varicocele	10 RCTs reporting natural pregnancy rates as an outcome measure	Surgical ligation or radiological embolization of the internal spermatic vein and untreated groups	The combined fixed-effect OR was 1.47 (95% CI: 1.05–2.05, very low-quality evidence), favoring the intervention. The number needed to treat for an additional beneficial outcome was 17, suggesting benefit of varicocele treatment over expectant management for pregnancy rate in subfertile couples in whom varicocele in the man was the only abnormal finding	Subgroup analysis after exclusion of the studies including men with normal semen analysis and subclinical varicocele (five studies) revealed favorable effect of treatment (combined OR: 2.39; 95% CI: 1.56–3.66; high statistical heterogeneity $I^2 = 67\%$). The number needed to treat for an additional beneficial outcome was 7

(continued)

Table 12.3 (continued)

Author, year, and reference	Population	Included studies	Interventions	Natural pregnancy after varicocelectomy	Remarks
Kim, 2013 [42]	610 infertile men from couples with otherwise unexplained subfertility	7 RCTs reporting natural pregnancy rates as an outcome measure	Surgical ligation and no treatment	The random-effects model showed an odds ratio (OR) of 1.90 (95% confidence interval [CI], 0.77 to 4.66; $p = 0.1621$). However, for subanalysis of three studies that included patients with clinical varicocele and abnormal semen parameters, the fixed-effects pooled OR was significant (OR, 4.15; 95% CI, 2.31 to 7.45; $p < 0.001$), favoring varicocelectomy	There were differences in enrollment criteria among the studies. Four studies included patients with clinical varicocele, but three studies enrolled patients with subclinical varicocele. Meanwhile, four trials enrolled patients with impaired semen quality only, but the other three trials did not

OR odds ratio, *REM* random-effects model, *FEM* fixed effects model

also reduction in sperm oxidative stress and sperm DNA fragmentation (SDF) [53]. Accordingly, Roque and Esteves [72] reviewed the effect of varicocele repair in DNA fragmentation and reinforced this idea after reviewing over 20 studies accounting for more than 1200 treated subjects. Despite using different SDF assays, various designs, and variable sample size, all studies reported significant decreases in SDF rates following varicocele repair in a follow-up period ranging from 3 to 12 months. The exact percentage of men who benefit from surgery is still poorly reported but is estimated to be around 78–90% by some authors [72].

Hence, even if traditional seminal parameters do not show a definite improvement after varicocele microsurgical repair, a positive effect has been demonstrated on other functional sperm tests, mainly among patients presenting varicocele of higher grades [73].

As for the effect of varicocele repair before assisted reproductive technology (ART), our previous observations indicate that treatment of clinical varicoceles can improve the outcomes of ICSI in couples with varicocele-related infertility [24]. In this study, the chances of achieving a live birth were significantly increased by 1.9-fold, while the chance of miscarriage was reduced by 2.3-fold when the varicocele was treated before ICSI [24].

Concerning the role of varicocele repair in men with NOA, a recent review with meta-analysis indicated that sperm return to the ejaculates was achieved in 44% (151/344) of the treated individuals [20]. This benefit is most notorious among men with testicular histopathology revealing hypospermatogenesis when compared with maturation arrest (OR 2.35; 95% CI: 1.04–5.29, $p = 0.04$) and Sertoli-cell only (OR 12.0; 95% CI 4.34–33.17, $p < 0.001$), and the procedure might avoid the need of surgical sperm extraction [20, 74].

Fertility Results

Current evidence indicates that varicocele repair may positively affect pregnancy rates. However, the literature still lacks powered randomized controlled trials with homogeneous populations to clarify the existent debate definitively.

A randomized clinical trial published in 2011 reported natural pregnancy rates of 32.9% in treated men versus 13.9% in the observation group (OR 3.04; 95% CI 1.33–6.95) [58]. Likewise, Marmar et al. in a meta-analysis including studies of various designs showed that the likelihood of natural pregnancy favored the varicocele repair group (odds ratio: 2.97; 95% CI 1.60–4.33) [75].

Another study showed that the type of infertility being either primary or secondary does not seem to impact fertility results at 1-year follow-up after microsurgical varicocele repair (42.1% vs. 31.4%, respectively) [57].

The most recent Cochrane compilation dates back to 2012 and included ten RCTs involving 894 men treated for infertility [76]. In this review, the authors included two trials involving men with normal semen analysis and three in men with subclinical varicoceles. The combined fixed-effect OR for the outcome of pregnancy was 1.47 (95% CI 1.05 to 2.05, very low-quality evidence), favoring the intervention. The number needed to treat (NNT) for an additional pregnancy was 17, suggesting a benefit of varicocele treatment over expectant management in infertile couples in whom varicocele was the only abnormal finding. A subgroup analysis excluding men with normal semen analysis and subclinical varicocele (five studies) also favored treatment (combined OR 2.39; 95% CI 1.56 to 3.66). In this case, the NNT for an additional beneficial outcome was 7. No studies reported live birth.

As for the surgical techniques, a 2009 meta-analysis that analyzed 4473 subjects who underwent microsurgery reported better natural pregnancy rates (41.9%, $p = 0.001$),

lowest recurrence (1.0% $p = 0.001$), and hydrocele formation (0.4%, $p = 0.001$) [77]. Pregnancy following either laparoscopic or microsurgery at 12-month follow-up has also been reported to be similar (29.7% vs. 40.5%, respectively) ($p = 0.34$) [59]. A more recent systematic review and meta-analysis published in 2018 compiled 23 studies with 1178 patients in the group of microsurgery and 1069 patients in the group of laparoscopic surgery [78]. The authors showed that microsurgical varicocele repair decreased complication rates (RR: 0.40, 95% CI: 0.21–0.75), recurrence rates (RR: 0.35, 95% CI: 0.22–0.55), and hospital stay (WMD: −0.53, 95% CI: −0.85 to −0.21), whereas it increased sperm concentration (WMD: 3.00, 95% CI: 1.23–4.76). No effects on operation time (SMD: 1.61, 95% CI: 0.71–2.51) and sperm motility (WMD: 2.38, 95% CI: 0.39–4.37) were observed, and no data was available concerning pregnancy outcomes.

Lastly, a 2019 prospective randomized study including 302 infertile patients with severe oligozoospermia and clinical (grade II/III) varicocele evaluated the role of preserving the testicular artery [79]. The authors showed that there was a statistically significant improvement in sperm density and motility at 3 and 6 months postoperatively, regardless of whether or not the artery was preserved. However, the greater seminal improvement occurred in patients who had the internal spermatic artery preserved. At 1-year follow-up, this group achieved significantly higher natural pregnancy rates (40% vs. 30%, $p = 0.03$). An artery-ligating varicocelectomy was an independent predictor of reduced natural pregnancy rates (hazard risk [HR] = 3.2, 95% CI 1.4–7.1, $p = 0.003$) [79].

12.2.2 Reconstructive Surgery of the Vas Deferens and Epididymis

12.2.2.1 Indications

Vasovasostomy and vasoepididymostomy are surgical procedures developed to bypass obstructions affecting either the vas deferens or the epididymis. In the United States, approximately 13% of married men aged 15–44 years report having had a vasectomy [80]. The number of men seeking a vasectomy reversal due to changes in marital status or reproductive goals is estimated to range from 2% to 6% [81]. In Brazil, approximately 200,000 vasectomies and 7000 reversals are performed annually [82] The vast majority of vasovasostomies and vasoepididymostomies is carried out to overcome obstructions secondary to vasectomy; however, other indications include correction of epididymal or vasal obstructions due to genital infections, iatrogenic injuries related to inguinal or scrotal surgery, especially during the early childhood years, and postvasectomy pain syndrome [83].

12.2.2.2 Preoperative Planning

Patient Evaluation

A detailed medical history must be taken and prognostic factors identified. The obstruction interval from vasectomy to reversal is critical for surgery outcomes. Patency and pregnancy rates for intervals up to 15 years are approximately 74% and 40%, respectively [84], whereas intervals longer than 15 years relate to lower patency and pregnancy rates. Furthermore, long-interval obstructions relate to higher incidence of epididymal obstruction; as a result, vasoepididymostomy (VE) is likely to be required.

A history of a previous vasectomy reversal attempt does not preclude a new one. Patency and pregnancy rates of 79% and 31%, respectively, are reported for repeated reversals [85]. Apparently, the history of conception with the current partner is the only significant predictor for a successful pregnancy. However, a history of genital/inguinal surgery should raise the concern about the possibility of iatrogenic inadvertent surgical obstruction. Repair of obstruction in the inguinal canal or retroperitoneum can be technically challenging.

A detailed physical examination should also be carried out. Small and soft testes might indicate impaired spermatogenesis. Indurate, irregular epididymis and the presence of hydrocele are often associated with obstruction and might suggest the need for vasoepididymostomy. Palpation of a granuloma in the vas deferens should be interpreted as a favorable prognostic sign. Its presence means that sperm has leaked at the vasectomy site preventing overpressure within the epididymis tubules and rupture [81, 84, 86]. If a vasal gap is detected, we advise our patients that a larger incision into the inguinal region might be needed to allow a tension-free anastomosis. Specific laboratory tests are not necessary before reconstructive surgeries. However, serum FSH might be informative concerning testicular reserve if testicular damage is suspected on physical examination. The clinical utility of antisperm antibody testing remains controversial; evidence suggests that late failures following reversals are likely to be technical rather than immunological [87, 88]. Besides, overall conception rates are acceptably high, and the presence of antisperm antibodies does not correlate closely with postsurgical fecundability [89].

We evaluate the female partner fertility carefully before any recommendation for reconstruction. For instance, the ovary reserve and the patency of the fallopian tubes must be evaluated and accounted for since they affect the chances of pregnancy. Also, we discuss the alternatives to vasectomy reversal with the couple. It has been shown that reversal outcomes in men with the same partners are significantly better than those with new partners. The proven fecundity of the couple, a shorter obstruction interval, and the firm decision to achieving conception are

critical factors for the higher success rate [81, 90]. By contrast, the female age greater than 40 years seems to be a negative predictor for success [91, 92].

12.2.2.3 Operative Aspects

Anesthesia

Vasovasostomy and vasoepididymostomy can be performed using local, regional, or general anesthesia. In the authors' practice, procedures are carried out on an outpatient basis. Total intravenous anesthesia with propofol (2,6-diisopropylphenol) coupled with the blockage of the spermatic cord using lidocaine hydrochloride solution is our preferred anesthetic method. A propofol induction dose of 3–4 $mg \cdot kg^{-1}$, followed by infusion of 60–100 $\mu g \cdot kg^{-1} \cdot min^{-1}$, is used under spontaneous or assisted ventilation with 2 $L \cdot min^{-1}$ of 100% O_2 on a face mask. An opioid such as fentanyl (1–2 $\mu g \cdot kg^{-1}$) or alfentanil (7–15 $\mu g \cdot kg^{-1}$) is given before the surgeon performs skin and subcutaneous infiltration with 1% lidocaine without epinephrine. Propofol is advantageous as it has antiemetic effects and allows patients to wake up with a sense of well-being and a clear mental state.

Incision

We make a 2-cm longitudinal scrotal incision in the anterior aspect of the scrotum on each side. The incision is made onto the palpable granuloma or the identified vasal gap. Only the vas ends are delivered through the skin incision. The incision might be extended to the inguinal region when (i) the vasectomy was performed high in the scrotum, (ii) a large segment was removed, or (iii) in repeat reconstructions with difficult vasal mobilization. The testis is only delivered if a vasoepididymostomy or a robotic-assisted anastomosis is to be performed.

Approaching the Vas

Microsurgical dissection is carried out at the region of the prior vasectomy site to free the vas and its vascular pedicle from surrounding scar tissue. Hemostasis is obtained with great care using either bipolar or hand-held thermal cautery units. After the vas has been mobilized and its scarred ends have been excised, patency of the abdominal vas end is confirmed with the introduction of a 24-gauge blunt tipped angiocatheter into the lumen and the injection of 20-mL sterile saline through the catheter. The ends of the vas must be adequately mobilized in order to allow a complete tension-free anastomosis. Either a microsurgical clamp or holding sutures can be used according to the surgeon's preference.

Vasal Fluid Examination

We examine the fluid from the testicular vas end both macroscopically and under the optical microscope for the presence of sperm. The presence of copious, clear, watery, or cloudy fluid and motile sperm is associated with excellent patency rates of 94%, opposed to only 60% when no sperm is found in the vasal fluid [81]. Thick toothpaste-like vasal fluid is suggestive of epididymal obstruction [81, 93]. The quality of sperm found in the intravasal fluid and the surgeon's microsurgical skills are the most critical factors to determine the type of reconstructive technique. Typically, the presence of sperm or sperm parts, and even a "dry" vas, is associated with adequate patency rates of about 70–80% following vasovasostomies [94, 95]. The vasoepididymostomy is a challenging surgical procedure that should only be attempted by experienced microsurgeons. A precise anastomosis of the vas (luminal diameter of 300–400 micrometers) to the epididymal tubule (150–250 micrometers) requires meticulous microsurgical technique and high magnification. Intraoperative sperm harvesting and cryopreservation can be offered during vasoepididymostomy [96].

Vasovasostomy Techniques

Attention to surgical details directly affects the success of reconstructive microsurgeries. These include the accurate mucosa-to-mucosa approximation, a water-tight tension-free anastomosis, preservation of the vasal blood supply and healthy tissue (mucosa and muscularis), and an adequate microscopic atraumatic technique.

Modified One-Layer Technique The modified one-layer technique described by Sharlip is the authors' choice for vasectomy reversal [97]. Our preference is to perform the anastomosis using a 9-0 nylon suture mounted on a taper-pointed needle and with the aid of a vas clamp (ASSI, catalog# MSPK-3678). The operation is performed entirely with the surgeon located on the patient's right side. The first suture is placed on the medial surface of the right vas (zero-degree position) (Fig. 12.3a). This suture is placed through the full thickness of the vas wall on the testicular side first taking a generous bite of adventitia and muscularis and a tiny portion of the mucosa. The suture is then passed into the corresponding zero-degree position of the abdominal side again taking a bite at the edge of the mucosa and a large portion of the muscularis/adventitia layer. This suture is tied and cut long, so it is easily identified as the procedure continues. The second suture is placed 180 degrees opposite to the first, again taking the full aspect of the vas wall, firstly on the testicular side and then on the abdominal one. This suture is also tied and cut long. A third full thickness suture is placed at the 60-degree position, one-third of the distance from the first to the second sutures. Before it is tied, a fourth suture is placed at the 120-degree position, two-thirds of the distance from the first to the second sutures. Third and fourth sutures are then tied after careful inspection of their

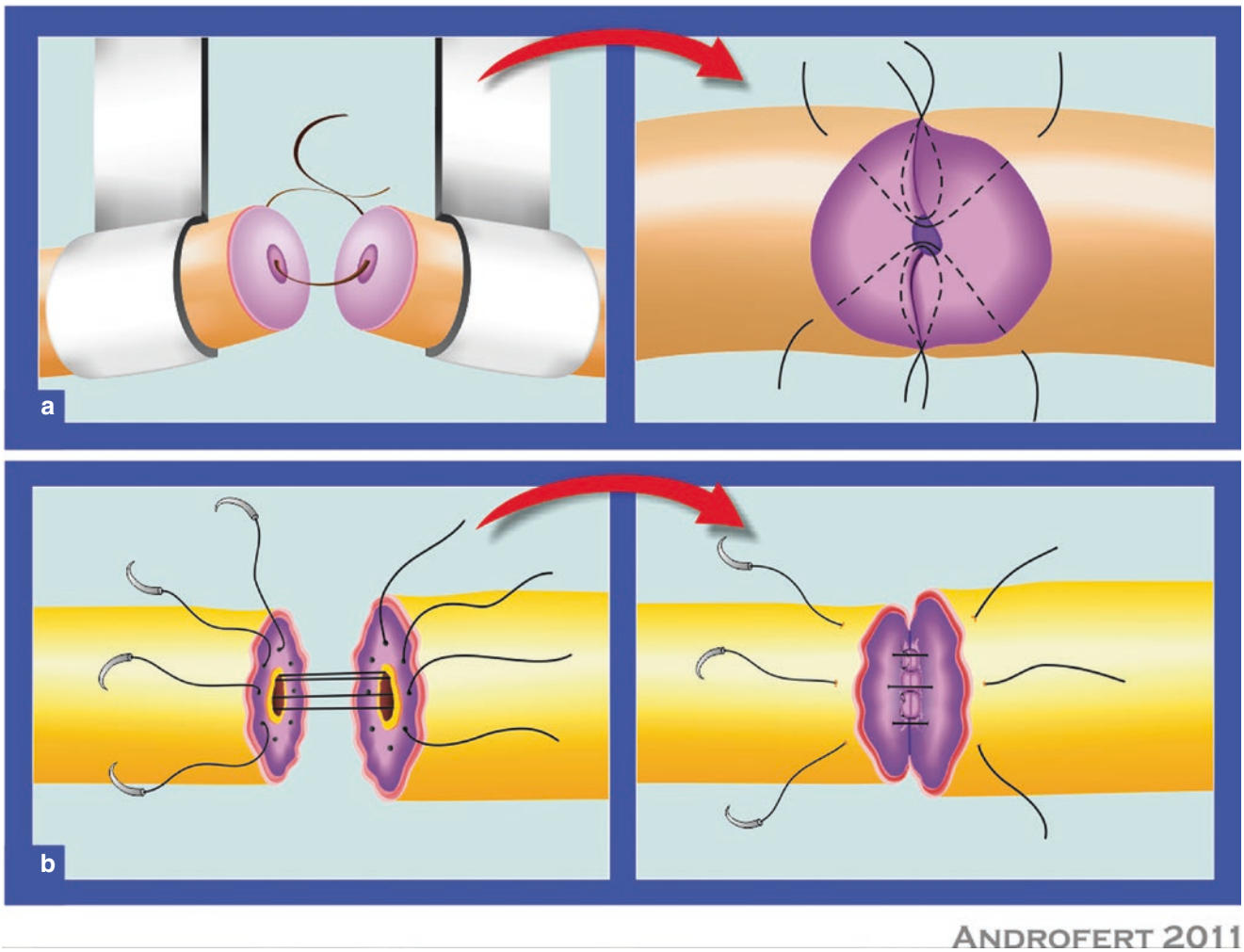

Fig. 12.3 Microsurgical vasovasostomy techniques. Illustration of the modified one-layer (**a**) and the modified two-layer (**b**) techniques. (Reprinted with permission, ANDROFERT© 2011. All Rights Reserved)

proper placement (Fig. 12.3a). A fifth suture is placed between these two at the 90-degree position, but only superficially through the muscularis. This completes the anastomosis of the anterior portion of the vas. At this point, four full thickness stitches and one muscular suture have been placed, and half of the total circumference of the vas wall is closed. The vas clamp is then rotated 180 degrees, and accidental back-walling and proper position of full thickness sutures are checked. After rotation of the vas, two full-thickness sutures are then placed at the 240-degree and 300-degree positions. These sutures are inserted and inspected before being tied. A final suture is placed in the muscularis at the 270-degree position. These complete the anastomosis, summing up eight sutures in total instead of twelve as described by Sharlip [97]. Upon anastomosis completion, the surrounding loose fibrous tissue is sutured over the anastomotic site alleviating tension. The scrotal incision is closed in the usual conventional manner.

Two-Layer Technique This technique, described by Belker, involves placing five to eight interrupted 10-0 nylon sutures in the inner mucosal layer and eight to ten 9-0 nylon sutures in the outer muscular and adventitial layer [98]. The use of an approximating clamp and a holding suture is recommended to stabilize vas ends for the anastomosis. Before the suturing begins, the surgeon looks straight down into the lumen of each end of the vas situated parallel to each other. As suturing proceeds, the transected ends of the vas bend toward each other, bringing the suture together without tension. Firstly, three posterior muscular layer sutures are placed in a row so that the knots are outside. Only 90 degrees of the circumference is approximated, leaving full access to the mucosa. Then, after three posterior mucosal sutures have been placed and tied, the far-corner and near-corner sutures are placed and tied until space remains for only two or three sutures in the anterior aspect of the anastomosis. These remaining stitches are then placed and left long and untied until back-walling can be safely ruled out. The sutures are

finally tied, and the closing muscular layer is sutured with caution to visualize the underlying mucosal layer sutures to prevent penetration of the lumen by the outer-layer ones. Placement of these sutures is more comfortable to perform from the assistant side toward the surgeon's side. Closure of scrotal incision is performed in the usual manner. A recent video shows in detail how the technique is performed by Hakki et al. [99].

Multilayer Microdot Technique This method, originally described by Goldstein, is preferred to treat markedly discrepant diameters in straight or convoluted vas [100]. Vasal ends are prepared with a 90-degree right angle cut, and methylene blue stain can be used to visualize the mucosal rings better. Planned needle exit points can be marked with microtip marking (Fig. 12.3b). Polypropylene monofilament 10-0 double-armed sutures with 70-micrometer-diameter taper-pointed needles are used for the anastomosis. Sutures are placed in an inside-out fashion eliminating the possibility for accidental back-walling. The mucosa and about one-third thickness of the muscularis should be included in each bite, symmetrically on each side of vas ends. Four initial sutures are placed in the anterior aspect of the vas and tied up (Fig. 12.2b). Three 9-0 sutures are then placed precisely in between the previously placed mucosal sutures, just above but not through the mucosa, sealing the gap between the mucosal sutures. The vas is then rotated 180 degrees, and four additional 10-0 sutures are placed completing the mucosal part of the anastomosis. Just before tying the last mucosal knot, vas lumen is irrigated with heparinized saline solution to prevent formation of clots. After completion of mucosal layer, 9-0 sutures are placed in between each mucosal suture again avoiding penetrating the mucosa itself (Fig. 12.2b). The sutures are placed but not tied until two or three more have been placed. Superficial additional adventitia 9-0 sutures should be placed if necessary. The procedure is completed by approximating the vas sheath with four to six 6-0 sutures.

Robot-Assisted Technique In recent years, some authors have shown the possibility to perform the classic above described techniques using robotic assistance. The robot can offer additional benefits of enhanced imaging (up to 100× magnification) and control of physiologic tremor [101, 102]. The use of robot-assisted reconstructive surgery for male infertility is out of the scope of this chapter (please refer to Chaps. 19 and 20 for more information).

Vasoepididymostomy Techniques

The procedure starts with the placement of a longitudinal incision in the upper scrotum. The testis is delivered through the incision, and the testis and epididymis are thoroughly inspected. The obstruction site may be often grossly visible as an area where the epididymis transitions from a firm, full caliber structure to a smaller and softer one. The distal end of the vas deferens is mobilized similarly as described for the vasovasostomy, but often a longer length is required to perform an epididymal anastomosis. At this point, the microscope is brought into the operating field. Currently, three variations of the technique have been used to connect the vas deferens lumen to a single epididymal tubule: end-to-end, end-to-side, and end-to-side intussusception techniques. Before the anastomosis, a dilated epididymal tubule must be identified immediately above the level of obstruction. The tubule is opened and the fluid inspected for the presence of motile sperm. If no sperm are identified, a more proximal site of the epididymis will be required for the anastomosis.

End-to-End Technique First described by Silber, the end-to-end VE is the most challenging anastomosis to perform [103, 104]. It involves dissection of a single epididymal tubule, complete transection, and anastomosis to the vas lumen. The epididymis is dissected off the testis for 3 to 5 cm to provide an adequate length to achieve a tension-free anastomosis. Initially, two 9-0 nylon sutures are placed at the 5 and 7 o'clock positions of the seromuscular surface of the vas, to secure the cut end of the distal vas to the epididymal tunica. Next, four double-armed 10-0 nylon sutures mounted in double-armed 70-micrometer fishhook-shape taper-pointed needle are placed in a quadrant fashion between the vas mucosa and the epididymal tubule (Fig. 12.4a). These sutures are not tied until all have been positioned. The anastomosis is completed by placing several interrupted 9-0 nylon sutures to approximate the seromuscular layer of the vas to the epididymal tunic layer.

End-to-Side Technique The end-to-side VE, popularized by Thomas, is performed by creating a small window in a loop of the epididymal tubule proximal to the obstruction and by suturing the end of the vas lumen to the open window [104]. The advantages over the end-to-end anastomosis include less dissection and bleeding during the anastomosis because hemostasis can be secured before opening the tubule. Moreover, only one tubule is opened making the identification of the patent tubule more precise and easier. With the tubule opened and sperm presence confirmed, three or four double-armed 10-0 nylon sutures are placed in a quadrant fashion through the edge of the epididymal tubule (Fig. 12.4b). The sutures are placed in the corresponding quadrant of the vasal mucosa and tied. The anastomosis is completed with additional 9-0 nylon sutures between the epididymal tunic and the seromuscular layer of the vas deferens. Finally, several 9-0 nylon sutures are used to anchor the vas

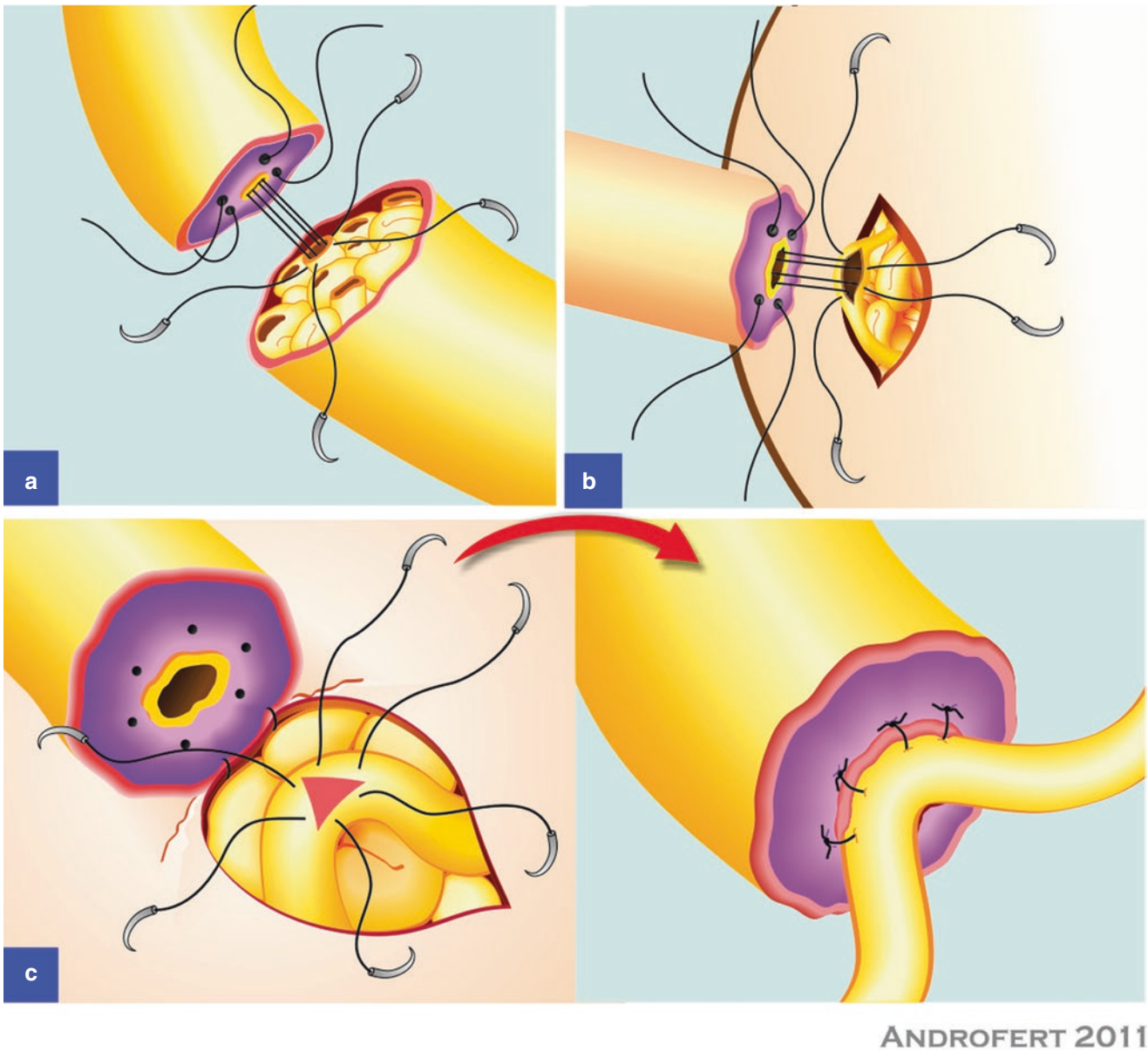

Fig. 12.4 Microsurgical vasoepididymostomy techniques. Illustration of the end-to-end (**a**), end-to-side (**b**), and triangulation end-to-side (**c**) anastomoses (see text for detailed description). (Reprinted with permission, ANDROFERT© 2011. All Rights Reserved)

deferens to the parietal layer of the tunica vaginalis. These final sutures are designed to prevent tension on the anastomosis and are placed well away from the vasoepididymostomy site.

Triangulation End-to-Side Vasoepididymostomy This technique was introduced by Berger [105] (with subsequent modifications by others [106, 107]). It is the simplest and fastest among the three techniques described in this chapter and the authors' choice for vasoepididymostomy. The intention is to combine the precision of the conventional end-to-side anastomosis with a simplified microsuture placement. Rather than a direct approximation of the epididymal tubule to the vas, this method involves pulling the epididymal tubule into the vas lumen. An opening window is made in the epididymal tunic corresponding to the vas diameter. Two 9-0 sutures are used to secure the muscular layer of the vas to the epididymal tunic to avoid tension on the anastomosis site. Three double-armed 10-0 nylon sutures are placed equidistantly in a triangular configuration in the desired epididymal tubule (Fig. 12.4c). Then, the epididymal tubule is carefully opened with microscissors or microknife between the positioned sutures. Once sperm are confirmed in the epididymal fluid, the needles are passed through the lumen of the vas in an inside-out fashion. The sutures are then tied, creating an

invagination of the epididymal tubule into the vasal lumen (Fig. 12.4c). Finally, additional 9-0 nylon sutures are placed to approximate the seromuscular layer of the vas to the epididymal tunic.

Marmar described a modification of the triangulation end-to-side VE [106]. In this technique, a single epididymal tubule is exposed, and two 10-0 nylon sutures mounted on double-armed 70-micrometer bi-curve needles are placed on the field. A needle from each suture is mounted on a styrofoam block and positioned parallel to the other with sufficient room for passage of the tip of a microblade between them. A microneedle holder is used to grasp both needles simultaneously and move them from the block to the field while maintaining the parallel arrangement. The tips of both needles are passed through a selected tubule at once. Both sutures are retracted laterally, and a tubulotomy is cut between them with a microknife. Then, all four needles from the epididymal sutures are individually placed into the mucosal lumen of the vas and out through the muscularis on the cut end. Needles are placed at the 8 and 10 o'clock positions on the left side and the 2 and 4 o'clock positions on the right side. Sutures are tied allowing the epididymal tubule to invaginate into the vas lumen. The anastomosis is completed with 3 to 4 additional 9-0 nylon sutures through the muscularis of the vas and epididymal tunic.

More recently, in 2014, two sutures of 10-0 nylon single-armed needles were used for longitudinal intussusception technique with satisfactory results [108, 109]. The first suture (5 cm long) is placed in an outside-in fashion through the mucosal layer of the vas deferens. Then, the needle is used to pierce the lateral aspect of the epididymal tubule and is placed longitudinally. The second suture (5 cm long) is placed identically and parallel to the first suture on the contralateral side of the epididymal tubule. The tubule is opened between the two parallel needles, and the fluid is examined for the presence of spermatozoa; the needles are pulled out and placed inside-out through the mucosal layer of the vas deferens, and the sutures were tied down making four-point fixation. In a series involving 22 patients, the mean operating time for unilateral and bilateral procedures is about 145 and 214 min, respectively. The reported patency rate was 59% (50% and 70% for unilateral and bilateral procedures, respectively); the overall paternity rate in a series was 36% [109].

12.2.2.4 Postoperative Care

Local dressing and scrotal supporter are kept for 48–72 hours and 2 weeks, respectively. Scrotal ice packing is always recommended to control local edema for the first 72 postoperative hours. Patients are counseled to restrain from physical activity and sexual intercourse for 1 or 2 months in cases of vasovasostomy and vasoepididymostomy, respectively. Oral analgesics usually suffice to control postoperative pain. Postoperative follow-up is aimed at evaluating improvement in semen parameters, complications, and natural or assisted conception. Patients are asked to provide semen specimens for analysis every 2 months after surgery until the semen parameters stabilize or pregnancy occurs.

Results

Microsurgical reconstruction of the vas remains a cost-effective, reliable, and effective means of restoring fertility in most men who have undergone vasectomy [110–114]. However, data comparing surgical reconstruction versus sperm retrieval/ICSI are neither randomized nor homogenous. Therefore, a proper understanding of the factors that can affect outcomes, overall cost, and the morbidity associated with each treatment modality, respective of the institution providing the treatment, is recommended. In experienced hands, reconstructive surgery of the male reproductive tract can be highly successful. Microsurgical techniques are superior to macrosurgical or loupe-assisted anastomoses [81, 115]. After microsurgery, 50–95% of the patients will have the return of sperm to the ejaculate, whereas 30–75% of couples achieve pregnancy without the need for ART.

Overall, historical patency/pregnancy rates following microsurgical vasovasostomy and vasoepididymostomy are 92%/55% and 78%/40%, respectively [75, 81, 84, 90, 93, 95, 103, 104–107, 116–126] (Tables 12.4 and 12.5). More recently, Majzoub et al. reported on their pregnancy data involving 171 consecutive patients after VR [125]. Overall patency rate (presence of any sperm in the ejaculate) was 91.8%, and spontaneous pregnancy occurred in 49.6% of couples with a mean duration of 1.3 ± 0.08 years. Most pregnancies occur within 24 months after surgery. Pregnancy rates are related to the time elapsed from vasectomy and reversal and female age. The likelihood of initiating a pregnancy after vasovasostomy is inversely proportional to the obstruction interval and is estimated to be less than 50% after 8 years according to EAU Guidelines on Male Infertility [15]. Other authors report that 30–40% of couples achieve a pregnancy after reconstructions performed in patients with obstruction intervals greater than 15 years as compared to >50% in shorter intervals [86, 93]. Although female partner's age does not affect patency rates after vasectomy reversal (VR), it does affect pregnancy rates (14% in women aged >40 years vs. 56% in those aged <39 years) [92]. Vasectomy reversal was shown to be feasible even in patients who failed percutaneous epididymal sperm aspiration (PESA). Marmar et al. reported limited epididymis trauma after PESA and up to 50% pregnancy rates after VR in this scenario among couples whose female partner was 37 years old or less [140].

Other factors that affect success rates include the gross appearance of vas fluid at the time of surgery, the presence or absence of sperm in the vas fluid and their quality, and the

Table 12.4 Vasovasostomy outcomes

Author	Year	N (total)	Technique	Patency rate (%)	Pregnancy rate (%)
Sharlip [116]	1981	n/a	Modified one layer	100	67
			Two layers	100	75
Lee [110]	1986	324	Modified one layer	85	50
			Two layers	91	52
Belker [81]	1991	1247	Modified one layer	89	57
			Two layers	86	51
Fischer [118]	2001	40	Modified one layer	88	n/a
			Two layers	90	n/a
Boorjian [84]	2004	159	Two layers	95	83
Chan [90]	2004	1048	Two layers	99	54
Hsieh [119]	2005	74	Modified one layer	91	43
Kolettis [95]	2006	34	Both	76	35
Schwarzer [120]	2012	958	Three layers	89	59
Van Dongen [121]	2012	162	Modified one layer	91.4	43.8
Safarinejad [122]	2013	112	Two layers	82.1	28.4
Crosnoe [123]	2014	561	Three layers	97.0	NR
Moon [124]	2015	263	Three layers	96.8	NR
Majzoub [125]	2017	139	Modified one layer or two layers	91.8	49.6
Marshall [126]	2017	60	One layer (robot assisted)	88.1	NR

length of the remaining segment adjacent to the epididymis. Recently, Scovell et al. performed a systematic review with meta-analysis comprising 1293 subjects with a mean obstruction interval of 7.1 years evaluating the prognostic value of finding sperm in the vasal fluid during vasectomy reversal. The unadjusted OR of postoperative patency was 4.1 times higher (95% CI 2.3–7.3) when intravasal sperm or sperm parts were found. Other vasal fluid characteristics such as consistency were not analyzed because of inconsistent reporting [142]. Mazjoub et al. also noted the presence of sperm or sperm parts during intraoperative vasal fluid analysis to be the only variable to significantly influence pregnancy rate [125]. As patency and pregnancy rates after the existing surgical procedures do not reach 100% and are technically demanding, efforts continue to be made to widen the options for reconstructive repair. Besides the intussusception vasoepididymostomy anastomotic techniques, the use of novel biomaterials/sealants and absorbable and non-absorbable stents and the use of robotics have been attempted [93, 102, 141–145].

The modifications to the conventional vasoepididymostomy techniques simplified and fastened the anastomoses. In a prospective study, Chan et al. reported overall patency and pregnancy rates of 84% and 40% using the intussusception technique [142]. These findings were confirmed by Schiff et al. who reported patency and pregnancy rates of approximately 82% and 45%, respectively, using simplified intussusception techniques [93]. It is suggested that anastomoses are more water-tight by using intussusception techniques; therefore, granuloma formation is decreased. Since pregnancy rates following vasoepididymostomy are below 50%

Table 12.5 Vasoepididymostomy outcomes

Author	Year	No. of patients	Anastomosis	Patency (%)	Pregnancy (%)
Dubin [127]	1985	46	End to end	39	13
Silber [128]	1989	139	End to end	78	56
Schlegel [129]	1993	93 17	End to end End to side	70	31
Berger [130]	1998	12	Intussusception	92	NR
Marmar [131]	2000	9	Intussusception	78	22
Chan [132]	2005	68	Intussusception	84	40
Schiff[a] [93]	2005	153	End to end End to side 3-suture Intuss 2-suture Intuss	73 74 84 80	
Kumar [133]	2010	24	Intussusception	48	N/A
Peng [134]	2012	73	Intussusception	71.7	33.3
Harza [135]	2014	36	End to side	77.7	22.2
Binsaleh [136]	2014	22	Intussusception	59	36
Hong [137]	2016	62	Intussusception	66.1	34.1
Peng [138]	2017	198	Intussusception	76.3	40.9
Yoon [139]	2019	2298	End to end/	61.1	26.9
			end to side		
			Intussusception	69.1	35.9

[a]p <0.05 comparing patency and pregnancy rates between the surgical approaches

and late failures occur in approximately 20% of the cases, it may be useful to retrieve sperm intraoperatively for cryopreservation, particularly in cases of difficult reconstruction. Sperm quality may deteriorate to the level of azoospermia or extreme oligozoospermia in approximately 20% of subjects after vasovasostomy at 1-year follow-up, thus making sperm cryopreservation advisable according to the EAU Guidelines on Male Infertility [15]. By contrast, a cost-analysis study demonstrated that sperm harvesting and cryopreservation during vasectomy reversal are not cost-effective [96].

The rationale of using sealants around the anastomotic site is to decrease operative time and to simplify the procedure without compromising success rates. Fibrin sealant stimulates the coagulation cascade producing a fibrin seal around the anastomosis. When mixed with thrombin and calcium, fibrinogen is converted to fibrin monomer, which in turn is converted to a stable cross-linked fibrin polymer [143]. Ho et al. achieved 85% patency rates and 23% pregnancy rates using three transmural 9-0 sutures and fibrin glue in a mean follow-up of 6.2 months [143]. There are concerns, however, about the potential contact of the glue with the vas lumen, which may result in possible obstruction, and also about the transmission of viral disease because fibrin glue is derived from pooled plasma [141]. The use of non-absorbable polymeric stent has been reported in the animal model. Preliminary results showed 100% patency rates in a follow-up of 39 to 47 weeks, and the total sperm count was significantly higher in the stented group [144].

The use of robotics is also novel. The rationale to add this technology to the already existing armamentarium relies on the possibility of physiologic static tremor correction, visual magnification (up to 100× when using a digital microscopic camera) and ergonomics [145]. Animal studies suggest that robotically assisted vasectomy reversal are easier to perform and yields better pregnancy rates than microsurgical reversal [146]. In a preliminary experience in humans, Parekattil et al. reported shorter operative time and higher postoperative sperm count for robot-assisted vasectomy reversal as compared to the microsurgical technique [145]. However, the advantages of the robot over an experienced microsurgeon are yet to be proven in larger series.

12.2.3 Transurethral Resection of the Ejaculatory Duct

12.2.3.1 Indications

Ejaculatory duct obstruction (EDO) is a potential surgically correctable cause of male infertility. Congenital obstructions are caused by atresia or stenosis of the ejaculatory ducts and utricular, Müllerian, and Wolffian duct cysts. Acquired obstructions can be secondary to trauma or infectious/inflammatory etiologies. Traumatic damage to the ejaculatory ducts might occur after removal of seminal vesicle cysts, pull-through operations for the imperforate anus, and even prolonged catheterization or instrumentation. Genital or urinary infection and prostatic abscess may also cause stenosis or complete obstruction of the ducts [147]. Prostatic infection may result in calculus formation and secondary obstruction, while tuberculosis produces genital devastation.

12.2.3.2 Preoperative Planning

Patient Evaluation

The diagnosis of EDO is usually made by combining the results of medical history, physical examination, semen analyses, and transrectal ultrasound. The clinical presentation may be highly variable; in addition to a history of infertility, complaints such as painful ejaculation, hemospermia, and perineal and/or testicular pain might be present. Nevertheless, some patients are entirely asymptomatic.

On physical examination, enlarged seminal vesicles or a mass might be palpable on rectal examination. Prostatic tenderness and/or epididymal enlargement might also exist. The serum levels of FSH and testosterone are usually normal.

Semen analyses reveal oligozoospermia or azoospermia, decreased motility, and decreased ejaculate volume. An acidic (pH <7.2) and low-volume (<1.5 mL) azoospermic ejaculate, associated with no detectable fructose, palpable vasa, and epididymal thickening, is virtually pathognomonic. However, the typical clinical picture might be complicated as unilateral, partial, and functional obstructions exist [147]. Post-ejaculate urinalyses are often performed to exclude retrograde ejaculation in patients with low-volume ejaculates.

High-resolution transrectal ultrasound (TRUS) evaluation using a 5–7 MHz biplanar transducer is recommended in all cases of suspected EDO. The exact definition of obstruction on TRUS, however, is still a matter of debate due to marked variability in the size and shape of the vas deferens, seminal vesicles, and ejaculatory ducts in fertile and infertile men. Common ultrasound findings include dilation of the seminal vesicles (defined as a cross-sectional width of greater than 1.5 cm) or ejaculatory ducts (defined as an internal duct diameter of greater than 2.0 mm), calcifications or calculi in the region of the ejaculatory duct or verumontanum, and midline or eccentrically located prostatic cysts [148–150]. Transrectal ultrasound-guided seminal vesiculography has been shown to provide excellent radiographic visualization of the ejaculatory ducts [151]. Also, TRUS-guided seminal vesicle aspiration and the presence of motile sperm in the aspirates can be used for diagnosis; since the seminal vesicles are not sperm reservoirs, the presence of sperm is sug-

gestive of obstruction [152]. A testicular biopsy can be done to document the presence of normal spermatogenesis. The authors' preference is to perform a "wet prep" using the percutaneous testicular sperm aspiration technique either before or at the time of surgery. The presence of motile sperm is highly indicative of ductal obstruction.

12.2.3.3 Operative Aspects

Anesthesia

Transurethral resection of the ejaculatory ducts (TURED) is performed using regional or general anesthesia.

Technique

Our choice is to perform the transurethral resection of the ejaculatory ducts (TURED), as described originally by Farley and Barnes [153], with minor modifications [147]. First, the obstruction is documented using intraoperative vasotomy and vasography. The vas is delivered using a small scrotal incision and dissected free of the associated perivasal vessels. A mixture of injectable saline and radiographic contrast material in a 1:1 ratio is injected into the abdominal end of the vas, together with methylene blue dye, by direct vas puncture with a 30-gauge lymphangiogram needle [147]. Vasography confirms obstruction, whereas dye injection confirms patency by visualization of the effluxing dye mixture during TURED. A 9-0 nylon suture is placed at the muscular layer of the vas to close the vasotomy site. Alternatively, transrectal ultrasound-guided seminal vesicle puncture and instillation of contrast can be used to confirm the obstruction.

TURED is performed with the patient in the dorsal lithotomy position. A resectoscope with a 24-French loop is used to remove a strip of tissue on the floor of the prostate just proximal to and including a portion of the verumontanum (Fig. 12.5). The ducts are considered adequately opened by visualizing its dilated portion and the dye efflux. An 18-F Foley catheter is left in place for 24 hours, and the patient is discharged the next day.

Notably, resection of the ejaculatory ducts is a hazardous procedure. The typical EDO patient is young and has a small prostate. Thus, TURED is carried out very close to the bladder neck, rectum, and sphincter. If a midline cyst is present, resection is performed to unroof the cyst completely. If not, the prostatic side of the verumontanum is resected until a dilated portion of the ejaculatory duct is seen. Resection is performed with pure cutting to avoid thermal injury to the proximal ejaculatory duct. We feel more comfortable placing a finger in the patient rectum to prevent rectal injury during resection and having methylene blue injected through the vasotomy site. Resection is completed by identification of free dye efflux into the urethra.

A movie illustrating the technical details of TURED using transrectal ultrasound-guided seminal vesicle puncture and instillation of methylene blue can be found at https://youtu.be/IoD7ssKOLhI [154].

12.2.3.4 Postoperative Care

An indwelling catheter is kept in place for 24–48 h, and patients are discharged the following day. Oral quinolone antibiotics and anti-inflammatory medication are pre-

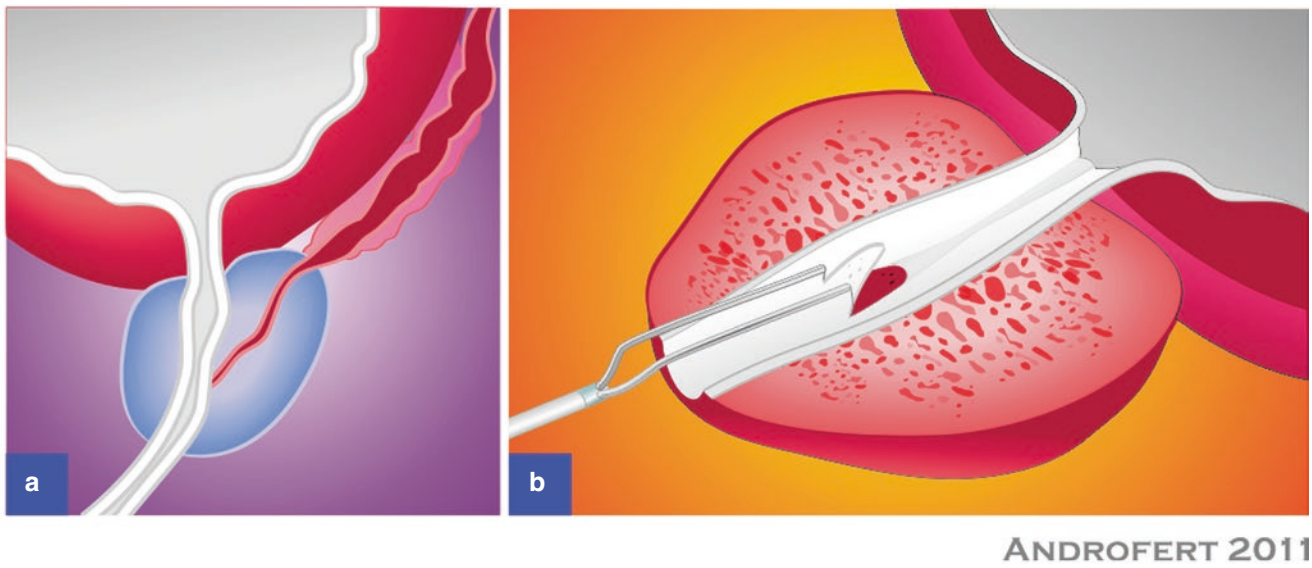

Fig. 12.5 Transurethral resection of the ejaculatory duct. Schematic representation of the ejaculatory duct entering into the prostatic urethra (**a**). A resectoscope loop is used to remove a strip of tissue on the floor of the prostate just proximal to and including a portion of the verumontanum (**b**). (Reprinted with permission, ANDROFERT© 2011. All Rights Reserved)

scribed for 5 days. A scrotal supporter is recommended for 1 week to avoid scrotal edema due to vasotomy. We ask our patients to have frequent ejaculation 3–4 weeks postoperatively, and patients are monitored with monthly semen analyses.

Results

Ejaculatory duct obstruction is a treatable cause of male infertility, but the diagnosis might be difficult to make, particularly in cases of partial obstruction. Transrectal ultrasound is valuable but not specific. Adjunctive procedures such as magnetic resonance imaging, chromotubation, seminal vesicle aspiration, seminal vesicle scintigraphy, and ejaculatory duct manometry might be used to confirm the diagnosis of EDO [155–157]. Transurethral resection of the ejaculatory ducts (TURED) remains the treatment of choice, but due to the risks and the possibility of urinary reflux into the seminal vesicle, less invasive approaches using balloon dilation coupled or not with transurethral incision of the ejaculatory ducts have been proposed with similar results and fewer complications [158, 159].

Furthermore, minimally invasive vesiculoscopy procedures have been introduced for treatment of EDO. Wang et al. used a ureteroscope to inspect the seminal vesicle cavity and remove calculi. In this procedure, the ejaculatory duct might be cleared without damaging the verumontanum [160].

In our earlier reported series of 14 patients with complete or partial obstructions, TURED results varied according to the etiology of obstruction, congenital or acquired. Semen quality improvement (ejaculate volume, sperm count, and motility) was observed in 83% of the patients in the congenital group. Natural pregnancy was obtained in 66% of these patients at an average of 5.7 months postoperatively. In the group of patients with secondary EDO, the seminal improvement was detected in only 30% of the individuals, and only one natural pregnancy was achieved. Two individuals (one in each group) conceived via assisted reproduction techniques. Complication rates were similar in both groups (33%) and included reflux of urine to the unroofed cyst cavity with consequent impairment of the semen parameters, retrograde ejaculation, and one case of epididymitis with obstruction. Rectal injury or incontinence was not reported in our case series [147].

El-Assmy et al. reported on a retrospective cohort of 23 infertile men with either partial or complete EDO secondary to midline cyst or post-inflammatory obstruction. The seminal improvement was seen in all patients with partial EDO and 23.5% of those with complete EDO. Natural pregnancy occurred in 13% of cases. Data suggests that men with complete EDO due to midline cysts do better after TURED compared with those with an inflammatory cause. On the other hand, seminal results do not seem to be affected by the cause of obstruction in partial EDO [161].

12.3 Conclusions

Surgical interventions to treat male infertility are within the scope of urologists with training in microsurgery and endourology. Varicocele repair is recommended to selected infertile men with clinical varicocele as a means to improve fertility and reproductive outcomes, both natural and assisted. Microsurgical reconstruction of the vas deferens is highly successful when properly indicated. However, surgeon's experience is a critical factor for successful outcomes. Vasoepididymoanastomosis is a challenging microsurgical procedure which has to be conducted only by urologists with optimal microsurgical skills. Ejaculatory duct obstruction treatment by transurethral resection of the ejaculatory duct or vesiculoscopy may yield satisfactory results fertility wise, in particular in cases of congenital obstruction.

12.4 Review Criteria

A search of studies examining the use of surgical techniques for varicocele repair, vasovasostomy, vasoepididymostomy, and ejaculatory duct obstruction in the context of male infertility was performed using search engines such as ScienceDirect, Ovid, Google Scholar, PubMed, and MEDLINE. The start date for the search was not specified, and the end date was January 2019. The overall strategy for study identification and data extraction was based on the following keywords: "varicocele," "DNA fragmentation," "semen analysis," "vasovasostomy," "vasoepididymostomy," "vasal reconstruction," "infertile men," "varicocelectomy," "infertility," "semen parameters," "pregnancy rate," "surgical treatment," and "ejaculatory duct obstruction." Articles published in languages other than English were not considered. Data that were solely published in conference or meeting proceedings, websites, or books were not included. Websites and book-chapter citations provide conceptual content only.

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Microsurgery for Male Infertility

13

Rajeev Kumar and Manoj Kumar

Keys Points

- Obstructive azoospermia is an important diagnostic category since a number of these cases can be treated surgically.
- Evaluation of infertile men aims at identifying the etiology, reversibility, and potential underlying medical conditions that may have manifest as infertility.
- Microsurgery is offered for bypassing an obstruction in the epididymis or the vas deferens and for correction of a varicocele. The evaluation and investigations are tailored to diagnose suitability for microsurgery and maximize successful outcomes.
- Two-suture intussusception vasoepididymostomy using 10-0 polyamide sutures is a quick and highly effective technique. Longitudinal suture placements allow better results than transverse placement.
- Two-layer anastomosis for vasovasostomy with a simplified 4-6 suture technique has excellent outcomes.
- Patient selection is key to success in microsurgical varicocelectomy.

13.1 Introduction

Obstructive azoospermia (OA) is one of the correctable causes of male infertility and is thus an important diagnostic category [1–3]. Microsurgery for infertility involves vasoepididymal anastomosis (VEA), vasovasal anastomosis, and microsurgical varicocelectomy. Reconstruction was first reported by Martin in 1902 when he described his side-to-side technique by bringing together the cut edge of tunica vaginalis of epididymis to the incised seromuscular layer of vas deferens, relying upon the creation of fistula between the open epididymal tubule and vas deferens, and demonstrated patency rates of 43% [4]. In 1936, Hagner reported his surgical outcomes using this technique with 60% patency rate [5]. In 1978, with improvement in magnification, Silber described his microsurgical approach for end-to-end epididymovasostomy and directly anastomosing the mucosa of the end of vas deferens to the end of single epididymal tubule [6]. In 1983, Fogdestam and Fall and Thomas described the end-to-side epididymovasostomy by anastomosing the end of the vas deferens to the side of an epididymal tubule with a patency rate of 64% [7, 8]. In 1998, Berger described the triangulation end-to-side intussusception technique by placing three double-armed 10-0 nylon sutures into the unopened epididymal tubule which reduced the operating time and produced a patency rate of 92% [9]. In 2000, Marmar modified the procedure by passing two needles simultaneously and transversely through the unopened epididymal tubule, with a small transverse tubulotomy placed between them with a microblade (transverse incision vasoepididymostomy [TIVE]) [10]. In 2003, Chan et al. [11] published a comparative report on three different intussusception techniques in rats and concluded longitudinal placement of sutures in the epididymal tubule (longitudinal intussusception vasoepididymostomy [LIVE]) resulted in higher patency rates.

13.2 Etiology and Evaluation

Microsurgery is currently offered for bypassing an obstruction in the epididymis or the vas deferens and for correction of a varicocele. The evaluation and investigations are tailored to diagnose suitability for microsurgery and maximize successful outcomes. The diagnosis of the obstruction is based on the two facts: first, the absence of sperm in the ejaculate (azoospermia), and second, the demonstration of normal production of sperm in the testis (on testicular histology) with normal FSH. Microsurgical reconstruction for obstruc-

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tive azoospermia may be offered to patients with vasal injury and vaso-epididymal junction obstruction.

1. *Vasectomy/vasal injury*: Vasectomy, as a cause of infertility, is easily diagnosed from history and examination, which reveals palpable nodules in both the vasa at site of the vasectomy. A semen analysis is obtained to confirm azoospermia, and additional testing is usually not required. Iatrogenic trauma during a hydrocele or hernia repair or retroperitoneal surgeries may cause OA. In such cases, formal vasography with injection of radiographic contrast into the vas deferens may be required to identify the site of the obstruction. This test should be done along with the actual surgical reconstructive procedure. Isolated diagnostic vasograms should not be performed as they may result in vasal scarring and injury.
2. *Vaso-epididymal junction obstruction (VEJO)*: The junction of the vas and the epididymis is susceptible to obstruction following infections and for unknown causes. It is a diagnosis of exclusion in men with normal sperm production, normal ejaculate volume, normal vas deferens, but with absence of sperms in the ejaculate. In a majority of patients, the etiology of VEJO cannot be determined, and the diagnosis is idiopathic obstruction (iVEJO). Fifteen percent of men with OA have intratesticular obstruction that is not amenable to reconstruction but cannot be diagnosed preoperatively. Acquired forms (post-inflammatory or post-traumatic) are more common than congenital forms. Its diagnosis is important because VEJO is one of the surgically correctable causes of azoospermia [12].

13.2.1 Reconstruction Versus Sperm Retrieval and In Vitro Fertilization/ Intracytoplasmic Sperm Injection (IVF/ ICSI)

As in other men with OA, men with iVEJO have the option of surgical sperm retrieval and IVF with ICSI. Treatment should thus be individualized to each couple. Microsurgical reconstruction is more cost-effective than sperm retrieval and IVF, which requires intervention in both partners [13]. A successful surgery allows subsequent pregnancies without further intervention and avoids the risks of complications of IVF including ovarian hyperstimulation, birth defects, multiple gestations, prematurity, and low birth weight [14].

On the other hand, considering the limited success with reconstruction, it should be offered cautiously in couples with advanced age of the spouse as female fertility declines significantly with age [15]. Long duration of obstruction, particularly when reconstruction is being considered post-vasectomy, is associated with poor outcomes [16]. Concomitant female factor abnormalities that require intervention such as tubal blocks would also be an indication for primary sperm retrieval and IVF instead of reconstruction (Table 13.1).

The overall fertilization rates with per injected oocyte after IVF/ICSI is 45–75%, and pregnancy rates range from 26% to 57%, when surgically retrieved epididymal or testicular sperms are used [18–24].

Table 13.1 Comparison of microsurgical reconstruction (vasectomy reversal and vaso-epididymal anastomosis) and in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI)

	Advantages	Disadvantages
Microsurgical reconstruction (vasectomy reversal and vaso-epididymal anastomosis)	1. Allows natural selection of best sperm during fertilization through natural intercourse. 2. If microsurgery is successful, it allows future conception without additional medical treatment. 3. Less cost – One-time procedure is less than the 20% cost of single cycle of IVF/ICSI [17]. 4. If microsurgery results in patency and not pregnancy, patients have options of IUI and IVF-ICSI using ejaculated sperm instead of other surgical sperm retrieval techniques.	1. This is the most challenging technique in urology; thus, results may depend on the microsurgical skills, experience, and training of the surgeon. 2. Surgical techniques may change over time which will influence the patency and pregnancy rate. 3. May not be available at many centers
In vitro fertilization/ intracytoplasmic sperm injection (IVF/ICSI)	1. It is the only treatment option in intratesticular obstruction, obstruction at multiple sites, and congenital bilateral agenesis of vas deferens (CBAVD). 2. It is preferred in advanced maternal age as fertility potential in female decreases after 35 years and limited after 40 year of age. 3. Females with tubal disease/tubal ligation, it is preferred as it avoids microsurgery in both the partners. 4. Previously failed microsurgical reconstruction in male	1. Risk for the female partner like ovarian hyperstimulation syndrome (OHSS) [14] 2. Risk for the fetus – prematurity, genetic abnormalities, and multiple gestations [14] 3. Complications during oocyte and sperm retrieval 4. Possibility of carrying or transmitting genetic defect to offspring related to male factor infertility 5. Increased cost of the treatment, and intervention in both male and female partners is required

13.3 Microsurgical Procedures

Microsurgical reconstruction is performed as day-care surgery under regional or light general anesthesia. An operating microscope with face-to-face attachments for surgeon and assistant along with an offset arm with facility for camera attachment and transmission is used. There has been a steady evolution in case selection and techniques over the years, and this has been accompanied by an improvement in outcomes.

13.4 Vaso-epididymal Anastomosis

Scrotal explorations for vaso-epididymal anastomosis form the most common microsurgical procedure performed at our institution. Nearly all such procedures are performed for men with idiopathic obstruction. An obstruction is suspected in men with normal volume azoospermia with palpable vas deferens and normal spermatogenesis on testicular FNAC (fine needle aspiration cytology). These men are counselled about the options of scrotal exploration and IVF. A presumptive prognosis for success of reconstruction is provided based on age, testicular size, serum FSH, and presence of scrotal pathology.

Two publications toward the end of the last century on techniques form the basis of our surgical approach (Table 13.2). Berger's [9] paper on the triangulation intussusception technique of end-to-side vasoepididymostomy was the first of these publications. The use of three double-armed sutures instead of multiple independent sutures for the inner layer, coupled with our increasing familiarity with an operating microscope, allowed us to attempt this technique in a number of patients with some success. A major problem that we faced with this approach was with the size of needles for the 10-0 suture. The 10-0 double-armed polyamide suture available to us was swaged on 200-μm needles. Placing three such needles in the small epididymal tubule proved difficult, and we would often manage to get only two sutures in place.

Marmar's [10] article in 2000 on the two-suture technique of vasoepididymostomy proved to be the turning point in our approach to this procedure. It was simpler to perform and afforded excellent results. We began using this technique in 2002 and have been using our modifications of it since then.

In 2003, Chan et al. [11] published a comparative report on three different intussusception techniques in rats and concluded that longitudinal placement of sutures in the epididymal tubule resulted in higher patency rates. Difficulty in placing our thicker needles transversely in the tubules had led us to this modification in our patients at about the same time, and we commented on our findings in a reply to their publication [25].

Table 13.2 Evolution in vasoepididymostomy technique at our center

Early 1990s: 6-0/7-0 sutures, non-mucosal anastomosis
Poor expertise in microsurgery
Limited equipment
Late 1990s: Berger's technique [9] of three suture intussusceptions
Thicker needles, inadequate space for three needles
Early 2000s: Marmar's technique [10] of two suture intussusceptions
Thicker needles
Thin tubules
Current: Modified, longitudinal technique of two suture intussusceptions [27]

13.5 Our Surgical Technique

Our initial microscope had a relatively high minimal height, and the height of the operating table and the eyepiece of the microscope made it impossible to sit and operate. Thus, all our microsurgeries were performed with the surgeon standing up. This initial practice has been continued, and we still perform all such surgeries standing up even after changing our microscope in 2009 to a Zeiss Opmi Vario® (*Carl Zeiss Micro Imaging GmbH, Germany*) with an S-88 stand. Our basic microsurgical instruments set consists of curved and straight micro-needle holders without ratchet, curved micro-scissors, straight iris scissors, Jeweler's toothed and non-toothed microforceps with a platform to assist suture tying, and Adson's toothed and non-toothed forceps. We do not use clamps for holding the epididymis or the vas deferens.

With the patient supine, a longitudinal incision is made in the scrotum at its anterolateral edge toward the upper end of the testis. The incision is deepened to expose the tunica vaginalis, which is incised to deliver the testis. Any adhesions within the tunical layer are divided to expose the anterior surface of the epididymis. The surface is inspected for calcifications/nodules and visibly dilated tubules. In patients with a previous hydrocelectomy, the tunical layer is obliterated, and an attempt is made, by feel, to identify the epididymis and incise layers of tissue above it to expose the epididymal tunic. The spermatic cord is palpated posterior-lateral to the testis to confirm the presence of the vas deferens and get a visual impression of its diameter. Occasionally, in men with a more distal obstruction, the vas may feel dilated and thick fluid in the lumen may be visible through its wall.

Before dividing the vas deferens, we inspect the epididymis through its tunica to identify dilated tubules. In men with clearly dilated tubules, we proceed to preparing the vas. However, if the epididymis does not show any dilated tubules and feels flabby in its entire length, we make an incision in the epididymal tunic and observe the individual tubules under the microscope (Fig. 13.1a). If there still appears to be a doubt about the presence of obstruction, one of the distal

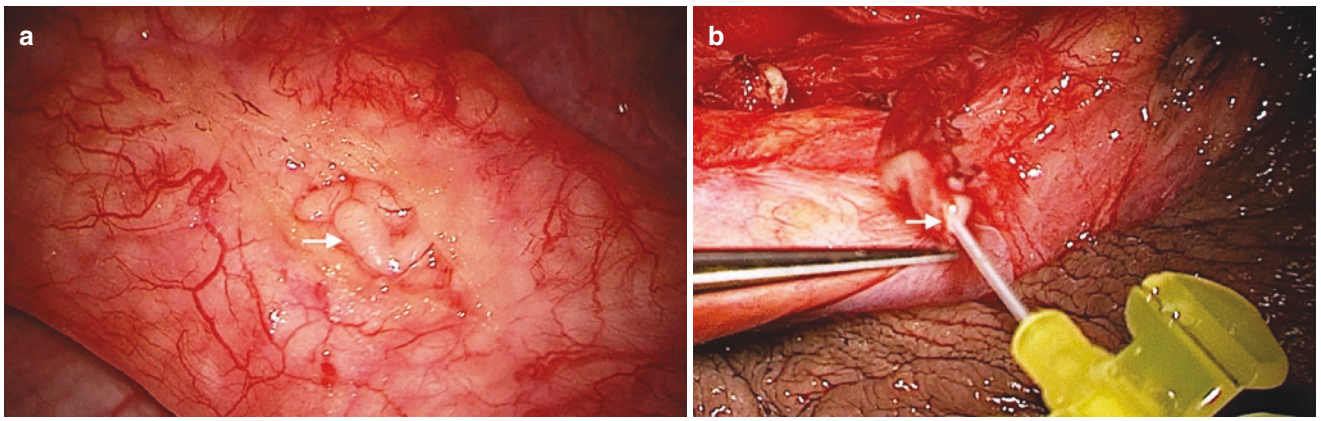

Fig. 13.1 (**a**) Intraoperative photograph of dilated epididymal tubule after opening the tunical layer of epididymis. (**b**) Vas deferens is isolated from the cord structures and transected, and a 24 G angiocatheter is inserted and flushed with saline to look for distal patency

loops of the tubule is incised, and the fluid is examined for sperms. If sperms are confirmed, a more proximal site is selected for the anastomosis.

The vas is isolated from the remaining cord structures with blunt dissection, maintaining a mesentery of blood vessels to the vas in its entire length. The junction of the convoluted and straight vas is identified, and a small segment of the vas at this site is elevated from its mesentery on a small hemostat. Using a sharp straight knife, the vas is hemisectioned at this level and inspected for any fluid that may suggest a more distal block. A 24 G angiocatheter is carefully inserted into the lumen of the distal vas and flushed slowly with up to 20 cm [3] of saline. Free flow of saline with no regurgitation is used as an indicator of distal patency, and the section of the vas is completed (Fig. 13.1b. If there is resistance to flow of saline, a 3-0 polyamide suture is threaded into the lumen to determine the level of block. If the block is at a short distance, the vas is exposed to the expected site of block, and a fresh vasotomy is made at that site and the procedure repeated. For more distant blocks, a formal vasography is performed.

Once distal patency of the vas is confirmed, further mobilization of the mesentery is performed, keeping a good amount of tissue and blood vessels around the vas. The periadventitial tissue is held gently with a hemostat until the first seromuscular sutures are placed.

The epididymis is reinspected and its tunica incised over the most visibly dilated tubules. Individual loops of the tubule are gently separated from their surrounding alveolar tissue until the selected tubule bulges above the rest. In our earlier cases, the sutures were placed transversally (Fig. 13.2a) [26] in the loop in a manner that they could be tied without crossing over of the sutures [27]. In our more recent cases, we place the sutures longitudinally (Fig. 13.2b) [26], again in a manner such that they can be tied to each other without crossing over.

The end of the vas is maneuvered to the site of the tunical incision. An 8-0 polyamide suture is placed from outside-in at the 5 o'clock position in the seromuscular layer of the vas. The needle is then passed through the epididymal tunic from inside out and tied. The procedure is repeated with another suture at the 7 o'clock position. The direction of placement of these sutures is important in that placing the suture along the direction of the vas helps provide traction to the needle as it enters the thick vas. Placement inside out on the epididymal tunic allows the needle to lift the tunic from the underlying tubules, preventing inadvertent injury to the tubules (Fig. 13.3a).

Once the vas is secured to the epididymis, the needle of a 10-0 polyamide double-armed suture is placed in the loop of the epididymal tubule. The needle is inserted at the end closer to the vas and exits at the opposite end. This needle may be placed transversally or longitudinally in the loop depending on the selected procedure. A second similar suture is placed, parallel to the first (Fig. 13.3b). The tubule between the two needles is incised using a microknife. It is important to ensure that the length of this incision does not extend beyond the entry and exit points of the two sutures. Fluid from the tubules is directly collected on a sterile microslide for examination under a light microscope. The procedure is continued if sperms are seen in the fluid. The needles are pulled through and kept separate from each other. The other ends of the sutures with their needles are now closer to the vas and are placed first into the vas lumen. These needles are placed at the 5 o'clock and 7 o'clock positions in the vas from inside the lumen, out through the muscular layer but not the full thickness of the vas. The two needles away from the vas are now placed similarly into the vas lumen at the 1 o'clock and 11 o'clock positions ensuring that the sutures are not entangled. The needles exiting at 7 o'clock and 11 o'clock in the vas belong to the same suture as do the 5 o'clock and 1 o'clock needles. The two

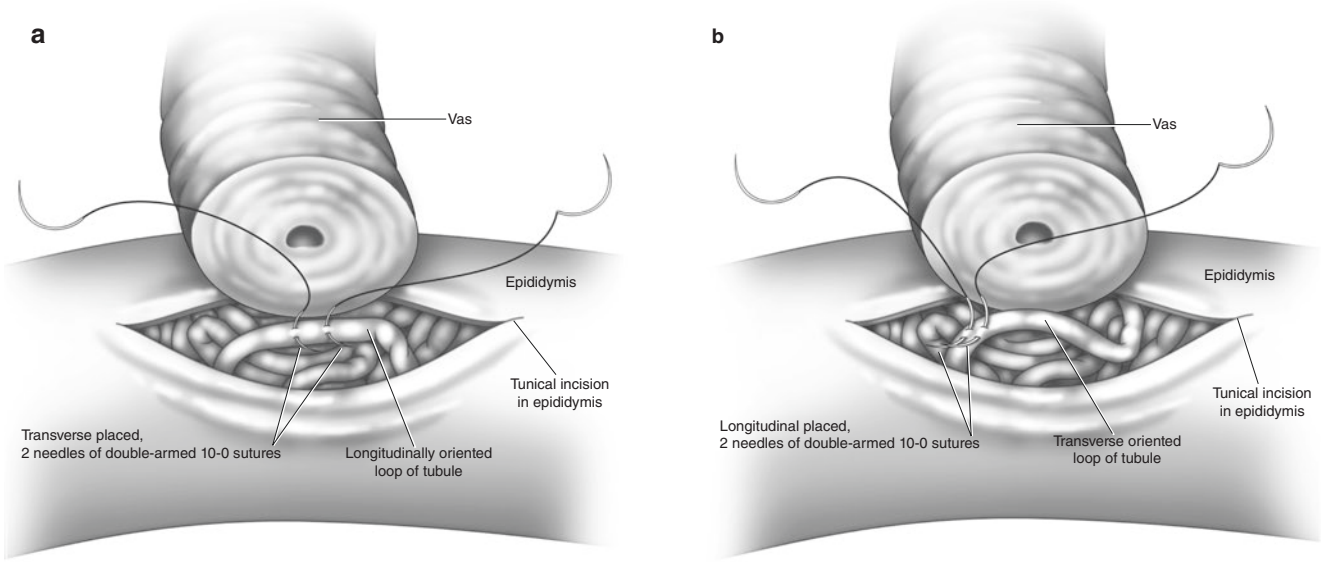

Fig. 13.2 (**a**, **b**) Transverse and longitudinal suture placement in the epididymal tubule for a vasoepididymostomy. Reproduced with permission by Kumar R [26]

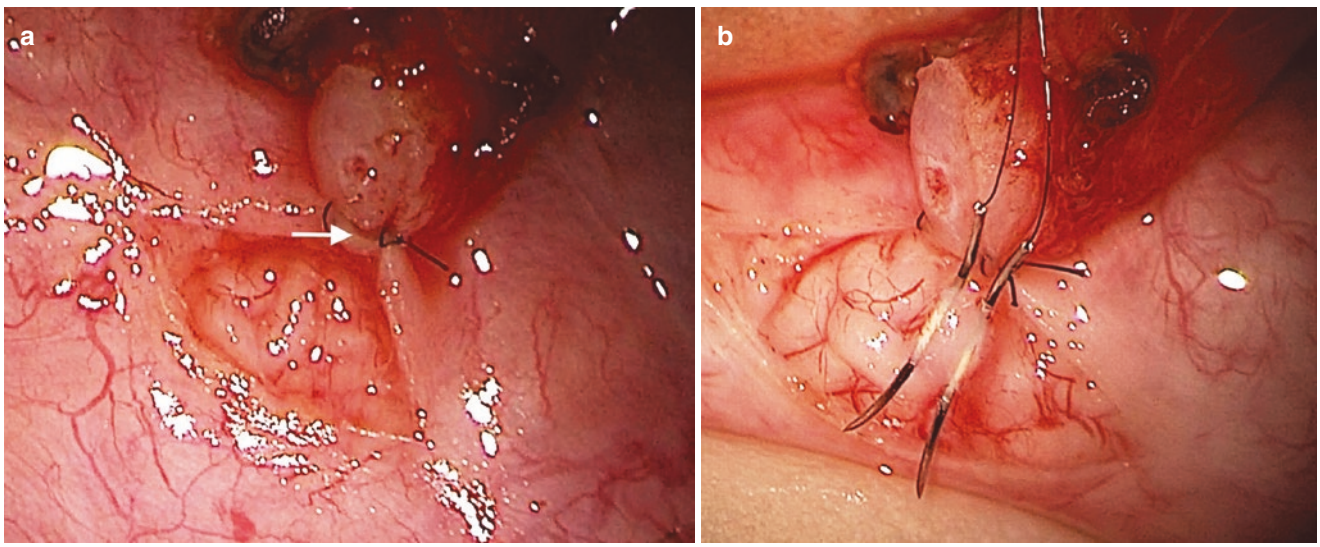

Fig. 13.3 (**a**) End of the vas is fixed with epididymal tunic using 8-0 polyamide suture. (**b**) Longitudinal placement of needles of two 10-0 polyamide sutures over the epididymal tubule and tubule incised in between using microknife

needles of each suture are held together and pulled to "hitch" up the tubule into the vas lumen (Fig. 13.4a). Both ends of the same suture are then tied to each other, intussuscepting the loop into the vas lumen. Additional 8-0 polyamide sutures are placed in the anterior layer of the vas and the epididymal tunic to secure the anastomosis. Two to three 8-0 polyamide sutures are also placed proximally in the serosal layer of the vas and the epididymal tissue to protect the anastomosis from cremasteric contractions (Fig. 13.4b). In case there are no sperms in the epididymal fluid, the sutures are removed and the procedure is repeated at a more proximal site.

All patients receive 3–5 days of antibiotics and are reviewed for suture removal after a week. A semen analysis is performed at 6 weeks and repeated every 3 months until patency is demonstrated. Patients who do not have a patent anastomosis by 1 year are advised to begin evaluation for IVF though they are counseled that delayed patency may occur even at 18 months. The modifications in our technique are described in Table 13.3.

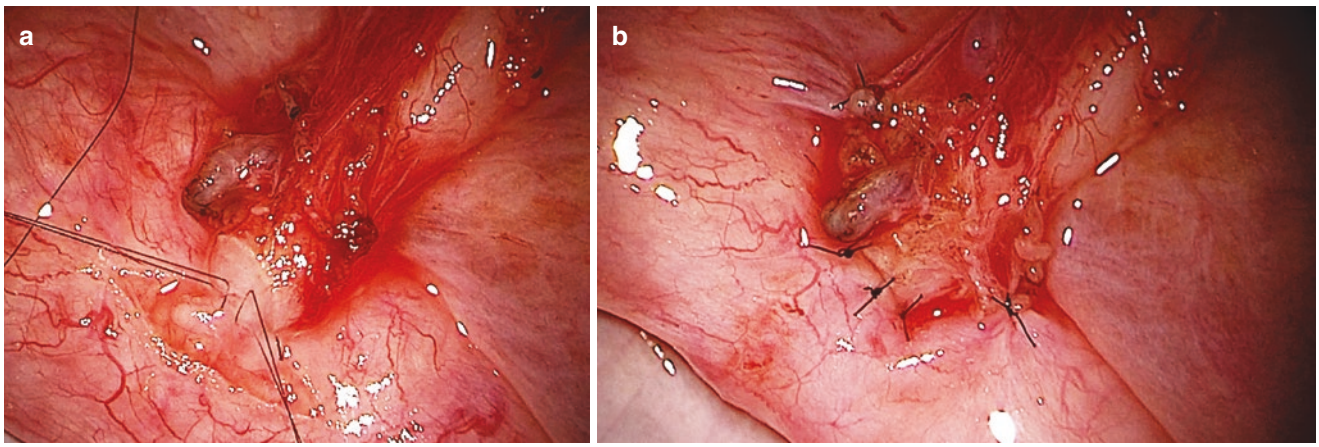

Fig. 13.4 (**a**) Two needles of each suture pulled together and tied to each other, thus intussuscepting the tubule into the vas lumen. (**b**) Final surgical aspect of vaso-epididymal anastomosis. Sutures are placed in the anterior layer of vas and epididymal tunic with 8-0 to secure the anastomosis

Table 13.3 Modifications to the vasoepididymostomy technique

1.Vas fixed to epididymal tunic prior to tubular sutures
Advantage: Limited manipulations required once the mucosal sutures are in place
Disadvantage: Vas sutures need to be taken down if there are no sperms in the epididymal fluid
2.Mucosal sutures placed sequentially, not simultaneously
Difficult to hold both 200-μm needles together in the micro-needle holder
3.Use of 200-μm needles on 10-0 suture instead of 70-μm needles
Cost and availability
4.Incision of tubule with needles in situ
Avoids inadvertent division of the suture material

13.6 Outcomes

Despite our stringent inclusion criteria for a VEA, we are able to perform an anastomosis in only about 60% of all explorations. Even among men where we are able to find sperms within the epididymis, in some, the tubules are extremely thin with minimal dilatation. In such cases, particularly where the sperms are found only in tubules in the head, a single tubule mucosal anastomosis is not feasible, and a non-mucosal anastomosis between the vas lumen and epididymal tunic with an incision in the tubules needs to be performed.

Among cases where a single tubule anastomosis is feasible, our success rates, on average, have been 50% in the form of a patent anastomosis, documented by return of sperms in the ejaculate. These rates have been higher at around 80% when the surgery has been performed bilaterally using the longitudinal suture placement technique and in men with motile sperms in the epididymal fluid. Further, technical satisfaction with the procedure was associated with higher patency rates [28].

Yoon et al. in recent meta-analysis of vasoepididymostomy reported that the mean patency rate was 64.1% and the mean pregnancy rate was 31.1% [29]. There are a number of reasons why our outcomes differ from those reported by other centers (Table 13.4) [30, 31]. The most important among these is an unclear etiology of obstruction. Most of our patients have primary infertility, and the diagnosis of obstruction at the vaso-epididymal junction is one of exclusion. Another potential reason is the use of thicker needles on the sutures. The 200-μm needles that we use are five times less expensive than the standard 70-μm needles, and this difference is often a major concern for our patients. Our attempts at preserving one side and operating unilaterally may also be causative since our outcomes among bilateral

Table 13.4 Operative results of vasoepididymostomy

Author	Year	Patients (n)	Anastomotic technique	Patency rates (%)	Pregnancy rates (%)
Martin and Hagner [4]	1902	–	Fistula technique	64	27
Silber [6]	1978	14	E-E (end to end)	86	–
Thomas [8]	1987	50	E-S (end to side)	66	49
Berger [9]	1998	12	Triangulation	92	–
Marmar [10]	2000	19	TIVE	77.7	–
Chan [11]	2005	68	Triangulation	84	42
Schiff [30]	2005	66	EE	49	20
		32	ES	37	40
		38	TIVE	68	46
		17 (total 153)	LIVE	67	44
Kumar [27]	2006	29	TIVE	48	–
Kumar [28]	2010	24	LIVE	48	–
Peng [31]	2014	73	LIVE	72	33

procedures have been much better. Finally, short follow-up of patients is a problem that plagues all our procedures. It is well known that patency may become apparent many months after a procedure, but most of our patients provide only one or at most two semen samples after the surgery. This may also be related to the previously discussed issues of social pressure and need for early outcomes. A number of these men possibly opt for assisted reproduction soon after the surgery, not willing to wait for a successful surgery [32].

13.7 Vasovasal Anastomosis

Vasectomy is an uncommon form of contraception in India, accounting for fewer than 5% of all contraceptive methods. A decision to undergo a vasectomy is usually taken after careful thought and requests for reversal are rare. The most frequent reason for seeking a reversal is the loss of a child [33]. This statistic has two important implications. First, the stress on success is likely to be greater in this group of patients than in those who already have living children, and second, most patients are interested in early patency during which they may father a child without significant concern about delayed closures. The low volumes of procedures also mean that there is inadequate training of surgeons. These factors have been instrumental in our attempts at simplifying the surgical technique of vasectomy reversal [34].

13.8 Our Surgical Technique

A semen analysis is obtained in all men seeking vasectomy reversal to confirm azoospermia. No additional investigations are requested if the testicular size is normal and both vas are palpable. A longitudinal incision is placed in the scrotal skin, as for the VEA procedure. The testis and the spermatic cord are delivered out of the scrotum, and the site of vasectomy is identified. Babcock forceps are used to hold the vas deferens above and below the site of vasectomy, and a short segment of the vas is stripped of its adventitial tissue. The distal vas is sharply divided and flushed with saline to test for patency, similar to the procedure previously described for a vasoepididymostomy. The proximal end of the vas is then divided and the fluid examined for sperms. In the absence of any fluid, a gentle barbotage of the proximal vas is performed with saline and this fluid is then examined. If no fluid or sperms are demonstrable, the epididymis is examined to look for a secondary block and a vasoepididymostomy is considered.

The adventitial tissue of the two ends of the vas is held with the tip of small hemostats to appose them to each other. Two 8-0 polyamide sutures are placed at the 5 and 7 o'clock positions in the seromuscular layers of the two ends and tied. The suture ends are left long and held in rubber shod hemostat clamps. The hemostats applied to the adventitia are removed. A double-armed 10-0 polyamide suture is placed, inside out at 6 o'clock in the mucosa of the distal vas, and the second needle is placed at the corresponding position of the proximal vas. The suture is tied. Three additional 10-0 polyamide sutures are placed at the 3, 9, and 12 o'clock positions in the mucosa of both ends of the vas. These three sutures are tied sequentially once all have been placed [35]. Two additional 8-0 polyamide sutures are placed in the seromuscular layer (Fig. 13.5a, b). Additional sutures may be placed in the seromuscular layer or the adventitia to stabilize the anastomosis. The postoperative advice and follow-up are as described for the vasoepididymostomy procedure.

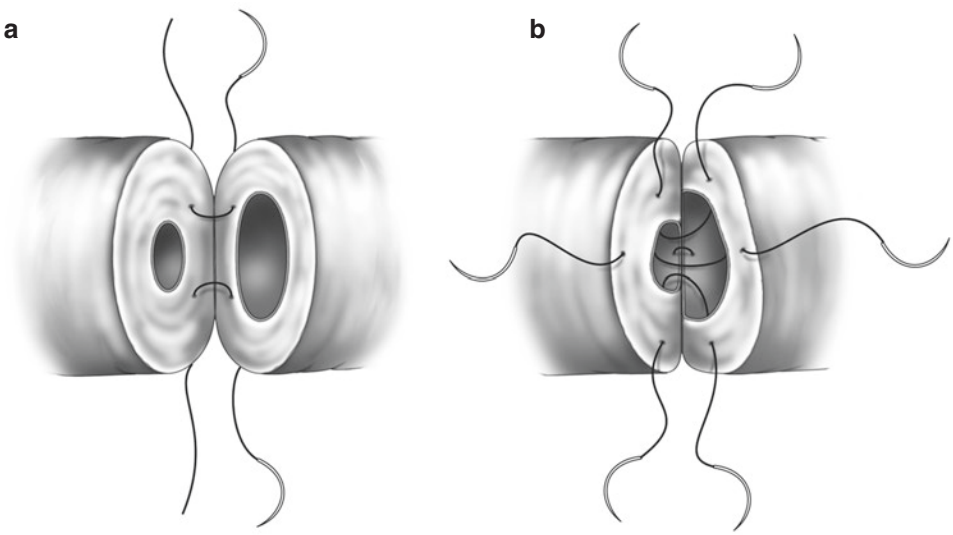

Fig. 13.5 (**a**, **b**) Suture placement technique for a 4 × 4 vasovasostomy. Reproduced with permission by Kumar R [26]

13.9 Outcomes

Vasectomy reversals have traditionally been a very satisfying procedure with excellent patency rates, usually above 90% [16]. The operative results of vasovasostomy are described in Table 13.5 [36–43]. Namekawa et al. in a recent review reported the overall patency rate of 87% and overall mean pregnancy rate of 49% following microscopic vasovasostomy and vasoepididymostomy for vasectomy reversal [44]. Our own practice of vasectomy reversals has been sporadic with fewer than 8–10 cases per year. The results were felt to be good, but a detailed record is not available. Since 2008, we began using the 4 × 4 technique described above exclusively following a study protocol. We have found this technique simple to perform with excellent outcomes. All eight patients described in our recent report have a patent anastomosis. Cases performed after the last accrual in this report are also patent. One of the potential problems with this technique is the possibility of sperm leakage and delayed closure. While this is certainly a theoretical possibility, as we stated earlier, most of our patients are keen on an immediate patency with little interest in delayed results. This procedure is simple and may be performed by a greater number of surgeons than the more difficult microdot technique. We feel the simplicity of the procedure with excellent early results, but unknown long-term outcomes may be an acceptable trade-off with the more difficult techniques that have proven long-term outcomes.

13.10 Microsurgical Varicocelectomy

We maintain a high threshold for case selection for varicocelectomy. Only men with clinically palpable varicoceles are accepted. This policy began even before the AUA and ASRM guidelines were published but were reinforced by these guidelines [45]. Surgery is performed for the side where the varicocele is palpable, and a Doppler of the scrotum is requested only where a clinical examination is doubtful.

13.11 Our Surgical Technique

The external inguinal ring is identified by insinuating a finger through the scrotal skin into the inguinal region. A 2-cm transverse incision is made in the skin overlying the external ring. Under magnification, this incision is deepened to expose the spermatic cord. The tissue around the cord and over the external ring is separated, and a small incision is made in the external ring to make it wider. This incision is directed along the inguinal canal. The cord is held with Babcock forceps and, under the microscope, is freed from the bed and surrounding tissues. The cord is then brought out onto the surface. The fossa is examined for any visibly dilated veins that are ligated and divided. The cord is held on the surface over a hemostat. The superficial layers of the spermatic fascia are divided longitudinally, closer to the cranial end of the cord. The artery is identified and isolated. All visible veins are individually identified and ligated and divided. The cord is then taken onto the surgeon's nondominant hand and scanned inside and outside the spermatic fascia to confirm that all major veins have been divided. We do not deliver the testis into the wound or ligate the gubernacular vessels.

13.12 Outcomes

We have previously reported our outcomes for subinguinal varicocelectomy [46]. Briefly, one-third of patients are able to father a child through natural conception after the surgery, and a majority will show improvements in seminal parameters. The procedure has resulted in downgrading the ART techniques required in a number of patients who have undergone this procedure [47].

13.13 Training and Credentials

Andrology and microsurgery procedures are performed as a part of the standard urological services available to all patients. Urology residents, during their 3 years of training,

Table 13.5 Operative results of vasovasostomy

Author	Year	Patients (n)	Anastomotic technique	Patency rates (%)	Pregnancy rates (%)
Bolduc [36]	2007	747	Single layer	86	33
Patel [37]	2008	106	Single/two	98	–
Kumar [35]	2010	8	Two layers	99	–
Schwarzer [38]	2012	1303	Multilayer	89	59
Li [39]	2013	34	Two layers	94	68
Mui [40]	2014	1229	Single/two	84	–
Chen [41]	2015	62	Two layers	56.5	25.8
Nyame [42]	2016	20	Single layer	93.3	–
		86(Total106)	Two layers	89.3	–
Wang [43]	2018	56	Two layers	87.5	42.5

are posted to assist in these surgical procedures. There are no fellowship programs in male infertility or andrology, and few would return for dedicated specialized training in microsurgery. This results in very little postresidency acquisition of skills or even skills maintenance because the number of procedures performed in urologic practice in the community is very limited.

13.14 Conclusions

The diseases responsible for male infertility and for cases undergoing exploration for microsurgical reconstruction at our center possibly differ from those reported in most Western literature. The largest majority of cases explored are for primary infertility with obstruction of unknown etiology. This has resulted in lower percentage of patients with a successful outcome. However, the socioeconomic factors around infertility management in our country dictate an attempt at reconstruction even when the expected outcomes are poor. In patients with favorable prognostic factors, the outcomes are generally good.

13.15 Review Criteria

An extensive search of studies on "microsurgery for male infertility" was performed using search engines such as PubMed, Google Scholar, ScienceDirect, and Ovid. The overall strategy for data extraction was based on the following keywords: "microsurgery for male infertility," "azoospermia," "varicocele," "vaso-epididymal anastomosis," "microsurgical-varicocelectomy," "vasovasal anastomosis," and "congenital bilateral absence of the vas deferens." Articles published only in English language were considered. The previous version of this chapter was revised.

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Advanced Techniques of Vasoepididymostomy

14

Wayland Hsiao and Marc Goldstein

Key Points

- Good anastomoses rely on healthy tissue and an accurate watertight mucosa-to-mucosa opposition in a tension-free anastomosis; setup is key.
- Anastomotic techniques include end-to-end, end-to-side, and end-to-side intussusception.
- Vasal length can be increased on the epididymal end or the vasal end or both. If these two maneuvers prove insufficient, orchiopexy should be considered as well as a crossed septal vasoepididymostomy in cases of unilateral testicular atrophy or absence.
- Our preferred anastomosis is the two-suture longitudinal, end-to-side intussuscepted technique.
- We have developed a single-arm version of vasoepididymostomy, which is useful when double-arm sutures are difficult to obtain.
- Vasoepididymostomy is the most challenging of all microsurgery and should only be performed by surgeons with sufficient training and adequate volume of microsurgery.

14.1 Introduction

The first vasoepididymostomy (VE) was reported in 1902 by Dr. Edward Martin at the University of Pennsylvania. His technique involved slashing across multiple epididymal tubules and anastomosis of the vas to the epididymal tunic in a side-to-side manner with four fine silver wires [1, 2]. Patency depended on the formation of a fistula. In 1909, Martin reported in a series of 11 patients with epididymal obstruction a patency rate of 64% and a pregnancy rate of 27% [3]. He proved that vasoepididymostomy was technically feasible and his approach is the foundation on which subsequent work was based.

With advances in surgical technique and the development of microsurgical techniques, modern vasoepididymostomy allows us to accurately approximate the mucosa of a single epididymal tubule to the mucosa of the vasal lumen [4]. With this increased precision, we have been able to achieve even higher patency and pregnancy rates [5, 6]. Microsurgical vasoepididymostomy, however, remains the most technically demanding procedure in all of microsurgery. In virtually no other operation are results so dependent upon technical perfection. Thus, microsurgical vasoepididymostomy should only be attempted by an experienced microsurgeon who performs a sufficient volume of microsurgery.

14.2 Vasoepididymostomy

Vasoepididymostomy is indicated in patients with obstructive azoospermia, and the decision to perform a vasoepididymostomy rather than a vasovasostomy is made intraoperatively. During vasectomy reversal, the testicular end of the vas is cut until a patent lumen is seen and the intravasal fluid is evaluated both grossly and with the aid of a 400× bench microscope. The presence of thick toothpaste-like fluid devoid of sperm, scant fluid in a patient without a sperm granuloma, or scant fluid with no spermatozoa seen on barbotage constitutes indications for vasoepididymostomy. For non-vasectomy-related obstruction, vasoepididymostomy is indicated when a testis biopsy reveals complete spermatogenesis and transection of the testicular end of the vas reveals no sperm even with barbotage.

The evolution of modern single tubule vasoepididymostomy techniques has progressed from the original end-to-end anastomoses described by Silber, end-to-side anastomoses

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described by Wagenknecht and Fogdestam, and end-to-side intussusception anastomoses first described by Berger. In all of these, the initial exposure and setup are similar. A high vertical scrotal incision is made about 3–4 cm in length aimed toward the external ring of the inguinal canal. In cases with inadequate length of the vas, the incision can be extended to the external ring if necessary and the external oblique aponeurosis can be incised and an inguinal dissection of the vas performed. After incision through the skin and dartos fascia, the testicle is delivered with the tunica vaginalis intact. Using a Babcock clamp, the vas is isolated and surrounded with a Penrose drain. The operating microscope is brought into the field. The junction of the straight and convoluted vas is identified and isolated. The vas is then dissected free of its investing sheath and blood vessels under the operating microscope to expose a clean segment of bare vas. The bare segment of vas is hemitransected with a 15° ultrasharp knife until the lumen is visualized. The vasal fluid is then sampled, placed on a slide mixed with media, covered with a coverslip, and examined using a bench microscope at 400× magnification. If no spermatozoa are seen, then an additional 0.1–0.2 ml of fluid is injected into the testicular end and that fluid is expressed back out by squeezing the testis and epididymis and the fluid examined under the bench microscope. Absence of vasal sperm on microscopic exam in a man with either a normal testis biopsy or a positive antisperm antibody assay [7] confirms the diagnosis of epididymal obstruction.

At this point, the abdominal end of the vas is checked for patency by cannulating the abdominal end of the vas with a 24-gauge angiocatheter and injecting 1 ml of lactated ringers. Smooth injection without resistance or backflow confirms patency of the abdominal end of the vas. If further confirmation is desired, then a Foley catheter can be inserted after injecting indigo carmine and the color of the urine inspected. Green or blue urine confirms the patency of the abdominal vas as well as the ejaculatory ducts.

Once epididymal obstruction is confirmed and the need for a vasoepididymostomy verified, the abdominal end of the vas is prepared for anastomosis by completing the transection using an ultrasharp knife drawn through a slotted 2, 2.5, or 3 mm nerve-holding clamp. The vas is cut until healthy vasal tissue is seen. The cut surface of the testicular end of the vas deferens is inspected using 15–25 power magnification and should look like a bullseye with the three vasal layers distinctly visible. A healthy white mucosal ring should be seen which springs back immediately after gentle dilation. This layer is surrounded by muscularis which should appear smooth and homogeneous. A gritty-looking muscularis layer may indicate the presence of scar/fibrosis. Healthy bleeding should be noted from both the cut edge of the mucosa and the surface of the muscularis. If the blood supply is poor or the muscularis is gritty, the vas is recut until healthy tissue is found. The vasal artery and vein are ligated with 6-0 Vicryl. Small bleeders are controlled with a microbipolar forceps set at low power. At this point, the tunica vaginalis is opened and the epididymis is inspected.

In patients with previous vasectomy, there are some minor variations, but the overall approach is similar. In these patients, the testicular and abdominal ends of the vas are identified and dissected free. The abdominal end is transected and checked for patency. After confirmation of abdominal end patency, the testicular end is then inspected and sectioned and intravasal fluid microscopically inspected. If examination of the fluid reveals no spermatozoa, even with barbotage, the need for vasoepididymostomy is confirmed. The tunica vaginalis is opened and the epididymis is inspected under the operating microscope. At this point, it is time to determine the site of anastomosis.

14.3 End-to-End Anastomosis

This is the original microsurgical technique introduced by Silber and it is the first technique to allow the anastomosis of a specific epididymal tubule to the vas. At its introduction, it was far superior to any method previously described. In this technique, the epididymis is dissected down to its junction with the convoluted vas. The epididymis is then serially sectioned until a large rush of fluid is noted (Fig. 14.1), indicating that the area of obstruction has been bypassed. The single tubule with gushing fluid is identified and anastomosed to

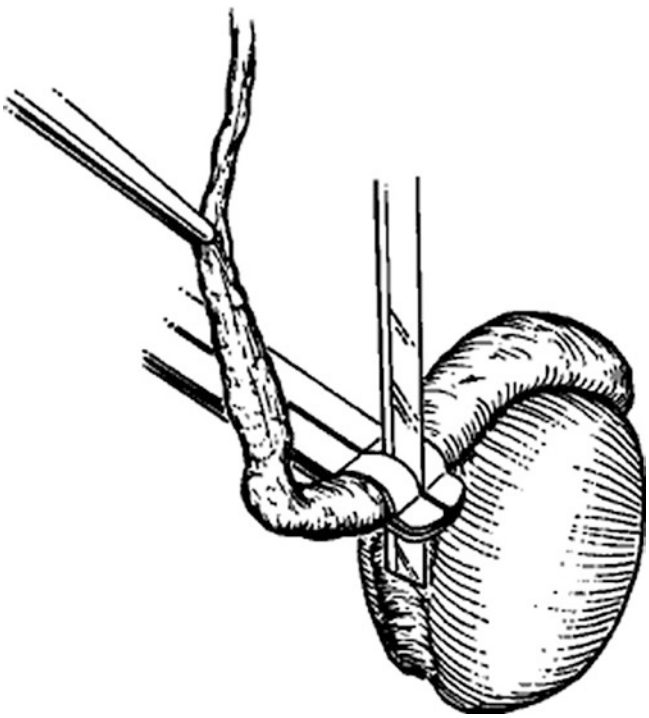

Fig. 14.1 Sectioning method employed in the end-to-end technique. (Reprinted from Goldstein [25], with permission of Elsevier)

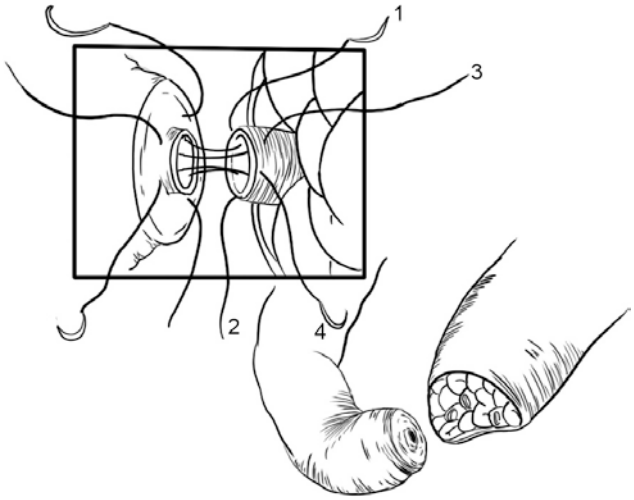

Fig. 14.2 End-to-end anastomosis showing anastomosis of a single epididymal tubule to the vasal lumen. Note that the outer vasal layers are then anastomosed to the tunica of the epididymis. (Reprinted from Goldstein [26]. With permission from Elsevier)

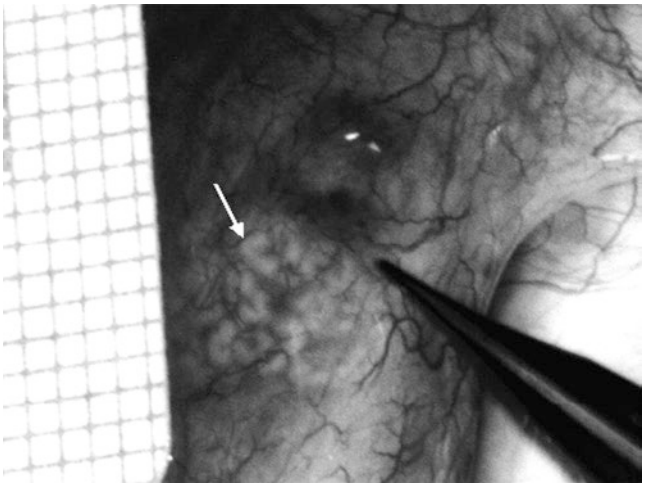

Fig. 14.3 Example of dilated epididymal tubules seen under the operating microscope

the vas with 3–5 interrupted 10-0 nylon sutures. The outer layer of the vas is anastomosed to the tunica of the epididymis with 9-0 nylon sutures (Fig. 14.2).

The advantages of this technique include the ability to dissect off the epididymis and rotate it to gain additional length if there are issues with short vasal length. A major disadvantage of this technique is that the outer diameter of the epididymal tunica is far larger than the outer diameter of the vas deferens, making a watertight closure exceedingly difficult. Also, the epididymal blood supply is invariably affected during transection. It also is more difficult to obtain clean, blood-free sperm for cryopreservation than it is with the end-to-side technique.

14.4 End-to-Side Techniques

End-to-side techniques of vasoepididymostomy improved on the end-to-end technique and have the advantage of being relatively bloodless and less traumatic to the delicate epididymis [8–11]. It requires minimal dissection of the tubule and allows the surgeon to easily tailor the size of the opening in the epididymal tubule. Also, this method allows for the preservation of all the epididymal branches of the testicular artery. Thereby, if another vasovasostomy is required, the blood supply to the intervening segment of vas can be preserved. In cases where the integrity of the testicular artery is in doubt (previous orchiopexy, previous non-microscopic varicocelectomy, or hernia repair), preservation of the deferential artery may be required for the maintenance of testicular blood supply.

The selection of an anastomotic site is a bit more involved with the end-to-side technique when compared with the end-to-end technique. After the vas has been prepared, the tunica vaginalis is opened and the testis delivered. Inspection of the epididymis under the operating microscope may reveal a clearly delineated demarcation above which epididymal tubules are markedly dilated and below which the tubules are collapsed. Often, a discrete yellow sperm granuloma is noted, above which the epididymis is indurated and the tubules dilated and below which the epididymis is soft and the tubules collapsed (Fig. 14.3). If the level of obstruction is not clearly delineated, a 70-μm tapered needle from the 10-0 nylon microsuture is used to puncture the epididymal tubule beginning as distal as possible and fluid sampled from the puncture site until sperm are found. At that level, the puncture is sealed with microbipolar forceps and the anastomosis is performed proximal to the puncture site.

An anastomotic site is selected where the epididymal tubules are clearly dilated. An avascular area is grasped with jeweler's forceps and the epididymal tunica tented upward. A 3–4-mm buttonhole is made in the tunica with microscissors to match the outer diameter of the vas. The epididymal tubules are then gently dissected until dilated loops of tubules are clearly exposed.

At this point an opening is made in the tunica vaginalis and the vas deferens end is brought through and secured to the tunic with two to three interrupted 6-0 prolene sutures to ensure that the vasal lumen reaches the opening in the epididymal tunica without tension and with some length to spare. The posterior edge of the epididymal tunica is then approximated to the posterior edge of the vas muscularis and adventitia with two to three interrupted sutures of double-armed 9-0 nylon. At the end of this step, the vasal lumen should be in close approximation to the epididymal tubule selected as the site for anastomosis. Proper positioning of the vasal segment and

proper setup are critical to the creation of a long-lasting tension-free anastomosis.

14.5 Anastomotic Technique

Once setup for the anastomosis is complete, the surgeon has a choice of anastomotic techniques which vary by the number of sutures placed, the order of suture placement, and intussusception of the tubule. We will discuss the classic end-to-side anastomosis as well as the various intussusception techniques.

14.6 Original End-to-Side

The classic end-to-side approach involves creation of a longitudinal incision along the selected epididymal tubule. This is done under 25–32× magnification. The intratubular fluid is microscopically inspected with the bench microscope. If no sperm are seen on microscopic exam, then the tubule is closed with a 10-0 suture and the overlying tunica closed with 9-0 nylon. A more proximal location is then identified and the setup for anastomosis is repeated. If sperm are found on microscopic inspection, it is safe to continue with the procedure. The extruded epididymal fluid is aspirated into glass capillary tubes and flushed into media for cryopreservation [12]. Diluted indigo carmine is applied to the field to highlight the edges of the epididymal tubule as well as the mucosal edges of the vas segment. Of note, we have previously shown that methylene blue and radiographic contrast are toxic to spermatozoa, while diluted indigo carmine is not [13]. Thus, it is our preference to use indigo carmine diluted 50% with lactate ringers for all vasograms and for emphasis of the mucosal edges.

Constant irrigation with saline or lactated ringers is required to keep the delicate epididymal tubule open and to visualize the edges. The posterior mucosal edge of the cut epididymal tubule is approximated to the posterior edge of the vasal mucosa with two interrupted sutures of 10-0 nylon double-arm sutures with 70-μm diameter tapered needles. After these mucosal sutures are tied, the anterior mucosal anastomosis is completed with two to four additional 10-0 interrupted sutures. The outer muscularis and adventitia of the vas are then approximated to the cut edge of the epididymal tunica with six to ten additional interrupted sutures of 9-0 nylon double armed with 100-μm diameter needles. The vasal sheath is secured to the epididymal tunica with three to five sutures of 9-0 nylon allowing for a straight course without kinks. The tunica vaginalis is then closed with 5-0 Vicryl and the dartos reapproximated with absorbable suture. The skin is closed in a subcuticular fashion.

14.7 End-to-Side Intussusception Technique

The next advance in vasoepididymostomy techniques came with the development of intussusception techniques. This method was first introduced by Berger in 1998 [14]. The setup is identical to that for the classic procedure. After the vas is fixed to the opening in the epididymal tunica, six microdots are placed on the cut surface of the vas to mark the sites of needle exit. The microdot technique ensures precise suture placement by exact mapping of each planned suture. The microdot method separates the planning from the placement of sutures [15]. Much as a civil engineer is consulted before workmen commence construction on a bridge, the microdot method allows the surgeon to completely focus on each individual task at hand. This results in substantially improved accuracy in suture placement as well as better suture spacing. Next, the epididymal tubule selected for anastomosis is dissected until it is free of surrounding tissue and displays prominently. Indigo carmine is applied to highlight the tubule. Using double-arm 10-0 nylon sutures with 70-μm tapered needles, three sutures are placed in the epididymal tubule in a triangular configuration. The needles are left in situ, creating a triangle of needles (Fig. 14.4). It must be remembered that the needle of the 10-0 suture is 70 μm in diameter while the suture material itself is only 17 μm. Thus, if the needles are pulled through prematurely, epididymal fluid and sperm would immediately leak through the suture hole causing the tubules to collapse, making placement of subsequent sutures and opening the tubules more difficult. Leaving the needles in situ also prevents accidentally cutting sutures when making the opening in the epididymal tubule.

After all three needles are properly placed, Berger originally described using a 9-0 cutting needle to lift the tubule and tear an opening in it. We prefer a 15° microknife to make an opening in the epididymal tubule in the center of the triangle. The three needles are then pulled through. The six needles are now

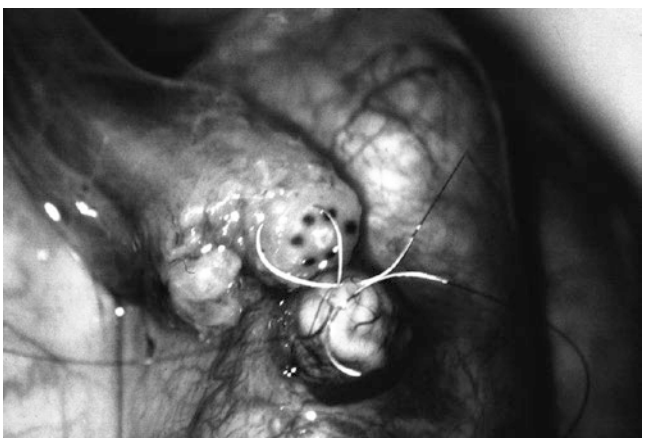

Fig. 14.4 Triangle of needles formed during the triangulation end-to-side intussusception technique introduced by Berger

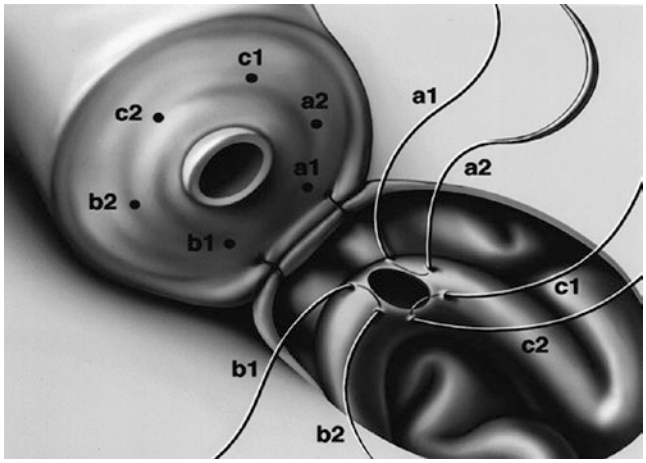

Fig. 14.5 End-to-side triangulation intussusception technique introduced by Berger. (Reprinted from Goldstein [25], with permission of Elsevier)

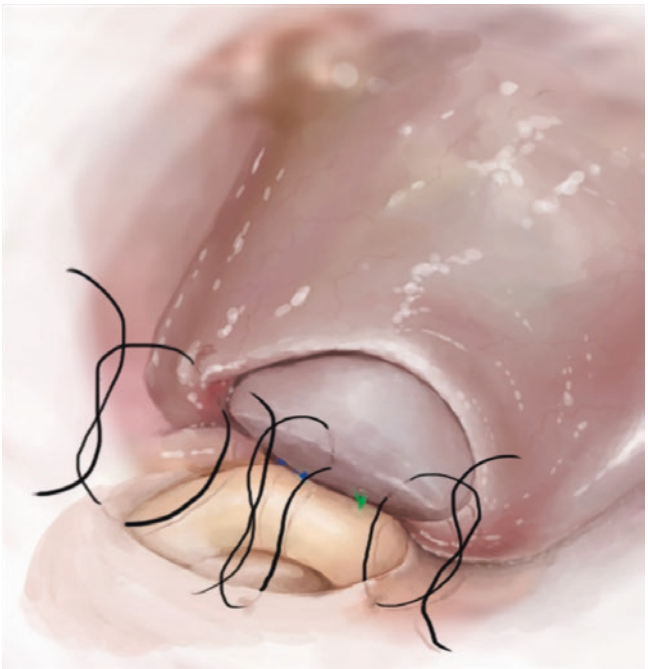

Fig. 14.6 Closure of the epididymal tunica should be done with 9-0 nylon sutures with particular attention paid to avoid incorporating any underlying tubules into the closure. (Reprinted from Goldstein [26]. With permission from Elsevier)

laid out to avoid tangling. The extruded fluid is inspected under the microscope for sperm. If sperm are seen, then the six needles are passed inside out the vas deferens exiting through the six previously placed microdots (Fig. 14.5). The sutures are then tied, intussuscepting the epididymal tubule into the vas lumen and thereby creating a watertight closure. Intussusception also allows the flow of fluid from the epididymal tubule into the vas to push the edges of the epididymal tubule against the vasal mucosa, further reinforcing the watertight nature of this anastomosis. The edges of the vas are then closed with interrupted 9-0 nylon sutures (Fig. 14.6). Limitations of the triangulation technique include the need for a relatively large tubule for the three needles to fit. Thus, this technique is not suitable for anastomosis to the efferent ductules or the proximal caput epididymis where the tubule is smaller.

14.8 Two-Stitch Longitudinal Vasoepididymostomy (LIVE Technique)

This is our currently preferred method of VE which allows for a two-stitch intussuscepted anastomosis. In this technique, four microdots are made on the vasal end. Two needles from two separate 10-0 double-arm sutures are then placed longitudinally in the tubule with care not to pull the needles completely through. The opening in the epididymal tubule is then made with a 15° microknife between the needles. After microscopic confirmation of the presence of spermatozoa, the needles are passed. The four needles are then passed through the vasal lumen and exiting the microdots (inside to outside). A 9-0 suture is placed to pull the anterior vas and adventitia toward the opening in the epididymal tubule bringing the vas mucosa into close approximation to the opening in the epididymal tubule. The lumen is irrigated with heparinized saline just prior to tying the mucosal sutures. Finally, the mucosal sutures are tied down (Fig. 14.7), allowing for the intussusception of the epididymal tubule. The outer layer is closed with interrupted 9-0 nylon sutures careful to not inadvertently incorporate any epididymal tubules when placing these sutures (Fig. 14.8). Again, by not pulling the needles completely through the tubule until the tubule has been incised, the tubule remains distended, which makes suture placement and incision of the tubule more accurate and reliable. Variations in this technique include

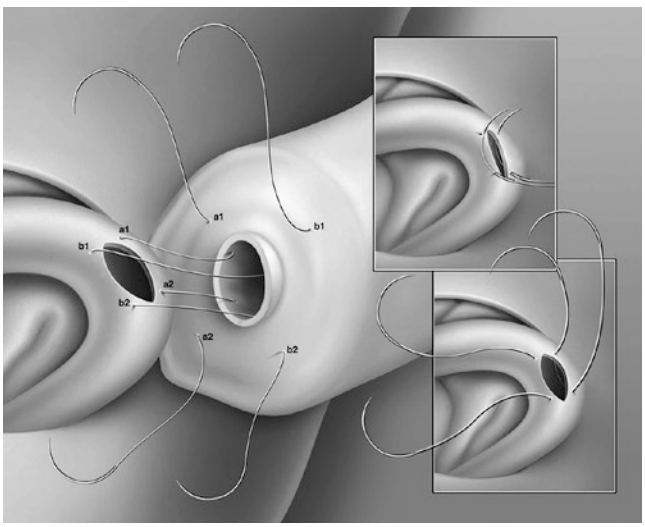

Fig. 14.7 Longitudinal intussuscepted vasoepididymostomy technique. Mucosal suture placement. (Reprinted from Goldstein [26]. With permission from Elsevier)

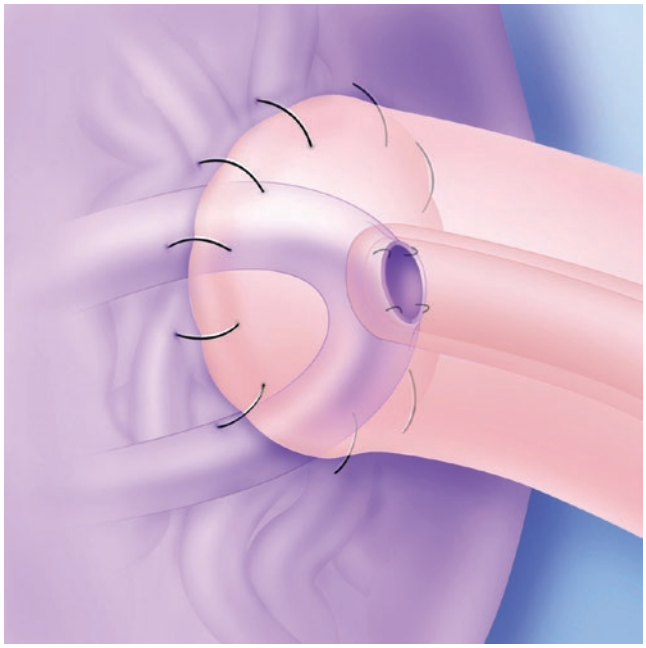

Fig. 14.8 Completed anastomosis for longitudinal intussuscepted technique. (Reprinted from Goldstein [26]. With permission from Elsevier)

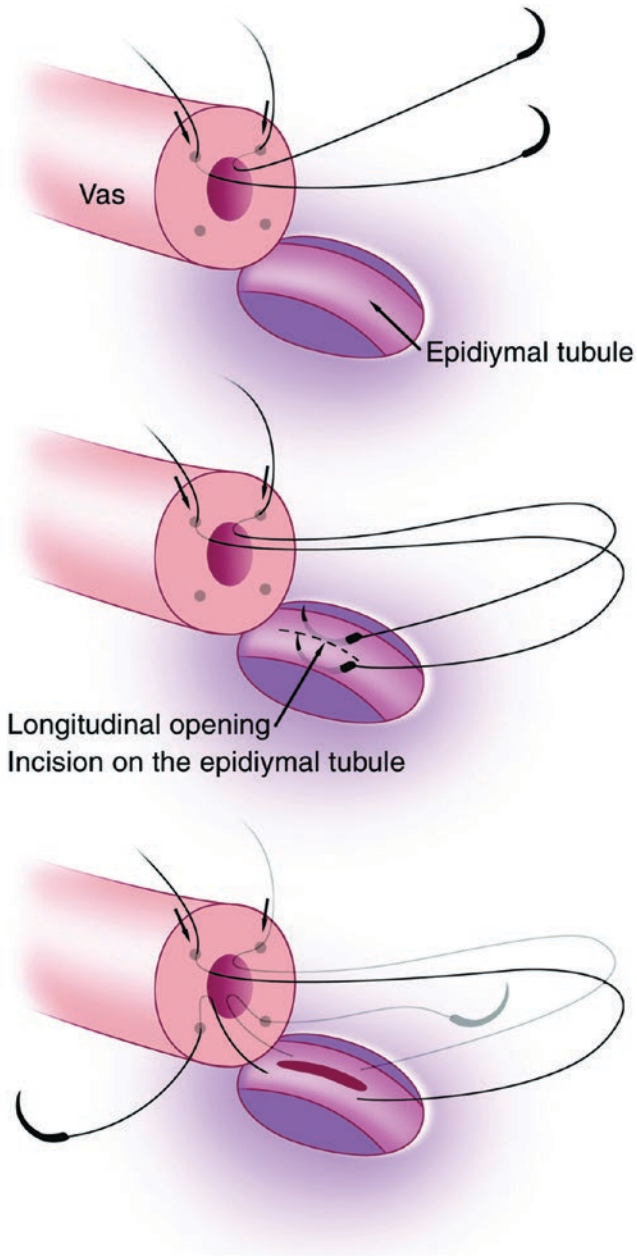

Fig. 14.9 Technique of single-arm vasoepididymostomy technique. Needles are passed outside in on the vas deferens. The needles are then passed longitudinally in the selected epididymal tubule and the cut made in the epididymal tubule. The needles are then passed and then placed inside out in the vas deferens. (Reprinted from Goldstein [26]. With permission from Elsevier)

mounting two needles in a single needle holder and placing them simultaneously transversely in the tubule as suggested by Marmar.

Of note, the cost of double-arm sutures can be high. In response to this, we have developed a single-arm technique of VE which we have found to be almost as effective as its double-arm counterpart [16]. It begins with the standard setup for VE. We then place four microdots in the vasal end. Two 10-0 single-arm nylon sutures are then passed through the microdots and exiting the vasal lumen (outside to inside). After this, the same two sutures are placed longitudinally in the selected tubule and the needles are not completely passed. After opening the tubule and confirming the presence of spermatozoa, the needles are pulled through and the needles passed through the vasal lumen and exiting the microdot (inside to outside) (Fig. 14.9). The sutures are then tied allowing the intussusception of the epididymal tubule. The outer sheath of the vas deferens is then approximated to the tunic of the epididymis with two to four interrupted 9-0 nylon sutures, removing all tension from the anastomosis.

14.9 Techniques When Vasal Length Is Severely Compromised

One of the most common problems that arise during vasoepididymostomy is inadequate vasal length, often due to a very destructive vasectomy. When there is inadequate length of the vas deferens to reach the dilated epididymal tubule without tension, a number of surgical techniques may be employed involving any of the following: freeing up the epididymis, increasing vasal length, fixation of the testis higher in the scrotum, or use of the contralateral vas deferens.

To gain length on the epididymis, the cauda and corpus epididymis can be dissected down to the vasoepidid-

ymal junction and then dissected off the testes as in the end-to-end operation. The epididymis is encircled with a small Penrose drain at the level of obstruction and dissected off of the testis up to the level of obstruction, yielding sufficient length to perform the anastomosis. Usually an avascular plane can be found between the tunica albuginea of the testis and the epididymis, and injury to the epididymal blood supply can be avoided. The inferior and, if necessary, middle epididymal branches of the testicular artery are ligated and divided to free up an adequate length of epididymis. The superior-epididymal branches entering the epididymis at the caput are always preserved, and this is adequate blood supply for the entire epididymis.

If the epididymis is indurated and dilated throughout its length, the epididymis is dissected all the way past the vasoepididymal junction. This dissection is often facilitated by first dissecting the convoluted vas to the vasoepididymal junction from below, and then, after encircling the epididymis with a Penrose drain, dissecting the epididymis to the vasoepididymal junction from above. In this way, the entire vasoepididymal junction can be freed up. This will allow preservation of maximal epididymal length in cases of obstruction near the vasoepididymal junction. After the epididymis is dissected off of the testis and flipped-up, a two-stitch longitudinal end-to-side intussusception anastomosis can be performed as described previously (Fig. 14.10).

Increasing vasal length can be done with extensive blunt dissection of the vas deferens off the spermatic cord toward the inguinal ring. If necessary, the external oblique aponeurosis is incised toward the internal inguinal ring and dissected with a finger sweeping motion. In extreme situations, the vas deferens can be rerouted medial to the vessels similar to the Prentiss maneuver employed during difficult orchiopexies [17]. An opening in the floor of the inguinal canal is made and the vas rerouted medially under the floor of the canal and right over the pubis.

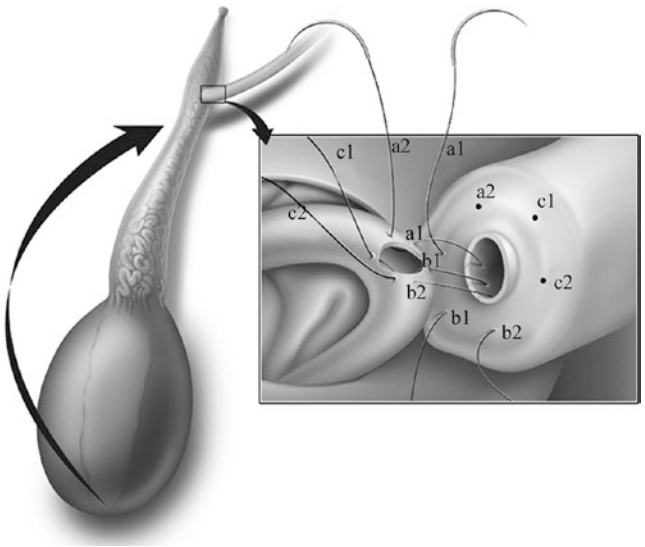

Fig. 14.10 Technique of dissecting the corpus and cauda epididymis to gain further length in cases of short vasal length. This is most helpful when the entire epididymis is dilated. (Reprinted from Goldstein [26]. With permission from Elsevier)

It is also possible to perform an orchiopexy positioning the testicle in a horizontal or even upside-down configuration to decrease the length needed. One must be careful to make sure the cord has no kinks in it and that the stitches do not damage the blood supply to the testis.

In cases where there is a unilateral atrophic testis or the contralateral testis is missing, it is possible to perform a crossed transseptal vasoepididymostomy. This is even more attractive if there is an ipsilateral hernia repair or where there is a second obstruction in the inguinal or abdominal vas. In this procedure, the contralateral vas is harvested as close to the vasoepididymal junction as possible. If vasal length is still inadequate, then the testicle can be pexed in the contralateral scrotal compartment to facilitate a tension-free anastomosis.

14.10 Long-Term Follow-Up Evaluation and Results

Microsurgical vasoepididymostomy in the hands of experienced skilled microsurgeon will result in the appearance of sperm in the ejaculate in 50–85% of men. Classic end-to-side or older end-to-end methods result in patency rates about 70% with a 43% pregnancy rate with a follow-up of 2 years [5, 18]. With intussusception techniques, patency rates are 70–90% with pregnancy rates of 40–45% [6, 14, 19–22]. Regardless of technique, pregnancy rates are higher the more distal the anastomosis is performed [23]. Therefore, one should always strive to make the anastomosis as distal as possible on the epididymis. The advantages and disadvantages of the main techniques we discussed are summarized (Table 14.1).

Another vexing problem is that of late anastomotic failure. With the older end-to-end or end-to-side methods, at 14 months after surgery, 25% of initially patent anastomoses have shut down [12]. With intussusception techniques, the late shutdown rates appear to be less than 10%, but long-term follow-up with these techniques has not yet been reported. Nevertheless, we recommend banking sperm both intraoperatively [24] and as soon as motile sperm appear in the ejaculate postoperatively after vasoepididymostomy, regardless of technique employed. In men with very low counts or poor sperm quality postoperatively and men who remain azoospermic, the sperm intraoperatively cryopreserved can

Table 14.1 Advantages and disadvantages of three main vasoepididymostomy techniques

Techniques	Advantages	Disadvantages
Intussusception (longitudinal intussusception vasoepididymostomy)	Virtually bloodless anastomosis Easier technique with dilated epididymal tubule	Unable to assess for sperm presence before anastomotic setup
End-to-side vasoepididymostomy	Able to assess for sperm presence prior to anastomotic setup No disruption of epididymal blood supply	Difficult to suture to collapsed epididymal tubule
End-to-end vasoepididymostomy	Technically easier inner layer anastomosis Able to assess for sperm presence prior to anastomotic setup Able to assess for sperm presence prior to anastomotic setup Able to mobility the epididymis off the testis for large gaps	Disruption of epididymal blood supply from inferior epididymal artery Difficult outer layer closure Can be difficult to identify proper tubule for anastomosis

be used for IVF with intracytoplasmic sperm injection. Persistently azoospermic men without cryopreserved sperm can opt for either a redo-vasoepididymostomy or microscopic epididymal sperm aspiration combined with IVF and intracytoplasmic sperm injection.

14.11 Conclusion

The modern evolution of vasoepididymostomy has been a remarkable journey. Since Martin's first attempts over 100 years ago, we have continued to make significant strides in the refinement of this surgical technique. Most recently, adoption of microsurgical techniques and intussusception methods of vasoepididymostomy have made this surgery progressively more effective. With the introduction of the two-stitch longitudinal intussusception method, anastomoses have become simpler and easier to teach with a decreasing risk of technical error.

Modern IVF–ICSI has opened up reproductive options for those couples desiring fertility. This has caused some to question the need for advanced reconstructive reproductive tract surgery. However, in the hands of experienced microsurgeons, vasoepididymostomy is a safe, effective method of reconstruction for patients, especially for those who do not want to undergo IVF or desire multiple children. In addition, vasoepididymostomy skills are crucial to have because of the possibility of finding secondary epididymal obstruction at the time of vasectomy reversal. It is of our opinion that any reproductive surgeon who performs vasal reconstruction must be capable of performing a vasoepididymostomy.

While vasoepididymostomy is already associated with good outcomes, we look forward to the future. Further technical refinements will most likely focus on the simplification of the vasoepididymostomy procedure, decreasing operative times and making the procedure more accessible to more surgeons. These developments will come from microsurgical models and animal models. Additionally, collaborative multi-institutional datasets may allow us to find better intraoperative or perioperative predictors of anastomotic patency and pregnancy. Intraoperative factor to evaluate and consider further research into would be factors at the anastomotic site: analysis of the gross fluid quality of epididymal fluid, the effect of microscopic motility, and sperm viability testing during reconstruction.

14.12 Review Criteria

An extensive search of studies examining the vasoepididymostomy outcomes in humans was performed using PubMed in December 2018. The overall strategy for study identification and data extraction was based on the following keywords: "vasoepididymostomy," "epididymovasostomy," and "infertility." Articles published in English were considered. Data that were solely published in conference or meeting proceedings, websites, or books were not included.

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Grafting Techniques for Vasectomy Reversal

15

Henry M. Rosevear and Moshe Wald

Key Points

- Microsurgical two-layered anastomosis to correct vasal obstruction remains the gold standard.
- The role of vasal stenting in the era of microsurgical two-layered anastomosis is limited.
- Biodegradable conduits to bridge segments of obstructed vas deferens where a primary microsurgical two-layered anastomosis is impossible remain investigational but hold significant long-term promise.

15.1 Introduction

Surgical reconstruction of the vas deferens is performed to remove an obstructive lesion that is present along its course. Obstruction can exist at various parts of the vas deferens and can be the result of a prior vasectomy, congenital anomaly, inflammation secondary to a urogenital tract infection, trauma, or a surgical misadventure during prior inguinal, pelvic, or scrotal surgery. While no official reporting system exists in the United States to monitor the number of vasectomies performed each year, a survey in 2002 estimated this to be 526,501, which is approximately consistent with data reported in 1991 and 1995 [1, 2]. An estimated 2–6% of all men, and up to 11% of men aged 20–24 at the time of vasectomy, request a vasectomy reversal [3]. It has been estimated that between 30,000 and 80,000 vasectomy reversals are performed annually in the United States, though as with vasectomies, reporting requirements are not standardized so the exact number is unknown [4].

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Congenital anomalies which can lead to obstruction of the vas deferens include congenital absence of the vas deferens, which is commonly associated with cystic fibrosis [5]. Partial vasal agenesis and congenital prostatic cysts can also lead to obstruction of the vas deferens [6, 7]. One example of a genetic disorder which can lead to obstructive azoospermia is Young's syndrome, which is characterized by chronic sinusitis and bronchiectasis as well as obstructive azoospermia [8]. In Young's syndrome, the obstruction usually occurs at the junction of the caput to corpus epididymis due to inspissated secretions. Inflammatory causes of obstruction are rare in the antibiotic era but include tuberculous epididymitis, gonorrheal urethritis progressing to obstructive epididymitis, and chlamydial epididymitis [9].

The success of a vasectomy reversal depends on several factors, only some of which can be controlled at the time of surgery. Factors which are independent of the method of reversal but which may influence subsequent conception include age and fertility potential of the patient's partner, length of obstructive interval, presence of antisperm antibodies, and high intravasal and epididymal pressure after the original obstruction [10–13]. Some factors which influence the success of the reversal are directly related to the technique chosen and include rate of stricture or scar development and granuloma formation [14]. The most commonly cited reason for these specific complications is an anastomosis made under tension, devascularization of the wall of the vas deferens, or a technical problem with the anastomosis leading to sperm leakage [15].

The current gold standard to surgically correct an obstructed vas deferens is a microscope-assisted two-layered vasovasostomy, but this has not always been so [16, 17]. Given the complex and time-consuming nature of this operation, new surgical techniques including robotics, modifications, and tools are continuously being explored. Some of these techniques include the use of fibrin glue, laser soldering, absorbable and nonabsorbable stents, and artificial conduits with or without specific growth factors added [18]. This chapter highlights the development of surgical grafting tech-

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niques for vasectomy reversal, including the use of stents and grafts, as well as the current clinical application of these devices and areas where further research is required.

15.2 Grafting Techniques in Reconstruction of the Male Reproductive Tract

15.2.1 Stents

As previously stated, several factors related to the success of a vasectomy reversal can be controlled at the time of surgery. From the 1950s to the mid-1970s, macrosurgical techniques for vasovasostomy were common and accepted as the gold standard. This technique allowed a primary anastomosis of the vas deferens to be created but was plagued with, by today's standards, low patency and pregnancy rates. According to a survey of the American Urological Association (AUA) members published in 1973, members at that time practicing vasovasostomy reported a 38% patency rate and a 19.5% pregnancy rate [19]. It should be remembered that the microsurgical techniques which are common today were not developed until the mid-1970s, and as such, stricture resulting in either partial or complete vasal obstruction was the most pressing technical complication of that period. To address this common complication, approximately 90% of urologists performing vasovasostomies at that time employed stents, with either silver wire or nylon suture being the most commonly reported [19]. The reason for the widespread use of stents can also be found in the 1973 AUA survey. Members reported that the pregnancy rate for non-stented reversals was significantly lower, at 10.9% compared to 19.9–26% for stented procedures, depending on the stent used. Numerous techniques had been developed in 1973 which maximized both patency and pregnancy rates. The main variation between these techniques was the use of loupes for magnification and/or the use of stent [20].

In the lexicon of the modern urologist, a stent most commonly refers to the hollow silicon tube that is used in the ureter for treatment of either intrinsic or extrinsic ureteral obstruction. A vasovasostomy stent, as it was originally used, was quite different. A stent in that sense was any foreign body, usually a piece of suture, the purpose of which was to maintain patency of the lumen of the vas deferens during and immediately after a macrosurgical (either with or without the supplemental use of loupes) primary anastomosis of the vas deferens. The simple goal of the stent was to prevent obstruction at the anastomosis site either because of a poorly placed suture at the time of surgery or as prevention of stricture or scar formation in the immediate postoperative period. In one example of this technique, a short section of 2-0 nylon suture is used as a stent to bridge the anastomosis, while 6-0 Prolene is used to actually complete the anastomosis [21]. In this technique, the nylon suture is removed before the operation is completed, and its purpose is to ensure that the vas deferens lumen remains patent during the procedure. In another variation on this theme, described by Dorsey, a zero monofilament suture is fed through a hollow needle introduced approximately 1 cm proximal to the site of the intended anastomosis [22]. This suture is then fed into the distal vas deferens. The anastomosis is then completed using 6–0 Ethiflex, and the proximal end of the stenting suture is brought through the scrotal skin and removed in 12–14 days. The goal of this stent in this technique is to ensure patency of the anastomotic site both during the procedure and in the immediate postoperative healing period. The success rates of these procedures were reported to be over 80% patency, which contrasts with the success rates reported in the 1973 AUA survey.

Even with the improvement in both patency and pregnancy rates reported by clinicians using stents during vasovasostomy, there were numerous known disadvantages of stents, especially with the use of exteriorized stents such as described by Dorsey [20]. The point of exit for the stent is a theoretical source of infection as well as a location where sperm can leave the lumen of the vas [20, 23]. Additionally, the location where the exteriorized stent left the lumen of the vas was identified in a publication by Fernandes as a common site of subsequent luminal obstruction (often instead of the primary anastomosis itself) [24]. To avoid the problem of an exteriorized stent, some groups have experimented with absorbable intravasal suture as a stent to bridge the anastomosis—the theoretical advantage being that these stents would slowly dissolve, maintaining the patency of the anastomosis both during the procedure itself and the postoperative healing period without need to be removed. In one experiment in a canine model, Montie et al. compared three groups: no stent, a Dexon intravasal stent, and a chromic intravasal stent [23]. Three to six months after the vasectomy reversal procedure, retrograde vasography was used to identify patency rates. Both absorbable stent groups had higher patency rates than the control (no stent) group, with the chromic group having the highest overall patency at 70% vs. 60% for the Dexon group and 50% for the no stent group. This concept was tested in a human clinical model by Rowland and colleagues a few years later, who found that intravasal absorbable stents (using 3-0 chromic) had higher patency rates than a group using exteriorized silkworm gut stents (86% vs. 67%, respectively) [25].

In 1975, Silber reported the first use of microsurgical vasovasostomy in humans [26]. His work, along with independent work by Owen, led the way to the modern microsurgical two-layered anastomosis [26, 27]. From a historical standpoint, it should be noted that it was Silber and his group who found through histologic and electron microscopic work

that stricture was more common than originally thought with macrosurgical anastomosis techniques [28]. Silber also popularized the two-layered closure based on his observation that this technique provided a better watertight mucosal approximation given the common discrepancies between proximal and distal luminal diameters of the vas deferens [29]. The techniques developed by these investigators have allowed the microsurgical two-layered vasovasostomy anastomosis to become the gold standard for vasectomy reversal, with success rates dependent on time since obstruction. These success rates may be as high as 97% patency and 76% pregnancy when the obstructive interval is less than 3 years and 71% patency and 30% pregnancy after 15 years of obstruction [13].

The reproducible success of this new technique resulted in a dearth of research into alternative techniques for many years. Even with its success, Silber's microsurgical technique was not perfect. The downside was that microsurgical anastomosis was a time-consuming operation best done by surgeons with specialized training using expensive operating microscopes. This prompted research into new techniques that would simplify the technique while maintaining the high patency and pregnancy rates. In 1989, Flam et al. reported work in a rat model on a hollow, absorbable polyglycolic acid tube [30]. In their experiment, they inserted a 10-mm-long by 0.5-mm outer diameter hollow stent into the lumen of the vas at the site of anastomosis on one side and completed the anastomosis with a single layer of suture (Fig. 15.1). On the contralateral side, they performed standard microsurgical anastomosis. They showed a trend toward improved patency in the stented vas deferens. Flam emphasized in his paper that sperm leakage at the site of the anastomosis could lead to secondary stricture and should be avoided. This work led to a clinical trial using absorbable intravasal stents conducted by Rothman and colleagues in 1996 [31]. This randomized study compared conventional two-layered microsurgical anastomosis to a modified approach using an absorbable polyglycolic acid stent without intraluminal sutures (Fig. 15.2). While the operative time was significantly reduced in the stented group (118 min vs. 137 min), both the patency and the pregnancy rates were lower in the stented groups (81% vs. 89% and 22% vs. 51%, respectively), and the authors concluded that intravasal stents should not be used.

More recently, Vrijhof et al. reported on a nonabsorbable stent in a rabbit model [14]. They theorized that the absorbable nature of the previously reported stents allowed strictures to develop at the site of the anastomosis once the stent had dissolved and that a nonreactive nonabsorbable stent would bypass this problem while simplifying the operation. Their stent was made of a biocompatible material designed to have both hydrophilic and hydrophobic characteristics. The stent also had a transverse ridge which was designed to minimize migration from the anastomotic site (Fig. 15.3). This group reported that all vasa were patent at the end of their study (39–47 weeks) and that total sperm count was higher in the stented group. No human data is available on this type of stent.

Fig. 15.1 Hollow polyglycolic acid stent (0.5-mm outer diameter) shown on a dime. (Used with permission from Flam et al. [30]. With permission from Elsevier)

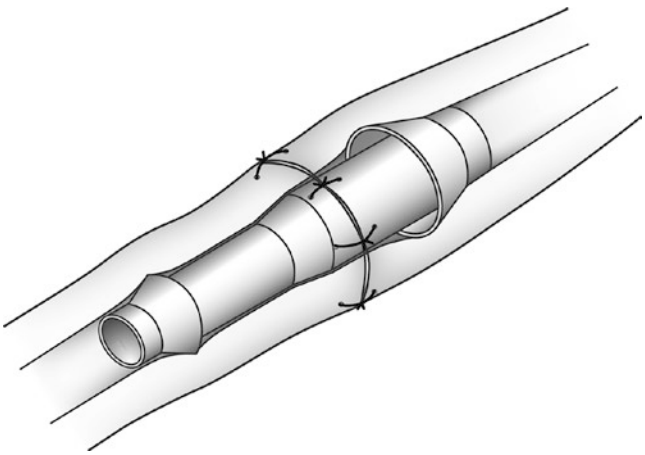

Fig. 15.2 Absorbable self-retaining polyglycolic acid stent

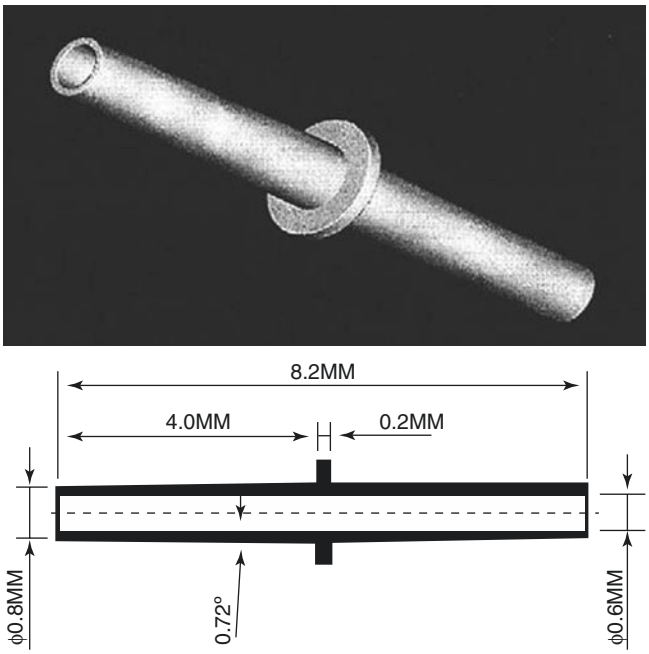

Fig. 15.3 Nonabsorbable polymeric stent with transverse ridge to minimize migration. (Reprinted from Vrijhof et al. [14]. With permission from Elsevier)

In the era of cost-conscious medicine, especially when many patients must pay out of pocket for vasectomy reversals, further research into efforts that simplify the present gold standard is appropriate with the caveat that patency and pregnancy rates should not be compromised. It is important to note that all of the absorbable and nonabsorbable stents which have been used to date in human studies have been well tolerated with no side effects and little to no inflammatory response.

In summary, stents were investigated as a method to improve patency rates in the era of macrosurgical vasovasostomy but were eclipsed by the application of the operating microscope to the field and the introduction of microsurgical two-layered vasovasostomy. Efforts to improve and simplify the microsurgical operation using absorbable stents have not improved overall patency or pregnancy rates. Recent efforts using nonabsorbable stents show promise in animal models but have not been tested in humans so their utility remains unproven. The ideal stent would, at a minimum, maintain the patency and pregnancy rates achieved through a conventional two-layered microsurgical anastomosis while decreasing the operative time, training, and cost required to achieve these results.

15.2.2 Conduits

The preferred method to bypass an obstructed portion of the vas deferens, regardless of the etiology of the obstruction, is surgical excision or exclusion of the obstructed segment and reanastomosis of the vas deferens using a microsurgical two-layered anastomosis. The goal of the operation is a watertight, tension-free, widely patent anastomosis. As described previously, numerous techniques have been suggested in an attempt to simplify this procedure while preserving its patency and pregnancy rates. The assumption in all of the previously described techniques is that the vas deferens could be sufficiently mobilized to allow a tension-free anastomosis. Unfortunately, cases exist where, due to the physical length of the obstruction, the vas deferens cannot be reconstructed in a watertight, tension-free manner. These cases present a clinical challenge because the resulting obstructive azoospermia is theoretically amenable to surgical correction. Presently, the only reproductive option available for these patients is surgical sperm retrieval. The technique of retrieving sperm from either the testicle or epididymis has been successfully reported in cases of obstructive azoospermia that is not surgically correctable but must be coupled with in vitro fertilization [32]. The hormonal manipulation, surgical interventions, risk of multiple gestations, and increased financial cost of in vitro fertilization make this solution less than ideal and create an intriguing field of research into reconstruction of the male reproductive tract.

Grafting of the male reproductive tract theoretically can take one of three forms. The first option is to use transplanted vas deferens with all of the complications, both technical and immunological, associated with such a procedure. The second option is to replace the obstructed segment of vas deferens with a tubular structure, the sole purpose of which is to simply allow passage of sperm in a distal direction. An analogous clinical problem can be found in vascular surgery where surgeons often replace diseased segments of vessels with either endogenous grafts such as the long saphenous vein or exogenous grafts such as a Teflon-coated endovascular stent. The third option involves tissue engineering. Tissue engineering as it applies to reconstruction of the male reproductive system involves the concept of creating an artificial conduit which serves as a scaffolding for the regrowth of the vas deferens itself. In a different biological system, polymer scaffoldings have been shown to facilitate peripheral nerve regeneration in segments as long as 1 cm [33]. Regardless of the method chosen to graft over the obstructed segment of vas deferens, the goal is to reestablish continuity of the male reproductive tract, allowing sperm to be present in the ejaculate and eliminating the need for assisted reproductive techniques (ART). It should be noted that even small amounts of ejaculated sperm could be a significant improvement, as this may allow for less invasive forms of ART [34].

The first reported experiment on the use of grafts in reconstruction of the male reproductive tract was by Romero-Maroto and colleagues in 1989 [35]. This group reported successfully autotransplanting a pediculated segment of vas deferens from one side to the contralateral in

rabbits. They reported good patency rates, but no data on pregnancies was noted. The clinical use of this technique is likely limited, as these subjects would likely be candidates for crossover vasovasostomies, a rare procedure with a high reported success rate [36], and given the questionable feasibility of harvesting a long vasal segment for reconstruction of the contralateral side.

Regarding the second option for grafting the male reproductive tract, Carringer et al. in 1995 reported patency rates in rats after either a vasal or vascular graft obtained from either the contralateral side of the same animal or from female rats, respectively [37]. In this study, three different lengths of grafts were used (0.5, 1.0, and 1.5 cm), corresponding to approximately 10%, 20%, and 30% of the entire vas deferens length. Patency was confirmed by direct examination of the graft 4 weeks postoperatively. The authors found an overall patency rate of approximately 40% in both surgical groups (vasal and vascular graft) with higher rates in the shorter segments. Pregnancy rates were not evaluated. No human clinical trials have been reported using either of these techniques. Questions on long-term patency of extensive artificial grafts remain unanswered, even in animal models, and should be further investigated.

The lack of a suitable allograft in humans for vasal reconstruction has led to research on the potential for biocompatible degradable polymer scaffolding for tissue engineering. As mentioned earlier, this model has been successfully applied to the clinical problem of peripheral nerve regeneration [33]. Additions to this technology, including micropatterned (grooved) inner lumens as well as target-specific growth factors, can increase the efficacy of this technology [38, 39]. The vas deferens is a good target for investigation because it has been shown to undergo spontaneous recanalization at the site of vasectomy [40].

Further evidence that may support tissue engineering of the vas deferens is the demonstration of elevated levels of selected growth factors at the vasectomy site in an animal model. Previous examination of vasectomy sites in rats using real-time polymerase chain reaction, enzyme-linked immunosorbent assay, and histopathological analysis demonstrated a 12-fold increase in platelet-derived growth factor beta and a ninefold increase in transforming growth factor beta [41].

Using the peripheral nerve regeneration model as a guide, biodegradable conduits made of d,l-lactide were studied for reconstruction of the reproductive tract in a rat model [42]. Biodegradable conduits with micro-patterned grooves on the inner surface were implanted in 47 rats following vasectomy (Fig. 15.4, scanning electron microscopy image). At 8 weeks postimplantation of the conduits, no evidence of recanalization was found. However, at 12 weeks, evidence of recanalization was noted in three of the remaining rats, with one showing a microcanal spanning the entire 0.5-cm conduit and the other two showing distinct epithelialized vas deferens microcanals at the conduit edges (Fig. 15.5) [42].

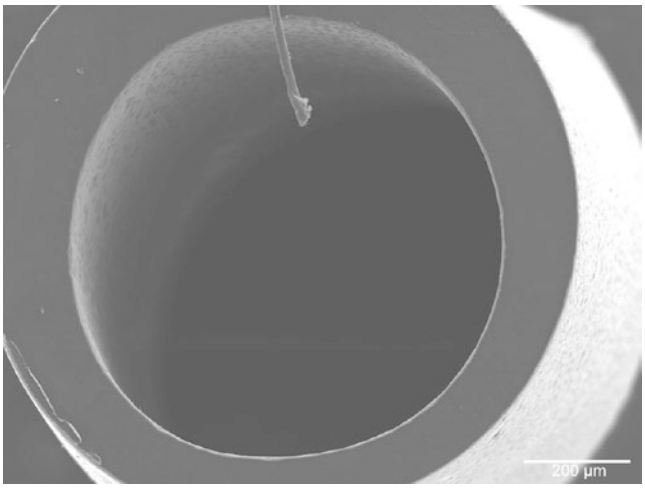

Fig. 15.4 Scanning electron microscope image of PDLA conduit. Bar = 200 µm. (Reprinted from Simons et al. [42]. With permission from Creative Commons License 4.0: https://creativecommons.org/licenses/by-nc-sa/4.0/)

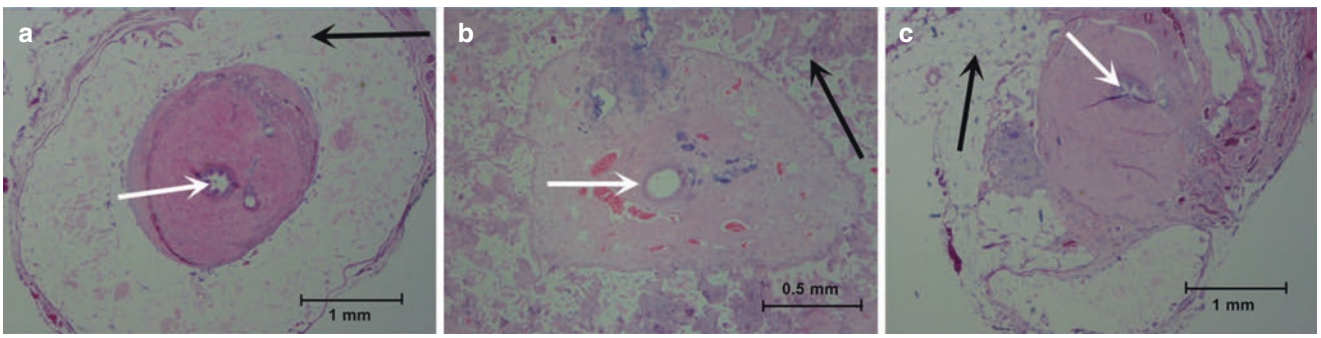

Fig. 15.5 (**a**) Evidence of microrecanalization at the midpoint of a 0.5-cm poly-(d,l-lactide) (PDLA) graft (magnification ×40). Bar = 1 mm. (**b**) Microcanal at the midpoint of a 0.5-cm PDLA graft (magnification ×200) Bar = 0.5 mm. (**c**) Microcanal at the interface zone of a 0.5-cm PDLA graft (magnification ×40). Bar = 1 mm. All panels: white arrows microcanals, black arrows graft. (Reprinted from Simons et al. [42]. With permission from Creative Commons License 4.0: https://creativecommons.org/licenses/by-nc-sa/4.0/)

Following the demonstration of microrecanalization of the vas deferens in this biodegradable graft model, attempts were made to identify ways to maximize this response (unpublished data). Based on the identification of elevated growth factor levels at the site of vasectomy, the effect of local microparticle-delivered growth factors on the rate of vasal recanalization in a biodegradable conduit model was examined. Delivering growth factors to a specific location in the body over a sustained period of time is not a simple task. Effective supplementation of growth factors selectively at the site of the grafted vas deferens may be compromised by the fact that the ability of growth factors to perform their function depends on their tertiary structure, which is susceptible to degradation if it is not protected from the local environment. Thus, delivery of a locally sustained concentration of growth factors requires the use of microspheres. The goal of a microsphere is to sequester the biologically active molecule and allow a controlled, sustained release of the molecule. The exact timing of the sustained release is a function of the characteristics of the microsphere into which it is placed. With these considerations in mind, a poly-(d,l-lactide) material was chosen for construction of the microspheres. As the biodegradable conduits used in this study were constructed of the identical material, noncovalent binding was assumed to keep the microspheres near the conduit. Reconstruction of surgically induced vasal gaps using biodegradable conduits soaked in microspheres containing TGF-beta and PDGF revealed an increase in the number of new microcanals in the graft but not in their length at 12 weeks postoperatively.

In an effort to further optimize the conditions for vasal recanalization, methods to increase the vascularity of the reconstructed vas deferens were investigated, based on an observation suggesting that neovascularization increased with time at the conduit to vasal border (unpublished data). To bolster this neovascularization and potentially increase the rate of recanalization, the effect of oral sildenafil citrate on recanalization in the biodegradable graft model was examined. Sildenafil citrate is a type 5 phosphodiesterase inhibitor that has been shown to promote neovascularization in other systems [43]. Rats received a daily dose of 5 mg/kg of oral sildenafil citrate following reconstruction of the vas deferens with a biodegradable graft. At 16 weeks, the rats on sildenafil citrate had a significantly increased number of microcanals (29 vs. 4) though the average length of the canals was constant at 2 mm. This observation was confirmed by an increase in staining for CD31, an endothelial marker. An ongoing study involves combining both oral sildenafil citrate with increasing the local concentration of TGF-beta and platelet-derived growth factors via microspheres. Areas of future research into this field include examining different substrates of which the conduit itself is composed and embedding the growth factors directly into the conduit to maximize local concentration.

15.2.3 Autografts

In addition to the earlier studies of allografts, vascular autografts, and vascularized (pediculated) vasal autografts for vasal reconstruction, more recent research has been conducted on the possible utilization of non-vascularized vas deferens autografts.

Kadioglu et al. [44] evaluated the possible utilization of a non-vascularized vas deferens autograft in a rat model. In this study, segments of isolated vas deferens, 2.5 cm in length, were used as bilateral autografts in 15 rats. Each autograft was implanted between the two transected ends of vas deferens using end-to-end anastomosis. Unlike the earlier study by Carringer [37], this study also assessed pregnancy outcomes. Fertility, sperm motility, and graft survival were evaluated and compared with a control group. After 3 months, 9 of the 15 (60%) rats were able to breed successfully and 24 (80%) vas grafts were patent and viable. Large granulomata were reported to be present at the proximal anastomosis sites in six (20%) autografts that failed. Unilateral minimal fluid leakage was reported in six (20%) of the proximal (testicular end) anastomosis sites in those rats that were able to breed. On semen analysis, forward motility was noted in 76% of sperm in the experimental group, compared to 78% in the control group ($p > 0.05$). The authors of this study concluded that vas deferens autograft can be successfully performed in a rat model with subsequent breeding capability [44].

However, these results were contradicted by a study by Nasir et al. that compared different autogenous graft materials for reconstruction of large segment vas deferens defect in a rat model. In this study, vas deferens, artery, and vein grafts were used to reconstruct 30% and 50% defects of the total vas deferens length. No patency was found in any of the grafts [45].

In addition to avoiding immunological difficulties, the utilization of isolated, non-vascularized vas deferens autografts may also allow more flexibility in the possible location of the implantation site along the reproductive tract, which might be limited when vascularized (pediculated) vasal grafts are used. More research would be required regarding the possible implementation of this technique for the reconstruction of long vas deferens defects.

15.3 Conclusions

Grafting of the male reproductive tract is an exciting new area of tissue engineering which may allow natural conception for patients with significant lengths of obstructed vas deferens. While stents had a significant and important role in increasing patency and pregnancy rates in the pre-microsurgical era, their role in the modern era of microsurgi-

cal two-layered anastomosis remains to be defined. To date, if the vasal obstruction is amenable to a primary watertight, tension-free anastomosis, microsurgical non-stented techniques remain the gold standard. Cases where a tension-free anastomosis is not possible because of the physical length of the obstruction remain problematic, but further research into autografts and tissue engineering in the form of implantable conduits holds much promise.

15.4 Review Criteria

An extensive search of studies examining grafting techniques for vasectomy reversal was performed using search engines such as ScienceDirect, OVID, Google Scholar, PubMed, and MEDLINE. The start and end dates for these searches were 1973 and January 2019, respectively. The overall strategy for study identification and data extraction was based on the following keywords: "grafting techniques," "obstruction of vas deferens," "vasectomy reversal," "epididymal obstruction," "partial vasal agenesis," and "vas deferens autograft." Articles published in languages other than English were also considered. Data that were solely published in conference or meeting proceedings, websites, or books were not included. Websites and book chapter citations provide conceptual content only.

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Mini-incision Vasectomy Reversal Using the No-Scalpel Vasectomy Instruments and Principles

16

Judith Dockray, Keith Jarvi, Ethan D. Grober, and Kirk C. Lo

Key Points

- Vasectomy reversal is a safe and established option for achieving pregnancy post-vasectomy.
- Microsurgical techniques are preferred to increase luminal patency rates.
- Mini-incision vasectomy reversal, as described below, has demonstrated decreased postoperative pain and faster return to normal activity with no compromise to outcome.
- Single mini-incision vasectomy reversal is a newer modification that is suitable for a subset of well-selected patients.

16.1 Introduction

Vasectomy reversal has a variety of described techniques, and these continue to evolve in current surgical practice. At the University of Toronto, we developed the "mini-incision" for vasectomy reversal using the principles and instruments used for the no-scalpel vasectomy. This has now evolved, in selected cases, to "single mini-incision."

By taking advantage of the compliance of the scrotal wall, a substantial length of vas deferens can be delivered through a mini-incision. By avoiding a longer incision, delivery of the testicle, and more extensive tissue dissection, we have shown a reduction in postoperative pain and a faster return to daily activities. This is achieved without compromising patient outcomes.

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16.2 History

Vasectomy remains the most commonly performed urological procedure in North America, with over 500,000 vasectomies performed annually in the United States alone [1–3]. In 1974, the no-scalpel vasectomy was introduced and provided surgeons with a technique that minimized discomfort and post-procedural morbidity without compromising patient outcomes [4–6]. However, between 2% and 11% of vasectomized men will ultimately request a reversal of their vasectomy for a variety of reasons such as a new partner or death of a child [3].

As with most surgical procedures, vasectomy reversal techniques are in constant evolution. The origin of the techniques used for vasectomy reversal dates back to 1902, when Martin performed the first documented vasoepididymostomy for a man with obstructive azoospermia secondary to gonorrhea [1]. In 1909 he published a series of 11 azoospermic men who underwent vasoepididymostomies with a patency rate of 64% and pregnancy rate of 27%. Martin's publication and the demonstrated effectiveness of his vasoepididymostomy technique helped to dispel the then widely held belief that such anastomoses were not worth pursuing given their technical difficulty and expected low success rates. Hagner subsequently reproduced these outcomes, in his series of 33 patients with reported patency and pregnancy rates of 64% and 48%, respectively. This solidified vasoepididymostomy as an effective technique in the management of obstructive azoospermia [1].

Quinby reported the first successful vasovasostomy in 1919 on a man who underwent vasectomy 8 years earlier [1]. O'Connor, Quinby's former assistant, subsequently published a series of 14 vasectomy reversals using Quinby's technique in 14 men, with an overall patency rate of 64% [1].

As the interest in family planning evolved over the ensuing decades, the rates of vasectomy increased substantially, as did the inevitable subsequent demand for vasectomy reversal.

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16.3 Vasectomy Reversal Techniques

The original descriptions for vasectomy reversal were open surgical techniques sometimes aided by optical magnification. The earliest described techniques used thin silver wire for the vasal anastomosis, which ultimately evolved into the use of nonabsorbable 4-0 to 6-0 sutures during the 1970s [2]. The reported patency rates for these techniques ranged from 79% to 88% with pregnancy rates of 34–50%. These techniques were largely abandoned when the operating microscope became widely available in most centers.

Silber and Owen are both credited with the first description of a microsurgical vasovasostomy in humans in 1977, although several authors had previously described this technique using animal models [2]. The anastomoses were performed with an operating microscope using 16–25 times magnification and 9-0 nylon sutures in a one- or two-layer closure.

Further evolution and refinements of the original techniques occurred, and by the 1980s, the two-layer closure techniques described the use 10-0 nylon for the mucosal anastomosis and 8-0 or 9-0 nylon for the seromuscular layer. Goldstein invented and introduced the microspike vas approximator clamp and microdot suture placement technique. This allowed for greater stabilization of the vasal ends and more precise placement of the 10-0 anastomotic sutures, particularly useful when the vasal lumens are of disparate caliber [1, 7].

The technique for vasovasostomy, described by Lipshultz et al., is probably the most commonly used among modern microsurgeons [2]. With this technique, the testicles and spermatic cords are delivered via a single 4–6-cm midline or bilateral 4–6-cm paramedian scrotal incision(s). The vasectomy site is identified, and the healthy testicular and abdominal ends of the vas are mobilized. Care is taken to preserve as much perivasal adventitia and vasal blood supply as possible. 5-0 absorbable stay sutures are placed superficially on both the testicular and abdominal vas 1–2 cm from the intended transection sites. The testicular vas is then transected and the expressed fluid is examined immediately with light microscopy at 100–400 times magnification. This serves to confirm patency by identifying the presence of sperm or sperm parts in the fluid. The abdominal vas is transected in an identical fashion and its patency confirmed with saline vasogram (or methylene blue vasography with temporary insertion of a Foley catheter). Hemostasis is managed with bipolar electrocautery to minimize vasal injury.

If the intraoperative findings are suitable for vasovasostomy (copious thin fluid, presence of sperm or sperm parts, and normal vasography), the two vasal ends are approximated and stabilized either by placement within a vas approximator or microvascular clamp or by placing 1–2 adventitial holding stitches at the 6 o'clock position. The operating microscope is now brought into the field, and a two-layer anastomosis is begun. A double-armed 10-0 nylon mucosal suture is tied at the 6 o'clock position. Three to five additional 10-0 mucosal sutures are placed around the circumference of the vasal lumen and tied. Interrupted single-armed 9-0 nylon sutures are circumferentially placed in the seromuscular tissue to complete the second layer.

A common modification to this technique eliminates the delivery of the testicle in an effort to minimize postoperative morbidity [7]. With this technique, a 4–6-cm incision is made in the upper scrotum angled toward the external inguinal ring along the path of the vas deferens. This allows for the easy identification of the vasectomy site and mobilization of the testicular and abdominal vasal ends. The anastomosis is then performed in an identical fashion to the previous description.

The patency and pregnancy rates reported in the literature are widely variable and dependent on a number of preoperative, operative, and postoperative factors that may or may not have been controlled. It is universally accepted that the microsurgical approach yields superior patency and pregnancy rates compared to traditional anastomoses given the ability to more precisely place significantly smaller and less obstructive sutures [3]. Unpublished data also demonstrates that surgeons with microsurgery training experience superior outcomes, with an average patency rate of 89% compared to 53% in inexperienced hands [3]. The Vasovasostomy Study Group reviewed the outcomes of 1469 contemporary microsurgical vasovasostomies [3, 8]. They demonstrated a 97% patency rate and 76% pregnancy rate in men less than 3 years from their vasectomy. As the interval from vasectomy increases, the rates decline with a 71% patency rate and 30% pregnancy rate when 15 years or more have elapsed since the vasectomy. The Vasovasostomy Study Group also determined that there was no statistically significant difference in patency or pregnancy rates between one-layer and two-layer anastomosis, with the decision based on surgeon preference and experience [1, 8].

The morbidity of vasovasostomy has been poorly examined with no published studies comparing the various techniques. Postoperative pain, swelling, bruising, and subsequent limitation of activity are all commonly seen after vasectomy reversal, especially if the testicle and tunica vaginalis are delivered. Most men are counseled to wear supportive briefs for 2 weeks, to take 1–2 weeks off work, and to limit themselves to light physical exertion for 3–4 weeks.

We have already discussed many technical modifications that have been developed in an attempt to improve surgical outcomes, but none specifically developed to help reduce the morbidity of the procedure.

16.4 Mini-incision Vasectomy Reversal (MIVR)

The mini-incision and no-scalpel techniques for performing vasectomy are familiar to most urologists and have been shown to reduce complication rates and decrease recovery

times without compromising vasectomy outcomes. In an effort to maintain the established effectiveness of microsurgical vasovasostomy, and to reduce postoperative morbidity, Jarvi et al. at the University of Toronto applied these principles to vasovasostomy [4, 9].

16.5 Technique of Mini-incision Vasectomy Reversal

The instruments required are a combination of those used for no-scalpel vasectomy and for traditional vasectomy reversal. The key additions are the inclusion of two ring vasectomy clamps and one sharp dissecting forceps, along with the normal microsurgical instruments required for the procedure.

Identical to the no-scalpel vasectomy, the vas deferens is palpated, manipulated, and stabilized through the scrotal skin in the mid to upper scrotum, using the three-finger technique previously described (Fig. 16.1). It is important to bring the vas deferens at least 1 cm lateral to the midline to be situated in the more pliable portion of the scrotal skin. The no-scalpel vasectomy ring clamp is then used to grasp the vas deferens either approximately 5 mm from the previous vasectomy site or directly onto the site of vasectomy occlusion (if possible) in an effort to minimize vasal injury (Fig. 16.2). Using the ring forceps, the abdominal vas is gently elevated to just below the scrotal skin, and a 15-blade scalpel is used to make a 1 cm skin incision directly over the vas (Fig. 16.3). This incision is deepened through the skin and dartos muscle layer using the combination of dissecting forceps plus diathermy, being careful not to injure the underlying vas. Once the vas is exposed, the second ring clamp is used to re-grasp the exposed vas within the incision and elevate it gently out of wound (Figs. 16.4 and 16.5). The vas is then carefully mobilized.

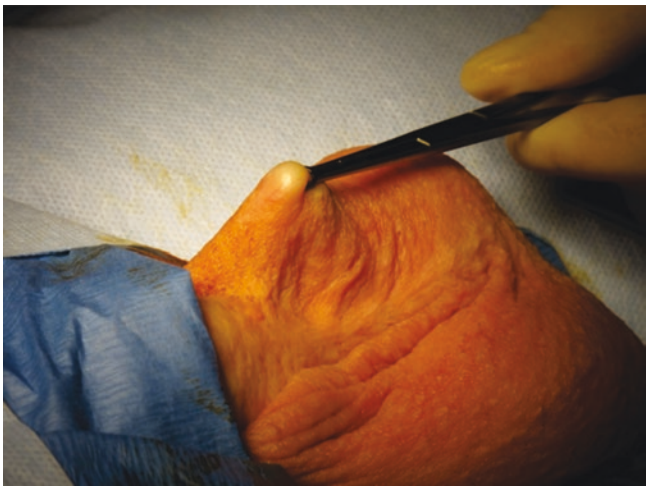

Fig. 16.2 Abdominal end of the vas deferens is grasped with a ring vasectomy clamp approximately 5 mm away from vasectomy defect. Clamp is then elevated

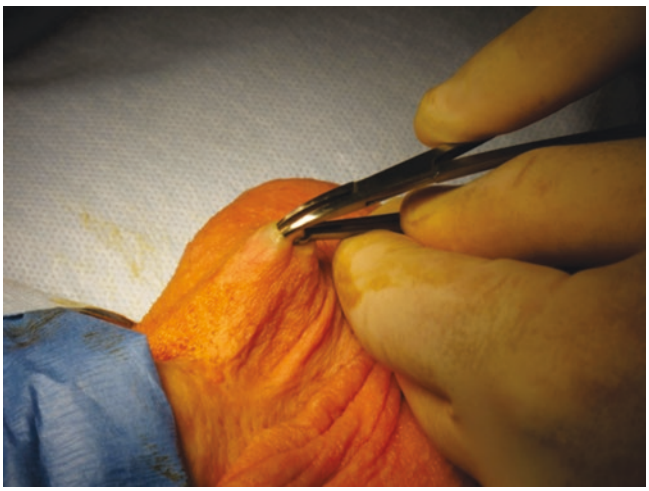

Fig. 16.3 Using a scalpel, the skin and dartos is opened directly over the vas deferens for a length of 8–10 mm

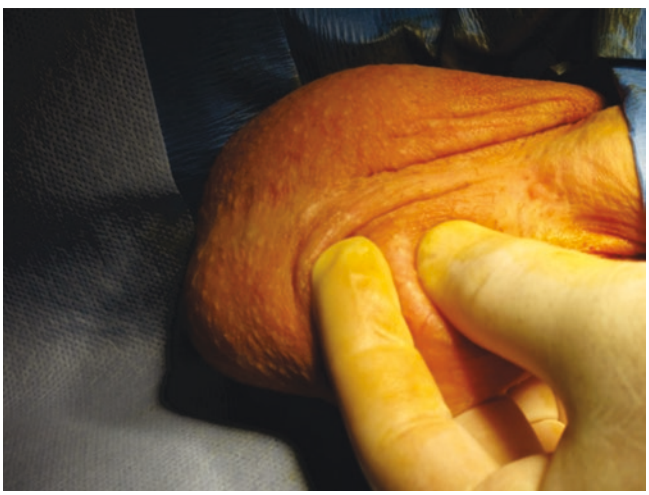

Fig. 16.1 The vas deferens is identified and secured using the three-finger technique

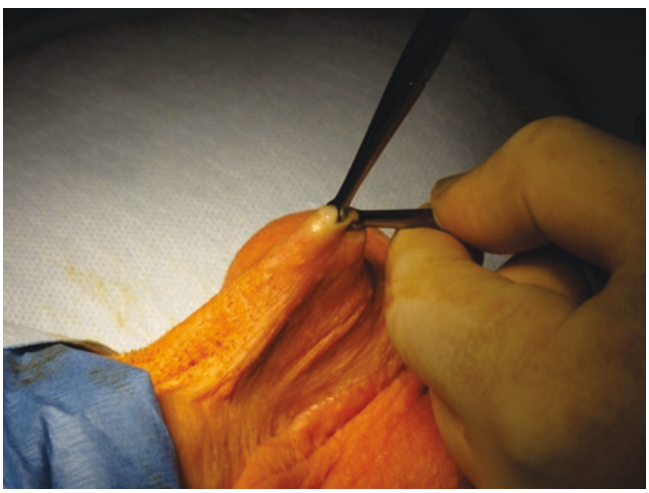

Fig. 16.4 A second vasectomy ring clamp is used to grasp the vas within the incision

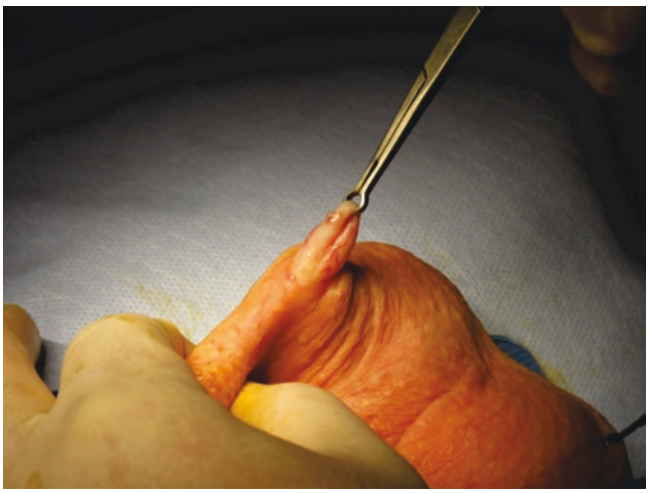

Fig. 16.5 The abdominal end of the vas is gently delivered through the incision

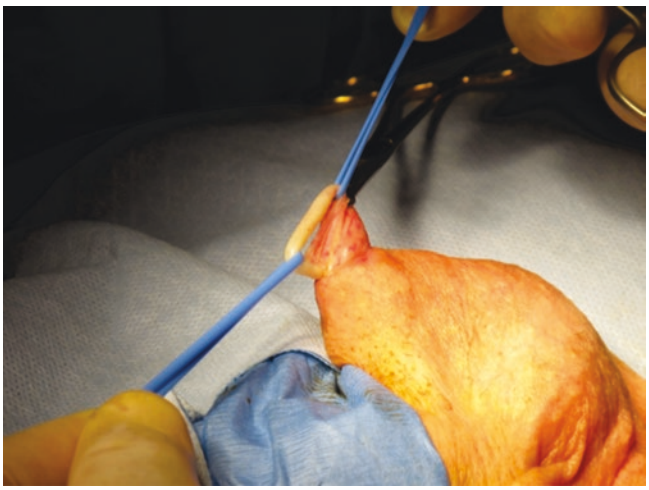

Fig. 16.7 The abdominal end of the vas is secured with vessel loops

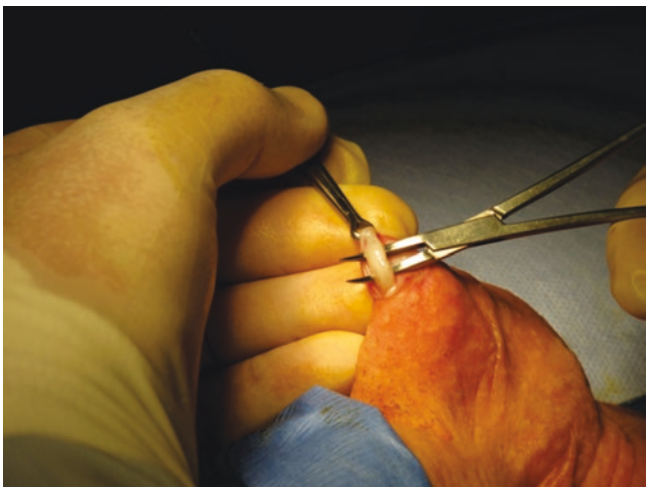

Fig. 16.6 A sharp dissecting forcep is used to isolate a small 1–1.5 cm perivasal window

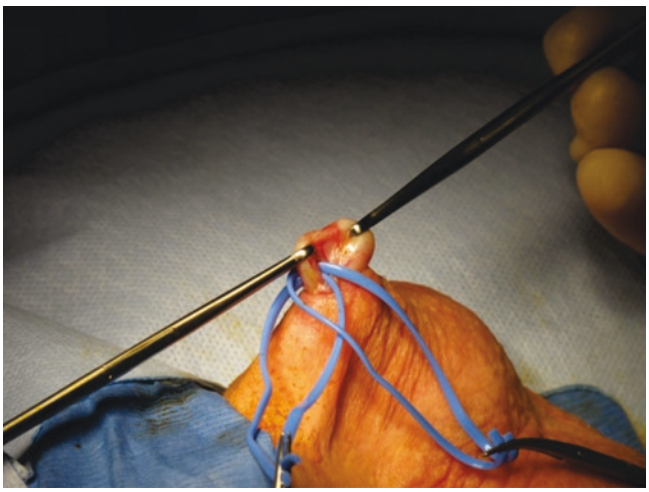

Fig. 16.8 With the abdominal end of the vas secured, the testicular end of the vas is delivered through the same incision and mobilized in an identical fashion to the abdominal end (note the vasectomy defect between the clamps)

A perivasal window is created with a combination of blunt and sharp dissection for a length of approximately 1 cm. Care is taken to preserve the vasculature within the perivasal adventitia. The vas is finally secured with a vessel loop (Figs. 16.6 and 16.7). At every step, meticulous hemostasis, with the judicious use of microscopic bipolar cautery, is essential as vessels in the dartos and subcutaneous layer may be difficult to control after they retract into the scrotum.

With the abdominal end of the vas mobilized and secured, the testicular end of the vas is palpated through the incision beyond the identified vasectomy site and is re-grasped with the ring forceps through the same mini-incision in the scrotal skin. The vas is gently delivered via the incision, mobilized, and secured in a manner identical to the abdominal vas (Fig. 16.8). Using this technique, a substantial portion of the vas can be delivered through the mini-incision given the inherent compliance of the scrotal skin (Fig. 16.9). Care must be taken during the mobilization of the testicular vas as the convoluted portion is often encountered and is very easily injured. Stay sutures of 5-0 Biosyn or PDS are placed through the superficial seromuscular layer of the vas approximately 5–10 mm away from the anticipated transection site on both the abdominal and testicular vas. These stay sutures have a dual function. They allow control of the vasal ends; aiding in positioning of the vas approximator and preventing retraction into the incision but can also can be tied after the anastomosis is complete, relieving tension on the repair.

With both vasal ends secured, each vas is then transected, stabilizing the vas over the forceps, as shown, which acts as

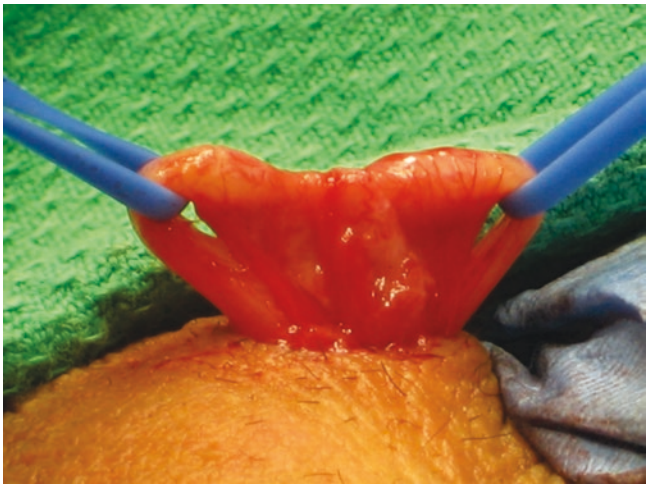

Fig. 16.9 Both vasal ends can be easily delivered through the mini-incision

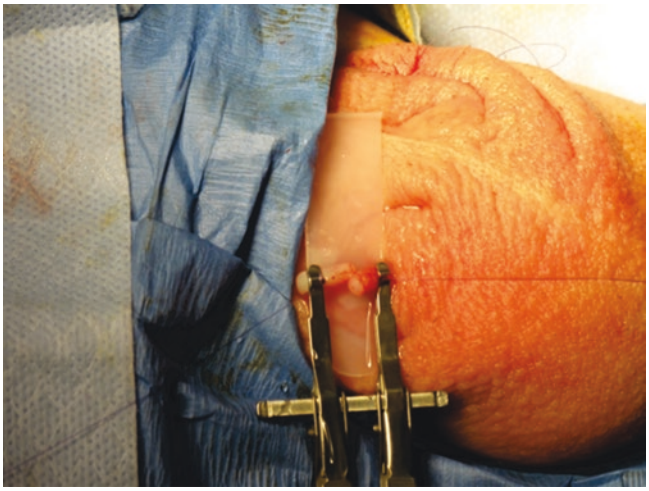

Fig. 16.11 The vasal ends are secured in place in a vas approximator clamp. A pre-cut plastic backboard is placed under the vasa and clamp to stabilize prior to anastomosis

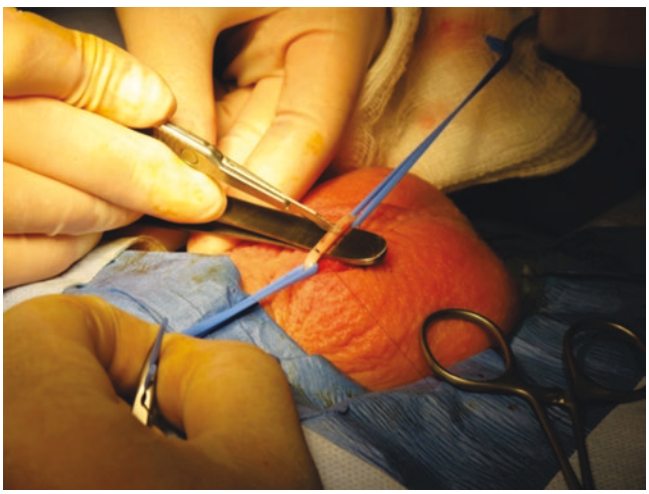

Fig. 16.10 Stay sutures of 5-0 Biosyn or PDS are placed superficially into the seromuscular layer of the vasal ends approximately 1 cm from the intended transection site. The ends are then transected with a scalpel

a backboard for cutting (Fig. 16.10). We recommend the use of a fresh 15-blade scalpel each time. A commercially available vas approximator clamp or small vascular clamp is then used to control the vasal ends and bring them into close proximity to each other just outside the incision. Finally, a solid, high-contrast backing is placed beneath both the vasal ends and the vas approximator clamp to improve visualization of the sutures and provide support during the microscopic anastomosis (Fig. 16.11). This can be easily fashioned from plastic available in the OR, e.g., light handle or diathermy holster.

The anastomosis is then performed under operating microscope magnification in a standard fashion. We begin by placing 4 × 10-0 double-armed nylon sutures through the mucosal and smooth muscle layer anteriorly in an inside out fashion on both vasa. Once all four anterior sutures are placed, they are tied. The second anterior layer is then completed by placing 3 × 9-0 single-armed nylon sutures between the tied 10-0 sutures incorporating the seromuscular layers only. Once the 9-0 sutures are tied, the vas approximator is rotated to expose the posterior wall of the vasa. The patency of the two vasal lumens is easy to assess visually under magnification and can also be confirmed by gentle probing with a jeweler forceps. Two to three additional 10-0 nylon sutures are then placed through the posterior mucosal layer depending on luminal size disparity between the two ends. Once they are tied, three additional 9-0 nylon seromuscular sutures are placed between the 10-0 sutures to complete the two-layer anastomosis. As previously mentioned, the 5-0 stay sutures can be tied loosely together to prevent tension on the anastomosis, or they can be removed at this point.

The vas is then returned to the scrotum and the operating microscope removed from the operative field. Hemostasis of the skin edges and dartos muscle is managed with electrocautery. Typically, only one stitch is needed for closure. The opening in the skin is typically 8–10 mm in length (Fig. 16.12). Postoperatively, a local incision block is performed using 5 cm^3 of 0.25% bupivacaine on each side. All patients are discharged home the same day with a prescription for 20 tablets of mild analgesia and are counseled to use a scrotal support for 7 days, refrain from sexual intercourse for 2 weeks, and avoid strenuous exercise and heavy lifting for 3 weeks. Office follow-up is arranged for 4 weeks postoperatively and semen analysis is arranged for around 3 months postoperatively and then every 3 months until pregnancy is achieved.

For primary or redo vasectomy reversals, the mini-incision approach is technically feasible in the majority of

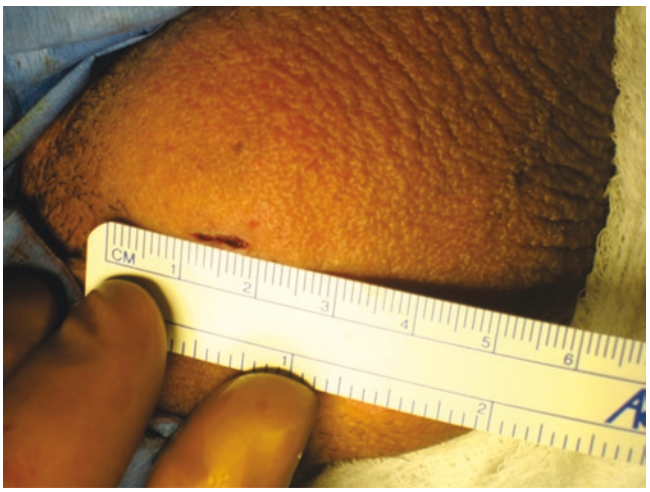

Fig. 16.12 Final incision length of <1 cm

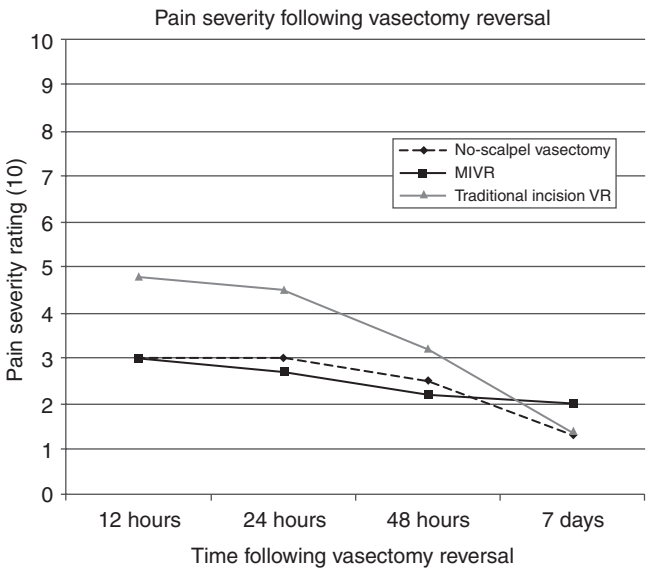

Fig. 16.13 Pain severity during the first 48 h following surgery was less among patients who received a bilateral MIVR compared to patients who received a traditional incision VR

men. Rarely, extensive scarring or very distal defects may preclude the use of the mini-incision technique.

16.6 Outcome of Mini-incision Vasectomy Reversal

Using a single surgeon's data, 164 consecutive vasectomy reversals from 2004 to 2010 were reviewed [9]. All patients were followed up 4 weeks after surgery. Patients were asked to quantify the number of days required for return of work and resumption of daily activities after surgery. Postoperative complications were recorded. Pain scores were documented using a validated post-vasectomy pain scale subsequently adapted to vasectomy reversals [10]. Semen analysis was also carried out at 2 and 4 months postoperatively and evaluated according to WHO 1992 criteria [11].

Of the 164 men, 139 underwent bilateral vasectomy reversal with 55% having a mini-incision technique. The patency rate for the mini-incision technique was 96% and was not statistically different from the patency rate of men who had undergone the traditional incision vasectomy reversal. Mean semen parameters also did not differ between the two incisions.

Fifty-three men completed the pain and recovery assessment including 20 men who underwent mini-incision vasectomy reversal. Reported pain severity in the mini-incision group was significantly less during the first 48 hours after surgery, compared to men who underwent vasectomy reversal using the traditional incision. By 1 week, there was no statistically significant difference in pain scores (Fig. 16.13).

Following the mini-incision vasectomy reversal, patients returned to self-reported "normal everyday activities" 2 days earlier compared to men following traditional incision vasectomy reversal. Time to return to work however was not different between the two groups and averaged 5 days for both.

To date over 3000 mini-incision vasectomy reversals have been performed by three different surgeons at the University of Toronto and is the preferred technique (>98%) for vasovasostomy.

16.7 Single Mini-incision Vasectomy Reversal (SMIVR)

Our newer modification of the above technique. For certain patients with a compliant scrotum and a short, favorably sited vasectomy defect, the vasa can be manipulated centrally allowing both VVs to be performed through a single midline incision (Fig. 16.14).

In a single surgeon's series of 320 patients [12, 13], the outcomes were comparable to bilateral MIVR. When comparing postoperative pain, 120 patients with SMIVR were compared to 200 with BMIVR. SMIVR patients reported significantly less pain immediately post-surgery and up to 1 week post-surgery. SMIVR patients reported quicker complete pain resolution, shorter duration of analgesic usage, and a faster return to work [13] (Fig. 16.15).

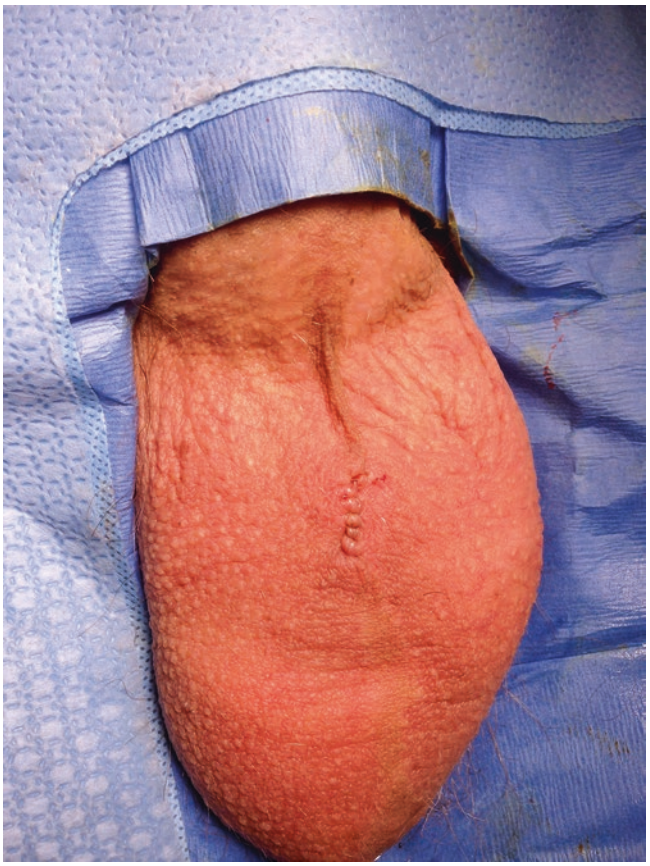

Fig. 16.14 Single mini-incision vasectomy reversal

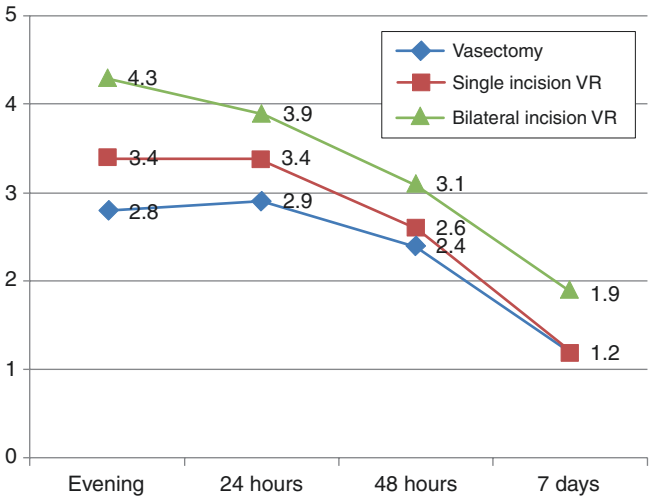

Fig. 16.15 Pain scores and functional recovery

16.8 Conclusion

Surgical techniques remain in constant evolution and vasectomy reversal is no exception. Mini-incision vasectomy reversal (MIVR) takes advantage of scrotal wall compliance and readily available surgical instruments. By avoiding delivery of the testicle and the associated tissue dissection, there are significant gains in reduction of postoperative pain and early return to normal activity. This is achieved without compromising outcome. Single mini-incision vasectomy reversal may be a viable alternative in certain patients with favorable anatomy.

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Office-Based Microsurgery Under Local Anesthesia for Male Infertility

17

Amir Toussi and Landon W. Trost

Key Points

- Male infertility procedures can safely be performed in the office setting without compromising outcomes and offer many advantages over general and monitored anesthesia such as reduced costs, faster recovery, convenience, real-time communication with the patient, and avoidance of complications from general anesthesia.
- MESA, TESE, and varicocelectomy can improve or provide sperm with better DNA fragmentation rates, resulting in overall enhanced outcomes with assisted reproductive techniques.
- Several modifications to practice are required to adequately perform procedures such as MESA/TESE, varicocelectomy, and vasectomy reversal in the office.
- The learning curve toward having an office-based male infertility surgical practice may be overcome via a slow transition from the OR to the office setting.
- The significant cost reduction in office-based vasectomy reversal reopens the debate and provides further support for cost-effectiveness over in vitro fertilization.

17.1 Introduction

The majority of urologic procedures involve intra-abdominal organs and require general anesthesia. However, infertility procedures are a unique subset of urologic procedures revolving around the external genitalia where pain can be fully controlled with local anesthesia and thus offer the ability to perform these procedures in the office.

Office-based procedures offer many potential advantages over general or monitored sedation, including elimination of risk of pulmonary or cardiac complications, absence of postoperative extended recovery, ability to communicate with the patient during the procedure, improved cost-effectiveness, greater efficiency, avoidance of side effects from general anesthetic medications, and improved convenience for patients and surgeons, among others.

In the office, cost is significantly reduced where the added expense of an anesthesiologist, ancillary staff, and facility fees are eliminated. As our healthcare transitions to value-based care, cost efficiency has become an important consideration in clinical practice, particularly in the setting of declining insurance reimbursements. This is especially the case with male infertility surgery, where the decision to proceed is often based on deductibles and insurance coverage rather than medical necessity alone. Cost reduction is also important in male infertility as most procedures are cash pay and couples are sometimes left choosing less effective options to limit costs.

Despite rapid development and significant improvements in assisted reproductive technology (ART), live-birth rates are suboptimal in many settings [1]. In order to improve treatment outcomes, recent research delving into molecular pathophysiology of male factors has identified sperm DNA fragmentation index (SDFi) as a critical contributing factor to successful live-births. SDFi represents the degree of DNA breaks present in sperm and varies from one sperm to another. Several intrinsic and extrinsic factors such as defective maturation, oxidative stress (varicoceles), lifestyle factors, prolonged stasis during epididymal transit, and systemic insults (diabetes, infection, cancer) have been linked to pathogenesis of increased SDFi [2, 3]. Clinically, high SDFi (>30%) is

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associated with longer time to achieve natural pregnancy, lower fertility potential, decreased pregnancy and delivery rates after intrauterine insemination (IUI), and decreased pregnancy rates with in vitro fertilization (IVF) [4–6]. Many treatment options have been proposed for high SDFi, and two relevant ones include varicocelectomy to reduce oxidative stress and retrieval of sperm directly from the testicles (testicular sperm extraction [TESE]) for use with ART. A recent meta-analysis revealed a 3.4% reduction in SDFi after varicocelectomy, which translated to higher natural and ART pregnancies [7]. In addition, SDFi is three- to fivefold lower in testicular sperm compared to ejaculated sperm, and a higher live-birth rate has been reported with testicular sperm over ejaculated sperm [8, 9]. Offering both of these procedures in the office can significant reduce the financial and time burden from couples undergoing ART.

Despite these advantages, there are limited data available on office-based male infertility procedures. The procedures described in these articles include hydrocelectomy, malleable penile prosthesis, microepididymal and testicular sperm aspiration (MESA/TESE), orchiectomy, spermatocelectomy, and varicocelectomy [1–15]. However, many of these were done with IV sedation or monitored anesthesia care and/or limited to underdeveloped countries [10–18].

This chapter is intended to describe our technique for commonly utilized infertility procedures in the clinic setting with a detailed focus on clinical setup, anesthetic application, description of the procedures including the complex vasoepididymostomy (VE), pitfalls, and troubleshooting.

17.2 Clinical Setup

17.2.1 Procedure Suite

The office procedure room is equipped with an operating microscope and a standard OR table. Two nurses were available during the majority of the procedure; one nurse handles and transfers the sterile instruments and assists during the procedure and the other nurse charts and handles any nonsterile equipment. No anesthesiologists are involved. The instruments, sutures, and equipment are identical between the office-based procedure room and the OR for all cases. With the patient's consent, the partners of the patient were allowed to be present and observe.

17.2.2 Pain Control

Prior to beginning the procedure, the patients are offered an oral pain medication (oxycodone 5 mg and acetaminophen 500 mg) and midazolam (5–15 mg depending on age and body habitus). This type of oral sedation is considered to be light anesthesia and is considered safe without need for anesthesia monitoring. In contrast, IV sedation with midazolam is considered moderate sedation, with most institutions requiring greater oversight for administration.

Patients receiving any form of sedation are required to have a driver prior to medication administration. For longer cases such as vasectomy reversal (VR), the patients are offered an additional 5–10 mg of midazolam 90 minutes into the procedure if the effects of the first dose had resolved or if desired. In general, the purpose of these medications is to help alleviate the anxiety and minimize bother associated with lying flat for extended periods rather than to enhance pain control.

Liposomal bupivacaine is the preferred medication for achieving optimal local pain control in our experience for several reasons. It is commercially available in 20 mL vials, and it can be diluted with normal saline to achieve a final volume of 80 mL without compromising efficacy. In addition, there is no period of delay required after its application, and it has a lasting analgesic effect for up to 72 hours (although most commonly <36 hours in our experience). In cases of MESA and TESE, it is applied to the incision (5–10 mL) and testicular cord (10 mL) on one or both sides. Varicoceles receive an additional 5–10 mL in the cord and external inguinal canal directly. For VR, 5 mL is used on the incision, 5–10 mL on each vas, and 5–10 mL on each testicular cord. The location and volumes of anesthetic administered are shown in Fig. 17.1. Judicious use is important and

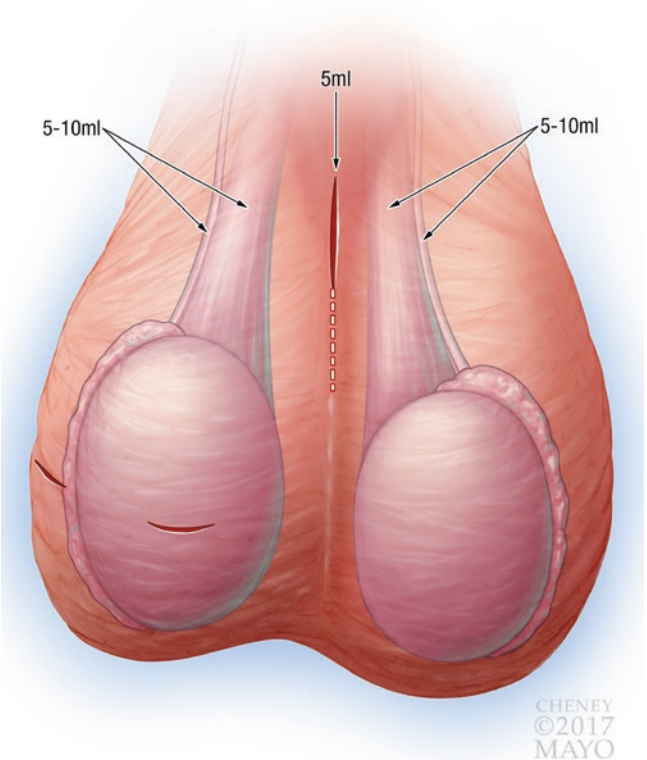

Fig. 17.1 Location and volumes of anesthetic administered. (Reprinted from Alom et al. [29] . With permission from AME Publishing Company)

Table 17.1 Medication and recommended dosing

	Dosing	Comments
Systemic[a]		
Antibiotics	Ciprofloxacin 500 mg	Prior to start of procedure. If allergic, replace with Bactrim or Cefdinir At the start of procedure
Pain control	Acetaminophen 500 mg Oxycodone 5 mg	At the start of procedure
Anxiolytic	Midazolam 5–15 mg	At the start of procedure. Age > 50 = 10 mg; age > 65 = 5 mg
Local[b]		
TESE/MESA	5–10 mL to the incision 10 mL to the cord	–
Varicocelectomy	5–10 mL to the incision 10 mL to the cord 5–10 mL to the external inguinal canal	–
VR	5–10 mL to the incision 5–10 mL to each vas 5–10 mL to each cord	–

[a]Patients without a driver are not offered oxycodone or midazolam
[b]Liposomal bupivacaine is diluted to a final volume of 80 mL using normal saline

it is preferred to have ≥20 mL remaining after all initial blocks are performed in case additional application is needed. The medication dosing and administration are summarized in Table 17.1.

17.3 Technical Description of Procedures

These procedures have previously been described in detail in the surgical literature, and our focus in this section entails modifications to enable performance in office setting under local anesthesia. See Fig. 17.2 for graphical depiction of suggested location of incisions.

17.3.1 TESE

After local anesthetic, a midline or transverse scrotal incision is made and extended for 1–2 cm. The testicle is grasped and rotated to position the epididymis posteriorly. The tunica vaginalis is incised and stay sutures are placed on each side of the incision to provide retraction. Two horizontal, parallel stay sutures are placed into the tunica albuginea of the testicle to elevate the testicle to the wound surface, and a 1 cm incision is made between the two sutures

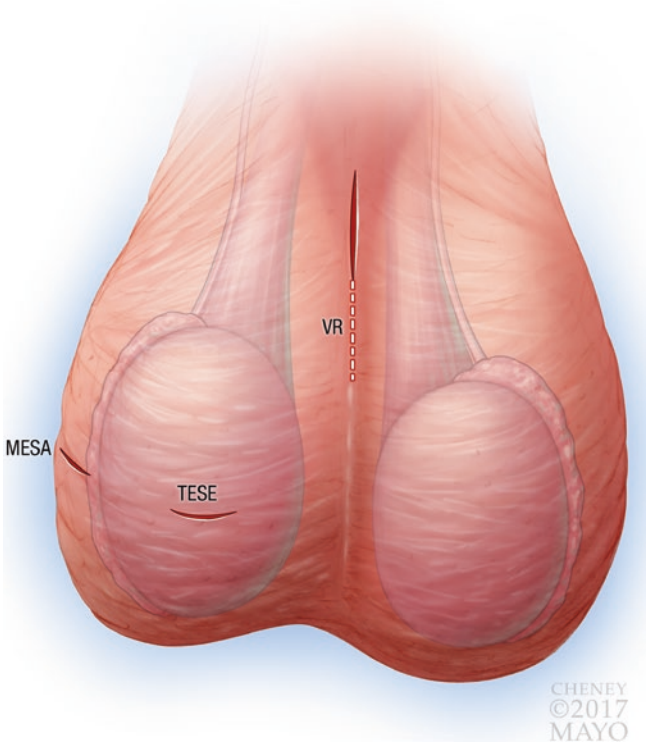

Fig. 17.2 Graphical depiction of suggested location of incisions. (Reprinted from Alom et al. [29]. With permission from AME Publishing Company)

(Fig. 17.3). The tunica is undermined using sharp scissors for 1 cm beyond the incision radially in all directions, and seminiferous tubules are expressed and excised. The tunica albuginea and vaginalis are sequentially closed by tying down the pre-placed sutures. We prefer this approach as opposed to the traditional approach of delivering the testicle to avoid the discomfort and pressure during replacement of the testicle back into its normal anatomic position within the scrotum.

17.3.2 MESA

Although traditionally MESA is performed where the testicle is delivered, we have elected to utilize a more minimally invasive approach without the need to deliver the testicle.

Following administration of the local anesthetic, a transverse scrotal incision is made on the lateral aspect of the scrotum. Once the epididymis is visualized, parallel stay sutures are placed on each side of the epididymis (Fig. 17.3). An eyelid retractor can be placed into the wound to allow better visibility. Alternatively, the pre-placed sutures can be pulled up to secure and bring the selected portion of the epididymis into view. The remainder of the procedure is performed in usual fashion.

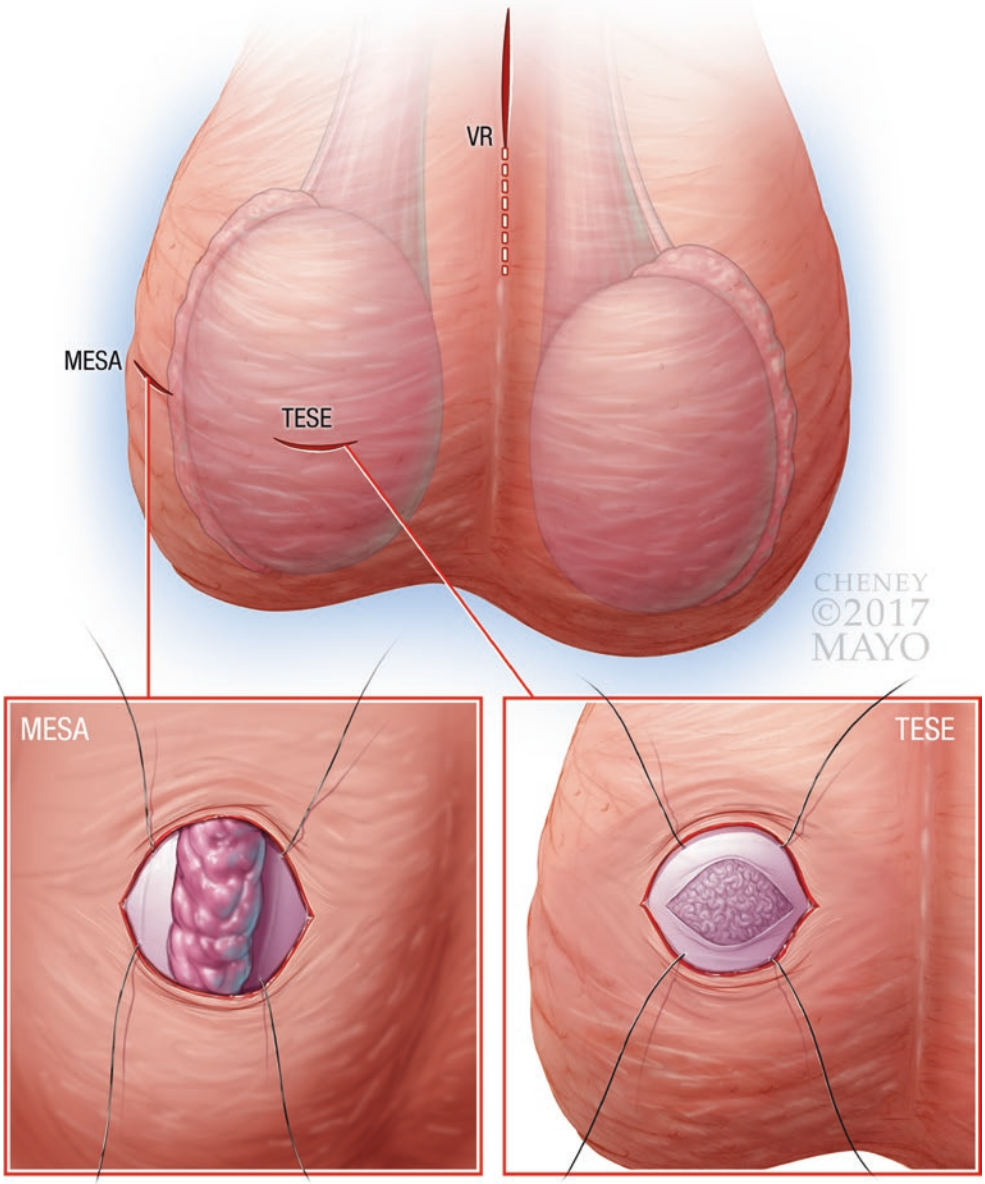

Fig. 17.3 Placement of stay sutures during TESE and MESA to facilitate exposure and closure. (Reprinted from Alom et al. [29]. With permission from AME Publishing Company)

17.3.3 Varicocelectomy

Although other approaches are feasible, we have preferred a microscopic subinguinal technique due to improved outcomes in regard to semen analysis parameters, recurrence rates, and hydrocele and hernia formation [19–24]. More importantly for an office-based setting under local anesthesia, it is associated with less intraoperative pain and faster recovery compared to the inguinal approach [25].

After application of local anesthesia, a standard transverse 2 cm skin incision overlying the external inguinal ring is made. The incision is carried through Camper's and Scarpa's fascia without incising the external oblique fascia. The spermatic cord is isolated near the pubic tubercle and gently elevated through the incision. A tongue depressor is placed under the cord as a backboard to secure it above the wound surface. The external and internal spermatic fascia are incised and opened. Using the surgical microscope, the veins are separated, doubly clipped, and divided. Care is taken to preserve the artery, and intraoperative micro-Doppler is used to confirm the artery prior to division of any structures. The cord is then returned to its bed and the incision is closed in two layers.

17.3.4 Vasectomy Reversal

Similar to TESE, after application of local anesthesia, a midline high scrotal incision is made (2–3 cm) after which

the vasa are brought through the wound. Once the vasa are isolated, the peri-vasal fascia is directly infiltrated with local anesthesia. Care must be taken to avoid extensive traction on the abdominal portion of the vas to limit sensations of flank, lower back, or inguinal pain from this maneuver. It is important, as this sensation cannot be addressed even with directed application of local anesthesia. No significant modifications are required during the dissection of the proximal and distal segments of the vasa, with the exception of granulomas, which often require additional direct administration of local numbing medication surrounding the granuloma.

Once the vasa are dissected, the testicular end of the vas is sampled to assess for presence of sperm. Fluid is also injected into the abdominal portion using a 24-gauge angiocatheter to assess patency. The volume of fluid instilled should be limited, as it may result in a burning sensation with volumes >1 mL.

If the decision is made to proceed with a vasovasostomy (VV), this is performed in the usual fashion. In our practice, we perform a double-layered anastomosis using 8-0 (outer) and 10-0 (inner) interrupted sutures without specific modifications. If a vasoepididymostomy (VE) is required, the scrotal incision is extended and the testicle is delivered. If a VE is required on both sides, it has been our preference to only deliver one testicle at a time. This limits the extent of the incision, amount of traction placed on the abdominal portion of the vas, time spent with testicle outside the body, and likelihood of venous congestion (facilitates pain control). Once the testicle is delivered, it should be kept moist by frequently dripping saline over the surface with a syringe. In our experience, the drying of the tunica sensitizes the testicle to sensations of testicular pressure, which are not easily managed with local numbing medicine. Similarly, care should be taken to avoid compressing the testicle during microsurgery, and gentle manipulation should be used to return it to the scrotum after completion of the VE. The VE is otherwise performed in usual fashion using an intussuscepted technique with parallel 10-0 sutures and 8-0 interrupted second layer. See Fig. 17.4 for graphical depiction of vasectomy reversal (vasovasostomy on the right and vasoepididymostomy on the left).

17.4 Tips and Tricks to Overcoming the Learning Curve

There is a clear learning curve in performing office-based surgical cases without monitored or general anesthesia. The critical learning points are centered on appropriate tissue handling and targeted application of local anesthetic. It is advisable that providers interested in transitioning their practice to an office-based setting consider performing these procedures in the operating room (OR) with anesthesia on standby if needed. The providers may also elect to slowly transition from general anesthesia to monitored care and finally to oral medications alone. In this setting, if deeper anesthesia is required, it can be done without rescheduling or disrupting the procedure. Similarly, providers may wish to step-wise transition from simpler procedures (vasectomy) to progressively more complex ones: MESA/TESE, varicocelectomies, and ultimately VR.

The following represents a list of several key factors that have been identified during our learning curve in the office setting. A summary of these key factors along with troubleshooting measures is shown in Table 17.2.

- Liposomal bupivacaine acts without a period of delay following administration and, as such, immediate feedback is available from patients if numbing is adequate or if additional infiltration is required.
- In general, we prefer liposomal bupivacaine as its duration of action is required for VR over other formulations such as lidocaine that wear off too rapidly.
- Liposomal bupivacaine cannot be diluted much beyond 60–80 mL without losing efficacy in the scrotum and penis.
- The numbing technique is critical to ensure judicious use of the local anesthetic to avoid reaching toxicity limits. Therefore, we recommend utilizing a smaller volume spread over larger area rather than inserting the needle into one location and depositing a large volume. Particularly for the spermatic cord where the nerves are in a wide distribution, a fan-like infiltration with repeated advancing and withdrawing of the needle can better achieve desired numbing effects.
- The adequate numbing of the vas requires direct instillation immediately adjacent to the vas. This technique can be practiced with vasectomy procedures before transitioning to VR.
- Adequate numbing of the testicle can only control the pain with dissection of the tunica vaginalis, vas deferens, and epididymis and not the sensation with excessive pressure. To reduce this pain and sensation, gentle handling of the testicle and periodic moistening are important.
- Patients with baseline addictions to high-dose narcotics and poorly controlled anxiety are better suited for procedures under general anesthesia. Also, patients with multiple previous surgeries in the location of interest (repeated vasectomies/VR, hernia repairs) may have altered nerve distribution and dysesthesias which pose difficulties to adequate local numbing.
- For longer cases such as VR, an iPad with games or movies can be used to entertain patients. Also, allowing their partner to be present in the room can provide some distraction for the patients.

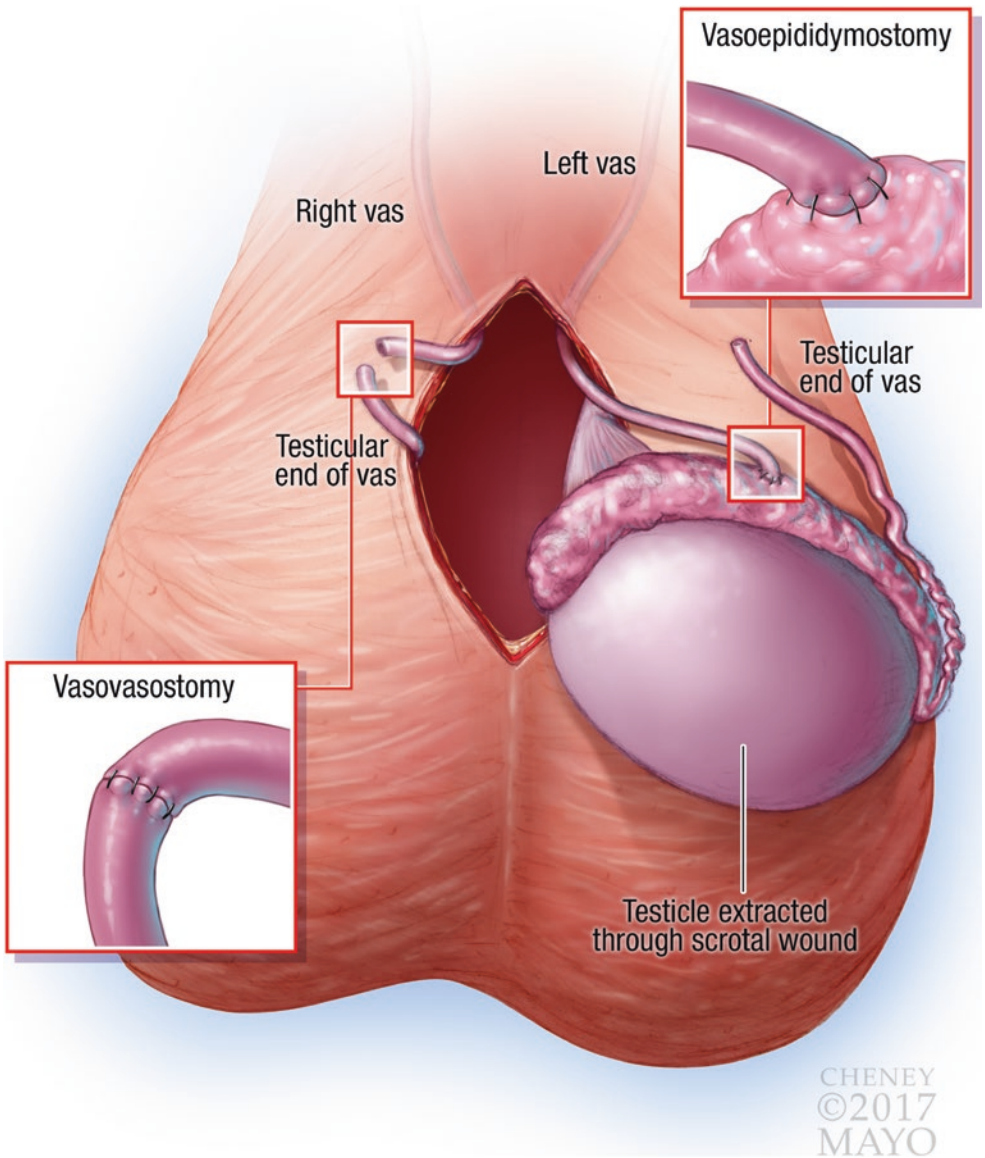

Fig. 17.4 Graphical depiction of vasectomy reversal (vasovasostomy on the right and vasoepididymostomy on the left). (Reprinted from Alom et al. [29]. With permission from AME Publishing Company)

Table 17.2 Key factors to achieving pain control and troubleshooting measures

Issue	Cause(s)	How to prevent	How to treat
Pain with grasping skin	Numbing is too deep	Visualize a "wheal" with numbing	Re-administer numbing medication more superficially
Pain with initial dissection or with grasping dartos muscle or septum	Numbing is not sufficient, is too superficial or deep, or needs to be re-applied	–	For unclear reasons, in some cases after a 3–4 hour procedure, the numbing must be re-applied to the skin prior to closure
Pain with dissecting the testicular tunica vaginalis	Cord block is inadequate (specifically, of the cremasteric fibers of the cord)	Perform a cord block[a]	Re-administer a cord block. If still insufficient, directly apply a small volume to the location where dissection will occur
Testicular (sharp) pain	Cord block is inadequate (specifically of the inner cremasteric sheath contents)	Perform cord block[a]	Re-administer a cord block. If still insufficient, directly apply a small volume (1 mL) inside the testicle
Testicular pressure	Drying of the testicle, compression of the testicle, grasping the tunica albuginea, vascular congestion	Limit pressure on the testicle, keep testicle moist throughout case, limit crushing of tunica with pickup instruments, assure adequate incision size to limit vascular congestion	Eliminate contributing factor(s)

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[a]Cord block is performed by injecting the testicular cord using a fan-like, and in-and-out motion, with small amounts of medication administered to a larger number of locations. The vas deferens should be included within the cord to assure that the entirety of the cord has been adequately treated

- Prior to any significant portions of the case, patients should be adequately warned about notable sensations that will be upcoming such as application of the numbing medication, grasping of the scrotum, pulling on the abdominal portion of the vas, and replacing the testicle back in the scrotum.

17.5 Financial Considerations

In our practice, the introduction of office-based procedures has permitted same-day surgeries, reduced costs for patients (office-based VR offered for $4550), and most importantly has not compromised technique or outcomes. The issue of cost saving is particularly relevant in the field of infertility, as many therapies are not covered by insurance and couples are often left making decisions based on cost rather than success of treatment modalities. Thus, transitioning infertility procedures to the office may increase access for a subset of patients who may have otherwise been excluded.

Although cost reductions are evident with all office-based procedures, the most significant one clinically is likely VR. Multiple publications have addressed the cost-effectiveness of VR versus IVF, and the preferred decision is complex and relies on factors such as indirect vs. direct costs, age of female partner, duration since vasectomy, success rates of specific VR surgeon and IVF clinic, duration of follow-up used to define success, rates of multiple gestations, potential for increased chromosomal abnormalities and subsequent care required, issues of diminished ovarian reserve, and couple preferences [26–28]. We have previously reported a 62% reduction with an office-based VR at our institution, which reopens the debate and provides further support for cost-effectiveness of VR over IVF in many scenarios [29].

17.6 Conclusions

Office-based surgical procedures performed under local anesthesia are a viable, safe, and cost-effective option for men seeking infertility procedures. We have offered technical modifications and key points to overcome the learning curve that may allow a smoother transition for surgeons interested in utilizing male infertility procedures to the office setting. This transition offers patients convenience, reduced risk with general/monitored anesthesia, increased availability, and allows for a faster recovery. Furthermore, the improved cost-effectiveness and emerging data on SDFi reopens debates on VR versus IVF as well as MESA/TESE and argues for a new debate on optimal cost-based treatment algorithms.

17.7 Review Criteria

An extensive search of studies examining office-based infertility procedures was performed using search engines such as PubMed, Google, and MEDLINE. The overall strategy for study identification and data extraction was based on the following keywords: “male infertility,” “cost-effectiveness,” “MESA,” “TESE,” “varicocelectomy,” “DNA fragmentation,” “office,” and “local anesthesia.” Only articles published in the English language were considered.

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Robotic Microsurgery for Male Infertility and Chronic Orchialgia

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Key Points

- Robotic assistance during microsurgery provides microsurgeons with many advantages: improved operative efficiency, elimination of tremor, scaling of motion, and enhanced imaging.
- Improved clinical efficiency appears likely with robotic assistance, and preliminary studies appear to support this concept.
- Novel treatment options for men with chronic testicular or groin pain are now available with this technology.
- Structured evidence-based platforms for the scientific development of this technology are necessary to protect patient safety. Groups such as RAMSES may provide guidance.

18.1 Introduction

Since the use of the operating microscope for microsurgery in 1975 [1], there has been a steady increase in the use of such technology in the operative management of male infertility and chronic testicular or groin pain [1–11]. Added to the reports relating to greater patency rates and fertility rates of vasovasostomy performed with the operating microscope [12], the concepts of magnification have been successfully applied to vasoepididymostomy and varicocele ligation. More recently, microscopic spermatic cord neurolysis has demonstrated applicability to the treatment of groin and testicular discomfort [13, 14]. These techniques require varying degrees of microsurgical skills and an array of supporting technology, neither of which may be part of many urologists' personal or technical armamentarium. The melding of improved visualization with magnification to an ergonomic platform that can be operated remotely has a significant application to testicular and reproductive surgery. Robotic assistance during surgical procedures has been utilized in a wide array of surgical fields with the abovementioned benefits [15–19]. This chapter covers the latest developments in the robotic microsurgical platform, robotic microsurgical tools, and current evaluations of various robotic microsurgical applications for male infertility and patients with chronic testicular or groin pain.

18.2 Novel Equipment

With any new field, the development of novel tools or instruments that can enable surgeons to create new solutions for existing clinical needs is of paramount importance. Below are some new products that enhance the ability to perform robotic-assisted microsurgery.

18.3 New Robotic Surgical Platform

Intuitive Surgical (Sunnyvale, CA) now offers an enhanced four-arm da Vinci-type Si robotic system with high-definition digital visual magnification that allows for greater magnification than the standard robotic system (up to 10–15×). The enhanced magnification capability allows the surgeon to position the camera 6–7 cm away from the operative field to avoid any local tissue effects from the heat emitted from the camera lighting (this was a problem with the older system, where the camera had to be placed within 2–3 cm of the operative field for microsurgery). This new system allows greater range of motion and better microsurgical instrument handling. The additional fourth arm has improved range of motion and positioning capabilities to provide the microsurgeon with one additional tool during procedures. The robot is positioned from the right side of the patient for microsurgical cases as illustrated in Fig. 18.1.

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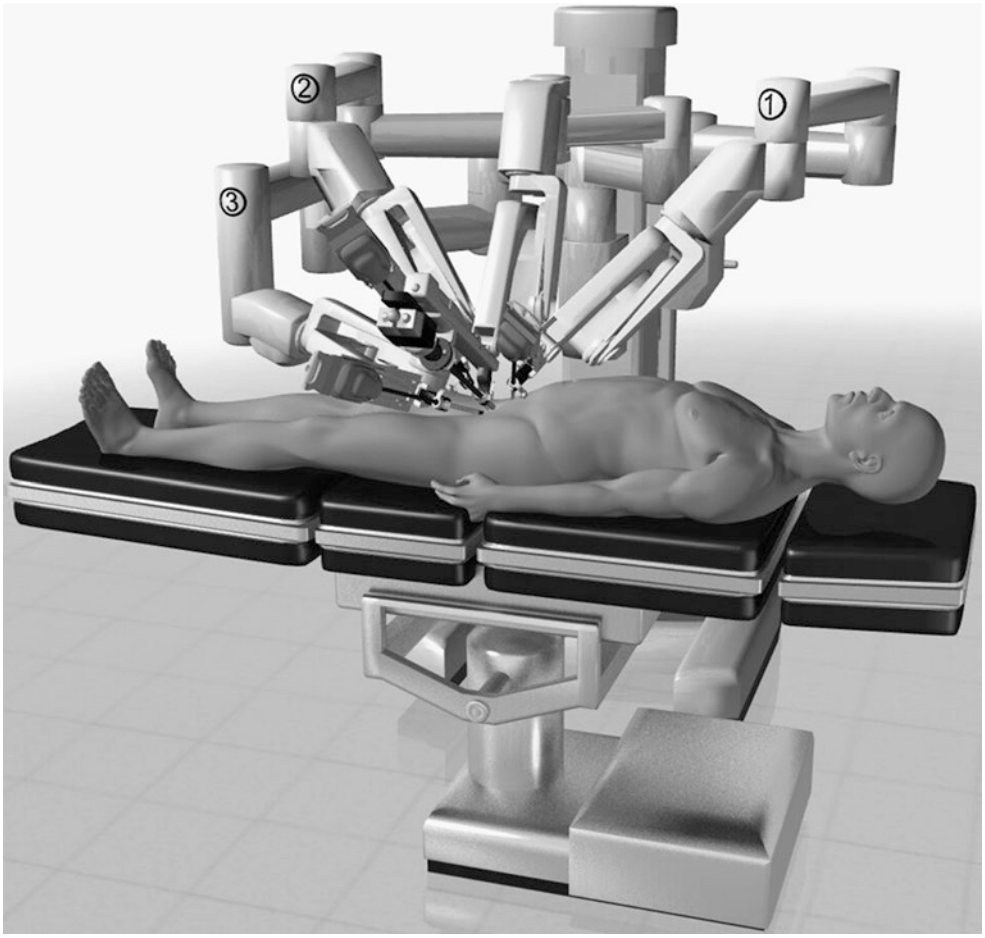

Fig. 18.1 Robotic platform positioning for microsurgical cases

18.4 Refined Robotic Doppler Flow Probe

Cocuzza et al. [20] have shown that the systematic use of intraoperative vascular Doppler ultrasound during microsurgical subinguinal varicocelectomy improves precise identification and preservation of testicular blood supply. During robotic microsurgical cases, the standard Doppler probe has to be held by a surgical assistant and cannot be manipulated readily with the robotic graspers. A new revised micro-Doppler flow probe (MDP) has been developed by Vascular Technology Inc. (Nashua, NH) that is designed specifically for use with the robotic platform (Fig. 18.2). This new probe allows for easy manipulation of the probe with the fourth arm and allows the surgeon to perform real-time Doppler monitoring of the testicular artery during cases such as robotic-assisted microscopic varicocelectomy (RAVx) and robotic-assisted microscopic denervation of the spermatic cord (RMDSC). This allows the surgeon to hear the testicular artery flow while dissecting out the veins and nerves with the other two robotic arms.

A recent prospective randomized controlled trial of the MDP was performed in 273 robotic microsurgical cases from July 2009 to September 2010: 67 robotic subinguinal varicocelectomies (RVx) and 206 robotic spermatic cord denervation procedures (RMDSC). The use of the MDP was randomized to 5 RVx and 20 RMDSC procedures. The primary endpoint was operative time, and secondary endpoint was surgeon ease in testicular artery localization and robotic grasper maneuverability. Operative duration was not affected

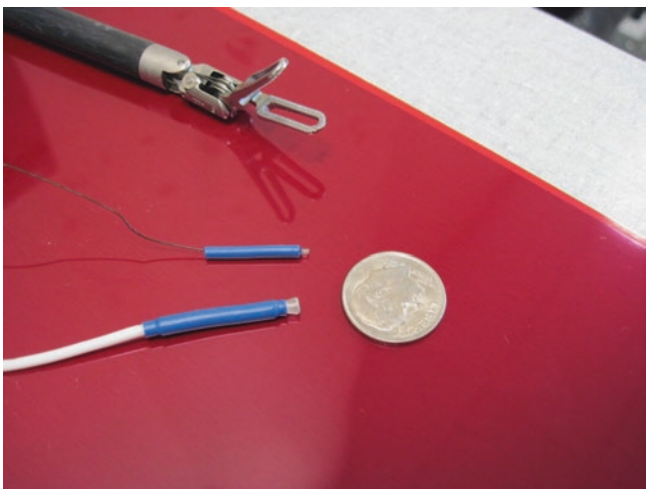

Fig. 18.2 Robotic micro-Doppler probe

by utilization of the MPD ($p=0.5$). The MDP was effective in identifying all testicular arteries within the spermatic cord in all cases. Due to the compact size of the MDP, maneuverability using the robotic grasper was significantly improved over the standard handheld Doppler probe. MDP allowed for full range of motion of the robotic arms allowing the surgeon to easily scan vessels from a wide range of angles. No complications from use of the MDP occurred. The new micro-Doppler probe for robotic microsurgical procedures appears to have performed effectively in this study.

Vascular Technology Inc. (Nashua, NH) has recently developed an even smaller microprobe that can detect flow through vessels at about 0.5-mm diameter (Fig. 18.2). This just expands further potential applications for this technology.

18.5 Enhanced Digital Visual Magnification

The miniaturization and development of advanced digital microscopic cameras (100–250×) allow even greater magnification than the standard robotic (10–15×) and microscopic (10–20×) magnification in use at this time. Our group is currently involved in clinical trials of a 100× digital camera (Digital Inc., China) that can be utilized via the TilePro™ da Vinci Si robotic system (Intuitive Surgical, Sunnyvale, CA) to allow the surgeon to toggle or use simultaneous 100× and 10–15× visualization. This provides the surgeon with unparalleled visual acuity for complex microsurgical procedures.

Karl Storz (El Segundo, CA) also offers a robotic arm platform to hold an optical mini-scope that offers 16–20× magnification that can then be used during the da Vinci robotic cases to provide an additional enhanced magnification view (routed through the da Vinci console).

18.6 Robotic Microsurgical Procedures

18.6.1 Robotic-Assisted Microscopic Vasectomy Reversal

A number of groups have developed robotic-assisted techniques to perform robotic-assisted microscopic vasectomy reversal (RAVV) in animal and ex vivo human models [21–25]. Some studies suggest that robotic-assisted reversal may have advantages over microsurgical reversal in terms of ease of performing the procedure and improved patency rates [23, 24]. A few groups have performed human robotic-assisted vasovasostomies using the initial da Vinci robotic system [26] (Intuitive Surgical, Sunnyvale, CA).

These efforts have been confirmed in human RAVV cases performed using the new da Vinci Si system [27, 28]. Our group performed a prospective control study comparing RAVV and robotic-assisted vasoepididymostomy (RAVE) to standard microsurgical vasovasostomy (MVV) and vasoepididymostomy (MVE) [29, 30]. Between August 2007 and February 2012, 155 vasectomy reversal cases were performed by a single fellowship-trained microsurgeon. The primary endpoint was operative duration. The secondary endpoint was total motile sperm count at 2, 5, 9, and 12 months postoperatively [30]. Case breakdown was as follows: 110 with robotic assistance, 45 pure microsurgical, 66 bilateral RAVV, 44 RAVE on at least one side, 28 bilateral MVV, and 17 MVE on at least one side. Selection of approach (robotic vs. pure microscopic) was based on patient choice. Preoperative patient characteristics were similar in both groups. The same suture materials and suturing techniques (two-layer 10-0 and 9-0 nylon anastomosis for RAVV; 10-0 nylon double-armed longitudinal intussusception technique for RAVE) were used in both approaches.

Ninety-six percent patency was achieved in the RAVV cases and 80% in MVV (>1 million sperm/ejaculate). There was a statistically significant difference in patency rates between the two groups ($p = 0.02$). Pregnancy rates (within 1 year post-op) did not differ significantly for the two groups: 65% for the RAVV and 55% for the MVV. Operative duration (skin to skin) started at 150–180 min initially for the first 10 cases for RAVV, but median operative duration was significantly decreased in RAVV at 97 min (40–180) compared to MVV at 120 min (60–180), $p = 0.0003$. RAVE at 120 min (60–180) was significantly faster than MVE at 150 min (120–240), $p = 0.0008$. Suture breakage and needle bending reduced significantly after the first 10 RAVV cases. Mean postoperative total motile sperm counts were not significantly higher in RAVV/RAVE versus MVV/MVE, but the rate of postoperative sperm count recovery was significantly greater in RAVV/RAVE. Similar outcomes have been reported by other groups as well [31, 32].

Further evaluation and longer follow-up are needed to assess its clinical potential and the true cost–benefit ratio.

18.6.2 Robotic-Assisted Microscopic Varicocelectomy

Although reports of robotic-assisted laparoscopic intra-abdominal varicocelectomy have been published [33], there are a number of publications that suggest that microscopic subinguinal varicocelectomy (MVx) may provide superior outcomes compared to intra-abdominal varicocelectomy [34–37]. Shu et al. were the first to publish on robotic-assisted microsurgical subinguinal varicocelectomy (RAVx) [38]. They compared standard microsurgical to robotic-assisted varicocelectomy and found that the robotic approach provided advantages in terms of slightly decreasing operative duration and complete elimination of surgeon tremor.

To further explore these findings, we performed a prospective randomized controlled trial of MVx to RAVx in a canine varicocele model by a fellowship-trained microsurgeon. The surgeon performed cord dissection and ligation of three veins with 3-0 silk ties. Twelve canine varicocelectomies were randomized into two arms of six: MVV versus RAVx. Procedure duration, vessel injury, and knot failures were recorded. The RAVx mean duration (9.5 min) was significantly faster than MVV (12 min), $p=0.04$. The duration for robot setup and microscope setup was not significantly different. There were no vessel injuries or knot failures in either group.

A review of our prospective clinical database of 97 RAVx cases from June 2008 to September 2010 (median follow-up of 11 months: range 1–27) is as follows. The median duration per side was 30 min (10–80). Indications for the procedure were the presence of a grade 2 or 3 varicocele and the following conditions: 10 with azoospermia, 42 with oligozoospermia, and 49 with testicular pain (with or without oligozoospermia, and failed all other conservative treatment options). Three-month follow-up was available for 81 patients: 75% with oligozoospermia had a significant improvement in sperm count or motility; one with azoospermia was converted to oligospermia. For testicular pain, 92% had complete resolution of pain (targeted neurolysis of the spermatic cord had been performed in addition to varicocelectomy). One recurrence or persistence of a varicocele occurred (by physical and ultrasound exam), one patient developed a small postoperative hydrocele, and two patients had small postoperative scrotal hematomas (treated conservatively). The fourth robotic arm allowed the surgeon to control one additional instrument during the cases decreasing reliance on the microsurgical assistant. The fourth arm also enabled the surgeon to perform real-time intraoperative Doppler mapping of the testicular arteries while dissecting the veins with the other arms if needed.

McCullough et al. [39] recently published at large series review of 258 cases with similar outcomes to the pure microsurgical technique. Robotic-assisted microsurgical subinguinal varicocelectomy appears to be safe, feasible, and efficient. The preliminary human results appear promising. Further evaluation and comparative effectiveness studies are warranted.

18.6.3 Robotic-Assisted Microscopic Denervation of the Spermatic Cord

Recent studies by Levine [13] and Oliveira et al. [14] have shown that microscopic denervation of the spermatic cord is an effective treatment option for men with chronic testicular pain. Our group has been developing a robotic-assisted microsurgical approach for the denervation of the spermatic cord (RMDSC) to assess if there may be any potential benefit over the standard microscopic technique.

Our group recently published a retrospective review of 872 cases (772 patients) who underwent RMDSC from October 2007 to July 2016 [40]. Selection criteria were as follows: chronic testicular pain (>3 months), failed conservative treatments, negative neurologic and urologic workup, and temporary resolution of pain with local anesthetic spermatic cord block. RMDSC was performed. Pain was assessed preoperatively and postoperatively using the subjective visual analog scale (VAS) and objectively with the standardized validated pain score (PIQ-6 by RAND). Follow-up data was available in 860 cases. Over a median follow-up of 24 months (1–70), 83% (718 cases) had a significant reduction in pain and 17% (142 cases) had no change in pain by subjective VAS scoring. Within the patients who had significant reduction in pain, 49% (426 cases) had complete resolution and 34% (292 cases) had a ≥50% reduction in pain. Objective PIQ-6 analysis showed a significant reduction in pain: 67% at 6 months, 68% at 1 year, 77% at 2 years, 86% at 3 years, and 83% at 4 years post-op.

RMDSC is an effective, minimally invasive approach with potential long-term durability for patients with refractory chronic orchialgia.

18.7 Single Port and Abdominal Robotic Microsurgical Neurolysis

Chronic groin pain can be debilitating for patients. Microsurgical subinguinal denervation of the spermatic cord (MDSC) is a treatment option for this pain. However, there are limited further options for patients who fail this treatment or who have phantom pain after orchiectomy. Our goal was to develop a single port and abdominal robotic microsurgical neurolysis technique to ligate the genitofemoral and inferior hypogastric nerve fibers within the abdomen above the internal inguinal ring.

We performed a prospective study of patients with chronic groin pain who either had failed previous MDSC or had phantom pain after orchiectomy. Primary endpoint was impact of pain on quality of life (PIQ-6 pain impact questionnaire from RAND) and secondary endpoint was operative robotic duration. PIQ-6 scores were collected pre-op and at 1, 3, 6, and 12 months post-op.

We completed 30 cases (five single ports) from June 2009 to September 2010. Elimination of pain occurred in 60% (18 cases), and a greater than 50% reduction in pain occurred in an additional 13% (4 cases) within 1 month post-op. Two of the failures were patients that had pain elimination for 6 months, but then pain returned thereafter. Median OR duration was 10 min (5–30). There were three complica-

tions: (i) one post-op scrotal hematoma that resolved with conservative measures, (ii) one patient had pain at one of the port sites, and (iii) one patient had pain that shifted from the groin to the leg. Single port and abdominal robotic microsurgical neurolysis appears to be an option for treatment in this difficult patient population. Further follow-up and evaluation are warranted.

18.8 Conclusion

The use of robotic assistance during microsurgical procedures is expanding. The application of this technology in other microsurgery fields apart from urology is also expanding, such as ophthalmology, hand surgery, and plastic and reconstructive microsurgery. The advantages of a stable microsurgical platform, ergonomic surgeon instrument controls, elimination of tremor, and magnified immersive 3D vision are all intuitively apparent. Further comparative effectiveness studies are ongoing and will be forthcoming on the true applicability of this new surgical platform. However, the preliminary results so far are quite impressive.

18.9 Review Criteria

A search of studies on robotic-assisted microsurgery in andrology was performed using search engines such as ScienceDirect, OVID, Google Scholar, PubMed, and MEDLINE. The overall strategy for study identification and data extraction was based on the following keywords: "robotic vasectomy reversal," "robotic varicocelectomy," "robotic denervation," "infertile men," "varicocelectomy," "vasectomy reversal," and "infertility." Articles published in languages other than English were also considered. Data that were solely published in conference or meeting proceedings, websites, or books were not included.

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Robotic Vasectomy Reversal: An American Perspective

19

Parviz Keikhosrow Kavoussi

Abbreviations

ASA	Antisperm antibody
CT	Computed tomography
FDA	Food and Drug Administration
FSH	Follicle-stimulating hormone
hCG	Human chorionic gonadotropin
RAVE	Robot-assisted vasoepididymostomy
RAVR	Robot-assisted vasectomy reversal
RAVV	Robot-assisted vasovasostomy
TURP	Transurethral resection of prostate
VE	Vasoepididymostomy
VR	Vasectomy reversal
VV	Vasovasostomy

Key Points

- Regardless of microsurgical or robot-assisted microsurgical approach to vasectomy reversal, it is paramount to have an in-depth knowledge of the surgical anatomy for acceptable outcomes.
- The anastomosis should be performed with a minimal touch, watertight, tension-free manner for optimal patency rates.
- Advantages to robot assistance: elimination of tremor, improved stability, seven degrees of freedom instrumentation, scalability of motion, 3D/HD visualization, manipulating three instruments and camera simultaneously, and faster times after learning curve met.
- Data show comparable outcomes with robotic vasectomy reversal compared to microsurgical vasectomy reversal with improved anastomosis and operative times once the learning curve is met and advantages including ergonomics and decreased fatigue.
- The learning curve for robot-assisted vasovasostomy reveals high-percentage patency rates very early across wide intervals of obstructive intervals, and 75 cases were required to optimize and plateau in anastomosis times and operative times.

19.1 Introduction

Approximately half a million men in the United States undergo vasectomy annually. Approximately 6% of men in the United States who have undergone vasectomy will choose to undergo vasectomy reversal (VR) [1] sometime in their lifetime, which is contributed to by the 50% divorce rate [2, 3]. VR was initially described in the 1930s and a significant improvement in patency rates was achieved with the use of the operative microscope for magnification to assist in the anastomosis, which was applied in the 1970s [4–6].

Americans have been dreaming of robots for decades. Regardless of if robots were dreamed up for science fiction or for practical purposes, the idea of robots mechanically assisting us with tasks has been a goal. Society has imagined robots performing different tasks to assist humans, and the idea that someday robots would do surgery for us was one of these tasks with great interest. That has not necessarily come to fruition; however, the use of robotics has advanced in assisting surgeons with procedures. The da Vinci® system was initially developed for use for gross surgical procedures and has advanced its use to the microsurgical platform. In male fertility surgery, this particularly lends itself to VR. VR was initially attempted without any technology and simply

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with the use of the naked eye and then evolved through numerous levels of technology starting with optical loupes, advancing to the use of operative microscopes, and now has progressed to the use of the robotic platform. The gain of seven degrees of freedom from the robotic endowrists allows for movements beyond those that the hand and wrist of a human being can make, scalability of motion, a less fatiguing ergonomic design for the surgeon which may enhance performance, and high-definition three-dimensional optimal visualization of a microsurgical field. After mastering the learning curve for robotic microsurgery, operative times improve as well.

19.2 History of Robotics in Surgery

Since 1942, when Isaac Asimov initially used the word "robotics" in his short story "Runaround," the ideas and imaginations of what robots may be able to do have captivated our society. This is especially true in the field of medicine in which robots have been portrayed in every form and medium including science fiction and cinematic films. The reality of robotics in medicine was developed in the mid-1980s when the PUMA system was developed in the United Kingdom for computed tomography (CT)-guided brain biopsy and for transurethral resection of the prostate (TURP) [7]. In 1992 the PROBOT was developed in London for TURP, with the ability to be programmed to resect based on specific prostate volumes [8]. In 1992 the ROBODOC utilized CT imaging programmed for the individual orthopedic patient to mill out precise fittings in the femur for hip replacements, which was developed in Germany [9]. The PAKY (Percutaneous Access to the Kidney) robotic arm system was developed at Johns Hopkins in 1997 [10–12]. In the 1990s in Germany, the ARTEMIS (Advanced Robotic Telemanipulator for Minimally Invasive Surgery) was engineered, which was the first master-slave manipulator system with instrumentation with six degrees of freedom. It was designed for cardiac surgery but was discontinued due to lack of funding [13]. In 1994 the AESOP (Automated Endoscopic System for Optimal Positioning) was developed in the United States which maneuvered laparoscopic trocars by surgeon voice commands. HERMES was the integrated operating room control system that allowed the complete integration of the robotic system, and in 2001, the ZEUS robotic system was introduced which combined the AESOP and HERMES systems as a master-slave device with a surgeon in a console controlling a separate robotic device [14, 15]. These robotic devices led up to the advent of the standard of care robotic device currently used in clinical surgical practice, the da Vinci® system developed by Intuitive Surgical.

19.3 Da Vinci® Robotic System

In the mid-1990s, the founders of Intuitive Surgical developed the da Vinci® robot system. The device included three primary components: a master-slave software driven system which controlled robotic instruments with seven degrees of freedom, a visualization system with three dimensions, and a safety monitoring system (Fig. 19.1) which is sensor-based to continuously reassess the performance of the device to optimize patient safety [16]. In 1997 the first robot-assisted surgery, a cholecystectomy, was performed by Jacques Himpens and Guy Cardiere in Brussels [17]. The Food and Drug Administration (FDA) approved the da Vinci® robotic system in 2000 with the components including a surgeon's console, a patient-side robotic cart with four arms manipulated by the surgeon, and the visualization system. The current da Vinci® robotic system offers three-dimensional, high-definition digital visual magnification up to 10–15× with instrumentation with seven degrees of freedom and scalability of motion.

19.4 Technology Hype (Gartner-Palmer Tech Hype Curve)

When a new technology is introduced in any field, especially surgery, the technology must be assessed and proven. Along with any technology, whether it is a smart phone, an electric vehicle, or a robotic surgical device, there tends to be a great deal of initial hype, a period of underachievement of expectations, and then a realization of what the technology can actually offer. The Gartner-Palmer tech hype curve can be applied to the da Vinci® robotic system. The hype cycle involves a technology trigger which gains media coverage and publicity. This is followed by a peak of inflated expectations, i.e., this device can do everything! This is followed by the trough of disillusion when the technology fails to deliver on certain expectations. This is followed by the slope of enlightenment as the benefit of the technology becomes more widely understood. Finally, the plateau of productivity is reached as there is more mainstream adoption of the technology and the applicability and relevance become clear (Fig. 19.2). This hype cycle was clearly evident with the advent of the da Vinci® robotic platform as the original design was for telesurgery for battlefield and remote environments and then became honed to be more useful for minimally invasive surgery. It may be argued that we are in a similar hype cycle with the application of robotics to microsurgery, especially with use for robot-assisted microsurgical vasectomy reversal (RAVR) [18].

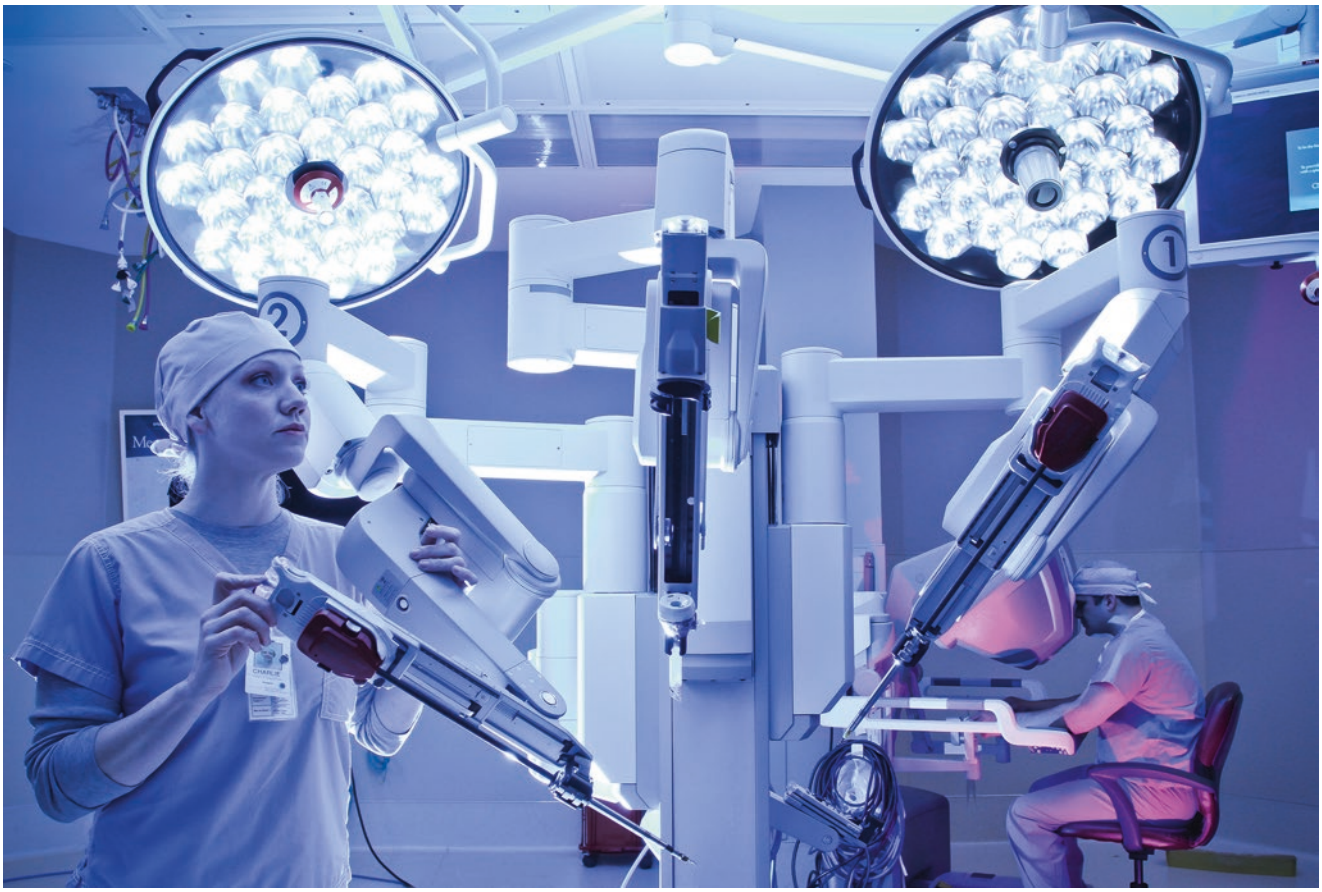

Fig. 19.1 Components of the da Vinci® robotic system including the surgeon console (background) and the bedside robotic cart demonstrating the robotic arms (foreground)

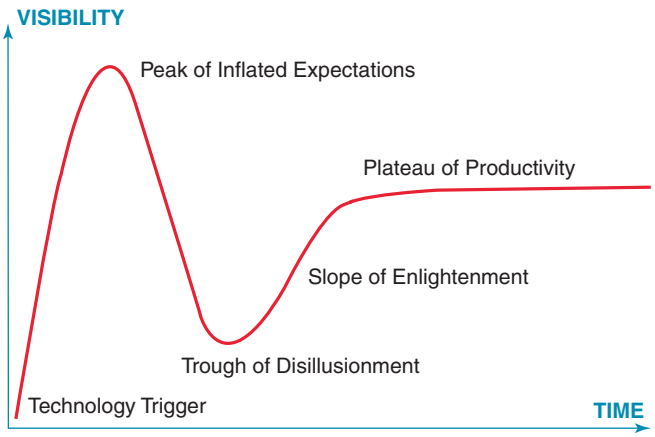

Fig. 19.2 The Gartner-Palmer tech hype curve demonstrating the technology trigger, the peak of inflated expectations, the trough of disillusionment, the slope of enlightenment, and the plateau of productivity

19.5 Anatomy

For successful outcomes of VR, regardless of whether it is performed as a RAVR, or with a pure microsurgical technique, the surgeons' understanding of the anatomy of the vas deferens and epididymis is paramount. The vas deferens extends from the distal end of the cauda of the epididymis. The mesonephric (Wolffian) duct is the embryological origin of the vas deferens. The vas deferens is a tubular hollow structure. The convoluted vas deferens is the tortuous segment exiting the epididymis for the first two to three centimeters of the vas deferens. From the cauda epididymis to the ejaculatory duct, where the vas deferens terminates, it measures between 30 and 35 cm in length. The vas deferens travels posterior to the spermatic cord vessels. The diameter of the lumen of the vas deferens varies between 0.2 and

0.7 mm depending on the segment of the vas deferens [19]. The primary arterial supply of the vas deferens is a branch off of the superior vesical artery known as the deferential artery [20]. The deferential vein is the venous drainage for the scrotal vas deferens which drains into the pampiniform plexus. The pelvic vas deferens' venous drainage is via the pelvic venous plexus. The lymphatic drainage of the vas deferens travels to the internal and external iliac lymph nodes.

Sperm reach the vas deferens by traveling through the epididymis from the testis. If the tightly coiled tubular epididymis was stretched out, the length would reach 12–15 feet. Three regions compose the epididymis including the caput (head), the corpus (body), and the cauda (tail). The caput epididymis is made up of 8–12 ductuli efferentes from the testis. The vas deferens is continuous with the most distal portion of the cauda epididymis. The caput and corpus epididymis receive their arterial supply from a branch of the testicular artery, which divides distally to supply the superior and inferior epididymal branches [21]. Arterial supply to the cauda epididymis is from branches from the deferential artery. Both cauda and corpus epididymis' venous drainage is through the vena marginalis of Haberer, which by means of the vena marginalis of the testis or through cremasteric or deferential veins drains into the pampiniform plexus [21]. The caput and corpus epididymis' lymphatic drainage are through channels that travel with the internal spermatic vein, draining to the preaortic nodes. The cauda epididymis' lymphatic channels join those leaving the vas deferens to drain into the external iliac nodes.

19.6 Preoperative Evaluation/Physical Examination

The level of spermatogenesis in the patient should be assessed prior to proceeding with RAVR. A proven history of fertility from before to the vasectomy is considered adequate. Prior to RAVR a complete physical examination with special attention to the genital examination should be performed. Testicular volumes and consistency should be assessed. Testicles with normal volume and firm consistency are indicators of good spermatogenesis, whereas small or soft testes may indicate spermatogenic dysfunction. The epididymis will commonly feel dilated on examination in men who have undergone vasectomy. Epididymal induration may indicate a level of epididymal obstruction and the need for robot-assisted vasoepididymostomy (RAVE). Counseling for a more extensive dissection to perform a tension-free anastomosis should be considered when a large vasectomy defect or gap is palpated on exam. When a sperm granuloma can be palpated at the testicular end of the vas deferens, it is associated with improved outcomes with VR. The sperm granuloma forms from leakage of sperm as a result of a pop-off valve-like mechanism decreasing intra-epididymal luminal pressures to protect the ductal system [22]. The surgeon should be prepared for a nonstandard incision approach when the scrotal exam reveals a very long vasectomy defect [23]. As hypogonadism has become a very common diagnosis, even in men during their fertility years, testosterone replacement therapy is being utilized to treat these men with an adverse impact on spermatogenesis. Men who plan on VR need an alternate medical regimen for treatment of hypogonadism in a manner that does not suppress spermatogenesis. Discontinuation of testosterone replacement with the addition of testicular salvage medical therapy with Clomiphene Citrate or human chorionic gonadotropin (hCG) should be administered for at least 3 months prior to RAVR for acceptable outcomes [24].

The timeframe since the vasectomy, known as the obstructive interval, plays a significant role on the type of RAVR required, robot-assisted vasovasostomy (RAVV) versus RAVE. Patency rates have been demonstrated to be impacted by the obstructive interval in many studies on VR, and generally the longer the interval since vasectomy, the more challenging the candidate is considered to be for VR [25, 26]. However, an experienced surgeon proficient in both RAVV and RAVE should still be able to offer RAVR to men with longer obstructive intervals. Patency rates remain high regardless of the type of VR performed in men with obstructive intervals over 10 years in technically skilled hands [27]. Several nomograms have attempted to predict the complexity of the VR required and patency success rates. These outcomes were assessed by factors including age of the men, the volume of the testicles, the presence or absence of a sperm granuloma, and the time interval since the vasectomy was performed [28, 29]. Inconsistent data has raised questions about the accuracy of the nomograms and their true predictive ability [30, 31]. Therefore, the recommendation is that VR only be performed by surgeons skilled in both VV and VE, as it is not typically possible to predict preoperatively if VE will be required [32, 33].

The patient's female partner should undergo a fertility evaluation with counseling on age-related impact on female fertility and ovarian reserve impact on the couple's fertility potential, prior to proceeding with VR [23, 34].

19.7 Preoperative Laboratory Testing

Although it is not considered to be common clinical practice, prior to VR, a semen analysis including an evaluation of the centrifuged pellet may be performed. Whole sperm is found in the centrifuged pellet 10% of the time, indicating improved outcomes suggesting that sperm will be encountered in the vas deferens at least unilaterally at the time of VR [35]. Transrectal ultrasound should be performed to assess the

possibility of a concomitant ejaculatory duct obstruction if a low semen volume is found during the semen analysis.

A serum follicle-stimulating hormone (FSH) should be obtained when there are indicators of a potential deficiency in spermatogenesis on physical examination, such as small, soft testicles. As an elevated FSH is a marker of spermatogenic dysfunction and a potentially poor outcome with VR, higher levels of assisted reproductive care may be warranted [36]. Serum antisperm antibody (ASA) testing is not recommended as a part of the routine preoperative evaluation prior to VR. Circulating ASAs are detectable in approximately 60% of men after bilateral vasectomy [37]. There is no proven value in testing ASAs preoperatively, and the impact of circulating ASAs on fecundability is at the very least questionable considering the high conception rate in couples following VR [1, 38–44].

19.8 Anesthesia

Local, regional, or general anesthesia may be administered for RAVR [23]. General anesthesia is the anesthesia of choice to minimize patient movement and to optimize patient comfort considering the level of meticulous tissue handling with RAVR for optimal results. Although it is possible to perform RAVR with local anesthesia with sedation, this results in more difficulty achieving optimal outcomes due to more patient motion. Local anesthesia or sedation poses even more of a challenge when a difficult anastomosis or a RAVE must be performed with longer operative times.

19.9 Positioning the Patient and the Robot

The patient is positioned supine on the operative table. After general endotracheal anesthesia is induced, all pressure points are padded, and a timeout is performed. The scrotum is shaved, prepared, and draped in a sterile fashion. After the vasa are prepared for the anastomosis, the operative robot is positioned from the patient's right side at a 90-degree angle with the zero degree camera placed directly over the operative field, perpendicular to the floor, once the vasa have been prepared and approximated for the anastomosis (Fig. 19.3).

19.10 Incision Approaches

The most direct access to the vasectomy defect is through a scrotal incision to isolate the ends of the vasa deferentia for anastomoses. In cases with high vasectomy defects or lengthy stretches of unusable vas or lengthy vas defects, the incision may be carried in the direction of the external inguinal ring. The testes may be delivered from the scrotum in cases where the vasectomy defect is in the proximal convoluted vas or adjacent to the epididymal cauda or when it is necessary to perform a RAVE. Mini-incision VR using no-scalpel vasectomy principles has been shown to have comparable patency rates with less postoperative pain and faster functional recovery [45, 46]. The author's approach of choice is performing RAVR through a mini-incision, using one longitudinal incision for bilateral anastomoses which is less than 1 cm in its entirety.

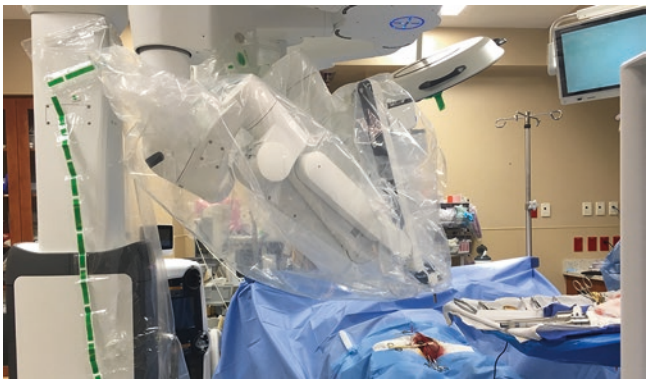

Fig. 19.3 Positioning of the robotic system at a 90° angle to the patient with the camera positioned directly above the operative field (view from console)

The surgical approach to RAVR will vary in men who have previously undergone varicocele repair or inguinal hernia repair. In men who have had a varicocele repair for hypogonadism or orchialgia with a vasectomy simultaneously, access through the previous incision after confirming that the vasectomy was performed subinguinally by previous operative report should be used for a subinguinal RAVR. When there is suspicion for a vas deferens obstruction from previous inguinal herniorrhaphy, performing the RAVR through the scar from the herniorrhaphy will lead to the vasal obstruction. Successful intra-abdominal RAVR has been reported following herniorrhaphy. This demonstrates an optimal example of the utilization of the robotic platforms ability to improve access to a difficult vas deferens reconstruction [47, 48].

19.11 Vas Deferens Preparation

Through a scrotal incision, the vasectomy defect is palpated and isolated, and the testicular and abdominal ends of bilateral vasa are isolated with vessel loops, through one small incision. The microvasculature supply to the vas deferens should be preserved, so care should be taken to not strip the vas deferens of the perivasal adventitia (Fig. 19.4). Mobilization is performed of the testicular and abdominal ends of the vas deferens, while keeping the vasal adventitia intact, in a manner to perform a tension-free anastomosis. The vasectomy defect site where the obstruction from the

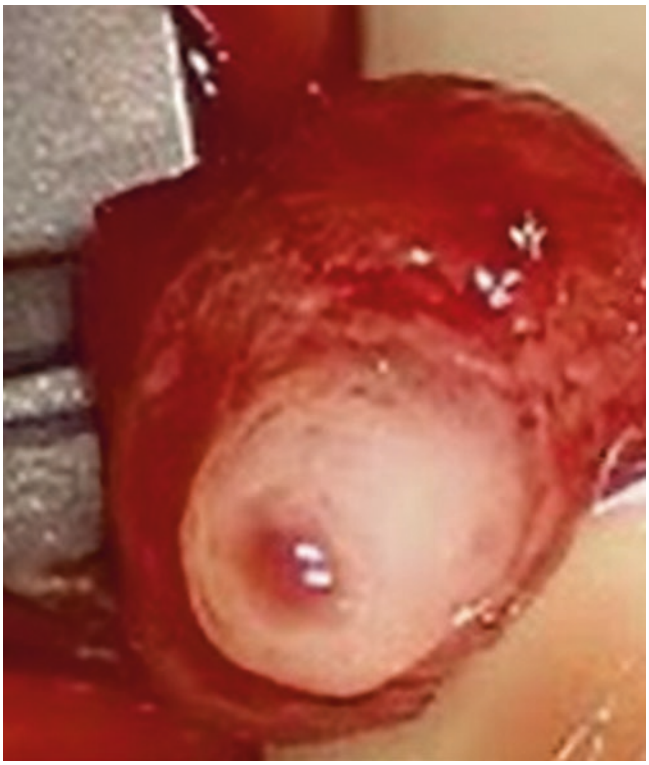

Fig. 19.4 The transected end of the vas deferens with the perivasal adventitia intact

original vasectomy is located may be excised or excluded. The deferential artery is preserved during this maneuver. Following isolation of the testicular and abdominal ends of the vas deferens, an exact 90-degree sharp division of the testicular end of the vas deferens is performed. The muscularis and the mucosa of the divided vas deferens should be inspected for a healthy anastomotic surface to minimize selecting an anastomosis site with fibrosis, scar, or an irregular edge. Vasal fluid obtained from the lumen of the testicular end of the vas deferens is applied to a glass microscope slide; the fluid is then diluted with a small amount of saline and is visualized with an inverted light microscope. The microscopic findings and the fluid quality obtained from the testicular lumen of the vas deferens indicate the need to perform a RAVV versus a RAVE. When whole sperm cells with tails are visualized microscopically (Fig. 19.5) or when copious, clear fluid from the lumen of the testicular end of the vas deferens without sperm microscopically are the findings, RAVV is the option of choice. When no fluid is present, barbotage is performed with 0.1 ml of saline injected through a 24-gauge angiocatheter sheath which is cannulated into the lumen of the testicular end of the vas deferens, and the barbotaged fluid is examined under the microscope for presence of sperm. RAVE should be performed when no

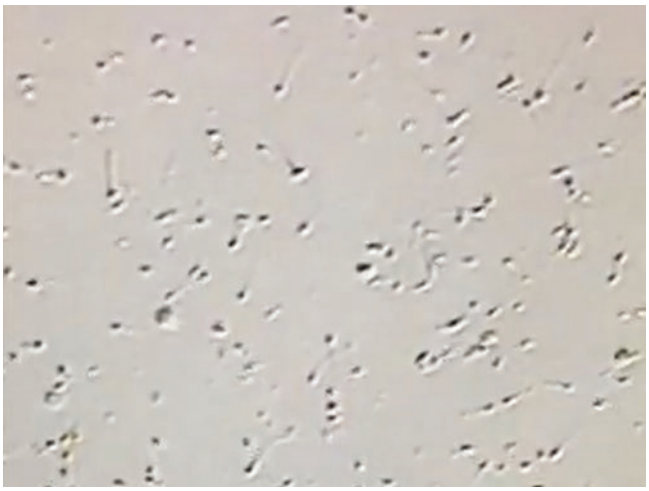

Fig. 19.5 Whole sperm visualized under the light microscope in the vasal fluid from the testicular end of the vase deferens

significant amount of vasal fluid or sperm are identified with barbotage. RAVE is also indicated when the vasal fluid appears toothpaste-like and thick and sperm are typically not found on examination of the fluid under the microscope. A patency rate of greater than 90% can be expected when fragments of sperm including sperm heads and/or short tails are visualized in the vasal fluid intraoperatively despite whether the fluid quality is good or bad. This 90% success rate exceeds that expected with VE and is the procedure of choice in this scenario [49, 50]. Vasal fluid sperm quality has been categorized in five grades. Grade 1 is categorized by visualization of motile normal sperm, grade 2 is mostly normal non-motile sperm, grade 3 is categorized by the majority of the findings being sperm heads, grade 4 is sperm heads only, and grade 5 is the complete absence of sperm or sperm fragments [51, 52].

Sharp division of the abdominal end of the vas deferens is performed at an exact 90 degrees. The lumen of the vas deferens is carefully cannulated with a 24-gauge angiocatheter sheath, and saline is injected through the lumen to demonstrate patency. Once abdominal vasal patency has been demonstrated, a Microspike approximating clamp is used to approximate the testicular and abdominal ends of the vas deferens, or they may be approximated with meticulously placed 6-0 polypropylene sutures in the adventitia of the two ends of the divided vasa to approximate the ends with an assistant's help. A metal ruler or tongue blade with a Penrose drain covering it is then positioned under the approximated ends as a template to perform the anastomosis. In instances when an attempt is made to inject saline through the lumen of the abdominal end of the vas deferens and there is high pressure or the saline does not easily flow, this indicates another site of obstruction further away in the abdominal end

of the vas deferens. By passing a 2-0 polypropylene suture gently through the lumen of the abdominal vas deferens, the site of distal obstruction can be isolated. If the obstruction is encountered within 5 cm of the initial site of obstruction where the initial vasectomy defect was found, dissection may be performed to the obstruction site which can be excised to perform a single anastomosis after adequate mobilization of both ends allows for an anastomosis which is tension-free. This often requires extending the incision. It is not recommended to perform multiple anastomoses on the vas deferens due to the increased risk for devascularization of the vas deferens resulting in failure.

19.12 Technical Aspects of Anastomosis

Robotic microsurgical skill is required for acceptable RAVR results. Improvements in outcomes are achieved by strictly adhering to microsurgical principles to perform the anastomosis. The luminal mucosa of the testicular and abdominal ends of the vasa must be perfectly approximated. A watertight anastomosis is necessary due to the inflammatory response antigenic sperm can induce with the potential to result in fibrosis and obstruction at the anastomotic site with sperm cells leaking out of the lumen in the general tissue of the scrotum [53]. Long-term success is dependent on a tension-free anastomosis. Abdominal and testicular vasa segment mobilization and placement of muscularis sutures for reinforcement can optimize long-term patency rates by minimizing tension on the anastomosis (Fig. 19.6). As little manipulation of the vasal ends with a minimal touch technique is crucial for a successful anastomosis, by not compromising microvascular supply by stripping the vas deferens of its adventitia with risk of devascularization of the vas deferens and increased chance of stricture.

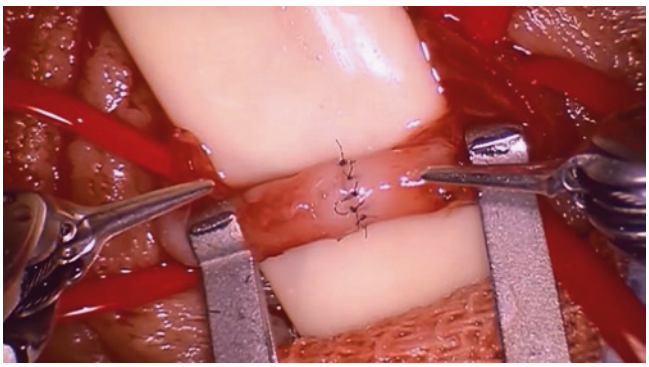

Fig. 19.6 The appearance of a watertight, tension-free anastomosis with the vasal adventitia intact upon completion of a robot-assisted vasovasostomy

19.13 The Da Vinci® Robotic Platform to Assist with Microsurgical Vasectomy Reversal

The robotic platform including a surgeon's console, a patient-side robotic cart with four arms manipulated by the surgeon, and the visualization system offers potential advantages over traditional microsurgery. These include elimination of tremor, improved stability, instrumentation with seven degrees of freedom, scalability of motion, three-dimensional/high-definition visualization, the ability for the surgeon to manipulate three surgical instruments and a camera simultaneously allowing less emphasis on a skilled microsurgical assistant, and faster anastomosis and operative times once the learning curve is met. Most notably the robotic platform has the ability to improve surgeon ergonomics and decrease surgeon fatigue. Replacing the surgical microscope with the robotic platform allows for more throughput with multiple microsurgical cases consecutively with lesser surgeon fatigue subjectively reported. This may ultimately extend the microsurgeon's longevity.

Optimal settings for the robotic platform include a 4× digital zoom, haptic zoom, a close-up working distance setting, and a three-dimensional viewer mode (Fig. 19.7).

19.14 Robot-Assisted Vasectomy Reversal

Magnification through the use of an operative microscope is the manner in which VR has traditionally been performed. More recently, da Vinci® robot assistance has been applied

Fig. 19.7 Robotic platform settings including a 4× digital zoom, haptic zoom, a close-up working distance setting, and a three-dimensional viewer mode

to VR. The use of robot assistance for VR was first applied to ex vivo human vas deferens with the findings of an absence of physiological tremor and similar patency rates revealing that the robotic platform is an option as an alternative technology to the operative microscope [54]. Next, RAVV and RAVE were shown to be feasible in the rat model with findings of stability improvement and reduction of extraneous motions during anastomotic suturing [55]. RAVV was then performed in an in vivo rabbit model with a multilayered anastomosis with similar patency rates, further progressing the potential role of the robotic platform use for microsurgery [56]. The first study published in humans who underwent RAVR revealed faster surgical times and early postoperative semen analyses demonstrating higher sperm counts than the cases performed microsurgically for VR [57]. A validating publication on RAVR in men compared to microsurgical VR revealed similar patency rates, surgical times, and sperm concentrations and total motile counts following RAVR. The average anastomosis time in the RAVV group was found to be statistically longer than the microsurgical group in the early robotic cases; this was only a 10 minutes longer average anastomosis time in the RAVR group, with arguable clinical significance [58]. The potential gains in using the operative robot for RAVR include absence of physiologic tremor, a more stable platform, ergonomic advantage for the surgeon resulting in less surgeon fatigue, motion scaling, three-dimensional high-definition real-time image, the surgeon being able to control the camera and three surgical instruments concomitantly, eliminating the need for a specialized assistant skilled in microsurgery, and the possibility of decreasing surgical times [58]. Although early studies show promise, large-scale prospective, randomized controlled trials are needed to validate the broader adoption of robot assistance for VR.

Advantages are gained for more challenging scenarios by use of the robotic platform. One of these scenarios in particular is use for intra-abdominal VR for men who have obstruction of the vas deferens secondary to a previous inguinal hernia repair or men who had a laparoscopic vasectomy performed simultaneously with another procedure [47, 48].

19.15 Robot-Assisted Microsurgical Vasovasostomy

RAVR mandates the same level of meticulous tissue handling principles utilized during microsurgical VR. The instruments used in both the left and right robotic arms are the black diamond microsurgical robotic forceps. A zero-degree camera is selected with 4× setting. A ratio of 3:1 is chosen for scalability of motion. The anastomosis for RAVV may be performed by two-layered or the author's preference of a modified one-layer anastomosis, for which four to eight sutures of interrupted 9-0 nylons are placed through the vasal lumens with full-thickness bites of each end of the vas deferens (Fig. 19.8) (Video 19.1). Microsurgical suture and needle handling techniques utilized with microsurgical instruments are applied in a similar fashion while using the robotic black diamond forceps to prevent bending needles or breaking sutures. After the transluminal sutures are placed, the muscular layer of the adventitia is approximated with interrupted 9-0 nylon sutures between the already placed full-thickness luminal sutures to decrease tension on the anastomotic sutures (Fig. 19.9) [59]. Once completed, the anastomosis should be watertight with luminal mucosal approximation and the vasal adventitia

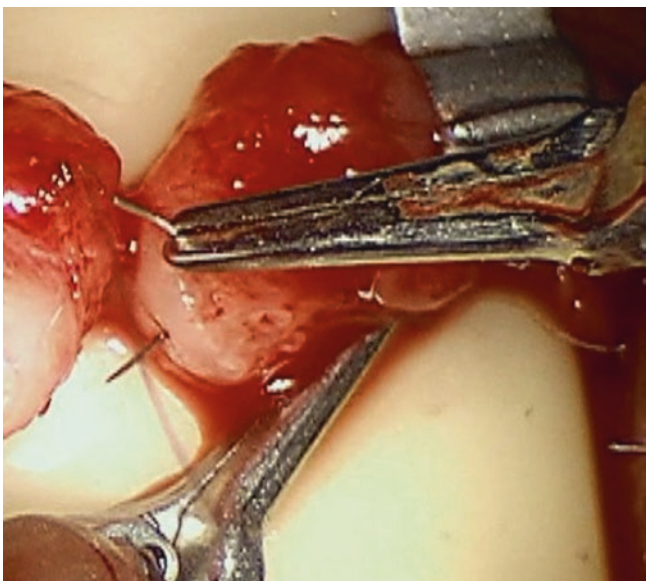

Fig. 19.8 Placement of a transluminal anastomotic suture with robot assistance

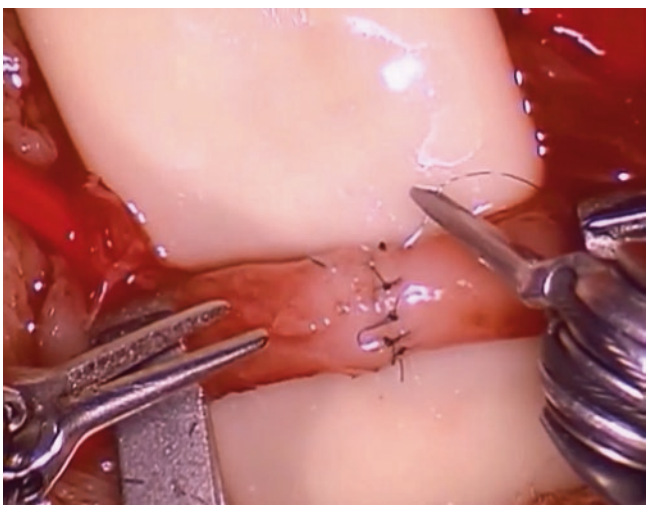

Fig. 19.9 Placement of interrupted suture in the seromuscular layer of the vas deferens between the transluminal anastomotic sutures with robot assistance

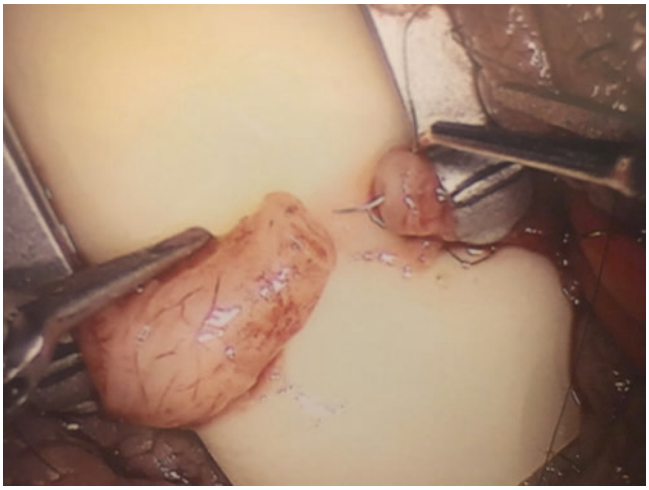

Fig. 19.10 Microscopically visible microvasculature in the adventitia of the vas deferens as a transluminal suture is being placed with robot assistance

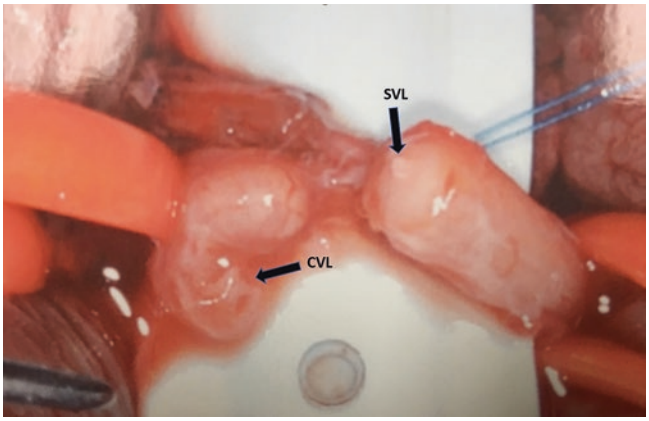

Fig. 19.11 Preparation for the anastomosis between the convoluted vas deferens and straight vas deferens lumens. The convoluted vas deferens lumen (CVL) is not concentric and is found at the end of the tortuous convoluted vas deferens. The straight vas deferens lumen (SVL) is demonstrated

should have been maintained to decrease the risk of stricture from micro devascularization (Fig. 19.10). Some surgeons have a preference for a complete two-layer anastomosis, which requires placement of five to eight interrupted 10-0 nylon sutures for approximation of the inner mucosal edges of the lumens of the vas deferens. This is followed by placement of seven to ten interrupted 9-0 nylon sutures in the muscular layer for tension-free reinforcement [52]. Robotic microsurgeons experienced in RAVV make up for the discrepancy between the diameters of the approximating lumens of the abdominal and testicular ends of the vas deferens visually while performing the anastomosis. Otherwise, placement of microdots with a marking pen with a microtip helps for planning the placement of sutures before the anastomosis is performed [60].

A successful anastomosis in the convoluted vas deferens during VV depends on extremely precise and meticulous techniques. When expertise and microsurgical principles are applied by skilled microsurgeons, patency rates are similar to those of anastomoses in the straight portion of the vas deferens, although the anastomosis in the convoluted vas deferens is technically more challenging (Fig. 19.11) [61, 62]. Most RAVVs may be performed through a single small incision, usually smaller than 1 cm longitudinally which improves the ease of recovery and is a cosmetically appealing incision with dissolvable sutures in the skin (Fig. 19.12). In difficult scenarios, a useful maneuver is the crossed trans-scrotal VV. This is used when an anastomosis of one testicular end of the vas deferens is made to the contralateral abdominal end of the vas deferens. This is useful in situations with a unilateral inguinal vas deferens obstruction associated with an atrophic testis on the contralateral side or obstruction of an inguinal vas deferens and epididymal obstruction on the contralateral side [2, 52, 63, 64]. Repeating a VR is a reasonable choice for men

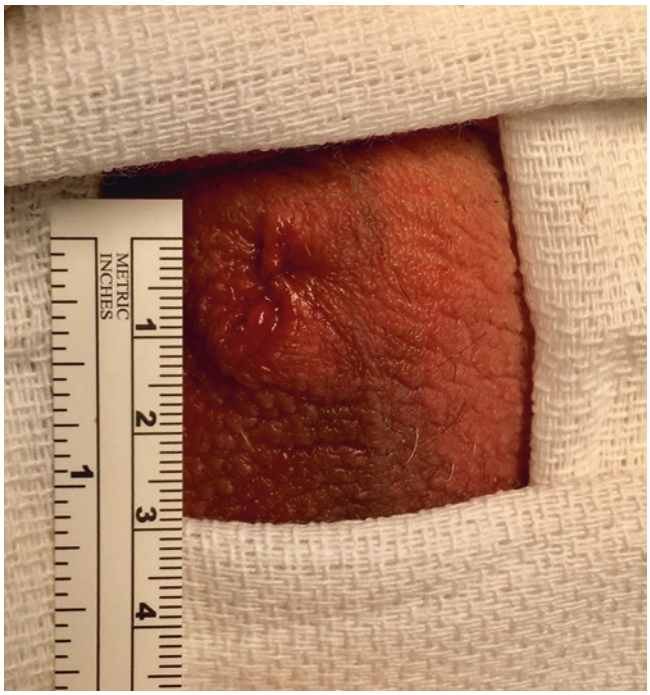

Fig. 19.12 Demonstration of a single, less than 1 cm incision after closure following bilateral robot-assisted vasovasostomies

who underwent an unsuccessful initial VR. Repeating a VR should include consideration of the time interval since vasectomy, the initial VR, and the skill and experience of the surgeon performing the redo VR [65, 66].

Postoperative patency is demonstrated by the presence of sperm in the ejaculate in up to 99.5% of men when VVs are performed after sperm is identified in the vasal fluid of at least one side [60]. Comparable patency rates have been demonstrated when comparing outcomes of one-layer VV and two-layer VV [67, 68]. Within 2 years of VR, pregnancy

rates of 52% have been reported and pregnancy rates up to 63% have been reported when excluding female factor and when based on female partner age and the time since vasectomy [22, 69–73]. Rates of spontaneous pregnancy in couples following VR are dictated by postoperative semen parameters and female factors and age of the female partner [67, 74].

19.16 Robot-Assisted Microsurgical Vasoepididymostomy

RAVE is a highly technically challenging surgery which should only be performed by skilled robotic microsurgeons with high levels of training and experience in this procedure who perform it commonly. In scenarios that require a VE, anastomoses are more likely to succeed and less technically challenging when they can be performed in the more distal tubules of the epididymis, as the wall of the epididymis progressively thickens more distally with more smooth muscle cells. Beyond the caput, the epididymis becomes a single microscopic tubule forming the corpus and cauda; therefore, blockage at any site will result in complete obstruction preventing sperm from reaching the vas deferens. Optical magnification is crucial to improve VE patency rates [75, 76].

VE has been performed in multiple manners. End-to-end and end-to side anastomoses have been used to perform VE [32, 33]. Anastomoses performed end-to-end have predominantly fallen out of favor. To perform an end-to-side VE, significantly dilated epididymal tubules are identified under magnification, and the muscularis and adventitia of the vas deferens are approximated to a specific opening in the epididymal tunica. This should be performed through a meticulous, atraumatic technique and should be relatively bloodless [33, 76–80]. A scrotal incision is made through which the testis is delivered. A vessel loop is used to isolate the vas deferens at the junction of the convoluted and straight vas deferens. The vas deferens is sharply divided at a 90-degree angle and preparation of the vas deferens is done as previously described above. The epididymis is inspected under magnification after incising the tunica vaginalis. The planned epididymal anastomotic site is identified proximal to the suspected site of obstruction, where dilated epididymal tubules are visualized. A tunical incision overlying the dilated tubules of approximately 3–4 mm is made using the tip of the robotic micro-Potts scissors. Exposure and gentle dissection of the epididymal tubules is performed. The selected dilated epididymal tubule is punctured with the sharp tip of a 10-0 nylon needle, and microscopic examination of the epididymal fluid is done to identify sperm. The vas deferens is anchored to the tunica of the epididymis with three to four 6-0 polypropylene interrupted sutures allowing for approximation of the vasal lumen to the opening of the tunica of the epididymis in a tension-free manner. With high magnification, 10-0 double-armed nylon monofilament sutures with tapered fishhook needles are utilized for approximation of the posterior mucosal edge of the epididymal tubule the posterior vasal mucosa. An additional two to four 10-0 nylon sutures are placed for the anterior mucosal anastomosis. An additional six to ten interrupted 9-0 nylon sutures approximate the outer muscularis and adventitia of the vas deferens to the incised edge of the epididymal tunica. Following completion of the anastomosis, the robotic system is undocked and the scrotal content is gently replaced into the tunica vaginalis followed by a multilayered closure of the scrotum.

The preferred technique for performing RAVE of the author is the two suture intussusceptions technique. Two needles of double-armed 10-0 nylon sutures are placed in parallel in the selected dilated tubule of the epididymis longitudinally, leaving them in place rather than pulling them through to prevent leaking of tubular fluid resulting in collapse of the epididymal tubule of interest. The sharp tip of a 10-0 nylon needle or the robotic microsurgical Potts scissor tips is used to create a longitudinal opening in the tubule between the previously placed and parallel placed needles. The epididymal fluid is examined under the visualization of an inverted light microscope for sperm. The needles of the preplaced 10-nylons are then passed inside out the vas deferens mucosa to perform the intussuscepted anastomosis (Fig. 19.13) [76, 80–82]. The muscularis and adventitia are anchored to the epididymal tunica as described above (Fig. 19.14).

Acceptable VE patency rates should be in the range 50% and 85% in the hands of experienced skilled microsurgeons [81, 83]. With techniques such as the classic end-to-side or older less frequently performed end-to-end anastomosis, respective patency and pregnancy rates of approximately 70% and 43% have been reported [75, 84].

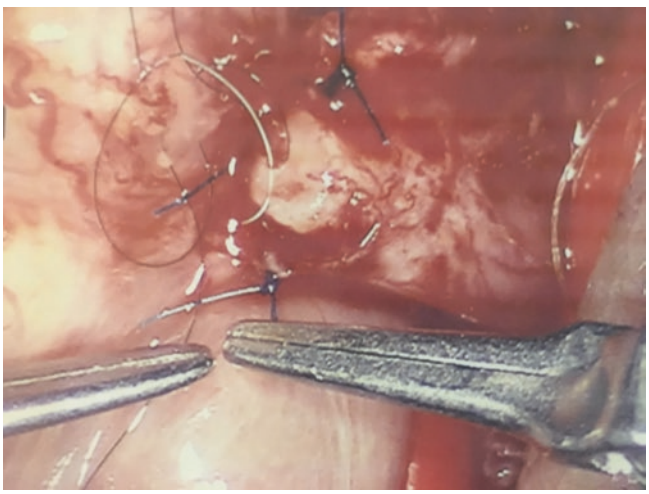

Fig. 19.13 Performing the anastomosis of a robot-assisted vasoepididymostomy with a two-suture intussuscepted technique

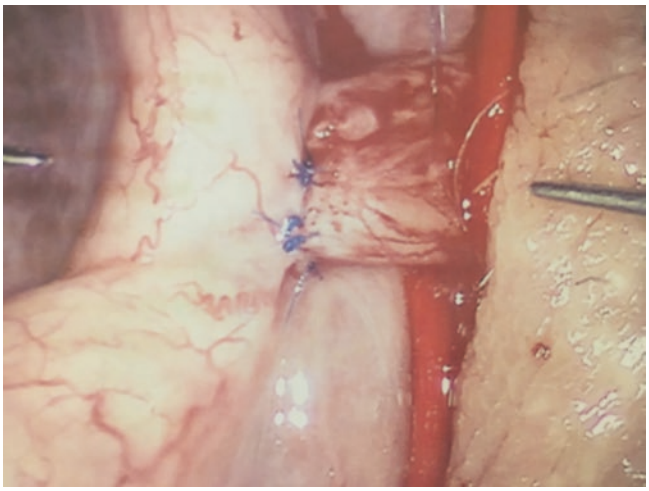

Fig. 19.14 Completed vasoepididymostomy anastomosis

The intussuscepted techniques offer high patency rates ranging between 70% and 90% [76, 80, 85].

19.17 Robot-Assisted Microsurgical Vasectomy Reversal Learning Curve

Once a new technology has been applied to a surgical procedure, and the safety and efficacy have been demonstrated, the learning curve should be assessed. A study evaluated the learning curve for a purely microsurgically fellowship-trained reproductive urologist with minimal robotic console training transitioning to RAVV for his first 100 cases of RAVV excluding RAVEs and redo operations from previous failures from other surgeons performing vasectomy reversal for uniformity. Endpoints were assessed at 25 chronologic case intervals and each group of 25 cases had similar variable obstructive intervals from very short to very long intervals since vasectomy. Patency rates were equivalent at 92% for the first three 25 case groups, and patency rates increased from 92% to 96% for the last 25 cases. Although this was not statistically significant, it may be argued whether it is clinically significant [86]. The same surgeon's anastomosis and operative times with pure microsurgical VRs were published in a previous study [45]. In comparison to the same surgeon's progression with RAVV, the operative times were faster than microsurgical operative times, even in the first 25 RAVV cases. The data revealed that excellent patency rates are feasible very early in the transition from pure microsurgical VV to RAVV across wide intervals of time since vasectomy and mean sperm concentrations in the initial semen analyses after RAVV are similar over time with more case volume. It was found that 75 RAVVs were necessary to reach optimal anastomosis times and operative times for pure microsurgeon without formal training in robot-assisted microsurgery [73].

19.18 Future of Evolution of Robot-Assisted Microsurgical Vasectomy Reversal

Future technology should include improvement of optics and instrumentation. Although the black diamond forceps which are currently the standardly used instruments for RAVR are adequate to perform microsurgery, they do not mimic the traditional microsurgical handheld instruments. Robotic instruments which replicate handheld microsurgical instruments would be optimal for advancement of robot-assisted microsurgery. The other major component would be improving magnification and decreasing pixilation at high magnification with the robotic visualization system.

19.19 Postoperative Care

Postoperatively following RAVR, the patient should be placed in a tight fitting scrotal supporter with fluff gauze. Antibiotics are not indicated postoperatively. Analgesics are offered. The majority of men are back to work 3 days following RAVR when their work is not physically strenuous. Restrictions include abstinence from sexual activity including ejaculation, avoiding strenuous activity and refraining from exercise for 3 weeks postoperatively. Semen analysis is performed at 6 weeks, 3 months, and 6 months following RAVR and then is repeated at 6-month intervals thereafter until the couple is successful conceiving or if assistance is needed to assist with conception. Failure of RAVR is clear if 6 months after RAVR the semen analysis continues to reveal azoospermia.

19.20 Complications

Rare complications of RAVR include hematoma and infection. A progressive decrease in sperm motility followed by diminishing sperm counts and ultimately azoospermia indicates a delayed stricture resulting in obstruction. After VR, delayed obstruction has been reported in 5% to 12% at 18 months postoperatively [85, 87]. As a safety backup, all men are recommended to cryopreserve sperm following RAVVR once motile spermatozoa are visualized in the semen as a precaution.

19.21 Conclusions

RAVR is a viable and effective option for patients interested in VR with potential advantages for the operating surgeon. Although there is a learning curve for operative time and anastomosis time to match those of pure microsurgical VR which is ultimately faster robotically, patency rates are similar very early in the series for microsurgeons trained in VR.

19.22 Review Criteria

An extensive search of studies on vasectomy reversal, vasovasostomy, vasoepididymostomy, and robot-assisted vasectomy reversal was performed using search engines including PubMed and Google Scholar. The start and end dates for these searches were 1938–2018, respectively.

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Robot-Assisted Vasectomy Reversal (Vasovasostomy)

20

George A. de Boccard

Key Points

- The outcome of vasectomy reversal dramatically improved with the use of an operation microscope.
- The precision of movements is increased with robotic assistance during microsurgery.
- The results of robot-assisted microsurgical vasectomy reversal are at least as good as with conventional microsurgery.
- New platforms dedicated to microsurgery with new concepts and new tools are being developed and will definitely make the difference.

20.1 Introduction

At the very end of the nineteenth and the early days of the twentieth century, vasectomy was used for eugenic and therapeutic purposes, but soon after World War I, it was adopted by liberal people, mainly workers and anarchists, seeking for sexual freedom, in Austria, Germany, France, and Spain [1]. Nowadays, this procedure is widely used as a contraceptive tool, and it is estimated that about 50 million men have relied on vasectomy for family planning. In Europe there is a great variation among countries, in the United States; vasectomy is used as contraceptive method by nearly 11% of married couples [2]. A minor indication for vasectomy is to prevent urogenital infections after prostatitis or prostatic surgery.

In the early years of the past century, Dr. Edward Martin, surgeon at the University of Pennsylvania, was the first to perform vasoepididymostomy in men who had obstruction secondary to epididymitis, but not vasectomy [3].

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The number of vasectomy reversals is rising because of the rising popularity of vasectomy combined with the continuing upward trend of divorce and remarriage, especially in industrialized countries. The younger the vasectomy is performed, the higher the percentage of reversal [4]. The first described postvasectomy reversal was performed as soon as in 1938 [5]. Most of the demand is related with the desire of a child in common in a newly formed couple, and in order to plan the more appropriate intervention to reach pregnancy, the chances of success in terms of patency of vas and pregnancy should be considered based on the experience of the surgeon combined with the access to an assisted procreation center and the experience of the team, patient's health history, age, results of physical examination, and reproductive capacity of his partner. A potential alternative is TESE or MESA for ICSI. Some men simply wish to restore a normal physical situation without any specific desire of pregnancy. In most men, vasectomy reversal is technically feasible, but success rates depend on a variety of factors determined by both male and female fertility factors [6].

Vasectomy reversal outcome has dramatically changed with the use of the operating microscope with optical magnification in 1971 by Earl Owen who performed the first microscopic vasectomy reversals. A number of procedures were then developed using various microsurgical techniques [7–9]. This chapter reviews the application of robot-assisted microsurgery for vasectomy reversal.

20.2 Robot-Assisted Vasectomy Reversal

Robotic assistance provides a number of benefits for the microsurgeon: elimination of tremor, scaling of motion, and stereoscopic magnification. The stable robotic platform with four arms eliminates the need for a microsurgical assistant. These are the main advantages for this technically challenging procedure.

Almost all centers performing vasectomy reversals are already equipped with a da Vinci robotic platform; therefore

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additional cost for a vasectomy reversal is only a few hundred dollars. Many studies have reported the results of robot-assisted vasectomy reversal in animal models and in humans: Fleming [10], Kuang et al. [11, 12], Schiff et al. [13], De Boccard [14], Parekattil et al. [15, 16], and Kavoussi [17].

20.3 Surgical Technique

The surgical technique has been previously described in detail [18]. The preparation of the vas deferens for robotic vasectomy reversal is identical to the one used for classical microsurgical technique. A scrotal median incision is made (about 3 cm in length). The previous vasectomy sites are identified, and the proximal and distal ends of the vas deferens are mobilized and exposed. The testicular side of the vas is transected, and the fluid pouring out from this end is evaluated on a slide under 200× microscope magnification for the presence of sperm cells. The inguinal vas end is flushed with saline to assess for patency. Both vasa are tested at first, and then the two ends of the right vas are marked with a long 4-0 suture and left inside the scrotum. The two ends of the left vas are now placed on the microspike approximator. A white multitubular drain is tailored and placed as a background for a better visualization of the sutures and a permanent drainage of fluids from the operating field (Fig. 20.1).

Once the vas ends have been safely installed, the arms of the robot (da Vinci Xi system, Intuitive Surgical, Sunnyvale, CA) are brought into the field. The robotic system is placed on the left side of the patient as in a robot-assisted left kidney surgery and the corresponding program is selected. The laser guidance is activated using the surgeon's thumb of the open hand as the target to position the optic trocar (0° Optic is placed in arm 2) on an imaginary line that mimics an abdominal wall approximately 20 cm over the actual operating field. Then the other instruments are placed on a row: Black Diamond microforceps in arms 1 and 3 placed with a 45° angle with the optic and Potts scissors in arm 4. A special

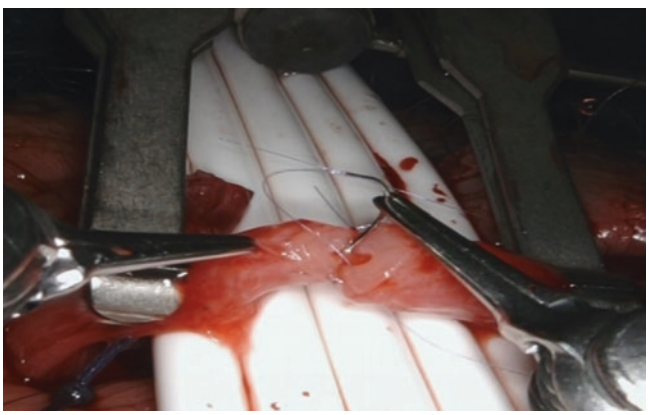

Fig. 20.1 Placing of the stiches with precision

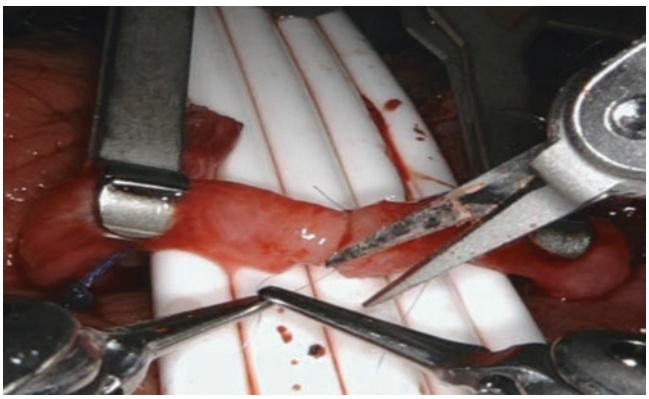

Fig. 20.2 Cutting of the threads without losing focus

attention is needed for the Potts scissors placement in order to avoid a conflict between arms 3 and 4. The scissors must appear in the field opposite of the camera. The surgeon goes then to the console. The anastomosis is now performed using polyglycolic acid (or Nylon^{R}) sutures. Two interrupted 9-0 sutures are placed posteriorly on the muscularis. Then the mucosa is sutured by six to eight 9-0 or 10-0 interrupted sutures. Finally the muscularis is secured by a corresponding number of 9-0 sutures. The use of the fourth arm with the Potts scissors improves the speed and efficiency of the procedure by maintaining a permanent focus during changing of tools (Fig. 20.2). During the anastomosis nothing should interfere with the movements, only flushing with saline in order to keep a clear view on the lumen is tolerated. After completion of the first anastomosis, the four arms are moved a few centimeter to the right of the patient, if necessary, manually by the nurse. The nurse then pulls on the 4-0 sutures marking the right vas and places its dissected end on the approximator with the white multitubular drain in the background again, and the anastomosis is performed the same way as described for the left side. At the end of the second side, the robot is pulled back. Undocking is not necessary since the trocars are not placed in the abdominal wall. The surgeon scrubs again, goes back to the field, and closes the scrotal incision with a 4-0 absorbable running suture in two layers.

Our first experiences in robot-assisted microsurgical vasectomy reversal started in 2003. Our cases between 2006 and 2009 were published previously, all 14 cases being patent after 6 months. Further publications by Parekattil et al. [16] and Kavoussi [17] demonstrated that the results of robot-assisted vasectomy reversal were at least equivalent to purely microscopic procedures.

The first advantage of the da Vinci system is its stability while placing sutures with the possibility to interrupt a movement and restart it at the very point it was left. The visibility is excellent with a stereoscopic view. The movement and zoom functions of the camera are easy without the need to

leave the tools as it is the case with many operative conventional microscopes. The movements of the arms are tridimensional allowing suturing in otherwise difficult angles with the feeling of having two right hands. The changing of tools, e.g., forceps to scissors, is done without losing focus. The forceps can keep on holding the needle while cutting the thread with the scissors. And last but not least, the surgeon sits comfortably, resting on his arms, thus avoiding many back and neck pain at the end of the day. The haptic feedback is not a concern, and it has been demonstrated that the feeling of the tension of the suture and the quality of the tissue is obtained visually and even literally felt in the hands after mastering the technique. In fact this already happens with conventional microsurgery when using smaller than 9-0 sutures.

The da Vinci surgical robotic system is already applied in almost all fields of surgery: it started with cardiac surgeons, was developed by urologists, and was soon adopted by gynecologists and general surgeon and now by plastic surgeons, ENT, and orthopedists [19]. The robot allows surgeons to significantly simplify the more complex reconstructive steps of laparoscopic and many open procedures. Almost all urological procedures are using da Vinci assistance, and we must admit that robotic technology is now the gold standard of minimally invasive surgery. And new applications for the robot are published every day as more centers report their results.

The precision of suture placement is enhanced with the elimination of the physiological tremor together with the magnification of the robotic camera (10×) even though it is not as high as that of the operating microscope. Enhanced control with motion reduction compensates for this difference. Data coming from the literature show that the precision of suture placement resulted in a more rapid and watertight anastomosis. Moreover, the robotic technology gives the surgeon an ambidextrous capacity allowing placing sutures without difference with the right or the left hand. Clinical confirmation is found in the percentage of patent anastomosis.

There is still a need for a specific microsurgical training in order to learn the techniques of suturing and the tissue response, but afterward the training curve specific to microsurgical procedure like vasectomy reversal is probably shorter than traditional microscopic techniques and benefits achieved with the surgical robot are acquired with a short learning curve. Experienced microsurgeons who already master robot-assisted surgery have an even shorter learning curve if any. A surgeon without expertise in microsurgical technique should participate in a rat microsurgery course and should have extensive lab animal microsurgery [20]. Vice versa, experienced microsurgeons need to learn to use the robot at first in a dry lab practicing suturing on different models ranging from the Konnyaku noodle [21] to the chicken thigh vessels.

The current disadvantages in using a robot for vasectomy reversal are the suboptimal instrumentation available, not originally designed for microsurgery, and the low magnification of the optic, but not the lack of haptic feedback. Those problems are about to be solved with the introduction of new robotic platforms especially designed for microsurgery like the Dutch Microsure™ system using a conventional microscope and classical microsurgical tools adapted to the robotic arms placed on a circle [22] or the Italian MMI system with its independent arms holding the finest robotic tools, driven by joysticks without a console [23]. Other systems are on their ways, dedicated to microsurgery and aiming to lower the costs of the procedure.

20.4 Outcome

With the first robot-assisted vasectomy reversal cases, we had the immediate feeling that the outcome would be at least as good if not better than with conventional microsurgery. This was soon demonstrated by animal studies [13], than by Parekattil et al. [16] and Kavousi [17] with two studies which allowed to achieve a 96% patency rate; however pregnancy rate differed only slightly with 65% in the robotic group against 55% in the manual group. Such results can be achieved manually only by longtime trained surgeons (Goldstein [24]). Contrary to some affirmations, the lack of haptic feedback doesn't lead to a less delicate handling of the tissue. The stability of the tools avoids useless manipulation, better placement, and thus more watertight anastomosis.

20.5 Costs

It has been shown by McCullough and Lipschultz [24] that costs can be hardly estimated since institutions as well as individual surgeons charge very different fees. The real cost of the robotic tools for a vasectomy reversal is approximately 450 USD since a single tool can be reused up to 10×.

The time in the operating room is the same or even less, and in an institution with a high number of robotic procedures, the cost of the robot itself for a vasectomy reversal becomes marginal.

20.6 Conclusion

Robot-assisted vasectomy reversal has become an attractive alternative to the microsurgical reversal vasectomy procedures. During the past years, this technique has reached maturity. Many publications can be found worldwide using as search terms vasovasostomy, vasectomy reversal, and robot on PubMed. This proves that this procedure has

become a standard associated with the wide diffusion in the United States and Europe of the da Vinci robots. New microsurgical platforms will improve the accessibility of the technique together with the results and probably diminish the costs, and in the coming years, the number of centers using this technology will certainly increase.

20.7 Review Criteria

An extensive search of studies on the origins of vasectomy and vasectomy reversal was performed using already published textbooks and search engines as PubMed and Google book. The start and end dates for searches on vasectomy were from 1890 through today, for vasectomy reversal from 1920 through today, and for robot-assisted vasectomy reversal from 2004 until today.

The research was based on the following keywords: vasectomy, vasectomies, male sterilization, male contraception, vasectomy reversal, vasovasostomy, vasovasostomies, microsurgery, microchirurgie, robot, robotics, robotique, and da Vinci. Research was made both in English and in French. Results in other languages (Spanish, German) were also considered. Book chapter citations and unpublished data like conferences or web videos were also considered and are included.

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Robotic-Assisted Varicocelectomy

21

Tung Shu and Run Wang

Key Points

- A varicocele is currently the most common surgically correctable finding identified in men being evaluated for infertility.
- Of several surgical approaches available for the treatment of varicocele, including the retroperitoneal approach (high ligation via open, laparoscopic, retroperitoneoscopic, single-incision laparoscopic, or robotic-assisted), the inguinal approach (open), and the subinguinal approach (open microscopic), the subinguinal microscopic approach offers the best outcomes, including shorter hospital stays, preservation of the testicular arteries and lymphatics, least number of postoperative complications, recurrence, and a higher number of pregnancies.
- Microscopic assisted varicocelectomy, however, takes longer time to perform because surgeons are unaccustomed to use micro-instruments and two-dimensional vision and are unable to see their own hands.
- By using indocyanine green with the SPY Elite fluorescence imaging system, the testicular artery can readily be identified in an efficient way with minimal learning curve required.
- With miniaturized wristed instruments, 3-D camera, and computer technologies, the da Vinci® Surgical System filters and translates surgeon's hand movements seamlessly into precise micromovements of the da Vinci instruments; dramatic improvements in tissue handling, time, and skill can be achieved.

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21.1 Introduction

A varicocele is defined as a meshwork of distended blood vessels in the scrotum. It is usually left-sided resulting from the dilatation of the spermatic veins or pampiniform plexus. It is currently the most common surgically correctable finding identified in men being evaluated for infertility and is observed in 8–16.2% of the normal male population and in 21–39% of infertile men [1, 2].

Several theories have been proposed to explain the observed pathophysiology of varicoceles. Semen quality uniformly declines in animals with induced varicoceles, even when there is only a left varicocele. The reduction in scrotal temperature after varicocele ligation supports a causative role of increased temperature on the infertility produced by varicocele. It has been hypothesized that varicoceles cause hypoxia, which might play a role in altering spermatogenesis in the varicocele patients [3]. A higher frequency of sperm cells with fragmented DNA has been reported in the ejaculate of subjects with varicocele, in comparison with fertile donors, a phenomenon that might be correlated with an increase in reactive oxygen species [4].

Numerous studies have reported the significant benefits on semen parameters with surgical treatment of varicocele. [4–8] Currently, there are several surgical approaches available for the treatment of varicocele [9–12], including the retroperitoneal approach (high ligation via open, laparoscopic, retroperitoneoscopic, single-incision laparoscopic, or robotic-assisted), the inguinal approach (open), and the subinguinal approach (open microscopic). Of these approaches, the subinguinal microscopic approach offers the best outcomes, including shorter hospital stays, preservation of the testicular arteries and lymphatics, least number of postoperative complications, recurrence, and a higher number of pregnancies [2, 12]. The microscopic assistance, however, takes longer time to perform because surgeons are unaccustomed to use micro-instruments and two-dimensional vision and are unable to see their own hands.

The da Vinci® Surgical System has helped surgeons overcome the limitations for both traditional open and conventional

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minimally invasive surgeries. With miniaturized wristed instruments, 3-D camera, and computer technologies, the da Vinci® Surgical System filters and translates surgeon's hand movements seamlessly into precise micro-movements of the da Vinci instruments. With added experience from using the da Vinci® Surgical System, dramatic improvements in tissue handling, time, and skill can be achieved [13, 14]. With preliminary experience, we have used the da Vinci® Surgical System to perform robotic-assisted subinguinal varicocelectomy in comparison with the standard microscopic approach for the treatment of varicocele [15].

21.2 Materials and Methods

Eight patients aged 29.1 ± 12.5 years underwent microscopic subinguinal varicocelectomies: seven patients with left-sided repair and one patient with bilateral repair. Eight patients aged 22.0 ± 8.0 years underwent robot-assisted varicocelectomies: seven patients with left-sided repair and one patient with bilateral repair.

All varicocelectomies were performed through inguinal incisions (Fig. 21.1a). The spermatic cord was exposed and delivered out of the wound with a Penrose drain placed underneath the cord structures (Fig. 21.1b). At this time, the da Vinci® Surgical System or operating microscope was brought in and placed above the surgical field (Fig. 21.1c). The testicular artery and vas deferens with vassal artery and small vassal veins were identified and isolated (Fig. 21.1d). Other veins within the cord were isolated (Fig. 21.1e) and ligated with 5-0 Vicryl sutures and divided (Fig. 21.1f). At the completion of the varicocelectomy, only the testicular artery, lymphatics, and vas deferens with its vessels remained.

In our later group of patients, in order to identify the testicular arteries in an accurate and efficient way, we adapted the usage of indocyanine green (ICG) intraoperatively with SPY Elite fluorescence imaging system, NOVADAQ®, Stryker Corporation. We were the first to incorporate the technology for varicocelectomy. Indocyanine green was injected intravenously. By using the SPY Elite fluorescence imaging system, a low-level light source excites ICG, and fluorescence is captured in real-time and displayed on the monitor (Fig. 21.1g). After varix ligation, ICG was used again to make sure all veins were ligated with no injury to the testicular arteries (Fig. 21.1h).

21.3 Results

The average operative time for the microscopic subinguinal varicocelectomy was 73.9 ± 12.2 minutes, whereas the robotic-assisted approach took 71.1 ± 21.1 minutes. Average follow-up time for the patients in the microscopic group was 34.3 ± 6.4 months, whereas it was 10.9 ± 7.1 months in the robotic-assisted group (Table 21.1).

In our experience, there was no difficulty in identifying and isolating vessels and the vas deferens with the robotic-assisted approach. With the aid of indocyanine green, time to identify the testicular artery was not decreased due to additional setup for the SPY Elite fluorescence imaging system. However, the accuracy of identifying testicular artery was beneficial. A short learning curve for tying with 5-0 sutures was required because of the lack of tactile sensation when using the da Vinci® Surgical System. Patients in both groups were able to resume daily activities on the day of surgery and full activities within 2 weeks. There was no intraoperative or postoperative complication. No recurrence of varicocele was observed in either group of patients.

21.4 Discussion

In 2006, we performed the first robotic-assisted subinguinal varicocelectomy using the da Vinci® Surgical System [15]. From our continuing experience, we believe that the robotic-assisted varicocelectomy can be safely and effectively performed when compared to traditional microscopic approach. There was not a significant difference with regard to operative time; however, with increased experience with the da Vinci® Surgical System, the time should decrease. When compared to the microscopic approach, the tremor is completely eliminated with the robot. More importantly, the advantage of decreased intraoperative and postoperative complications experienced with microsurgical approach is maintained with the robotic approach.

Our goal, ultimately, is to have all urologists feel comfortable to perform a microscopic varicocelectomy especially in hospitals where a robotic system is available but not a surgical microscope. The biggest challenge for a novel urologist performing a varicocelectomy is to confidently identify the testicular artery. By using indocyanine green SPY Elite fluorescence imaging system, we were the first to incorporate the technology for varicocelectomy, the testicular artery can be readily identified in an efficient way with minimal learning curve required. Of note, we advocate using indocyanine green for either robotic-assisted or microscopic-assisted subinguinal varicocelectomy.

We are currently studying the cost-effectiveness and efficacy with regard to the improvement of semen quality and pregnancy for patients with infertility with our described robotic-assisted subinguinal varicocelectomy.

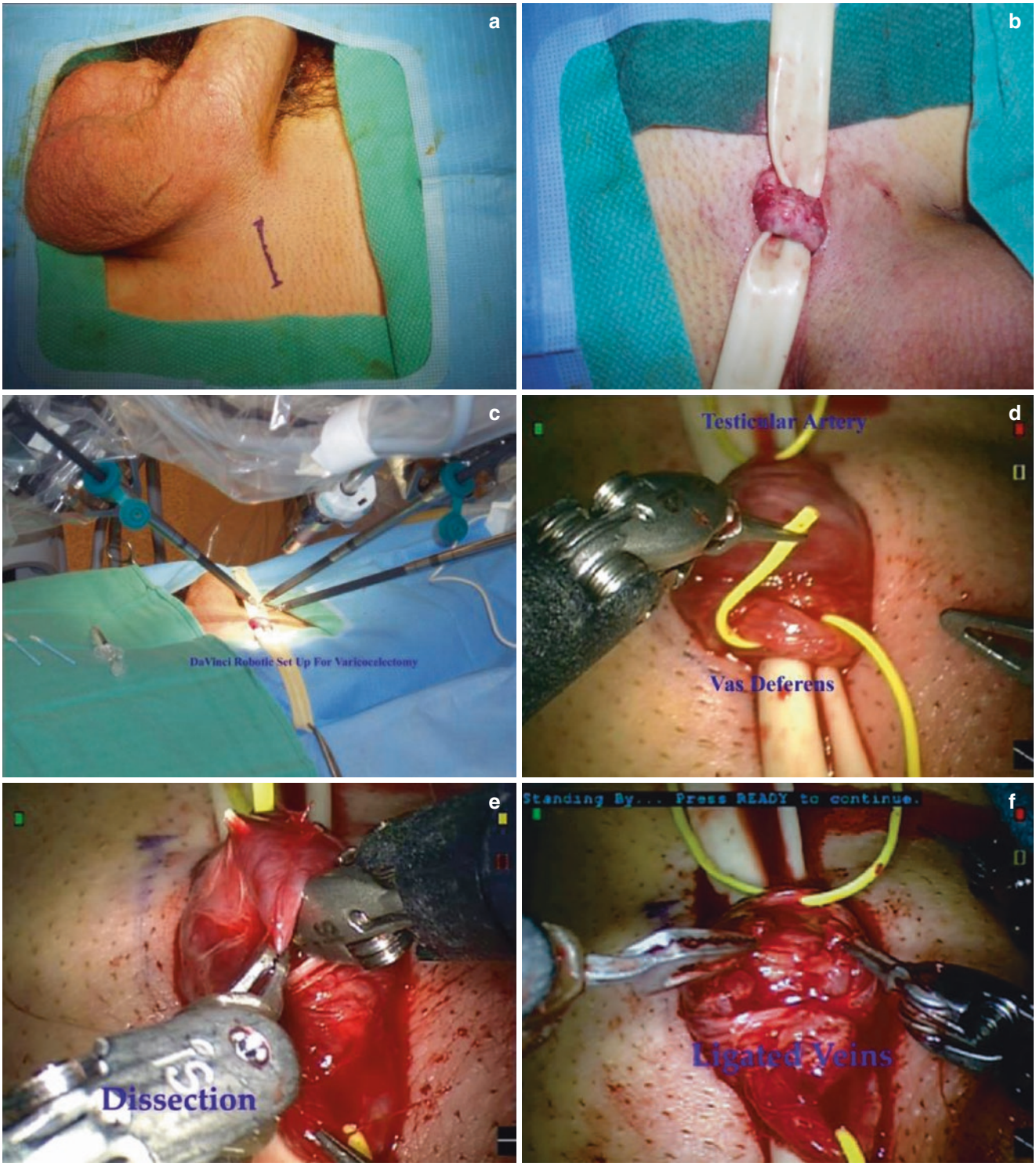

Fig. 21.1 (**a**) Subinguinal incision. (**b**) Spermatic cord exposure. (**c**) Da Vinci robot setup. (**d**) Testicular artery and vas deferens isolation. (**e**) Spermatic vein isolation. (**f**) Spermatic vein ligated and divided. (**g**) By using indocyanine green, the testicular artery can readily be identified. (**h**) Post-varicocele ligation, using indocyanine green

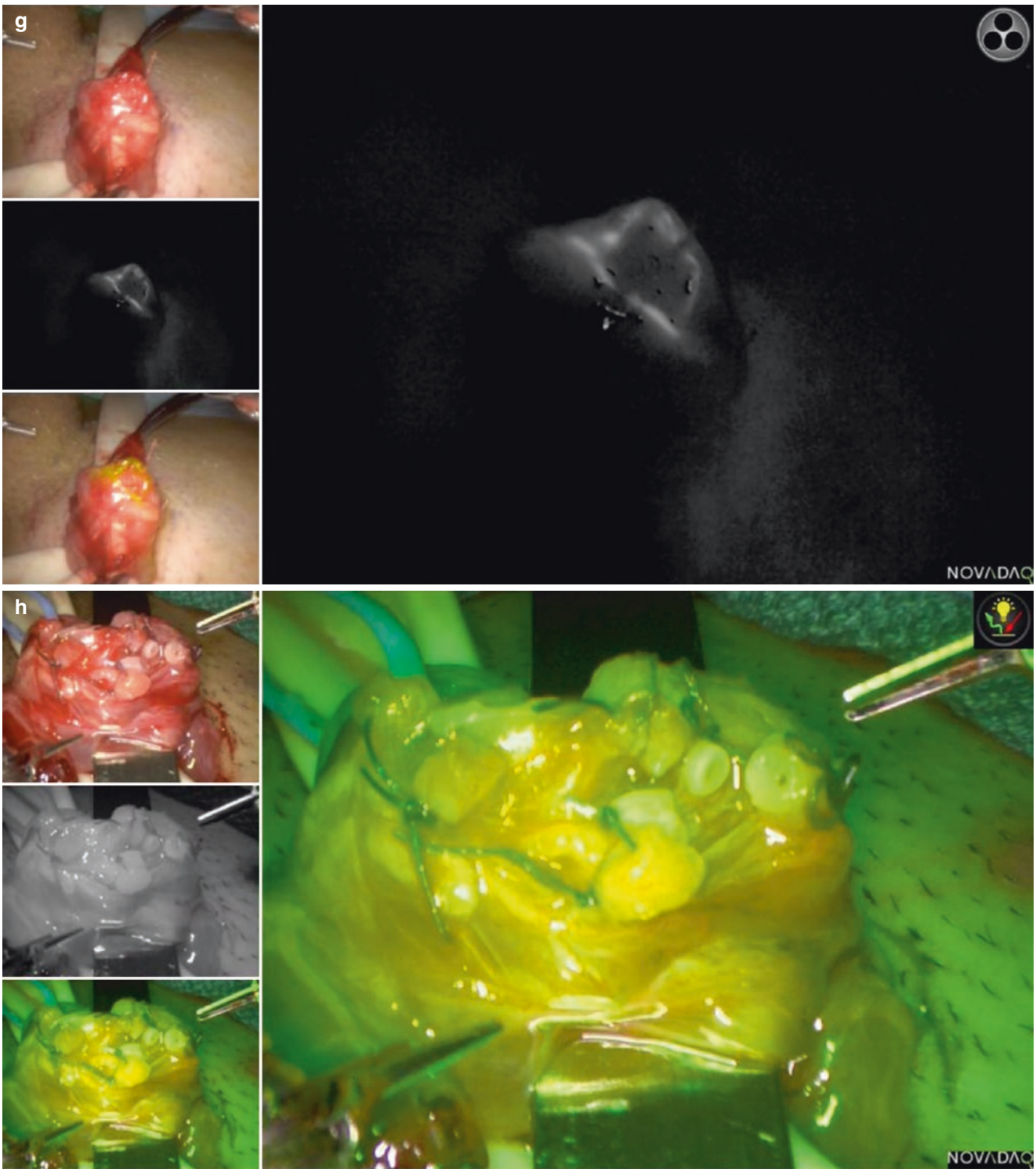

Fig. 21.1 (continued)

Table 21.1 Data of microscopic- and robot-assisted varicocelectomies

	Age (years)	Average operative time (min)	Follow-up time (months)
Microscopic technique 8 patients, 9 varicocelectomies	29.1 ± 12.5	73.9 ± 12.2	34.3 ± 6.4
Robot-assisted technique 8 patients, 9 varicocelectomies	22.0 ± 8.0	71.1 ± 21.1	10.9 ± 7.1

21.5 Conclusion

With the popularity and versatility of the da Vinci® Surgical System, hospitals throughout the world have purchased at least one system if not more for multispecialty usage. With da Vinci® Surgical System, urologist will not have to undergo a microscopic training course or a fellowship in order to perform any microscopic surgery. In the case of varicocelectomy, robotic assistance would allow any urologist to take care of a common urologic condition efficiently and effectively. By adding indocyanine green intraoperatively with SPY Elite fluorescence imaging system, the testicular artery can be readily identified in an efficient way with minimal learning curve required.

21.6 Review Criteria

An extensive search of studies examining robotic-assisted varicocelectomy was performed using search engines such as ScienceDirect, OVID, Google Scholar, PubMed, and MEDLINE. The start and end dates for these searches were Jan 2000 and Jan 2019, respectively. The overall strategy for study identification and data extraction was based on the following keywords: "varicocele," "robotic-assisted microsurgery," "varicocelectomy," and "male infertility." Articles published in languages other than English were also considered. Data that were solely published in conference or meeting proceedings or websites were not included. Websites and book-chapter citations provide conceptual content only.

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Management of Fertility Preservation in Male Cancer Patients

22

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Key Points

- Improvement in cancer treatment has men living beyond their reproductive years.
- All cancer therapies are potential threat to a man's reproducible potential.
- It is not possible to predict whether a man will recover spermatogenesis after cancer treatment.
- Sperm cryopreservation is the best cost-effective means if preserving man's fertility.
- With availability of ART procedure such as ICSI, it makes sperm freezing a very viable option in cancer patients.
- Barriers to sperm banking still exist, but these can be overcome by patient and provider education.

22.1 Introduction

Cancer is a common disease process that can significantly challenge individuals of all ages [1]. Of those patients who are diagnosed with cancer, 9% of them are younger than 45 years old, while 1% are younger than 20 years of age [2]. In the past 20 years, advancement in cancer therapies has significantly improved survival outcomes for young children and older adults. However, these improvements are not demonstrated in the reproductive aged population of adolescent and young adult [3].

Chemotherapy, radiation, and surgery are the primary treatment modalities for cancer. However, the potential toxicities have detrimental effects on a number of biological processes. One of the major drawbacks of cancer treatment is infertility [4, 5]. The cause of infertility may be secondary to the underlying malignancy or cancer treatment itself. Cancer affects male infertility through various mechanisms. Men are known to be at high risk of gonadal dysfunctions after cancer treatment due to the direct effect of treatment on the testes where the germ cells, Sertoli cells, and Leydig cells are present [6]. These cells are responsible for spermatogenesis and testosterone production. Any insult to these cells will lead to hypogonadism, altered semen parameters, and subsequent infertility [6].

These patients should be counseled and managed by a multidisciplinary team of healthcare specialists such as medical oncologists, urologists, reproductive biologists, and psychologists who are familiar with the cancer treatment and its complications, particularly infertility in the patients of reproductive age group. Fertility preservation must be recommended before the initiation of cancer treatment especially to those patients whose fertility is likely to be compromised [7]. The aim of this chapter is to discuss the impact of cancer on male fertility and available options for fertility preservation.

22.2 Incidence of Cancer

Adolescents and young adults are defined as men from 15 to 39 years at the time of detection of cancer diagnosis. In the United States, around 700,000 of these patients are diagnosed with malignancy each year [5]. Data from the US National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) database report that male adolescents, 15–19 years old, have had an annual increase of 0.67% in the incidence of cancer for all sites during the period of 1975–2012 [8]. This included a remarkable increase in thyroid cancer

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(1.59%), testicular cancer (1.55%), and non-Hodgkin's lymphoma (1.38%). These findings imply an overall increase of cancer incidence by more than 25%. During 2008–2012, the incidence of cancer among this male age group for all sites was 22.9 per 100,000. From birth to age 19, an estimated 15,780 new cases of malignancy were detected in 2014 in the United Sates, reflecting an annual incidence of 186.6 per one million [9]. Likewise, 1 in 285 children will be diagnosed with malignant disease before they reach age 20. However, roughly 1 in 530 young adults (20–39 years) will become a childhood cancer survivor [9].

Overall, the estimated new cases of cancer in 2018 were 1.73 million in the United States (SEER), and the estimated number of deaths is 609,640 from all cancer types. Based on 2011–2015 cases, the number of new cases of cancer of any site was 439.2 per 100,000 men. During a man's lifetime, approximately 38.4% of them will be diagnosed with malignancy of any site. In New Zealand, 1541 new primary cancers were detected in men 25–29 years old between the period of 2000 and 2009 [10]. Men in this age group have twice the risk of developing cancer compared to men of 15–24 years old.

22.3 Quality of Life Concerns and Reproductive Aspirations in Men with Cancer

All men of reproductive age should be provided with adequate details about the impact of cancer therapies including their effect on future fertility. Green et al. [11] determined the effect of treatment of childhood cancer on their reproductive potential through questionnaires. 941 out of 6224 men (15–44 years) expressed their desire to father a child 5 years after being diagnosed with cancer. Radiation therapy greater than 7.5 Gy to the gonadal organs caused a decrease in initiating a pregnancy (hazard ratio = 0.12). Similar results were reported in higher cumulative alkylating agent dose score or treatment with cyclophosphamide (hazard ratio = 0.48) or procarbazine (hazard ratio = 0.48). Likewise, Armaund et al. [12] mailed postal questionnaires to 484 cancer survivors to address their concerns after cancer treatment. Men who had not initiated a pregnancy at diagnosis and were young expressed a desire to father a biological child 3–7 years after cancer diagnosis. On the other hand, 17% who did have a desire for children prior to cancer treatment changed their decisions about their wish of children posttreatment.

An online focus group discussion with childhood survivors was conducted by Nilsson et al. in Sweden [13]. A total of 134 participants including 66 males who were diagnosed with cancer before the age of 18 years and at least 5 years after the diagnosis were included in this program. Patients were concerned about the negative impact of risk of cancer treatment on infertility, their well-being, and intimate relationship with their partner. In addition, they also raised their concerns on their reluctance of being a parent due to the known consequences of having a malignant disease. In another study, 15 participants finished a 2-month web-based program focused on sexual problems and fertility distress 1–5 years after the diagnosis of cancer [14]. Although a small number of participants were included in the program, all subjects expressed their disturbed sexual functions and concerns regarding infertility.

In an exploratory qualitative study by Flink et al. [15] including 27 individuals with newly diagnosed cancers, "concern for future fertility" was the top reason for fertility preservation, while "parenthood" was the top priority [15]. Although financial constraints may hinder the utilization of fertility preservation, 50% still pursued it despite this barrier [15].

In a cross-sectional survey done on individuals at least 18 years old who banked sperm or testicular tissue before the start of cancer treatment [16], among the respondents that included 131 males, almost two-thirds who completed the questionnaires expressed desire to have a child at the present and in the future. Ninety percent of men who attempted to conceive had a child since completing cancer therapy. At the time of completion of the survey, 27% of men used their cryopreserved sperm. Although evidence is limited on the reproductive experiences of these patients, this may help those patients contemplating fertility preservation. These findings can be used to counsel male cancer patients with high risk of infertility before the initiation of cancer treatment.

22.4 Cancer in Children and Adolescents

In the United States, 18 in 100,000 children and adolescents are detected with malignancy each year [8]. It is well-recognized that young individuals diagnosed with malignancy must be provided biological, psychological, and reproductive health information [17]. Treatment of cancer in childhood and adolescence usually results in a good outcome with a 5-year survival rate for all malignancies of 80% [18]. Despite this effective treatment in this age group, modern cancer therapies may affect their reproductive ability and reduce fertility potential.

In a population-based matched cohort study in Sweden by Armuand et al. [19], male adolescent cancer survivors' ability to have a child measured had lower hazard ratio than childhood cancer survivors (0.56 vs. 0.70). The authors concluded that cancer survivors during childhood or adolescence had a lower chance of having a first live birth. In another study comprising 43 patients diagnosed with cancer between ages 14 and 18 years old who underwent cancer treatment

for at least 6 months, 3 major concerns were raised by these patients [20]. These include infertility issues, emotions when discussing infertility, and treatment options to address infertility. Fifty percent of males expressed their uncertainty about their possibility to father a child. Five males decided to cryopreserve their sperm before the anticancer therapies. It is important to recognize these treatment-associated reproductive risks in order to advise male childhood cancer survivors to improve their fertility and quality of life in the future.

22.5 Impact of Cancer Therapies on Male Fertility Potential

The main treatment options for malignancy are chemotherapy, radiotherapy, and surgery. Complete understanding of these therapeutic modalities is valuable in order to choose the most effective treatment with least deleterious sequelae [21].

22.5.1 Radiation Therapy

Radiotherapy is used as a treatment option for men diagnosed with cancer. However, radiation therapy can result in gonadotoxic effects leading to transitory or permanent detrimental effect on male reproductive potential. A dose as low as 0.1 Gy radiation can affect the rapidly dividing spermatocytes because these cells are most sensitive to radiation effects. Severe oligozoospermia and azoospermia may be observed at doses up to 0.65 Gy. Permanent sterilization can be observed at doses higher than 1.2 Gy [22].

Although considered to be the most resistant cells to radiation effect, Leydig or interstitial cells are vulnerable to damage from radiation compared to its effect from chemotherapy. Higher doses of radiation can lead to Leydig cell failure which can substantially compromise the spermatogenesis. Radiation dose less than 20 Gy can cause Leydig cell dysfunction leading to elevated luteinizing hormone (LH) levels [23]. However, radiation doses of 20–30 Gy can lead to primary hypogonadism requiring testosterone supplementation [24]. Normal testosterone production can still be seen in most men who underwent <20 Gy fractionated radiation treatment to their testis. On the other hand, young men with testicular relapse of acute lymphoblastic leukemia who received >24 Gy fractionated radiation had high risk of Leydig cell dysfunction [25].

22.5.2 Chemotherapy

Cytotoxic chemotherapeutic drugs can actively target the rapidly dividing cells. This will affect both malignant and normal cells [26]. Spermatocytes are rapidly dividing cells during spermatogenesis that are at the highest risk for the cytotoxic effects of drugs within the germinal epithelium [27]. Due to lack of cell division, more mature spermatids are less sensitive to chemotherapy. Likewise, Leydig cells are more resistant to cytotoxic drugs but when exposed to higher doses can lead to elevation of gonadotropin levels.

In an earlier study examining the use of PEB (cisplatin 20 mg/m^2, etoposide 100 mg/m^2, and bleomycin 15 mg/m^2) for the treatment of testicular cancer, conventional dose showed statistically higher sperm density compared to high-dose PEB (median 5.83 million/ml vs. 0.0005 million/ml, $p = 0.008$). In addition, a lower number of patients who had azoospermia were also reported (19% vs. 47%) [28].

Van Beek et al. [29] evaluated long-term gonadal sequelae in 56 males with childhood Hodgkin's lymphoma after the combination treatment with ABVD or EBVD (adriamycin/epirubicin, bleomycin, vinblastine, dacarbazine) with or without MOPP (mechlorethamine, vincristine, prednisone, procarbazine). Sperm concentration was significantly reduced in men who received MOPP compared to men who were treated without MOPP (1.05 million/ml vs. 49.5 million/ml, $p < 0.05$). In this treatment group, both median gonadotropin levels were significantly elevated ($p < 0.001$). In another study comprising 49 men with Hodgkin's disease treated with chemotherapy, azoospermia was observed in 42 men, while sperm concentrations below 1 million/ml were documented in 7 men [30]. Although decreased sexual drive and activity was reported in a majority of patients during treatment, it returned to normal after completion of chemotherapy. Green et al. [31] performed semen analysis on 214 adult male survivors of childhood cancers who were given alkylating agents for their chemotherapy. Azoospermia and oligospermia were observed in 25% and 28% of patients, respectively. Normal spermatogenesis was demonstrated in 89% of patients when cyclophosphamide equivalent dose less than 4000 mg/m^2 was used [31]. Nevertheless, the potential of chemotherapy affecting sperm chromatin and aneuploidy cannot be ruled out which is a major concern of embryo development [32, 33].

22.5.3 Surgery

Unilateral radical orchiectomy is considered the gold standard for the treatment of men presenting with testicular mass. This surgical procedure has a negative impact on semen quality in men with testicular cancer. Semen parameters were poorer in men with nonseminomatous testicular cancer compared with seminomatous testicular cancer. In a study by Liguori et al. [34], the median sperm concentrations before and after surgery for men with nonseminomatous testicular cancer (17.9 million/ml and 8.16 million/ml) were lower compared to men with seminomatous testicular cancer

(35.47 million/ml and 23.99 million/ml). In another study, the median sperm concentration decreased from 17 million/ml to 7 million/ml. This occurred in 30 out of 35 men who underwent radical orchiectomy and azoospermia was observed in 9% of patients [35].

Any surgical procedure performed within the pelvic cavity and retroperitoneum can cause parasympathetic nerve damage which is responsible for erection. Injury to the sympathetic nerves of the hypogastric and pelvic plexuses may lead to seminal emission and ejaculation dysfunction. Surgery, particularly retroperitoneal lymph node dissection (RPLND) as a treatment option for testicular cancer, can be a contributing factor for the development of infertility. Retrograde ejaculation is the main andrological complication in this type of surgery. In a study by Matos et al. [36] examining 297 men who underwent non-nerve-sparing RPLND, fertility rate was only 37% compared to 62% who underwent nerve-sparing RPLND. On the other hand, a fertility rate of 77% was demonstrated in men in whom RPLND was not performed. Likewise, lower abdominal surgeries for colorectal, urinary bladder, and prostate gland cancers may also lead to ejaculatory and erectile dysfunction [37]. Radical prostatectomy in particular results in the removal of a portion of the vas deferens and seminal vesicles, both of which are integral for the transport of semen outside the body.

22.5.4 Immunotherapy and Other Modalities

Immunotherapeutic agents can cause negative gonadal impact resulting in infertility. Interferon alpha has not been extensively studied with regard to its effect on male fertility. In a case report of a 38-year-old man with relapsing stage III cutaneous melanoma treated for 5 years with interferon alpha 2a (9 million units 3× a week), semen analysis revealed azoospermia and higher follicle-stimulating hormone (FSH) (14.6 mU/ml) levels. Azoospermia was observed even after 1 year of stopping the immunotherapy [38]. This is believed to be secondary to degeneration of spermatogenic cells and complete atrophy of the seminiferous tubules as demonstrated in an animal study [39]. Further studies are needed in order to conclude the negative effect of interferon alpha on male infertility.

Immunosuppressive agents used for kidney transplantation including sirolimus and everolimus can produce deleterious effects on the testis with resultant impairment of the gonadal functions. These are rapamycin inhibitors that act on the inhibition of a stem cell factor/c-kit-dependent process in immature germ cells such as spermatogonia. Almost all of the studies included in the review of Huyghe et al. [40] showed decreased level of testosterone and increased levels of LH and FSH after immunotherapy. Both transplanted patients and healthcare providers should be aware of the potential detrimental effect of these immunomodulators before commencement of the treatment.

Imatinib, a tyrosine kinase inhibitor, plays a significant role in the regulation of germ cells in the gonadal development. However, this drug can cause adverse effects on testosterone production and spermatogenesis. Shash et al. [41] reported a 36-year-old male diagnosed with chronic myeloid leukemia and treated with imatinib 400 mg/day. Despite the treatment, he fathered 2 children during the 22 months of treatment period. On the other hand, oligospermia was observed when an adolescent received a higher dose of imatinib (800 mg/day) for hypereosinophilic syndrome [42]. Likewise, prolonged administration of imatinib for chronic myeloid leukemia in an 18-year-old male resulted in severe oligozoospermia [43]. These case reports document the potential adverse effect on the derangement of semen parameters in men receiving treatment with tyrosine kinase inhibitors especially when these are given before the complete maturation and development of the gonadal organs and at higher doses.

22.6 Fertility Preservation Counseling for Men, Couples, or Family

There is an increase in cancer survivorship due to the improvement in cancer therapies. Despite of this achievement, infertility is a growing concern among men diagnosed with cancer especially young adults. That is why it is important to understand the value of fertility preservation prior to initiation of cancer treatment [44].

The only established technique for fertility preservation in men is sperm cryopreservation [45]. This is considered as an established method of fertility preservation that should be offered to children or adults of reproductive age before initiation of any cancer treatment [46].

The American Society for Reproductive Medicine (ASRM) [21] and American Society of Clinical Oncology (ASCO) [47] have formal recommendations to patients and healthcare providers regarding the detrimental impact of cancer therapies on reproductive potential and the option for fertility preservation. Sperm cryopreservation should be offered when necessary, although in some areas of this field, controversies still arise. An international recommendation from physicians with expertise in the field of fertility preservation in patients with malignancy was made in 2015 [48]. There were ten recommendations made including the recommendation that physicians discuss, as early as possible, with all patients of reproductive age their risk of infertility from the disease and/or treatment and their interest in having children after cancer, and help with informed fertility preservation decisions. Sperm cryopreservation and embryo/oocyte cryopreservation are standard strategies for fertility

preservations in male and female patients. These recommendations provide data addressing the cancer survivors' concerns about their fertility, although there are issues that still remain controversial.

Fertility preservation is not a well-documented option of concern for male cancer patients who undergo treatment. Of the 231 records comprising 4 cancer centers, only 26% documented infertility risk discussion, 24% documented fertility preservation option discussion, and 13% documented referral to fertility specialist [49]. Likewise, records were less likely to document infertility risk discussion, fertility preservation option discussion, and fertility specialist referral for patients aged 40 years old ($P < 0.001$, <0.001, and 0.002, respectively) and those who already had children (all $p < 0.001$) [49]. In another study, only 29% ($n = 201$) of male with cancers were counseled regarding their fertility risks after chemotherapy, while 11% attempted sperm cryopreservation. Of these 11%, 87% successfully banked their sperm [50].

A prospective, single-group observational study by Klosky et al. [51] on 146 adolescent males demonstrated 53.4% attempted to collect a semen sample (collection attempt) and 43.8% had successful sperm banking. Likelihood of collection attempt increased following consultation with fertility specialist ($p = 0.007$), Tanner stage >3 ($p = 0.003$), and recommendation of parent to bank ($p = 0.007$). On the other hand, history of masturbation ($p = 0.025$), banking self-efficacy ($p = 0.012$), parent ($p = 0.010$), and medical team ($p = 0.008$) recommendation to bank were associated with increased likelihood of completion of sperm banking [51].

Even though the healthcare providers are aware of the adverse effects of anticancer treatment on reproduction, men are less informed about their option of sperm banking particularly in the developing countries where banking options are limited. It is the role of the healthcare provider to educate and address the fertility concerns and options available for sperm banking to the family of the patient. In a study of oncologists' compliance with guidelines on cancer treatment-related infertility, 83% of patients were informed about this concern [52]. However, compared to women, men are less informed regarding the awareness of fertility-related concerns (OR, 3.57; 95% CI, 1.33–9.60; $p = 0.012$).

Although some of the studies show less awareness of fertility preservation, others are practicing it as part of the treatment options in cancer therapies. There is an increase from 1.73 to 5.57 per 100,000 persons per year in the overall incidence of sperm cryopreservation according to a French sperm banks between 1990 and 2013 [53]. In 2020, the projected incidence will increase to 6 per 100,000.

It is well documented that both type of malignancy and associated treatment may negatively affect male infertility. Although fertility preservation in young patients diagnosed with cancer has raised controversial concerns ethically and legally, sperm cryopreservation is still the management strategy for men before the initiation of anticancer treatment. Referral for fertility counseling and sperm banking can address fertility preservation in men diagnosed with cancer at the time of initial treatment. Members surrounding the patient play an important role in their treatment. In a systematic review by Taylor et al. [54] on the perspective, experiences, and preferences of adolescents, parents, and providers regarding fertility preservation, all these individuals were considered as key stakeholders in a shared decision-making process in every step of the treatment of the cancer patient.

22.7 Sperm Parameters and Semen Quality in Men with Cancer

Cancer and its treatment modalities can negatively affect sperm production and gamete quality. Pretreatment semen parameters in men with malignancy were extensively studied by Williams et al. [55]. During a 5-year period from the cryopreservation laboratory, 409 men with a total of 717 semen samples showed a normal range for semen volume (mean = 2.8 ml), sperm density (mean = 47.4 million/ml), and sperm motility (mean = 50%). However, testicular cancer patients had statistically reduced semen quality compared to men with other causes of malignancies. The authors recommended pretreatment cryopreservation for this set of patients before the initiation of gonadotoxic therapies. In a retrospective study ranging over a 15-year period in Korea, 66 cases of male cancer patients (age range = 19–58 years) evaluated the semen parameters prior to chemotherapy [56]. Testicular cancers accounted for 47% of cases. Among the different types of malignancy, sperm concentration (42.3 ± 48.6 million/ml, $p = 0.033$) and viability (52.4 ± 15.5%, $p = 0.012$) showed significant difference. On the other hand, semen volume (2.0 ± 1.3 ml, $p = 0.127$), motility (30.2 ± 19.1%, $p = 0.075$), and morphology (14.1 ± 10.2%, $p = 0.549$) demonstrated no significant difference. In another study, Negoro et al. [57] reviewed the records of 257 men (germ cell tumors = 113; hematological disorders = 111) with cancers who underwent cryopreservation. Of these, 25 men had successful reproductive outcomes. Men with germ cell tumors have significantly lower average sperm concentrations compared to men with hematological disorders (32.6 vs. 46.1 million/ml, $p < 0.05$). Other semen parameters showed nonsignificant results [57].

Paoli et al. [58] conducted a longitudinal study examining the sperm quality of 519 men [adolescent (13–17 years) = 50; adults (18–51 years) = 454] to determine if Hodgkin's lymphoma itself or its various treatment modalities caused impairment of sperm production impairment. Sperm concentration was normal in 75% of patients. Males (13–17 years old) had significantly lower sperm concentra-

tion (63.7 + 69.9 million/ml) and volume (1.8 + 1.3 ml) compared to other age groups, although mean semen parameters were still considered as normal. A significant decrease in sperm concentration and total number of sperm were noted after 6 ($P < 0.001$) and 12 months ($p < 0.01$) of treatment with ABVD (doxorubicin, bleomycin, vinblastine, and dacarbazine). Likewise, a significant reduction was also reported in progressive motility ($p < 0.001$) after 6 months of treatment [32].

Hotaling et al. [59] documented the raw and test-thaw semen parameters in 1010 samples from men with newly diagnosed cancer after cryopreservation. Men with prostate cancers showed best mean total motile count of raw (155.1 million) and post-thaw (53.2 million) semen quality. On the other hand, men with lymphoid leukemia showed worst raw total motile count (26.8 million), while men with myeloid leukemia had the worst post-thaw total motile count (6.9 million). Men with testicular cancer ($p = 0.0314$) and lymphoid leukemia ($p = 0.0291$) demonstrated statistically significant decreased proportion of semen samples with total motile count more than 5 million.

Although testicular cancer represents only 1% of all newly diagnosed male malignancies, it is still the most frequent cancer in adolescents and young adults in developed countries. A systematic review by Djaladat et al. [60] showed an association between testicular cancer and abnormal semen parameters even outside the treatment effects of orchiectomy, chemotherapy, and radiation.

22.8 Semen Collection for Male Cancer Patients

Patients must be encouraged to initiate and complete sperm cryopreservation before starting any cancer therapy that affects the reproductive system. Semen for sperm cryopreservation is generally obtained by masturbation. For many men, this may be an embarrassing or uncomfortable process due to cultural or religious limitations. Men may collect a semen sample at home or another location than the clinic, provided that they keep the specimen at body temperature and return it to the lab within approximately 45–60 minutes after collection. Lubricants should be avoided as they can contaminate the specimens. The entire specimen should be collected, in a widemouthed specimen container to avoid any spill out. The adolescent male population is one in which extremely careful counseling and tactful, age-appropriate instructions are necessary, as these patients are at risk for emotional distress from this process. Parents should be included in discussions, although separate sessions with the adolescent are useful. Unfortunately, no guidelines exist for the best approach to semen cryopreservation in the adolescent male, but individual institutional strategies are available [61].

At the time of diagnosis, many cancer patients are admitted in hospital as indoor patients, and this may be the appropriate time that they are offered sperm cryopreservation option. Some men are quite ill and debilitated by their cancer and are unable to produce a sample. In these cases, surgical/electroejaculatory sperm retrieval can be offered. A semen analysis is performed on all samples prior to cryopreservation. Semen parameters should be documented, and multiple collections are recommended depending on the concentration and number of motile sperm seen in the ejaculate.

22.9 Techniques for Sperm Cryopreservation

There are many steps in the process of successful preservation and storage of sperm for future use. Technical oversight in any step can adversely affect the final reproductive potential of the patient's sample.

22.9.1 Sperm Preparation Prior to Cryopreservation

Semen samples of men diagnosed with malignancy are of poor quality. Sperm washing allows the separation of the sperm and seminal plasma before cryopreservation. Sperm preparation by density gradient concentration method is another way to perform sperm washing protocol [62]. In this method, two different layers of density gradients are layered in a test tube. The high density gradient (lower phase) is layered at the bottom and the lower density (upper phase) is layered on top. The semen sample is carefully layered on the top. After centrifugation for 20 minutes, followed by resuspending the pellet, a highly motile sperm fraction is obtained that is cryopreserved.

22.9.2 Slow Freezing

Manual slow freezing usually takes to 2–4 hours to complete. Cleveland Clinic's method of controlled, slow freezing involves the complete liquefaction of the semen sample by placing the specimen in an incubator at 37 °C (Fig. 22.1) [63]. Cryovials are labeled and color coded. Freezing medium equal to 25% of the original specimen volume is gradually added to the centrifuge tube with a sterile pipette, and the specimen with the freezing medium is gently rocked for 5 minutes on a test tube rocker (Fig. 22.2). This is repeated until the added freezing medium equals that of the original

Fig. 22.1 Incubator set at 37 °C and depiction of sample undergoing liquefaction. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2014–2019. All Rights Reserved)

Fig. 22.2 Sample placed on a test tube rocker for 5 minutes after the addition of Test-yolk buffer. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2014–2019. All Rights Reserved)

specimen volume (Fig. 22.3). The cryodiluted patient sample is added to pre-labeled cryovials using sterile serological pipettes (Fig. 22.4). The labeled vials are placed in a labeled cryocane and covered with a cryosleeve (Fig. 22.5). The cryocane with 2 cryovials is placed upright in a freezer at −20 °C for 8 minutes (Fig. 22.6). Following this, the canes are removed from the -20 °C freezer and placed upright in LN_2 vapor tank (–80 °C) for a minimum of 2 hours (Figs. 22.7 and 22.8). The vials are exposed to LN_2 vapors only. The canes are flipped after 24 hours and plunged in LN_2 (–196 °C) and placed in long-term storage until ready for use (Fig. 22.9).

After 24 hours, the test vial is removed; the cap is loosened and placed in the incubator at 37 °C. The sample is mixed and analyzed using the computer-assisted semen analyzer for count, motility, curvilinear velocity, linearity, and amplitude of lateral head movement. Sperm cryosurvival is calculated by examining the percentage motility of post-thaw specimen to that of the pre-freeze specimen. The number of inseminations possible from the frozen specimen is calculated based on the fact that 15–20 million are required for one insemination [64].

The major drawback with this technique is that ice crystals can form within cells if the cooling rate is too fast. Additionally, slow cooling can shrink the cells due to water osmosis and cooling rate [65]. Slow, staged freezing using automated, computerized methods limits cryodamage of low-quality sperm [66]. However, automated freezers are time-consuming and expensive, requiring up to five times more liquid nitrogen [67].

Slow freezing technique, as proposed by Behrman and Sawada, [68] is widely applied for sperm cryopreservation. This technique progressively cools the sperm over a period of 2–4 hours. Using a programmable freezer, this can be done either manual or automatic. Slow freezing is based on dehydration which happens during cooling process. Ice masses containing crystalline water are created. Compared to rapid freezing, this technique allows the sperm to slowly adjust to lower temperature. As a result, it causes less osmotic strain on the cellular membrane of the sperm [69].

Using the manual technique, this is simultaneously performed by lowering the semen temperature while putting cryoprotectant in a stepwise manner [70]. The optimal initial cooling rate of the semen sample from room temperature to 5 °C is 0.5–1 °C/min. Before the sample is plunged into liquid nitrogen at –196 °C, the sample is frozen from 5 °C to –80 °C at a rate of 1–10 °C/min. Although slow freezing is a commonly used method for sperm cryopreservation, another technique such as vitrification is a faster, easier, less toxic, and less expensive method [71].

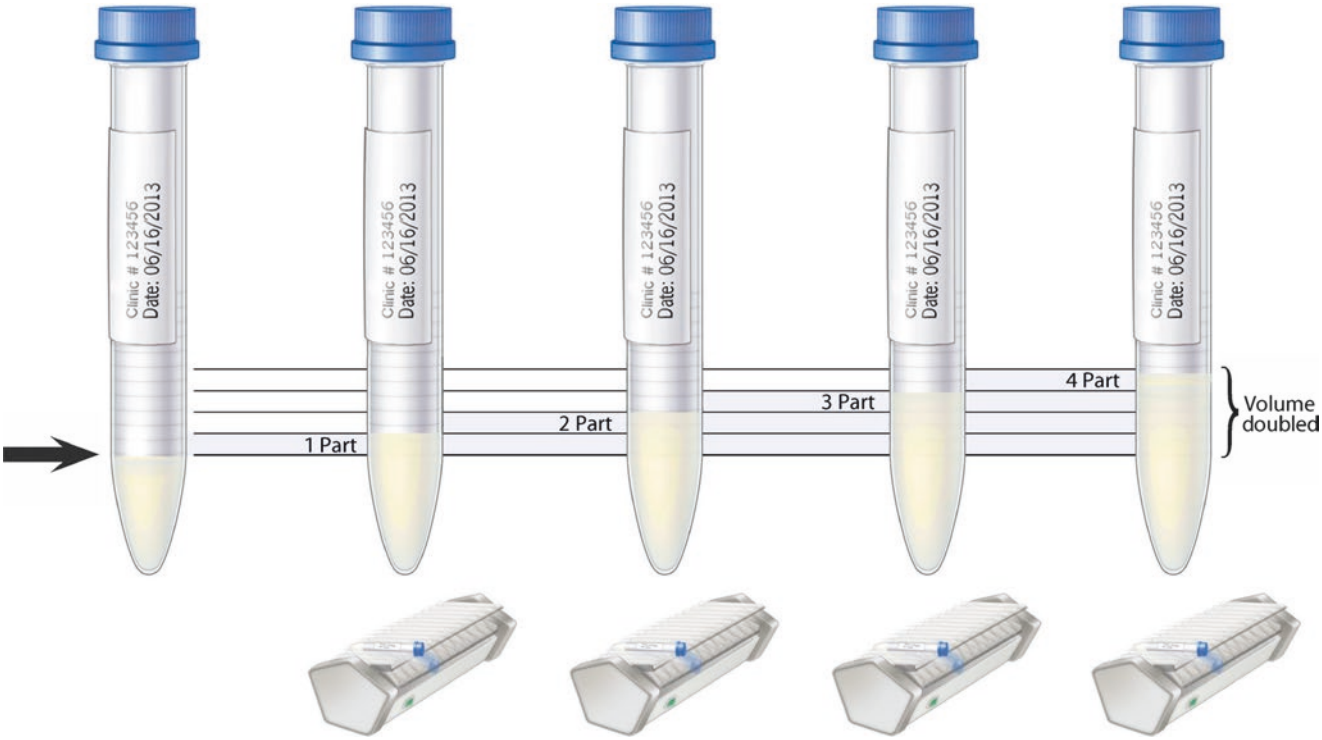

Fig. 22.3 Stepwise addition of Test-yolk buffer to patient sample. Volume of Test-yolk buffer equal to 25% volume of patient sample—added 4 times, or until total volume in test tube has doubled. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2014–2019. All Rights Reserved)

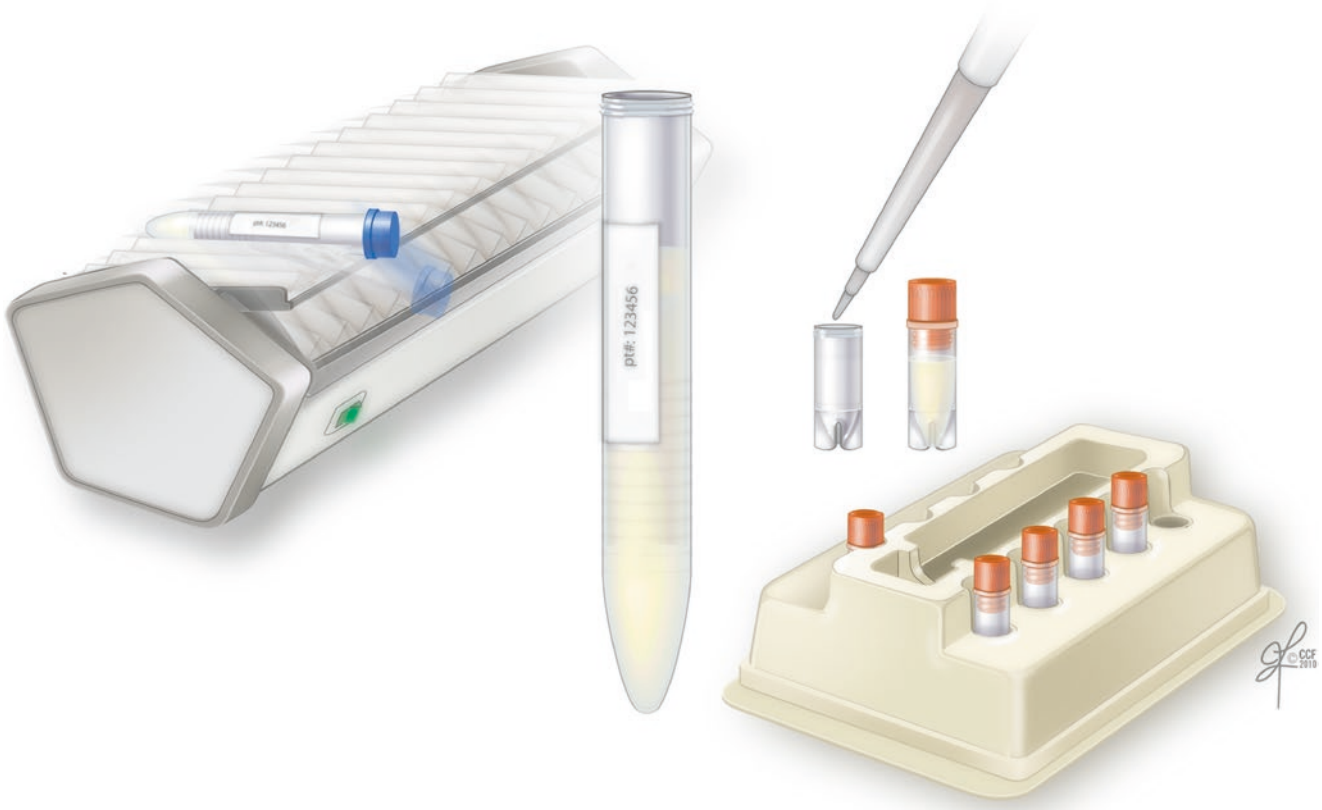

Fig. 22.4 Even distribution of cryodiluted patient sample into cryovials using a sterile serological pipette. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2014–2019. All Rights Reserved)

Fig. 22.5 Loading of the cryovials on a cryocane. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2014–2019. All Rights Reserved)

Fig. 22.6 Placing the cryocanes in a freezer at –20 °C. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2014–2019. All Rights Reserved)

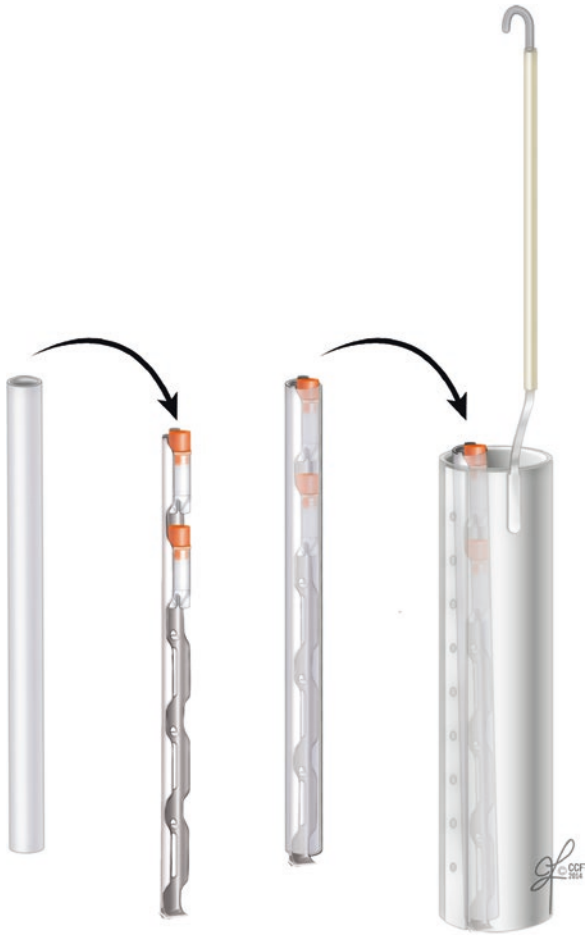

Fig. 22.7 Placing the canes in a canister before lowering in LN_2 vapors. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2014–2019. All Rights Reserved)

Fig. 22.8 Cryotank canister containing cryocanes and cryovials added slowly, upright into cryotank. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2014–2019. All Rights Reserved)

Fig. 22.9 Long-term storage of semen sample in liquid nitrogen tank. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2014–2019. All Rights Reserved)

22.10 Rapid Freezing

Rapid freezing protocols are commonly used for sperm cryopreservation and provide better post-thaw motility and cryosurvival than slow freezing protocols in non-oncologic controls [72]. The Irvine Scientific method is a fast and convenient cryopreservation method that can be used to rapidly freeze and store sperm for a longer term. With this method, the entire volume of freezing medium is added at one time, and the specimens are immediately immersed in liquid nitrogen [64].

This rapid freezing technique requires direct contact between the straws and nitrogen vapors for 8–10 minutes before plunging into liquid nitrogen at –196 °C. Due to the formation of extracellular ice crystals, it lessens the cryoprotectant toxicity and osmotic damage as well [69]. The semen sample is first mixed with equal volume of cryoprotectant. It is placed in a vial or straw and then in nitrogen vapor at −96 °C for 2 hours. Finally, it will be immersed in liquid nitrogen at −196 °C. Compared to slow freezing method, rapid freezing showed superior motility and survival outcomes after thawing [64].

22.11 Sperm Vitrification

Recently, sperm vitrification has been advocated as an alternative to slow freezing method. Vitrification, an ultrarapid freezing method, may offer improved results compared to rapid freezing protocols [73–76]. The vitrification technique is advantageous in that it requires no equipment and is straightforward, quick, and inexpensive. It is more commonly used to freeze oocytes and embryos. Spermatozoa are osmotically fragile, and the use of high concentrations of permeable cryoprotectants is cytotoxic, drastically reduces spermatozoal motility, and compromises sperm DNA quality. Cooling can be achieved using either liquid nitrogen or liquid nitrogen vapor phase. Vitrification—either with no cryoprotectant or very low levels of cryoprotectant—has been reported [77, 78]. In normospermic samples, no significant difference was reported in the sperm recovery rate and motility rate between spermatozoa cryopreserved without any cryoprotective agent and those preserved with sucrose [78]. Furthermore, the authors reported a higher viability and lower DNA damage than those cryopreserved with sucrose using a cryotop carrier. Lack of cryoprotectant is compensated by the use of high cooling rates achieved by directly plunging samples into liquid nitrogen [~720,000 K/min]), and the use of an extremely small sample volume increases the surface area for exchange of heat [79].

This technique rapidly cools the semen sample at a rate of more than 1000 °C/min without the risk of ice formation [69]. A comparative experimental study was done by Riva et al. between slow freezing and ultrarapid freezing method [80]. They showed that slow freezing had lower progressive motility and higher nonprogressive and immotile sperm count compared to ultrarapid freezing. In addition, the former had significantly higher sperm DNA fragmentation (47.3 ± 13.4% vs. 14.6 ± 4.6%, $p < 0.05$).

In another study, vitrification was not superior to rapid freezing in men with normozoospermic sample. In 30 normal ejaculate samples, Agha-Rahimi et al. demonstrated no significant difference in sperm DNA fragmentation between the two techniques (15.7 ± 4.4% vs. 16.6 ± 5.6%, $p > 0.05$) [81]. Progressive motility and viability showed nonsignificant relations as well. According to the latest meta-analysis

comparing vitrified and conventional cryopreserved samples [82], post-thawed sperm following vitrification demonstrated significantly higher total motility (weighted mean difference (WMD) 6.98; 95% CI, 2.94–11.02; $p < 0.0001$) and progressive motility (WMD 4.59; 95% CI, 0.78–8.39; $p = 0.02$). Sperm DNA fragmentation did not show any significance between the groups being compared. However, due to the small number of studies available, the authors suggested a large well-conducted study in order to validate these findings accurately.

22.12 Options for Home Banking: An Innovation-Based Approach

An attractive alternative option for patients who are unable to travel or for those who find providing a semen sample at a sperm bank emotionally challenging is the introduction of a home sperm banking kit called NextGen. With this kit, patients can collect a semen sample in the privacy and comfort of their home and ship it overnight to a sperm banking facility. Cryosurvival rates were examined in sperm collections from patients with and without cancer—both on-site and off-site—using remote collections with the NextGen kit [83]. Pre-freeze and post-thaw sperm motility, total motile sperm, and percent cryosurvival rates were compared. Cryosurvival rates were similar between the NextGen and on-site collection samples in both infertile men (53.14 ± 28.9% vs. 61.90 6 ± 20.46%; $p = 0.51$), respectively, and men with cancer (52.71 ± 20.37% vs. 58.90 ± 22.68%; $p = 0.46$). Cancer patients can bank sperm effectively using the NextGen kit.

22.13 Cryopreservation of Epididymal or Testicular Tissue: Indications and Techniques

The advancement and development of assisted reproductive technology (ART) particularly intracytoplasmic sperm injection (ICSI) using the surgically retrieved testicular and epididymal sperm have dramatically altered the reproductive potential of men with obstructive azoospermia (OA) and nonobstructive azoospermia (NOA) [84]. In intrauterine insemination (IUI) and in vitro fertilization (IVF), use of ejaculated sperm is standard practice due to availability of enough large number of live and motile sperm even after thawing. In addition, less contamination with blood cells and cellular debris can be observed in ejaculated sperm sample. However, some conditions and scenarios will dictate the use of cryopreserved testicular and epididymal sperm in the management of male infertility.

With the current advancement in the field of cancer treatment, the overall outcome of a patient with malignant disease has improved significantly. In male children and adolescents diagnosed with cancer, in whom spermatogenesis has not yet been initiated, cryopreservation of testicular tissue before the start of cancer treatment like chemotherapy and radiation will offer reproductive potential in the future [85]. In prepubertal boys, cryopreservation of the testicular tissue is the only way of preserving the future fertility [86]. Keros et al. cryopreserved testicular tissues from five prepubertal boys before the initiation of gonadotoxic treatment [87]. There were no clear structural changes observed in the fresh and cryopreserved testicular tissue samples. For young boys whose sperm production is not yet evident, cryopreservation of immature testicular tissue is an option [88].

Azoospermia is the main reason for attempting sperm retrieval from the testis or epididymis. In men with NOA, a wide list of possible etiologies and severity of the underlying conditions can be faced by the specialist. This may prove a challenge in obtaining enough sperm sample for cryopreservation. History of chemotherapy or radiation exposure can contribute to NOA [84]. The gold standard of sperm retrieval for men with NOA is microdissection testicular sperm extraction (microTESE) [89, 90]. In a recent meta-analysis of the comparison of different sperm retrieval techniques, microdissection TESE had a 1.5 times more probability of sperm retrieval rate (SRR) compared to conventional TESE [91]. On the other hand, SRR was twice more likely with conventional TESE compared to testicular sperm aspiration (TESA).

Sperm can be surgically retrieved from the testis or epididymis in patients with OA [92]. Contrary to men with NOA, retrieval is restricted to the testis. Testicular sperm retrieval can be performed by an open testicular biopsy or testicular sperm aspiration (TESA). The conventional method of testicular extraction provides the best chance of acquiring large number of tissues. However, Schlegel revolutionized this technique into microTESE removing significantly less testicular tissue (9.4 mg vs. 720 mg) with superior SRR compared to conventional technique [90]. Multiple testicular sperm extractions can cause irreversible damage and to some extent can lead to testicular atrophy [90]. On the other hand, testicular sperm aspiration is a relatively fast and easier method of testicular sperm retrieval [85]; however, SRR is lower compared to open testicular biopsy especially in men with NOA [93]. Retrieval of epididymal sperm can be done by percutaneous epididymal sperm aspiration (PESA) or by microsurgical epididymal sperm aspiration or by fine needle aspiration (FNA). These procedures are good options for men with obstructions at the level of the vas deferens or epididymis and offer good SRR [84].

22.14 Fertility Preservation in Prepubertal Boys: Experimental Techniques with IRB Guidelines

Although ICSI using frozen sperm has revolutionized infertility treatment, it is not applicable in prepubertal boys since spermatogenesis has not begun. In such cases preserving spermatogonial stem cells (SSCs) can be recommended to prepubertal patients diagnosed with cancer and who are at a risk of losing their SSCs or are at a risk of developmental genetic disorders [94]. Testicular tissue with spermatogonial stem cells may be used in the future for fertility preservation in men and boys prior to treatment, as these cells are capable of self-renewal, proliferation, and repopulation of the seminiferous tubules [95]. Although germ cell transplantation has become an important research tool in rodents and other animal models [96–102], the clinical application in humans remains experimental. The technique of autotransplantation of SSCs in testicular tissue has shown promising results in animals and nonhuman primates. However, cryopreserving the testicular tissue containing SSCs is the first step in translating SSC-based cell therapy into clinical practice, and it is important to evaluate their quantity and functionality as a translational therapy.

Regarding testicular grafting as another exciting strategy for fertility preservation in males prior to gonadotoxic therapy, both autologous and xenologous transfer of immature tissue revealed a high regenerative potential of immature testicular tissue and generation of sperm in rodents and primates [97]. Like germ cell transplantation, however, further research is needed before an application in humans can be considered safe and efficient. Despite current limitations with regard to generation of sperm from cryopreserved male germ line cells and tissues, and since future improvements of germ cell transplantation and grafting approaches are likely, retrieval and cryopreservation of testicular tissue prior to therapy should be offered to young men with cancer who are at high risk of fertility loss, as this could be their only option to maintain their fertility potential after treatment [103, 104]. Additionally, prepubertal testicular tissue from boys facing gonadotoxic treatment may be cryopreserved under special conditions. Doing so may offer fertility preservation for young patients in the future [87].

A potential concern about using spermatogonial stem cells and testicular grafts is the theoretical risk of restoring cancer cells back into the recipient. This effect has been demonstrated in leukemic rat models [104]. But efforts have been made to reduce this risk using telomerase in culture [105]. The use of embryonic stem cell technology to treat infertile men is also under investigation; however, significantly more translational research is needed, before these technologies are applied to the treatment of human male infertility [106].

Experimental protocols for cryopreservation of human testicular tissues from boys recently diagnosed with cancer have been developed [107, 108]. Another protocol has been introduced [108] for testicular tissue banking by collecting biopsies from both testis of prepubertal boys diagnosed with cancer and undescended testicles. While experimental testicular tissue banking is an option for future fertility applications in humans, the in vitro germ cell differentiation using cryopreserved testicular tissue has been demonstrated [109, 110].

22.15 Sperm Quality After Cryopreservation

Sperm quality in cancer patients (before freezing and after thawing) is generally worse than those of healthy donors [59]. Prostate cancer patients show the best pre-freeze total motile count (TMC) (155.1×10^6), whereas lymphoid leukemia the worst (26.8×10^6). Of all the semen parameters, compared with the controls, motility was most affected in cancer patients ($<5 \times 10^6$ TMC). Sperm quality also depends on the abstinence time between semen collections. When the cancer treatment is urgent, the normal abstinence time can be reduced from 2 to 5 days to more frequent collection schedule, sometimes multiple ejaculations within a day or two [111].

In a recent report, men with testicular germ cell tumor (TGCT) had a sperm survival rate of only 44.8% and had the lowest odds of a post-thaw total motile cell (TMC) above 5 million compared with controls and men with other types of cancers; they also had the lowest odds of successful intrauterine insemination (IUI) [59]. Men with seminoma demonstrated higher sperm concentration, TMC, and percentage motility than those with nonseminomatous germ cell tumor (NSGCT) [112, 113]. On the other hand, NSGCT histology was associated with a higher post-thaw TMC (OR: 4.3) than seminoma. The association between TGCT histology and cryosurvival is not clear and may be related to testicular development, Sertoli cell function, or gene and protein expression [114, 115]. Semen quality is comparable in repeated ejaculates from cancer patients [116]. However, conflicting results have been reported between cancer stage and semen parameters [59, 117, 118].

22.16 Procedure for Post-thaw Sperm Preparation

When semen is cryopreserved, a small aliquot of the sample is frozen separately, thawed, and reanalyzed after the initial freeze. This “test thaw” allows the post-thaw survival to be determined as it can vary among individuals and even among different ejaculates from the same person [61]. Post-thaw

sperm motility is a good representation of the entire ejaculate and gives a reliable estimate of the total motile sperm count for that sample in the future [119].

22.17 Number of Ejaculates Stored Prior to Use of Cryopreserved Sperm

The number of vials cryopreserved is determined by the quality of sperm, health of the individual, the type of cancer, time to start cancer therapy, and the type of ART procedure that will be required to achieve a pregnancy [120]. Therefore, optimizing the post-thaw TMC and cryosurvival is important [121]. While sperm quality is poor in cancer patients and may not be optimal for IUI, sperm of poor quality can be used for intracytoplasmic sperm injection (ICSI).

Cancer patients have worse parameters compared to controls, so this must be taken into consideration for the post-thaw total motile count for IUI [59]. As a rule of thumb, given a 50% cryosurvival from conventional cryopreservation, many laboratories would consider a pre-thaw motile sperm count of 10 million necessary to obtain approximately 5 million sperm after thawing, and this number is adequate for IUI [122–124]. This would be the ideal number of sperm frozen per vial so that each vial could be used with each subsequent IUI. Sperm concentration of >5–10 × 10^6 sperm is predictive of successful IUI [122, 125]. It is recommended that patients with TGCT freeze a minimum of 15 vials (about 1 million/vial) before oncological treatment, thereby providing adequate sperm for two IUI attempts. A similar number (>5×10^6 TMC) is needed to freeze for conventional IVF. If the sperm count or other parameters are lower than this, the samples can be aliquoted into a number of vials to be used for intracytoplasmic sperm injection [59].

22.18 ART Outcomes in Cryopreserved Sperm

Success rates of IVF and ICSI treatment using cryopreserved sperm are almost as high as fresh semen [126–128]. In cancer patients, using cryothawed sperm, the pregnancy rate per cycle and per couple in the IUI group was 11% and 32%, while it was 37% and 68% in the ICSI group. ICSI has been suggested as the preferred method of treatment for achieving pregnancies using cryopreserved sperm in cancer patients [129–134].

Naysmith et al. assessed the effect of cancer treatments on the natural and assisted reproductive potentials of men. Semen samples were analyzed before and after cancer therapy. Twenty-seven percent of the men had abnormal semen parameters before treatment, whereas 68% of the samples were abnormal after cancer treatment. Twenty-three percent of men developed azoospermia after treatment. Sperm cryopreservation prior to treatment improved the fertility potential of 55% of the patients. The authors commented that improving awareness and education of patients and providers on the impact of cancer and cancer treatments on fertility is essential. They also stressed that with the advent of ICSI, all men with cancer should be offered pretreatment sperm cryopreservation, as even in men with very low sperm concentrations, the chances of conception are very reasonable [135].

Schmidt et al. [136] reported their experience with couples referred for ART because of male factor infertility due to cancer and cancer treatment. Most of their patients had testicular cancer and lymphomas. Ninety percent of the men had adjuvant treatment with chemo- and/or radiation therapy. Perhaps, most impressively, semen was cryopreserved in 82% of the men prior to treatment. Following cancer therapy, 43% of the men had motile spermatozoa in the ejaculate, while 57% were azoospermic. Both fresh and cryopreserved sperm were used, and the clinical pregnancy rates per cycle were 14.8% after IUI, 38.6% after ICSI, and 25% after ICSI-frozen embryo transfer, with corresponding delivery rates of 11.1, 30.5, and 21%. Cryopreserved semen was used in 58% of the pregnancies. Of note, the delivery rate per cycle was similar after use of fresh or cryopreserved sperm. The authors concluded that male cancer survivors have a good chance of fathering a child by using either fresh ejaculated sperm or cryopreserved sperm and that ICSI be used as a first choice, given the better success rates with ICSI as well as the need for overall higher total motile sperm counts for IUI which are not always available post-thaw [129]. These reports of successful pregnancies with cryopreserved sperm in male cancer survivors are supported by numerous other studies [133, 137–143].

Van Casteren et al. reported their experience with ART using cryopreserved semen of cancer patients [130]. Five hundred and fifty-seven male cancer patients banked 749 semen samples. Out of the total group of 557 men who cryopreserved semen, 218 (39%) returned for semen analysis after cancer treatment. Motile sperm were found in 155 (71.1%) of these 218 men. Twenty of these 218 men reported a natural pregnancy. While only 42 of the cancer survivors (9.6%) ultimately requested the use of their banked semen, half of these men were successful in having live births using IVF/ICSI [130] indicating the importance of sperm cryopreservation before cancer therapy.

22.19 Utilization of Cryopreserved Samples

A few studies have looked at the utilization of cryopreserved sperm by male cancer survivors. In one study of 258 men, only 18 returned for treatment [144]. Ginsburg et al. found

that at their fertility center, 19 male cancer survivors underwent a total of 35 IVF cycles, and 11 of these cycles used cryopreserved semen [142]. In two studies, Magelssen et al. and Edge et al. looked at posttreatment paternity in 1388 testicular cancer survivors. Four hundred and twenty-two of these men had cryopreserved semen after orchiectomy. Overall, only 29 men (7%) used their cryopreserved semen for ART, while 67 men (17%) fathered at least one child with fresh semen [145, 146]. Lastly, according to a study by Saito et al., if male cancer survivors resume spermatogenesis following treatment, none would choose to use their cryopreserved sperm. Even if the cryopreserved sperm was not used, as in most cases, a positive psychological effect of having banked sperm was achieved [147]. The utilization of frozen samples remains low [148]. It is difficult to know the fertility status of the patients who do not come forward for follow-up testing, those conceiving naturally, those with no intention of conceiving, and those who may have psychological reasons for not participating. Because utilization of banked specimens is low, sperm banks should be carefully managed to ensure that resources are targeted to the patients in most need.

22.20 Challenges, Barriers, and Safety Issues of Sperm Cryopreservation

When cryopreserved samples leak in liquid nitrogen, there is a potential for cross-contamination to occur [149]. Regulatory bodies have issued current good tissue practice (CGTP) guidelines to prevent any adverse events resulting from these risks. All facilities offering sperm banking are regulated by the FDA and American Association of Tissue Banks (AATB) guidelines and are required to be registered with these bodies. The FDA has issued guidelines for businesses that provide human cells, tissues, and cellular- and tissue-based products; these establishments must follow the requirements in the CGTP regulations.

Crawshaw et al. [150] reported five main challenges of sperm banking in young cancer patients: attributes of professionals, skills of professionals, consent issues, issues relating to the effects of the process on the young men, and follow-up services. This study outlined the difficulties in building and maintaining an adequate knowledge and skills base in this field and lack of appropriate training [151]. Challenges also arise about what is to be done with stored materials in the event of the patient's death.

There are a number of barriers that can prevent patients from receiving fertility preservation counseling: lack of time during a patient visit, anxiety at the time of diagnosis, conflicting cultural or religious views, loss to follow-up during referral to a specialist, an inadequately communicated sense of seriousness regarding fertility loss, and a physician who is poorly informed about fertility preservation options [152].

Naturally, discussing potential fertility loss can cause patients to become very anxious—58% of patients felt that their levels of anxiety had affected their ability to think about fertility. It may be beneficial instead for physicians to discuss fertility preservation options initially at the time of diagnosis and again perhaps a week later but still before the start of treatment. Cultural or religious views that oppose masturbation or artificial insemination, for example, may also prevent fertility discussions from occurring productively [153].

Physicians who do not work in reproductive medicine may have a suboptimal knowledge of the effects of cancer and associated treatment on fertility, and this may be the limiting reason why many patients do not receive fertility counseling [107, 154]. The American Society of Clinical Oncology (ASCO) in their 2006 guidelines recommended sperm banking as a standard part of care, but it is still not implemented as often as it should be. Educating both physicians and other healthcare providers to integrate sperm banking awareness among the difficult discussions with their patients is vital [155].

Counseling adolescent patients poses an additional challenge to healthcare providers [154]. Doctors must work to alleviate patient anxiety by considering biological issues and acknowledging the psychological needs and individual situation when counseling young cancer patients [156]. Uncertainty on how to navigate the legal and ethical issues may discourage them from appropriately counseling these patients.

Many patients and their families make use of open and spontaneous discussion about fertility [154]. Such discussions can encourage patients to look to the future and reassure them that the aim of cancer therapy is cure. Sperm banking options must be on a case-by-case basis, especially in instances where a patient may be too sick with a very poor prognosis to produce a semen sample—in this case, sperm cryobanking may not be worth the effort.

Future infertility is not a problem of an individual, but of the couple, and a product of a family. As a result, decisions regarding fertility preservation can impact others besides the patient. This is true in case of minors, where these patients have little autonomy to decide sperm banking without their parents' approval [107]. In other instances, some families may not be comfortable discussing masturbation, sexuality, or reproduction issues with their son. The boys themselves may feel particularly uncomfortable talking openly about these issues or providing a sample via masturbation, or they may not fully understand the future implications of a more immediate loss of fertility if they are too young to be thinking about having children. Asking them to produce a sample when accompanied by a parent may unduly embarrass them.

Another serious ethical issue is the postmortem period in cases where the patient did not use his semen sample. Who does the sample belong to? Each clinical circumstance is unique, and each individual patient's diagnosis, prognosis, current desires and future hopes, relationship status, and (specifically in adolescents) maturity level must be taken into account when discussing fertility preservation options.

22.21 Factors Preventing Individuals from Banking

While options for fertility preservation or sperm banking are available, and more patients continue to be referred by their oncologists to discuss their fertility options, knowledge regarding the medical application of fertility preservation is still lacking particularly in the developing countries and in remote areas of developed countries. Even when patients were properly informed regarding infertility risk and cryopreservation, 42–54% did not use sperm banking [157]. Despite the well-established link between antineoplastic therapy and infertility, only 18–24% of young men with cancer were reported to have banked their semen prior to treatment [158]. Studies have shown that only 5–10% of patients who bank their semen prior to treatment return for IVF treatment using their cryopreserved specimens [137].

The psychological consequences of banking/not banking among patients in need of fertility preservation, particularly in the context of oncofertility, have been reviewed recently [159]. In another report, a significant variability was reported in the practice of fertility preservation for patients with cancer [160]. In that study, a 36-item survey was sent to board-certified reproductive endocrinologists. The results showed that 83% of the participants reported counseling men with sperm banking with 22% recommending against sperm banking for men previously exposed to chemotherapy. Overall, 79% of the respondents reported knowledge of the American Society of Clinical Oncology fertility preservation guidelines—knowledge that was associated with providers offering gonadal tissue cryopreservation in both men and women diagnosed with cancer, suggesting variability in how reproductive endocrinologists manage fertility preservation in cancer patients.

Effective promotion of sperm banking involves adequate communication regarding the severity and personal risk for infertility, assessment of the importance of having children, emphasis on the benefits of banking, and addressing possible obstacles such as cost, misperceptions, or cultural and other factors [161].

There are various factors for underutilization of sperm banking among young men. Some of the common factors cited were:

(i) Priority

Sperm banking is not usually a priority for patients who have already completed their family and for those that do not want to have children or for patients who are too young to understand the impact [161].

(ii) Cost

Presumed high cost by healthcare professionals is a major factor in sperm banking for patients [162]. In most instances, banking is not covered or is partially covered by insurance agencies. As cancer, itself, may already have a profound financial impact, many patients have concerns regarding the cost of sperm banking and continued long-term storage of specimens [161]. A survey of patients revealed that financial constraints were a major obstacle for 7% of cancer survivors who chose not to bank sperm [158]. Cost may play an even larger role in younger patients with limited or no income [161].

(iii) Time interval

The urgency to start therapy as soon as possible is also a major factor that prevents young patients from sperm banking [163]. Patients with leukemia have a relatively short time interval between initial diagnosis and the initiation of gonadotoxic therapy [164].

(iv) Lack of information

Very few men diagnosed with cancer bank their specimens, and the most common reasons in one study were lack of information or the attitude of the oncologist and practices regarding banking of sperm before cancer treatment [165]. There is a lack of education/counseling services by healthcare professionals [166] and limited use of cryopreservation by urologists and gynecologists in most fertility programs due to a lack of information regarding the effectiveness of gamete cryopreservation and a lack of agreement on the best universal method.

(v) Psychosocial issues with sperm banking—anxiety and emotional stress

A diagnosis of cancer at any stage of life (young or old) can provoke a life-altering crisis. The diagnosis itself and the threat of infertility both can cause a tremendous stress on these individuals [158, 165, 167]. Schover et al. [158, 165] noted some interesting psychological aspects in cancer survivors: (1) they may experience a higher level of infertility distress than healthy controls with (2) adolescents being more distressed than adults, (3) women are more often distressed than men, (4) those with inheritable cancers are more frequently distressed than those with noninheritable cancers, (5) a lower quality of life might be associated with less concern with regard to infertility, (6) cancer survivors might view their relationship with children more positively and (7) be more likely to prefer adoption or third-party donation, and (8) overall, they may lack accurate risk knowledge [167, 168]. These factors need to be acknowledged by healthcare professionals and utilized in proper care and treatment of these patients.

Surveys show that failure of physicians to provide patients with sufficient information in a timely manner is one of the

main reasons patients fail to utilize cryopreservation [169]. This could include situations in which the option was not presented entirely [158], the actual risk of infertility was downplayed by the physician [161], or patients who expressed interest failed to receive counseling and referral to a sperm bank [158]. These investigators reported that although more than 90% of oncologists felt that male patients at risk for infertility should be offered sperm banking [158], only 52% discussed the option with their patients [169]. Providing the patient with accurate information may help restore the patient's perception of the benefits of sperm banking. Rates of cryopreservation might be further improved by presenting sperm banking as a standard practice to patients and their families.

22.22 Counseling and Ethical Considerations

Counseling patients on fertility preservation, especially at the time of an initial oncologic diagnosis, can be challenging. The counseling should be offered by the oncologist or the physician who is delivering the diagnosis [130]. The important task of the oncologist is to clearly explain, in a compassionate manner, the disease, possible lines of therapy and probable implications of the disease, and its effects on male infertility. It is important to highlight the role healthcare practitioners play in patient decision-making with regard to fertility preservation [153]. Early and open communication with patients and implementation of a multidisciplinary oncofertility team are vital.

Because cancer and cancer treatment both adversely affect spermatogenesis, the onus is on the oncologist to discuss the effects on fertility and fertility preservation options and stress the importance of cryopreservation. It is the responsibility of the reproductive medicine specialist to ensure that other physicians are aware of the relatively good pregnancy outcomes that can be achieved with cryopreserved semen [130]. Educating physicians outside reproductive medicine as to when to refer a patient to a fertility specialist and discuss sperm banking and subsequent future fertility options is vital [130]. Oncologists should also be aware of contemporary ART treatment options available such as ICSI that require single healthy sperm for fertilization.

One organization, SaveMyFertility.org, is dedicated to increasing awareness of fertility preservation options among both providers and patients by providing informative materials that facilitate and stimulate discussions on the importance of sperm cryobanking (save my fertility.org). It is recommended that physicians initiate and guide sessions on fertility preservation with their patients. An informed nurse and other healthcare provider staff, however, may be helpful in providing continuing support throughout oncologic treatment [155].

22.23 Cost-Effectiveness of Fertility Preservation in the ART Era

Presumed high cost by healthcare professionals is a major factor in sperm banking for patients [162]. A survey of patients revealed that financial constraints were a major obstacle for 7% of cancer survivors who chose not to bank sperm [165]. Cost may play an even larger role in younger patients with limited or no income [161]. A recent study by Gilbert et al. [114] reported the cost-effectiveness of pretreatment fertility preservation vs. posttreatment fertility management in patients with testicular cancer. Sperm cryopreservation before chemotherapy or radiation therapy remains the most cost-effective strategy for fertility preservation across a range of possible costs associated with surgical sperm retrieval and ART.

22.24 Future Research Strategies

Sperm cryopreservation prior to undergoing chemotherapy or radiotherapy remains the most cost-effective strategy for fertility preservation, across a range of possible costs associated with surgical sperm retrieval and in vitro fertilization/intracytoplasmic sperm injection [114]. As part of education and informed consent before cancer therapy, healthcare providers (including medical oncologists, radiation oncologists, gynecologic oncologists, urologists, hematologists, pediatric oncologists, and surgeons) should address the possibility of infertility with patients treated during their reproductive years (or with parents or guardians of children) and be prepared to discuss fertility preservation options and/or to refer all potential patients to appropriate reproductive specialists. Sperm cryopreservation is considered a standard practice [170]. Other fertility preservation methods should be considered investigational and should be performed by providers with the necessary expertise. According to the latest ESHRE-ASRM Expert Update on fertility preservation [45], semen cryopreservation is the only established method of fertility preservation in men diagnosed with cancer at this stage.

Testicular tissue cryopreservation can be offered under IRB guidelines as an experimental procedure in prepubertal boys, because fertility restoration strategies by autotransplantation of cryopreserved testicular tissue have not been tested for safe clinical use for humans. Genetic stability of long-term culture of human SSCs from two cancer patients has been reported [171]. Fertility restoration strategies using autotransplantation of cryopreserved testicular tissue have not been tested for safe clinical use in humans [172]. Using proteomic platform to understand the underlying alterations in major reproductive pathways affecting the fertilizing

potential in cancer patients cryobanking their specimens before cancer treatment can shed further light on future management strategies in these men [173].

22.25 Conclusion

Improvements in cancer treatments have resulted in more men living into their reproductive years, and fertility is an important measure of quality of life in this patient population. However, all cancer therapies—chemotherapy, radiation, and surgery—are potential threats to male reproductive potential. The type of treatment(s) and individual susceptibilities to the deleterious effects of these treatments make it next to impossible to predict whether or not a man will recover spermatogenesis after therapy and what will be the status of fertility potential in these individuals. Stem cell transplantation technologies may hold promise in the future but are unavailable for use in humans at this time.

Many men in various stages of life could benefit from utilizing sperm cryopreservation. This makes sperm freezing a very viable option for cancer patients. Fertility preservation options should be discussed at an early stage during treatment planning for cancer. Efforts continue to optimize sperm freezing methods including recent research efforts in providing cryoprotectant-free sperm vitrification and refining the cooling and warming protocols to obtain optimal outcomes with vitrification. Assisted reproductive techniques such as ICSI offer a promising solution to enhance the rate of successful fertilization and subsequent pregnancy.

Barriers to sperm banking still exist, but the sensitive nature of this option can be overcome by patient and provider education, as well as deliberate, coordinated strategies at comprehensive cancer care centers to make fertility preservation for male cancer patients a priority during pretreatment planning. By all indications, the role for fertility preservation is only likely to increase over the next several decades. Providing fertility preservation options to these survivors is therefore imperative. It is important to offer the patient the option of fertility preservation rather than precluding his chance of fathering a biological child.

22.26 Review Criteria

An extensive search of studies examining the relationship between cancer and fertility preservation was performed using search engines such as Google Scholar and PubMed. The start and end dates for these searches were September 1985 and December 2018, respectively. The overall strategy for study identification and data extraction was based on the following keywords: “male infertility,” “cancer,” “chemotherapy,” “gonadotoxicity,” “sperm retrieval,” “sperm banking,” “semen parameter,” “fertility preservation,” “freezing techniques,” and “challenges in sperm cryopreservation.” Articles published in languages other than English were excluded. Data published in conference or meeting proceedings, websites, or books was also excluded.

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Clinical Management of Men with Nonobstructive Azoospermia due to Spermatogenic Failure

23

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Key Points

- Azoospermia denotes the lack of any sperm after examination of centrifuged semen specimens.
- Medical history, physical examination, and endocrine profile (FSH and total testosterone serum levels) are helpful to establish the type of azoospermia (obstructive vs. nonobstructive). Together, these factors provide a > 90% diagnostic accuracy.
- Clinical parameters and hormone testing results are unreliable markers for determining the chances of sperm acquisition in men with nonobstructive azoospermia due to spermatogenic failure (NOA-SF).
- NOA-SF men desiring fertility should be screened for Y chromosome microdeletions. Retrieval attempts should not be offered to men with AZFa and/or AZFb microdeletions. AZFc microdeletions will be invariably transmitted from father to son by ICSI.
- Hormonal therapy and microsurgical varicocele repair might increase sperm retrieval success in selected patients with NOA-SF.
- Micro-TESE is the technique of choice for SR in NOA-SF. The method increases the chance of retrieving testicular sperm for ICSI and minimizes testicular damage.
- Sperm fertilizing potential is lower in NOA-SF men than other male infertility categories. Compliance with state-of-the-art laboratory techniques and quality control is essential when handling testicular tissue taken from NOA-SF men.
- Men with NOA-SF have a reduced chance of achieving a live birth by ICSI compared to men with other infertility categories. The short-term profile of resulting offspring by testicular sperm ICSI does not seem to be adversely impacted by the spermatogenic failure.

23.1 Introduction

Azoospermia affects approximately 1% of all men and 10–15% of infertile males. Its definition relies primarily on the examination of the centrifuged pellet confirming a complete absence of spermatozoa in the ejaculate [1, 2]. Spermatogenic failure (also termed nonobstructive azoospermia [NOA] or secretory azoospermia) is a severe form of male infertility [3]. However, the testes of men with NOA might contain areas exhibiting full spermatogenic activity. In such cases, sperm production is minimal precluding sperm to appear in ejaculates. Among men desiring fertility, sperm retrieval (SR) remains the only viable option as treatment to restore spermatogenesis is not effective. The goal of SR is to harvest viable testicular sperm for intracytoplasmic sperm injection (ICSI) [4–6]. Testicular sperm taken from men with NOA are capable of fertilizing the oocyte and induce normal embryo development, thus allowing such men to achieve biological parenthood [7–9].

In this chapter, we discuss the clinical management of men with NOA due to SF according to the algorithm used in our center. Figure 23.1 depicts a brief overview of the consecutive steps, which will be detailed further.

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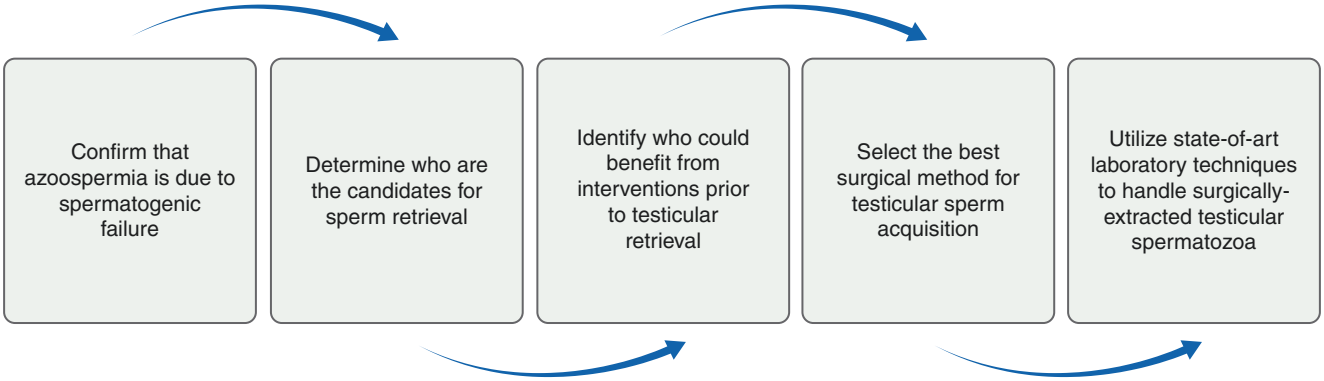

Fig. 23.1 Suggested step-by-step approach for the clinical management of men with nonobstructive azoospermia due to spermatogenic failure seeking fertility. (Reprinted with permission, ANDROFERT© 2018. All rights reserved)

23.2 Step 1: Confirm the Diagnosis of NOA due to Spermatogenic Failure

23.2.1 Semen Analysis

The diagnosis of azoospermia primary relies on the results of semen analysis. Thus, a rigorous method should be applied to reduce analytical error and enhance precision [2, 10]. The semen of men with NOA due to SF has normal volume (>1.5 ml; 95% confidence interval 1.4–1.7) and pH (~7.2), therefore confirming that the seminal vesicles are functional and that the ejaculatory ducts are patent [11].

The azoospermic semen on an initial examination should undergo centrifugation as a few spermatozoa might be present in the pellet [2]. An earlier study conducted in the 1990s showed that about 23% of men with azoospermia had spermatozoa in the pellet [12]. Centrifugation forces of >1000 *g* for a minimum of 10–15 minutes are needed to pellet the cells [13]. The examination of multiple semen specimens rather than a single specimen is vital for establishing the diagnosis of azoospermia. The reason relates to the reports of transient azoospermia secondary to toxic, environmental, infectious, or iatrogenic conditions [14, 15]. Examination of multiple semen analyses is also critical given the sizeable biological variability observed in semen specimens from the same individuals [10, 14, 15]. Finding viable sperm in the pellet could allow sperm injections to be performed without the need of SR. However, a 2015 small cohort study involving 57 patients with transient azoospermia showed that fertilization rates and embryo development were decreased when only immotile sperm were found in the ejaculate and used for ICSI. Nevertheless, pregnancy outcomes were not affected [16]. At our andrology laboratory, the azoospermic semen is centrifuged at 3000 *g* for 15 minutes, followed by a careful examination of the resulting pellet (Table 23.1).

23.2.2 Medical History

Nonobstructive azoospermia (NOA) relates to genetic and congenital abnormalities, postinfectious exposure to gonadotoxins, medications, varicocele, trauma, endocrine disorders, and idiopathic diseases. Thus, a detailed medical history should be carried out to underlie these conditions. Information should be obtained concerning: (a) diseases during childhood and puberty (e.g., viral orchitis, cryptorchidism); (b) previous surgeries, in particular, those involving pelvic/inguinal regions and genitalia; (c) history of genital trauma; (d) history of infections (e.g., orchiepididymitis and urethritis); (e) physical and sexual development; and (f) exposure to gonadotoxic agents (e.g., radiotherapy, chemotherapy, and steroid abuse) [1, 9].

23.2.3 Physical Examination

The affected patients have normal epididymides and palpable vasa deferentia, but the testes are often small (<15 ml in volume) and soft. However, men with NOA associated with spermatogenic maturation arrest (MA) have well-developed and normal-volume testes [17, 18].

23.2.4 Endocrine Profile

The pulsatile secretion of pituitary gonadotropins controls spermatogenesis. Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) stimulate the Sertoli cells and Leydig, respectively, promoting differentiation of immature germ cells to mature spermatozoa within the seminiferous tubules and androgen production. FSH levels higher than twice the normal upper limit is a reliable indicator of spermatogenic failure [19]. Indeed, FSH levels are often high in

Table 23.1 Interventions and recommended actions in the clinical management of azoospermic men with nonobstructive azoospermia seeking fertility

Clinical management step	Intervention	Action	Interpretation
Differential diagnosis	Medical history, physical examination, endocrine profile (FSH and testosterone levels at a minimum; LH, prolactin, thyroid hormones, and estradiol are added as needed), and examination of pelleted semen in multiple occasions Testicular biopsy could be considered in selected cases in which the differential diagnosis could not be determined	Confirm that azoospermia is due to spermatogenic failure, and identify men with severely impaired spermatogenesis with presence of few sperm in the ejaculate	A differential diagnosis between obstructive azoospermia, hypogonadotropic hypogonadism, and spermatogenic failure should be established as management varies according to type of azoospermia
Determination of proper candidates for sperm retrieval	Y chromosome microdeletion screening using multiplex (PCR) blood test The basic set of PCR primers recommended by the EAA/EMQN for the diagnosis of Yq microdeletion includes: sY14 (SRY), ZFX/ZFY, sY84 and sY86 (AZFa), sY127 and sY134 (AZFb), sY254 and sY255 (AZFc)	Deselect men with microdeletions involving subregions AZFa, AZFb, and AZFb+c	Approximately 10% of men with NOA-SF harbor microdeletions within the AZF region SR success in men with YCMD involving the subregions AZFa, AZFb, and AZFb+c are virtually nil and such patients should be counseled accordingly SR success in men with AZFc deletions range from 50% to 70% Genetic counseling should be offered to men with AZFc deletions because testicular spermatozoa used for ICSI will invariably transmit the deletion from father to son
Identification of patients who could benefit from medical therapy or varicocele repair before sperm retrieval	Serum levels of total testosterone and estradiol	Medical treatment with gonadotropins, aromatase inhibitors, or clomiphene citrate should be considered for the patients with hypogonadism (TT < 300 ng/dL) or T/E ratio < 10	Patients should be counseled that the evidence of a positive effect of medical treatment remains equivocal
	Physical examination to identify the presence of clinical varicocele and analysis of testicular biopsy results (if available)	Microsurgical repair of clinical varicocele	Microsurgical varicocele repair might increase SR success Patients with testicular histopathology indicating Sertoli-cell-only are unlikely to benefit from varicocele repair Evidence of a positive effect of varicocele repair is limited, and patients should be counseled accordingly
Selection of the most effective surgical method for testicular sperm acquisition	Analyses of testicular biopsy results (if available) and of whether sperm have been obtained in previous treatment and by which method	Microdissection testicular sperm extraction is the SR method of choice Conventional testicular sperm extraction may be considered in cases of previous success with TESE, particularly when testicular histopathology indicates hypospermatogenesis	Micro-TESE in NOA-SF is associated with higher SR success (42.9–63%) than conventional TESE (16.7–45%) The lower tissue removal facilitates sperm processing and lessens testicular damage
State-of-the-art laboratory techniques to handle surgically extracted testicular spermatozoa	Extraction of a minimum volume of tissue by micro-TESE facilitates tissue processing and search for sperm Testicular tissue preparation techniques include mechanical and enzymatic mincing and erythrocyte lysis	Sterile techniques, stable pH and temperature, and high laboratory air quality conditions useful to optimize micromanipulation efficiency and safety Excess sperm not used for ICSI should be cryopreserved for future attempts	Spermatozoa collected from NOA-SF men are often compromised in quality and are more fragile than ejaculated counterparts The reproductive potential of such gametes used for ICSI is differentially affected by NOA-SF

EAA European Association of Andrology, *EMQN* European Molecular Genetics Quality Network, *ICSI* intracytoplasmic sperm injection, *micro-TESE* microdissection testicular sperm extraction, *NOA-SF* nonobstructive azoospermia due to spermatogenic failure, *PCR* polymerase chain reaction, *SR* sperm retrieval, *T/E* testosterone to estradiol ratio, *TESE* testicular sperm extraction, *TT* total testosterone

men with NOA due to SF. By contrast, total testosterone levels are either low (<300 ng/dl) or around the lower limit [1, 20] in about 45% of the affected patients [21, 22]. A decrease in testosterone levels is likely indicative of Leydig cell deficiency, which may be associated with high luteinizing hormone (LH) levels [22, 23].

In most cases, however, LH levels will be within normal limits. Testosterone secretion follows a circadian cycle with an early morning peak between 8 and 10 am; it is, therefore, essential to instruct our patients to collect blood specimens accordingly. Bioavailable testosterone (free or unbound testosterone) levels may vary depending on sex hormone-binding globulin (SHBG) and albumin concentrations.

Notably, low testosterone levels could result from obesity, metabolic dysfunction, or thyroid dysfunction [24, 25]. Obesity can increase peripheral serum levels of estradiol and estrone due to increased peripheral aromatization of testosterone and androstenedione under the influence of aromatase [26]. High estradiol levels have a direct inhibitory effect on testosterone biosynthesis and might reduce the pulse amplitude of cyclical pituitary LH and FSH secretion [24, 27]. Furthermore, obese men have excessive circulating leptin that exerts an effect on steroidogenesis mediated by its adverse action on specific Leydig cell receptors, thus reducing the serum levels of androgens further [28].

Men with NOA-SF can also have a normal endocrine profile. The control of FSH and LH secretions relies on the quantities of spermatogonia and Leydig cells, respectively, which are well-preserved in men with maturation arrest. Indeed, patients with diffuse spermatogenic maturation arrest and 10% of those diagnosed with the Sertoli-cell-only syndrome (SCOS) present with non-elevated endogenous gonadotropins [17, 18]. Nevertheless, the combination of a testis long axis < 4.6 cm and FSH levels >7.6 IU/l was shown to predict NOA in 89% of men [29].

23.2.5 Hypogonadotropic Hypogonadism

Despite being rare, hypogonadotropic hypogonadism (HH) falls in the category of nonobstructive azoospermia (NOA). HH is characterized by failure of spermatogenesis due to lack of appropriate stimulation by gonadotropins. By contrast, spermatogenic failure relates to an intrinsic testicular impairment [30]. Unlike SF, men with NOA due to HH have remarkably low levels of pituitary gonadotropins (FSH and LH < 1.2 mUI/ml) and androgens (TT < 300 ng/dl), usually associated with signs of absent or poor virilization. NOA due to HH includes both patients with congenital forms of HH and those whose sperm production is suppressed by excess exogenous androgen (e.g., anabolic steroid abuse). The patients with HH benefit from specific hormonal therapy. These patients usually show remarkable recovery of spermatogenic function with exogenously administered gonadotropins or gonadotropin-releasing hormone [30].

23.2.6 Testis Biopsy

Testis biopsy is the "gold standard" test for confirmation of azoospermia due to SF. The common histopathology phenotypes include hypospermatogenesis, germ cell maturation arrest, germ cell aplasia (Sertoli-cell-only syndrome), tubular sclerosis, or a combination of those. The specimens are placed in fixative solutions such as Bouin's, Zenker's, or glutaraldehyde. Importantly, formalin should not be used as it may disrupt the tissue architecture. We only perform testicular biopsies when we could not establish the differential diagnosis between obstructive azoospermia and NOA. In these cases, we use either a percutaneous or an open "window" technique without testis delivery [1, 31]. When we find mature sperm on a wet examination, we routinely offer the patient sperm cryopreservation [31, 32].

Histopathology results have also been used to predict the chances of finding testicular sperm on retrievals. Patients with SCO have lower SR rates (19.5%) than those with MA (40.3%, $p = 0.007$), and these rates in both categories are lower than that of hypospermatogenesis (100.0%, $p < 0.001$) [33]. However, performing a testicular biopsy in men with NOA for diagnostic purposes remains controversial. Firstly, due to testicular heterogeneity, none of the histopathology phenotypes can predict with certainty who may or may not have sperm retrieved [33, 34]. Secondly, extraction of testicular parenchyma might inflict additional harm as men with NOA usually have small testes and abnormal androgen production. Lastly, biopsied specimens might contain mature spermatozoa that would be wasted after fixation and staining. Thus, despite the clinical utility of histopathology data for counseling, caution should be applied to recommend it for men with NOA routinely. If one opts to do so, it is advisable to also perform a wet examination of the extracted specimen and freezing the testicular parenchyma in case sperm is identified.

23.3 Step 2: Define Who Are Possible Candidates for Sperm Retrieval

The uncertainty of sperm acquisition in men with NOA due to SF makes prognostic factors desirable (Table 23.1). In a study involving 60 men with SF, we found that infertility etiology, testicular volume, serum levels of pituitary gonadotropins, and testicular histopathology results have low accuracy overall to predict who will have a successful SR [35]. In this study, the areas under the ROC curve for FSH, testosterone,

and testicular volume as regards a successful SR were 0.53, 0.59, and 0.52, respectively. Others have shown that a combination of testicular volume, FSH levels, and histopathology results yielded a diagnostic accuracy of 74% [36]. Sperm retrieval success varies from 25% to 70% in cryptorchidism, post-orchitis, Klinefelter syndrome, radio-/chemotherapy, and idiopathic infertility [37–41].

By contrast, genetic screening of the long arm of the Y chromosome has proved to be of value not only to identify those patients in whom Yq microdeletions is the cause of NOA but also to predict the chances of retrieving sperm [4, 42–49]. The region Yq11 at the long arm of the Y chromosome, known as "azoospermia factor" (AZF), clusters 26 genes involved in spermatogenesis regulation [46, 49–51] (Fig. 23.2). About 10% of men with azoospermia due to spermatogenic failure harbor microdeletions within the AZF region that might explain their condition [46, 51]. In practical terms, finding a microdeletion within the AZFa or AZFb region means that the odds of harvesting sperm from the seminiferous tubules are virtually nil, irrespective of the method used for sperm acquisition [52]. By contrast, patients with isolated AZFc microdeletions often harbor residual spermatogenesis, with SR success ranging from 50% to 70%.

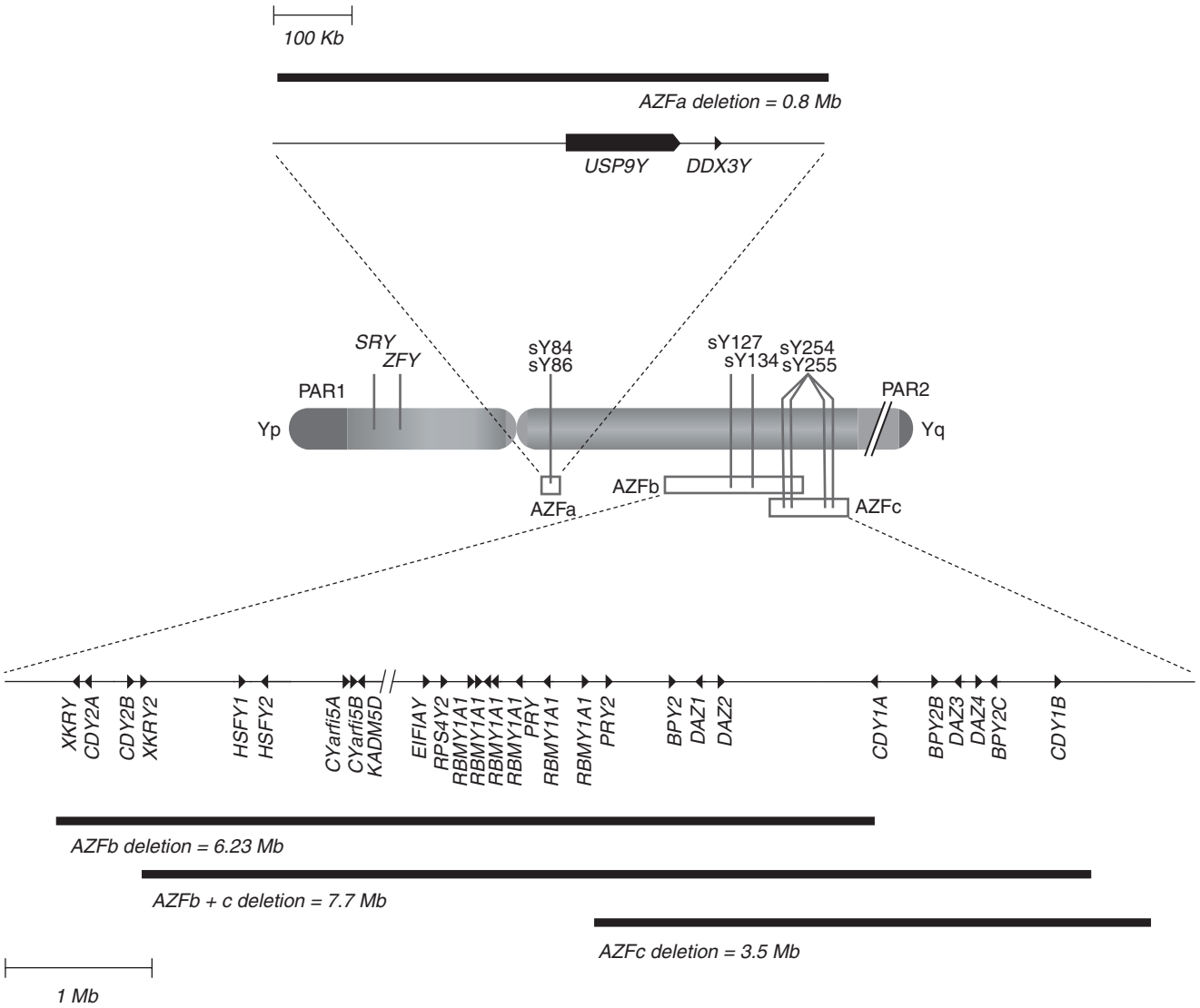

Fig. 23.2 Human Y chromosome map depicting the AZF subregions and gene content. The AZFa region maps from approximately 12.9–13.7 Mb of the chromosome and contains two single copy genes, USP9Y and DDX3Y. AZFb spans from approximately 18–24.7 Mb of the chromosome and AZFc from approximately 23–26.7 Mb. Both regions contain multiple genes as depicted in the bottom of the figure. The location of the basic set of sequence-tagged sites primers to be investigated in azoospermic men with spermatogenic failure, according to the European Association of Andrology and the European Molecular Genetics Quality Network 2013 guidelines, are identified by solid vertical lines. (Reprinted with permission, ANDROFERT© 2018. All rights reserved)

23.4 Step 3: Define Who Can Benefit from Interventions Before Sperm Retrieval

23.4.1 Medical Therapy

Some studies have examined the effect of therapies to boost testosterone production in men with NOA-SF and hypogonadism. Testosterone is essential for spermatogenesis [53, 54], and its levels are over 100-fold higher in the testes than in the serum [55]. Thus, boosting testosterone production could restore intratesticular androgenic bioactivity that is essential to sustain spermatogenesis when combined with adequate Sertoli cell stimulation with FSH [56]. Clomiphene citrate, gonadotropins (human chorionic gonadotropin and FSH), and aromatase inhibitors [22, 24, 38, 57–61] are among the tested medical interventions. Table 23.2 [22, 58, 59, 61–65] summarizes the existing studies and their main outcomes. Although an overall beneficial effect has been observed, the evidence is currently limited and based mostly on case series. Hence, a firm conclusion on the role of medical and surgical intervention therapy in men with spermatogenic failure and azoospermia cannot be drawn yet. Randomized controlled trials are needed to precisely assess the impact of such interventions on sperm production and sperm retrieval outcomes.

Table 23.2 Summary of studies concerning medical therapy in NOA-SF

Study	Design	Study group	Control group	Medication	Main findings
Pavlovich et al. (2001) [58]	Case series	43 men with T/E ratio < 10	N/A	Testolactone, 50–100 mg twice daily for a mean of 5 months	None of the 12 men who completed 3 months of treatment had sperm in ejaculate; T/E ratios were restored to normal range (>10) in all treated men
Hussein et al. (2005) [63]	Prospective cohort	42 men with favorable histology (hypospermatogenesis or maturation arrest)	N/A	CC, 50 mg every other day for men for 5 months; dose titrated by 25-mg increments until target T levels between 600 and 800 ng dl^{-1} were achieved	64.3% of the men had sperm in posttreatment semen analysis (mean density of 3.8 million ml^{-1} and motility of 20.8%). All of the men ($n = 15$) who remained azoospermic had success at SR
Selman et al. (2006) [64]	Prospective cohort	49 men with normal endocrine and genetic profile in whom diagnostic testis biopsy showed maturation arrest and no sperm on wet examination	N/A	rec-hFSH, 75 IU SC on alternate days for 2 months and then dose increased to 150 IU and hCG (2000 IU SC twice weekly) added for another 4 months	None of the patients had return of sperm in ejaculate; posttreatment SRR were 21.4%
Ramasamy et al. (2009) [59]	Case series	56 men with nonmosaic Klinefelter syndrome and T levels lower than 300 ng dl^{-1}	N/A	Testolactone (50–100 mg) or anastrozole (1 mg) were used orally, alone, or combined with subcutaneous (SC) hCG (up to 2500 IU 3× week) for at least 3 months	SRR were increased by 1.4-fold (77% vs. 55%; $p = 0.03$) in men who had an elevation of 150 ng dl^{-1} T levels from baseline after medical therapy compared to ones who did not
Reifsnyder et al. (2012) [22]	Retrospective cohort	307 men with T levels lower than 300 ng dl^{-1}	41 men with T levels lower than 300 ng dl^{-1}	AI (50 to 100 mg testolactone orally twice daily or 1 mg anastrozole daily), hCG (at a dose of 1500 to 2000 IU SC 2-3× a week SC), or CC were used, alone or combined, for at least 2 to 3 months before surgery	None of the patients had return of sperm in ejaculate; SRR were not different in treated (51%) and untreated (61%) men; SRR were not different between treated males with T levels lower than 300 ng dl^{-1} (51%; $n = 307$) and untreated ones with T levels greater than 300 ng dl^{-1} (51%; $n = 388$)

Table 23.2 (continued)

Study	Design	Study group	Control group	Medication	Main findings
Shiraishi et al. (2012) [65]	Prospective cohort	28 men with idiopathic[a] NOA who had negative SR	20 men with idiopathic[a] NOA who had negative SR	At least 6 months after the first SR attempt, patients received treatment with hCG (5000 IU SC 3× weekly) for 3 months. If FSH levels decreased after hCG (<3 mIU ml^{-1}), recombinant FSH (rec-hFSH, 150 IU SC 3× weekly) was added for 2 months	Sperm was obtained at the second SR attempt in 6 (21%) of the 28 treated men ($p < 0.05$), but in none of the untreated men
Hussein et al. (2013) [61]	Prospective cohort	612 unselected men	116 unselected men	CC (50 mg every other day) alone or combined with hCG (5000 IU SC twice a week) and hMG (75 IU SC once weekly) were administered for an average of 5.4 months	Sperm were found in ejaculates of 10.9% of the treated males; in patients who remained azoospermic, SRR were higher in those who received medical therapy compared with controls (57.0 vs. 33.6%, $p < 0.001$)
Hu et al. (2018) [62]	Prospective cohort	25 NOA men with failed SR by TESE and hypospermatogenesis on testis histology (treatment group)	10 NOA men with failed SR by TESE and hypospermatogenesis on testis histology (no treatment; control group)	Goserelin (3.6 mg 1× every 4 weeks for 24 weeks). hCG (2000 IU IM 1×/week started 4 weeks after G and was given for a total of 20 weeks). hMG (150 IU IM 2×/week) was started 4 weeks after hCG and was given for a total of 16 weeks	6 months after treatment, SR was repeated, and sperm were acquired in 2 of 25 treated patients. Both of them received medical therapy, and sperm was found in the ejaculate in one of them Increase in inhibin B levels was found in 25% of treated group

AI aromatase inhibitor, *CC* clomiphene citrate, *FSH* follicle-stimulating hormone, *G* Goserelin, *hCG* human chorionic gonadotropin, *hMG* human menopausal gonadotropin, *IM* intramuscular, *IU* international units, *NOA* nonobstructive azoospermia, *rec-hFSH* recombinant human FSH, *SC* subcutaneous, *SR* sperm retrieval, *SRR* sperm retrieval rates, *T* total testosterone, *T/E* testosterone to estradiol ratio, *TESE* testicular sperm extraction, *N/A* not applicable

[a]Exclusion criteria were men with Klinefelter syndrome, testis volume less than 4 ml, T levels less than 200 ng dl^{-1}, varicocele and cryptorchidism

23.4.2 Varicocele Repair

Varicocele is found in 4–13% of men with NOA-SF [66, 67]. The surgical repair of clinical varicoceles, mainly using microsurgical techniques, has been carried out in an attempt to improve sperm production in such men [66–68]. The goals are to increase the chances of finding sperm in the semen or retrieving sperm from the testis.

A 2016 review systematically evaluated the role of varicocele repair in men with NOA [69]. Sixteen studies accounting for a total of 344 men reported data related to sperm return to the ejaculate post repair of clinical varicoceles. The age and follow-up duration of the treated population were 32.5 years (± 2.3) and 12.4 months (± 5.5), respectively. The proportion of patients with postoperative return of sperm to the ejaculate was 44% (range 20.8%–55.0%). Postoperatively, the mean sperm count and motility were 1.8 million/mL (SD 1.6, 95% CI 0.9–2.7) and 23% (SD 15%, 95% CI 12%–33%), respectively, and the interval between varicocele repair and appearance of sperm in postoperative ejaculate varied from 4.5 to 11 months. Three studies provided surgical sperm retrieval (SR) data between treated and untreated men with varicocele and NOA and therefore were meta-analyzed [69]. The observed pooled effect size involving 400 patients indicated that SR success was significantly higher in the treated varicocele group than in the untreated one (OR 2.6, 95% CI 1.7–4.1, $I^2 = 0\%$, $P < 0.0001$). The time interval between varicocele repair and SR was 10.8 months (SD 11.1, range 3–23.6 months). Two studies involving 140 couples in total reported data on pregnancy by ICSI using testicular sperm harvested from the seminiferous tubules [70, 71]. These studies included a control group of men with NOA and untreated varicocele for comparison. The estimated pooled increase in the odds of achieving a clinical pregnancy and a

live birth by ICSI using testicular sperm from treated men were 2.2 (95% CI OR 0.99–4.83, $P = 0.05$, I^2 0%) and 2.1 (95% CI OR 0.92–4.65, $P = 0.08$, $I^2 = 0\%$), respectively [69]. The results discussed above were corroborated by Kirby et al.'s meta-analysis, which showed that SR rates (OR = 2.5) and pregnancy rates (OR = 2.3) were significantly higher in persistently azoospermic men after varicocele repair [72].

23.5 Step 4: Use the Optimal Method for Harvesting Testicular Sperm

The primary goal of sperm retrieval in NOA-SF are to obtain the optimal quantity of good quality sperm, which can be used for ICSI promptly or cryopreserved for future ICSI attempts (Table 23.1). Retrieval methods should also minimize testicular damage thus preserving androgen activity and the chance of repeated retrieval attempts.

Both microdissection testicular sperm extraction (micro-TESE) and conventional TESE have been used to retrieve sperm from NOA-SF men. Conventional TESE involves open single or multiple testicular biopsies taken at random, which are processed and examined for the presence of sperm [5, 9, 36, 73]. Since the prediction of both the existence and the geographic location of the islets of normal spermatogenesis is not possible before SR, more than one specimen is usually required to find sperm. TESE with multiple biopsies results in higher SRR than fine-needle aspiration (TEFNA), a variation of testicular sperm aspiration (TESA), particularly in cases involving SCO and maturation arrest [74]. A disadvantage of TESE is that removal of large fragments of testicular tissue might affect the already compromised androgen production, transiently or permanently [75]. Added to this, laboratory processing of such large quantities of testicular tissue taken by TESE is time-consuming and labor-intensive [31, 32, 76].

Microdissection testicular sperm extraction (micro-TESE) is a microsurgical method of sperm retrieval [76]. Micro-TESE is preferred over TESE due to the higher SR rates and lower surgical complications such as hematoma formation, fibrosis, and testicular atrophy. Also, the lower tissue removal facilitates sperm processing and lessens testicular damage [5, 32, 41, 57, 73, 76–80]. Micro-TESE aims at identifying focal areas of sperm production within the testes, based on the size and appearance of the seminiferous tubules, with the aid of the operating microscope (Fig. 23.3). Such areas are selectively extracted thus allowing minimal tissue removal, which has been shown to be 50- to 70-fold less when compared with conventional TESE [5, 73, 76]. The use of optical magnification also reduces the chances of vascular injury by proper identification of testicular blood supply, thus reducing the chances of hematoma formation and testicular devascularization [41]. Although a decrease in

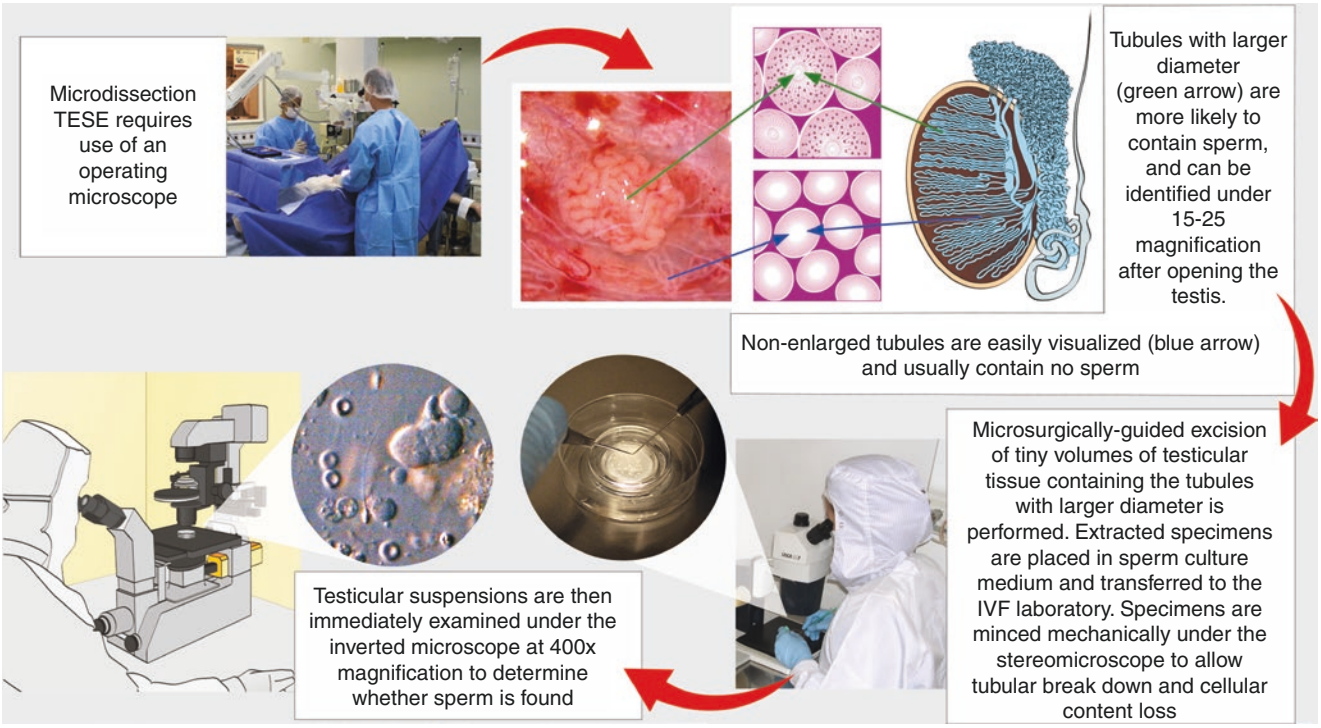

Fig. 23.3 Microdissection testicular sperm extraction. Flowchart illustrates the consecutive steps from the microsurgical procedure to the laboratory processing of testicular specimens. (Reprinted with permission, ANDROFERT© 2018. All rights reserved)

serum testosterone has been documented after removing testicular parenchyma by micro-TESE, especially in men with severely compromised androgen activity such as those with Klinefelter syndrome [38], testosterone levels return to pre-surgical values in 95% of the subjects within 18 months following surgery [81].

In a controlled study involving sixty men with SF, we compared SR rates between micro-TESE and conventional single-biopsy TESE [35]. The SRR was significantly higher with micro-TESE (45 vs. 25%; $p = 0.005$) both overall and after stratifying the patients by testicular histopathology phenotype (hypospermatogenesis 93 vs. 64%; maturation arrest 64 vs. 9%; Sertoli-cell-only syndrome 20 vs. 6%; $p < 0.001$). Controlled studies have corroborated our results by showing that micro-TESE has higher sperm recovery and lower complication rates (below 5%) than conventional TESE [77–80]. We have recently reported our updated experience involving 356 patients with SF who have undergone micro-TESE. SRR was 41.4% overall [7] and 100.0, 40.3, and 19.5% according to the histopathology phenotypes of hypospermatogenesis, maturation arrest, and SCO, respectively [33]. Micro-TESE might rescue approximately one-third of the cases that failed in previous retrieval attempts with conventional TESE or TESA and is particularly useful for men with spermatogenic failure presenting the worst-case scenarios [57, 76]. Lastly, a recent systematic review involving seven comparative studies and 1062 patients confirmed that micro-TESE in SF was associated with a more favorable sperm retrieval rate ranging from 42.9% to 63% compared with 16.7–45% in conventional TESE [82].

23.6 Step 5: Laboratory Handling of Testicular Sperm

The laboratory handling of surgically retrieved gametes requires special attention because spermatozoa collected from NOA-SF men are often of reduced quality and more fragile than ejaculated counterparts [83]. Both sperm DNA fragmentation and aneuploidy rates are higher in testicular sperm obtained from men with spermatogenic failure compared with ejaculated sperm obtained from infertile men with various etiology categories [84, 85]. Consequently, lower fertilization, embryo development, and pregnancy rates have been achieved when ICSI is carried out with gametes retrieved from men with SF [7, 83].

From the surgeon's perspective, attention should be taken to deliver the best-quality extracted sample as possible for the laboratory personnel, with minimal or no contaminants such as red blood cells and harmful microorganisms. Micro-TESE allows extraction of a minimum volume of tissue, which is advantageous. The searching process in lower-volume testicular tissue increases laboratory efficiency and minimizes missing spermatozoa in the sea of cells and noncellular elements [32]. Testicular tissue preparation techniques have been developed to increase sperm retrieval rates that have been used to handle these specimens, including mechanical and enzymatic mincing. These techniques promote tubular wall breakdown and cellular content loss [32, 86, 87].

The laboratory team must master the processing techniques to minimize iatrogenic cellular damage during sperm preparation (Table 23.1). State-of-the-art laboratory practice standards, including sterile techniques and laboratory air quality conditions, are of utmost importance to optimize micromanipulation efficiency and safety assurance [32, 88]. Critical aspects to be controlled include (i) centrifugation force and duration; (ii) exposure to ultraviolet light and temperature variation; (iii) laboratory air quality conditions; (iv) dilution and washing steps; and (iv) quality of reagents, culture media, and disposable materials. At our center, we perform sperm retrieval and all related laboratory steps involved in the handling of testicular specimens in controlled environments. The latter includes tissue processing, microinjection of surgically extracted sperm, the culture of embryos generated from such procedures, and cryopreservation. At Androfert Center, the IVF and andrology laboratories, the operating theater in which oocyte and microsurgical sperm harvestings are carried out, and embryo transfer rooms were constructed according to cleanroom standards for air particles and volatile organic compounds [89].

Lastly, whenever possible, techniques to improve sperm fertilizing potential should be applied, including the use of chemical stimulants and methods to select viable sperm for ICSI. The latter is particularly important when only immotile spermatozoa are available as their vitality may be reduced. After a successful SR in NOA-SF, cryopreservation of surplus testicular sperm is highly recommended because such patients often require more than one ICSI treatment to establish pregnancy, but repeated retrieval attempts are not always possible. Cryopreservation of testicular sperm from NOA-SF men can be done with either conventional straws and liquid nitrogen (LN) vapor techniques or low-volume straws and ultrafast LN freezing [90–92]. In a meta-analysis of 11 studies and 574 ICSI cycles, there was no statistical difference between fresh versus frozen-thawed testicular sperm with regards to fertilization rates (RR 0.97, 95% CI 0.92–1.02) and CPRs (RR1.00, 95% CI 0.75–1.33) [93].

23.7 Outcomes of ICSI

The clinical outcomes of ICSI using surgically extracted testicular sperm from NOA-SF men are lower than ejaculated and obstructive azoospermia counterparts [7, 83, 94–96]. Apparently, such sperm have a higher tendency to carry deficiencies in centrioles and genetic material, which might affect

their potential to activate the oocyte and to trigger the formation and development of a normal zygote and a viable embryo [84, 85].

In an early series involving 330 patients with different infertility conditions including 53 azoospermic men with SF, we examined ICSI outcomes according to the source of spermatozoa and the type of azoospermia. We found that 2PN fertilization rates were significantly lower with testicular sperm from NOA-SF men than from those with ejaculated and testicular/epididymal sperm of men with obstructive azoospermia (52.2, 71.1, and 73.6% in SF, ejaculated sperm, and OA, respectively; $p < 0.05$). Embryo development and pregnancy rates are also negatively affected by SF [35]. In two series involving a larger cohort of NOA-SF men, we compared ICSI outcomes and analyzed the health of offspring according to the source of sperm and the type of azoospermia. In one study, 182 women underwent ICSI using sperm from partners with NOA-SF, and the outcomes were compared with a group of 182 and 465 women whose partners had OA or where non-azoospermic. Live birth rates after ICSI were lower in the SF group (21.4%) compared with the OA (37.5%) and non-azoospermic sperm (32.3%) groups ($p = 0.003$). In this report, 326 live births resulted in 427 babies born. Despite a tendency towards poorer neonatal outcomes in the azoospermia categories, differences in gestational age, preterm birth, birth weight, and low birth weight were not noted [96]. In another series, we compared 365 azoospermic men with SF who underwent micro-TESE with 40 men with SF who used donor sperm for sperm injections due to failed retrieval and 146 men with OA who underwent percutaneous sperm retrieval. The SR rate in SF was 41.4%, and the results were lower than the OA group (100%, adjusted odds ratio 0.033, 95% CI 0.007–0.164, $p < 0.001$). Live birth rates were lower among couples whose male partners with SF had their own sperm used for ICSI (19.9%) than those who used donor sperm (37.5%, adjusted odds ratio 0.377 [95% CI 0.233–0.609, $p < 0.001$]) and obstructive azoospermia (34.2%, adjusted OR 0.403 [95% CI 0.241–0.676, $p = 0.001$]). Neither the miscarriage nor the newborn parameters (gestational age, birth weight, malformation rate, perinatal mortality) of infants conceived were significantly different among the groups [7]. Although the data on the health of resulting offspring after ICSI using the sperm of men with azoospermia due to SF is reassuring, only five studies have compared to date the neonatal profile of such babies [7, 8, 96–98]. The limited population analyzed calls for continuous monitoring, and studies on the physical, neurological, and developmental outcomes of children conceived are limited [99].

23.8 Complete Aspermatogenesis

Aspermatogenesis relates to either a complete lack of germ cells or its presence as immature forms only. The condition affects 25–45% of NOA-SF men [100]. Spermatids are the earliest male germ cells with a haploid set of chromosomes. The use of spermatids for ICSI has been attempted when mature sperm are not available, but more than two decades since its introduction, the clinical efficacy of spermatid injection in humans has been merely anecdotal [101, 102]. Besides, ethical and safety concerns related to potential transmission of genomic imprinted disorders have driven the ban of spermatid injection in the United Kingdom. In the USA, spermatid injection is considered as an experimental procedure [103]. In 2015, Japanese scientists reported the birth of 14 babies after refinement of spermatid injection by activation with electric current [104]. The resulting offspring were found to have no notable physical, mental, or epigenetic problems. However, the number of children born to date is too small and the follow-up period too short to consider this approach a safe option.

Current efforts focus on the differentiation of preexisting immature germ cells or the production/derivation of sperm from somatic cells [105]. In humans, generation of human haploid-like cells from pluripotent stem cells of somatic origin has been achieved using in vitro sperm derivation. Despite promising, these methodologies are experimental, and the in vitro production of gametes is a highly complex process which has not been translated to humans yet [100].

23.9 Conclusions

The clinical management of infertile men with azoospermia starts with a proper diagnostic workup that allows the differentiation between NOA-SF and other types of azoospermia. Patients with NOA-SF should be screened for Y chromosome microdeletions. Currently, sperm retrieval (SR) should be offered only to men with no Yq microdeletions or microdeletions involving the AZFc region solely. Nevertheless, genetic counseling should be given to NOA-SF patients with AZF Yq microdeletions as the use of their testicular sperm for ICSI will inevitably transmit the deletion from father to son. Before sperm retrieval, medical therapy to boost endogenous testosterone production and microsurgical repair of clinical varicoceles can be offered to selected men. These interventions could increase the sperm retrieval likelihood of success or eventually allow the finding of a few sperm in the ejaculate, which might be used for ICSI. Micro-TESE is the SR technique of choice for NOA-SF men. The method not only increases the chance of retrieving testicular sperm for ICSI but also minimizes testicular damage. Compliance with state-of-the-art laboratory techniques is essential to both avoid jeopardizing the sperm fertilizing potential and improve ICSI outcomes when handling testicular specimens extracted from NOA-SF men. The likelihood of harvesting sperm for ICSI is reduced in men with NOA-SF compared to those with obstructive azoospermia. These patients are also at a reproductive disadvantage concerning ICSI outcomes when compared to other

male infertility categories. However, the short-term profile of infants conceived after sperm injection does not seem to be negatively affected by NOA-SF. Current research efforts on biotechnology techniques aiming at producing artificial gametes could rescue fertility in men whose germ cells are entirely lacking or present only in immature forms.

23.10 Review Criteria

An extensive search of studies examining the relationship between nonobstructive azoospermia and infertility, with a focus on spermatogenic failure, was performed using search engines such as ScienceDirect and PubMed. The end date for these searches was September 2018. The overall strategy for study identification and data extraction was based on the following keywords: "non-obstructive azoospermia," "spermatogenic failure," "azoospermia," "sperm retrieval," "infertility, male," "assisted reproductive technology," "intracytoplasmic sperm injection," "semen parameters," "reproductive outcomes," and "pregnancy rate." We have not included articles published in languages other than English. Data that were published in a conference or meeting proceedings, websites, or books were used to provide conceptual content only.

Conflict of Interest The authors have nothing to disclose.

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Novel Approaches in the Management of Klinefelter Syndrome

24

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Key Points

- Klinefelter syndrome is the most common sex chromosomal anomaly in men that occurs mostly due to meiotic nondisjunction, but most of the cases remain undiagnosed.
- The diverse phenotypic expression in its presentation requires a great deal of conjecture from the clinician caring for such patients.
- Early diagnosis and treatment of boys with KS should be emphasized as it can have beneficial effects on their physical, social, and academic development.
- Cryopreservation of sperm is offered to the adolescent KS patients who are able to provide a semen sample.
- Pharmacological therapy with aromatase inhibitors prior to micro-TESE has the highest sperm retrieval rates in azoospermic men.

24.1 Introduction

Klinefelter syndrome (KS) was initially described in a report published by Klinefelter et al. in 1942 on nine men, who had sparse facial and body hair, enlarged breasts, and small testes [1]. The genetic basis of KS was discovered in 1959 when an extra X chromosome was found in the genetic profile of those patients [2]. The occurrence of KS ranges from 85 to 223 per 100,000 live-born males. Hence, it is the most frequent sex chromosomal anomaly [3]. It is encountered in approximately 3–4% of infertile males and the occurrence rises to 10–12% in patients with azoospermia [2]. It is estimated that only 25–40% of the subset of patients with KS are ever diagnosed and only around 10% of these patients are identified during their childhood and adolescent years [4]. The reason behind the underdiagnosis of KS can be explained by the wide variability in its presentation requiring a great deal of conjecture from the clinician caring for such patients.

The management of patients with KS is still provided by endocrinologists who may not particularly focus on the reproductive aspects of this disease. That being said, KS patients can have a good chance of fathering their biologic children provided that proper fertility-preserving treatment is offered to them. While a subset of KS patients can still produce sperm, testicular sperm can be retrieved from azoospermic KS patients and used in assisted reproductive procedures. The latter finding has triggered the interest of reproductive specialists to explore methods that could increase the reproductive outcome of such patients.

24.2 Genetic Background

Initial research on the genetic profile of patients with KS showed that they typically carry a 47XXY karyotype [2]. Further studies have shown that this karyotype is held by 85–90% of males with KS, while in the remaining patients, a mosaic karyotype (46,XY/47,XXY), a supernumerary chromosome (47,XXY/48,XXXY, 48,XXYY, 48,XXXY, 49,XXXXY), or structurally abnormal sex chromosome can be present [5, 6]. A national survey done in Denmark on 4477 patients with KS and a cohort study done in the UK showed that the karyotype 47XXY was present in 86.3% of

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patients, while the frequencies of the remaining karyotypes are listed in Table 24.1 [4, 5].

Meiotic nondisjunction is the basic genetic defect in patients with KS. The improper separation of the sister chromatids during the process of cell division results in the abnormal number of chromosomes in daughter cells that is called aneuploidy. It is generally accepted that most trisomies in autosomal chromosomes occur due to nondisjunction in the maternal meiotic division, whereas the paternal errors occur in less than 10% of the patients [7]. It has been suggested that the paternal origin of a chromosomal aneuploidy having an extra Y chromosome should impart an effect on the phenotype of KS patients having this chromosomal anomaly. However, most of the studies could not find any association, whereas only a few were able to find some effect on phenotypic traits due to the parental errors including particularly an effect on the time of onset of puberty [8] and the waist and height ratio to the arm span [9]. Differences in the communication skills and motor function were also found [10] as well as correlations with some psychiatric morbidities such as schizotypal and autistic traits [11].

Table 24.1 Karyotype distribution among 4477 Klinefelter syndrome patients

47XXY	3863 (86.3%)
46XY/47 XXY	383 (8.5%)
48 XXXY	157 (3.5%)
49 XXXXY	66 (1.5%)
48,XXY + trisomy of chromosome 18	5 (0.1%)
47,XXY/48,XXXY	2 (<0.01%)
Undetermined	1 (0 < 0.01%)

Based on data from Ref. [4 and 5]

When the sex chromosomal aneuploidy is due to maternal origin, it can occur due to nondisjunction of the homologous chromosomes in the first or second meiotic division, whereas in a sex chromosomal aneuploidy with an extra X chromosome due to paternal error, the meiotic nondisjunction must occur in the first meiotic division as if it occurs in the second stage of meiosis, then it will result in only two possibilities that are either XX or YY gametes (Figs. 24.1 and 24.2). On the contrary, the mosaic forms of KS are either a result of the loss of an additional X chromosome in a 47XXY genotype or due the nondisjunction of the homologous chromosomes in mitotic division at early stages of cell division in a normal 46XY zygote; this process is named as trisomy rescue.

Bojesen et al. proved that maternal age was an important risk factor for KS. The authors found that there was a fourfold rise in the occurrence of KS cases if the age of the mother was more than 40 years [5]. However, paternal age has not been considered highly influential on the prevalence of KS [12]. Recent work by Fonseka et al. confirmed that paternal age has a little effect on sex chromosomal trisomies and an even lower effect on autosomal aneuploidy [13]. However, some studies depicted that paternal age was influential on fathers who had an offspring with paternally inherited chromosomal aneuploidy [14].

Patients with KS exhibit a broad variety in the phenotype that vary from being normal or almost normal to having developmental issues at an early age and fertility problems. It is not known why patients with KS present on either end of the phenotypic spectrum. This variation in the phenotype can

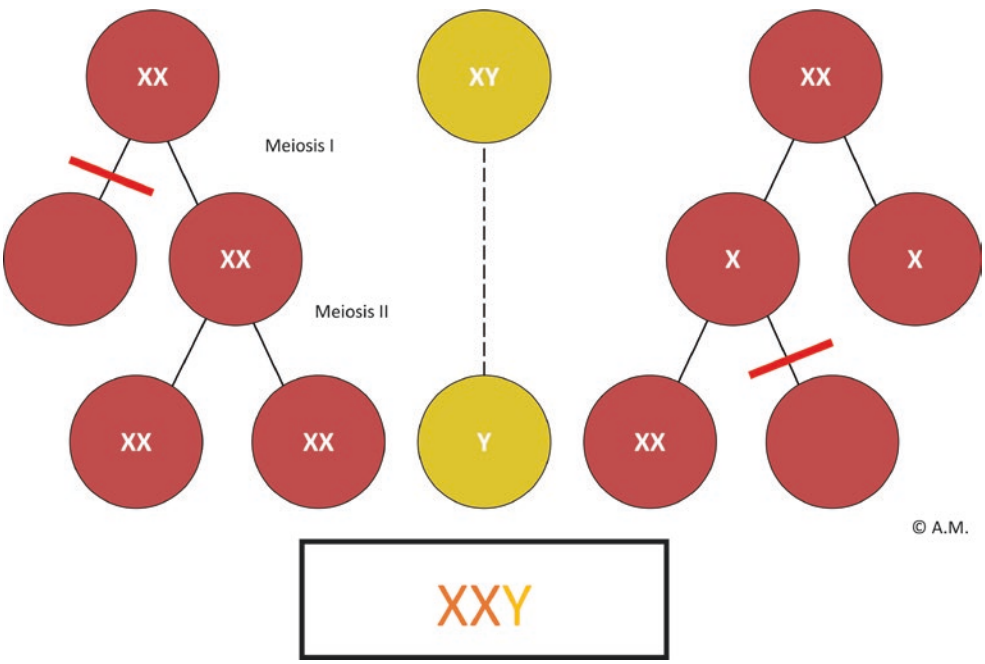

Fig. 24.1 47XXY of maternal origin

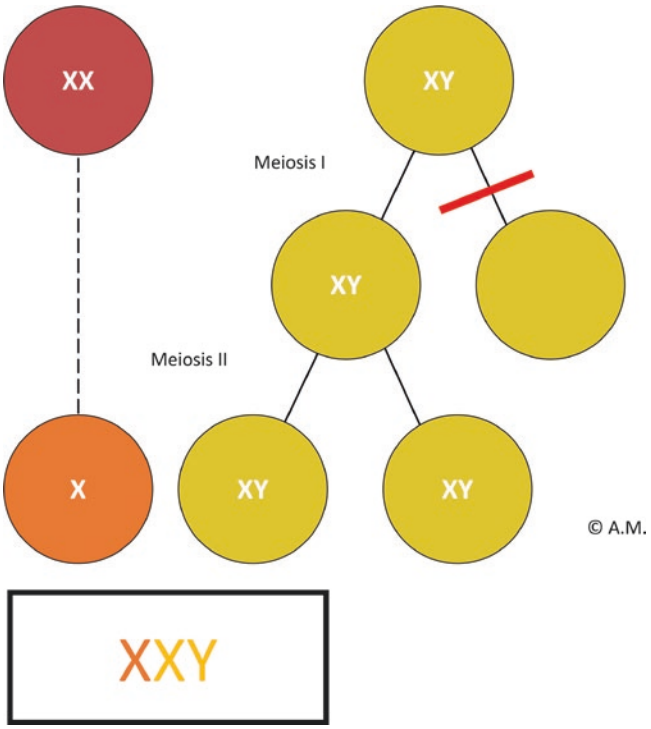

Fig. 24.2 47XXY of paternal origin

be described by a phenomenon called genomic imprinting, in which certain genes are expressed in the phenotype, while others are silenced depending on their parent of origin [10]. Gene polymorphisms occurring after the inactivation of the X chromosome provide more explanation. Gene polymorphisms in the form of CAG repeat sequence encode the androgen receptor (AR) which is located on the X chromosome. The relationship between the functional response of the AR and CAG repeat sequence has an inverse relation. To further clarify, the longer the AR CAG repeat sequence, the less marked is the androgen effect. Nonrandom or skewed inactivation of the X chromosome is seen in patients with KS in which the shortest AR CAG repeat sequence is inactivated [15]. Studies have shown that there is a negative correlation between the length of the CAG repeat sequence and penile length [16], higher education and strong relationships [15]. KS patients with shorter CAG repeat sequence respond better to androgen therapy. On the other hand, longer length of the CAG repeat sequence is related to enlarged breasts, deranged anthropometric measures such as body height and arm span, and poor bone density and testicular volume [15]. The variety in the phenotype of KS patients might be clarified by the concept of nonrandom inactivation of the X chromosome. Recent studies have shown that DNA methylation profile in patients with KS is related to the changes in the brain and blood tissue. It might be possible that the mechanism of DNA methylation plays a role behind the broad phenotypic spectrum in KS patients [17, 18].

24.3 Endocrine Function and Spermatogenesis in KS

Recent studies show that the initial embryonal development of the testicles is similar in infants having KS and infants with normal 46XY karyotype. Germ cells originate from the yolk sac and then migrate to the urogenital ridge [19]. The secretory function of the Leydig and Sertoli cells that originate from the mesenchyme is initiated after this migration of germ cells [19]. Work done by Hunt et al. showed that the mitotic proliferation of the germ cells that undergo migration to the urogenital ridge is reduced as the testicular development progresses [20]. This was further confirmed by Mikamo et al. who demonstrated that during the first year of an infant's life, the number of spermatogonia gradually declines from 24% to 0.1% compared to the control value [21]. Studies performed on boys with KS during their prepubertal age have demonstrated that a number of spermatogonia are markedly decreased. They also found evidence of arrest in maturation at the spermatogonium and the early primary spermatocyte stage [22]. Furthermore, the ability of the immature Sertoli cells to transform into the mature Sertoli cells is also reduced [23].

This creates a conflict on how spermatogenesis occurs in KS patients. To clear this conflict, two hypotheses have been given. The first hypothesis suggests that the spermatogonia of the 47XXY patients are able to complete the process of meiosis that clarifies the increased rates of aneuploidy in the sex chromosomes [22, 24]. The second hypothesis states that the compromised testicular environment in KS patients results in errors in the meiotic cell division that results in a higher rate of aneuploid sperm, while some traces of the normal 46 XY spermatogonial stem cells may still exist [25]. The histopathologic examination of the testicular tissue of adult patients with KS has shown patches of hyalinization of the seminiferous tubules and areas showing extensive fibrosis [26] intermixed in larger areas containing more matured cellular structure [27]. However, as the majority of patients with KS are non-mosaic, this fact can reject the second hypothesis.

Unlike spermatogenesis, the endocrine function appears to be normal in patients with KS until the age of puberty. Previous studies have shown evidence that KS patients in their prepubertal age have normal serum levels of luteinizing hormone (LH), follicular-stimulating hormone (FSH), testosterone, and inhibin B before the beginning of puberty [28–30]. The level of serum testosterone rises initially, then the level plateaus, and afterward it remains at the lower limit of the normal range constantly during the entire puberty [29, 30]. However, this level of serum testosterone is adequate for boys having KS to develop during puberty and attain adequate secondary sexual characteristics [28, 31].

Boys with KS in the prepubertal and pubertal age mostly have high levels of estradiol, despite the presence or absence of gynecomastia, with a predisposition to have a higher ratio of estradiol/testosterone ratio during puberty [28, 32]. The levels of inhibin B in KS patients show an early rise before the beginning of puberty just like normal boys, but due to the rise in levels of serum testosterone, they drop rapidly [23, 29]. A gradual increase is seen in the serum levels of FSH and LH in patients with KS as they progress from the midpuberty onward. The rise in FSH levels appears early and is more marked than LH levels [28, 29, 32].

24.4 Clinical Manifestations

The clinical presentation of KS patients varies according to the age of the patient and his phenotypic expression. It is difficult to recognize KS male patients before the onset of puberty because of the endocrine reasons discussed in the previous section. Physical anomalies that are present in KS patients including increased length of the lower limbs and mild decrease in the testicular volume often go unnoticed. After the onset of puberty, a variety of symptoms of deficiency of androgens characterize the syndrome. These symptoms in KS patients can be classified into the following:

Consequences of Hypogonadism A recent study determined a link between the levels of testosterone in the prenatal period and the adult phenotypic features in normal men showing that the levels of testosterone in the umbilical cord were associated with more masculine features. They also showed that the levels of testosterone in the adult period did not seem to affect such features [33]. This might be the case in patients with KS, but still no study has been conducted to prove this association. Most of the patients are able to attain a normal penile size, but about 70% of patients have complaints of decreased libido and potency as they approach the age of 25 years [34].

Gynecomastia In the first description of KS by Klinefelter et al., all the patients had gynecomastia, and it was considered to be a characteristic trait [1]. However, later studies showed that it was present in about one-third of the patients [35]. Gynecomastia was due to the deficiency of testosterone or the decreased ratio of testosterone to estradiol [36].

Anthropometry and Body Composition An increase in the anthropometric measures such as hip, waist circumference, body weight, total and abdominal fat, and total fat percentage was found in a cohort study done on 73 males when they were compared to age-matched controls [9]. The height of patients with KS is higher when compared to normal men with a mean of 5–7 cm. However, this increased height is attributed to the relative hypogonadism in KS patients during puberty that causes delayed epiphyseal closing resulting in longer leg length [9]. A recent study conducted on adult KS males in Korea found that 57% of them were having normal BMI [37]. The increase in BMI is most likely due to a trend of general increase in BMI in the general population. BMI as an indicator of fat mass of the body should be interpreted cautiously in patients with KS, as they have an increased height and lower lean body mass. However, KS males have an increased truncal fat percentage, evaluated by dual-energy X-ray absorptiometry, for any given BMI [9, 38].

Bone Mineralization Patients with KS have altered bone mineralization secondary to the degree of hypogonadism they most likely have, placing them at higher risk for bone fracture and osteoporosis [4, 39, 40]. Bone mineral density (BMD) has been found to be decreased in KS patients in many clinical studies but without any clear correlation with the levels of serum testosterone [41, 42]. Furthermore, some clinical studies detected the presence of decreased levels of 25-hydroxyvitamin D and decreased muscle strength in KS patients [41, 43]. Lower levels of insulin-like growth factor 3 (INSL3) have been detected in KS patients. While this hormone is known to correlate with osteocalcin, a marker of bone formation, no clear relationship was found between the levels of BMD and INSL3 [44].

Diabetes and Metabolic Syndrome A threefold rise in the occurrence of diabetes in KS patients has been found in epidemiological studies of both mortality and morbidity [38, 40]. A study conducted on 39 male patients with KS found that the average age at diagnosis of diabetes mellitus was 27.1 years, with a prevalence of 12.5% [45]. The prevalence of diabetes was higher in the KS patients when compared to the control group that comprised of men with idiopathic hypogonadotropic hypogonadism. The authors proposed that the deficiency of testosterone alone cannot clarify the increased prevalence of diabetes in KS patients. The extra X chromosome might play a role in the development of diabetes as the prevalence of diabetes is higher in patients with more than normal X chromosomes [45]. The prevalence of type 1 diabetes is also higher in KS patients as the levels of autoimmune antibodies directed against diabetes-specific autoantigens in patients with KS were 8.2% compared to less than 1% in controls [46]. The incidence of metabolic syndrome in KS seems relatively higher when compared to other populations of men with hypogonadism. This suggests that metabolic syndrome is related to a more specific genetic background [47]. A recent study conducted in Germany including 132 patients with KS reported higher level of triglycerides and lower levels of HDL compared with controls [48].

The risk of diabetes and metabolic syndrome significantly rises with hypogonadism. However, this relationship seems to be related more to the adiposity rather than the deficiency of testosterone because insulin sensitivity improved in obese patients only and did not improve the sensitivity of insulin in lean patients [49].

Cardiovascular Disease Mitral valve prolapse and impairment of the diastolic function of the ventricles and the chronotropic function of the heart are more common among KS patients [50–53]. Clotting defects are also seen in KS patients that can lead to thromboembolic events. The high BMI and low levels of testosterone levels that are characteristically seen in KS patients are related to elevated levels of plasminogen activator inhibitor-1 (PAI-1) which can have detrimental effects on fibrinolysis [54, 55]. Di Minno et al. documented an increase in the activity of platelets among KS patients [56]. Increased levels of C-reactive protein, LDL cholesterol, and triglycerides combined with low levels of HDL cholesterol are thought to be factors that can aggravate the risk of getting IHD in KS patients. Adiponectin is a secretory protein that is specific for adipocytes and is protective against hypertension, and diabetes [57] is negatively correlated with the levels of testosterone in the body [58]. Interestingly, a study conducted by Swerdlow et al. found a slightly decreased mortality rate in KS patients (standardized mortality rate (SMR) (95% CI); 0.7 (0.5–0.9)) [4], whereas another study conducted by Bojesen et al. noticed an increased incidence of IHD in KS (hazard ratio (HR) (95 % CI); 1.71 (1.28–2.29)) [40].

Cancer The risk of malignancies in KS patients is almost similar to that of the normal population [40, 59]; however, certain malignancies such as breast cancer, mediastinal tumors, and hematological disorders are more likely to occur in KS patients, while KS has a protective effect against prostate cancer. The risk of breast cancer in patients with KS increases to 50-fold [60]. Most of the mediastinal germ cell tumors arise before 30 years of age and have been seen in more than 40 patients [61]. Non-Hodgkin lymphoma and leukemia are the hematological malignancies mostly related to KS [62]. Significantly decreased risk of prostate cancer is attributed to the decreased levels of testosterone [59]. A study done in the UK in 2017 showed that diabetes and increased body fat have a protective effect on prostate cancer, so they could be the reasons behind the decreased risk of prostate cancer in KS patients [63].

Cognitive Disturbances KS patients commonly exhibit verbal deficits, detected in approximately 70–80% of patients [64]. KS patients were compared to dyslexic children in intellectual activities planning, concept formation, problem-solving, and switching tasks [65].

Table 24.2 Frequencies of psychiatric illnesses in Klinefelter syndrome patients

Learning Disorders	65%
Attention deficit hyperactivity disorder	63%
Depressive disorders	24%
Psychotic disorder	8%
Schizophrenia	2%

Based on data from Ref. [69]

Psychiatric Disturbances Epidemiological studies have shown that people with chromosomal aneuploidy having an extra X chromosome as in KS have a higher incidence of psychiatric illnesses when compared to cytogenetically normal population [66]. Children of school age with KS exhibit low self-confidence, apprehension, temperament disorders and problems of socialization [67]. Work done by Ross et al. demonstrated that the presence of problems in early life with poor socialization often resulted in psychiatric disorders [68]. A study conducted on 51 KS boys based on the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR; APA, 2000) revealed the presence of psychiatric disorders in them [69]. The frequencies are shown in Table 24.2.

24.5 Diagnosis

KS diagnosis requires clinical suspicion and a high level of awareness about its clinical presentation such as sexual dysfunction, delayed puberty, low levels of testosterone, and infertility. The condition should be investigated when signs and symptoms of hypogonadism are seen during the initial evaluation of male fertility. Clinical findings such as decreased facial and body hair with enlarged breasts are usually evident. Genital examination reveals small testes with a firm consistency. In a study conducted in Japan, atrophy of prostate is seen in 30% of the cases on digital rectal examination [70].

The testes of KS patients undergo slow degeneration during childhood, which progresses during puberty resulting in fibrosis and hyalinization of the seminiferous tubules, which is a characteristic feature of KS [71]. This evidence of degeneration of testicular tissue in early life was supported by Wikstrom et al. who observed that only 50% of KS males in their peripubertal age had germ cells in their testes [23]. In another study, the same authors observed that the germ cells are arrested at the early stages of cell division [72]. As a result, during ejaculation, only 8.3% of KS males are able to produce sperm [73]. Endocrine evaluation of adult KS patients exhibits lower levels of serum testosterone in about 80% of patients, secondary to impairment of Leydig cells during puberty [36]. The serum concentrations of sex-hormone-binding globulin (SHBG) are high in KS patients,

which further aggravates the reduction of free testosterone. On the contrary, the testosterone/estradiol ratio is disrupted due to the higher levels of estradiol in KS patients compared to normal men. The concentrations of LH and FSH are raised emphasizing a hypergonadotropic hypogonadism picture. The levels of FSH are consistent demonstrating the coherent damage of seminiferous tubules [74], which is secondary to decreased levels of inhibin B [29].

Early diagnosis and treatment of boys with KS should have beneficial effects on their physical, social, and academic development [75]. Unluckily, only 10% of men having KS are identified at a young age, the time during which treatment may be most beneficial. To screen KS patients, children with learning difficulties or those having developmental problems should be the target population so that timely detection and prompt treatment can be done [75].

Testing for Barr bodies by taking sample from the buccal mucosa, which corresponds to the inactive supernumerary X chromosome, is an obsolete method [76]. Currently, the diagnosis is confirmed through cytogenetics by the chromosomal analysis of lymphocytes [76]. Skin fibroblasts or testicular biopsy samples might be required if a normal karyotype is reported to confirm chromosome mosaicism. Microdeletion analysis of the Y chromosome is also indicated in patients having KS, as some reports have suggested that KS patients are at an increased risk for such deletions [77].

KS patients are at an increased risk of osteoporosis and osteopenia due to androgen deficiency so DEXA scan should be regularly done to check bone density. If the presence of osteopenia or osteoporosis is confirmed, then further laboratory tests should be done that include serum calcium, serum phosphorus, parathyroid hormone (PTH), and vitamin D3.

KS patients should be informed that they are at an increased risk of breast cancer, and they should be educated on performing self-breast examinations regularly. They should also be encouraged to seek medical advice when they feel a change in the consistency of breast tissue or if they notice any discharge from the nipples.

24.6 Management

A multidisciplinary approach toward the management of KS patients is essential to encounter the multiple features of the disease. However, attaining fertility is the fundamental goal while treating KS patients, the clinician should not undermine other consequences of the disease that can have an impact on the quality of life and health of the patient. Some factors must be taken into account while managing a patient with KS that include the presenting complaint of the patient, the age at which the diagnosis was made, and the status of the fertility of the patient.

24.6.1 Managing Hypogonadism

24.6.1.1 In Adolescents and Adults

The hypogonadism in KS patients needs to be treated by testosterone replacement (TRT) at some point during the course of the therapy. The impact of TRT on fertility in men with KS is still not known in studies done till date. In a study conducted by Schiff et al. [78], TRT was given to five patients for a time span ranging from 2 to 14 years, but sperm retrieval was successful in only one patient through testicular sperm extraction (TESE). Another study conducted by Ramasamy et al. observed a worse sperm retrieval rate (SRR) in patients who had received TRT [79]. Recent treatment practice statements recommend to initiate TRT in KS patients in the age of early-to-midpuberty, or at the time of commencement of hypogonadism [80, 81, 2], to ascertain the completion of puberty and to minimize the long-term undesired effects of androgen deficiency. Some physicians choose to initiate TRT therapy even at an earlier age trying to correct the relatively decreased penile length in KS boys [82]. There are no established protocols for TRT therapy in KS patients. Clinical Practice Guidelines for treatment of hypogonadal men can be used to determine dosage according to age [83].

Some reports have emphasized that hormone replacement therapies can enhance sperm production and can decrease the harmful effects of exogenous testosterone. In study of 10 patients who were administered topical testosterone for a period of 1–5 years with an aromatase inhibitor in combination before doing micro-TESE, sperm retrieval rate (SRR) was 70% [84]. In another study on relatively larger sample size of 21 non-mosaic KS patients, the sperm retrieval rate (SRR) was 57%, which was comparatively less [85]. Aksglaede et al. investigated 22 studies in which the overall sperm retrieval rate was 50% [71]. The levels of testosterone need to be monitored after TESE, as it takes more than 12 months for the testosterone levels to return to its normal level [86]. The combination of intramuscular injections of human chorionic gonadotropin or clomiphene citrate are other available options that can be used. These regimens are not specifically used in patients with KS; however, some evidence can be deduced from their use in men with androgen deficiency. A retrospective study conducted on 26 men documented no differences in the semen parameters of the patients after 1 year of treatment with human chorionic gonadotropin (HCG) in combination with TRT; surprisingly 35% of the couples became pregnant spontaneously [87]. In another study, the lower doses of HCG were used and they were successful in sustaining adequate intratesticular levels of testosterone [88]. A disadvantage that is encountered after the continuous use of HCG is that FSH replacement therapy should also be started after some time so that the process of spermatogenesis is maintained. In hypogonadal men, clomiphene citrate has been acknowledged as an equitable

alternative to exogenous testosterone. In spite of having a lower efficacy than testosterone in its injectable form, clomiphene citrate is efficient to achieve comparable symptom relief [89]. A new estrogen receptor modulator—enclomiphene citrate—was successful in normalizing the endogenous production of testosterone and restoring the sperm counts by restoring the functioning of the hypothalamic-pituitary-testicular axis [90]. Concerns about the safety regarding the use of clomiphene and its derivatives for a longer period of time arose. Moskovic et al. worked on this issue by doing a follow-up of 46 patients being treated with clomiphene citrate for more than 12 months. He was unable to find any adverse effects in that group of patients, so making it clear that it can be used in appropriate patients for longer-term therapy [91]. Still more studies are required to ensure the safety of patients on a larger population for a longer period of time.

24.6.2 Fertility Management

24.6.2.1 Peripubertal KS Boys

Based on prior knowledge, there is a progressive decline in the testicular function that begins at puberty. Some researchers suggest to seek fertility preservation at an early age even before staring TRT. In a study conducted on adolescents aged 12–20 years, Mehta et al. were able to confirm the presence of sperm in the ejaculates of 70% of the patients [92]. Research on testicular tissue samples in this age group are justifiably scarce. In a case report by Damani et al., successful sperm retrieval was done on a boy aged 15 years [93]. Work done by Aksglaede et al. [71] confirmed that the loss of germ cells in the testicular tissue occurs after the age of 10 years. Wikstrom et al. [23] examined testicular tissue samples in 14 boys with KS aged 10–14 years and found germ cells in only half of the patients, proposing that testicular dysfunction is present in the peripubertal period in KS patients. Furthermore, all the samples that contained germ cells were from boys aged less than 12 years with normal testicular volume according to their age and normal serum FSH and serum inhibin B concentrations. Researchers have predictive markers that would allow sperm retrieval to be done at the most accurate time to ensure its success. As the levels of serum inhibin B levels are in the normal range during the prepubertal and early pubertal periods [29], it makes it an optimal marker to exhibit the number and integrity of Sertoli cells. Unfortunately, this notion was not supported as the levels of inhibin B even within the normal range did not correlate with the presence of spermatogonia [22]. The levels of anti-Mullerian hormone also did not correlate with spermatogenic activity even though its levels rise during prepuberty and early puberty in KS boys and decrease subsequently [94]. Insulin-like factor 3 (INSL3), a marker of Leydig cell function, also did not correlate with spermatogenic activity as it is within normal levels before the onset of puberty in KS patients and decreases subsequently [72]. In a study conducted on both children and adults, Rohayem et al. determined that if the levels of testosterone are above 7.5 nmol/l and the levels of LH are below 17.5 U/l the sperm retrieval rates by mTESE are higher [95]. This concept was supported by another study conducted generally on men with azoospermia; low levels FSH and LH combined with higher levels of serum testosterone were acknowledged as positive predictive markers. However, in KS patients, the predictive value of serum testosterone still remains unsettled [96].

24.6.2.2 Cryopreservation in Adolescents

Moral concerns arise when attaining sperm from patients in the adolescent age via masturbation. Practices such as idiosyncratic masturbation or anejaculation that are frequent in this age group would further exacerbate the challenge. The clinician should be capable to handle this sensitive issue by actively involving parents and dealing with the patients according to their respective ages. Cryopreservation of sperm is offered to the adolescent KS patients after their detailed counselling with their parents. Current guidelines support sperm banking for all males who are at or above Tanner stage III, particularly if progressive motile sperm are found on semen analysis [97].

Stimulatory techniques can be used if masturbation cannot be executed. Penile vibratory stimulation or electroejaculation can be attempted under anesthesia. Surgical Sperm Retrieval techniques can also be used of which the most commonly used are percutaneous epididymal sperm aspiration (PESA) or testicular sperm aspiration (TESA). Schlegel et al. conducted a study to compare the SRR in patients undergoing standard TESE and micro-TESE. He determined a statistically significant difference in the SRR that was 45% in standard TESE as compared to 63% in micro-TESE, and he also found a difference in the spermatozoa yield that was 160,000 in micro-TESE as compared to 64,000 in standard TESE [98].

Furthermore, micro-TESE has decreased the damage to the blood supply of the testicles and decreased the amount of testicular tissue needed. Most of the research related to the use of these procedures in adolescents is done on cancer patients for fertility preservation. In a study conducted on the attitudes of patients and their parents on sperm banking of boys who are undergoing chemotherapy for cancer treatment, Van den Berg H et al. noticed that 70% of them were in the favor of using electrostimulation or masturbation to obtain sperm for sperm banking [99]. Invasive procedures for sperm retrieval in adolescent age are not advocated as the sperm retrieval rates are higher in adulthood, and sperm retrieval can have a negative impact on the endocrine system.

A new modality for the preservation of fertility has been proposed in which the spermatogonial stem cells (SSCs) are preserved from testicular tissue cryopreservation. These cells can be used later for the restoration of spermatogenesis or for in vitro maturation into a viable sperm [74]. Since the procedure is still experimental requiring surgery on minors, its utility is still not justified. Further studies should be done on this procedure to ensure its practicality as well as factors that can recognize potential patients for whom this procedure can be beneficial.

24.6.2.3 Adult KS Men

During the past two decades, treatment of infertility associated with KS has undergone considerable advancements making it feasible for KS patients to father their biologic children. Spontaneous pregnancy is a rare outcome in KS patients, so to achieve pregnancy, intracytoplasmic sperm injection (ICSI) is considered to be a more realistic approach. Few case reports have reported successful pregnancies in KS patients after ICSI was performed on ejaculate sperm [100]. In spite of that, most patients present with azoospermia and would require sperm extraction procedure before doing ICSI. Sperm retrieval procedures have been used in KS with nonobstructive azoospermia over the past two decades with SRR ranging from 30% to 70% [84, 101]. Microsurgical TESE has higher success rate as shown in the recent reviews [102]. In almost 50% of all the published cases of patients with KS in which TESE was utilized for sperm retrieval and ICSI, pregnancy and live birth were attained [102].

Hormonal therapy is advocated in KS patients prior to their scheduled sperm retrieval procedure. We have previously determined that the results of SRR in patients with prior hormonal stimulation were better compared to patients without prior hormonal stimulation [101]. Pharmacological treatment modalities such as the use of HCG, clomiphene citrate, and aromatase inhibitors have been tried in patients with KS [78]. Some studies have proposed the notion that aromatase inhibitors should be preferred based on the characteristic features of the disease, specifically the disturbed testosterone/estradiol ratio and increased truncal fat mass [101]. In controlled studies, these medications have been found to improve semen parameters such as sperm concentration and semen motility and also increase the testosterone-to-estradiol ratio [103]. However, aromatase inhibitors should not be used for more than 2 months prior to surgery as they can result in tachyphylaxis.

Several reports have tried to consider the prognostic factors for successful TESE. The baseline levels of testosterone and response to medical treatment are usually beneficial, although this concept is not supported by some studies. The levels of FSH and inhibin B have been thought of as prognostic factors for adequate spermatogenesis in normal men with no chromosomal abnormality [104]; however, this concept is not of much importance in KS patients. In reality, patients with inhibin B levels even below the detectable limit have undergone TESE successfully [105]. As testicular function deteriorates with age, the age of the KS patients undergoing TESE is of significant importance to the outcome [106]. One study determined a cut-off age of 32 years to be a well-established predictive parameter of successful sperm retrieval in KS patients [79, 107]. Nevertheless, the age factor has not been persistent among studies [71, 101].

24.7 Genetics Risks to Offspring

The advancements allowing us to overcome the infertility issues of KS patients have raised concerns regarding passing genetic anomalies to the offspring. In a survey conducted on KS patients, most of them expressed a desire to father children [108]; however, 70% of them acknowledged to have concerns about the safety of transmitting chromosomal anomalies to their offspring [108]. Semen analysis of the ejaculates of KS patients have revealed a high percentage of sperm with abnormal morphology [109]. Consequently, due to high percentage of abnormal sperm, it has been proposed that patients with KS have significantly greater ratio of chromosomal aneuploidy when compared to normal males [75]. On the contrary, most of the offspring biologically fathered by KS men have been healthy and normal with no chromosomal abnormalities [110], proposing that only the normal haploid sperm may be capable of fertilizing an ovum and results in a pregnancy. In a cohort study of 42 men, Schiff J et al. noticed that the incidence of phenotypic or chromosomal anomalies did not rise in the boys fathered by KS patients [78]. Nevertheless, the conception of offspring with an extra chromosome with the karyotype 47,XXY has been reported [111].

In order to protect the offspring of KS men from chromosomal abnormalities, it is recommended to have preimplantation genetic diagnosis (PGD) of embryos. Staessen et al. [112] have proposed a notion that PGD should be compulsory; the morphology of the embryo sometimes does not exclude any underlying genetic anomaly. In their study, they assured that frequency of normal embryos was 54% in KS patients, which was lower than the normal controls who had a frequency of 77.2%. They also determined that the anomalies can occur on both autosomes and sex chromosomes. PGD is not commonly available due to its expense and requirement of skilled workforce for its operation. Not to mention that PGD may be unacceptable in certain religious groups. In conclusion, KS couples undertaking ICSI should get PGD done unless there is an objection about its use.

24.8 Conclusion

KS is the most common sex chromosomal abnormality that has a significant impact on the patients' health and fertility. It is characterized by testicular failure and hypergonadotropic hypogonadism, which worsens as the age progresses. Early diagnosis can prevent the negative impact of the androgen deficiency on KS patients. Adolescents concerned about fertility should be asked to provide a semen sample if possible and should be offered sperm cryopreservation. Invasive procedures for sperm retrieval in adolescent age are not advocated as the sperm retrieval rates are higher in adulthood and sperm retrieval can have a negative impact on the endocrine system. Hormonal therapy is advocated in KS patients prior to their scheduled sperm retrieval procedure as it can produce superior results. KS couples undertaking ICSI should get PGD done unless there is an objection about its use.

24.9 Review Criteria

An extensive research exploring Klinefelter syndrome was performed using search engines such as ScienceDirect, OVID, Google Scholar, PubMed, and MEDLINE. The start and end dates for these searches were December 2000 and December 2018, respectively. The overall strategy for study identification and data extraction was based on the following keywords: "Klinefelter syndrome," "male infertility," "treatment," "testosterone," "genetics," "semen parameters," and "assisted reproduction." Articles published in languages other than English were also considered. Data that were solely published in conference or meeting proceedings, websites, or books were not included.

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Assisted Reproductive Technology and Its Impact on Male Infertility Management

25

Alice Rhoton-Vlasak, Joseph M. Kramer, and Elizabeth Plasencia

Key Points

- In vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) have become the standard of care for severe male factor infertility.
- Semen analysis is the standard by which each male patient is initially assessed in order to categorize his level of contribution to the couple's sub- or infertile condition.
- There are a variety of methodologies for selection and processing of sperm, some of which may be better suited for particular cases based on concentration, motility, and morphology.
- Insurance coverage for IVF and fertility preservation services remain limited in the United States.
- In cases of severe male factor infertility, a genetic, hormonal, and urological workup should be completed.

25.1 Introduction

Approximately 12–15% of sexually active couples are infertile. The etiology of infertility is likely multifactorial. Previous work has estimated that 50% of infertility is attributed to the female, 30% to the male, and 20% to both the male and female. Male factors are partially or completely responsible for infertility in up to 50% of cases. Recent advances, mainly in the assisted reproductive technologies (ART), have allowed some couples with severe male factor infertility to establish a pregnancy. The most significant advance is in vitro fertilization (IVF) with intracytoplasmic sperm injection (ICSI) [1, 2]. ICSI was introduced in the early 1990s as one of the most dramatic technological breakthroughs in assisted reproductive technology. After its introduction, the technique was rapidly incorporated into the routine clinical practice of fertility centers throughout the world [3].

Previously, these couples had at best only a remote chance of establishing a pregnancy due to severely reduced sperm concentration, compromised sperm function, or uncorrectable obstruction. Donor sperm or adoption may have been the only option for family building. As a result of the high success of IVF/ICSI, and as a by-product of the fact that fertility visits are often initiated by the female partner, the practice of modern ART can oftentimes proceed without a complete evaluation of the male partner. Instead, almost any couple, even those with a severe male factor, after being evaluated by only a reproductive endocrinologist, can theoretically become pregnant using IVF/ICSI [4]. Male evaluation is critical, as male factor infertility has been shown to independently affect reproductive outcomes, including the setting of assisted reproductive medicine [5].

There has also been increasing attention placed on male factor infertility as it relates to patient health. Currently, there are still many barriers to care, including geographic, financial, socioeconomic, political, and awareness. This chapter outlines updates and future advances in male infertility care, beyond the use of standard IVF with ICSI. Potential additions to culture media, sperm treatments, and new sperm selection techniques will be discussed.

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25.2 Male Infertility Workup and the Role of Urologists

Despite the trend toward unilateral care of the female partner in the reproductive health community, it is important to consider the couple as a whole unit. According to the National Survey of Family Growth (NSFG), as many as 27% of males do not complete an evaluation among couples seeking infertility care [6]. Lack of male investigation or limited urologic services can lead to potentially reversible, life-threatening, and/or genetic conditions being missed. Management of men with obstructive or nonobstructive etiologies that request or require sperm retrieval techniques also needs to be coordinated [7]. A study by Kolettis and Sabanegh evaluated potential factors that could be missed by bypassing a urological evaluation in infertile male patients. The study took place at two academic infertility practices. This study showed that significant medical pathology was discovered in 33/536 (6%) of patients. A total of 27 patients had genetic abnormalities, including cystic fibrosis mutations in 24 and karyotypic abnormalities in 3. Of the remaining six patients, one had testicular cancer, one had prostate cancer, three were found to have diabetes mellitus, and one had hypothyroidism [8]. Any couple proceeding directly to ART without even a basic urologic evaluation may not have their underlying medical pathology detected or discussed with them. Semen quality has also been recognized as a possible marker of male health [9].

Despite growing literature as above, there remains some debate about the importance of a urological evaluation. At first glance, this does not seem a problematic notion at all. If infertile couples can be afforded the opportunity to have a child in a time-efficient, cost-efficient, and safe manner, it can be concluded that this event represents an ideal outcome. However, when determining whether the male patient even needs to be evaluated given the availability of IVF/ICSI, several simple, conceptual problems do present themselves. Indeed, a large volume of data exists that argues that failure to evaluate the male is less safe for male patients, their partners, and their offspring and can be less cost-efficient. There is existing medical evidence in support of the idea that an evaluation of the male partner is mandated by both patient safety and cost-effectiveness [4]. According to the American Society for Reproductive Medicine (ASRM) committee opinion, when screening shows abnormal male reproductive history or abnormal semen analysis, a specialist in male reproductive health should be involved [10]. Review of this information will highlight the importance of infertility and ART centers having male infertility specialists in their practice area to optimize male factor patients' workup and treatment. The ideal infertility practice would involve a multidisciplinary clinic with both reproductive endocrinology and infertility (REI) and urology specialists. Other important clinical infertility team members would include embryologists, psychologists, and nurse specialists.

A multidisciplinary approach to male infertility patients including a urologist results in safer outcomes for couples. ART safety is a topic that has been extensively studied since the initial use of IVF in 1978 [11]. Safety issues that must be detected by a male infertility evaluation include detection of endocrine, genetic, or malignancies underlying an infertility diagnosis [8]. A thorough male infertility evaluation may also uncover genetic abnormalities, which could have implications for the health of future offspring. The identification of genetic conditions, such as Klinefelter's syndrome, microdeletions of the Y chromosome, and cystic fibrosis/congenital absence of the vas deferens, in patients with male infertility had profound implications for the offspring as well [12–14]. A complete workup of a man with obstructive azoospermia could lead to the finding of his being a carrier of a mutation for the cystic fibrosis membrane regulator (CFTR) gene. This has important implications for testing his partner to see whether she is a carrier or not. Obviously, if both were carriers of the cystic fibrosis gene, there is a higher chance they could have an offspring with this condition. Therefore, especially in the case of cystic fibrosis, it is imperative that the partner of an affected patient be screened to adequately counsel the couple on the risks of potentially passing this condition on to their anticipated offspring.

In much the same way, the identification of any genetic condition through a routine evaluation of male infertility is doubly important for the reason that couples can be counseled regarding the passage of these conditions to their offspring. Male offspring of patients with Azoospermia factor (AZF) region Y chromosome microdeletions can be expected to have the same deletion and are, therefore, likely to be subfertile or infertile themselves [4]. A review of these causes of male factor infertility highlights the importance of the complete urologic evaluation of the male partner to result in a safer outcome for the male patient, his partner, and their potential offspring.

There is a growing body of literature that has shown an association of male infertility and long-term health consequences, including risk for malignancy [15]. The rates of testicular and high-grade prostate cancer seem to be at least double the risk of the general population. There is also an association between infertile men and cancer risk in first- and second-degree relatives. Testicular cancer risk has been shown to be 52% higher in first-degree relatives of infertile men, and there is a two- to threefold increase in childhood cancer among siblings of infertile men.

Another reason for emphasizing the importance of a urologic evaluation in male infertility is that there is proven cost efficiency of evaluating and treating underlying male infertility, especially in cases where an anatomic or hormonal abnormality is found and correctible, thereby even allowing attempts at natural conception [4]. Medical or surgical treatment can potentially eliminate the need for ART or decrease the level of assistance needed. In the case of male varicocele, surgical correction can result in improvement in semen parameters, which improved the chances of conception naturally, with IUI or with assisted reproduction [16]. A cost analysis of effectiveness by Garceau considered four studies where IUI and IVF/ICSI were compared; a significant improvement in cost efficiency was observed in the IUI cohort across all studies, even when comparable populations were considered. The only exceptions to this conclusion were cases of severe male factor infertility or tubal obstruction. Certainly, in cases with treatable causes of male infertility and no single female factor that precludes IUI, it would seem reasonable to offer treatment to the male partner [17].

Fertility preservation is another service that is crucial to male well-being and emotional health. Every year in the United States, 9,000 males between 15 and 35 years of age are diagnosed with cancer. Male children less than 15 years old represent another 4,000 cancer victims annually. Cure rates are only increasing, given new research and innovative technology, increasing the number of males who will need sperm preservation therapy or ART later in life. It is currently recommended that all patients who are about to undergo treatment for cancer be counseled by a fertility specialist. There are multiple organizations, such as LIVESTRONG, the Alliance for Fertility Preservation, RESOLVE, the Oncofertility Consortium, and ASRM, which are currently attempting to make fertility preservation the standard of care for these patients [18].

It is clear that it is cost-effective to support treatment of reversible causes of male infertility and that safety is improved where there is a male urologic evaluation. There is also an increasing demand for oncofertility counseling and therapy. The existing evidence supports the rigorous evaluation of the male partner. Both the ASRM and the American College of Obstetricians and Gynecologists (ACOG) guidelines for best practice on infertility are in agreement on this point [19, 20]. Despite the significant advances in ART, there remains a very important role of the urologist in the evaluation of male infertility in order to maximize the safety for both the male and female partners and their offspring. Furthermore, it is cost-effective, especially in the setting of the identification of a treatable or reversible cause of male infertility.

25.3 Multidisciplinary Clinical Care

A multidisciplinary clinic system with reproductive endocrinologists and urologists working side by side is the optimal way to offer the highest level of reproductive care to infertile couples. A multidisciplinary clinic such as this has been highly effective and well received by patients. A study by Nangia [7] assessed the spatial distribution of ART centers and male infertility specialists by location, driving distance from ART centers, and potential male population in need of these resources. It was a cross-sectional study which found that a disparity of urology male infertility specialists exists in the United States, with large areas of the country being underserved and the overserved based on the location of the ART centers. The study identified 197 male infertility specialists and 390 ART centers (Figs. 25.1 and 25.2). On a state level, the highest male population in the reproductive years was seen in California, Texas, and Florida. The highest male populations per male specialists were found in Oregon, Tennessee, and Oklahoma. The Northeast region had the highest number of male specialists within ART service areas. The distribution of male population per male specialist was independent of a state's mandated insurance status for ART services. This study highlights the importance of proximity of male specialists to ART centers for the overall care of the couple with male factor infertility. This is related to the common understanding and interaction between urology and ART specialists regarding joint management or procedures for the couple. Lack of this joint care can potentially lead to significant biases, incomplete evaluation, and poorly informed discussion about treatment choices [21].

Offering the highest level of male infertility care could involve use of an interdisciplinary clinic with a urologist and reproductive endocrinologist. Decision trees could be used to enhance interdisciplinary teamwork within the reproductive medicine clinic. A common scenario would involve an infertility patient coming to clinic with her partner with known male factor infertility. If a reproductive endocrinologist and urologist hold clinic on the same day, the female history, exam, and testing could be discussed and performed while also allowing the couple to see the urologist to begin a focused male infertility evaluation. A collaborative interaction between these two specialists could allow coordination of any genetic or hormonal testing. Ultimately, collaboration between these two specialists could lead to a decision regarding whether or not the best treatment involves management of an underlying medical condition causing male infertility, a correctible underlying cause, which could be treated with medications or surgery allowing attempts at pregnancy with IUI, or whether ART and ICSI are needed. At our institution, we had such an interdisciplinary clinic, which allowed the

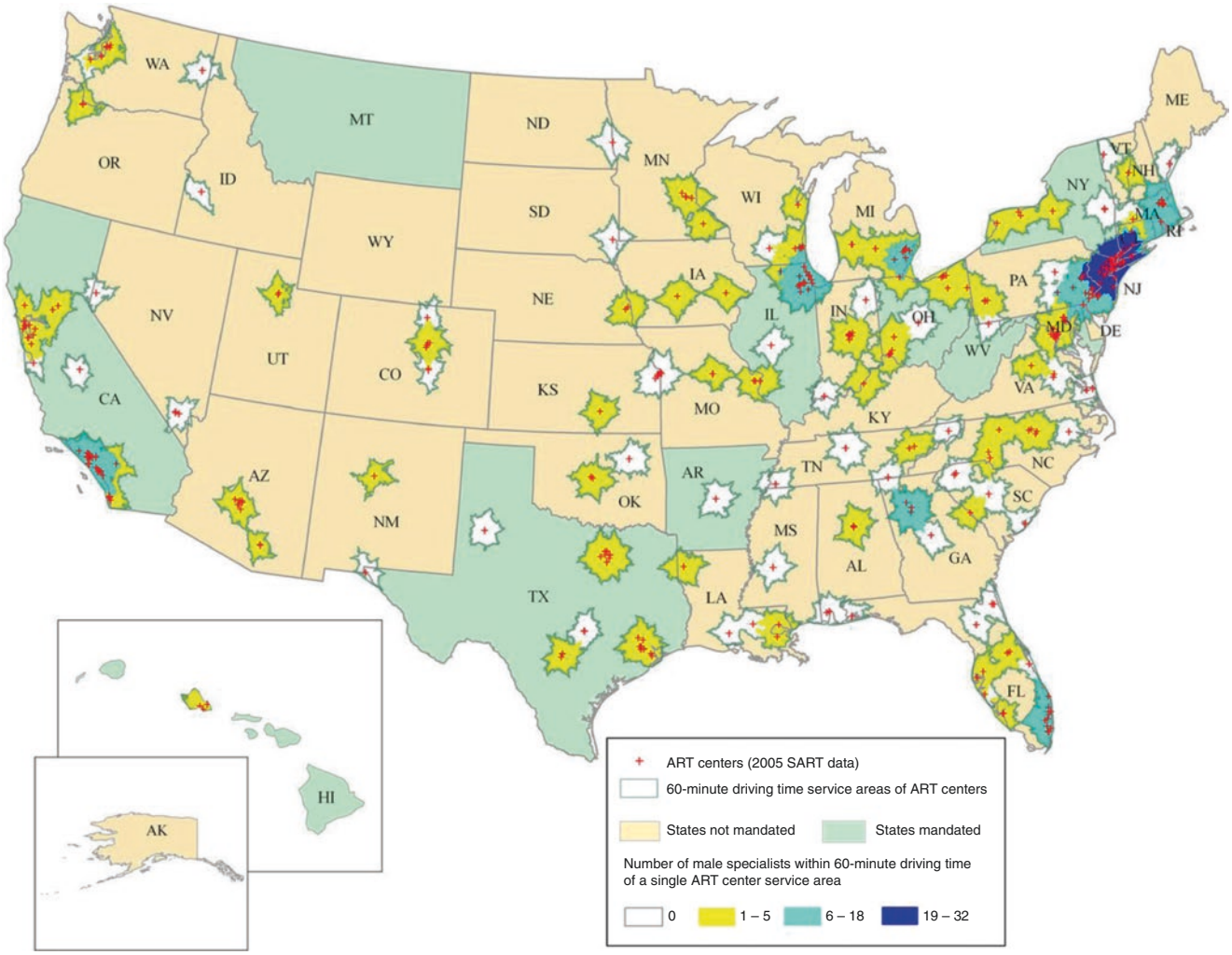

Fig. 25.1 Number of male specialists within 60-min driving distance of a single ART center. (Reprinted from Nangia et al. [7]. With permission from Elsevier)

coordination of many complicated male infertility cases, even in terms of scheduling fresh sperm aspiration procedures on the same day of oocyte retrievals and ICSI. On the other hand, having the immediate availability of a urologist in our clinic allowed patients referred for severe oligospermia to be evaluated and then treated for underlying hormonal conditions with resolution of their poor sperm counts, therefore, allowing them the chance at a natural conception and bypassing the use of ART. Options regarding surgery such as varicocelectomy can also be discussed at this time, and potential differences in cost depending on treatment strategy can be discussed.

A large barrier to care currently is the insurance coverage for male and female infertility. Infertility was recognized as a disease by the ASRM in 2008; however, access to infertility treatments continues to be viewed as an elective option and not a basic right in the United States [18]. Currently, infertility coverage is up to the discretion of the state. Dupree et al. [22] determined which states required insurance coverage for infertility. There were 15 states that mandate insurance coverage for infertility. Of these, only six states (California, Connecticut, Massachusetts, New York, New Jersey, and Ohio) included male infertility evaluation or treatment. Certain male treatments such as cryopreservation or vasectomy reversal are specifically excluded in some of these states (Fig. 25.3). While mandated health insurance coverage was associated with increased use of ART, according to a study by Griffin and Panak, no evidence of overutilization of ART by patients with low chance of reproductive success was found [23].

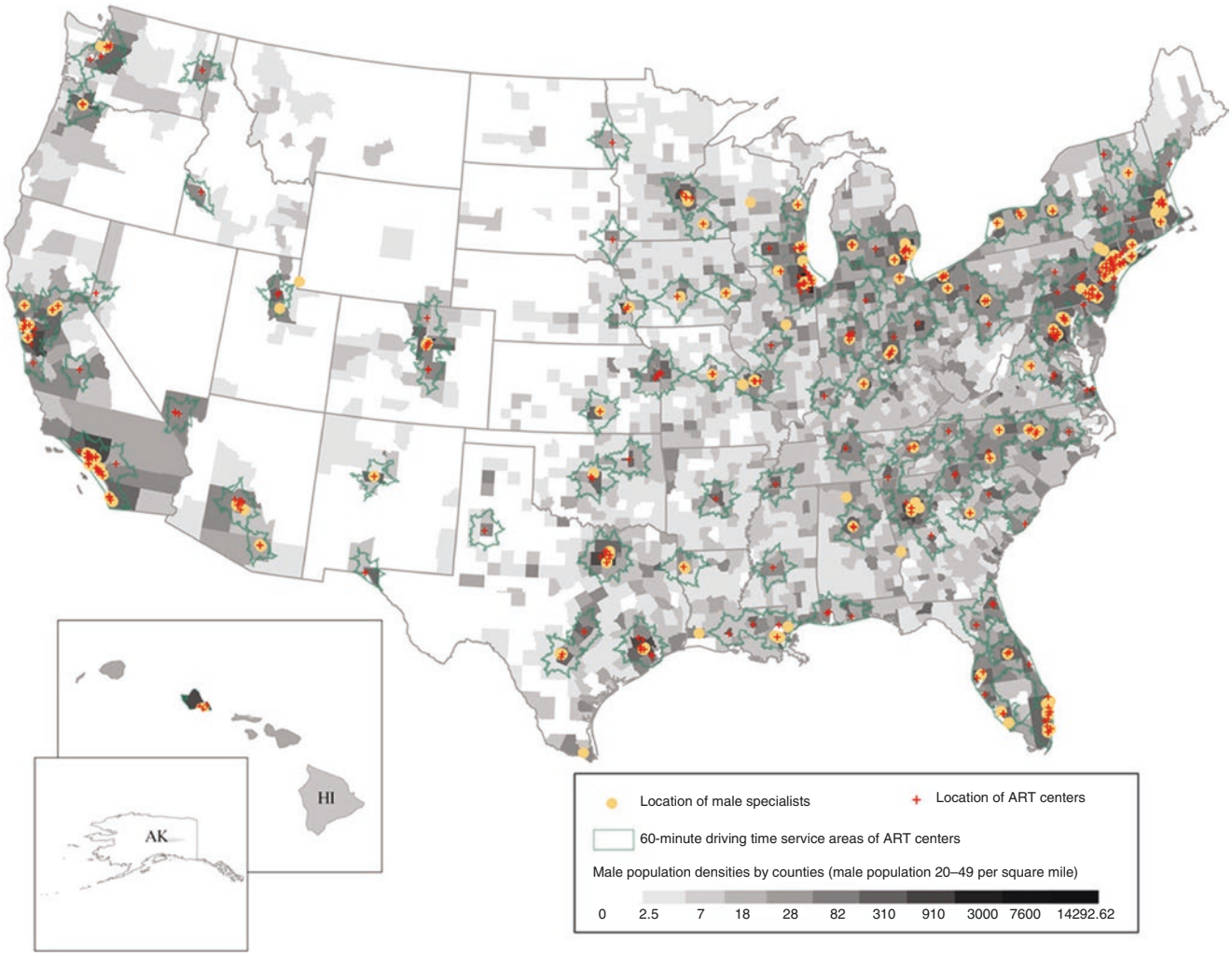

Fig. 25.2 Distribution of male population in the reproductive years, urology male infertility specialists, and ART centers with service areas within the United States. (Reprinted from Nangia et al. [7]. With permission from Elsevier)

25.4 The Techniques and Impact of the ART Laboratory on Male Infertility

The treatment of the male, within the context of a couple, may not always allow for the months of treatment necessary for long-term hormonal interventions or, for that matter, vas reversals following years of sterility. These situations are best assessed by the urologist in collaboration with the treating infertility specialist. The array of available treatment options is also a matter of the overall abilities of the ART laboratory and its ability to bring to bear the necessary technology appropriate for the specific patient treatment requirements.

With the availability of various ART/ICSI procedures now used in clinical settings, the treatment of combined male and female infertility issues, or just those associated with the male partner only, becomes a choice of expediency. The primary road to expediency is of course due to the age of the female.

From the viewpoint of what technologies are now available for the treatment of the male's contribution to the couples' infertility issues, we should look closely at the ability to firstly assess the conditions that directly impact the patient's sperm fertilizing potential and secondly the steps required to overcome this disability.

The semen analysis procedure is the standard by which each male patient is initially assessed in order to categorize his level of contribution to the couple's sub- or infertile condition [24]. Aspermia, azoospermia, and/or severe asthenospermia all place this patient in line for some type of assisted

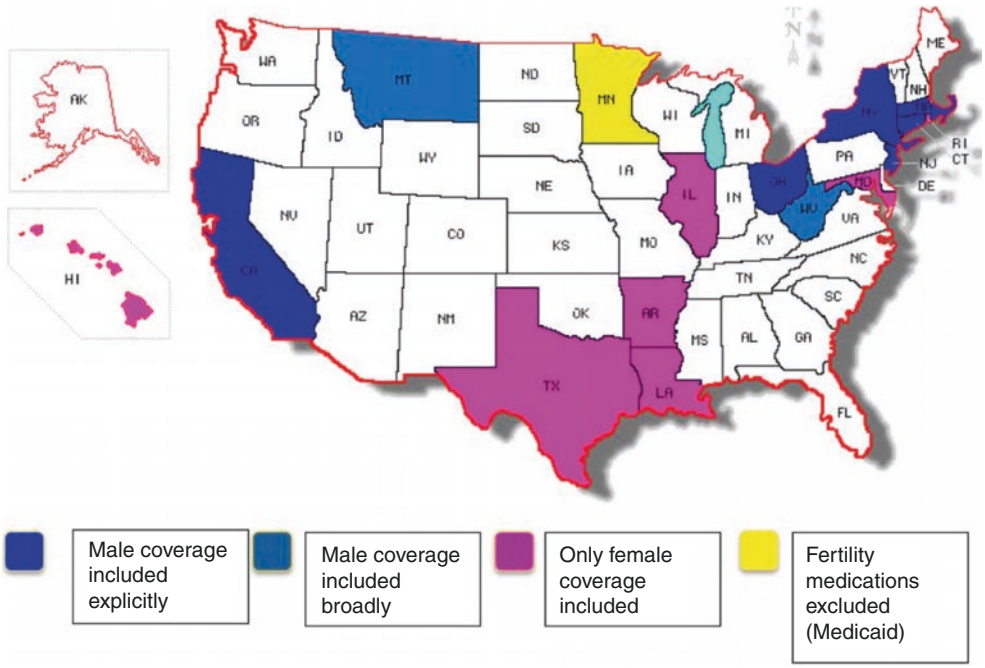

Fig. 25.3 Mandated health insurance coverage spectrum for male factor infertility. (Reprinted from Mehta et al. [18]. With permission from Elsevier)

reproductive treatment or intervention. It may also be useful to address the use of donor sperm under these circumstances as well. However, all of the abovementioned conditions are potentially treatable. In addition to these most serious conditions, semen volume, coloration, viscosity, and pH are often included in an initial assessment of a semen sample [25]. Further assessment of various vital sperm characteristics is also essential; these will include sperm concentration, sperm motility, sperm morphology, antisperm antibodies, and in the case of severe asthenozoospermia, vital staining tests [26]. The question, however, is: do these assessments in aggregate, or any one of them individually, actually allow the physician to determine whether or not these sperm can carry out normal fertilization?

There is also controversy as to the meaningfulness and significance of attached cutoff values. The WHO laboratory manual for the examination and processing of human semen, fifth edition, published by the World Health Organization in 2010, generated debate even before publication [27–31]. However, there exists a very predictive diagnostic test that the couple could undergo in order to answer this question, and that is in vitro fertilization. Since this diagnostic test would be considered far too expensive and invasive, it tends not to be used for that purpose, although IVF procedures sometimes turn out to answer that question. Because of the potential outcome called "failed fertilization," many ART programs prefer to utilize ICSI micromanipulation as their first-round defense to treat male infertility.

If there was a validated, reasonably priced sperm diagnostic test able to determine the fertilization capacity of the male patient's sperm sample, how might it change the clinical decision-making and the use of invasive ART practices like ICSI? One might also ask "what are present-day ICSI usage criteria?". One very broad axiom in use today is "once ICSI always ICSI," and this covers almost any treatment proposal if a program always starts a protocol using ICSI. What then are the prospects of being able to determine the ability of sperm function in order to avoid the use of micromanipulation or prepare for its expedient use if the procedure is necessary? Following a decision to employ micromanipulation, is it also necessary to choose a particular sperm within the available sample which will ensure proper embryonic development?

The following procedures or tests are offered in order to assist in the diagnosis and treatment planning of the male infertility patient. These tests may be available through collaboration with reproductive endocrinologists, urologists, and in vitro laboratory embryologists.

25.5 Oxidative Burden and the Use of Antioxidants

Since sperm function relies heavily on sperm membrane integrity and intramembrane lipid fluidity, the potential oxidative impact of oxygen radicals and peroxides, generated within the reproductive tract, can irreversibly damage the sperm's ability to undergo membrane alterations and respond to ionic signaling required during spermatogenesis [32–34], the process of membrane fusion at the time of fertilization. It is possible to measure antioxidant levels and capacity of seminal fluid in order to determine whether sperm is adequately protected during the collection process [33, 35–37].

There have also been studies reporting favorable outcomes using oral antioxidants as enhancers of sperm function [38–40]. Low levels of vitamin C are associated with an increase in oxidatively modified DNA base, 8-hydroxydeoxyguanosine in sperm DNA [41]. However, the type and mix of antioxidants used in this prophylactic manner have not been adequately resolved [33, 42].

25.6 Use of Antioxidant Additives During Fertilization and Embryo Culture

As well as employing antioxidants during sperm production and processing, selected compounds have also been utilized to enhance culture conditions during fertilization and embryonic culture [43, 44]. In vitro culture systems lack many of the protective elements found in follicular fluids and the fallopian tube environment. The higher levels of oxygen generally found within in vitro culture systems is of concern due to its ability to overwhelm what little antioxidative capacity resides in commercial media with unlimited sources of deleterious oxygen radicals. Therefore, the first strategy that should be put into place to reduce these reactive elements is to lower the oxygen content within culture incubators to levels thought to be present in the female reproductive tract (5–7%).

The application of various compounds that may be able to destroy or convert dangerous levels of reactive radical species when added to culture systems have been explored [45]. To date, none seem to be convincingly more efficient or useful than added taurines and cysteines. Vitamins such as vitamin C and vitamin E have been used as culture antioxidants in a number of human and animal systems [43]. However, these additives have not shown consistent beneficial results in order to warrant their inclusion. Because current culture systems are static in nature, it may be that a cascade of oxidative reducing elements (vit E®, lipoic acid®, vit C) may be required within a dynamic culture environment such as those proposed using microfluidics where oxidized compounds can then be removed [46].

25.7 Selection of Sperm

Among the fundamental procedures within the ART laboratory is the attempt to replace the natural selective processes occurring within the female reproductive tract in which semen is reduced to a population of viable motile sperm capable of fertilizing an egg. Integral to this process is the removal of seminal plasma to allow for capacitation of sperm. Over the past few decades, numerous techniques have been described for selecting sperm cells for use in ART, each of which has advantages and disadvantages.

25.7.1 Centrifugation

Density gradient centrifugation (DGC) is the primary processing step to separate seminal plasma containing nonmotile cells from motile sperm. After liquefaction, the semen sample is typically laid on top of a discontinuous gradient composed of silica particles within a conical tube and centrifuged at maximum of 300 × *g* for 20 min. During the centrifugation process, motile sperm are aided by gravitational force and move toward the end of the tube, forming a soft pellet. Nonmotile sperm cells, leukocytes, and seminal fluids are mostly trapped within the upper columns of the gradient. Following DGC, the sperm pellet is typically resuspended in a fresh wash medium and either subjected to an additional round of centrifugation (wash step) or utilized in a swim-up procedure. As a whole, DGC centrifugation is highly effective with normozoospermic samples but less useful for cases where sperm counts are below normal ranges.

Simple sperm wash alone is the preferred method for oligozoospermic samples. Semen is gently mixed with wash medium and centrifuged for 5 min to separate sperm cells from seminal plasma. Unlike DGC, the resulting sperm pellet will contain a greater concentration of nonmotile cells, including leukocytes. Motile sperm can easily be further selected during ICSI.

The major concern with sperm centrifugation is the potential damage to sperm cell function. Aside from physical damage, the removal of antioxidant-rich seminal plasma during centrifugation combined with potential concentration of ROS producing immature germ cells and peroxidase-positive leukocytes may negatively affect mitochondrial membrane potential and DNA integrity [47–49]. For DGC, a trade-off between total sperm recovery and functionality of sperm recovered is proportional to the g-force and duration of centrifugation. In terms of oxidative stress, DGC, when carefully monitored for g-force and duration, has been shown to be effective in reducing oxidative stress in processed sperm [50, 51].

25.7.2 Swim-Up

The swim-up procedure selects motile sperm by allowing those with good mobility to naturally migrate into a layered column of medium over a period of 1–2 h. The concentration of sperm aspirated from the upper layer of medium is generally too little from raw semen to be useful for IUI or conventional IVF. However, when swim-up is utilized following a first round of DGC, the recovery rates with normozoospermic samples are sufficient for most ART procedures. In addition, the combination of DGC and swim-up yields higher motility and lower DNA fragmentation than either processing method alone [52].

25.7.3 Electrophoresis

Electrophoresis, a traditional molecular laboratory technology to separate macromolecules based on size and charge, can be utilized to separate sperm [53]. During sperm maturation, sperm naturally develop a negative net charge. When placed into an electrophoresis instrument, sperm with negatively charged membranes will migrate toward the anode. Several studies have shown that when compared to positively charged sperm, a sperm with negatively charged membranes is more likely to be morphologically normal and have lower levels of DNA damage [54–56]. Furthermore, there is some evidence to suggest negatively charged sperm are associated with higher embryo development and higher pregnancy rates [56]. While these studies are promising, the technology may be limited for use with microelectrophoresis chambers designed for use during ICSI.

25.7.4 Magnetic-Activated Cell Sorting

Magnetic-activated cell sorting (MACS) is a sperm preparation method that isolates immunologically labeled sperm using a high-gradient magnetic field. This technology has been used for the selection of acrosome-reacted sperm [57], removal of leukocytes within semen [58, 59], and separation of apoptotic sperm [60]. Non-apoptotic sperm sorted by MACS based on the expression of phosphatidylserine are also associated with decreased DNA fragmentation [61–63] and improved motility, morphology, and viability [63–65]. In addition, there is data to suggest the use of MACS and ICSI improves embryo quality in select couples [66]. More studies are warranted, however, to define which populations would benefit from MACS technology.

25.7.5 Microfluidics

Newer approaches with microfluidic devices are showing promising results in the selection of sperm in a clinical setting [67]. Unlike traditional microfluidic devices that used complex pump systems or gravitational flow to sort motile and morphologically normal sperm [68], these newer chemical- and flow-free chips use small volumes and microchannels to constrain the migration of slow and nonmotile cells. Initial studies have shown these devices to select sperm with good motility and reduced levels of DNA fragmentation [67], although the concentration is lower compared to traditional DGC. Future studies will need to evaluate whether the quantity of highly motile sperm recovered can be suitable for procedures other than ICSI.

25.8 Further Selection for ICSI

25.8.1 Sperm-Hyaluronic Acid Binding

During conventional insemination, sperm must pass through a hyaluronic acid (HA)-rich extracellular matrix of cumulus cells in order to access the egg. Healthy normal sperm contain the enzyme hyaluronidase, which aids in breaking down the extracellular matrix. This principal interaction between sperm and HA has led to the development of Physiological ICSI (PICSI), a commercially available sperm-HA binding assay that can be used to further select healthy sperm to use for ICSI. Several studies suggest the utilization of PICSI increases characteristics such as minimal DNA fragmentation, normal shape, low frequency of chromosomal aneuploidies, as well as high DNA chain integrity [69, 70]. There is also some evidence that using PICSI may increase the pregnancy rate and reduce the miscarriage rate when the overall level of HA binding is low in a sperm sample.

25.8.2 High-Magnification Sperm Selection ICSI

Intracytoplasmic morphologically selected sperm injection (IMSI) is the combination of motile sperm organelle morphology examination (MSOME) and ICSI. The typical inverted microscope used for ICSI has a total magnification of 200–400×, which is not sufficient for an embryologist to select sperm based on strict morphological examination during injection. In contrast, with IMSI utilizing a 100× oil objective and 1.5× optical enhancer, the digital magnification enhancement can produce a high-resolution (6,000–10,000×) real-time imaging of sperm in an ICSI dish. Utilizing this enhanced visualization of sperm morphology, especially taking into account head vacuolization and midpiece abnormalities, has led to successful outcomes for severe male factor patients [71–73]. Additionally, live birth success has been correlated to types and grades of sperm abnormalities viewed under high magnification [74]. Unfortunately, this procedure tends to be time-consuming and technically challenging.

25.9 Additional Sperm Processing

25.9.1 Culture and Supplementation

Temperature and duration of incubation of sperm after processing until insemination can impact the quality of sperm and potential success of ART. While short-term incubation of sperm at 37 °C is suggested to improve sperm capacitation

[75], longer periods (>2 h) of storage at room temperature or body temperature (37 °C) have been shown to reduce motility and viability [76–78]. Processed samples stored for >2 h. are also associated with reduced IUI success [79, 80]. Additional causes for concern are studies that show prolonged incubation of processed sperm at 37 °C is correlated with increased DNA fragmentation [78, 81]. In many ART clinical programs, timing is usually at the convenience of the patient and physician, and the duration from semen collection, processing, and utilization may often be 4 h or more. To lessen the risk of declining sperm function, busy ART laboratories should consider lower temperatures throughout processing until time of use.

As in the case of treating a patient with supplements of vitamins and other antioxidants in order to reduce the effects of oxidative stress on systems responsible for sperm production and processing, the use of vitamins, metal chelators, Glycerophospholipid (GPL) micelles, proteins, and disulfide compounds in the medium used to process sperm samples for assisted reproductive treatments has shown to be useful for sperm viability and function [82, 83]. Sperm membrane stability is preserved by reducing or eliminating peroxidation, and nuclear DNA is protected from damage by highly reactive radical species [44]. These protective compounds will help to enhance in vitro embryo development.

25.9.2 Cryopreservation

Semen and sperm cryopreservation has a long history in both human and domestic animal reproduction [84], and for the most part, glycerol has been used as the primary ingredient responsible for retention of viability post-thaw. Additional components have since been added to enhance viability and thawed motility through incorporation of processed egg yolk in an attempt to stabilize membranes through a low-cost source of phospho- and glycolipids, such as phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn), phosphatidylinositol (PtdIns), and phosphatidylserine (PtdSer) [85]. Since typical semen specimens tend to comprise many millions of sperm, a return rate of 50% of the initial live sperm content is considered very acceptable to most users.

On the other hand, if the sperm sample has been obtained through invasive urological procedures and is to be used in expensive assisted reproductive treatments, then each sperm takes on a significant value to both the patient and the laboratory staff. Cryopreservation under these conditions requires a system that not only maintains high levels of viability but also allows for the efficient retrieval of the few thawed sperm that may have been obtained [86].

A number of semi- and/or micro-sperm freezing systems have been proposed ranging from the use of harvested empty zonae [87] or cryo-devices [88] for the storage of one or two sperm to a Cell Sleeper unit [89] housed in a standard cryovial, allowing storage of anywhere from a few to a few hundred collected sperm. The use of these sperm cryo-storage systems can improve the use of limited number of sperm available for couples with severe male factor infertility.

25.10 Use of Testicular Sperm

Extraction of testicular sperm for treatment of azoospermia is routine for modern ART laboratories. These expensive urological procedures are usually performed in outpatient surgical centers or occasionally within the clinical office. Extracted testicular tissue and fluid are then transported to the ART laboratory for further processing. Methods, such as microdissection with fine needles or mincing with homogenizing pestles, can be used to tease sperm from extracted portions of seminiferous tubules [90, 91]. Recovered sperm can then be used for ICSI or cryopreserved for future use.

Aside from treatment for azoospermia, testicular sperm extraction is a controversial option for couples struggling with infertility in which the male partner has a high level of sperm DNA fragmentation. While initial efforts to decrease DNA fragmentation should focus on lifestyle modifications and antioxidant treatment [92], there is evidence to suggest testicular sperm extraction can be used to select healthy sperm with nuclear DNA integrity [93].

25.11 Stem Cells

For young men affected with testicular failure arising from malignant and nonmalignant conditions, there is growing optimism for restoration of fertility with use of spermatogonial stem cells (SSCs). Isolation of human SSCs was demonstrated over a decade ago [94], and the transplantation of SSCs has been shown to be successful in mice [95] and nonhuman primates [96]. Cryopreservation technologies currently available in the ART laboratory can easily be adopted for preservation of testicular tissue that could be used for isolation of SSCs for transplantation or possibly used for in vitro gametogenesis.

In vitro gametogenesis is attractive, in particular for survivors of malignant disease that may be at increased risks for reintroducing potentially malignant cells during SSC transfer. Differentiation of SSCs into sperm has been demonstrated with animal models using organ culture systems, embryonic stem cells (ESC), induced pluripotent stem cells (iPSC), and germline stem cells (GSC) [97]. While it is only a matter of time before this technology proves successful in humans, studies are needed to verify paternal-specific patterns of epigenetic imprinting in in vitro produced sperm that could have short- and long-term concerns for children derived with this technology.

25.12 Conclusions

Over the past 40 years, the development and integration of ART has transformed the practice of reproductive medicine. While many of the advances have improved overall IVF success, there is no debate that the single greatest tool for treating male infertility was the introduction of ICSI. With that said, the success of IVF and ICSI is in part limited by the quality and health of the sperm. As discussed in this introductory chapter, well-established methods and recent technological advances will be focal areas of improvement as we look to extend the clinical reach of the infertility specialist and urologist.

With this acknowledgment of the key role played by the advancing technologies derived from research and delivered in the ART clinical laboratory, the initial patient assessment and diagnosis of both the male and female must still set the stage for treatment workup and outline the long-term algorithmic pathway. There is perhaps more appreciation today that there is an impact of general chronic poor nutrition on reproductive health, and attendant conditions that release systemic inflammatory by-products can often take their toll on reproductive function. High technology is not required to address these problems in initial phases of treatment. Even if a standard semen analysis cannot guarantee assessment of the total fertilizing capacity of the male's semen, if performed correctly, it is able to highlight various obvious physiological problems which need to be addressed.

25.13 Review Criteria

Search of studies examining the relationship between male factor infertility, availability of urologist, and laboratory methods for selecting and examining sperm was performed using search engines such as ScienceDirect, Ovid, PubMed, and MedLine. The start and end dates for these searches were January 2000 through October 2018, respectively. The overall strategy for study identification and data extraction was based on the following keywords: "male infertility," "sperm selection," "oxidative stress and sperm," "male urology services," "state infertility mandates," and "sperm selection for ICSI." Articles published in languages other than English were not considered. The previous book chapter and citations remained, where appropriate, based on the current chapter updates.

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Part II

Sperm Physiology and Metabolism

Fuel/Energy Sources of Spermatozoa

26

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Key Points

- Both the oxidative phosphorylation and glycolysis pathways generate energy in spermatozoa, of which oxidative phosphorylation is a more efficient pathway for ATP production compared to glycolysis.
- Mitochondrial oxidative phosphorylation is the primary energy source for sperm motility, while glycolysis in the sperm head and tail is the main source of ATP along the flagellum.
- Sperm energy metabolism is supported by the unique anatomical and physiological characteristics of spermatozoa, as well as coordination between Sertoli cells and spermatogonial cells during energy production.
- Clear understanding of sperm energetics will help improve the medium for longer in vitro storage of spermatozoa while maintaining sperm function in robust conditions.
- Deeper knowledge on sperm energy metabolism would be useful in developing non-hormonal contraceptives based on reduction or cessation of sperm energy generation.

26.1 Introduction

Spermatozoa are haploid cells that are highly specialized. The cellular processes of the spermatozoa, such as motility, hyperactivation, capacitation, and acrosome reaction, are necessary for fertilization to be successful. The energy required for sperm to carry out these functions is provided by a small, water-soluble molecule called adenosine triphosphate (ATP) [1]. Removal of a phosphate group (hydrolysis) from the ATP molecule forms adenosine diphosphate (ADP) in a reversible reaction. The interconversion of ATP and ADP (phosphorylation) provides energy for the cell, and as such, ATP acts as the "energy currency" of the cell [2].

The production of ATP typically occurs via two metabolic pathways: oxidative phosphorylation and glycolysis. Between the two, ATP synthesis happens 15 times more efficiently via oxidative phosphorylation than it does via glycolysis [3, 4]. Oxidative phosphorylation involves the respiratory chain and ATP synthase found at the inner mitochondrial membrane [5]. For every molecule of oxidized glucose, 30 molecules of ATP are generated via the process of oxidative phosphorylation. The availability of ADP is the primary rate-determining factor for oxidative phosphorylation and therefore the generation of ATP [3, 4]. Glycolysis involves the breakdown of six carbon monosaccharides via a sequence of enzyme-catalyzed reactions that yield two to three molecules of pyruvates. For every molecule of glucose oxidized, there is a net yield of two molecules of ATP generated via glycolysis. Subsequently, pyruvate is further oxidized whereby CO_2 and an acetyl group (of acetyl-coenzyme A) is formed, which is then oxidized completely to CO_2 via the citric acid cycle [3, 6].

Metabolic processes such as glycolysis and the citric acid cycle yield electron donors, namely the reduced form of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$). Besides glycolysis, NADH and $FADH_2$ are also formed by the citric acid cycle and fatty acid oxidation. Each of these energy-rich molecules contains two electrons with a high transfer potential, i.e., an electron-motive force [2]. Reduction of molecular oxygen to water by these electrons releases plenty of free energy, which can be utilized

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to produce ATP. Conversion of the electron-motive force into the proton-motive force is facilitated by electron-driven proton pumps, which are transmembrane complexes with multiple oxidation-reduction centers. The proton-motive force is subsequently converted into the phosphoryl transfer potential [2].

The process of oxidative phosphorylation yields ATP due to the transfer of electrons from NADH or $FADH_2$ to molecular oxygen (O_2) via a sequence of electron carriers, i.e., the electron transport chain. Electron flow from the NADH or $FADH_2$ to O_2 through the protein complexes within the inner mitochondrial membrane causes protons to be pumped out of the mitochondrial matrix, which leads to an uneven distribution of protons. The resulting transmembrane electrical potential and pH gradient generate a proton-motive force. As the protons flow back to the mitochondrial matrix through the ATP synthase enzyme complex, ATP is synthesized [2].

However, it is still debatable as to which of these metabolic pathways has the most crucial role to energy production in the spermatozoa. Mammalian spermatozoa require energy for its motility, which is generated by the beating of the lengthy flagellum that makes up more than 90% of the total length of the spermatozoa [7]. Thus, all mammalian sperm are faced with a similar challenge in that the energy (intracellular ATP) generated must be transferred along the whole length of the flagellum to ensure efficient movement [8]. Loss of forward progressive motility due to depletion of energy sources/disorder in motion characteristics or both could greatly impede sperm function and very likely jeopardize fertilization. Therefore, spermatozoa must implement various metabolic strategies to ensure sufficient energy is supplied from its fuel machineries to support its high motility requirement [1].

The chapter will review the unique functional ultrastructure of sperm that support its fuel machineries, sperm energy metabolism and pathways involved, potential mechanisms by which spermatozoa obtain the energy to fuel its motility, hyperactivation, capacitation, and acrosome reaction necessary to achieve successful fertilization as well as the impact of fuel depletion on sperm function. This chapter also highlights how understanding the concepts of energy metabolism in spermatozoa could improve in vitro sperm storage and contribute toward the development of energetics-based, nonhormonal contraceptives.

26.2 Functional Ultrastructure of Sperm: Fuel Machineries

26.2.1 Flagellar Ultrastructure

The flagellum of the mammalian sperm has three distinctive regions, namely the midpiece, the principal piece, and the terminal endpiece (reviewed in Ref. [9]). The axoneme extends centrally throughout the flagellar length. The surrounding outer dense fibers also extend at different lengths down the flagellum. The mitochondria are organized in a helix surrounding the outer dense fibers and axoneme in the midpiece. The midpiece serves as the energy powerhouse of the sperm where mitochondria are exclusively localized (reviewed in Ref. [10]). About 72–80 mitochondria are localized within a mature spermatozoon, and they comprise about 15–22% of the total cellular volume [11]. These mitochondria play a role in the acrosome reaction and oocyte penetration [12].

The mitochondria can be divided into four distinct subcompartments, namely, the (i) outer mitochondrial membrane, (ii) inter-membrane space, (iii) inner mitochondrial membrane, and (iv) matrix. However, the mitochondrion of the sperm differs morphologically and functionally from that of its somatic counterparts [13, 14]. This could be due to the tight wrapping of the mitochondria around the axoneme of the spermatozoon, which results in a mitochondrial capsule. This capsule is made up of selenoprotein and disulfide bridges [15], which provide stability to the mitochondrial sheath [16, 17].

The principal piece makes up most of the length of the spermatozoa and is a region devoid of mitochondria [4]. Within the principal piece lies a unique cytoskeletal structure known as the fibrous sheath, which is specific to the mammalian sperm [18]. The fibrous sheath is located just beneath the plasma membrane as two vertical columns attached by lateral ribs. Not only does the fibrous sheath have a structural function, but it also plays an important role in sperm motility. The short endpiece begins where the fibrous sheath terminates (reviewed in Ref. [10]).

26.2.2 Spermatozoa ATPases

Ion-exchanging ATPases have a crucial role in sperm motility, hyperactivation, chemotaxis, and acrosome reaction. These necessary functional modifications of the spermatozoa leading to fertilization appear to be regulated by the different ATPases. Moreover, the location of the ATPase within the cell seems to reflect its function [19].

Na^+/K^+-ATPase is a transmembrane protein involved in the active exchange of three intracellular sodium ions for two extracellular potassium ions across the plasma membrane. In mammals, four α (catalytic subunit of the enzyme) and three β (enzyme localization and maturation) subunits have been identified as isoforms of Na^+/K^+-ATPase [20]. The α4 along with α1 isoforms are expressed in the midpiece of the sperm flagellum [21]. Selective inhibition of the Na^+/K^+-ATPase α4 isoform by ouabain resulted in decreased sperm motility [21, 22]. Activity of the Na^+/K^+-ATPase α4 isoform contributes indirectly to the maintenance of intracellular calcium levels and sperm pH [22].

Calcium is a key cellular second messenger essential for sperm motility and other sperm functions. Plasma membrane Ca^{2+}-ATPase (PMCA) pumps out calcium ions against the transmembrane electrochemical gradient to maintain low intracellular calcium ion concentrations. In the sperm, calcium ion homeostasis is controlled by PMCA located at the head and principal piece of flagellum [23]. The importance of PMCA function in calcium homeostasis for sperm motility has been shown by several studies. Deficiency of PMCA isoform 4 located in the sperm tail led to impaired sperm motility and hyperactivity [24]. The antioxidant quercetin caused inhibition of PMCA activity leading to dose-dependent decreases in sperm motility [25]. Sperm incubated with cadmium displayed lowered sperm motility due to inhibition of PMCA and axonemal dynein-ATPase activity [23].

Dyneins are specific ATPases of the sperm flagellum that provide energy for the sliding movement between the microtubule doublets. Besides their vital role in the whipping movement of the flagellum, dyneins also contribute to transport along the sperm axoneme within the flagellum [26].

In summary, activities of the Na^+/K^+-ATPase and Ca^{2+}-ATPase enzymes in human spermatozoa contribute to the maintenance of the membrane gradient required for sperm motility (reviewed in Ref. [27]). The dynein ATPases that are associated with the axoneme serve as the main consumers of ATP in spermatozoa [28, 29]. Therefore, energy must be transferred from the mitochondria at the midpiece down the length of the flagellum.

26.2.3 Unique Properties of Sperm Glycolytic Enzymes

Glycolysis in mammals supplies, albeit less efficiently, a high-throughput local production of ATP along the length of the principal piece. Once the substrates for glycolysis penetrate the plasma membrane of the flagellum, they are acted upon by the highly abundant glycolytic enzymes, which are firmly bound to the fibrous sheath cytoskeleton [30].

The fibrous sheath of the principal piece acts as a scaffold onto which multiple glycolytic enzymes anchor along the length of the flagellum [30]. Tethering of the glycolytic enzymes could potentially hinder protein function in several ways, including by interfering with a necessary conformational change or obstructing the binding site of a substrate. To prevent these unwanted events, mammalian sperm-specific variants of several enzymes in the glycolytic pathway have domains that distinguish them from their somatic cell isoforms [10].

Three such sperm-specific isozymes are the glyceraldehyde 3-phosphate dehydrogenase-S (GAPDS), phosphoglycerate kinase-2 (PGK2), and lactate dehydrogenase-C4 (LDH-C4). These isozymes are encoded by genes which are expressed exclusively during spermatogenesis [31–33]. In mammalian sperm, the GAPDS isozyme has a novel N-terminal proline-rich domain that binds it tightly to the fibrous sheath cytoskeleton [34]. Compared to its somatic isoenzyme, the sperm-specific GAPDS is far more stable and does not perform non-glycolytic functions [35].

Other sperm cell glycolytic enzymes also have its distinctive functional or structural properties [36–38]. The unique characteristics of these sperm glycolytic enzymes may play a vital role in ensuring an adequate localized supply of ATP down the length of the flagellum, which is essential for sperm motility [39].

26.2.4 Seminal Plasma Components

Following ejaculation, mammalian sperm gain energy from the nutrients available in the seminal fluid and female reproductive tract. With the testis being an exception, the other tissues of the male reproductive tract are able to produce fructose and sorbitol [40]. Secretions of the seminal vesicles, a major accessory reproductive gland in humans, contain a high amount of fructose. However, seminal plasma fructose concentrations vary in different species. While a high amount of fructose was detected in the bull and ram, almost none was present in the dog and stallion [41].

Fructose undergoes glycolysis to form pyruvate. Sorbitol can be reversibly converted to fructose via the NAD-sorbitol dehydrogenase enzyme and to glucose via the NADPH-aldose reductase enzyme [7]. The presence of the enzymes required for the production of both sorbitol and fructose in human spermatozoa has been verified using Western Blot analysis [42]. Between the two substrates, fructose is the principal reducing sugar and therefore the main fuel source for spermatozoa in human seminal plasma [43]. Therefore while still in the seminal fluid, human spermatozoa utilize fructose as an energy supply to meet its metabolic needs. Within the female reproductive tract, human spermatozoa switch to using glucose as its energy source [44].

26.3 Energy Production in Spermatozoa

A spermatozoon is made up of two distinctive regions: the head and the flagellum. The sperm head comprises the acrosome, equatorial segment, and post-acrosomal region. Also present within the sperm head is the nucleus containing the compacted paternal genome. As discussed in Sect. 26.2.1, the sperm flagellum can be distinctively divided into the midpiece, the principal piece, and the terminal endpiece (reviewed in Ref. [9]).

As is the case with all eukaryotic cells, the generation of energy in the spermatozoa is also compartmentalized [45]. In the spermatozoa, the generation of ATP via oxidative phosphorylation takes place in the mitochondria of the midpiece, while glycolysis occurs in the head and fibrous sheath within the principal piece of the flagellum.

26.3.1 ATP Production via Oxidative Phosphorylation

The process of oxidative phosphorylation occurs in the mitochondria, which is available within the mitochondrial sheath in the midpiece of the mammalian sperm [13]. The midpiece is the only region of the spermatozoa that contain mitochondria, which are the most efficient producers of cellular energy in the form of ATP. The inner mitochondrial membrane folds to form structures known as cristae, which are the principal sites for oxidative phosphorylation and ATP generation.

Sperm mitochondria contain specific isoforms of proteins and isozymes such as cytochrome C [46, 47], hexokinase subunit VIb of the cytochrome C oxidase [48], and lactate dehydrogenase (LDH) [49]. All of these isozymes are however absent in somatic cell mitochondria [50].

26.3.2 ATP Production via Glycolysis

The process of glycolysis contributes greatly toward the production of ATP in the mammalian sperm. The head and principal piece of the spermatozoa contain glycolytic enzymes but lack the necessary respiratory enzymes for oxidative generation of ATP. Therefore, ATP production in these areas can only take place glycolytically [7].

The fibrous sheath in the principal piece of the sperm flagellum contains several glycolytic enzymes that are sperm-specific, namely, glyceraldehyde-3-phosphate dehydrogenase (GAPD), phosphoglucokinase isomerase, phosphofructokinase, hexokinase, and LDH isozyme [4, 38, 45, 51].

The localization of a unique ADP/ATP carrier protein termed sperm flagellar energy carrier (SFEC) along with the glycolytic enzymes on the fibrous sheath of human spermatozoa appears to play a vital role in sperm motility [4].

26.3.3 Metabolic Coordination of Sertoli Cells and Germ Cells in Spermatogenesis

The intricate process of spermatogenesis relies heavily on the firm metabolic cooperation between the developing germ cells and the Sertoli cells. Besides providing physical support to the developing germ cells, Sertoli cells are also involved in the maintenance of an appropriate metabolic [52] and ionic [53, 54] milieu necessary for sperm differentiation and functional maturation.

Germ cells rely strongly on lactate for its development and survival [55]. In addition to its role as an energy source, lactate also confers an anti-apoptotic effect on the germ cells [56]. Sertoli cells preferentially metabolize glucose, which is converted from pyruvate into lactate during glycolysis. Lactate production by the Sertoli cells commences with the uptake of glucose, either actively by sodium-dependent glucose transporters or passively by glucose transporters [57]. Thereby, the transport of glucose through the plasma membrane plays a role in the regulation of lactate secretion by Sertoli cells.

Glucose metabolism in Sertoli cells is subject to intricate control by several hormones, including insulin, 5α-dihydrotestosterone (DHT), follicle-stimulating hormone (FSH), 17-estradiol (E2), and thyroid hormones [58–60]. Tight hormonal control of these metabolic processes involves a variety of complex signaling cascades that are yet to be fully elucidated [61]. Moreover, the precise molecular mechanisms coordinated by the respective hormone during Sertoli cell glucose uptake and metabolism are not fully known [62], although studies have begun to shed some light on the matter.

FSH and insulin stimulate lactate production [63] by regulating the activity of enzymes involved in either carbohydrate metabolism and/or glucose transport regulation [64]. Since FSH induces androgen receptor expression, it may also regulate the sensitivity of Sertoli cells toward androgens [65, 66]. Insulin stimulates various Sertoli cell functions including lactate production, uptake of free nucleosides, synthesis of DNA and protein, transferrin secretion, and glycide metabolism [59, 67–69].

Not only do the sex steroid hormones DHT and E2 modulate glucose utilization and lactate production in human Sertoli cells [70], but they also regulate the apoptotic signaling of Sertoli cells [71]. With thyroid hormone receptors being present in Sertoli cells [72], it is probable that the thyroid hormones have a regulatory role in Sertoli cell proliferation and lactate production.

Locally produced factors such as transforming growth factor alpha, tumor necrosis factor alpha, interleukin 1 alpha, basic fibroblast growth factor, and epidermal growth factor [73–75] have been proposed to increase lactate production by enhancing glucose uptake along with total LDH activity [55]. Endocrine disruptors are also reported to modulate glucose metabolism in Sertoli cells [76].

Glucose metabolism in Sertoli cells is a well-controlled process essential for normal spermatogenesis. As such, any compromise in the Sertoli cells' ability to metabolize glucose could jeopardize the energy supply to the germ cells, which would then disrupt spermatogenesis and subsequently impact male fertility [62].

26.4 Energy Utilization by Spermatozoa

Biological energy fuels all the physiological functions of spermatozoa from the time of deposition in the female reproductive tract till achievement of successful fertilization. Mammalian fertilization involves multiple courses of processing mainly due to the semen deposition site being distant from the fertilization site in the female genital tract [77, 78]. It requires synchronized spermatozoa functions including robust sperm motility, capacitation, hyperactivation, and finally acrosome reaction [79]. These vital spermatozoa actions are all energy driven, which explains the prime importance of biological energy in male gamete functions. In the subsequent section, energy utilization for sperm functional processes is discussed.

26.4.1 Sperm Motility and Flagellar Movements

Sperm flagellum contains the apparatus for motility that aids the forward propelling action of a spermatozoon until it penetrates an oocyte [80]. The movements of the microtubules, especially the dynein "arms" present in the eukaryotic flagellum, enable the flagellum to bend to bring about "flagellar movements" [81]. The regulation of these flagellar movements is quite complex, and several research studies have attempted to put forth the mechanisms involved [82].

The rhythmic flagellar beating is powered by the axonemal protein, dynein [83]. As dynein ATPases get activated, the externally placed axonemal doublet microtubules slide which cause bending of the flagellum [7]. Thus, dynein generates the required force for flagellar movements by converting the chemical energy obtained via ATP hydrolysis into the desired mechanical energy [84].

As mitochondria are heavy organelle, they are located in the midpiece of the sperm cell and not in the flagellum to prevent mechanical restrictions in flagellar movements [85]. Mitochondrial electron transport chain (ETC) mediates efficient ATP production, but it is not yet clear whether the mitochondrial ATP are the ones that sufficiently diffuse along the full length of spermatozoa flagellum to fulfill the energy requirement of flagellar beating.

Researchers, especially the biophysicists [86], have calculated the ATP diffusion rate from the midpiece to the flagellum in mammals like bulls and sea urchins. They suggested that the rate of ATP diffusion into the flagellum is sufficient to support its required beating pace. This explains the hypothesis that sperm motility is gauged by mitochondrial membrane potential, reduction of which leads to decreased sperm motility and fertilization ability [87]. This had also been demonstrated by Paoli et al. [79] who found that nonlinear sperm motility positively correlates with mitochondrial membrane potential in asthenozoospermic patients.

Modulations in the activities of the mitochondrial respiratory chain enzymes have also been reported to affect sperm motility [88]. The mitochondrial ETC has two mobile electron carriers (cytochrome C and coenzyme Q) and four complexes (I, II, III, and IV). Each of this essential machinery of mitochondria finds importance in electron transport and activation of ATP synthase to generate ATPs. As such, alterations of these components are suggested to be the basis of idiopathic asthenozoospermia [88].

This hypothesis of mitochondrial ATP generation and sufficient diffusion to the flagellum for its robust beating is contradicted by the views of Tombes and Shapiro [89]. Their observations suggest that ATP in spermatozoa is produced by the mitochondria or cell body that is located far from the sperm flagellum through glycolysis or oxidative phosphorylation. But this generated ATP does not suffice the energy requirement for proper flagellar movements owing to the poor ATP diffusion capacity to the area of high energy demand. However, this hypothesis is still not confirmed in human spermatozoa [11]. Moreover, as the flagellar movements are ATP driven, it is needed to prevent accumulation of the products of its hydrolysis such as ADP, inorganic phosphate (Pi) and hydrogen ions that may impose kinetic and thermodynamic stress to the spermatozoa [90].

These contradictory hypotheses have led to several other studies that have suggested an alternative pathway of sperm energy generation. The hypothesis proposes the presence of an ATP source in proximity to the site of ATP utilization for flagellar movements. This may be achieved through the glycolytic pathway by the glycolytic enzymes present in the fibrous sperm flagellar sheath [45]. In addition, mammalian spermatozoa have several options of carbohydrate to use as a substrate for production of ATP [42, 91]. This explains the views that the flagellum may generate its own ATP required for its movement independent of mitochondrial activity [45].

Glucose is the most important substrate for ATP generation and reportedly induces increased flagellar beating frequency. Mukai and Okuno, in their study, used a glucose analogue (2-deoxyglucose—DOG), to hinder spermatozoa glycolysis. It was observed that DOG inhibited pyruvate and lactate activities but did not affect mitochondrial respiration (confirmed by a fluorescent probe) [92], while the study outcome indeed led to a reduction in ATP content and low sperm motility [92]. This study provides the evidence that glycolysis is a key energy contributor for sperm motility, which, if suppressed, results in reduced sperm motility even though the mitochondrial substrates are unaffected.

The fact that DOG did not disrupt ATP synthesis in the mitochondria can be explained by the unaltered ATP concentration in the presence or absence of carbohydrate metabolism

along with DOG. This may indicate that ATP production by normal mitochondrial respiration is not adequate to meet the energy demand required for sperm flagellar motility. These facts firmly support glycolysis as the preferred energy source, particularly for sperm motility.

Spermatogenic-specific enzyme isoforms required for glycolysis are present along the fibrous sheath of the sperm flagellum, among which a key enzyme is the GAPD enzyme. Mouse spermatogenic cells contain the GAPD-S [39], while the human has its ortholog as GAPD-2. The GAPD is the first catalytically active enzyme evident to be present as attached to the fibrous sheath of the flagellum of spermatozoa [45]. Being a key glycolytic regulatory enzyme, it aids spermatogenesis and is vulnerable to the adverse effects of environmental factors that may affect male fertility [33]. According to a study, GAPD-deficient mice displayed lower sperm motility compared to the control group with working GAPD-S genes [39]. There have been similar observations with GAPD-2 genes in humans [33].

Another evidence to suggest that mitochondrial respiration does not bear a role in sperm motility is put forth by the study that used carbonyl cyanide m-chlorophenylhydrazine (CCCP), an uncoupler inhibiting mitochondrial ATP synthesis [92]. Moreover, lactate dehydrogenase-C (LDH-C), a glycolytic enzyme that is used to catalyze pyruvate to lactate conversions [50], is essential for sperm motility in anaerobic respiration. LDH-C gene disruption in mice displayed neither tyrosine phosphorylation nor hyperactive motility that is required for capacitation [93]. It has also been proposed that oxidative phosphorylation is not needed for increasing sperm motility in hyperactivation [94]. By exogenous supplementation of glucose and pyruvate, it was seen that the glycolytic pathway is the predominant source of ATP for sperm motility and capacitation [95].

26.4.2 Sperm Capacitation, Hyperactivation, and Acrosome Reaction

Fertilization is a multistep process that occurs in a synchronized manner [96]. Sperm are deposited in the female reproductive tract at the time of copulation, and this is followed by a sequence of events, which is collectively called capacitation. It occurs before successful fertilization is possible [12]. Capacitation refers to modifications in cellular and biochemical processes of sperm in response to specific stimuli necessary for acrosome reaction to take place [12]. Incubation of sperm in the capacitation medium showed that 10–20% of the sperm had adopted motility patterns that resembled the state of hyperactivation [97]. These important events of spermatozoa activation require an efficient and adequate supply of ATP [98]. Spermatozoa capacitation leads to modifications at the sperm head surface and also along the length of the flagella [99]. Energy-aided activation of spermatozoa results in a successful acrosome reaction, thereby releasing hydrolytic enzymes from the acrosome vesicles, followed by sperm binding and successful penetration of the oocyte.

The molecular events involved in acrosome reaction begin with signals from the oocyte, which activate G-proteins present in the sperm. This leads to a rise in intracellular Ca^{2+} levels activating specific kinases and phosphorylation of several other proteins. Intracellular rise in Ca^{2+} level can be achieved by the influx of Ca^{2+} either through the cell membrane protein channels (proteins of CatSper family) or release of Ca^{2+} from the endoplasmic reticulum and other organelles [100]. The redundant nuclear envelope (clustered membrane vesicles) [101] is also a vital Ca^{2+} ion store located at the base of the mitochondrial sheath in sperm midpiece. Increase in Ca^{2+} levels in spermatozoa in response to acrosome reaction stimuli enhances the sperm flagellar beating intensity resulting in increased hyperactivation [102].

Therefore, adenosine triphosphate is a critical player in the maintenance of acrosome integrity and induction of acrosome reaction because it is needed for ATPase activity, the formation of cyclic adenosine monophosphate, and phosphorylation.

Mitochondria are essential to mediate tyrosine phosphorylation in capacitated mammalian spermatozoa [103]. The Ca^{2+}-ATPase-aided secretory pathway in the spermatozoa midpiece may mediate the clearance of excess intracellular Ca^{2+} released from the redundant nuclear envelope [104]. Moreover, spermatids express an abundant level of selenium-dependent phospholipid hydroperoxide glutathione peroxidase whose activities greatly increase in the post-pubertal testis [105]. Mature spermatozoa express this only at the midpiece region [15], which embeds the mitochondrial helix and contributes in mitochondrial function and sperm maturation [106]. Tyrosine phosphorylation in human sperm capacitation still warrants detailed research.

As previously discussed, mammalian spermatozoa depend on the availability of glucose, pyruvate, and lactate for its fuel [107], and these are all present in the oviductal fluid [108]. In pigs and sheep, the oviductal fluid seems to contain lactate, which acts as a prime substrate for ATP production that aids in spermatozoa activity. In mice, glucose and pyruvate have been observed to bring about and sustain vigorous sperm motility [109]. Thus, metabolic substrates used for ATP generation are species specific, which consequently determines the preferential metabolic pathway for production of ATP in spermatozoa to obtain flagellar movements and adequate capacitation [17].

As a back-up arrangement of sperm fuel, if there is the unavailability of such glycolysis substrates, metabolism of respiratory substrates is triggered in spermatozoa [92]. These respiratory substrates undergo gluconeogenesis in the sperm midpiece producing glucose as the main end product. Now glucose may diffuse from the midpiece to the regions of fla-

gellum which can again utilize glucose to produce ATP for its movements. Bull spermatozoa rely upon oxidative phosphorylation to generate energy for capacitation, while human spermatozoa appear to depend on glucose-mediated ATP [110]. Despite glucose-derived ATP acting as an immediate source of energy for sperm capacitation, mitochondrial function was also found to heighten during this time [111].

Mitochondrial functionality of spermatozoa during capacitation had been assessed in sperm samples, pre- and post-swim-up treatment, with different times of incubation. Mitochondrial respiratory activity was significantly higher in the spermatozoa incubated under capacitating states than those in spermatozoa before swim-up treatment. This observation suggests that sufficient ATP has been generated through oxidative phosphorylation during capacitation [112].

26.5 Consequences of Fuel Depletion in Sperm Functions

It is well known that human or animal spermatozoa require biological energy for their normal physiological functions. It has been shown in fish spermatozoa that a substantial reduction in ATP leads to decrease in flagellar beat frequency from almost 60–20 Hz within just 20 sec [113].

The ionic composition of the sperm dilution medium is also a determining factor in such experiments. This has been evident from observations that motility was initiated in trout spermatozoa without adding any external calcium, and flagellar movements stopped abruptly in spite of the presence of adequate ATP to sustain sperm motility. Whereas when sperm medium contained calcium ion of about 10 mM, flagellar movements did not abruptly cease. Instead, both movements and the ATP concentration gradually decreased as all the ATP became depleted. This suggests that the axonemal machinery (and/or the dynein-ATPase) activation is associated with calcium ion-dependent cAMP regulation. The fact that ATP content in sperm does not get totally depleted and their ATPase activities are lower in the recovery phase as compared to their motility phase supports this interpretation. Moreover, sperm with recovered ATP at levels close to that in quiescent sperm are unable to swim or hydrolyze ATP to gain energy unless there is again an external supply of sufficient Ca^{2+} [113].

It has been put forth that progressive depletion of ATP in fish spermatozoa, owing to its consumption by movements of sperm axoneme, may modify the shape of the flagellum, for example, by stiffening of the flagellar distal part [114]. This flagellar stiffening may be the result of a deficiency of local ATP in the distal part of it which is quite distant from the mitochondrial ATP generation site [115]. This may lead to blockade of the dyneins to render it in a state of rigor, perhaps by making the distal portion of the sperm axoneme to become very rigid. This action in combination with alterations in intracellular ionic concentration directly affects dynein activity [116]. The local axoneme regulators also contribute to this rigidity, such as the hydin, and the acentral pair protein of cilia and flagella [117].

26.6 Knowing the Energy Sources of Spermatozoa Contributes Toward Improving:

26.6.1 Handling of Gametes In Vitro: Cryopreservation

Spermatozoa storage should assure that the functionality of the sperm is retained along with its longevity. As is the case with horse sperm storage, it is preferred to be stored before the artificial insemination stage to ensure greater longevity as the nature of ovulation in mares is asynchronous [118]. If artificial insemination is needed within 12 h of semen collection, the sperm can be kept at room temperature. However, if sperm longevity is needed, they are either chilled (up to 72 h) or cryopreserved (for an indefinite time) in order to cease sperm metabolism. This helps to reduce ROS production as well as decrease acidification of the storage medium by accumulated metabolic products, such as carbon dioxide and lactic acid from oxidative phosphorylation and glycolysis. Since it becomes arduous for the sperm to tolerate the stress of chilling or cryopreservation, improved storage medium needs to be developed to extend sperm longevity [119].

Cryopreservation is currently the lone viable in vitro sperm storage method, used for more than 72 h of preservation. The process of cryopreservation and repeated thawing of the spermatozoa, mainly via osmotic stress, decreases their acrosomal integrity, sperm viability, motility [120, 121], and even formation of DNA lesions on vital genes that mediate fertilization and embryonic development [122]. Moreover, the cryoprotectants render the cryodiluent to be hyperosmotic, which can dehydrate the spermatozoa via exosmosis [123]. This dehydration is required to retain sperm viability after thawing. However, extreme hyperosmolarity leads to stress due to the loss of excess fluids from spermatozoa via membrane water channels to balance the osmolarity [124]. These result in sperm membrane damage [125], DNA disintegration [126], and excess ROS generation causing alterations mimicking those during capacitation [127].

26.6.2 Handling of Gametes In Vitro: Ambient Temperature Storage

Sperm storage in ambient temperature will help to prevent the stress that the sperm needs to combat owing to chilling or cryopreservation. Development of an advanced sperm stor-

age medium is required, preferably one that renders sperm storage temperature to be ambient where they can be stored for at least a week. This would allow export of the stored sperm to other geographical locations that would transmit new genetics into isolated areas, without any loss of fertility that is caused by chilling and cryopreservation methods. For ambient temperature storage, additions of animal-derived products are not required and thus sperm can easily be transported without biosecurity risks.

The implications associated with using higher temperatures for in vitro sperm storage should be considered. Among these, the first complication is the bacterial growth in the storage medium, which is a nutrient-rich semen extender. While collecting semen from a horse, it is inevitable that the bacteria contaminating the penis will also contaminate the ejaculate [128]. These bacterial strains, or at least some of them, can potentially affect sperm vitality and motility. Besides it obviously happening in ambient temperature, this can occur even during storage at 4 °C [129] and in cryopreservation [130].

To mitigate the adverse effects of these bacteria in the sperm storage medium, development of various antibiotic formulations is under research [130]. It has been put forth that sperm storage medium containing 50 μg/mL streptomycin, 0.25 mg/mL gentamicin and 50 IU/mL penicillin is able to inhibit bacterial growth for up to one week at ambient room temperature [131]. Apart from this, the second concern is that if metabolism in stored sperm is not ceased by chilling or cryopreservation, the continued oxidative phosphorylation will lead to excessive ROS generation [132]. These will eventually disrupt sperm functions [133, 134].

The protective measure that has been attempted to nullify the effects of ROS on sperm is by antioxidant supplementation in the storage medium, but it did not show the anticipated results in horse semen [135], and even in some occasions, had adversely affected sperm function [136]. However, in human spermatozoa, antioxidant supplementation showed positive results [137], which may be attributed to their alternative ATP production mode.

Of late, carnitine has been gaining popularity for its antioxidant properties [138], with L-carnitine being able to significantly reduce mitochondrial ROS production and oxidative damage in horse spermatozoa during in vitro storage [138, 139]. However, L-carnitine supplementation alone may not be able to prevent ROS-mediated cell damage in totality, and thus there is a need for combination treatment to completely scavenge ROS [138]. Further investigations are needed to come up with appropriate antioxidant supplementations for a sperm storage medium in ambient temperature.

Since mitochondrial metabolism serves as the prime ROS generating source, in order to minimize oxidative damage to sperm while in a storage medium, it may be beneficial to utilize mitochondrial antioxidant to regulate sperm mitochondrial bioenergetics. L-carnitine meets this requirement but as discussed before, the overall protection can be achieved through the combination of L-carnitine with additional antioxidants. Such additional antioxidants could possibly be the coenzyme Q_{10} (a vital integral component of the ETC), an antioxidant that strongly combats ROS-induced lipid peroxidation [140], and melatonin, a pineal hormone which acts as a free radical scavenger, decreases nitric oxide production in mitochondria, and mediates bioenergetic functions via regulation of respiratory complex and influx of calcium ions as well as by increasing mitochondrial permeability [141].

Yet another implication of storing spermatozoa in ambient temperature is ATP depletion through continued metabolism, thus gradually reducing sperm motility [142] and ultimately triggering sperm cell death [143]. If ambient temperatures are to be used for sperm storage, it is essential to support mitochondrial energy generation to avoid ATP depletion. There is also a need to prevent the increased pressure on ATP-dependent pathways vital for cell functions, such as the regulation of ionic influx or efflux [144]. If sodium chloride in the storage medium is replaced by non-ionic, organic osmolytes such as carbohydrates, amino acids, and betaines, the pressure on the Na^+/K^+-ATPase pump can be reduced which in turn will decrease ATP utilization in the medium [144].

Research has shown that a combination of pyruvate (the prime energy source for oxidative phosphorylation), L-carnitine [145], and an organic, non-ionic osmolyte [146] could maintain sperm motility and viability at ambient temperature for up to 72 h [138]. In the case of horse spermatozoa, it consists of a number of proteins of beta-oxidation whose inhibition can reduce sperm motility [147]. L-carnitine, being vital for beta-oxidation and also acting as an osmolyte and an antioxidant, induces mitochondrial ATP generation by transporting acetyl groups of pyruvate to the mitochondrial matrix and buffering the free CoA. Thus, the importance of L-carnitine for maintenance of sperm quality in vivo is well recognized [148].

Moreover, in the body, spermatozoa contain the highest intracellular L-carnitine concentrations as androgen stimulates the epithelial cells to produce and secrete L-carnitine in high concentrations into the epididymal lumen [149]. This accounts for an L-carnitine concentration of up to 2000-fold higher than that of blood [150]. Hence, L-carnitine is definitely a molecule of high importance in male fertility. Oral L-carnitine supplementation showed higher pyruvate uptake by spermatozoa [151], which suggests that L-carnitine is associated with overall sperm metabolism.

26.6.3 Non-hormonal Male Contraception

Due to the uncertainty and higher failure rates of physical contraception and disadvantages of hormonal contraception, non-hormonal contraception affecting sperm functions

are becoming a priority in current contraception research. Chemicals and drugs affecting sperm motility have been used as useful tools for non-hormonal male contraception. For instance, EPP055 is an agent that is found to have anti-sperm motility functions and has been used in different animal species as non-hormonal contraception. EPP055 affects a sperm surface protein, EPPIN, to cause loss of forward and progressive motility. EPPIN binds to SEMG1 on the sperm surface and leads to a substantial decrease in intracellular pH, which in turn reduces internal calcium levels [152–154].

Therefore, the normal physiological cascades for fertilization mediated by increased internal pH to activate CatSper calcium channel gets disrupted. This leads to reduced calcium influx, which then inhibits cAMP production to mediate pathways for ATP production. This, in turn, prevents normal sperm capacitation and hyperactivation. Sperm motility remains low at least 78 h after infusion but it fully recovers by the 18th day after the treatment is ceased, which indicates its potential efficacy as a non-hormonal contraceptive agent [154].

Soluble adenylate cyclase (sAC) is also an interesting target to inhibit sperm activities. sAC is required to produce cAMP in the sperm cytoplasm to aid capacitation and hyperactivation [155]. sAC inhibitors thus potentially inhibit cAMP production and thereby down-regulate energy generation to bring about sperm motility. The sodium-hydrogen exchanger that is needed for sAC expression is found in sperm and thus is also a target for non-hormonal contraception [156].

Sperm motility is maintained via proper regulation of sperm volume and concentration of potassium chloride. In this regard, the co-transporters and ion-specific channels are crucial entities which can be targeted for contraception [157]. The SFEC is also a potential contraception target as it mediates the transportation of ATP into the principal piece of sperm for energy generation and utilization to activate flagellar movements for capacitation [4].

26.7 Conclusion

Energy generation and utilization in spermatozoa, though an integral part of sperm functionality, still remains partly ambiguous. This may be due to the unique anatomical and physiological characteristics of spermatozoa. This chapter thus discussed the unique functional ultrastructure of sperm that supports its energetics. It puts forth the preferred metabolic pathways in spermatozoa, both glycolytic as well as mitochondrial oxidative phosphorylation, to generate energy as per utilization. The coordination of Sertoli cells and spermatogonial cells in terms of energy metabolism is described for a clear understanding of energy sources for spermatogenesis. The chapter also vividly presents the possible mechanisms by which spermatozoa fuel their own hyperactivation, capacitation and acrosome reactions until fertilization is obtained. The proper concepts of energy metabolism help in improving the in vitro storage medium for spermatozoa as well as in evaluating the efficacy of non-hormonal contraceptions, based on cessation or reduction of sperm energy generation. Further interventions are encouraged for precise understanding of sperm energetics as that will help in designing a better medium for longer storage of spermatozoa while keeping their functionalities in robust conditions.

26.8 Review Criteria

An extensive search of studies examining the source, production and utilization of energy by spermatozoa was performed using search engines such as PubMed, MEDLINE, OVID, Science Direct, and Google Scholar. The start and end dates for these searches were July 2018 and December 2018, respectively. The overall strategy for study identification and data extraction was based on the following keywords: “sperm energy metabolism,” “sperm energetics,” “sperm fuel,” “sperm fuel machinery,” “sperm energy utilization,” “oxidative phosphorylation,” “glycolysis,” “ATP,” “sperm ATPases,” “sperm glycolytic enzymes,” “spermatogenesis,” “sperm function,” “hyperactivation,” “capacitation,” “acrosome reaction,” “sperm motility,” “sperm storage,” “cryopreservation,” “male contraception,” and “non-hormonal male contraception.” Articles published in languages other than English were also considered, provided that the abstract was in English. Data that were solely published in conference or meeting proceedings, websites, or books were not included.

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Physiological Role of ROS in Sperm Function

27

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Key Points

- ROS generation is a universal property of spermatozoa of all mammalian species.
- ROS can be endogenously generated or induced by exogenous sources.
- ROS mediate some essential intracellular signaling cascades needed for normal sperm functions.
- ROS pay essential role in sperm production, maturation, capacitation, and acrosome reactions.
- Excess ROS production leads to redox imbalance and OS inducing oxidative damage to the sperm.

27.1 Introduction

Male reproductive functions present a complex physiological mechanism, which are still far from complete understanding. Among the multivariate factors playing roles in mediating reproductive functions in men, reactive oxygen species (ROS) find immense importance in recent interventions from both physiological and pathological aspects [1]. The reports on the decline in semen quality over the last few decades have added to the necessity of a better understanding of sperm functions [2–5]. It is evident that most of the disruptors of sperm functions, be it endogenous, environmental, or lifestyle mediated, may operate via unregulated ROS. This leads to a condition of oxidative stress (OS) [6, 7] with generation of reactive species (oxidants) superseding total antioxidant capacity (reductants) in the seminal plasma.

ROS are, as oxygen metabolism by-products, highly reactive oxygen derivatives that are obviously toxic beyond physiological levels [8–10]. However, at normal physiological concentrations, they mediate not only essential bodily functions but also male reproductive functions [11]. ROS facilitate vital intracellular signaling cascades for appropriate sperm functions such as maturation, hyperactivation, and capacitation as well as acrosome reaction (AR) [11, 12]. In addition, they play a crucial role in the fertilization process.

This chapter provides distinct concepts on the vital contribution of ROS in normal sperm physiology by mainly elucidating (a) the endogenous and exogenous sources of ROS in male reproductive tract and (b) the physiological role of ROS in different phases of spermatozoa production, maturation, and attainment of maturity, until fusion with the female oocyte and fertilization.

27.2 Reactive Oxygen Species

An atom is in its ground state when its outermost shell has two complementary paired electrons with opposite spin directions. In such condition, the atom is stable and inert enough not to react with its surroundings. If atoms or molecules miss one electron, i.e., have only one free electron in the outer orbit, they are called radicals. Atmospheric oxygen does not occur in atomic form, but in molecular form as diatomic oxygen (O_2), and as per its electronic conditions, oxygen is a diradical with two free unpaired electrons in the outer orbit. This electronic condition causes the chemical reactivity of oxygen.

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Oxygen is required for cellular respiration and for sustenance of survival. If oxygen is reduced, it leads to the generation of an extremely reactive oxygen metabolite, the superoxide anion ($•O^{2-}$), which is also a radical. This metabolite may interfere with various cellular functions. If this reduced metabolite further gains an electron, it is converted to peroxide (O_2^{2-}) which is not a free radical [13]. $•O^{2-}$ dismutation produces hydrogen peroxide (H_2O_2), another endogenous electronically not charged oxidant. It is found in abundance and, due to the fact that it is not a radical, relatively less reactive. However, due to its uncharged character, H_2O_2 can penetrate plasma membrane just like water.

A number of metabolic reactions in the human body produce H_2O_2 that includes, but not limits to, the peroxisomal pathway by monoamine oxidases and β-glycolate [14]. Both $•O^{2-}$ and H_2O_2 are susceptible to chains of transformations by Fenton and Haber-Weiss reaction to form the highly reactive hydroxyl radical (OH•). The Haber-Weiss reaction consists of an $•O^{2-}$-mediated reduction of ferric (Fe^{3+}) to ferrous ion (Fe^{2+}) and O_2, followed by the Fenton reaction, in which Fe^{2+} catalyzes the conversion of H_2O_2 to OH• and OH^- [15]. The superoxide anion may also react with nitric oxide (NO) forming peroxynitrite ($ONOO^-$). NO generation is aided by the enzyme nitric oxide synthase (NOS). NO is a reactive radical consisting of odd electron number [16]. Other ROS species, such as organic peroxyl and alkoxy radicals and ozone, may also be present but do not bear much biological significance [17]. ROS include all free radicals with an oxygen atom, and many people include hydrogen peroxide, which is not a radical, but a reactive oxygen derivative. As oxidants, these molecules capture electrons from adjacent cellular structures to reach ground state and in the process render the donator molecules (reductants) as free radicals. These chains of reactions continue to amplify the level of disruption in the neighboring cellular components [18].

27.3 Origins of ROS in Male Reproductive Tissues

ROS generation is a universal property of all cells including spermatozoa of all mammalian species. There are two proposed mechanisms of ROS production by spermatozoa: (a) via the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system in the sperm membrane and/or (b) via the nicotinamide adenine dinucleotide-dependent redox reaction in the sperm. Spermatozoa are mitochondria-rich cells owing to their constant requirement of energy for their motility [19]. ROS are mostly contributed by electron leakage from mitochondria, induced by various factors that disrupt the electron transport chain. Elevated numbers of dysfunctional spermatozoa in seminal fluid are a strong inducer of ROS generation. The prime ROS produced in human spermatozoa is $•O_2^-$ that via dismutation produces H_2O_2. In presence of transition metals, iron and copper, H_2O_2 and $•O_2^-$ undergo the Haber-Weiss reaction to yield the most reactive and toxic metabolite, $•OH^-$. This may initiate lipid peroxidation in sperm membranes followed by the chain of reactions impairing sperm functions [20, 21].

27.4 Endogenous Sources of ROS in Seminal Plasma

27.4.1 Leukocytes

Peroxidase-positive leukocytes, such as the polymorphonuclear leukocytes (50% ~ 60%) and macrophages (20% ~ 30%), originate from the seminal vesicles and the prostate gland. During urogenital infections or inflammations, these cells may generate even 100 times more ROS higher than in normal conditions as part of the immune response. In turn, the production of NADPH via the hexose-monophosphate shunt also increases [1]. An inflammation-induced rise in pro-inflammatory mediators and decreased antioxidant capacity may trigger respiratory burst leading to OS [22]. Leukocytospermia refers to a condition in which semen contains greater than one million peroxidase-positive leukocytes per milliliter of semen and is associated with high degree of impairments to sperm functions [23].

27.4.2 Immature Spermatozoa

Normal physiological events in sperm maturation include extrusion of excess cytoplasm. However, when spermiogenesis is disrupted, spermatozoa retain excess cytoplasm around the midpiece, thus impeding its functions (excess residual cytoplasm, ERC).

Immature spermatozoa with distorted head morphology and cytoplasmic retention are thus a major source of seminal ROS [24]. The retention of the excess cytoplasm in immature sperm is the cause of surplus of metabolic enzymes, such as the glucose-6-phosphate dehydrogenase (G6PD) and NADPH oxidase, and NADPH oxidase as enzymes intimately involved in free radical production via the intermediate NADPH formation [25]. G6PD is a key enzyme to catalyze the hexose-monophosphate shunt, mediating the reaction with $NADP^+$ and glucose-6-phosphate to form reduced NADPH. Thus, ERC may activate the NADPH system via the hexose-monophosphate shunt, paving the way for ROS production and OS [26].

Normal spermatozoa are able to generate ROS by means of two different sources: NADPH oxidase in their plasma membrane and NADH-dependent oxidoreductase (diaphorase) of their mitochondria [27, 28]. NADH diaphorase is a

major participant of the high ATP-generating Krebs cycle. This biochemical pathway is common to almost every aerobic organism that yields high energy, and one of the functions of this cycle is acetate oxidation. The Krebs cycle yields three NADH molecules from NAD^+. NADH transports electrons to be utilized for generation of via mitochondrial electron transport chain and, in the process, leads to generation of some ROS as well. However, ROS generation mediated by sperm mitochondrial has been reported to be considerably low [28].

27.4.3 Sertoli Cells

Sertoli cells have also been documented to be able to generate ROS [29–31]. Studies have shown that addition of so-called scavestrogens (synthetic steroidal estrogens with potent antioxidant properties [32]) inhibits the production of reactive oxygen species from Sertoli cells [31]. Scavestrogens potentially scavenge free radical scavengers and can restrict iron-induced cell damage in vitro [31]. Thus, it may be suggested that Sertoli cells, in normal in vivo conditions, aid spermatogenesis mediated by ROS production. Further investigations are required to clarify the role of Sertoli cells in ROS production.

27.4.4 Varicocele

Varicocele, an abnormal venous dilation in the pampiniform plexus around the spermatic cord, affects about 40% of male partners among overall infertile couples and is one of the leading causes of male subfertility and infertility [33]. There are several mechanisms explaining the pathophysiology of varicocele-induced alterations in sperm functions. However, it has been put forth that the most common mechanism includes varicocele-mediated testicular hyperthermia and hypoxia in causing OS-induced sperm dysfunctions [33, 34]. A meta-analysis has confirmed a higher incidence of oxidative stress parameters such as ROS and lipid peroxidation in semen samples from varicocele-affected infertile patients as compared with healthy fertile donors [33, 35]. The levels of seminal ROS levels have suggested to have direct correlation with the grade of varicocele [36].

27.5 Exogenous Sources of ROS in Seminal Plasma

27.5.1 Radiation

Mobile phone radiation greatly induces ROS generation in seminal plasma and leads to deleterious effects on semen quality. It triggers sperm DNA damage and epigenetic modifications disrupting sperm motility, count, and vitality [37, 38]. Electromagnetic waves, via the cytosolic charged molecules, lead to alterations in the process of intracellular electron transfer along sperm membranes and thereby impair sperm functions [39]. Radio frequency waves influence male fertility via thermal as well as nonthermal mechanisms. Testes are susceptible to disruptions by electromagnetic energy as testicular temperature regulation depends mostly upon surface conduction unlike other organs that mainly depend on blood flow for temperature regulations. Radiation may lead to raised scrotal temperature and affect spermatogenesis, which is sensitive to even a 1 °C increase in its temperature. The nonthermal effects of radiation are mediated through oxidative stress or changes in cell membrane potential affecting spermatogenesis and inducing spermatozoa apoptosis. Chronic exposure to radiation also brings about degeneration of Leydig cells [40].

27.5.2 Lifestyle Factors

Smoking is a modifiable lifestyle factor that severely disrupts the balance of ROS production and antioxidant defense. Smoking may lead to an increase in seminal leukocyte concentrations by 48% that accounts for the increase in seminal ROS by 107%. The seminal antioxidant capacity gets diminished accompanied by an increase in 8-OHdG concentrations (a potent oxidative damage biomarker) [41, 42]. Furthermore, smoking elevates lead and cadmium concentrations in semen and blood. In turn, this increases ROS production affecting sperm functions [39]. Germ cell apoptosis and DNA damage are generally common in smokers [43]. Alcoholism is another crucial factor that paves the way to excess seminal ROS generation and reduction in antioxidant capacity. Acetaldehyde, produced as a by-product through ethanol metabolism, yields ROS reacting with sperm cellular components. This significantly reduces the percentage of functional spermatozoa [9].

27.5.3 Toxins

Industrialization and domestic sophistications are responsible for increasing environmental toxins and endocrine disruptors in an individual's immediate environment. These intrude the body and may potentially induce excessive testicular ROS generation and OS, which in turn impairs morphology and functions of sperms. Exposure to environmental toxins leads to ROS-induced germ cell apoptosis [44]. For example, increased use of plastics has led to rise in exposure to phthalates which in addition to toxic metals such as lead, manganese, cadmium, chromium, and mercury afflict sperm quality, count, and spermatogenesis [5, 45].

27.6 Physiological Role of ROS on Different Sperm Functions

As discussed earlier, ROS can be detrimental to sperm functions when their seminal levels exceed physiological limits. Nonetheless, at regulated levels, ROS mediate some essential sperm functions including sperm maturation, capacitation, hyperactivation, acrosome reaction, and sperm-oocyte fusion [11, 28, 46–50].

27.6.1 Sperm Transformational Stages

The spermatozoon nucleus comprises of a haploid paternal genome so that it complements the female haploid genome present in the oocyte. Fertilization involves nuclear fusion of male and female gametes resulting in the formation of a diploid organism [28].

Formation of mature, normal spermatozoa from developing spermatids is the primary criterion toward successful fertilization. After production of immature germ cell in the seminiferous tubules of the testis, the cells migrate to the epididymis where they are stored and mature under the influence of the adjacent epithelial cells. This maturation phase in the epididymis transforms nonmotile spermatozoa into motile germ cells able to fertilize an oocyte. Spermatogenesis further comprises of nucleus condensation, a process which is finalized by the oxidation of protamine sulfhydryl groups to disulfide bridges, which further stabilize the sperm nucleus. During ejaculation, secretions of different accessory glands are mixed with the spermatozoa to form semen [28].

Semenogelin, a secretion from the seminal vesicles, is the most abundant seminal protein which aids semen coagulation upon ejaculation [51]. Prostate-derived prostate-specific antigen (PSA) later degrades the semen coagulum. ROS may also play a vital role here in the process of seminal coagulation and liquefaction by mediating redox reactions. Hamada et al. had shown that higher ROS levels in patients accounted for increased semenogelin levels in their semen [52]. On contrary, Chatterjee et al. [53] had also reported that $\bullet O^{2-}$ amplifies the process of semen liquefaction. The discrepancies in scientific reports of the role of ROS may be due to the multifarious actions of ROS at the same time. Alternatively, it may owe to the use of a higher concentration of $\bullet O^{2-}$ in vitro in the study by Chatterjee et al. as compared to its in vivo levels [28].

Mature spermatozoa are highly polarized and compartmentalized, comprising of a head with very little cytoplasm and condensed chromatin in nucleus and a flagellum (tail) separated in midpiece, principal piece, and end piece. The acrosome is part of the anterior sperm head, taking up an area between 50% and 70%. It is an inactive, enzyme-filled structure, which derives from the Golgi apparatus and is enclosed by its membrane. The midpiece of the spermatozoon is referred to as its "powerhouse" owing to the presence of a profuse number of mitochondria that generate energy for flagellar movements [54]. The flagellum contains the axoneme as motility-generating structure and the outer dense fibers (ODF), which contribute to the stability, rigidness, and efficient energy conversion of the flagellum [55–57]. In addition, the ODF are responsible for the typical "whiplike" flagellar motion of mammalian spermatozoa [58, 59].

Following ejaculation, mature spermatozoa may enter the female reproductive tract. This event marks the onset of several physiological changes in spermatozoa that render them potent for recognition and binding to zona pellucida of the oocyte. This reversible, preparatory process, which takes a few hours to complete, is termed capacitation. The cellular alterations taking place during this phase attribute to increased sperm plasma membrane fluidity, efflux of cholesterol, activation of adenylyl cyclase and increased intracellular cyclic AMP (cAMP) and calcium levels, membrane hyperpolarization, increased intracellular pH, tyrosine (Tyr) phosphorylation of proteins, and hyperactivation of the spermatozoon [60, 61].

On completion of the capacitation process, spermatozoa approach the oocyte and acrosome reaction (AR) can be initiated [28]. This process involves membrane hybrid vesicle formation between the sperm plasma membrane and out acrosomal membrane resulting in exocytosis of the acrosomal enzymes following the sperm penetration of the zona pellucida [62]. In contrast to capacitation, acrosome reaction is an irreversible process that not only goes along with morphological but also physiological changes of spermatozoa. Physiological triggers of acrosome reaction are the zona pellucida glycoprotein 3 (ZP3) or progesterone [63].

From the production of spermatozoa in the testes, until their fusion with oocyte, spermatozoa pass through different specialized maturational steps to attain their functional competence to successfully fertilize oocytes. The morphological integrity of the matured spermatozoon and their physiological functions are facilitated by extracellular cues from their immediate environment, in which ROS play a vital role as stimulatory factors that are required by the male germ cells to attain their complete functional competence [64].

27.6.2 Maturation

The epididymis is the site of spermatozoal maturation. The maturation steps include sperm membrane alterations,

membrane protein rearrangement, enzymatic modulations, and nuclear remodeling [11]. These steps are mediated via appropriate cell signaling pathways modulated by levels of seminal ROS [28, 49]. Since smaller-sized protamines replace the histones as nuclear proteins in mammalian spermatozoa, it is possible to highly condense the DNA in an array-type structure instead of supercoiled solenoids [65]. The cysteine residues of the protamines have inter- and intramolecular disulfide bonds between them to impart chromatin stability [66]. This formation of disulfide bond is aided by ROS to ensure chromatin stability and prevent damage to the chromosomal DNA. ROS, especially peroxides, also play a role in the mitochondrial capsule formation, which is formed by network of proteins with several disulfide bonds preventing proteolytic degradation of mitochondria [11, 67].

27.6.3 ROS as Signal Transducers

ROS are well-suited as intracellular signaling molecules for their tiny size, ubiquitous nature, and short half-life. They mediate all vital physiological functions of sperm such as maturation, motility, activation, capacitation, and acrosome reactions [28]. ROS actions are mainly carried out through the regulations of the redox status of cysteine residues. The redox status of thiol groups determines enzymatic activity, which acts via activation of adenylate cyclase (AC) by increasing the intracellular cyclic AMP (cAMP) concentration. cAMP in turn activates protein kinase A (PKA), thereby triggering various downstream cell signaling pathways specific to different phases of spermatozoa maturation [11].

27.6.4 Motility and Hyperactivation

Hyperactivation refers to a state of exaggerated nonlinear motility in sperm with relatively high-amplitude asymmetric, whiplash-like, flagellar movement, and increased sidewise displacements of sperm head [68]. It is suggested to be a part of the capacitation process and is essential for successful attainment of sperm oocyte fusion and fertilization. ROS mediate upregulation of sperm hyperactivation processes [49]. The underlying mechanism includes initiation of capacitation and hyperactivation by induction of Ca^{2+} and HCO_3^- influx possibly through inactivation of plasma membrane Ca^{2+}-ATPase (PMCA) and cytosol alkalization. Calcium ions and ROS, specifically $\bullet O_2^-$, mediate AC activation and increased production of cAMP which activates PKA. This process triggers NADPH oxidase and thereby further upregulates ROS generation. Moreover, PKA leads to phosphorylation of serine (Ser) and Tyr residues, thereby activating protein tyrosine kinase (PTK). PTK phosphorylates the subsequent tyrosine residues in the axoneme fibrous sheath and the sperm flagellum cytoskeleton. ROS, especially H_2O_2, trigger phosphorylation of tyrosine by activating PTK and inhibiting phosphotyrosine phosphatase (PTPase) from dephosphorylating the Tyr residues. These ROS, especially superoxide, inducing phosphorylation of tyrosine residues are suggested to be the final step in sperm hyperactivation [11, 39].

27.6.5 Capacitation

Capacitation is the final functional processing of spermatozoa maturation, which potentiates them and renders them competent for fertilizing an ovum. Extensive research has suggested essential functions of ROS at physiological levels in facilitating capacitation. There is supportive evidence that ROS are vital for the amplification in phosphorylated tyrosine residues (P-Tyr) of proteins (molecular weight ~100 kDa) [69]. ROS possibly potentiates this process by mediating cell signaling pathways, which involve an increase in intracellular cAMP levels followed by activation of PKA and phosphorylation of mitogen-activated *protein* kinase (MEK) and the downstream regulatory proteins. The overall impact of ROS-mediated activation of fibrous sheath proteins orchestrates the physiological process of sperm capacitation and the male germ cells acquiring the required potency to execute acrosome reaction [39, 70].

Ca^{2+} and HCO^{3-} influx via the inactivation of an ATP-dependent Ca^{2+} regulatory channel (PMCA) and cytosol alkalization may initiate the possible biochemical pathway in regulation of sperm capacitation and hyperactivation. ROS, especially $\bullet O^{2-}$, along with Ca^{2+} activate AC, followed by production of cAMP, which phosphorylates PKA. PKA in turn induces ROS generation by activating membrane-bound NADPH oxidase. PKA also phosphorylates serine (Ser) and tyrosine (Tyr) residues that participate in protein tyrosine kinase (PTK) activation and subsequent phosphorylation and activation of threonine-glutamate-tyrosine residues of fibrous sheath proteins surrounding the axoneme [70, 71], which is the flagellar cytoskeletal component. ROS, especially H_2O_2, highly induces Tyr phosphorylation by triggering PTK activity and via inhibition of phosphotyrosine phosphatase (PTPase) activity that acts to dephosphorylate Tyr residues [11, 28] (Fig. 27.1).

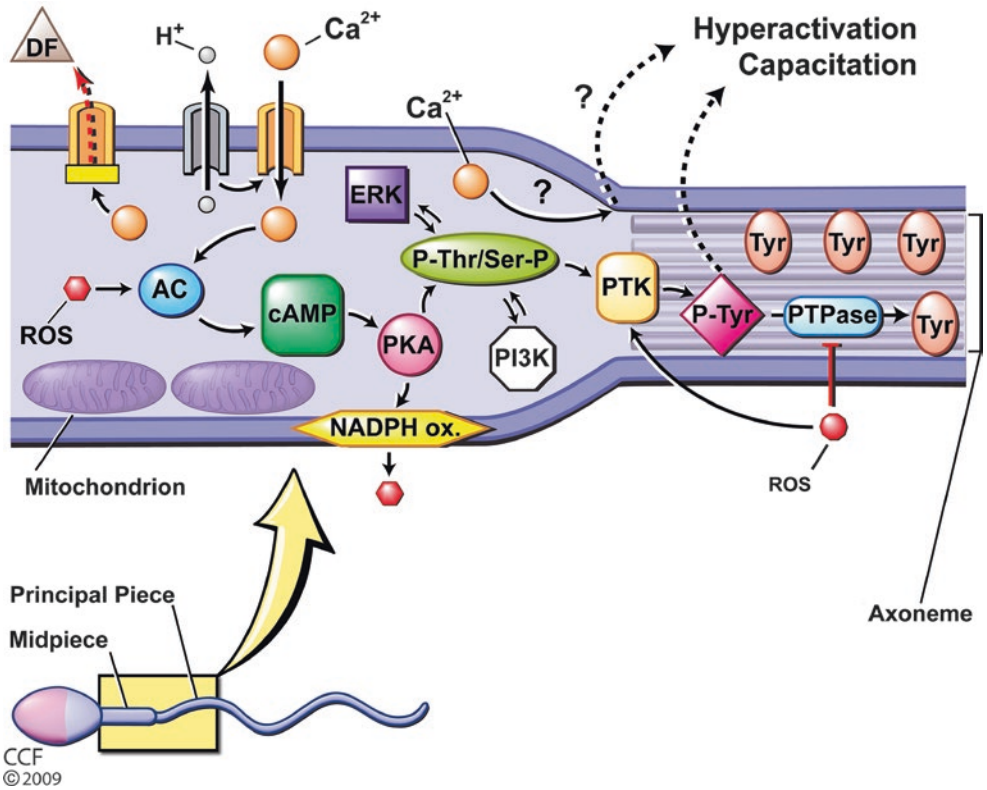

Fig. 27.1 Biochemical pathway mediated by ROS to regulate sperm capacitation and hyperactivation. PMCA, plasma membrane Ca^{2+} ATPase; ROS, reactive oxygen species; AC, activate adenylate cyclase; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; PTK, protein tyrosine kinase; PTPase, phosphotyrosine phosphatase (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2009–2019. All Rights Reserved)

27.6.6 Acrosome Reaction

The primary criterion for successful fertilization is sperm-oocyte fusion followed by penetration of sperm head through the zona pellucida. To accomplish the same, hyperactivated spermatozoa need to cross the layer of cumulus cells to reach the zona pellucida of oocyte. The completion of acrosome reaction (AR) refers the end in maturational stages of the spermatozoon as at this point it acquires the ultimate fertilizing ability. ROS facilitate the acrosome reaction possibly by induction of phosphorylation of specific plasma membrane proteins [11, 28]. The anterior part of the sperm head gets modified to release the acrosomal enzymes as the sperm comes in contact with the zona pellucida. Acrosin, a trypsin-like serine proteinase, is an essential enzyme for acrosome reaction and sperm-zona penetration as it facilitates dissolving the zona pellucida, thereby creating a pore for the sperm to penetrate [67].

Induction of the AR can take place via both physiological and nonphysiological stimulators, including ROS, the zona pellucida itself, or progesterone [72]. It is initiated by Ca^{2+} influx and Ca^{2+} release from the acrosomal calcium store as occurred during capacitation. This leads to production of diacylglycerol (DAG) and inositol triphosphate (IP3) from phosphatidylinositol-4,5-bisphosphate (PIP2). This is followed by activation of actin-severing proteins, triggering acrosomal fusion with plasma membranes, and subsequent exocytosis of acrosomal contents. DAG eventually phosphorylates protein kinase C (PKC), leading to yet more Ca^{2+} influx that activates phospholipase A2 (PLA2) (Fig. 27.2). The huge quantity of membrane fatty acids release during AR elevates sperm plasma membrane fluidity required for its fusion with oocyte [28, 39].

27.6.7 Sperm-Oocyte Fusion

ROS essentially participate in increasing the plasma membrane fluidity during sperm-oocyte fusion by mediating the activation of biochemical cascades for capacitation followed by successful acrosome reaction. Along the entire duration of spermatozoa capacitation, ROS impede the deactivation of PLA2 via inhibition of protein tyrosine phosphatase activity. Thus, the activated PLA2 potentially cleaves the secondary fatty acid from the membrane phospholipid triglycerols and enhances the plasma membrane fluidity [73].

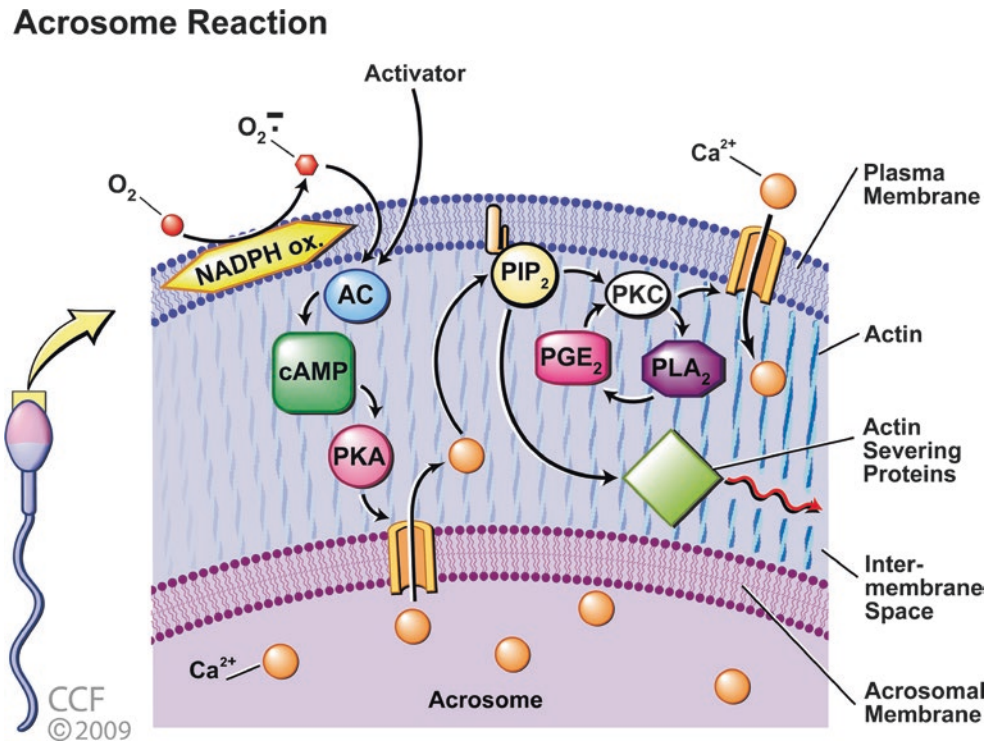

Fig. 27.2 Biochemical pathway mediated by ROS to regulate the acrosome reaction (AR). ZP, zona pellucida; PIP_2, phosphatidylinositol-4,5-bisphosphate; DAG, diacylglycerol; IP_3, inositol triphosphate; PKC, protein kinase C; PLA_2, phospholipase A_2(Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2009–2019. All Rights Reserved)

27.7 Conclusion

ROS are essential for sperm functions at all levels of sperm production, storage, and fertilization when their concentrations are within physiological limits. As discussed, both endogenous and exogenous sources of ROS contribute to the levels of ROS in seminal fluid. ROS ensure the morphological modulation of spermatozoa by being mediators of key intracellular signaling pathways that aid sperm chromatin condensation, motility, chemotaxis, capacitation, hyperactivation, and acrosome reaction to attain successful fertilization. On the other hand, excess ROS generation is resulting in deleterious effects on the male reproductive system by induction of OS. Therefore, it may be suggested that complete sperm functionality with utmost fertilization capacity is attained by a well-balanced processes of generation and scavenging of ROS.

Further research is needed to unveil the proteins that are expressed and phosphorylated in spermatozoa in response to oxidative stress and how ROS mediate specific intracellular signaling pathways. Proper concepts regarding the intricate oxidative balance playing essential physiological roles in sperm functions find immense importance in the diagnosis, prevention, and treatment of male subfertility and/or infertility.

27.8 Review Criteria

An extensive literature search has been performed to find the relationship between oxidative stress and male infertility using search engines such as Science Direct, OVID, Google Scholar, PubMed, and MEDLINE. The overall strategy for study identification and data extraction was based on the following keywords: “oxidative stress,” “reactive oxygen species,” “infertile men,” “infertility,” “semen parameters,” and “assisted reproduction” and the names of related oxidative stress markers and specific ROS assessment methods. Articles published in languages other than English were also considered. Data that were solely published in conference or meeting proceedings, websites, or books were not included. Websites and book chapter citations provide conceptual content only.

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Sperm Physiology and Assessment of Spermatogenesis Kinetics In Vivo

28

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Key Points

- Adult men produce millions of spermatozoa daily, dependent on adequate testicular environment and hormonal control. Proper functioning of the hypothalamic-pituitary-gonadal axis, which is regulated via the release of gonadotropin-releasing hormone (GnRH) is essential to sustain both spermatogenesis and steroidogenesis.
- FSH and LH action in germinal cell development is affected by androgen and FSH receptors on Leydig and Sertoli cells, respectively. While FSH acts directly on the germinative epithelium, LH stimulates secretion of testosterone by the Leydig cells.
- Spermatogenesis is a complex process that leads to the transformation of primordial germ cells into mature spermatozoa, starting with diploid cells and completing with highly specialized haploid cells, and this process is estimated to take 64 days.
- In vivo, ejaculated sperm are unable to fertilize until they have undergone capacitation, which allows the acrosome reaction to take place when spermatozoa approach or contact the oocyte.

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28.1 Introduction

Spermatogenesis and steroidogenesis depend on the proper functioning of the hypothalamic-pituitary-gonadal axis, which is regulated via the release of gonadotropin-releasing hormone (GnRH). Hypothalamic neurons secrete GnRH in a pulsatile form; the latter is then transported to the anterior compartment of the pituitary gland where it binds to specific receptors located in the gonadotropic cells to modulate the synthesis and secretion of pituitary gonadotropins, the follicle-stimulating hormone (FSH), and luteinizing gonadotropins (LH). These hormones are secreted into the systemic circulation and act on the testes. FSH acts via receptors located in the Sertoli cells, whereas LH stimulates Leydig cells to produce testosterone, an essential prerequisite for male fertility and maintenance of spermatogenesis [22, 50, 108]. Steroids and gonadal peptides are subsequently secreted into the systemic circulation to modulate the hypothalamic and pituitary hormone secretion [64, 118].

Spermatogenesis is a complex process that leads to the transformation of primordial germ cells into mature spermatozoa, starting with diploid cells and completing with highly specialized haploid cells [57]. This is a continuous process throughout the reproductive life that is controlled by paracrine, autocrine, endocrine, genetic, and epigenetic regulators, occurring within the seminiferous tubules [82]. The complete process comprises (i) the proliferation of spermatogonia, (ii) the differentiation of spermatogonia into spermatocytes, (iii) production of spermatids through the meiotic division of spermatocytes, (iv) maturation of round spermatids, and lastly, (v) release of mature spermatozoa into the lumen of the seminiferous tubules [104].

Although the resulting spermatozoon acquires its final shape and size in the testis, it only achieves full potential for natural fertilization after passage through the epididymis, in a process known as epididymal maturation and capacity building. Finally, sperm capacitation takes place after contact with the female reproductive tract [40, 64, 118].

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In this chapter, we will present and discuss the critical features of sperm physiology, spermatogenesis, and steroidogenesis.

28.2 The Testis: Structure and Function

The primary functions of the testis are the synthesis of steroid hormones (steroidogenesis) and the production of mature sperm (spermatogenesis). The testis performs its functions through the coordination and interrelation between its various cell types, such as Sertoli cells, Leydig cells, peritubular myoid cells, and germ cells [7].

The testicles are outside the abdominal cavity and lie within the scrotum. Thermoregulatory mechanisms maintain the testicular temperature at approximately 2–3 °C lower than the body temperature. Since male germ cells are thermosensitive, this location is critical to maintaining adequate spermatogenesis. Changes in the mechanism of temperature control can lead to infertility, as it occurs in cases of varicocele and cryptorchidism. The testicular parenchyma is covered by a capsule of connective tissue (the tunica albuginea), and externally to the albuginea, there is the vaginal tunic [110]. The testicular and epididymal blood supply originates from three main arterial branches, namely, the internal spermatic testicular artery, deferential artery, and external or cremasteric spermatic artery. The spermatic artery is a branch of the abdominal aorta, originating distally from the renal artery. It is associated with a network of veins that anastomose and form the pampiniform plexus. The vascular arrangement of this plexus facilitates the exchange of heat and small molecules between arteries and veins. In healthy men, the heat exchange that occurs in the spermatic cord causes the intratesticular temperature to be lower than the corporal temperature. Inside the testicular capsule, the testis is divided into about 200–300 lobules by septations arising from the tunica albuginea [82]. In each lobe, there are seminiferous tubule loops, each loop ending in the rete testis network. This network is connected with the efferent ducts, thus allowing sperm to reach the head of the epididymis. Within each lobe, the testis is divided into two compartments, that is, (i) intratubular compartment, which is composed of the seminiferous epithelium and contains two cell types, the sperm cells at different stages of differentiation and the Sertoli cells, and (ii) peritubular or interstitial compartment, which is composed of Leydig cells, connective tissue cells, mast cells, macrophages, nerves, blood vessels, and lymphatics; in human testicles, the interstitial tissue represents 20–30% of the testicular volume [94].

28.2.1 Seminiferous Tubules

The seminiferous tubules are long, enveloped structures that arise and terminate in the rete testis network, an anastomotic network of tubules that drains into the efferent ducts. The total length of the existing 600–1200 tubules in the human testicle is approximately 250 meters. The rete testis network coalesces to form 5–10 efferent ducts, which will carry the testicular fluid and spermatozoa to the region of the head of the epididymis. The seminiferous tubules provide a unique environment for the production of germ cells through their germinative elements (sperm cells at various stages of development) and the "supporting cells," which harbor and provide a physiological basis for male germ cells differentiation. "Supporting cells" include Sertoli cells and basement membrane cells, both remaining in intimate contact with the sperm cells. The germinative elements comprise a population of epithelial cells, including the primordial germ cells, spermatogonia, spermatocytes, and spermatids [94]. Each seminiferous tubule consists of a basement membrane covered by Sertoli cells interspersed with germ cells at various stages of maturation. The germ cell maturation, better known as the spermiogenesis process, occurs in a centripetal way, with the most immature cells present in the periphery and migrating toward the lumen as they progress through the sequential stages of maturation [5] (Fig. 28.1). In the pre-pubertal phase, the seminiferous tubules are called seminiferous cords, being constituted only by Sertoli cells and spermatogonia. As the testosterone production by Leydig cells increases after puberty, the remaining germline cells appear [14].

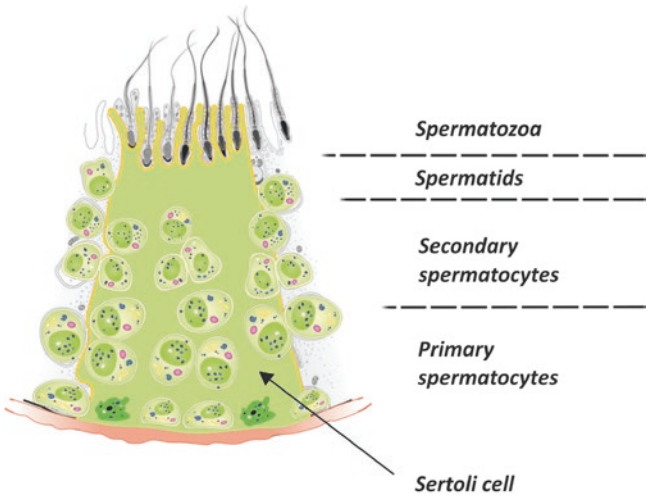

Fig. 28.1 Schematic representation of a histologic cross-section of a single seminiferous tubule depicting its cell components. (Reprinted from Ref. [119] with permission from Elsevier)

28.2.2 Sertoli Cells

Sertoli cells—the true epithelial cells of the seminiferous epithelium—are pyramidal cells that rest on the basal membrane of the seminiferous tubules with their filamentous ramifications extending toward the lumen of the tubules. They provide a structural support to the seminiferous epithelium and

surround the sperm cells, being responsible for both maintenance of the hematopoietic barrier and development of the germ cells. The Sertoli cells also have functional properties related to the secretion of hormones (anti-Müllerian hormone and inhibin) and phagocytosis. The Sertoli cells produce a large amount of fluid that provides not only a suitable environment for the spermatozoa but also helps to move the immotile sperm from the seminiferous tubule to the epididymis [110].

The Sertoli cell population is established early in postnatal development and is the main determinant of both sperm production and release during adulthood. At puberty, the Sertoli cells go through a period of proliferation, in which FSH acts as an important regulator [14]. Tight-junctions are found between adjacent Sertoli cells, which make the physical basis for the hematopoietic barrier. The Sertoli cells support the development of germ cells by creating a suitable microenvironment in the adluminal compartment of the seminiferous epithelium; they also facilitate the migration of the differentiating germ cells to the tubular lumen [28, 110].

The Sertoli cell cytoskeleton consists of actin filaments, intermediate filaments, and microtubules. Actin filaments are composed of actin monomers and, through their binding to myosin, allow motions between the cells. The actin filaments are found predominantly as components of tubulobulbar complexes and ectoplasmic specializations. The tubulobulbar complexes are attached to the spermatids, on the apical surface of the germinal epithelium, and also between the Sertoli cells along the basolateral membrane. It is speculated that these complexes have a role in shaping the spermatid head. The ectoplasmic specializations are located in the apical region of the germinal epithelium in association with the spermatids, but are also found in the basolateral membrane between Sertoli cells and the basement membrane. They are responsible for the adhesion between Sertoli cells and between maturing spermatocytes and spermatids [69, 81]. Microtubules are tubular polymers composed of α and β tubulin, involved in the assembly and release of elongated spermatids. Their polarity allows the movement of spermatids and other substances toward the apical membrane [6].

Sertoli cells express androgenic and FSH receptors, allowing spermatogenesis to be sustained indirectly by functional stimulation [95]. They also express aromatase (CYP19), which converts testosterone derived from Leydig cells into 17-β-estradiol. Sertoli cells produce androgen-binding protein (ABP), which acts as the intracellular carrier of androgens, thus helping to maintain high androgen levels both in the adluminal compartment as well as in the lumen of seminiferous tubules. Furthermore, the Sertoli cells play an essential phagocytic role, eliminating residual bodies, which correspond to sperm cytoplasmic excess eliminated during spermiogenesis.

Inhibin B is a glycoprotein secreted by Sertoli cells. The secretion of inhibin B is controlled by gonadotropins, Sertoli cells, Leydig cells, and germ cells. Before puberty, Sertoli cells are the predominant cell type in the seminiferous tubules, whereas in the adult testis germ cells prevail. This pattern may be necessary for the maintenance of inhibin B secretion and consequent inhibition of FSH before the onset of puberty. Inhibin acts by negatively regulating the synthesis and release of FSH by the anterior pituitary. The expression and secretion of inhibin B correlate with Sertoli cell activity, sperm number, and spermatogenesis pattern and is inversely related to FSH levels [21, 71].

28.2.3 Hemato-testicular Barrier

The hemato-testicular barrier divides the seminiferous epithelium into basal and apical (adluminal) compartments. Spermiogenesis and spermiation occur in the apical compartment, while the spermatogonia renewal and the early stages of spermatogenesis (up to the prelopten stage of the spermatocyte) occur in the basal compartment [28]. The blood-testicular barrier has the function of blocking the passage of substances from the blood to male germ cells. It allows Sertoli cells to control the supply of nutrients to germ cells and to create an immunologically safe environment for spermatogenesis, thus avoiding an autoimmune reaction by defense cells against germline cells. The blood-testis barrier, owing to its continuous characteristics, facilitates the dynamic interaction between germ cells and Sertoli cells, as well as the migration of germ cells to the adluminal surface [110].

Sertoli cells adhere to germ cells to form a highly specialized epithelium, in which the various junctions (tight junctions and adherens junctions) between adjacent Sertoli cells allow creation of an immunological barrier within the seminiferous tubules. The immunological barrier seems to have three different levels, the first being formed by tight junctions between Sertoli cells. The other two levels are located in capillary epithelial cells and peritubular myocyte cells. The blood-testicular barrier is the only human immunological barrier in which tight junctions and adherens junctions are located contiguously and working together. The proper functioning of this barrier is crucial for the maintenance of male fertility [6].

28.2.4 Peritubular Microenvironment

Several layers of peritubular tissue and a complex capillary network provide adequate nutrition for the seminiferous tubules. Separating the interstitial tissue from the tubule, there is a first adventitious layer comprised of fibrocytes. The next layer consists of myoid cells intercalated by connective tissue, whereas the third layer is composed of a large amount of collagen adjacent to the basement membrane underlying

the seminiferous epithelium. Peritubular myoid cells have a contractile function and secrete various substances such as fibronectin and collagen type 1. The peritubular compartment also contains Leydig cells, which are steroidogenic stromal cells [94]. The peritubular cells are distributed concentrically in layers around the seminiferous tubules, separated by collagen fibers. These cells produce extracellular matrix, connective tissue proteins (collagen, laminin, vimentin, fibronectin), and proteins related to cellular contractility such as smooth muscle myosin and actin. The peritubular cells also synthetize adhesion molecules, such as nerve growth factor (NGF) and monocyte chemoattractant protein 1 (MCP-1) [93]. Secretion of the above-mentioned factors is regulated by tubular necrosis factor-α (TNF-α), which in turn is produced by mast cells; therefore, an interaction between peritubular and mast cells is suggested. It has also been shown that the number of mast cells increases in the testis in certain infertility cases [11]. Peritubular cells have contractility properties that aid in the transport of sperm through the seminiferous tubules. Peritubular contractility is regulated by oxytocin, prostaglandins, androgens, and endothelin [90, 107, 115]. Endothelin is, in turn, modulated by the relaxant peptide adrenomedullin produced by Sertoli cells [90]. Peritubular cells also secrete insulin-like growth factor-1 (IGF-1) and cytokines that modulate Sertoli cell function [107]. Due to the complex interactions between peritubular cells and other cellular elements, it has been suggested that these cells have a role in fertility. In fact, loss of contractility markers, tubular fibrosis and sclerosis, and an increased number of mast cells are seen in conditions involving derangements of spermatogenesis leading to subfertility [11, 52, 88]. Peritubular and interstitial fibrosis, in association with spermatogenic damage, have also been demonstrated in the testis of vasectomized men [87].

28.2.5 Steroidogenesis

Leydig cells are located in the interstitial space of the testis and are responsible for testosterone production, which is essential for proper spermatogenesis. The differentiation of Leydig cells is determined, at least in part, by peritubular and Sertoli cells, which secrete leukemia inhibitory factor (LIF), platelet-derived growth factor-α (PDGF-α), and other factors that trigger Leydig stem cells to proliferate and migrate into the interstitial compartment of the testis, where they differentiate into the so-called progenitor Leydig cells. Subsequently, growth factors and hormones (LH, IGF-1, PDGF-α, among others) transform the Leydig stem cells into immature Leydig cells and, finally, into adult Leydig cells, which is primarily responsible for the production and secretion of testosterone [100].

In adults, binding of LH to its receptors on Leydig cells activates cyclic AMP and a cascade of intracellular events, including increased gene transcription and steroidogenic enzymatic activity, which culminates with production of testosterone. LH binding to high-affinity receptors on the plasma membrane of Leydig cells results in hydrolysis of cholesterol esters, increased expression of steroidoenic genes, and expression of LDL and HDL receptors. LH also has a long-term trophic effect, which promotes the growth and proliferation of Leydig cells. The lack of stimulation by LH, in turn, results in a decrease in steroidogenic enzymatic activity and, as a consequence, cellular atrophy [39].

Testosterone is the main product secreted by the testis, with a daily production rate of 5–7 mg. It is estimated that the intratesticular content of testosterone is 100 times greater than that in the peripheral circulation. Reduced testosterone levels might result in low spermatogenic activity, leading to oligozoospermia or even azoospermia. Testosterone is transported into the bloodstream linked to albumin or sex hormone-binding globulin (SHBG). Only about 2% of total testosterone circulates freely in the bloodstream. Plasma testosterone levels are closely correlated with LH levels [91, 116].

The major substrate for androgen synthesis is cholesterol. The limiting point of the steroidogenesis rate is the transfer of cholesterol from external mitochondrial membrane to internal mitochondrial membrane, where bioconversion to pregnenolone by the cytochrome P450SCC (side-chain cleavage) enzyme occurs. This transfer depends on the synthesis of steroidogenesis regulatory protein (StAR) and mitochondrial translocator protein, synthesized after the binding of LH to its receptors on Leydig cell surface. Mitochondrial enzymes cytochrome P450SCC or CYP11A1 (cytochrome P450, family 11, subfamily A, polypeptide 1) transforms cholesterol into pregnenolone, a process limited by the availability of cholesterol substrate. In the so-called delta-4 pathway, pregnenolone is converted to progesterone by 3β-hydroxysteroid dehydrogenase, which in turn is converted to 17α-hydroxyprogesterone and androstenedione by 17α-hydroxylase or CYP17A; androstenedione is finally converted to testosterone by cytochrome P450c17 (Δ4 pathway: pregnenolone → progesterone → 17α-hydroxyprogesterone → androstenedione). In the delta-5 pathway, pregnenolone is hydroxylated to 17α-hydroxypregnenolone and dehydroepiandrosterone by 17α-hydroxylase or CYP17A, which in turn are converted to androstenediol by cytochrome P450c17; finally, androstenediol is transformed into testosterone by 3β-hydroxysteroid dehydrogenase (Δ5 pathway: pregnenolone → 17α-pregnenolone → dehydroepiandrosterone → 5-androstenediol). Testosterone can be converted to estradiol by aromatase or to dihydrotestosterone by 5α-reductase. LH stimulates the transcription of genes that encode the enzymes involved in the steroidogenic pathways to testosterone [22].

28.2.6 Spermatogenesis

Spermatogenesis is a highly efficient and coordinated process that results in the production of mature spermatozoa. The entire spermatogenic process is thought to require approximately 74 days, but recent studies suggest that the duration in normal men might vary between 42 and 76 days, taking approximately 64 days in humans. Every 16 days, a new group of spermatogonia enters the process of spermatogenesis [42, 77, 94].

Spermatogenesis is classically divided into three phases: (1) a proliferative or mitotic phase, in which the primitive germ cells/spermatogonia pass through a series of mitotic divisions, forming new stem cells or forming cells that will originate the spermatocytes; (2) meiotic phase, in which spermatocytes pass through two consecutive divisions to produe spermatids (haploid cells); and (3) spermiogenesis, in which spermatids differentiate into spermatozoa [5, 97].

Spermatogonia are specialized diploid cells located in the basal membrane of the seminiferous tubules. They are the precursors of all other types of germ cells and are present in the testicles throughout the lifespan. To maintain the supply of mature sperm, spermatogonia must perform three functions: (1) differentiation, (2) self-renewal, and (3) control [27]. From the least to the most differentiated, spermatogonia can be divided into (based on their heterochromatin contents) dark type A (Ad, dark) and pale type A (pallid) spermatogonia.

Ad-type spermatogonia have the ability to perform mitotic divisions, generating new Ad cells, which are considered reserve spermatogonia. Spermatogonia type Ap is located in the basal compartment of the seminiferous tubules and are the precursors of type B spermatogonia: the latter can differentiate into spermatocytes. During spermatogenesis, meiosis occurs sequentially and without interruption. The spermatogonia type B passes through the process of mitosis originating the primary spermatocyte (diploid cell), which is the most abundant cell of the spermatogenic lineage and that will undergo meiosis. This cell spends most of the time in prophase I, which is the first stage of meiosis I and where gene recombination occurs. At the end of meiosis I, the two daughter cells are called secondary spermatocytes, containing a haploid number of chromosomes with each chromosome containing two chromatids. A second division begins (meiosis II) and gives rise to four spermatids, with a haploid number of chromosomes with a single chromatid in each. The spermatids, still rounded, undergo a process of transformation and remodeling, known as spermiogenesis and spermiation, which result in the formation of spermatozoa, a tiny elongated cell in which it is possible to distinguish three regions: head, middle part, and tail [5, 14, 94] (Fig. 28.2).

Androgens play a crucial role in the initiation and maintenance of spermatogenesis [67, 92]. Through their binding to androgen receptors (ARs), they regulate the end of meiosis and the transition from spermatocytes to round spermatids

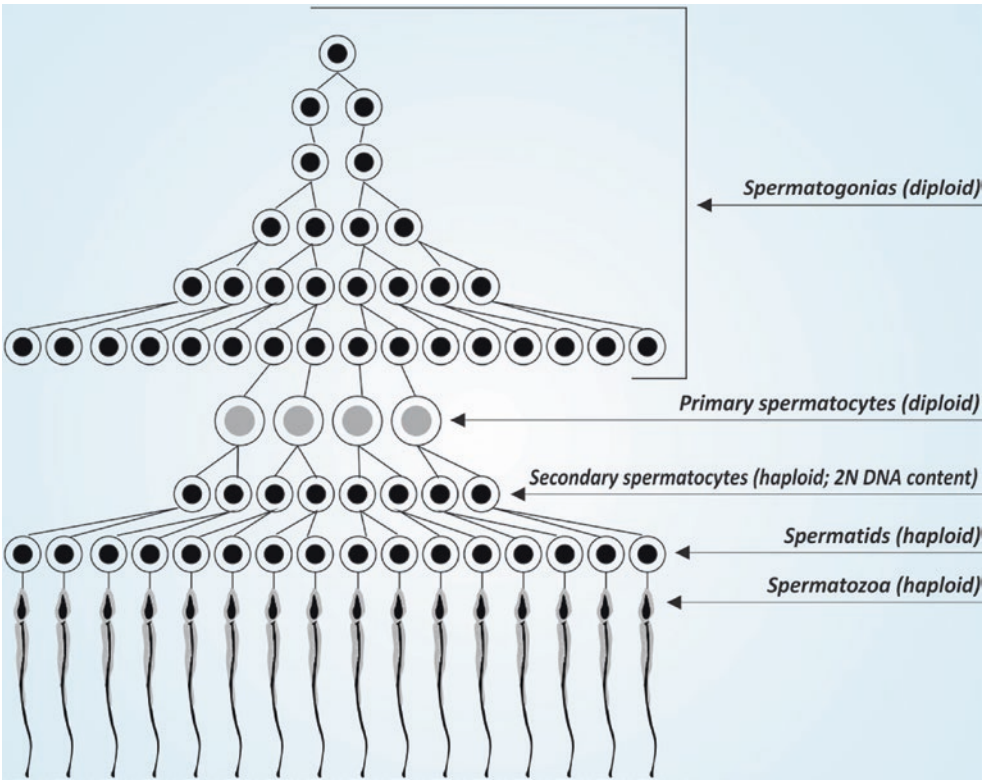

Fig. 28.2 Schematic representation of cell division stages during spermatogenesis. (Reprinted from Ref. [119] with permission from Elsevier)

[36]. The location of ARs in the epididymal tissue is of vital importance for the production of a physiological environment that allows sperm maturation. The androgenic action is mediated by aromatase, an enzyme that catalyzes the conversion of androgens to estrogens. Several growth factors and cytokines are also involved in this complex process of spermatogenesis [13, 23, 117].

28.2.7 Spermiogenesis and Spermiation

Spermiogenesis refers to the period in which the germ cell undergoes a process of metamorphosis acquiring various organelles and accessory structures, such as the acrosome and the flagellum. During this period, extensive changes occur in the cytoplasm and nucleus of spermatids [94]. The acrosome originates from the Golgi complex and consists mainly of hydrolytic enzymes, including proteases, hydroglycolases, and esterases, such as hyaluronidase, cathepsins, and acrosin [1]. These enzymes will be subsequently secreted to aid the passage of sperm through the barriers that protect the oocyte, thus aiding in fertilization. During spermiogenesis, cell volume decreases at the expense of cytoplasmic volume reduction due to the elimination of residual bodies containing Golgi complex remnants, ribosomes, mitochondria, and lipid droplets. Part of the residual bodies are phagocytosed by the Sertoli cells, and part is released into the lumen of the seminiferous tubule. The residual body should not be confused with the "cytoplasmic droplet," which consists of the excess of cytoplasm present in the mature spermatozoa and that will be released only in the lumen of the epididymis, thus maximizing sperm motility. The mitochondria migrate to the base of the future sperm mid-piece along with the microtubules derived from the centrioles, to constitute the mitochondrial sheath. The microtubules form the structure of the flagellum, which is critical for sperm motility. The accumulation of mitochondria is crucial for the energy supply necessary for flagellar movement [5, 14]. Early spermatids contain two centrioles arranged at right angles. The distal centriole that is oriented parallel to the long axis of the sperm cell originates the axial filament of the flagellum, denominated axoneme. The axoneme consists of microtubules organized in the classic 2 + 9 formations (2 central microtubules surrounded by a circular array of 9 microtubule pairs). The axoneme is formed early during spermiogenesis and can already be seen as a protrusion from the elongated spermatid [5]. The spermiation process refers to the release of germ cells into the lumen of seminiferous tubules as mature spermatozoa and removal of the last traces of cytoplasm (particles/cytoplasmic excess) [5, 14].

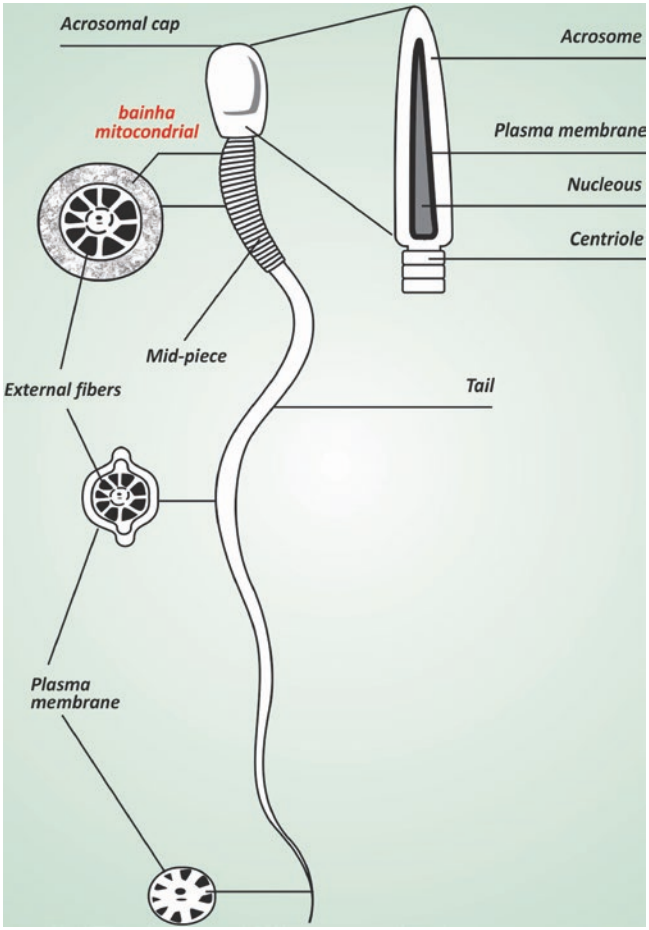

Fig. 28.3 Schematic representation of a mature human spermatozoon showing its components, the head, midpiece, and tail. (Reprinted from Ref. [119] with permission from Elsevier)

Spermatozoa are the end product of spermatogenesis. Each spermatozoon has a length of approximately 60 μm. The final structure of the spermatozoon includes three regions: head, mid-piece, and tail (flagellum). The oval head, approximately 4.5 μm long and 3 μm wide, is subdivided into an acrosome and post-acrosome region. Most of sperm head volume is occupied by the ovoid nucleus containing the highly compact chromatin. The acrosome results from the fusion of Golgi complex vesicles and contains hydrolytic enzymes necessary for sperm penetration through the outermost layers of the oocyte during the fertilization process. The mid-piece is a highly organized segment consisting of helically distributed mitochondria surrounded by dense fibers, being responsible for the supply of ATP for flagellar movement. The tail (flagellum) consists of a central axoneme, composed of microtubules surrounded by dense fibers that extend from the head to near the end region of the flagellum. The axoneme consists of 2 central microtubules surrounded by nine microtubule pairs, with the typical 9 + 2 configurations [5] (Fig. 28.3).

28.3 The Epididymis: Structure and Function

The epididymis is vital for sperm function, with the following roles, namely, maturation, transport, concentration, and storage [94]. Sperm that emerges from the testis are functionally immature. Maturation occurs during transit through the epididymis and female reproductive tract [40]. After release in the lumen of seminiferous tubules, spermatozoa, immersed in testicular fluid, migrate through the network of efferent ducts and reach the epididymis, a tubular organ composed of the head, body and tail regions [106]. The head of epididymis consists of 10–15 efferent ducts and the proximal segment of the epididymal duct. The lumen of the efferent ducts is broader and more irregular near the testis, becoming narrower and oval near the junction with the epididymal duct. The diameter increases as it advances through the other epididymal regions. Externally to the basal lamina of the efferent and epididymal ducts, there are contractile cells, which decrease in number and are replaced by smooth muscle cells in the tail of the epididymis. There are regional differences in epididymal portions concerning anatomy, innervation, vascularization of the tubules, and histology, suggesting that the epididymis is a succession of different tissues [94].

28.3.1 Sperm Maturation

As they progress through the various epididymal regions, spermatozoa undergo biochemical and molecular changes. Motility and fertilizing potential are obtained during this process, both of which increase considerably from the head to tail of the epididymis. Testicular spermatozoa are unable to fertilize the oocyte unless injected directly into its cytoplasm by intracytoplasmic sperm injection (ICSI) [106]. Sperm maturation depends on the secretory capacity of epithelial cells, which creates a unique microenvironment that induces biochemical changes in both sperm cytoplasm and membrane. These modifications enhance sperm motility, stabilize the chromatin, and allows acquisition of binding and fusion sites with the zona pellucida.

28.3.2 Sperm Transport

The transit of the spermatozoon through the epididymis takes 2–6 days. Therefore, the maturation process occurs rapidly compared to other species. Sperm transport through the epididymis relies on the hydrostatic pressure of testicular fluid and the rhythmic and spontaneous contractions of the contractile tissue surrounding the ducts [105]. After transiting through the head and body of the epididymis, the spermatozoa are stored in the tail. In humans, approximately 55–65% of epididymal sperm are found in the tail region [106]. During ejaculation, millions of sperm are expelled from the epididymis. The epididymal fluid containing sperm mix with secretions from the seminal vesicle and prostate, thus making the semen. Seminal fluid provides an adequate environment for short-term sperm survival after ejaculation [14].

28.4 Sperm Function

28.4.1 Hyperactivation

Hyperactivated spermatozoa exhibit an extremely vigorous but nonprogressive motility pattern, as a result of Ca^{2+} influx, which promotes increased flagellar curvature [70] and vigorous lateral movement of the sperm head [80]. Proteasome participates in activating calcium channels that also leads to increased membrane fluidity and permeability [58, 75, 78, 114]. These events are followed by or occur simultaneously with (i) a decrease in net surface charge, (ii) devoided area of intramembrane protein and sterols, and (iii) increased concentrations of anionic phospholipids [73, 114]. Hyperactivated motility is essential for sperm penetration into the intact oocyte-cumulus complexes both in vitro and in vivo [61, 112].

28.4.2 Capacitation

In vivo, ejaculated sperm are unable to fertilize until they undergo capacitation, which allows the acrosome reaction to take place when spermatozoa approach or contact the oocyte [46, 55, 61, 103, 111]. Capacitation is a time-dependent phenomenon, with the absolute time course being species-specific [79]. It prepares the sperm to undergo the acrosome reaction with the accompanying release of lytic enzymes and exposure of membrane receptors, which are required for sperm penetration through the zona pellucida and fusion with the oolema [79]. Sperm transport through the female genital tract can occur quite rapidly (times as short as 15–30 min have been reported in humans), whereas capacitation may take from 3 h to 24 h [79]. It is speculated; therefore, that capacitation is not completed until after the spermatozoa have entered the cumulus oophorus. This delay is physiologically beneficial because spermatozoa do not respond to acrosome reaction-inducing signals until they approach the zona pellucida, preventing premature acrosome reactions that lead ultimately to the sperm inability to penetrate the egg vestments [48, 79]. Sperm capacitation is a

post-ejaculatory modification of the sperm plasma membrane, which involves mobilization and/or removal of surface components, such as glycoproteins, decapacitation factor, acrosome-stabilizing factor, and acrosin inhibitor. Sperm capacitation involves major biochemical and biophysical changes in the membrane complex and energy metabolism. The presence of high concentrations of cholesterol in the seminal plasma, which maintains a high cholesterol concentration in sperm membranes, seems to be the most important factor for inhibiting capacitation [33]. Capacitation is associated with increased membrane fluidity caused by the removal of cholesterol from sperm plasma membrane via sterol acceptors present in the female tract secretions [66, 80].

28.4.3 Acrosome Reaction

The acrosome reaction is a stimulus-secretion coupled exocytotic event in which the acrosome fuses with the overlying plasma membrane [19, 113]. The multiple fusions between the outer acrosomal membrane and the plasma membrane result in the release of hydrolytic enzymes (mostly acrosin) and exposure of new membrane domains, both of which are essential for fertilization. The hydrolytic enzymes released from the acrosome digest the zona pellucida, allowing the spermatozoa to penetrate the oocyte [18]. The acrosome reaction (AR) seems to be physiologically induced by natural stimulants such as the follicular fluid (FF), progestin, progesterone, and hydroxyprogesterone [73]. Follicular fluid and cumulus cells have protein-bound progesterone that has been identified as one of the most important acrosome reaction-inducing agents [19, 102]. Follicular fluid stimulates the acrosome reaction in a dose-dependent manner [20, 102]. A link between locally produced estradiol by ejaculated spermatozoa, acrosome reaction and sperm capacitation has been described [12]. Moreover, evidence indicates that environmental estrogens can significantly stimulate mammalian sperm capacitation and acrosome reaction [2].

The acrosome reaction is probably initiated when ligands produced by the oocyte bind to the receptors on the spermatozoa. This signal is transduced intracellularly via second messengers, ultimately leading to exocytosis [15]. A number of second messenger pathways have been identified in human spermatozoa, including those that result in the activation of cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), and phospholipid dependent protein kinases [16, 37, 38, 114]. These kinases are called, respectively, protein kinase A, protein kinase G, and protein kinase C. It is possible that these pathways interact to assure an optimal response at the correct place and time during the fertilization process. Although the concentration of cGMP in ejaculated human semen is almost seven times lower than cAMP, it is speculated that both nucleotides have a similar role in the AR since their dependent protein kinases are closely related proteins [17, 51, 89]. The AR can also be induced by artificial stimulants that cause an increase in sperm intracellular calcium [43, 114].

28.4.4 Sperm-Zona Pellucida Binding and Penetration

The spermatozoon binds to the zona pellucida (ZP) with its intact plasma membrane after penetrating into the cumulus oophorus. Sperm binding occurs via specific receptors to ZP glycoproteins located over the anterior sperm head [18]. Glycosylation of ZP glycoproteins is important in sperm–ZP interaction. It is believed that human ZP glycoprotein-3 (ZP3) has a central role in initiating the acrosome reaction [63]; however, it has been recently demonstrated that human ZP1 and ZP4 are also implicated in the process [30, 49].

Acrosome integrity is crucial for normal fertilization. A high proportion of sperm with intact acrosomes is seen in ejaculates of normal men. In such individuals, ~5–20% sperm cells may exhibit spontaneous acrosome reactions that are of no clinical significance [43]. Conversely, several abnormal conditions affecting the sperm acrosome might lead to decreased fertilization ability. Acrosomeless round-headed spermatozoa (globozoospermic spermatozoa) are unable to fertilize the oocytes, and increased percentages of morphologically abnormal acrosomes were related to fertilization failure in assisted reproductive technology (ART) using conventional in vitro fertilization (IVF) [62].

The AR is a time-dependent phenomenon that cannot take place prematurely or too late [34]. Premature acrosome reaction and the inability of the spermatozoa to release the acrosomal contents in response to proper stimuli (acrosome reaction insufficiency) have been associated with idiopathic male infertility [101]. Although the cause of premature acrosome reaction is unknown, the premature (stimulus-independent) initiation of acrosomal exocytosis seems to be related to a perturbation of the plasma membrane stability. In this situation, the acrosome reaction might not involve a premature activation of the receptor-mediated process, but rather reflect an inherent fragility of the sperm membrane, leading to a receptor-independent acrosomal loss [44]. Antisperm antibodies (ASA) can adversely affect the ability of sperm to undergo capacitation and acrosome reaction [31]. Chang et al. [26] reported reduction in fertilization rate either by IgG directly bound to sperm or IgM present in female serum. The combination of IgG and IgA may have a synergistic negative effect on fertilization [35, 60, 74, 76].

Toxic substances to sperm can also impact the AR. High concentrations of dietary phytochemicals, such as genistein,

isoflavone, and β-lapachone, were shown to suppress the AR in a dose- and time-dependent manner in the rat model [33]. Inhibition of AR by genistein seems to involve the protein kinase C pathway, while β-lapachone has a direct cytotoxic effect on sperm cell membrane. It is suggested that genistein and β-lapachone may impact male fertility via AR suppression in high doses and AR induction in low doses [65]. Calcium channel blockers may also interfere with the AR exocytotic event. Sperm incubation with different blockers, such as trifluoperazine (calmodulin inhibitor), verapamil (Ca^{2+} channel inhibitor), and nifedipine (voltage-dependent Ca^{2+} channel inhibitor), significantly reduced the ability of hamster sperm to undergo the acrosome reaction [45].

Recent publications on acrosome reaction focused on the biochemical and functional aspects of sperm-oocyte fusion. It has been demonstrated that in humans, ZP1 in addition to ZP3 and ZP4 binds to capacitated spermatozoa and induces acrosomal exocytosis [30, 49, 53, 54]. The ZP3-induced acrosome reaction involves the activation of T-type voltage-operated calcium channels (VOCCs), whereas ZP1- and ZP4-induced ARs involve both T- and L-type VOCCs. Chiu et al. reported that glycodelin-A, a glycoprotein present in the female reproductive tract sensitizes spermatozoa to the zona pellucida-induced acrosome reaction in a glycosylation-specific mechanism involving the activation of the adenylyl cyclase/PKA pathway, suppression of extracellular signal-regulated kinase activation and up-regulation of zona pellucida-induced calcium influx. It is therefore suggested that glycodelin-A might be important in vivo to ensure full responsiveness of human spermatozoa to the zona pellucida [29].

28.4.5 Chromatin Condensation/ Decondensation and DNA Integrity

During spermatogenesis, significant morphological and functional changes occur in the nucleus of germ cells. In round spermatids, the nucleus is found in a central position, but as the spermatid lengthens, it moves to a more eccentric position and undergoes a significant condensation, reaching 10% of its initial size, thus favoring sperm hydrodynamics. The sperm chromatin is a highly organized and compact structure, consisting of DNA and heterogeneous nucleoproteins [3]. At this point, the chromatin is insoluble and condensed, which is essential to protect genetic integrity during transport of the paternal genome through the male and female reproductive tracts [41]. The compact sperm chromatin also ensures that the paternal DNA is delivered into the oocyte, enabling the fusion of two genomes and development of a genetically normal embryo [96, 109].

In round spermatids, the nuclear DNA is arranged around nucleosomes composed of histones as in other somatic cells. However, in mature spermatozoa, histones are replaced by protamines, which are small proteins rich in arginine. Protamination is responsible for both condensation of the sperm DNA and resistance to denaturation [5]. The role of protamines relates to condensation of spermatic nucleus transforming it into a more compact and hydrodynamic form, protection of the genetic code, maintenance and repair of sperm DNA integrity, and genetic imprinting [85].

The human sperm nucleus contains two types of protamine, namely, protamine 1 (P1) and protamine 2 (P2), expressed in a ratio of 1:1. It has been shown that changes in expression of P1 and P2 are associated with male infertility [10, 24], and that altered levels of protamine relates to an increased susceptibility to sperm DNA damage [25]. For a spermatozoon to be fertile, it must be capable of undergoing decondensation at an appropriate time during the fertilization process [9]. Thus, in sperm with normal chromatin structure, condensation and decondensation processes are essential for sperm fertilizing ability [41].

Protamination of sperm DNA is critical as it allows a compacted paternal genome to be transported through the female genital tract. Furthermore, DNA integrity is essential for the accurate transmission of paternal genetic information. A number of in vivo and in vitro studies supports the importance of DNA integrity in fertilization, early embryo development, implantation, and pregnancy outcomes [4, 98, 99]. Abnormal spermatogenesis, varicocele, inflammatory processes, and other conditions associated with excessive oxidative stress might cause sperm DNA integrity defects, leading to functional modifications that might impact reproductive outcomes negatively, both in a natural conception or even assisted conception [86].

28.5 Assessment of Spermatogenesis Kinetics In Vivo

28.5.1 The Past

The identification of the seminiferous epithelial cycle in mammals was among the most important discoveries in the field of spermatogenesis in the past century [32, 68]. The experimental approach used animals to determine the duration of the germinative cycle. It consisted in analysing the rate of disappearance of germ cells from the seminiferous tubules after testis irradiation. When administered in proper doses, X-rays destroy a large percentage of spermatogonia, which results in a progressive loss of spermatocytes and spermatids in the seminiferous tubules. Naturally, this method could not be applied to humans.

With the advent of radioactive tracers and the development of radioautography, it became possible to perform quantitative analyses of the tubular cross-sections containing

labeled cells. The method involved radiographic analysis of sequential biopsies after the testes have been injected with tracers. Tritiated thymidine, which is selectively incorporated into the nuclei of cells preparing for mitosis or meiosis, became the label of choice and was extensively used to time the cycle.

In 1963, a human study conducted by Heller and Clemont evaluated seven vasectomy candidates who received injections of tritiated thymidine, followed by serial testicular biopsies [56]. Based on this study, it was estimated at 16 days the transition from spermatogonia to spermatocytes. The total duration of the three phases of spermatogenesis combined, namely, (1) proliferation of spermatogonia to produce diploid spermatocytes; (2) meiotic division, which originates spermatids; and (3) cytological transformation, which leads to mature spermatozoa, was estimated at 64 days. The transit time through the epididymis was estimated at 5.5 days, and this estimation derived from previous animal studies with pigs [47]. The common knowledge that spermatogenesis would take approximately 3 months to complete comes from these data, which have been used for the past five decades to guide treatment of male infertility [47].

The aforementioned studies could never be repeated in humans, due to its toxicity and invasiveness. Nevertheless, their findings have helped investigators to understand different cellular events that occur during spermatogenesis. For instance, although a clear-cut cycle of the seminiferous epithelium was difficult to describe in humans due to an apparent mixing of germ cells, the analysis of serial sections in well-fixed biopsies revealed typical cell associations into six stages. A mixing of germ cells at the interface of adjacent cell associations was present, including a frequent absence of one or more germ cell generations. Of note, the number of germ cells at the same developmental stage was relatively small and occupied restricted tubular areas [32].

28.5.2 The Present

In 2006, Misell and colleagues described a nontoxic and noninvasive method for measuring spermatogenesis in vivo. The overall mean time to detection of labeled sperm in the ejaculate was 64 ± 8 days [77]. They performed the study using a stable isotope labeled with 70% enriched heavy water (^2H_2O) and analysed DNA isotopic enrichment in ejaculated sperm by gas chromatography/mass spectrometry (GC/MS). The authors characterized the kinetics of human spermatogenesis in vivo in a group of healthy men with normal sperm production [77]. The volunteers had a daily intake of ^2H_2O for 3 weeks in order to achieve and maintain a body water enrichment of approximately 1.5%. This level of body water enrichment has been previously shown to allow adequate label incorporation for subsequent analysis of DNA synthesis [72, 83, 84]. In their experiment, a total of 11 healthy men with normal sperm concentrations ingested deuterated (heavy) water (^2H_2O) and semen samples were collected every 2 weeks for up to 90 days. Label incorporation into sperm DNA was quantified by gas chromatography/mass spectrometry, allowing the calculation of the percentage of new cells in ejaculates.

The aforementioned study was the first noninvasive direct kinematic measurement of human spermatogenesis in vivo. In contrast to the study of Heller and Clermont, in which spermatogenesis was estimated to take 64 days excluding epididymal transit time, Misell and colleagues showed that spermatogenesis duration was shorter. The authors showed that the appearance of new sperm in ejaculates of normal men occurred at a mean of 64 days, but this value included epididymal transit time.

The time to new sperm in the ejaculates varied from 42 to 76 days, as observed by Misell and colleagues, which denotes a significant interindividual variation, thus contradicting the current belief that spermatogenesis duration is fixed among individuals. While in one subject the time lag to detect greater than 33% of new sperm was 42 days, in all others it took at least 60 days. All subjects achieved greater than 70% new sperm in the ejaculate by day 90, but plateau labeling was not attained in most men, thus suggesting rapid washout of old sperm in the epididymal reservoir. Although these findings can be related to a variation in spermatogenesis per se, it could also be possible that epididymal transit time influenced the results, since a separate analysis of spermatogenesis and epididymal transit duration was not possible using their methods. Indeed, it has been speculated that epididymal transit time varies due to the rate of passage through the epididymis cauda, which is, in turn, influenced by ejaculatory frequency [8]. Furthermore, it has been suggested that men with high testicular sperm production have shorter epididymal transit time than men with lower testicular sperm output [59]. This difference might be explained by a direct association between the production of sperm and fluid, since testes that produce more sperm also produce more fluid, so the movement of spermatozoa along the epididymal duct could be more rapid.

Misell and colleagues data also suggest that in normal men sperm released from the seminiferous epithelium enter the epididymis in a coordinated manner with little mixing of old and new sperm before subsequent ejaculation. It is a novel concept since it had been suggested that because of mixing, in any segment of the epididymal duct, the population of sperm would be heterogeneous in age and biological status. Their kinetic data showed a sharp increase and a subsequent steep decrease in sperm enrichment in men with complete labeling curves, thus indicating that the sperm age is not heterogeneous. If significant mixing of young and old sperm had occurred, the slope of the labeling curve would

have been far more gradual. These kinetic data suggest that the epididymal reservoir is purged of old sperm fairly rapidly and completely in normal men.

28.6 Conclusions

Spermatogenesis is a highly organized and complex sequence of differentiation events that generates genetically distinct male gametes for fertilization. Sperm production is a continuous process, initiated at puberty and continuing throughout life, which occurs in the seminiferous tubules within an immune privileged site. Spermatozoa released from the seminiferous tubules into the epididymis undergo post-testicular maturation. Before fertilization can occur, spermatozoa must undergo further biochemical changes via capacitation and acrosome reaction, both of which occur after ejaculation. Recent knowledge that originated from a novel direct measurement of human spermatogenesis kinetics in vivo indicates that the entire sperm production process is shorter than previously believed. Moreover, there is large individual biological variability concerning the duration of spermatogenesis. Both spermatogenesis and steroidogenesis depend on the proper functioning of the hypothalamic-pituitary-gonadal axis which is regulated through the release of GnRH. Any event that affect spermatogenesis and steroidogenesis might impact sperm quantity and/or quality, with a potential adverse effect on fertility.

28.7 Review Criteria

A thorough search of medical literature was conducted with the MEDLINE, EMBASE, Science Direct, and Scielo databases until January 2019. We used relevant terms, related to "sperm physiology," "spermatogenesis," "steroidogenesis," and "sperm kinetics."

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Origins of Sperm DNA Damage

29

Ralf Henkel and Kristian Leisegang

Key Points

- Male inf ertility is a major issue of concern as it affects more people during their lifetime as a common disease, diabetes mellitus.
- Among these infertile men, the prevalence of sperm DNA damage is up to 80%.
- DNA damage modifications include (i) the chemical change of bases as 8-oxo-2′-doxyguanosine (8-OHdG), (ii) a single-strand break, (iii) double-strand break, (iv) a base missing from the DNA backbone, (v) modifications in purine, pyrimidine, and deoxyribose, (vi) introduction of abasic sites, and (vii) DNA cross-linking.
- Causes of sperm DNA damage are chromatin remodeling, oxidative stress, varicocele, scrotal hyperthermia, or poor lifestyle habits.
- Medication and aging also contribute to sperm DNA damage.

29.1 Introduction

Globally, an estimate of 7% of the males at reproductive age are infertile [1], amounting to an overall contribution of about 50% of all cases of couple infertility [2]. Clinical studies show that oxidative stress is the cause of the male infertility in about 40% of unselected cases and in up to 96% in patients with spinal cord injuries [3–5]. Oxidative stress, in turn, has been widely accepted as a major cause of male infertility and sperm DNA damage [6].

Although standard semen analysis comprising of the analysis of sperm concentration, motility, viability, and normal sperm morphology is still the cornerstone of andrological diagnostics, it has been proven to be very limited in its clinical value [7, 8], as it does not detect the male germ cells' functional capabilities. Consequently, in about 20% of infertile men, abnormalities in standard sperm parameters are not detected [9] and resulting in high numbers of idiopathic infertile men [10]. Therefore, it is crucial to identify and validate reliable, evidence-based new male fertility markers with high predictive value in order to improve andrological diagnosis [11]. One of these promising tests is sperm DNA fragmentation/damage, which is, despite some debate and negative results [12–15], currently advocated as it is a more stable parameter with lower biological variability compared to sperm count or motility [16]. It appears that concise and strict guidelines are necessary in order to establish sperm DNA fragmentation/damage as a robust clinical test [17].

29.2 What Is Deoxyribonucleic Acid Damage?

While a mutation is a change in the sequence of standard base pairs, DNA damage is defined as the occurrence of an abnormal chemical structure of the DNA and causes structural changes in the DNA that prevents replication mechanisms from functioning and performing properly [18]. Sperm DNA damage is the consequence of modifications of the molecular structure of the DNA [19]. These modifications include (i) the chemical change of bases as 8-oxo-2′-doxyguanosine (8-OHdG), (ii) a single-strand break, (iii) double-strand break, (iv) a base missing from the DNA backbone, (v) modifications in purine, pyrimidine, and deoxyribose, (vi) introduction of abasic sites, and (vii) DNA

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cross-linking [20, 21]. As a consequence, gene transcription can be either blocked or induced, final transduction pathways can be induced, errors in DNA replication can occur, attrition of telomeric DNA can increase, and the genome can become unstable [22–24].

There are two main types of DNA damage, namely, endogenous DNA damage caused by assaults of reactive oxygen species (ROS) and exogenous damage caused by radiation, thermal disruption, certain toxins, or mutagenic substances. These can be further classified into subgroups according to the nature of the endogenous cellular processes involved and according to the type of exogenous agent (UVA light, UVB light, ionizing radiation, etc.). The extent of natural and environmentally caused DNA damage occurring per day has been reported between 10,000 and 1,000,000 lesions per cell on a daily basis [25]. Although in spermatozoa repair mechanisms for DNA damage are highly effective, these are only functional during mitosis and meiosis before the last 3 weeks of spermatogenesis. Since the male germ cell has no repair mechanisms from this point in time, sperm are highly susceptible to DNA damage during spermiogenesis, the process in which haploid round spermatids differentiate and morphologically transform into elongated, highly polarized spermatozoa. During this process, the whole chromatin is remodeled from a voluminous toroid structure with histones as nuclear proteins to an extremely condensed and compacted chromatin organization where the negative charges of the DNA are neutralized by basic protamines to allow fitting the entire genome into the small space of about 5 × 2.5 μm of a human sperm head. Sperm with defective DNA can therefore fertilize oocytes [26, 27] and the repair of the sperm DNA damage would then be left to the ability of the oocyte to repair at least some of these damages [28]. If repair is not possible, this can lead to pregnancy failure, malformations, or early childhood cancer [29].

29.3 Spermatogenesis and Chromatin Packaging

Spermatogenesis is the process in which male germ cells are produced in the testes. This process can be divided into three steps, the proliferative, maturation, and differentiation. The latter step is also called spermiogenesis, which, in turn, is divided into four phases, Golgi phase, Cap phase, acrosome phase, and maturation phase. Spermiogenesis is a highly complex process, during which sperm DNA is undergoing major multistep changes including an exchange of histones as nuclear proteins by so-called transition proteins and finally by protamines resulting in a dramatic compaction of the male genetic material. This process is also characterized by a transform of the male germ cells from round cells into elongated ciliated spermatozoa [30].

29.4 Mechanisms of DNA Damage

There are three main theories explaining the origins of sperm DNA damage, namely, (i) reactive oxygen species (ROS) causing oxidative stress, (ii) sperm chromatin packaging, and (iii) apoptosis.

29.4.1 Reactive Oxygen Species

Reactive oxygen species (ROS) are highly reactive oxygen derivatives with half-life times in the nano- to milli-second range causing oxidative stress. Oxidative stress can be caused by endogenous and exogenous factors such as smoking, alcohol abuse, radiation, or environmental toxicants. As most important endogenous factors, male genital tract infection/inflammations, obesity, and diabetes mellitus are to be mentioned. Like any other somatic cell, spermatozoa also produce their own ROS as a result of electron leakage from the mitochondrial electron transfer chain. In cases where leakage exceeds the normal physiological levels, elevated levels of superoxide are produced which are dismutated into hydrogen peroxide leading to membrane damage through lipid peroxidation which triggers even more damage to mitochondrial membrane causing even more ROS leakage. Eventually, this will be resulting in a vicious cycle [31] with the sperm cell entering the intrinsic apoptotic pathway with externalization of phosphatidylserine as another early marker of apoptosis and eventually sperm nuclear DNA damage as a late sign of apoptosis [32].

ROS from any source, intrinsic from the sperm cells themselves or from leukocytes, initiate lipid peroxidation (LPO) of membrane lipids, not only in mitochondrial membranes but also in all other cell membranes. LPO is characterized as a radical chain reaction by which polyunsaturated fatty acids present in the membranes will be oxidized and a variety of lipid metabolites including lipid peroxyl radicals, alkoxyl radicals, malondialdehyde, 4-hydroxynonenal (4HNE), and acrolein are produced [33, 34]. 4HNE can bind to mitochondrial proteins and trigger the generation of ROS, thereby forcing the sperm cell to enter the vicious cycle of mitochondrial membrane damage and apoptosis. This process is eventually leading to oxidative DNA adduct formation, DNA strand breakage, and cell death [35]. These aldehydes are also powerful oxidants themselves that can exacerbate mitochondrial electron leakage with more production of ROS and can thereby not only cause damage to the sperm DNA but also to other sperm functions.

29.4.2 Sperm Chromatin Packaging

This transformation of the sperm nucleus with the packaging of the chromatin is a critical process to reduce the size of the nucleus from the normal somatic size to a small size of the spermatozoon. During this process, about 90–95% of the histones are eventually replaced by protamines [36]. In order to replace histones by protamines, the DNA nicks are created to provide relief of torsional stress, thereby facilitating histone disassembly and aiding chromatin rearrangements [37]. These nicks disappear completely at the time when chromatin packaging is completed [37–40]. McPherson and Longo [37] hypothesized that chromatin packaging required an endogenous nuclease, topoisomerase II, to create and ligate nicks in order to facilitate protamination [41]. In accordance with this hypothesis, it has recently been shown that topoisomerase II plays a major role in linking DNA replication to chromosome condensation and that it interplays with a large protein complex, i.e., condensation, which has key functions in mitotic chromosome assembly and organization [42, 43]. In addition, it has also been shown that topoisomerase II is present in the human seminiferous tubules [44] which are the location of spermiogenesis. This enzyme is also mainly involved in the DNA repair of elongating spermatids [45]. Furthermore, poly(ADP-ribose) polymerase inhibits topoisomerase II. This polymerase is activated as a result of DNA strand breakages [46].

The DNA remodeling that occurs during spermatogenesis is unique in that it produces a cell type in which the nucleus is transcriptionally inactive and a large part of the cell is stripped off. It is therefore not surprising that the human ejaculate normally contains a heterogenous population of spermatozoa that possess a variety of abnormalities at the nuclear, cytoskeletal, and organelle levels. In the human, it is well known that the chromatin of the mature sperm nucleus can be abnormally packaged [47]; furthermore, there is a strong relation between abnormal chromatin packaging and sperm nuclear DNA damage [48–51], and there is a strong association between the presence of nuclear DNA damage in the mature spermatozoa of men and poor semen parameters [38, 52]. This process of histone replacement is facilitated by hyper-acylation of histone tails loosens the chromatin structure causing DNA strand breaks and making the DNA more susceptible to damage [53, 54]. In turn, once this process is finished and stabilizing intra- and inter-molecular disulfide bridges in the protamine molecules are formed during the epididymal passage [55, 56], this high rate of cross-linking affords sperm DNA with a high amount of protection against assaults and compensates for missing DNA repair mechanisms in sperm [57]. Changes in the DNA repair mechanisms can have serious consequences for the genomic integrity of the male germ cells.

29.4.3 Apoptosis

The process of apoptosis is also known as programed cell death. In vertebrates, two apoptotic pathways have been proposed, namely, an intrinsic, mitochondrial pathway and an extrinsic, death receptor-mediated pathway [58, 59], triggered by Fas and other members of the TNF family activating the initiator caspase-8 to trigger executing caspase-3. In the intrinsic pathway, mitochondria are playing a major role with many stimuli involved integrating the death signals via members of the BCL-2 family and caspase activation with subsequent release of cytochrome c [60]. In mammalian sperm, however, phosphatidylinositol 3-kinase (PI3K) normally prevents the cell from entering this pathway, and sperm can only initiate the intrinsic pathway, if PI3K is inhibited. Initiation of this pathway will eventually lead to elevated mitochondrial ROS production, the cell undergoing apoptotic changes, and oxidative DNA damage [61].

Aitken and coworkers [21] pointed out that in contrast to somatic cells, spermatozoa are highly compartmentalized by their structural differentiation into head, midpiece, and tail. In addition, sperm have only one enzyme, 8-oxoguanine DNA glycosylase, for the base excision (BER) pathway available located in nucleus and mitochondria, hence shortening this pathway as compared to somatic cells. Since downstream factors such as apurinic endonuclease-1 are missing, abasic sites at the DNA that have already been affected by 8-hydroxy-2′-deoxyguanosine are formed, this is resulting in DNA strand breaks. Such DNA base changes are mutagenic and cause DNA damage [62].

29.5 Etiological Factors of DNA Damage

Apart from exogenous factors such as radiation, environmental toxins such as pesticides or other chemical exposure, there are numerous endogenous factors that can cause sperm DNA damage (Fig. 29.1). Among them are heat exposure, genital tract infections/inflammations, poor nutrition, obesity, and diabetes mellitus the most common factors.

29.5.1 Varicocele and Increased Testicular Heat

Varicocele is an abnormal tortuous enlargement of the pampiniform venous plexus in the scrotum. These veins drain blood from the testes back into the body. Considering that, together with the testicular, deferential, and cremasteric arteries, the pampiniform venous plexus forms a countercurrent mechanism that cools down the blood coming from the

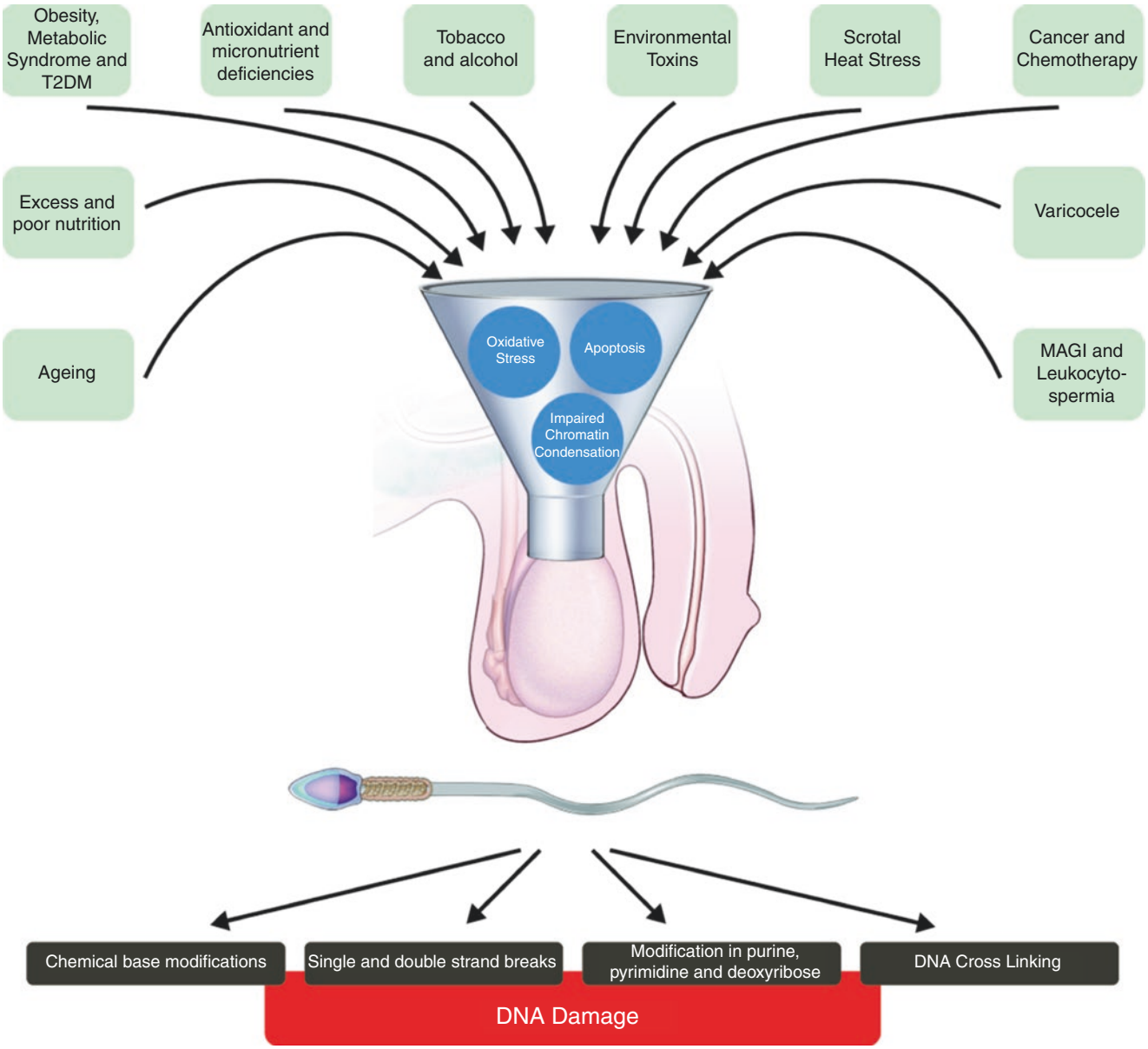

Fig. 29.1 Etiological factors of sperm DNA damage: Sperm DNA damage can be caused by numerous endogenous and exogenous factors such as aging, poor lifestyle, environmental toxins, infections, or some medical treatments. As a direct result, testicular and/or seminal oxidative stress is induced which will lead to apoptosis and impaired chromatin condensations. Apart from induction of lipid peroxidation and direct damages to proteins, oxidative stress also initiates various forms of DNA damage including base modifications, DNA strand breaks, modifications in purine and pyrimidine, as well as DNA cross-linking. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2019. All Rights Reserved)

aorta from body core temperature of 37 °C to about 35 °C. Globally, varicocele represents the most common cause of primary and secondary male infertility with an estimated 35–40% and up to 80%, respectively [63, 64]. Varicocele affects male fertility via several pathophysiological mechanisms as it can impair spermatogenesis significantly by elevating scrotal temperature, causing hormonal disturbances, testicular hypoperfusion, hypoxia, a reflux of toxic metabolites, or an accumulation of cadmium [65]. Heat stress causes significant increases in mitochondrial, cytoplasmic, and plasma membrane ROS production [66, 67].

The most often discussed pathophysiological mechanisms of varicocele are oxidative stress and testicular heat stress, and oxidative stress seems to be associated with all mentioned pathophysiological mechanisms [65, 68]. Although the presence of elevated seminal ROS levels is quite common with a reported prevalence between 25% and 40% in unselected cases of male infertility [3, 4] and up to 96% in patients with

spinal cord injuries [5], varicocele exacerbates high ROS levels and oxidative stress [69]. The latter association is closely related to the size of the varicocele with higher levels of oxidative stress in patients with larger varicocele and is evident not only by increased levels of ROS but also by increased levels of markers of sperm DNA damage [70–72].

Scrotal hyperthermia, caused by either varicocele or other sources of heat stress, such as hot bathes, welding, sedentary positions, or tight underwear, is negatively affecting Leydig cell and Sertoli cell function with significantly lower intratesticular testosterone concentrations and lower androgen-binding protein activity, respectively, in animals with varicocele and therefore higher testicular temperature [73, 74]. In contrast to Leydig and Sertoli cells and spermatogonia A, spermatogonia B, pachytene spermatocytes, and early spermatids are considered even more thermosensitive as these cells have not been exposed to higher temperatures in the uterus [75, 76]. Thus, scrotal heat stress is affecting the whole process of spermatogenesis leading eventually to high levels of sperm DNA damage [77]. Scrotal cooling as a therapeutic option has been shown to improve sperm motility and morphology [78].

29.5.2 Genital Tract Infection and Inflammation

According to Nieschlag [79], male genital tract infections and inflammations are the third single most common cause of male infertility, following idiopathic male infertility and varicocele. Other authors indicate a prevalence ranging from 6% to 15% in andrological outpatient clinics [80] or even up to 30.1% [81]. Considering that infections are mostly caused by bacteria, the infection itself is potentially treatable with antibiotics and the inflammation with anti-inflammatories to relieve obstruction of the excurrent genital ducts as a consequence of the infection [82]. A problem with which clinicians are confronted is that male genital tract infections are often asymptomatic [83, 84]. In addition, men do not readily consult a doctor. Hence, the diagnosis and treatment are often coming late.

Urogenital infections trigger leukocyte infiltration of the infected organ with a subsequent release of high amounts of ROS and pro-inflammatory cytokines such as IL-6, IL-8, or tumor necrosis factor alpha [85, 86]. In turn, these cytokines directly and indirectly exert their detrimental effects on sperm functions and sperm DNA integrity through oxidative stress and lipid peroxidation [87–89].

29.5.3 Poor Nutritional Intake

Poor nutritional intake affects the quality of semen through spermatogenesis [90–92]. Food beneficial for male fertility includes increased antioxidant fruits and vegetables, seafood (especially omega 3 polyunsaturated fatty acids), whole grains, nuts, seeds, vegetable oils, and micronutrients (e.g., β-carotenes, folate, vitamin C, vitamin E, selenium, and zinc) [91, 92]. This can be achieved through adherence to the Mediterranean or similar "prudent" diets, which improve fertility parameters in men across a BMI range [93–95]. Modern Western obesogenic diets have been correlated with poor semen quality and reproductive outcomes in males. This is represented by high-energy and nutrient-poor food and drinks, including increased refined sugars, trans-fatty acids, red meat, processed foods, soy and alcohol, and reduced fiber, fruits, vegetables, polyunsaturated fatty acids, micronutrients, and antioxidants [90]. These nutritional exposures lead to OS and increased spermatozoa DNA fragmentation index compared to males with favorable diets. Epigenetic modification is further demonstrated in spermatozoa and transferred to the offspring, although causality is not demonstrated [96–98] increasing transgenerational disease risk, including metabolic disorders and cancer [98]. Animal models demonstrate that high-fat diets, low-protein diets, reduced folate, and exposure to pesticides and herbicides cause DNA damage, are mutagenic, and modulate DNA methylation, chromatin remodeling and small coding RNA functions [97].

Excess macronutrient intake, including fats, carbohydrates, and proteins, contribute to systemic ROS. Increased energy consumption increases OS further through increased NADPH oxidase activation and reduced endogenous antioxidant defenses [99, 100]. This results in inefficient germ cell metabolism and impaired spermatogenesis with impaired mitochondrial function and DNA integrity [101, 102].

Westernized overnutrition of energy dense foods conversely lead to micronutrient deficiencies that increase reproductive tract OS and have a detrimental effect on sperm, including DNA fragmentation [103]. Many important nutrients for spermatogenesis and genomic stability include carotenoids, vitamin C, folate, vitamin E, zinc, selenium, copper, glutathione, α-lipoic acid, N-acetylcysteine, and coenzyme Q10 [104]. These can be acquired through adequate nutritional sources or over-the-counter supplements, which are associated with improved semen quality [96]. Particularly, vitamins A, C, and E have been found to improve DNA stability in spermatozoa [105, 106]. Conversely, over-supplementation with carotenoids and ascorbic acid can result in DNA damage and DNA adduct formation [107]. Uncontrolled over-supplementation with antioxidants may result in reductive stress which is regarded as dangerous as oxidative stress [108]. Furthermore, excessive antioxidants may result in the antioxidant paradox, inhibiting redox-regulated functions such as chromatin condensation, acrosome reaction, capacitation, and oocyte binding [109].

Macronutrient undernutrition also remains a significant public health concern, and underweight males are associated

with subfertility and poor semen parameters [110]. Chronic undernutrition and protein energy malnutrition (PEM) cause increased OS in the reproductive tract [111] and also negatively affect embryonic development causing poor testicular structure and infertility as an adult [112]. Within this context, appropriate caloric restriction that does not result in micronutrient deficiency (MND) reduces systemic OS and chronic disease risk and increases the life span [113]. This is in part mediated by increasing DNA repair mechanisms reducing inflammation and OS markers. This has a positive effect on DNA integrity, reducing cellular apoptosis [113]. However, the impact on male reproduction of appropriate dietary restriction (DR) is not yet elicited [114, 115].

29.5.4 Obesity and Metabolic Syndrome

Obesity is described as an excess accumulation of visceral adiposity, and clinically defined by a body mass index (BMI) > 30 [116]. Increasing BMI is associated with increased morbidity and mortality through numerous metabolic, endocrine, and immune mechanisms [116]. Metabolic syndrome (MetS) further describes a clustering of five obesity phenotypic comorbidities: increased waist circumference, hypertension, increased triglycerides and glucose, and/or reduced HDL-cholesterol. Any three of these five parameters diagnostically define MetS [117]. Both obesity and Mets have significantly increased in recent decades, alongside an increasing obesogenic environment and concurrent decline in semen parameters [116, 118]. Well-described complications of obesity and MetS include cardiovascular disease (CVD), type 2 diabetes mellitus (T2DM), neurodegeneration, malignancies (including colorectal and prostate), nonalcoholic fatty liver disease, and obstructive sleep apnea [116, 117]. Furthermore, in males, hypogonadism, erectile dysfunction, and poor semen quality have been reported [119].

The pathophysiology and comorbidities are due to underlying mechanisms including chronic systemic inflammation, OS, hyperinsulinemia, adipose tissue dysfunction and altered adipokine secretion, and hyperestrogenemia and hypogonadotropic hypogonadism. Testicular inflammation and ROS have been demonstrated to impair testicular spermatogenesis and steroidogenesis and epididymal sperm maturation and cause DNA fragmentation. This is further transmitted to the offspring through epigenetic modification, although this is variable [118, 120]. Furthermore, impaired fertility in males has emerged as a predictor of metabolic comorbidities commonly mediated by OS, inflammation, and hypogonadism [121].

Obesity is correlated with sperm parameter defects, including damaged DNA in spermatozoa [120]. Within this context, animal and human studies directly associate increased adiposity with reproductive tract OS and resulting alterations in sperm DNA. Altered OS genes in testicular tissues include antioxidant defenses such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase, and Nrf2 [122–124]. Increased paternal adiposity therefore has negative impact on DNA integrity alongside epigenetic modulation [116]. Studies consistently report an association with excess adiposity and DNA damage in numerous tissues, including hypothalamus, skeletal muscle, liver, pancreas, prostate, and testes [116, 125]. Adiposity-induced sperm DNA damage and epigenetic modification increase risk for reduced live birth rates and ART success, recurrent pregnancy loss, impairments in embryo development, and health effects on the offspring [116]. Obesity alters the environment for spermatogenesis, resulting in poor sperm quality, altered membrane lipids, and DNA fragmentation mediated particularly through OS and inflammation [120]. Similarly, MetS patients have increased DNA fragmentation in sperm, demonstrated in animal and human studies [119, 126–129]. However, the overall understanding of the impact and mechanism of obesity on DNA damage remains relatively poor.

Epigenetic programming resets methylation marks in DNA, regulating monoallelic gene expressions and establishing paternal-specific methylation in normal development. Sperm DNA methylation and noncoding RNA modification are proposed to transfer metabolic and health derangements to the fetus and offspring through paternal obesity, which is emerging as a further public health concern [120, 130, 131]. Sperm genes that have been identified to undergo inappropriate differentially methylated regions in paternal obesity include MEG3, NDN, SNRPN, and SGCE/PEG10. These are involved in embryonic and fetal development and tumor growth [130]. Current evidence further suggests that weight loss prior to fertilization may prevent some of the negative effects of obesity and MetS on the offspring [116]. However, the quality of human studies is generally low, with numerous confounding factors relevant to environment, nutrition, and lifestyle that require further investigation [132].

29.5.5 Diabetes Mellitus

Diabetes mellitus (DM) describes chronic hyperglycemia due to insulin dysregulation, resulting in impaired carbohydrate, protein, and fat metabolism among numerous other dysfunctions [133]. Type 1 (T1DM) is characterized by hypoinsulinemia due to a chronic autoimmune response in β-cells of the pancreas [134]. Type 2 (T2DM) is characterized by chronic hyperinsulinemia due to unfavorable lifestyle factors and environmental exposures related to obesity and metabolic syndrome [117].

There is a significant increase in DM in recent years, particularly T2DM, including younger men of reproductive age

and an alarming increase in adolescents and even children [135]. As glucose and insulin are important in spermatogenesis and steroidogenesis, DM negatively affects reproductive and sexual functions and is an established cause of reduced semen parameters (particularly sperm concentration, motility, normal morphology), DNA fragmentation, acrosome reaction, and increased apoptosis in humans and animals [136]. DM causes a negative alteration in spermatogenesis and testicular tissue structure on histology, particularly in the epididymis [137, 138]. T1DM more specifically reduced ejaculate volume due to epididymal voiding and reduced motility due to mitochondrial damage. T2DM is associated with inflammation and ROS generation, negatively affecting motility and DNA fragmentation [137]. Mechanisms of DM-induced testicular dysfunction include endocrinopathy, inflammation, glycation, neuropathy, and OS [138].

Evidence strongly demonstrates significant DNA damage in DM, including genomic and mitochondrial DNA, mediated by increased OS [136–140]. Importantly, DM also results in epigenetic modification during spermatogenesis, and these are inherited by the offspring [136]. T2DM has been more strongly associated with OS-induced sperm DNA damage, complicated by metabolic comorbidities. This increases testicular ROS generation and reduces endogenous and exogenous antioxidants, alongside increased enzymatic glycation end products, sperm lipid peroxidation, mitochondrial dysfunction, and DNA fragmentation [137]. In T2DM, OS has been suggested to impair male fertility, targeted potentially at the epididymis, resulting in greater percentage of spermatozoa with impaired MMP, DNA fragmentation, and in late apoptosis [137]. Epigenetic modification through impaired DNA methylation and histone modifications has also been demonstrated with significant impact on embryo development and offspring health [136]. Importantly, significant investigation into the mechanisms and potential novel therapeutic targets are required in clinical and preclinical studies.

29.5.6 Alcohol and Tobacco

Tobacco continues to be socially acceptable, affecting up to 30% of males globally [141]. Although the negative effects of these practices are well defined, the negative impact on reproduction is generally underappreciated [142]. It is difficult to do experimental studies in humans due to ethical considerations, and therefore this evidence relies on observation data, retrospective data, and preclinical studies [142]. Exposure to numerous chemicals through tobacco consumption clearly reduces male fertility parameters from available literature [143–146], up to 22% decline in standard semen parameters compared to nonsmokers [141]. These mechanisms are unclear and involve ROS, mitochondrial, and chromatin damage, inhibition of acrosome reaction and capacitation, spermatozoa morphological alterations, and smoking-induced hypoxia [142, 147, 148]. Tobacco is established to cause DNA damage in numerous tissues, including spermatozoa DNA and chromatin condensation alongside aberrant DNA methylation [148–153]. This appears to be strongly mediated through increased ROS and reduced endogenous antioxidants [148].

Chronic and heavy alcohol consumption is known to impair spermatogenesis, negatively affect semen parameters and reduce testosterone synthesis through reduction in testicular size and Leydig cell numbers [154–157]. As with tobacco smoking, heavy alcohol use also appears to increase seminal leukocyte concentration, potentially mediating poor fertility [158]. However, the effect of moderate alcohol consumption on male reproduction appears minimal, with only ejaculate volume seemingly affected, and acute alcohol intake appears to have minimal impact compared to chronic consumption [156, 159, 160]. In fact, some studies suggest an improvement in reproductive parameters and even ART outcomes with moderate consumption [160]. Semen parameters appear to be negatively affected above 20 or 25 units per week [161–163]. Several studies have indicated that the alcohol may induce spermatozoa DNA damage and apoptosis indices associated apoptosis in spermatozoa and Leydig cells [148, 163–166]. This is mediated through increased ROS and reduced endogenous antioxidant mechanisms [148]. However, these findings have not been consistent [167, 168]. Ethanol-induced DNA damage appears to upregulate Fas system, alongside increased p53 and caspase activation, cytochrome c translocation, and apoptosis in germ cells [164, 169]. Furthermore, ethanol exposure induces epigenetic changes that are transferred to the next generation, including inheritance of alcohol-related pathology [170]. The role of paternal alcohol exposure is emerging as an important factor in the risk and development of alcohol-related growth defects in the fetus, mediated through alterations in sperm DNA methylation profile [171].

29.5.7 Cancer and Chemotherapy

Incidence of testicular cancers are increasing in young men, necessitating chemotherapy and radiotherapy [172]. Chemotherapy treatment has been well established in germ cell testicular carcinoma and lymphoma, where chemotherapy (e.g., coadministration of bleomycin, etoposide, and *cis*-platinum) significantly improves 5-year survival rates above 90% [173, 174]. Patients with testicular cancer and lymphoma, prior to onset of chemotherapy, have been shown to have reduced sperm DNA integrity and compaction, even in the setting of and normospermic standard semen analysis [172, 175–177]. Interestingly, male infertility is a risk factor

for cancer, particularly testicular germ cell cancer, prostate cancer, lymphoma, and melanoma. Although poorly understood, it is hypothesized that common mediators include testicular dysgenesis syndrome, chromosomal abnormalities (including Y chromosome abnormalities), and reduced DNA integrity through damaged DNA repair mechanisms [178].

The current chemotherapy treatment further negatively affects testicular function, reduces spermatogenesis, and damages spermatozoa chromatin integrity [173, 174, 177, 179]. This is influenced by dose and type of chemotherapy [180]. Furthermore, damage to DNA integrity and compaction following chemotherapy in cancer survivors persists for up to 24 months in patient survivors, with additional damage to seminal parameters during and post-treatment [174, 179]. Importantly, adolescent chemotherapy has been suggested to permanently reprogram the spermatogenic stem cell epigenome, with the potential to transfer this epigenetic inheritance to the offspring [181].

Alongside chemotherapy, radiation treatment in cancer causes DNA damage in spermatozoa, for 2 years following treatment. Combination treatment is more genotoxic than either alone [182]. This necessitates the need for appropriate reproductive and fertility counseling on the effects of cancer and chemotherapy, including cryopreservation advice based on DNA integrity assessments in cancer patients undergoing chemotherapy [175, 177, 179].

29.5.8 Pollution and Environmental Toxins

Air pollution has significantly increased in recent decades, particularly in urban and industrialized regions, increasing disease risks. Although associated with poor semen quality, any association with male reproductive system and infertility remains relatively unclear [183, 184]. Current evidence demonstrates a negative effect of air pollution exposure on sperm morphology defects, with less evidence relevant to sperm concentration, motility, and DNA fragmentation [184]. The latter has been demonstrated in only a handful of studies where air pollution was associated as a significant cause of spermatozoa DNA fragmentation [185–187], with other studies showing no association [188–190]. Polycyclic aromatic hydrocarbons (PAH) particularly, through air or nutritional sources, are increasingly known to damage DNA through the production of PAH-DNA adducts [191]. This includes chronic exposure to environmental air pollutants particle matter (nitrates, sulfates, ammonium, carbon metals, and organic material suspended as liquid drops in the air), nitric oxide (NO), carbon monoxide (CO), carbon dioxide (CO_2), and ozone (O_3) alongside increased blood, urine, and seminal plasma markers for exposure including polycyclic aromatic hydrocarbons (PAH), nitrogen dioxide (NO_2), lead (Pd), and cadmium (Cd) [183]. Pollution exposure is also reported to induce polymorphisms in spermatozoa DNA, including XRCC1, which further mediates adverse effects on PAH-DNA adducts [192]. However, the evidence remains weak in this regard and any potential mechanisms and causal relationships are poorly investigated [183].

29.5.9 Medications and Recreational Drugs

Numerous medications are associated with a negative impact on the male reproductive system. Drugs that have a good body of evidence for male infertility include testosterone, antiandrogenic drugs, sulfasalazine, anabolic steroids, cyproterone acetate, opioids, tramadol, GhRH analogues, and sartan. Drugs with known negative impact on DNA integrity include sulfasalazine azathioprine, mycophenolate mofetil, and methotrexate [193]. Antidepressant medication negatively affects sperm parameters, including sperm concentration, motility, and morphology [194, 195]. Selective serotonin reuptake inhibitors (SSRI) medication may have negative effects on DNA integrity mediated through OS [196–198]. Methylenedioxymethamphetamine (MDMA), ecstasy, and opioid use are associated with increased OS in the ejaculate and sperm DNA damage [197, 199]. However, the evidence is very weak currently for most medications and DNA integrity.

29.5.10 Aging

Aging is associated with a gradual decline in various biological tissue structure and function alongside a declining quality of life [200]. Importantly, aging is an independent risk factor for obesity, CVD, T2DM, neurodegeneration, and common malignancies (including breast, colon, and prostate carcinoma) [200]. This is strongly mediated by a gradual age-related shift toward OS, particularly through declining endogenous antioxidants [201]. In fact, OS remains a leading theory in the mechanism of aging and age-related pathophysiology [128, 202]. Male reproductive function gradually declines with age. This includes hypogonadism, erectile dysfunction, and testicular dysfunction, alongside increased risk for chronic prostatitis, benign prostatic hyperplasia, and prostate cancer [203].

There is a decline in quantity and quality of sperm produced in aging men, although generally men continue to produce spermatozoa throughout their life span [204]. Elderly men have reduced germ cell quality and quantity than younger men [205]. Aging increases DNA damage through OS in germ cells [205]. Again, young men typically have improved percentage of sperm with intact DNA and chromatin quality compared to older men [206]. An increase in abnormal gametes with DNA damage is apparent, particularly due to reduced ability to repair DNA damage, further generating ROS. This accelerates apoptosis rates through

OS-induced caspase activation and germ cell apoptosis alongside declining spermatogenesis and sperm DNA integrity [207, 208]. Increased paternal age has demonstrated increased DNA fragmentation and single-gene mutations in sperm [209]. This is mediated through variations in single nucleotides that arise from DNA replication errors through ROS. Additional risks include environmental toxin exposure, endocrine, and immune changes, aging, and genetic abnormalities in germ cell lines [210].

In addition to increased risk of pregnancy complications, evidence demonstrates increased disease susceptibility in the offspring of aging males [211–213]. Increased paternal age has been associated with epigenetic transfer to the offspring, increasing risk of congenital defects, cancers (e.g., lymphoma, prostate), and neuropsychiatric disorders including autism, bipolar disorder, and schizophrenia [209]. This is transferred through accumulation of increased rates of DNA mutations through spermatogenesis [209, 212, 214]. Age-related DNA methylation of spermatozoa is apparent in more than 100 genes currently, including genes associated with risk for neuropsychiatric disorders [212, 215]. In fact, DNA methylation describes clear patterns in the sperm epigenome that can be used as a reliable predictive marker of the paternal age of the donor [211]. However, causal relationships have not been found. With increased life expectancy and many couples opting for parenting at older ages, the impact and mechanisms of aging on male reproduction and infertility require further attention and clinical consideration in management of older males wanting to conceive [211, 216].

29.6 Conclusion

Sperm DNA damage has been recognized as a major molecular reason for male infertility and has also been identified as a more stable and therefore more reliable diagnostic parameter than standard semen analysis. In recent years, it became evident that, apart from genital tract infections and inflammations, certain poor lifestyle behaviors and aging lead to an excessive production of reactive oxygen species and thereby oxidative stress. Due to the high reactive potential of the oxidants driving these processes, the sperm nuclear material can be significantly damaged which can not only lead to fertilization or pregnancy failure but also to more serious problems for the offspring as early childhood cancer was reported as a result of sperm DNA damage. These issues do not only include the direct or indirect damage to the DNA through oxidative assaults but also epigenetic changes which in turn can contribute to and exacerbate the problem. Although recent reports brought more light into the etiology of sperm DNA damage and its molecular mechanisms, more work has to be done to fully understand its implications and more importantly the pathophysiology in order to develop rational treatment option for these patients.

29.7 Review Criteria

An extensive search of studies examining the impact of infections on male fertility was performed using search engines such as Google Scholar and PubMed. The searches were not limited for time. Yet, the most recent records were preferred. The overall strategy for study identification and data extraction was based on the following keywords: “sperm DNA damage,” “oxidative stress,” “varicocele,” “male infertility,” “obesity,” “environmental factors,” “lifestyle,” and “sperm DNA fragmentation.” Articles published in languages other than English were not considered. While books were included in the search, data that were solely published in conference or meeting proceedings or websites were not included. Websites and book chapter citations provide conceptual content only.

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Seminal Oxidation-Reduction Potential

30

Mohit Rana and Ashok Agarwal

Key Points

- Oxidative stress (OS) has extensive implication on male infertility due to its ability to induce lipid peroxidation of the spermatozoa's membrane.
- Previous methodologies have been developed to assess how reactive oxygen species affect sperm directly or indirectly by decreasing total antioxidant capacity (TAC). However, these methods are time-consuming and technically demanding and require expensive instruments. Nitroblue tetrazolium (NBT) was an assay to avoid these pitfalls, but the uncertainty of what causes it to indicate OS calls into question its diagnostic accuracy.
- The MiOXSYS system was developed to allow an easy determination of oxidation-reduction potential without requiring an expensive instrument and laborious methodologies.
- Preliminary tests validated the MiOXSYS system's sensitivity to OS, its reliability, and reproducibility.
- The most accurate way to determine an oxidation-reduction potential cutoff is to assess semen with abnormality versus semen without abnormality. Several investigations have led to the refinement of the current cutoff of 1.34 mV/10^6 sperm/mL.
- Global studies on this cutoff value show a deviation cutoff ORP, suggests further investigations into how ORP and OS changes reflect ethnic-specific pathology.

30.1 Introduction

Currently, male infertility is assessed globally by performing a routine semen analysis, which is the cornerstone of andrology. However, it has received criticism in its ability to assess male infertility [1]. The controversies surrounding basic semen analysis stem from three discrepancies. The first is the failure of the World Health Organization (WHO) fifth edition semen analysis results to predict male fertility [2–5]. The second is the lack of inclusion of Middle East, Latin American, Asian, and African countries in the formation of the WHO fifth edition guidelines [6]. The exclusion of Northern and sub-Saharan African countries is particularly negligent as these countries have the highest global prevalence of infertility [7]. The third is the failure to assess the functional health of the sperm as semen analysis does not necessarily reflect the sperm's ability to fertilize the oocyte [5, 8].

Male fertility functional tests can provide a variety of information regarding the health of the sperm. Acrosome reaction testing can indicate if spermatozoa are able to undergo the necessary acrosome reaction to fertilize the oocyte [9]. Once sperm have prematurely undergone acrosome reaction, they cannot fertilize the oocyte, which indicates a pathological process in the semen [10–12]. Zona sperm penetration assay assesses if spermatozoa are capable of penetrating the zona pellucida membrane of the oocyte [13]. Inability of the spermatozoa to penetrate the zona pellucida indicates a pathological process either in capacitation, acrosome reaction, or zona pellucida binding/penetration [13, 14]. As reactive oxygen species (ROS) initiates acrosome reaction and facilitates zone pellucida penetration, an overproduction of ROS could cause premature initiation of either process and facilitate infertility, and subsequent functional tests could determine the etiology of infertility [9, 15, 16]. However, with the advent of intracellular sperm injection (ICSI), both functional tests provide little clinically useful information as ICSI is the resolution to both aforementioned pathologies [8]. Since ICSI bypasses

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all biological barriers preventing sperm, an emphasis has been placed on sperm DNA fragmentation (SDF) as one of the last sperm factors that can influence fertilization success. One pathology which directly increases SDF is oxidative stress (OS).

30.2 Oxidative Stress

OS is the condition when ROS overwhelms spermatic antioxidants [17–20]. Sperm production of ROS was first investigated in 1943 when sperm incubated in high oxygen concentrations had improved motility with the addition of catalase [21]. While elevated ROS production decreases sperm viability, a small amount of ROS is needed to induce physiological processes of capacitation and acrosome reaction [22–24]. Therefore, ROS have both a beneficial and detrimental role in sperm function (Fig. 30.1).

Reactive oxygen species traditionally are free radical oxygen molecules with an unpaired electron but can also act as powerful oxidizers [25]. Hydrogen peroxide is an example of a powerful oxidizer and is significant in male infertility as it is more stable than other forms of ROS [26]. Free radical ROS are subdivided into categories based on the oxygen functional group that contains the radical [22, 23]. More clinically relevant subtypes of free radicals with regard to male infertility are peroxyl (ROO^-), hydroxyl (OH), and superoxide ($\cdot O_2^-$) [27, 28]. While ROS technically only have oxygen as the reactive atom, it has become an umbrella term to include other nonoxygen-based radicals too. Reactive nitrogen species (RNS) are involved in OS [29]. RNS subdivided into groups based on the functional nitrogen group

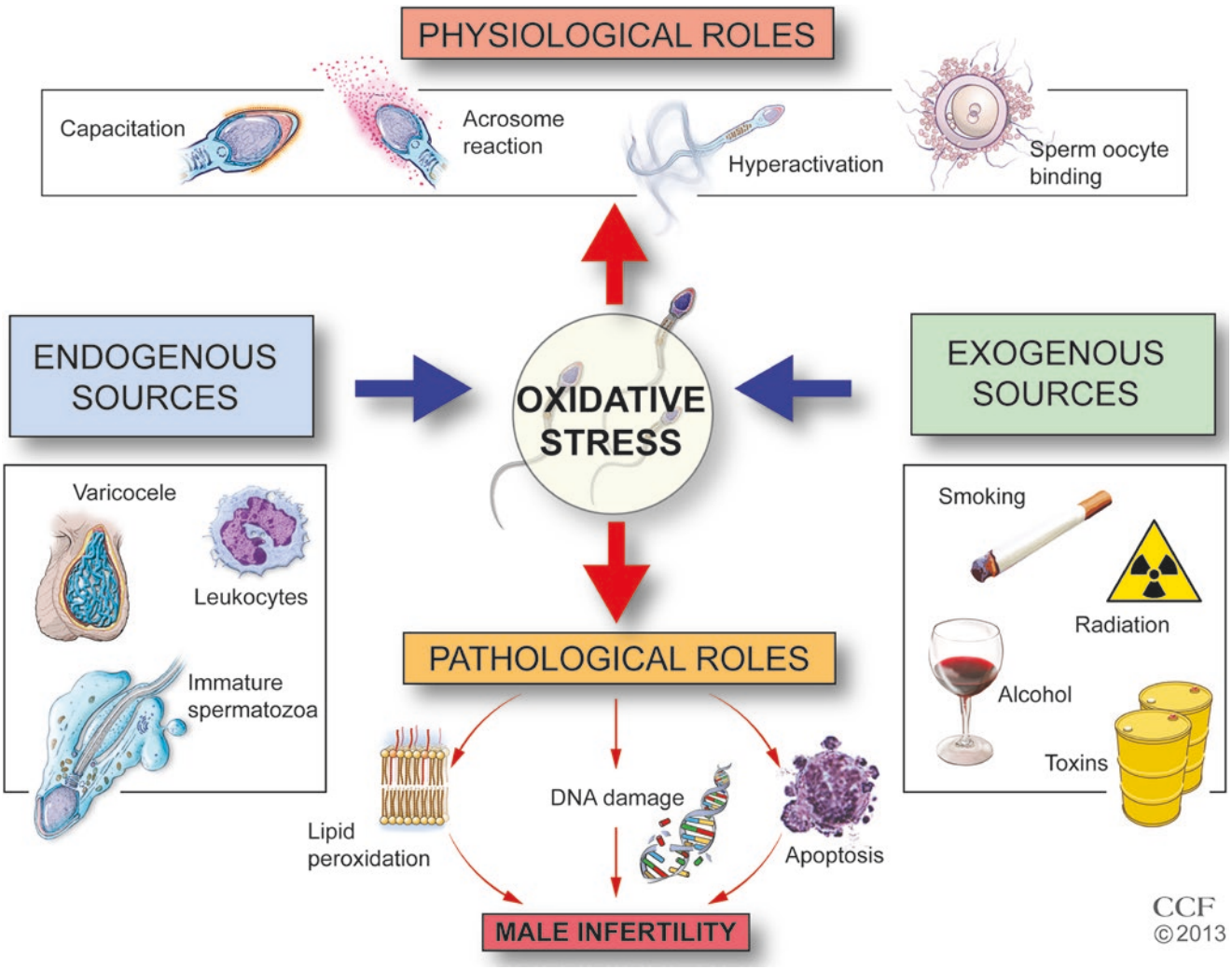

Fig. 30.1 An overview of how reactive oxygen species are involved in physiological processes, such as capacitation and acrosome reaction. When ROS, from either endogenous or exogenous etiologies, are overabundant, pathological processes develop, thus leading to male infertility. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2013–2018. All Rights Reserved)

attached to the radical, too [22]. Clinically relevant RNS are nitric oxide (NO) which, in reaction to superoxide, produces peroxynitrite ($ONOO^-$) [28, 29, 30].

Elevated reactive oxygen species are present in 20–80% of all male infertility patients [31], and antioxidant concentrations are depleted compared to fertile men [17, 18]. Sources of ROS can be either exogenous or endogenous to the spermatozoa. Endogenous sources of ROS are varicocele, diabetes mellitus type 2, metabolic syndrome, infection, immature spermatozoa, and elevated body temperature [26, 32–35]. Exogenous sources of ROS are alcohol abuse, drug abuse, mobile phone use, radiation, heavy metal exposure, prolonged exposure to high temperatures, and semen processing [36–40].

Spermatozoa can metabolically form superoxide, hydroxyl, and nitric oxide radicals [41]. In fertile men, metabolic ROS production is sequestered by enzymatic and low molecular antioxidants [42]. However, in idiopathic infertility, OS causes depletion of antioxidants and allows propagation of ROS-mediated damage [43, 44]. As spermiogenesis removes excess cytoplasm, deregulation of this process produces immature spermatozoa which retain an abundance of cytoplasm [17, 45]. The cytoplasm contains NADPH oxidase which continually produces ROS [46]. Leukocytospermia is a condition where leukocytes, particularly granulocytes, are excessively present ($>1 \times 10^6$/mL) in semen, which results in significant increase in ROS production [47, 48]. Leukocytes are particularly destructive as they produce up to 100 times the ROS compared to that of immature spermatozoa [49]. Sperm are susceptible to ROS-mediated damage by a variety of pathways. The first is the composition of the sperm plasma membrane primarily being comprised of polyunsaturated fatty acids which undergo electrophilic reactions with ROS, thus resulting in lipid peroxidation [18, 50–52]. Reactive oxygen species can destabilize the mitochondrial membrane potential which results in increased ROS production and depletion of adenosine triphosphate [53–56]. The most significant effect of ROS-mediated infertility is the increase in sperm DNA damage. In the presence of ROS, spermatic DNA forms base adducts which results in single-strand DNA breaks as spermatozoa lack the enzymes to repair a basic site (Fig. 30.2 [57–59]). High levels of SDF results in the failure of sperm to fertilize the oocyte in natural conception and assisted reproductive techniques [60–63]. Therefore, assays to determine ROS levels and their effects on antioxidant concentrations have been developed to understand and diagnose ROS-mediated infertility.

Assays to determine ROS in infertile men are evaluated by chemiluminescence, total antioxidant capacity (TAC), and, more recently, nitroblue tetrazolium (NBT) [31, 64–69]. Each methodology showed different pathologies with male infertility and presented with their own advantages and disadvantages (reviewed in Table 30.1). Chemiluminescence utilizes luminol as an ROS probe to determine concentrations of ROS either intracellular or extracellular to spermatozoa [70]. Elevated ROS results in lipid peroxidation of the sperm membrane, acrosome-reacted sperm, axoneme damage, and DNA damage [18, 50, 57, 71, 72]. TAC assays determine how well a semen sample can suppress the effects of ROS. Assays for the measurement of TAC utilize a source of ROS propagation and a probe which is sensitive to ROS-induced photon emission. The probe utilized can be either chemiluminescent, such

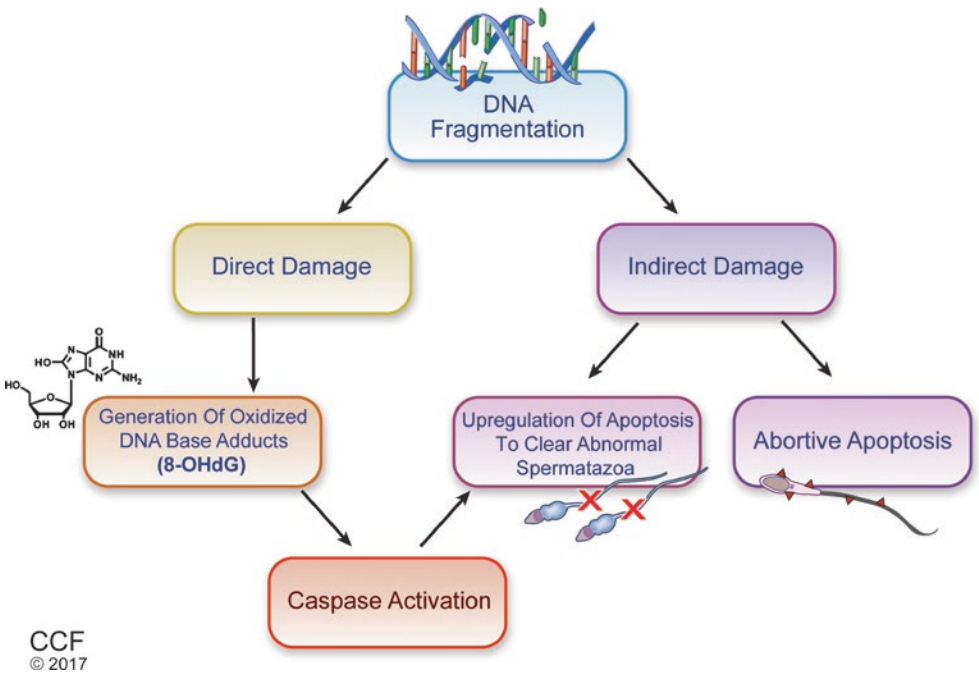

Fig. 30.2 OS can directly lead to formation of 8-OHdG base adducts. As spermatozoa are unable to repair these adducts, spermatozoa shall enter apoptosis when fragmentation is elevated. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2013–2018. All Rights Reserved)

Table 30.1 Current assays used to determine OS in semen

Assay	Description of finding	Advantages	Disadvantages	References
Chemiluminescence (ROS)	Commonly utilizes lucigen or luminol probes which react with ROS and emit a photon	The assay directly measures both intracellular and extracellular concentrations of ROS	Time-consuming, equipment is expensive, semen age affects the results, must be conducted in the dark to avoid interference with external light sources	[31, 64, 70]
Total antioxidant capacity (TAC)	The semen's ability to suppress chemiluminescence or colorimetric expression when exposed to a source of ROS determines the relative amount of antioxidant in the sample	Measures antioxidant concentration in semen Colorimetric method allows for a quick result	Requires expensive equipment; the length of the assay can change the results obtained	[66, 67, 121, 122]
ROS-TAC score	A statistical model used to determine semen quality and ability to compensate for OS	ROS-TAC scores are easy to comprehend, wholesome indicators of OS in semen	Time-consuming, requires calibration for the center's demographics prior to diagnostic capability, requires both a plate reader and a spectrophotometer	[19, 43, 123]
Nitroblue tetrazolium (NBT)	NBT is a clear, yellowish probe which turns indigo when exposed to ROS	Requires a fluorescent microscope, significantly reduces testing costs	NBT has questionable specificity for only ROS to cause the color changes	[69, 76, 124]
Thiobarbituric acid reactive substances (TBARS)	Able to detect lipid peroxidation by detecting MDA adduct formation	MDA formation is stable and is additive over time, showing OS damage over time Flow cytometer allows determination of 5000 sperm over the 200 from microscope evaluations	Requires a flow cytometer and strict quality control to produce reliable results	[125, 126]
Oxidation-reduction potential (ORP)	Provides a measurement of redox balance or ORP through an electrochemical measurement of all known and unknown oxidants and antioxidants	The only assay that measures redox balance exclusively. System procedure requires very little technician involvement and is easy to standardize	Affected by high levels of semen viscosity. Cannot be tested in stored samples containing sperm preparation media, buffer, or cryoprotectants	[77, 83, 112]

as luminol, or colorimetric, such as 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) [66, 73]. The antioxidants in a semen sample will prevent either a photon emission or a color shift and, thus, produce the TAC. Low TAC values indicate a decrease in antioxidants present within the semen and subsequent pathological processes.

While chemiluminescence and TAC methodologies have advanced the pathological understanding of OS, they have poor diagnostic characteristics. Both methodologies are time-consuming, laborious, and require skilled personnel and expensive instruments [74, 75]. To circumvent these limitations, NBT was explored as an alternative to chemiluminescent techniques. To assess OS with NBT, a bright field microscope is required and visual acuity to determine sperm that have minimum color from those with dark-blue staining [68, 69]. While NBT has questionable specificity for oxidation strictly by ROS, the low cost of the assay allows NBT to be economical for developing nations, thus allowing OS to be standardized across the globe [46, 76]. Given the clinical significance of OS in male infertility evaluation, there is a need for a simple, cost-effective, and robust methodology.

30.3 The MiOXSYS System

The MiOXSYS system (Fig. 30.3) is an innovative system for diagnosing OS-induced infertility. The principle is to determine oxidation-reduction potential (E^{ORP}) of the Nernst equation (Eq. 30.1) [43, 43, 44]. The MiOXSYS system measures E^{ORP} utilizing a galvanostatic measurement of a working solution [74, 77]. Galvanostatic measurements are not innovative in their own right, as they were originally described in 1941 [78]. The original use of these systems was to measure potential changes in electrolytic processes. The most widespread application of electrolytic chemistry was in municipal water supply chlorination [79–81]. Biological application of oxidation-reduction potential was observed in donor-acquired organs for transplantation, as ORP can be used to monitor ischemia-induced organ injury [82]. However, there was a limited use for ORP in biological samples due to the large volume of specimen required, the large size of the analyzers, and the impracticality of handling multiple samples in a timely manner [74, 80]. The MiOXSYS System enables the scope of ORP to be applied to semen and other biological samples:

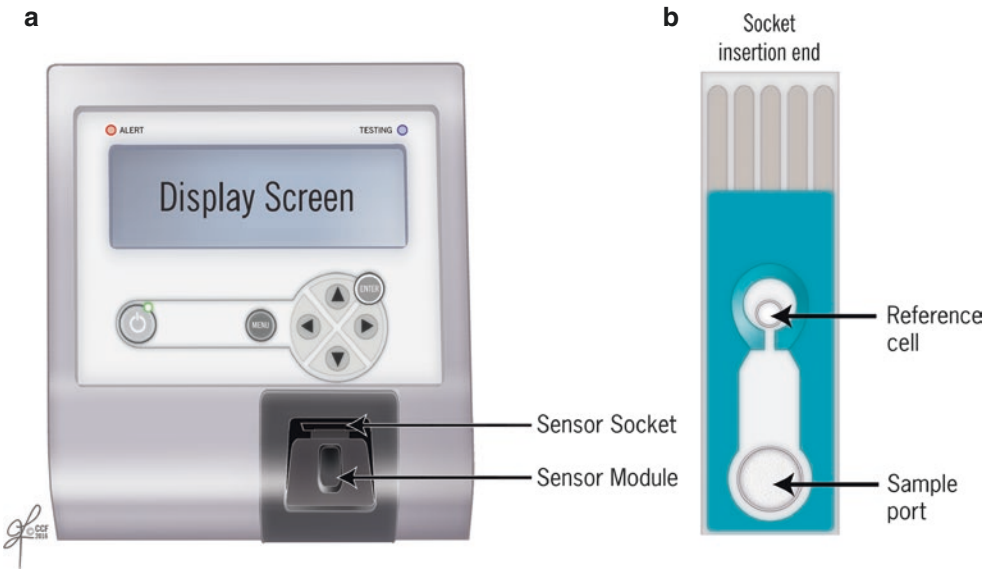

Fig. 30.3 (**a**) The MiOXSYS instrument is a compact and simple instrument. (**b**) Due to the use of disposable sensors, semen samples can be applied to the sample port and through capillary action connect the reference cell to a working circuit. Once these circuits are connected, the instrument assesses the resistance of the sample and generates an ORP value. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2013–2018. All Rights Reserved)

$$E(\mathrm{ORP}) = \frac{E^0 - \mathrm{R}\cdot\mathrm{T}}{n\cdot\mathrm{F}\cdot\ln\dfrac{[\mathrm{Ox}]}{[\mathrm{Red}]}} \qquad (30.1)$$

E (ORP) is the oxidation-reduction potential of the sample (in mV), E^0 is the standard reduction potential of hydrogen, n is the number of electrons exchanged (in moles), T is the absolute temperature (in degrees kelvin), F is Faraday's constant, R is the universal gas constant, [Ox] is the concentration of oxidations in the sample (in moles), and [Red] is the concentration of reductants in the sample (in moles).

The MiOXSYS System utilizes two primary components: a disposable single use platinum based sensor and a small galvanostat analyzer. The test sensors allow 30 μL of sample which is applied to the sensor port and connects the working circuit to the reference circuit [83]. The MiOXSYS analyzer is compact and requires very little laboratory bench space. The MiOXSYS system procedure takes approximately 5 minutes and requires very limited hands on time. The MiOXSYS System applies a low voltage current to the sample and the electron activity is measured in millivolts (mV). Results from the MiOXSYS system require the ORP to be normalized by the concentration [77].

30.4 Oxidation-Reduction Potential and Male Infertility

While other methods determine OS either by measuring ROS, TAC, or both, ORP is an independent measure of OS. Oxidation-reduction potential cutoff values were validated as a clinical marker of OS in infertile males (reviewed in Table 30.2). The first study was to establish if ORP can determine semen quality according to the WHO fifth edition guidelines.

30.4.1 Semen Quality and Oxidation-Reduction Potential

Prior to the diagnostic validation of the MiOXSYS system, a series of analytical studies were undertaken to determine the reliability and repeatability in a clinical laboratory setting. Agarwal et al. [77] established the ORP does not significantly vary between the time of collection and 120 min after liquefaction, validating ORP is stable in semen up to 2 h. In addition to the stability of time and ORP, the difference in ORP between semen and seminal plasma was determined. Agarwal et al. [43] validated that both semen and seminal plasma were time-independent and had ORP values correlating with each other.

Additional investigations examining the influence of oxidants on biological semen samples being analyzed by the MiOXSYS System were performed. Agarwal et al. studied the effects of cumene hydroperoxide in fresh and frozen semen samples. The study also investigated whether freezing of semen samples induced exogenous OS, which increased ORP [87]. Both motility and viability decreased significantly in a dose-dependent manner when exposed to cumene hydroperoxide. The ORP readings increased significantly in a dose-dependent manner, as well, but failed to show a significant correlation with the decrease in motility and viability [87]. In addition, it was validated that an increase in ORP is indicative of OS.

Table 30.2 Determining a cutoff value for ORP to diagnosis male infertility

ORP cutoff ($mV/10^6$ sperm/mL)	Pathologic indication	Sensitivity	Specificity	PPV	References
1.635	> 4 abnormalities on semen analysis				[91]
3.29	Abnormal morphology of < 4%		89.1	85.7	[92]
1.57	Able to detect at least one abnormality on semen analysis				[109]
1.42	one abnormality on semen analysis across nine different centers	97.1	43.7	94.2	[116]
1.48	Abnormality on semen samples from a neat semen sample	60	75		[77]
2.09	Abnormality on semen analysis with seminal plasma	46.7	81.8		[77]
1.57	Detect one abnormality and able to determine oligozoospermic with greatest accuracy among patients	70.4	88.1	95.5	[105]
1.38	One abnormality on semen analysis with a better odds ratio than 1.41	63.3	87.8	97.6	[89]

ORP oxidation-reduction potential, *PPV* positive predictive value

Initial clinical validation of ORP was performed using semen samples from fertile donors and infertile patients. Cutoff value of 1.36 $mV/10^6$ sperm/ml was established with a sensitivity 69.6%, specificity 83.1%, positive predictive value 85.3% and negative predictive value 65.9% using MiOXSYS analyzer [106]. ORP levels were lower in fertile controls (1.03 $mV/10^6$ sperm/mL) compared with infertile patients (5.49 $mV/10^6$ sperm/mL) [89]. In fertile donors, ORP values were negatively correlated with sperm concentration [77]. Whereas, in infertile patients, ORP values were negatively correlated with both concentration ($r = -0.883$) and total motility ($r = -0.369$) [43]. Arafa et al. also reported a negative correlation between the ORP levels and semen parameters such as sperm concentration, total sperm count, total motility, progressive motility, and normal sperm morphology [89, 90]. Poor semen parameters with increased ORP indicates a state of OS.

Elbardisi et al. [91] divided patients into those who had an abnormality in at least one semen parameter ($n = 364$) and those with no abnormalities on semen analysis ($n = 64$) according to WHO fifth edition guidelines. ORP readings from the seminal plasma between abnormal and normal semen parameters revealed ORP did not correlate with motility but did so with progressive motility and morphology [91–94]. Seminal plasma ORP was most effective at differentiating patients with >4 abnormalities and was not effective in patients with a single abnormality on semen analysis [91]. Majzoub et al. [93] determined ORP values between infertile patients who had a significant correlation between abnormal morphology, particularly sperm head defects ($r = 0.34$), and SDF (measured by sperm chromatin dispersion assay; $r = 0.73$). Arafa et al. [95] assessed ORP's ability to correlate semen parameters and SDF. SDF was evaluated by Halosperm and significantly correlated with ORP ($r = 0.351$). Interestingly, patients with lower ORP values had higher total motility [95]. Subsequently, ORP correlated significantly with SDF values, it serves as a surrogate marker for centers without the capabilities to determine SDF [96].

While ORP comparisons between fertile and infertile patients assess how semen quality varies between patients of all etiologies to fertile controls, other studies determined if the MiOXSYS system can differentiate ORP values from patients with known OS-induced pathologies. Semen cryopreservation diminishes post-thaw sperm survival due to cryo-injury which is partially caused by OS induced by the increased metabolism during the thawing process [97]. The post-thawed semen samples produced higher ORP values which negatively correlated with post-thaw total motility, total semen counts, and cryo-survival [98]. Roychoudhury et al. [99] determined that grade 3 varicocele had significantly higher ORP levels compared to other varicocele grades in idiopathic infertile patients and fertile controls. Saleh and Agarwal [100] confirmed this difference between varicocele and idiopathic infertility. In addition, patients with varicocele had ORP levels which correlated with total motility, total motile count, progressive motility, abnormal morphology, and leukocyte concentration [100]. Varicocelectomy improves OS markers in male infertility patients [101–103], and, as expected, ORP reflects this in post-surgery patients [104]. Patients who had undergone varicocelectomy had significantly reduced ORP three months post-operation [104]. For patients with leukocytospermia, empiric doxycycline treatment reduced ORP by 56% after completion of the treatment [105]. With ORP reflecting poor semen quality, investigations were pursued on establishing various cutoff values to diagnose different aspects of male infertility.

30.4.2 Determining a Cutoff to Diagnose Infertile Men

In determining a cutoff value for the MiOXSYS system, a fundamental question prior to establishing a value is how many readings are required to produce a reliable result. To produce a reliable result, the MiOXSYS system requires a single reading. ORP values determined in duplicates change insignificantly by 0.1 $mV/10^6$ sperm/mL, indicating one reading is accurate [89]. Assaying how the observer can induce a change in the reading is an important aspect of any clinical test as readings can vary between a single

observer (intraobserver) and multiple observers (interobserver). Readings between a single observer and multiple observers vary by 3.61% by 8.39%, respectively, validating that the MiOXSYS system produces a reproducible and reliable diagnostic marker [106, 107]. Clinicians are reported with only a single ORP reading.

A cutoff ORP value for men with infertility versus fertile controls can be determined in a multitude of ways (reviewed in Table 30.2). One method to determine an ORP cutoff is to compare its values from fertile men and infertile men and establish a cutoff which distinguishes with the greatest accuracy between those two populations. Agarwal et al. [77] determined such a cutoff by evaluating both semen samples and seminal plasma from fertile and infertile men. The cutoff value for semen is 1.48 mV/10^6 sperm/mL with an accuracy of 78.9%. However, there is a difference between semen and seminal plasma cutoffs as seminal plasma cutoff is 2.09 mV/10^6 sperm/mL with an accuracy of 72.9%.

Reports have examined a second method to determine an ORP cutoff which can distinguish between semen without an abnormality and those with an abnormality according to WHO fifth edition guidelines [108]. When comparing men with completely normal semen parameters to men with at least one abnormality on semen analysis, an initial cutoff of >1.57 mV/10^6 sperm/mL indicates a patient has one abnormality on semen analysis and has OS [105, 109]. The cutoff values have a sensitivity of 70.4%, specificity of 88.1%, and a positive predictive value (PPV) of 95.5% to distinguish between semen with no abnormality and semen with at least one abnormality. Further refinement by Arafa et al. [110] determined a cutoff of 1.42 mV/10^6 sperm/mL that distinguishes infertile men with at least one abnormality with a specificity of 78% and PPV of 95.7%. A cutoff of 1.64 mV/10^6 sperm/mL differentiates 96% of infertility patients with greater than four abnormalities on semen analysis [91].

For a more specific cutoff value, ORP values which can distinguish abnormal morphology were further investigated as morphology has the highest predictive power to differentiate between infertile and fertile men [108]. A cutoff of 3.29 mV/10^6 sperm/mL distinguishes semen with high abnormal morphology [92]. When the sample size was increased from 400 infertile men to 1168, the cutoff was refined to 1.73 mV/10^6 sperm/mL with a sensitivity of 76%, specificity of 72%, and PPV of 69.2% [111].

To determine which method to utilize for a definitive cutoff value, Arafa et al. [89] compared fertile and infertile men. A cutoff of 1.36 mV/10^6 sperm/mL was determined with ORP from men with one abnormality on semen analysis. A second cutoff was determined by comparing infertile versus fertile ORP values regardless of semen analysis results which produced a cutoff of 1.41 mV/10^6 sperm/mL. By comparing which cutoff had a better odds ratio in distinguishing fertile from infertile men, the more accurate cutoff was determined as the ORP with an abnormality on semen analysis [89]. An ORP based on a semen abnormality by semen analysis can differentiate between normozoospermic and oligozoospermic men but cannot differentiate with asthenozoospermic men [108]. The cutoff of 1.36 mV/10^6 sperm/mL was further evaluated and has a sensitivity of 69.6%, specificity of 83.1%, and PPV of 85.3% [106]. Men with ORP values greater than 1.36 mV/10^6 sperm/mL had negatively correlated concentration ($r = -0.823$), total count ($r = -0.728$), motility ($r = -0.485$), and normal morphology ($r = -0.238$). While the cutoff value was determined, validation with more than one individual infertility center is required to validate the utility on a global scale.

30.4.3 Global Validation

The first multi-center was completed by a center in the USA and in Qatar. The main objective was to determine the ORP values in infertile men and fertile donors at both the centers and to establish a cutoff value. Both populations had a significant difference between fertile controls and infertile patients in concentration, motility, progressive motility, and normal morphology [112, 113]. To determine a cutoff, patients and controls were divided with the presence of one abnormality on semen analysis. Interestingly, the study found a difference between normal morphology and motility between infertile patients, validating the criticism of subjectivity in semen analysis [114, 115]. The cutoff was 2.26 mV/10^6 sperm/mL for the United States and 1.42 mV/10^6 sperm/mL for Qatar. When the two populations were combined, the cutoff was determined to be 1.42 mV/10^6 sperm/mL [112]. Infertility centers from the United States, Qatar, Japan, the United Kingdom, Turkey, Egypt, and India (nine in total) determined a cutoff of 1.34 mV/10^6 sperm/mL [112]. The data from the centers revealed ORP has the highest predictive value to differentiate fertile from infertile men, compared to traditional semen analysis [116].

30.4.4 Oxidation-Reduction Potential and In Vitro Fertilization

Continuing this trend, two studies evaluated whether ORP can determine the effect of various culture media on sperm. To determine if culture medium responses to OS are distinguishable by ORP, Panner Selvam et al. added varying concentrations of ascorbic acid and cumene hydroperoxide to culture media [117]. The effect on media shows ascorbic acid reduced ORP and cumene hydroperoxide increased

ORP. When comparing baseline ORP values of various media, a trend emerged; ART media had lower ORP values than sperm wash media and cryopreservation media had ORP values in between [117]. The findings suggest the antioxidant capacity of ART media is higher than that of cryopreservation and sperm wash media. Comparing the effect of ART media versus a neat semen sample after 1 h revealed polyvinylpyrrolidone (PVP) had a lower ORP than either hyaluronic acid or neat semen [118]. The results show PVP allows for better antioxidant capacity over a prolonged time compared to neat semen.

30.5 Five-Year Outlook

While the MiOXSYS system provides a novel method to determine OS, the system requires further validation within the IVF setting prior to universal acceptance.

Subsequently, the effect of ART media and semen is interesting; however, determining the physiological ORP of sperm will allow a more comprehensive understanding of how changes in ORP affect sperm. Understanding how media causes semen to deviate from physiological ORP allows the development of media which maintain semen quality longer. Subsequently, media refinement would minimize the effect of OS on successful fertilization after cryopreservation and assisted reproductive technique.

Oxidation-reduction potential correlates with SDF determined using Halosperm assay. As different methods to evaluate DNA fragmentation assess different aspects of SDF, investigating if ORP correlates with TUNEL and/or SCSA is required [119, 120]. This will better reassure the robustness of ORP as a surrogate marker for SDF.

30.6 Conclusion

OS and idiopathic male infertility has been well documented in the literature. Evaluating redox balance in a patient's semen has been explored previously, but the techniques are labor intensive, time-consuming, and unable to be standardized in routine clinical practice. The MiOXSYS System provides a rapid and accurate measure of redox balance in a simple and standardizable method. The MiOXSYS system cutoff based upon semen analysis abnormality produces the highest predictive power to distinguish between fertile and infertile men.

30.7 Review Criteria

An extensive review of the literature regarding OS, male infertility, and oxidation-reduction potential was performed using PubMed, Google Scholar, and Medline databases. Articles were initially assessed for the following keywords: "oxidation-reduction potential," "MiOXSYS," "male infertility," "semen analysis," "sperm functionality," and "oxidative stress." Only articles published in English were reviewed.

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Part III

Common Conditions and Factors Affecting Male Reproductive Health

Varicocele

31

Daniel Suslik Zylbersztejn and Sandro C. Esteves

Key Points

- Approximately 8% of men in reproductive age seek for medical assistance for fertility-related problems. Of these, 1–10% carries conditions that compromise the reproductive potential, and varicocele accounts for 35% of these cases.
- Epidemiology data show that approximately 35% of men with primary and 80% of men with secondary infertility have varicocele. The higher frequency of varicoceles in both the elderly and in men with secondary infertility suggests that it is a progressive disease. Bilateral varicoceles are more common than previously reported.
- Impaired drainage or pooling of blood around the testicles leading to increased scrotal temperature, hypoxia, increased testicular pressure, reflux of renal and adrenal metabolites, excessive oxidative stress (OS), and decreased pH in the spermatozoa cytosol and seminal plasma are the leading theories to explain the detrimental effects of varicocele on spermatogenesis.
- The concept that varicocele causes infertility relies on three main aspects: (i) the increased incidence of this condition among infertile men, (ii) the association of varicocele with reduced semen parameters and testicular size, and (iii) the improvement of semen parameters and pregnancy rates after surgical repair of clinical varicoceles.
- Varicoceles diagnosed by physical examination, which is the preferred diagnostic method, are termed "clinical." Clinical varicoceles are graded according to the size. When a varicocele is not palpable, but other diagnostic methods detect a retrograde blood flow, the varicocele is termed subclinical. The significance of a positive test result using adjuvant diagnostic techniques in infertile men remains uncertain.
- Varicocele treatment is indicated for men with clinically palpable varicocele and abnormal semen parameters. Open microsurgical inguinal or subinguinal techniques are considered the best treatment modalities because they result in higher natural pregnancy rates and fewer recurrences and postoperative complications than laparoscopic, radiologic embolization and macroscopic inguinal or retroperitoneal varicocelectomy techniques. There are no absolute predictive factors for successful varicocele repair, and existing evidence does not support the recommendation for treating infertile men with subclinical varicocele (SCV).
- Recovery of spermatogenesis can be achieved after repair of clinical varicocele in infertile men with nonobstructive azoospermia (NOA). Testicular histopathology is predictive of success, and men with maturation arrest (MA) and hypospermatogenesis (HS) are more likely to ejaculate motile spermatozoa after surgery.
- Functional markers for oxidative stress and DNA integrity are impaired in infertile men with clinical varicocele but may be significantly improved after varicocele repair.
- Surgical repair of varicocele increases the chance for either natural or assisted conception in infertile couples whose male partner has a clinical varicocele. Also, the chance of retrieving testicular sperm

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for ICSI is optimized in nonobstructive azoospermic men with treated clinical varicocele.
- Lifestyle modifications may benefit reproductive health. When considering therapeutic measures to treat varicocele-associated infertility, counseling toward lifestyle modifications should be strongly encouraged.
- Varicocelectomy treatment can be offered to men with clinical varicocele and hypogonadism as a means to improve androgen production and potentially avoid testosterone replacement therapy.
- Soon, proteomics and genomic studies might help identify the ideal candidates for varicocele treatment. Such studies might also be able to identify those individuals who need early interventions to avoid deterioration of testicular function.

31.1 Introduction

Approximately 50 million couples suffer from infertility worldwide and the percent of infertility that is attributable to men ranges from 20% to 70% [1]. Varicocele is a common finding mong men with infertility complaint. In a group of 2875 infertile couples attending our tertiary center for male reproduction, we found the presence of varicocele in 21.9% of the male partners.

The first reports concerning the existence of varicose veins surrounding the testes date back to the first century AC [2]; however, the association between varicocele and infertility was only suspected at the end of the nineteenth century when surgical occlusion of dilated veins was shown to improve semen quality [3]. Later, in 1952, Tulloch was the first to report that bilateral varicocele repair in a male with azoospermia resulted in an increase in sperm concentration that resulted in a natural pregnancy [4]. In 1965, MacLeod first reported that most semen specimens obtained from infertile men with varicocele had decreased sperm count, decreased motility, and increased abnormal forms [5].

The term "varicocele" derives from Latin *varix* (a dilated vein) and Greek *kele* (tumor). It comprises the dilation of the veins of the pampiniform plexus that drain the blood from the testicles. Typically, small one-way valves prevent the backward blood flow. Valve abnormalities or vein compression by adjacent structures can cause vein dilation.

The pathophysiology of varicocele and its impact on the male reproductive potential have been debated for the past 50 years. Varicocele is still one of the most controversial issues in the field of male infertility, especially regarding why, when, and to whom treatment should be applied. Varicocele repair is considered the treatment of choice for varicocele-associated infertility, but its effectiveness still elicits heated debate. Although the ultimate endpoint for the treatment of male factor infertility is a live birth, efforts to maximize the couple's fertility potential by improving testicular function should not be overlooked. This chapter discusses the current concepts and controversies regarding the epidemiology, pathophysiology, diagnosis, treatment, and significance of clinical and subclinical varicoceles (SCV) in male infertility. It also reviews the management of varicoceles in azoospermic patients and the indications of varicocelectomy in the era of assisted reproductive technology (ART).

31.2 Epidemiology

Approximately 7% and 10–25% of prepuberal and postpubertal males have a varicocele, respectively [6, 7]. Varicoceles are found in up to 43% of adult males [8]. The prevalence of varicocele increases over time, and it is estimated that a 10% rise in incidence occurs for each decade of life. Approximately 35% of men with primary and 80% of men with secondary infertility have varicose veins [1, 9]. The higher frequency of varicoceles in both the elderly and in men with secondary infertility suggests that the condition is progressive. Although the frequency of unilateral left-sided varicocele has historically been reported to be approximately 85–90%, recent data indicate that bilateral palpable varicoceles are found in more than 50% of the affected subjects [6].

Whether or not a varicocele might be inherited remains equivocal. Some authors have described an increased prevalence of varicocele in men whose first-degree relatives also have varicose veins [10–12]. Others, however, studying adolescents, could not find a direct association in first-degree relatives, although it has been acknowledged that young men with high-grade varicocele might have a hereditary predisposition [13]. Further studies are needed to establish the relationship between varicocele and genetic inheritance conclusively.

31.3 Pathophysiology

The etiology of varicocele formation is likely to be multifactorial. The right internal spermatic vein inserts directly into the inferior vena cava at an acute angle, while the left one inserts into the left renal vein at a right angle. It is also suggested that a partial obstruction of the left spermatic vein due to the compression of the left renal vein between the aorta and the upper mesenteric artery might be the causative factor in some cases (the "nutcracker" phenomenon). An increase in the hydrostatic pressure of the left spermatic vein may be transferred to the venous plexus of the spermatic cord causing its dilation [14]. Moreover, primary insufficiency of the

internal spermatic and subsequent malfunction of the external spermatic and cremasteric vein valves may lead to regression of blood [15, 16]. Also, there seems to be an inverse relationship between the occurrence of varicocele and body mass index [17]. Moreover, intense physical activity (2–4 h daily, 4–5 times per week) over several years appears to worsen the semen quality of men with varicoceles and abnormal semen parameters [18].

A fivefold increase in the hydrostatic pressure of the spermatic veins has been observed in men with varicocele compared to controls [19]. Microscopic evaluation of the spermatic vein fragments reveals alterations in the longitudinal muscle layers and a decrease in the number of nerve elements in the vessel wall [20]. These findings suggest a defective contractile mechanism of blood transport through the pampiniform plexus that may lead to a reversal of the pressure gradient and cause a hypoxic status.

Several theories aim to explain the impact of varicoceles on testicular function, but none of them can fully elucidate the variable effect of varicocele on human spermatogenesis and male fertility [14]. Proposed mechanisms include hypoxia and stasis, testicular venous hypertension, elevated testicular temperature, increase in spermatic vein catecholamine leading to testicular underperfusion, and increased oxidative stress (OS) [21]. It is believed that reflux of warm blood from the abdominal cavity to the varicose veins increases the scrotum temperature, but the mechanism by which temperature influences spermatogenesis is not clearly understood.

Germ cell apoptosis and subsequent oligozoospermia, a common phenomenon in men with varicocele, can be attributed to increased scrotal temperature, increased intratesticular cadmium concentration, and reduced levels of androgens [22, 23]. An increased concentration of toxic metabolites inside the testicles (e.g., catecholamines from the kidney and adrenal glands) can cause chronic vasoconstriction of the intratesticular arterioles, contributing, along with impaired venous return caused by valve insufficiency, to persistent testicular underperfusion and subsequent dysfunction of the spermatic epithelium [24]. Biopsies of varicocele-affected testicles showed a decrease in E-cadherin and alpha-catenin in the Sertoli–Sertoli junction and subsequent disruption of the blood–testis barrier that can contribute to the pathology and impairment in sperm production [25]. However, histopathologic findings typical of varicocele have not been observed [26].

Excessive oxidative stress (OS) is often seen in infertile men with varicocele. High production of reactive oxygen species (ROS) in the reproductive tract impairs both the fluidity of the sperm plasma membrane and the integrity of deoxyribonucleic acid (DNA) in the sperm nucleus. Abnormal high levels of sperm DNA damage are associated with a decrease in several fertility markers including fertilization rate, embryo cleavage rate, implantation rate, pregnancy rate, and live birth rate [27]. Fertility impairment in men with varicocele might result from decreased pH in the spermatozoa cytosol and seminal plasma [28]. According to this hypothesis, testicular underperfusion diminishes cell oxygen and glucose supply to the metabolically active tissues. Under conditions of low glucose supply, the flux through the pentose phosphate pathway is markedly decreased, as well as the provision of reductants to the antioxidant system. Indeed, the drastic fall in the reduced nicotinamide adenine dinucleotide phosphate/oxidized nicotinamide adenine dinucleotide phosphate (NADPH/NADP+) ratio leads to an impairment in the tissue antioxidant capacity because glutathione regeneration is retarded. Under such pathologic circumstances, ROS production surpasses the antioxidant capacity and causes increased oxidative stress [24]. Spermatozoa are susceptible to damage by oxidative stress through ROS, especially lipid peroxidation [27], owing to the low amount of cytoplasm and abundance of polyunsaturated fatty acids in the sperm plasma membrane. Lipid peroxidation damages the membrane function in sperm head and midpiece, thus not only decreasing sperm motility but also intracellular pH, partly because of malondialdehyde-mediated reactions.

Malondialdehyde, produced by the peroxidation of polyunsaturated fatty acids, reacts with spermine, a polyamine essential for sperm activity, forming Schiff bases. It results in a further decrease of pH as well as in a direct impairment of spermine-dependent cellular functions. The optimum pH for ROS scavenging by the enzymatic antioxidant systems ranges from neutral to slightly alkaline, but their activity is markedly depressed in low pH. It has been observed that antioxidant enzyme activity is significantly impaired in infertile men with varicocele, and it may further diminish sperm motility [27].

The hypothesis mentioned above is novel and adds to other proposed mechanisms for defective sperm function in men with varicocele, such as the peroxidation of the unsaturated fatty acids in the sperm plasma membrane and the impaired acrosome reaction and DNA integrity induced by ROS [24]. However, it has also been speculated that individual differences may exist; therefore, the mechanism described above may not be deterministic. If, for example, the glucose supply is less restricted or the accessory glands are particularly efficient, the accumulation of ROS and acidification of the seminal plasma could be minimized. These observations might help us understand the variable effect of varicocele on male fertility.

31.4 Infertility

The concept that varicocele causes infertility relies on three main aspects: (i) the increased incidence of this condition among infertile men, (ii) the association of varicocele with

reduced semen parameters and testicular size, and (iii) the improvement of semen parameters and pregnancy rates after surgical repair of clinical varicoceles.

In a large observational study involving 9034 men, it was observed that 25.6% of men with abnormal semen analysis had varicocele. It was also noted that total sperm count and testosterone levels were lower in men with varicoceles than those without varicoceles. Also, the testicular size was significantly reduced at the varicocele side as compared to the contralateral one in the cases of unilateral varicoceles [29]. Surgical repair of varicocele was shown to restore testicular temperature in both animals and humans [24].

The hypothesis that varicocele can cause testicular damage was further confirmed on pubertal boys in which the reduction in the size of the ipsilateral testis was restored by surgical repair of varicocele [30]. Despite the proven association between varicocele and infertility, it is still unclear the reasons why about two-thirds of men with varicocele retain their fertility [3, 31] and why fertility potential is not always improved after surgical varicocele repair [32, 33].

31.5 Diagnosis

Currently, physical examination with the patient standing in a warm room is the preferred diagnostic method (Fig. 31.1). Varicoceles diagnosed by this method are termed "clinical" and graded according to the size. It is essential to ask the patient to perform a Valsalva maneuver during the examination. Large varicoceles (grade III) are varicose veins seen through the scrotal skin (Fig. 31.2). Moderate (grade II) and small-sized varicoceles (grade I) are dilated veins palpable without and with the aid of the Valsalva maneuver, respectively [34].

Physical examination is limited by a sensibility and specificity of about 70% compared to other diagnostic modalities [35, 36]. Interobserver and intraobserver variabilities have been observed among practitioners. Indeed, the physical examination can be inconclusive or equivocal in cases of low-grade varicocele and in men with a history of previous scrotal surgery, concomitant hydroceles, or obesity. Imaging studies may be recommended when the physical examination is inconclusive. When a varicocele is not palpable but retrograde blood flow is detected by other diagnostic methods such as venography, Doppler examination, ultrasonography, scintigraphy, and thermography, the varicocele is termed subclinical [35, 37].

The gold standard method to diagnose blood reflux into the veins of the pampiniform plexus is the percutaneous venography of the spermatic veins; however, it is not routinely used because of its invasiveness [35, 37]. Among the noninvasive diagnostic modalities, color Doppler ultrasound (CDU) is the best diagnostic tool. The commonly accepted CDU criterion for varicocele (maximum vein

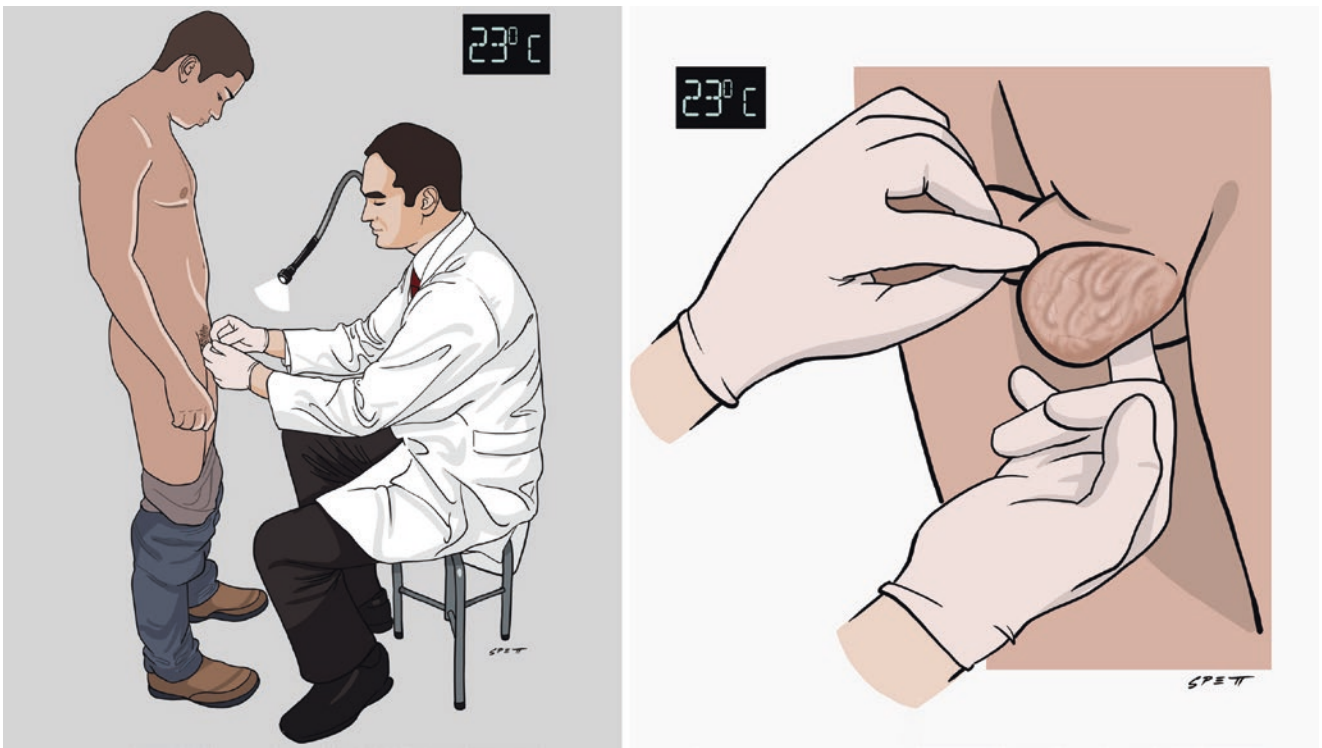

Fig. 31.1 Illustration depicting the scrotal examination of a young male with a varicocele. Adequate illumination and room temperature are essential to allow proper assessment of clinical varicoceles (Reprinted with permission from ANDROFERT. All Rights Reserved)

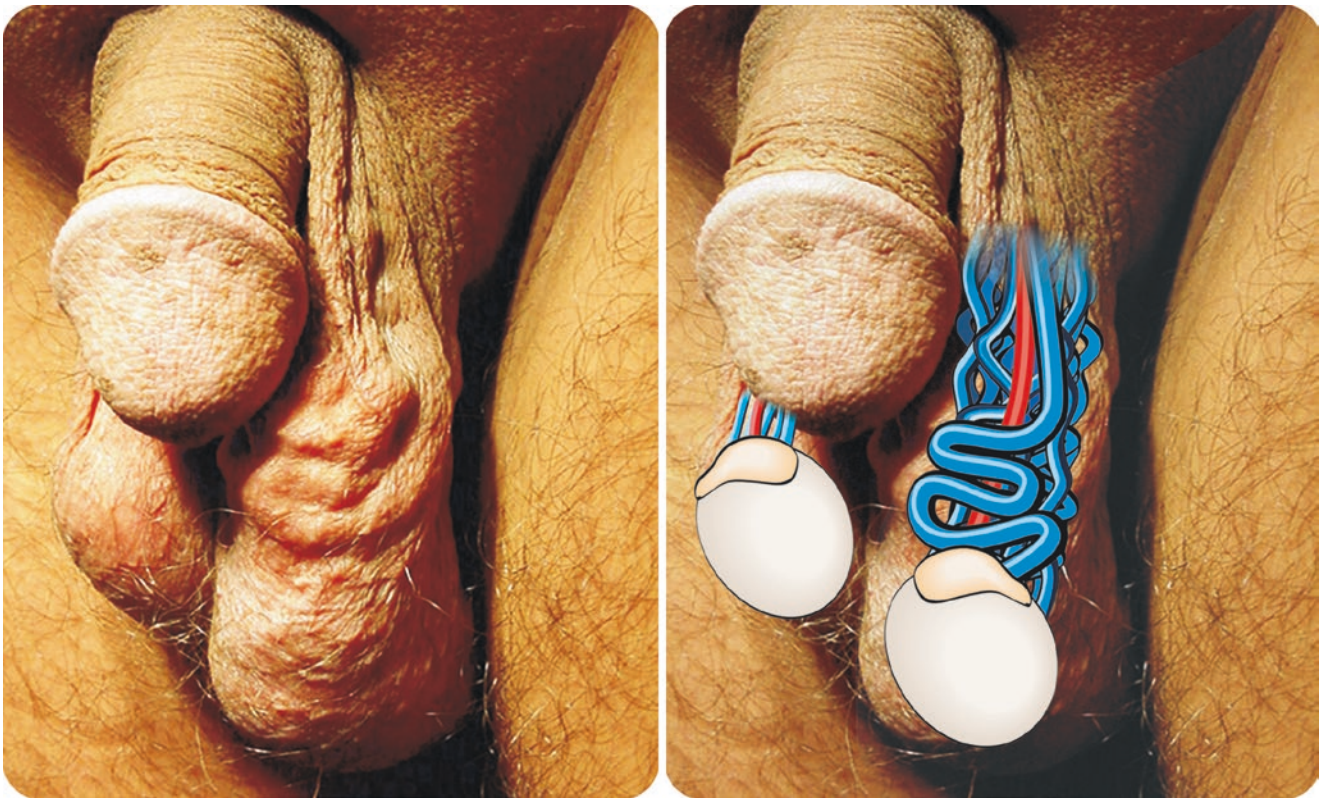

Fig. 31.2 Photograph of a large left varicocele (grade III) seen through the scrotal skin (left). The illustration depicts varicose veins on the left spermatic cord and normal-sized veins on the right side (right) (Reprinted with permission from ANDROFERT. All Rights Reserved)

diameter of 3 mm or greater) has a sensitivity of about 50% and specificity of 90% compared to physical examination [38].

Notably, a scoring system, incorporating the venous diameter, the presence of a venous plexus, and the change of flow on Valsalva maneuver, yields sensitivity and specificity greater than 85% when compared to physical examination [38] or venography [36]. Besides, a pencil-probe Doppler (9 MHz) stethoscope is an inexpensive tool that may aid in the diagnosis of the varicocele. The patient is examined in the upright position, and a venous "rush" representing blood reflux is heard with or without the Valsalva maneuver. Although simple and easily performed in the office, Hirsh et al. demonstrated that more than 50% of men without clinical varicoceles exhibited Valsalva-maneuver Doppler-positive reflux [39]. Despite that, Doppler examination has been advocated as a useful tool to examine the contralateral spermatic cord to determine if a subclinical varicocele exists when a clinical varicocele is found on the other side [40]. Unfortunately, none of these adjunctive diagnostic methods can differentiate between clinical and subclinical varicoceles. The significance of a positive test result using any of these adjuvant techniques in infertile men remains uncertain.

31.6 Treatment

The varicocele treatment in infertile men should aim to achieve the most significant improvement concerning the fertility status, with lower rates of complications such as recurrence or persistence, hydrocele formation, and testicular atrophy. Current recommendations suggest that treatment should be offered for couples with documented infertility whose male partner has a clinically palpable varicocele and abnormal semen analysis. Additionally, an adult male presenting with palpable varicocele and abnormal semen analyses who is not currently attempting to achieve conception but has a desire for future fertility is also a candidate for varicocele repair [41]. The ideal treatment must combine low complication rates with the highest seminal improvement to either increase the chances of natural conception or to optimize assisted conception outcomes. Along these lines, the ideal surgical technique should aim for ligation of all internal and external spermatic and cremasteric veins, with preservation of spermatic arteries and lymphatics. Only the inguinal or subinguinal microsurgical approaches allow the ligation of both the internal and external spermatic veins (Table 31.1).

An increase in natural pregnancy rates after the treatment of varicocele is difficult to ascertain due to a variety of factors

Table 31.1 Treatment results of varicocele repair in infertile men. Vein ligation sites, postoperative recurrence, hydrocele formation, and natural pregnancy rates among different techniques[a]

Technique	Internal spermatic vein ligation	External spermatic vein ligation	Recurrence rate	Hydrocele formation rate	Natural pregnancy rate
Retroperitoneal high ligation	Yes	No	7–35%	6–10%	25–55%
Laparoscopic	Yes	No	2–7%	0–9%	14–42%
Embolization	Yes	No	2–24%	NR	20–40%
Macroscopic inguinal	Yes	Yes	0–37%	7%	34–39%
Microscopic inguinal or subinguinal	Yes	Yes	0–0.3%	0–1.6%	33–56%

[a]Vein ligation sites, postoperative recurrence, hydrocele formation, and natural pregnancy rates among different techniques (values are expressed as range)

that includes the lack of a uniform posttreatment follow-up interval and, in particular, female factor confounders. Moreover, varicocele repair in men with inadequate lifestyle only solves part of the problem. Lifestyle modifications are likely beneficial in the context of fertility restoration in men with varicocele [42–44]. As such, when therapeutic measures to treat varicocele-associated infertility are considered, counseling toward lifestyle modifications should be strongly encouraged. This strategy, along with the cause-specific treatment, is more likely to lead to a marked improvement in the male reproductive health as compared to varicocele repair alone.

The role of medical therapy in varicocele-related infertility is poorly understood, and well-designed studies are rare. In one study, the use of L-carnitine in combination with the nonsteroidal anti-inflammatory agent cinnoxicam did not affect semen parameters of infertile men with clinical varicoceles [45]. Likewise, the use of clomiphene citrate in men with subclinical varicoceles showed no benefit [46]. By contrast, kallikrein therapy for three months (600 units orally per day) improved sperm motility and morphology in a small group of infertile men with left-sided varicocele and asthenozoospermia [47]. The use of menotropin in association with varicocelectomy yielded better improvement in sperm parameters than varicocelectomy alone [48]. Daily oral administration of pentoxifylline, zinc, and folic acid for three months was shown to improve sperm morphology in men with varicocele-associated infertility [49]. Also, a combination of vitamins and minerals was shown to significantly improve sperm count in men with persistent oligozoospermia after varicocele embolization, although natural conception rates were not increased in a one-year follow-up period [50].

Currently, varicoceles are treated either by surgery (open with or without magnification and laparoscopy) or percutaneous embolization of the internal spermatic vein. Although techniques vary, the central concept is the occlusion of the dilated veins of the pampiniform plexus. The high retroperitoneal (Palomo), radiologic, and laparoscopic approaches are performed for internal spermatic vein ligation, while the inguinal (Ivanissevich) and subinguinal approaches also allow the ligation of the external spermatic and cremasteric veins that may contribute to the varicocele (Table 31.1).

Percutaneous embolization is accomplished in approximately 90% of the attempts. It is associated with faster recovery and minimal pain as compared to the standard surgical approaches, but with higher recurrence rates (Table 31.1). Embolization requires interventional radiologic expertise and has potentially serious complications such as vascular perforation, coil migration, and thrombosis of pampiniform plexus [51–54]. Nonetheless, percutaneous embolization may have a role in the treatment of persistent or recurrent varicoceles previously treated by surgery [55].

Laparoscopic varicocelectomy provides higher magnification with a low incidence of hydrocele formation. However, external spermatic veins, the second most frequent cause of varicocele recurrence, are not ligated, leading to a recurrence rate of approximately 5% [52]. The laparoscopic approach requires extensive training, and the cost of instrumentation is high. It might be argued that it is more invasive than an open microsurgical approach, requiring general anesthesia and placement of a urethral catheter [56, 57]. Complications include intestinal and vascular injuries that occur in approximately 8% of the cases [52]. In a recent meta-analysis, microsurgery was compared to laparoscopy; the authors concluded that microsurgery resulted in a more considerable increase in sperm concentration and a significant decrease in hospital stay, complication, and recurrence rate [58].

Open surgical varicocele repair is often performed using a retroperitoneal, inguinal, or subinguinal approach (Fig. 31.3). High ligation of the internal spermatic vein can be easily performed via the retroperitoneal approach, but it is associated with high recurrence and hydrocele formation rates (Table 31.1). Inguinal and subinguinal approaches are advantageous as they allow the ligation of external spermatic veins. Internal and external spermatic veins can be identified via inguinal/subinguinal approaches macroscopically, but the use of magnification facilitates identification and preservation of internal spermatic artery and lymphatics, which may prevent testicular atrophy and hydrocele formation, respectively [59] (Fig. 31.4).

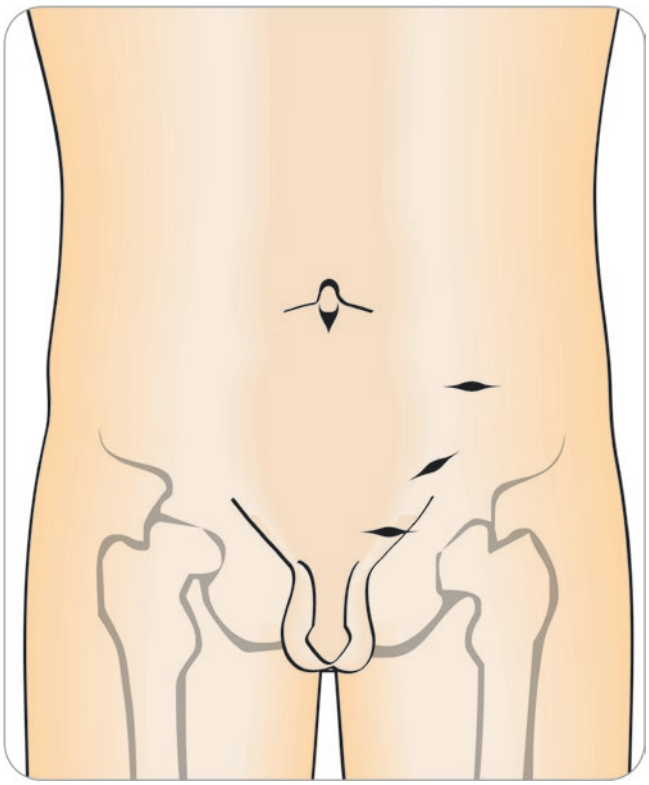

Fig. 31.3 Incision sites used for subinguinal, inguinal, and retroperitoneal open surgical varicocele repair. In the subinguinal approach, a transverse incision is made just below the level of the external inguinal ring. An oblique incision is made along the axis between the anterior superior iliac spine and the pubic tubercle for the inguinal approach. In the retroperitoneal approach, a transverse incision is made medial to the anterior superior iliac spine (Reprinted with permission from ANDROFERT. All Rights Reserved)

Microsurgical varicocelectomy can be performed via an inguinal or subinguinal approach with similar results; the reported recurrence and hydrocele formation with these approaches are below 2% (Table 31.1). The main advantage of the subinguinal over the inguinal approach is that the former obviates the need to open the aponeurosis of the external oblique, which usually results in more postoperative pain and a longer time before the patient can return to work (Figs. 31.3 and 31.4).

The urologist who opts to treat varicocele using microsurgery should obtain appropriate training. It is also essential to have adequate microsurgical instruments and a binocular operating microscope with foot-control zoom magnification. Microsurgical varicocelectomy, either using inguinal or subinguinal approaches, requires more skill as compared to other surgical modalities because a higher number of internal spermatic vein channels and smaller-diameter arteries are seen at the level of the inguinal canal. It is believed that subinguinal microsurgical varicocelectomy requires more microsurgery skills because it is associated with higher number of arteries and internal spermatic veins with a smaller diameter as compared to the inguinal approach [52]. However, histomorphological studies were unable to find differences in number and wall thickness of spermatic cord veins and arteries between the subinguinal and inguinal levels [60].

A systematic review including 4473 individuals was performed to define the best treatment modality of palpable varicocele in infertile men [52]. The authors concluded that open microsurgical inguinal or subinguinal varicocelectomy techniques resulted in higher natural pregnancy rates and fewer recurrences and postoperative complications than laparoscopic, radiologic embolization and macroscopic inguinal or retroperitoneal varicocelectomy techniques in infertile men.

Overall, varicocelectomy studies report significant improvements in one or more semen parameters in approximately 65% of men [61]. The mean time for semen improvement and natural pregnancy after surgery is approximately five and seven months, respectively [62]. However, it is still unknown why fertility potential is not always improved after varicocelectomy. Studies evaluating predictors for successful varicocele repair would aid in the identification of the best candidates for treatment, but to date, few reports exist, and results are conflicting [52, 61, 63–68]. From the existing data, it seems that infertile men either with higher preoperative semen parameters or undergoing varicocele repair for large varicoceles are more likely to show postoperative semen parameter improvement [61, 67].

Men who achieve a postoperative total motile sperm count greater than 20 million seem to be more likely to initiate a pregnancy either naturally or via intrauterine insemination [65]. On the other hand, reduced preoperative testicular volume, elevated serum FSH levels, diminished testosterone concentrations, and subclinical varicocele, as well as the presence of Y chromosome microdeletions, seem to be negative predictors for fertility improvement after surgery [21, 63, 64, 66, 68–70]. Notably, paternal age does not seem to have significant influence on the reproductive outcomes of men with varicocele-associated infertility [71]. In the presence of bilateral palpable varicocele, it is recommended to perform surgery on both sides at the same operative time [72].

In our practice, when a clinically palpable varicocele is identified in one side, the contralateral cord is examined using a pencil-probe Doppler (9 MHz) stethoscope to determine if a subclinical varicocele exists. If so, it is treated at the same time as the coexistent clinical varicocele. This strategy relies on the observation that altered blood flow after varicocelectomy may unmask an underlying venous anomaly and result in clinical varicocele formation [40, 73]. In the early years, we used loupe magnification to facilitate the ligation of the dilated varicose veins. However, this method is not good enough for identification of both testicular arteries and lymphatics. With loupes, we found that instillation of papaverine

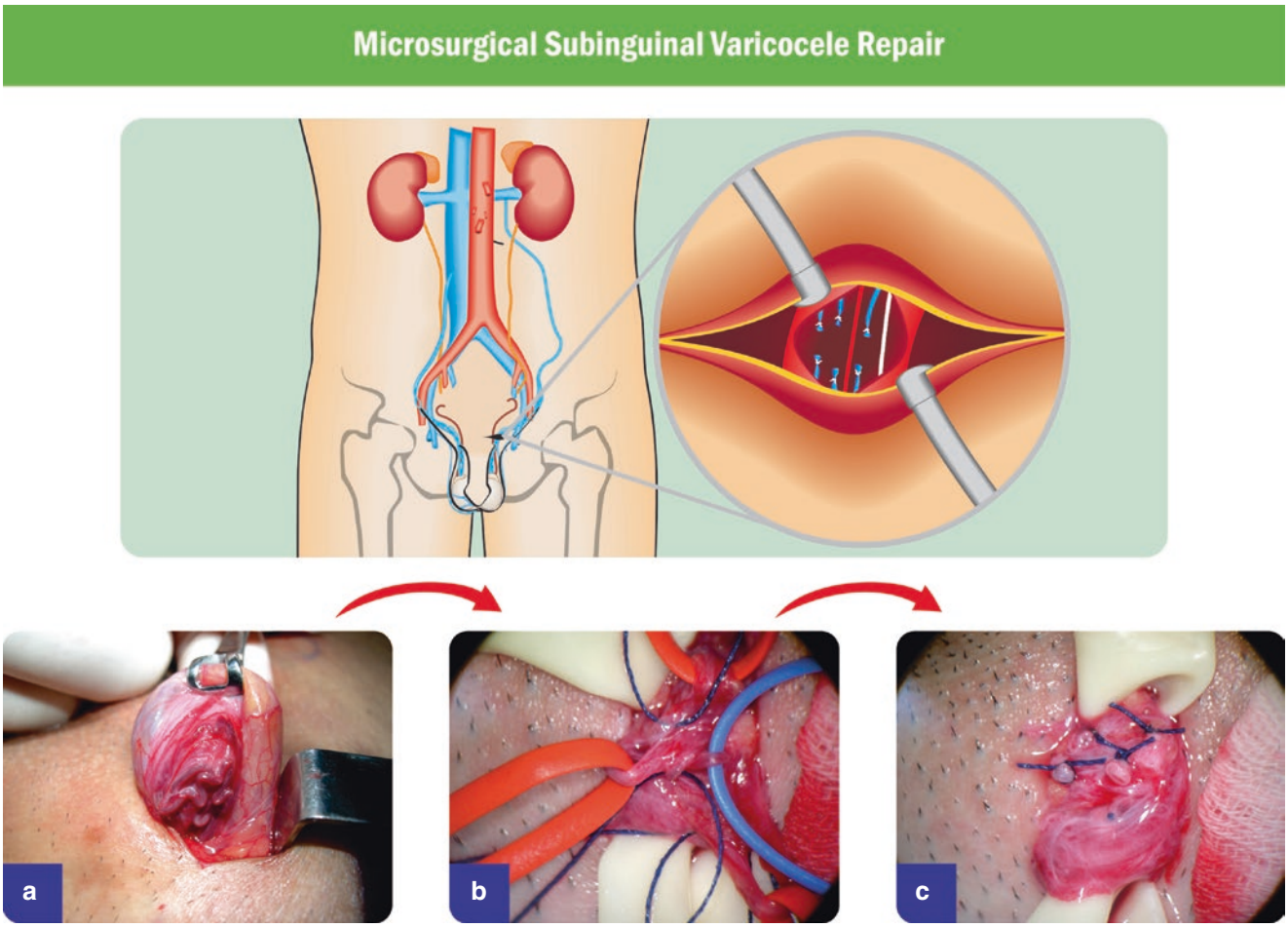

Fig. 31.4 Subinguinal microsurgical varicocele repair. A 2-cm transversal skin incision is made immediately below the external inguinal ring (upper). The muscle layers and the inguinal canal are not violated. The lower panels show intraoperative photographs of the spermatic cord structures. The spermatic cord is exteriorized and the cremasteric veins are identified and ligated (**a**). In panel (**b**), the spermatic cord is dissected to allowing the identification of testicular artery (blue vessel loop), dilated varicose veins (red vessel loops), and lymphatics (blue cotton sutures). The testicular artery and lymphatic channels are preserved, whereas the dilated veins are ligated with nonabsorbable sutures and then transected (**c**) (Reprinted with permission from ANDROFERT. All Rights Reserved)

was needed in most cases to aid in the identification of arterial pulsations, thus increasing the operative time. Also, recurrence seems to be higher when loupe magnification is used in association with the inguinal or subinguinal approach to repair varicoceles (Table 31.2). Our current surgical method is the subinguinal microsurgical varicocele repair with the use of an intraoperative pencil Doppler (9 MHz) to aid in the identification of the artery pulsations.

An earlier 2011 meta-analysis found that microsurgical techniques are associated with higher natural pregnancy rates (mean, 44.7%; range, 33.8%–51.5%) and reduced rates of hydrocele formation [74]. More recently, in 2017, these results were corroborated by Yuan et al. [75].

In later years, varicocele has also been treated using robotic surgery. In addition to the ergonomic benefits, the primary advantage of robotic surgery is tremor elimination which is typical for inexperienced surgeons performing microsurgical varicocele repair. However, the total cost of robotic treatment is still very high when compared to microsurgery, thus limiting its general clinical utility [76–79].

31.7 Subclinical Varicocele

Subclinical varicocele (SCV) refers to the presence of retrograde blood flow that cannot be detected by physical examination of the spermatic cord during Valsalva maneuver and requires adjunctive tests for diagnosis, such as Doppler examination, color Doppler ultrasound (CDUS), scrotal thermography, isotope imaging, or venography [35–39].

The role of subclinical varicocele as a cause of male infertility remains debatable. Currently, the evidence does not support the recommendation for treating infertile men with subclinical varicocele [32, 46, 69, 80]. The optimal

Table 31.2 Results of microsurgical subinguinal varicocelectomy in a group of 862 men with varicocele and infertility

Type of magnification	Loupes	Operating microscope
No. of procedures	101	761
Male age in years; mean (range)	32.4 (24.0–63.0)	36.1 (24.0–56.0)
Varicocele side; N (%)		
Unilateral Bilateral	51 (50.4) 50 (49.6)	266 (34.9) 495 (65.1)
Varicocele grade[a]; N (%)		
Grade I Grade II Grade III	14 (13.9) 48 (47.5) 39 (38.6)	3 (19.0) 199 (51.8) 112 (29.2)
Endocrine profile; mean ± SD		
Serum FSH (mUI/mL) Serum testosterone (ng/dL)	5.7 ± 8.8 523.8 ± 547.1	7.1 ± 6.2 398.1 ± 522.6
Mean operative time; minutes (range)		
Unilateral Bilateral	78.6 (50–90) 101.1 (80–150)	71.0 (45–92) 105.2 (85–140)
No. of veins ligated; mean (range)		
Left side Right side	4.8 (2–7) 4.2 (2–6)	8.2 (2–11) 6.2 (2–11)
Vein diameter in millimeters; mean (range)		
Left side Right side	3.2 (1–6) 2.8 (1–4)	3.0 (1–5) 2.6 (1–5)
Testicular artery identified; %	84.1[b]	96.5
Improvement in seminal parameters[c]; %	60.4	65.9
Recurrence rate; N (%)	3 (2.9)	9 (0.1)
Hydrocele formation rate; N (%)	1 (1.0)	1 (0.0)
Other complications; N (%)	2 (1.9)[d]	5 (0.0)[e]
Pregnancy; N Natural; N (%) IUI and ART; N (%)	85[f] 20/69 (28.9) 4/16 (25.0)	562[f] 180/562 (32.0) 140/282 (49.6)

[a]The largest varicocele grade is reported in cases of bilateral varicocele
[b]Instillation of papaverine for identification of artery pulsation needed in 85% of the cases
[c]≥15% improvement from baseline preoperative values in at least one of the semen parameters (sperm count, progressive motility, strict morphology)
[d]Testicular hematoma (1 case); testicular atrophy (1 case)
[e]Testicular/subinguinal hematoma or skin infection
[f]Reported number of patients who were assessed for pregnancy
IUI intrauterine insemination, *ART* assisted reproductive technology

management of infertile men with a unilateral clinical varicocele and a subclinical one at the contralateral side remains to be elucidated. Zheng et al. compared the efficacy of bilateral and left unilateral varicocelectomy in a group of 104 infertile men with left clinical and right subclinical varicoceles, and found that bilateral varicocelectomy had no benefit over the left clinical varicocelectomy [81]. In their study, however, a retroperitoneal approach was used for vein ligation, which was shown to be associated with a high recurrence rate [52]. Elbendary et al., in a prospective trial, studied a group of 145 infertile men with clinical left and subclinical right varicoceles [82]. Patients were randomized to undergo either unilateral inguinal repair of clinical varicocele or bilateral repair of both clinical and subclinical ones. Although a significant improvement in sperm parameters was observed in both groups, the magnitude of change in sperm count and motility and the natural pregnancy rates were significantly higher in the group of men who had bilateral varicocele repair. Their findings are in agreement with earlier studies suggesting that bilateral varicocelectomy is more effective than unilateral for such patients [73, 83]. It is also postulated that altered blood flow after unilateral clinical varicocelectomy may unmask an underlying contralateral venous anomaly that may result in a clinically manifested varicocele [40, 73].

As far as the subclinical varicocele is concerned, a 2018 meta-analysis showed that repairing SCV does not improve pregnancy outcomes, albeit it is associated with a slight increase in total motile sperm count [84]. Since most of the existing studies are retrospective, heterogeneous, and low powered, further research is needed to elucidate whether or not repairing SCV might add any benefit concerning fertility.

As already said, the subinguinal microsurgical varicocelectomy using the operating microscope is the method of choice to treat varicocele-associated infertility at our institution (Table 31.2 and Fig. 31.4). The subinguinal approach provides excellent results, and the surgical intervention can be performed on an outpatient basis using intravenous anesthesia in association with spermatic cord blockade with lidocaine [85].

31.8 Azoospermia

Nonobstructive azoospermia (NOA) comprises a spectrum of testicular histopathology resulting from various causes that include environmental toxins, medications, genetic and congenital abnormalities, trauma, endocrinologic disorders, and idiopathic disorders. Men with NOA have historically been the infertile men most difficult to treat, but since the advent of in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI) and surgical methods for testicular sperm extraction (TESE), many pregnancies have been achieved with the use of testicular sperm. However, only about 50% of men will have sperm present at the time of TESE [86].

Varicoceles are found in approximately 5% of men with azoospermia, but it is still debatable whether varicoceles can cause or contribute to azoospermia [85]. There has been a renewed interest in varicocele repair in azoospermic men resulting from the introduction of ICSI. Success rates vary, and no predictors of success have been definitively identified because of the small numbers in the case series [85, 87–93].

The first meta-analysis, published in 2010, examined the impact of varicocele repair to recover spermatogenesis in NOA men [94]. A total of 233 infertile men with clinical varicocele and NOA were analyzed in a mean postoperative follow-up of 13 months. Motile sperm was found on postoperative ejaculate in 39% of men. Pregnancies were achieved in approximately 26% of men with sperm in the ejaculate, 60% unassisted and 40% with the assistance of IVF. Postoperative mean sperm density and motility were 1.6 million and 20%, respectively. Levels of serum follicle-stimulating hormone (FSH) and testosterone, testis size, patient age, varicocele grade, and surgical technique did not appear to affect outcomes, but the limited number of patients precluded conclusions. Histopathology was the only predictor of success. Postoperative appearance of sperm in the ejaculate was significantly higher in patients with biopsy-proven hypospermatogenesis (HS) or maturation arrest (MA) than Sertoli cell only (odds ratio 9.4; 95% confidence interval [CI] 3.2–27.3). Combined success was 48% with HS or MA compared to 11% with SCO (Table 31.3). Unfortunately, randomized control trials are lacking, and studies included in the meta-analysis of Weedin et al. were case series without a control group that did not undergo varicocele repair. Although an argument can be made that a control group would remain azoospermic, it is not rare to observe that NOA men occasionally ejaculate small quantities of motile sperm despite any intervention. Therefore, one cannot exclude that the appearance of a minimal number of motile sperm in the ejaculates after varicocele repair may be merely coincidental.

Moreover, reports including men with germinal cell aplasia who ejaculated motile sperm after varicocele repair are intriguing [91]. It is unlikely that men lacking any sperm precursor within the testicle may benefit from treatment, but the only way to investigate this relationship is to repeat the testicular biopsy after the surgical repair of varicocele. In one series [85], patients with SCO were re-biopsied six months after varicocele repair, and testicular histopathology results remained unchanged. The persistence of SCO after surgery denotes that varicocele coexisted with primary testicular failure, which of course was not affected by the surgery. However, testicular biopsy has many limitations and may not reflect the most advanced site of spermatogenesis due to the heterogeneity of sperm production within the testicle; therefore, it is still possible to retrieve sperm from men whose testicles exhibit SCO [95].

Even with the improvement in spermatogenesis in up to half of the NOA patients with favorable testicular histopathology after varicocele repair, intracytoplasmic sperm injection (ICSI) will be necessary for most couples to initiate a pregnancy [85, 94]. However, the use of motile ejaculated sperm is preferred for ICSI, since their fertilizing ability is higher than that of sperm retrieved from the testis [96], except when the proportion of ejaculated sperm exhibiting DNA fragmentation is elevated [97].

Nonetheless, continuing azoospermia after varicocele repair is still a potential problem, and sperm extraction before ICSI will be inevitable for many individuals. Results of testicular sperm extraction (TESE) for men who remain azoospermic after varicocelectomy are scarce and conflicting [98, 99]. Schlegel et al. reported sperm retrieval (SR) rates of 60% per attempt using testicular microdissection (micro-TESE) in men with NOA and varicocele, regardless of whether the previous varicocelectomy had been done [99]. It is questionable, however, if the inclusion of patients with subclinical varicocele biased their results since the benefit of treating subclinical varicocele is debatable [69, 80]. On the other hand, Inci et al., also using micro-TESE, reported a 2.6-fold increase in the chances of retrieving testicular sperm for ICSI after repair of clinical varicoceles [98]. Unfortunately, testicular histopathology results were not available in their study. Therefore, it cannot be excluded that higher retrieval rates were obtained after varicocelectomy because of patients with favorable histopathology patterns for successful sperm retrievals, such as the ones exhibiting hypospermatogenesis or maturation arrest, which biased this group [100, 101].

In the past years, additional studies have been added to the literature. Zampieri et al. evaluated the effect of varicocelectomy in two different groups of patients. In one group (19 patients), the patients were subjected to TESE three months after the varicocele repair, whereas in the other group (16 patients), varicocele repair was carried out during microsurgical TESE. The sperm retrieval success rate was significantly higher in group 1 (57.8%) than in group 2 (25%, $p < 0.05$), thus suggesting that varicocelectomy might

Table 31.3 Results of meta-analysis on varicocele repair in azoospermic men

	Wendin et al. [94]	Esteves et al. [103]
No. of patients	233	344
Mean age	30.1	32.5
No. (%) of patients with sperm in postoperative ejaculate	91/233 (39.0)	151/344 (43.9)
Mean postoperative sperm count ($\times 10^6$/mL)	1.6 ± 1.2	1.8 ± 1.6
Interval for sperm appearance in ejaculates (months); range	NR	4.5–11.0
Outcome according to histopathology[a]		
Hypospermatogenesis Maturation arrest Sertoli cell only	30/55 (54.5) 24/57 (42.0) 5/44 (11.4)	27/48 (56.2) 18/51 (35.3) 6/62 (9.7)
Relapse to azoospermia; N (%)	11 (4.6)	NR
Natural pregnancy; N (%)[a]	14 (6.0)	12/88 (13.6)
Mean follow-up (months)	13.3	12.4

[a]Not all studies reported data

enhance the chances of retrieving sperm in selected men with NOA [102].

In a 2016 systematic review comprising 18 studies, we summarized the scientific knowledge regarding the effect of varicocelectomy in men with NOA (Table 31.3). The pooled estimates indicated a 2.6-fold increase in the sperm retrieval rate among treated patients compared to untreated patients. Moreover, a total of 44% of men subjected to varicocelectomy had sperm return to the ejaculate, thus potentially allowing ICSI to be performed without the need for testicular biopsy [103]. In the study mentioned above, there was a trend toward higher pregnancy rates and live birth rates by treating a varicocele before sperm retrieval in men with NOA. Our results have been corroborated by a concurrent meta-analysis [104]. Notwithstanding the above findings, further well-designed studies are needed to understand the real benefit of varicocelectomy in azoospermic patients better.

31.9 Hypogonadism

Since varicocele can negatively impact the testicular function, research has been conducted to investigate the role of varicocele repair on androgen production. Indeed, varicocele is associated with hypoxia on the Leydig cells and, consequently, reduction in testosterone production [105–107]. In a 1984 study, varicocele induced testicular hyperthermia and inhibited 17-alpha-hydroxyprogesterone aldolase, an enzyme involved in the conversion of 17-hydroxyprogesterone to testosterone [108]. In another study, it was shown that in the presence of varicocele, free radicals exerted deleterious effects on Leydig cell mitochondria, inhibiting the expression and function of the steroidogenic acute regulatory (StAR) protein, thus contributing to a reduction in testosterone [109].

In recent years, this matter has gained renewed interest as it has been suggested that testosterone levels are linked to the overall health condition, risk of diseases, and life expectancy. In a 2011 case–control study, Tanrikut et al. showed that men with clinical varicocele have significantly lower testosterone levels than those without varicocele. Moreover, the authors showed that testosterone levels increased after microsurgical varicocelectomy regardless of the age of the patient, varicocele grade, and laterality. The authors concluded that varicocele was an independent factor of androgen deficiency and its repair might provide beneficial effects concerning testosterone production [110].

Along the same lines, a 2018 review concluded that varicocelectomy is a real option to improve androgen levels in older hypogonadal males, in particular when one does not desire continuous testosterone therapy and continuous laboratory monitoring [111].

31.10 Oxidative Stress Markers

Infertile men with clinical varicocele have significantly lower sperm production and quality than normal controls and fertile men with varicocele [112]. Such differences are observed not only in the conventional sperm parameters, such as sperm count, motility, and morphology, but also in the novel functional markers for oxidative stress and DNA integrity.

Seminal plasma total antioxidant capacity (TAC) and sperm mitochondrial activity are decreased [27, 113, 114], while the frequency of sperm exhibiting abnormal DNA integrity and chromatin immaturity is increased [113, 115] in ejaculates of infertile men with varicocele. Although elevation of scrotal temperature is seen in both fertile and infertile men with varicocele, an increase of oxidative stress (OS) is only observed in the latter, thus indicating that a disturbance of the OS scavenging system is likely to play a significant role in the pathophysiology of varicocele-associated infertility [27, 116].

Novel noninvasive contrast imaging has shown that intratesticular microcirculation perfusion, which is altered in men with clinical varicocele, affects spermatogenesis [117]. Molecular biology studies show that high cadmium content and hypoxic conditions induce overexpression of metallothionein, a metal-binding protein that protects against cell apoptosis, in internal spermatic veins of infertile men with clinical varicocele [118]. Ichioka et al. determined the distribution of antioxidant enzyme gene genotypes in infertile men with varicocele. Their preliminary data suggest that genetic polymorphisms in the glutathione S-transferase T1 gene may affect individual response to varicocelectomy [119].

When clinically palpable varicocele coexists with impaired semen quality, surgical repair is the best treatment option. Varicocele repair can improve spermatogenesis and fertility, and it offers better cost-effectiveness as compared to ART [120]. Several meta-analyses demonstrated a beneficial effect of varicocelectomy on the fertility status of infertile men with clinical varicocele [112, 120–122]. Agarwal et al. examined the effect of varicocelectomy on semen parameters and demonstrated that sperm concentration increased by 9.7 million/mL (95% CI 7.34–12.08, $p < 0.001$), sperm motility increased by 9.9% (95% CI 4.90–14.95, $p < 0.001$), and WHO sperm morphology increased by 3.1% (95% CI 0.72–5.60, $p = 0.01$) after varicocelectomy [112]. Ficarra et al. reviewed randomized clinical trials for varicocele repair and found a significant increase in the pregnancy rates for patients who underwent varicocele treatment (36.4%) compared to ones having no treatment (20%) [121].

Similarly, Marmar et al. reported a significantly higher pregnancy rate (33%) after varicocelectomy compared to the group of patients having no surgery (15.5%) [122]. In their

study, the chances of obtaining a natural conception were 2.8 times higher in the varicocelectomy group as compared to the group of patients who received either no treatment or medication. Additional studies demonstrated that markers of sperm function were also significantly improved after varicocele repair [123–128]. It has been reported that seminal oxidative stress may be attenuated by varicocelectomy in infertile men with varicocele, but this beneficial effect is not always associated with an improvement in the conventional sperm parameters [124–126]. Sperm DNA integrity is also increased six months after repair in infertile men with clinically palpable varicocele [126, 128]. Smit et al. reported a decrease in sperm DNA fragmentation (SDF) after varicocelectomy and an association between DNA fragmentation index and the ability to conceive either naturally or via assisted reproduction [127].

In the last ten years, many studies investigated the role of varicocele repair to improve sperm chromatin integrity. A 2012 meta-analysis concluded that varicocelectomy alleviates sperm DNA fragmentation [129]. In a recent prospective controlled study, Aahathal et al. recruited 29 infertile men with abnormal seminal parameters, high DNA fragmentation index, and clinical varicocele. All of them underwent microsurgical varicocelectomy. The control group comprised of six normozoospermic healthy fertile men. The authors found that the surgical repair was associated with a significant decrease in sperm DNA fragmentation index, supporting the hypothesis that varicocelectomy improves spermatogenesis and reduces oxidative stress [130].

Recently, in 2018, we performed a recent systematic review to summarize the existing evidence concerning the role of varicocelectomy on sperm DNA fragmentation (SDF) [131]. We concluded that repair of clinical varicoceles was unequivocally associated with reduction of SDF. Among the studies evaluating pregnancy rates, partners of couples achieving pregnancy had lower SDF than counterparts who remained childless.

31.11 Varicocelectomy, ICSI, or Both?

Even though natural pregnancy remains the litmus test for evaluating varicocele treatment success, many patients with varicocele-related infertility will require ART due to the severity of sperm abnormalities and/or the presence of a significant problem affecting the female partner. The indication of varicocele repair before IVF/ICSI is still unusual, but increasing evidence indicates that varicocele repair before ART might be beneficial in selected case scenarios.

Varicocele repair in men with non-obstructive azoospermia with favorable testicular histopathology might help to increase the likelihood of finding sperm in ejaculates [94]. Although small sperm numbers are usually seen after varicocele repair in this case scenario, presence of sperm in the ejaculate allows the possibility of IVF/ICSI without the need for surgical sperm retrieval (SR). Moreover, SR success in patients who remain azoospermic after varicocelectomy increases, and as a result, the couple's chance of having a baby increases as well [98].

Varicocelectomy also has the potential to obviate the need for ART or to downstage the level of ART needed to bypass male factor infertility [123]. Esteves et al. showed that surgical treatment of clinical varicoceles might also improve the outcomes of assisted reproduction in couples with varicocele-related infertility [131]. The authors enrolled 242 infertile men with treated and untreated clinical varicoceles who underwent intracytoplasmic sperm injection (ICSI) and found significantly higher live birth rates after ICSI in the group of men who underwent microsurgical varicocele repair before ART (46.2%) as compared to the ones undergoing ICSI in the presence of a clinical varicocele (31.4%). In this study, the chances of achieving a live birth (odds ratio = 1.87; 95% CI 1.08–3.25; $p = 0.03$) by ICSI were increased, while the chance of miscarriage occurrence after obtaining a pregnancy by ICSI was reduced (odds ratio = 0.433; 95% CI 0.22–0.84; $p = 0.01$) if the varicocele had been treated before assisted conception.

A 2017 recent review clarified the role of varicocelectomy before ART. The authors concluded that varicocele repair could improve spermatogenesis and, therefore, decrease the complexity of the method of assisted conception needed to achieve a pregnancy [132].

31.12 Future Diagnostic Approach

Although the varicocele onset begins at puberty, the disease is rarely diagnosed at this stage. The reasons stem for the lack of awareness and the uncommon visit of the adolescent to the urological office.

Notwithstanding, varicocele is a time-progressive disease; thus, the sooner the diagnosis, the better the outcome concerning treatment to restore fertility and androgen production.

As far as the adolescent varicocele population is concerned, it is well known that just a small part of these individuals will show deterioration of their fertility potential in adulthood. Thus, it would be ideal to identify which adolescents benefitted from early surgical treatment. In this population, a semen analysis is not a reliable test for the evaluation of the varicocele detrimental effect because the hypothalamic-pituitary-testicular axis is still immature. Moreover, the current clinical criteria for recommending surgery based on testicular growth retardation are suboptimal, since these findings relate to an already inflicted testicular damage [133–135].

Since 2010, researchers have been focused on studying the seminal proteomic approach of adolescents and adults with varicocele. Indeed, both sperm and seminal plasma are rich in proteins [136, 137].

Proteomics is a new field of study that aims to analyze globally the set of proteins expressed in a cell or tissue, i.e., the proteome. Protein expression is dynamic and can vary according to the conditions that the environment imposes at a given moment. Therefore, the study of proteins can generate critical information, including the understanding of which proteins are expressed, their levels of expression, when they are produced, and the responses expressed by the cells in different situations or treatments. The study of the proteome represents a way to evaluate protein functions and to investigate metabolic processes to better understand the functioning of a cell or tissue at the molecular level [138–140].

The search for a protein marker in the seminal plasma, an early indicator of impairment to spermatogenesis, could make possible to recommend varicocele repair before it inflicts damage to testicular function as a whole and spermatogenesis in particular. The discovery of specific biological protein marker might be used as a routine during the workup of adolescents and adults with varicocele to understand better who are at higher risk of infertility, thus allowing better clinical management [141, 142].

31.13 Conclusions

Varicocele is a common disease affecting males with fertility issues. The central pathophysiology mechanism of varicocele-related infertility involves excessive oxidative stress, which impairs sperm quality and quantity through a variety of nonexclusive mechanisms. The diagnosis of varicocele mainly relies on the physical examination since improvements in fertility endpoints after varicocele treatment are typically seen only among men with clinically palpable varicoceles. Among treatment modalities, microsurgical varicocele repair is the method of choice due to the achievement of better sperm quantity/quality with fewer complications. Varicocele repair has been recommended to selected men with non-azoospermia and before assisted reproductive technology (ART) to increase success rates of sperm retrieval and ART outcomes, respectively. Notwithstanding, it is still unknown why many men remain fertile despite a varicocele and why improvement after varicocele repair is not universal. Given the complex nature of varicocele-related infertility, counseling toward lifestyle modifications should be strongly encouraged in addition to varicocele treatment.

Besides fertility, varicocele repair can be offered to men with clinical varicocele and hypogonadism as a means to improve androgen production and potentially avoid testosterone replacement therapy. Emerging tools, including proteomics studies, might help identify the ideal candidates for varicocele treatment.

31.14 Review Criteria

A systematic search using PubMed/Medline, Scielo, EMBASE, and Google Scholar was performed to identify eligible studies. The following terms and descriptors were used: "varicocele," "microsurgery," "oxidative stress," "sperm DNA fragmentation," "adolescents," "testosterone," "proteomics," "infertility," "azoospermia," "subclinical varicocele," "varicocele repair," and "varicocele treatment." We also carried out a search using the PubMed Search Builder for the following words: "varicocele/surgery," "varicocele/diagnosis," and "varicocele/classification." The newest and more relevant studies were used to update our chapter for the second edition of this textbook.

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Infection in Infertility

32

Ralf Henkel

Key Points

- Male infertility is a major issue of concern as it affects more people during their lifetime as a common disease, diabetes mellitus.
- Among these infertile men, the prevalence of male genital tract infection is reportedly between 20% and 40%.
- Many pathogens, including bacteria, protozoa and viruses, are sexually transmitted and require treatment of the couple.
- Among the sexually transmitted bacteria, infections with *C. trachomatis* and mycoplasms are most prevalent, while *E. coli* causes the most prevalent non-sexually transmitted male genital tract infection.
- Among the viral infections, mumps and HIV infections are the most important as mumps can cause permanent infertility due to mumps orchitis and HIV can be carried by spermatozoa from the testis or epididymis.

32.1 Introduction

Globally, due to population growth, the absolute numbers of couples suffering from infertility has increased from about 42 million in 1990 to 48–50 million in 2010 [1], which is an estimated 15% of couples. The male partners are solely responsible for the infertility in 20–30% of the cases with an overall contribution of 50% [2]. Although the percentages of primary and secondary infertility did not change in most parts of the world [1], infertility has to be regarded as a serious global health issue. On the other hand, human fecundity is reportedly declining in both developed and developing countries [3–5]. Reasons for this remarkable decline are manifold and include socio-economic changes [4], changes in lifestyle with higher prevalence of obesity [6, 7] or environmental pollution [8]. In general, data on the prevalence of infertility, i.e. the inability of a sexually active, non-contracepting couple to achieve pregnancy within 1 year's time [9], vary considerably between 3% and 25%, of which 15% seek for medical assistance [10, 11]. Infertility is a couple problem as both male and female partner contribute more or less equally, with prevalence reported for male infertility between 30% and 50% [12]. Approximately 7% of all men are confronted with fertility problems during their reproductive lifetime, thus making male infertility a problem, which has an even higher prevalence than diabetes mellitus with an overall estimate of 2.8% in the year 2000 and 4.4% in 2030, and which is considered a common disease [13, 14].

However, according to Article 16 (1) of the United Nations Universal Declaration of Human Rights, 'Men and women of full age, without any limitation due to race, nationality or religion have the right to marry and found a family' [15] enshrines the human right of healthy reproduction. In turn, this requires clinicians and scientists to find solutions to this increasing problem. Potentially correctable causes of male infertility are genital tract infections [16], which play a major role in male infertility. Infections and inflammations are not only seriously affecting spermatogenesis and sperm transit during ejaculation as can be seen in clinical findings in cases of oligozoospermia, asthenozoospermia or azoospermia [17, 18] but also the cause of dysfunctional male accessory glands [16] and significantly impaired sperm functions [19, 20]. These changes can be triggered in various ways, namely direct action of the pathogens on spermatozoa and sperm functions [21] or indirectly by inducing inflammatory processes in the seminal tract by activating leukocytes [22]. Globally, the prevalence of male genital tract infection-related infertility varies between 10% and 20% in non-selected cases and amounts up to 35% in a large study comprising more than 4000 patients consulting for infertility

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Table 32.1 Diagnostic methods of several pathogens. A variety of different diagnostic methods are generally available. However, some of the laboratory methods are challenging and a general lack of standardization can be noticed

Pathogen	Diagnostic methods	References
Chlamydia trachomatis	Clinical presentation of symptoms *C. trachomatis* culture with immune-fluorescent staining of reticulate bodies	
	PCR amplification of bacterial rDNA in semen	[25, 26]
	Nucleic acid hybridization tests	[27]
Mycoplasma hominis	Real-time PCR	[28]
M. genitalium	PCR	[29–32]
Ureaplasma urealyticum	Standard semen culture PCR should be the diagnostic method of choice	[26]
U. parvum	Standard semen culture, PCR	
Neisseria gonorrhoeae	Clinical presentation Gonococcal culture	[33, 34]
	Nucleic acid hybridization tests	[27]
Escherichia coli		
Viruses	Clinical presentation of symptoms	
CMV	PCR	[35]
HSV	PCR	[36]
HIV	PCR	
Protozoa *Trichomonas vaginalis*	PCR	[37]
Treponema pallidum *Trypanosoma brucei* *T. cruzi*	PCR	[38]
Schistosoma spp.	Serological tests	[39]
	PCR	[40]

[23]. It also appears that bacterial infections have a more detrimental effect in fertility-compromised patients than in fertile men [24], indicating that the impact of such bacterial genital tract infections may have to be differentiated.

From a diagnostic point of view, a number of techniques including the microscopic, molecular and serological tests are available. However, these laboratory methods are sometimes challenging and lack standardization (Table 32.1).

32.2 Pathogens Causing Male Genital Tract Infections

Male urogenital tract infections can be classified according to the kind of microorganism causing the infection and the location, namely the testis (orchitis), epididymis (epididymitis), prostate (prostatitis) or urethra (urethritis). These pathogens can be bacteria, viruses, protozoa or even parasites. The most prevalent bacterial pathogens are *Chlamydia trachomatis*, *Ureaplasma urealyticum*, *Neisseria gonorrhoeae*, *Mycoplasma hominis*, *Mycoplasma genitalium* or *Escherichia coli* [41]. While the former bacterial pathogens are sexually transmitted, *E. coli* is regarded as the most common cause of non-sexually transmitted urogenital tract infection, particularly of epididymo-orchitis or prostatitis where it is the cause of 65–80% of the cases [42]. Furthermore, viral infections like mumps virus, human papillomavirus (HPV), herpes simplex virus (HSV) and particularly human immunodeficiency virus (HIV) have also been associated with increased seminal leukocyte concentrations [43]. The latter virus can infect the testes and male sex accessory glands [44]. With the recent outbreak of Zika virus infections in French Polynesia in 2013 and in Brazil in 2015, a new threat for human reproduction has emerged [45].

The pathological mechanisms involved in these infections include not only the attraction of leukocytes but also the release of toxic substances. All of these increase the release of reactive oxygen species (ROS) and thus oxidative stress in the whole body including the male reproductive system. Consequently, spermatogenesis and sperm functions are compromised leading to sub- or infertility (Fig. 32.1).

32.3 *Chlamydia trachomatis*

C. trachomatis is a Gram-negative bacterium that is worldwide one of the most frequently sexually transmitted bacterial pathogens, accounting for an estimated 92 million new urogenital infections per year [47]. This number may be underestimated because of the high asymptomatic nature of the pathogen, with approximately 70–80% of women and up to 50% of men infected being asymptomatic [48]. Yet, in males, this infection can become even more symptomatic than in females [49]. Reportedly, in symptomatic men, the prevalence of infections varies from 4% to 10% [50]. Another report found a prevalence of up to 35.9% in men and 38% in women [51]. Since a high number of chlamydial infections remain undiagnosed, the pathogen can even be transferred to the newborn during delivery accounting for 25–50% of conjunctivitis and 10–20% of pneumonia in newborns, thus posing a health risk on the offspring as well as enormous costs for diagnosis and treatment on countries' health systems. For the United States, these costs estimate to $2.2 million for each 500 cases [48].

In men, *C. trachomatis* has been associated with prostatitis, epididymitis and urethritis. The bacterium has been detected in the testis including Leydig cells [52], the prostate [53] and even epididymis and seminal vesicles [54]. Apart from the lesions triggered by the infection and the implications and acute inflammation can cause in the male genital

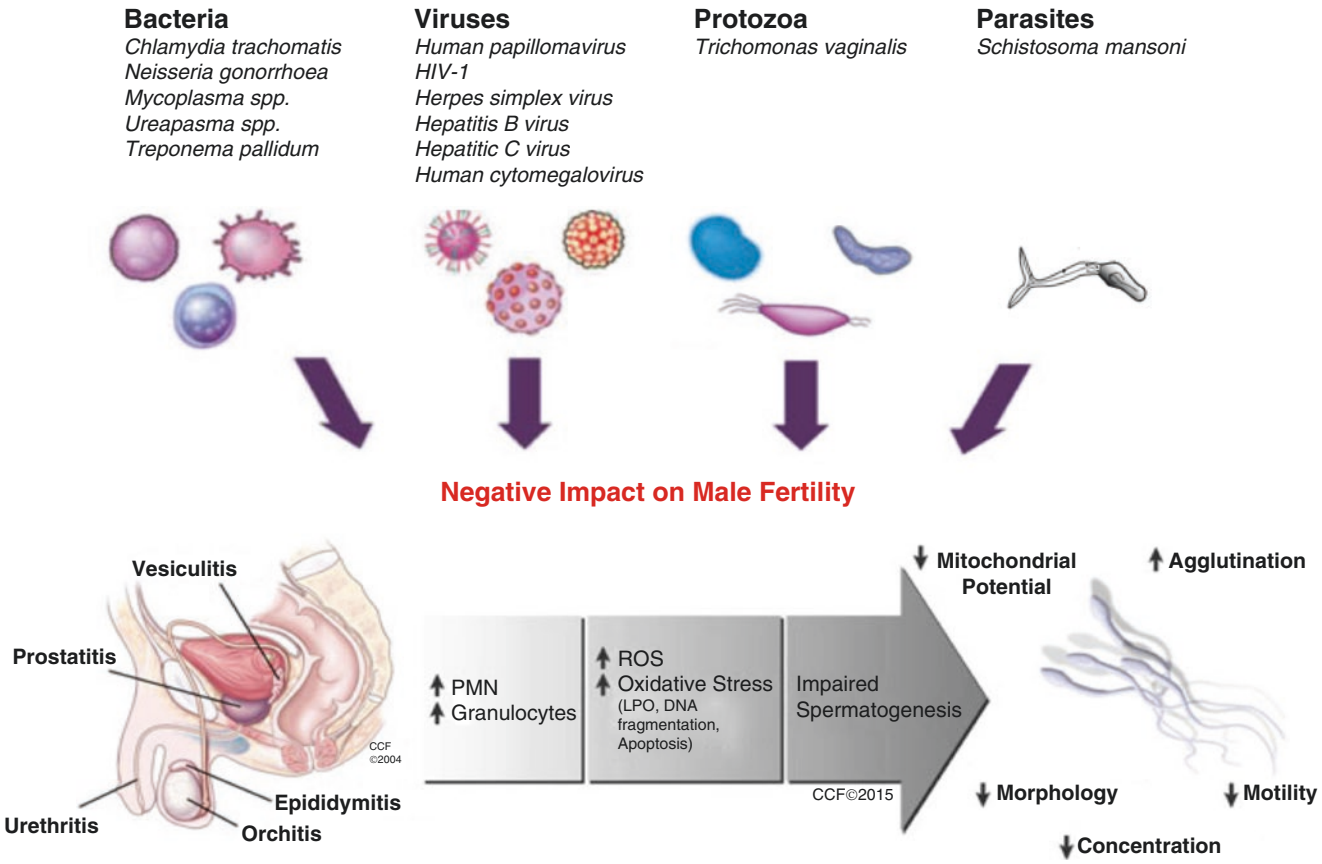

Fig. 32.1 Impact of various pathogens on male reproductive functions leading to low sperm count, motility and compromised sperm functions. (Adapted from Ref. [46] with permission from Springer Nature)

tract, reports on the influence of chlamydial infections on male fertility are inconsistent. While some authors [55, 56] found no significant association, most others have shown a direct negative influence of the chlamydial infection on male fertility [53, 57, 58]. The direct effect on sperm parameters such as sperm concentration, viability, normal sperm morphology and motility is also controversially reported with most studies showing clear negative effects [59–62]. Even acrosome reaction appears to be negatively affected in *Chlamydia*-infected males [63, 64].

In vitro studies by Hosseinzadeh et al. [65–67] even indicate that the pathogen directly causes changes in sperm proteins and premature cell death induced by lipopolysaccharides (LPS) secreted by Chlamydiae. These LPS can bind to the 54 kD CD14 glycolipid-anchored receptor which is implicated in the regulation of T- and B-cell activation and function [68]. LPS are also reported to trigger ROS production, which, in turn, cause a decrease in sperm motility [69].

The discrepancies in findings may be due to the fact that in numerous cases, chlamydial infections are accompanied by other microbial infections making a clear association of infertility with the chlamydial infection difficult [70]. Besides, the duration of the infection can also influence the results of the studies; the method by which the infection is detected might also be important [64] as various detection methods for the pathogen in asymptomatic cases appear challenging, and especially the sensitivity and specificity of serological markers were regarded as problematic [48]. For that reason, recent studies suggest polymerase chain reaction (PCR) amplification of bacterial rDNA in semen as this approach seems to be promising especially to identify asymptomatic patients [25, 71].

32.4 Mycoplasms

Mycoplasms and ureaplasms belong to the family of *Mycoplasmataceae*, which are widely distributed in vertebrate species. They are the smallest bacteria replicating in culture and are lacking a cell wall [72, 73]. Five species colonize the male urogenital tract and may contaminate the semen during ejaculation, namely *Ureaplasma urealyticum*, *U. parvum*, *Mycoplasma hominis*, *M. genitalium* and *M. fermentans*, of which only the first four are pathogenic.

32.5 *Ureaplasma urealyticum, U. parvum*

Both these microorganisms normally colonize the male urethra and contaminate ejaculates during the ejaculation [74]. Although both *U. urealyticum* and *U. parvum* are pathogenic species, it is particularly *U. urealyticum* that is reported to cause non-gonococcal urethritis, pelvic inflammatory disease or infertility [16, 75, 76]. Incidences of infection between the two pathogens vary considerably between 5% and 42% for *U. urealyticum* [77] and 2.9% for *U. parvum* in a Tunisian population [51]. There is no difference in the effect of the infections by either *U. urealyticum* or *U. parvum* on seminal parameters such as volume, pH, sperm concentration or motility [51]. Yet, in preparation for assisted reproduction in the embryology lab care must be taken with the sperm separation since simple washing of sperm does not entirely remove these bacteria from the sperm suspension [78].

In the past, the association between ureaplasma infections and male infertility was discussed controversially [79, 80]. In a recent large study including 346 selected subjects, Wang et al. [81] showed that ureaplasma infections cause higher seminal viscosity, decreased sperm concentrations and lower pH. Although this study could not find any further effect on other sperm parameters, Potts et al. [80] found significantly increased seminal reactive oxygen species (ROS) levels and Reichart et al. [82] higher levels of sperm DNA damage. These discrepancies might be due to the fact that *U. urealyticum* shows a distinct energy metabolism-dependent effect on sperm activity [83]. According to this theory, sperm motility is impaired by the bacterium at low pH since it competes with sperm mitochondrial energy production. On the other hand, at higher pH, sperm motility will even be enhanced as ureaplasma stimulates glycolysis.

The prevalence of male genital ureaplasma infections among infertile men varies considerably from 10% to 40% [84], most probably as a result of different diagnostic methods used in various studies and different population groups examined. Like in patients with chlamydial infections, detection of *U. urealyticum* and mycoplasms is particularly problematic in asymptomatic patients because these patients may shed fewer colony-forming units (CFU; organisms) to be detected in a standard culture assay. Therefore, PCR should be the diagnostic method of choice [26].

32.6 *Mycoplasma hominis, M. genitalium*

Although the direct impact of these two bacteria on male fertility was debated in earlier years, recent reports associate *Mycoplasma hominis* and *M. genitalium* with genitourinary infections and infertility [85, 86]. Nevertheless, it appears that the pathological mechanism is elusive [87]. Reportedly, frequencies of infection are with 10.8% for *M. hominis* and 5% for *M. genitalium*, respectively, lower than for other pathogens [76]. However, for patients presenting with recurrent urethritis, *M. genitalium* seems to be with 19–41% the predominant pathogen [88]. Both species can attach to and penetrate human sperm plasma membrane [89, 90], which might have a significant long-term impact on male fertility on the onset of pregnancy and the health of the offspring. In the case of *M. hominis*, this could be due to dysfunctional spermatozoa [77]. While the first may contribute to the distribution of the bacterium to the female to cause cervicitis and endometritis [91] or an alteration of the plasma membrane affecting acrosome reaction [92], the latter might particularly be caused by the sperm DNA damage triggered by the infection [57].

32.7 *Neisseria gonorrhoeae*

These Gram-negative, immotile cocci grow in pairs (diplococci) and cause one of the most common infectious diseases in men leading to urethritis, prostatitis and epididymitis which in turn may impair male fertility. These bacteria have pili on their surface, which facilitate attachment to other cells [93]. In sperm, an asialoglycoprotein receptor has been identified that recognizes and binds lipopolysaccharides in gonococcal membranes [94]. Since chlamydial lipopolysaccharides can cause death of human sperm by inducing apoptosis [95], it is conceivable that Neisseria lipopolysaccharides might also induce such reactions. Yet, this has not been shown thus far.

Even though its incidence has been declining in Western countries during the past decades, 150–400 new infections per 100,000 are still recorded in Europe per year [93]. Presumably, due to socio-economic and behavioural factors, these numbers are much higher in Third World and developing countries, with highest numbers in Sub-Saharan Africa and South and Southeast Asia [96]. Imudia et al. [97] found a prevalence of 0.4% for *N. gonorrhoeae* infections in women presenting for infertility treatment. Considering that this pathogen is sexually transmitted, one can assume that the prevalence in the male partners would be similar. However, according to a newer study by Abusarah and co-workers [77], the prevalence of *N. gonorrhoeae* infections is about 6.5% in infertile men while no infection was diagnosed.

32.8 *Escherichia coli*

E. coli is a Gram-negative bacterium that belongs to the family of *Enterobacteriaceae* and is responsible for most urogenital tract and male accessory gland infections between 65% and 80% of type I and type II prostatitis [98] with patients presenting with type II prostatitis having signifi-

cantly lower sperm vitality and total and progressively motility, while seminal volume and sperm concentration were not affected [99]. In contrast to other uropathogens like *Enterococcus* or *Staphylococcus saprophyticus*, *E. coli* has significant direct negative effects on sperm motility [100]. An in vitro study by Köhn et al. [92] revealed that this microbe can even negatively affect sperm functions like acrosome reaction. This might be due to morphological alterations, particularly on the acrosome and flagellum, seen after exposure of human sperm to the pathogen [101]. Studies by Fraczek et al. [102, 103] show that in patients with seminal bacterial contamination (*E. coli*, *Bacteroides ureolyticus*), not only the sperm membrane stability may be compromised but also mitochondrial membrane potential and sperm DNA integrity [104]. Since the latter effect is even more pronounced in sperm fractions after density gradient centrifugation, inflammatory mediators might be directly responsible for the damage [103]. Another reason for the impaired fertility may be an epididymal obstruction [105] caused by uropathogenic *E. coli* [106].

Due to the direct interaction of bacterial pili with the sperm plasma membrane [107], *E. coli* is interfering with sperm motility [108]. A study by Schulz and co-workers [109] demonstrated two mechanisms by which *E. coli* affects spermatozoa, the described direct interaction and by action of soluble factors that induce apoptosis and a breakdown in the mitochondrial membrane potential. Potential candidate substances causing these cellular reactions might be α-haemolysin and Shiga-like toxin as these have already been associated with sperm motility loss [110] and apoptosis in Hep-2 cells [111], respectively. In a mouse model, Kaur and Prabha [112] found that sperm-agglutinating *E. coli* rendered infected females infertile.

32.9 Viruses

A number of viruses are also able to infect all parts of the male genital system, namely the testes (e.g. mumps virus, HIV-1), epididymis (e.g. Coxsackie virus), seminal vesicle (e.g. cytomegalovirus [CMV]), prostate (e.g. HPV, HSV, HIV-1) and the semen (e.g. HSV, HPV, HIV) [113]. In a recent study including 241 asymptomatic patients attending an infertility clinic for semen analysis, viral DNA was detected in 45 patients: CMV (8.7%), HPV (4.5%), HHV-6 (3.7%), HSV (3.6%), Epstein–Barr virus (0.4%) and hepatitis B virus (0.0%) [114]. Recently, another viral infection made headlines as it not only replicates and persists in the male reproductive tract but was also detected in semen 6 months after the infection: the Zika virus [115, 116].

While some of the viral infections like HSV or HIV-1 are associated with poor semen and sperm quality [43, 117], this could not be confirmed for CMV and human herpes virus type 6 (HHV6) [114]. Thus, the latter viruses appear not to cause male infertility. However, in patients with viral infections affecting male fertility, leukocytospermia (>1 × 10^6 leukocytes/ml ejaculate) is strongly associated with the infection as well as with elevated levels of inflammatory markers like polymorphonuclear (PMN)-elastase or interleukins [114].

A matter of concern is the high number of globally more than 65 million people infected with HIV, out of which a growing number of HIV-positive people are seeking for assisted reproduction to have children without infecting the partner or the offspring. Since a few years, some IVF centres started treating infected couples and received acceptable pregnancy rates, the worst in couples where both partners were infected [118]. In these patients, not only the risk of vertical transmission from a seropositive mother to her unborn child has to be dealt with but also the fact that semen is a vector of viral propagation and sperm can bind and incorporate the virus via a CD4-independent receptor and/or the HIV co-receptor CCR5 [119, 120]. Despite it being generally accepted that motile sperm are not productively infected by HIV, sperm can carry viral particles deriving from the testis or epididymis [121, 122]. In view of the fact that seminal leukocytes shed different viral strains than those in the blood [123], the question rises whether infected leukocytes and free virions contaminating the semen are of different origin, and an infected testis might represent a special reservoir for the virus as this area is resistant to antiviral drugs due to the blood–testis barrier [122]. Therefore, special care must be taken when separating sperm for assisted reproduction, particularly for intracytoplasmic sperm injection (ICSI).

Studies on Zika infections confirm the risks of replication in the male reproductive system [124]. Most Zika virus patients remain asymptomatic; however, patients can suffer from fever, myalgia, arthralgia and conjunctivitis [125]. Although the disease is benign in most cases, death was reported in chronically sick patients, and its implications should not be underestimated [126]. The virus causes testicular damage, testicular inflammation and epididymitis in a male mouse model [127, 128]. In pregnant women, congenital microcephaly and Guillain–Barre syndrome (GBS) have been reported in the offspring [129, 130].

Transmission of this virus occurs mostly via mosquitoes, especially *Aedes aegypti* and *Aedes albopictus*. However, sexual [131, 132] and perinatal [133] transmissions have also been observed. Recently, Zika virus has been detected in the amniotic fluid and umbilical cord blood after conception by in vitro fertilization [134]. However, additional testing for Zika before assisted reproduction does not exclude the risk of getting infected during pregnancy. One also has to consider that the risk of getting infected is high, but the risk of microcephaly in the offspring would not be higher than the risk of a miscarriage or birth defects due to other pathogens [135].

32.10 Protozoa

The most common non-viral sexually transmitted disease is trichomoniasis. *Trichomonas vaginalis*, a motile flagellated non-invasive parasitic protozoon, infects about 248 million people every year [136]. Despite this high number of new annual infections, trichomoniasis is only poorly studied with respect to its virulence properties, pathogenesis and immunopathogenesis [137]. *T. vaginalis* is regarded as a relatively rare cause of male infertility [138] with cohort-dependent prevalences between 0.009% and 38.8% [139]. In the male, the protozoon inhabits the urethra, epididymis and the prostate and is regarded a major cause of prostatitis with *T. vaginalis* being the cause in 10.5–19% [140, 141]. The pathogen has also been associated with balanoposthitis, epididymo-orchitis and non-gonococcal urethritis [142]. In prostate tissue from patients with benign prostatic hyperplasia, a high detection rate was observed [143]. The interaction of *T. vaginalis* with spermatozoa seems to be direct via adhesion and phagocytosis of sperm [144] as well as via the secretory extracellular polymeric substances from *T. vaginalis*, which significantly decreased motility, viability and functional integrity and thereby decreased fertilization rate in a mouse model [145].

32.11 *Treponema pallidum*

Treponema pallidum is a highly contagious pathogen causing syphilis, and despite relatively cheap antibiotics being available, there is still an excess of 10 million people globally infected with about 30% of the patients developing tertiary syphilis [146]. However, the reasons how the pathogen can evade a patient's immunological response is still not clear [147]. On the one hand, *T. pallidum* has a sufficient amount of lipoproteins to activate macrophages and dendritic cells [148–152]. On the other hand, pathogen-associated molecular patterns are not freely available to Toll-like receptors to activate the host's innate immune system, because these lipoproteins are not readily exposed on the outer surface of the bacterium [147]. The restricted exposure of the pathogen to innate and adaptive immune response is not sufficient to keep the pathogen under control, thus resulting in a substantial number of bacteria evading leukocytotic phagocytosis [147].

Despite *T. pallidum* infecting the male genital system, a direct detrimental effect has not been reported. Nevertheless, numerous complications affecting male fertility are described. Among these, epididymitis, chronic obstructive endarteritis and interstitial inflammation are the most common and can lead to small, fibrotic testicles [153].

32.12 *Trypanosoma spp.*

In this context, two species of trypanosomes are important to mention, *T. brucei*, the pathogen of sleeping sickness, and *T. cruzi*, the pathogen of the Chagas disease, affecting estimates of 70 million and 18 million people, respectively [154, 155].

With respect to male fertility, clinical signs of an infection with *T. brucei* include scrotal dermatitis, orchitis, periorchitis, thrombosis of the pampiniform plexus vessels, degeneration of the seminiferous tubules and testicular degeneration. In addition, studies have shown that trypanosomes can damage the pituitary, thereby affecting the hypothalamus–pituitary–gonadal axis [156] with patients demonstrating pituitary fibrosis being described [157], which, in turn, can lead to significant impairment of spermatogenesis and hypogonadism [155]. Impotence and decreased libido have been reported in 70% and 45.5% of the cases [158].

For *T. cruzi* causing Chagas disease, very little has been published, and it seems that the effects on the male reproductive system are similar to that caused by *T. brucei*. In a study by Lamano Carvalho et al. [159] including 34 patients, 35% presented with oligo- or azoospermia. The same group reported a decrease in Leydig cell numbers [160]. Moreira et al. [161] reported no changes in the LH levels in patients with Chagas disease with testosterone levels being normal in some patients. Yet, individual LH levels in the patient group receiving clomiphene citrate were abnormally scattered suggesting hypothalamic dysfunction similar to that in *T. brucei* infections [162]. More recent reports on *T. cruzi* effects on the reproductive system report rather on female reproduction as the parasite reduces female fertility and induces foetal death in a mouse model [163].

32.13 *Schistosoma spp.*

Another group of parasites that can also cause male infertility is *Schistosoma*, flatworms of the genus of trematodes, which are responsible for schistosomiasis (bilharziasis), a disease, which, following malaria, has devastating socio-economic consequences [164]. Although this disease is affecting globally more than 230 million people [165], not many reports are published on its impact on male fertility. In 1995, a case of a traveller returning from Africa and South America was diagnosed having had a *Schistosoma haematobium* infection in the genital tract with *Schistosoma* ova microscopically demonstrated in the semen [166]. The authors concluded that infertility may develop in cases with Schistosoma infection of the genital organs. Omer [167] reported that 13% of infertile Sudanese men with oligozoo- or azoospermia had a history of schistosomiasis, which is due to testicular infarction with partial or complete occlusion

of the spermatic venous plexus by the parasites' ova and subsequent granuloma formation [168]. Abdel-Naser et al. [169] report another case of azoospermia due to schistosomiasis.

32.14 Male Genital Tract Infections

32.14.1 Orchitis

As per definition by the European Association of Urology, the term *orchitis* describes an inflammatory lesion of the testicle, which is associated with a leukocytic exudate inside and outside the seminiferous tubules, resulting in tubular damage [17]. The condition can be a reason for spermatogenic arrest and testicular atrophy leading to low seminal sperm concentration and poor sperm quality [17, 170]. An acute infectious orchitis is characterized by an abrupt onset of severe pain with visible swelling of the affected testicle as well as of the inguinal lymph nodes and can be accompanied by fever. Further symptoms are similar to those of testicular torsion and can include haematuria and blood in the ejaculate. Subacute and chronic inflammatory conditions, however, often remain asymptomatic [171]. Yet, an orchitis may lead to intratesticular obstruction, which is the case in about 15% of obstructive azoospermia [17].

In contrast, non-infectious inflammations of the testis may occur in patients with testicular seminoma where predominantly CD8-positive T-lymphocytes infiltrate the tumour tissue, and macrophages are found in the fibrovascular septae and at the periphery [18]. Such infiltration of activated T-lymphocytes into testicular tissue is also indicative of a significant disturbance of the local immunoregulation [172]. Thus, due to an impaired blood–testis barrier, these inflammatory cells overcome the testicular immunosuppressive mechanisms and the formation of anti-sperm autoantibodies under such conditions would be conceivable, particularly in cases of prolonged inflammatory processes as in chronic orchitis [173]. On the other hand, except for a few cases with positive titres for autoantibodies in patients with a mumps history, only little evidence is available for this relationship [174, 175].

As reported in a large study, the prevalence of an isolated orchitis is 0.42% among relatively low testicular pathologies [176]. However, due to retrograde ascending infectious lesions, a 'non-specific' orchitis triggered by *Pneumococcus* spp., *Salmonella* spp., *Klebsiella* spp. or *Haemophilus influenza* is in most cases associated with an epididymitis as epididymo-orchitis. The close vicinity of the different compartments as well as the ascending nature of the infections makes a distinction between inflammations, i.e. isolated epididymitis vs. epididymo-orchitis, very difficult in the clinical routine [173].

Sexually transmitted bacteria like *C. trachomatis* and *N. gonorrhoeae* are the cause of the acute infection in men younger than 35 years, while *E. coli* is the predominant trigger in older men [18]. On the other hand, an orchitis can also occur after haematogenous dissemination of pathogens like the Coxsackie B or the mumps virus [17] as a complication of a systemic viral infection. For instance, the mumps virus may affect the testes (mumps orchitis) in 20–30% [177] and lead to infertility in 13% of the cases with unilateral orchitis and in 30–87% of patients with bilateral orchitis [178, 179]. However, clinicians should realize that an epididymo-orchitis can also develop secondary to the mumps infection, even in the absence of a parotitis [180]. While bacterial infections as described above cause a 'non-specific' orchitis, *Mycobacterium tuberculosis*, *M. leprae*, *Treponema pallidum* or *Brucella* spp. may cause a 'specific', predominantly granulomatous orchitis [17].

32.14.2 Epididymitis

Epididymitis, an inflammation of the epididymis, is a painful, feverish, almost unilateral condition with tender swelling of epididymis and scrotum. Based on the duration of the symptoms, epididymitis may be sub-classified as acute, subacute and chronic. In the latter, symptoms are present for more than 6 weeks. In cases of acute infectious epididymitis, an involvement of the testicle due to ascending canalicular bacterial infections can represent a complication which may occur in up to 60% of affected patients as epididymo-orchitis [181]. Although up to 35% of patients consulting for fertility problems present with male genital tract infections [23, 182], data on the prevalence of epididymitis/epididymo-orchitis vary considerably from 0.29% of all consultations [183] to 20% of all urologic admissions in an US Army setup [184].

As for orchitis, *C. trachomatis* and *N. gonorrhoeae* are the most common cause of epididymitis in sexually active men younger than 35 years. In contrast, Gram-negative *Enterobacteriaceae*, of which *E. coli* is the predominant pathogen, are aetiologically responsible for the disease in older men [16, 181], which are also particularly at risk of having urethral strictures, bladder neck obstruction or benign prostate hyperplasia (BPH) resulting in increased voiding pressure to empty the bladder resulting in a reflux of contaminated urine into the excurrent genital ducts and subsequent infection [185].

Potential risk factors for epididymitis include sexual activity, strenuous activities like lifting heavy goods, bicycle and motorbike riding or extended periods of sitting at job or travelling [186]. Even traumatic events such as accidents or scrotal traumas and iatrogenic injury to the epididymis during surgeries can be a cause of epididymitis.

Major problems for male fertility may arise particularly in patients with epididymitis as this disease appears to have a greater influence on semen quality and male fertility than an infection/inflammation of the prostate or seminal vesicle [187]. In addition, in quite a number of patients, the diagnosis of chronic epididymitis is extremely difficult as these patients do not feel discomfort and their health is not compromised [173]. Due to a *silent* nature of the infection/inflammation, epididymitis will only be diagnosed once these patients appear in an andrological clinic consulting for infertility. Eventually, inflammatory lesions of the epididymis can result in dysfunction of the organ and ultimately in obstructive azoospermia, which is the most common cause for this condition [17].

In patients with an acute epididymitis, a semen analysis is not recommended [17]. In cases of chronic epididymitis, semen parameters may be dramatically affected with lower sperm count, motility and seminal α-glucosidase. In contrary, many patients can present with leukocytospermia, i.e. leukocyte count of more than 1×10^6/ml [188], elevated seminal levels of polymorphonuclear granulocyte elastase and atypically stained sperm flagella [173].

32.14.3 Prostatitis

Despite the numbers of urological consultations for prostatitis outnumbered those for benign prostate hyperplasia or prostate cancer [189], prostatitis has been called 'the third most important disease of the prostate gland' [190]. Epidemiological studies revealed an estimated prevalence of 4–11% for prostatitis, which then represents the most common urological diagnosis in men younger than 50 years [191]. A large study with more than 600 men included suggests that only about 5–10% of these cases are of bacterial origin [192]. Yet, more recently, Bjerklund Johansen et al. [193] and Nickel et al. [194] showed that only about 50% of all patients with chronic prostatitis (bacterial and abacterial) respond positively to antibiotic treatment.

According to the classification system suggested by Drach et al. [195], prostatitis was divided into four clinical syndromes, namely, acute, chronic bacterial and chronic abacterial prostatitis and prostatodynia. Considering that this system was never validated and left clinicians confused about diagnostic and therapeutic strategies, particularly since many cases without infection have pathogenic processes outside the prostate, the National Institutes of Health (NIH) introduced a new system classifying prostatitis [196, 197] (Table 32.2). Even though many clinicians diagnose 'prostatitis', it rather represents diverse clinical symptoms ranging from acute bacterial infection to chronic pelvic pain and should actually be referred to as 'prostatitis syndrome' as patients present with a variety of urogenital, perineal and perianal complaints [190, 198].

Table 32.2 NIH classification and definition of the categories of prostatitis

NIH classification		Definition
Category I	Acute bacterial prostatitis	Acute infection of the prostate gland
Category II	Chronic bacterial prostatitis	Recurrent infection of the prostate
Category III	Chronic abacterial prostatitis/CPPS	No demonstrable infection
Category IIIA	Inflammatory CPPS	White blood cells in semen/EPS/voided bladder urine-3 (VB-3 or postprostatic massage)
Category IIIB	Non-inflammatory CPPS	No white blood cells in semen/EPS/VB-3
Category IV	Asymptomatic inflammatory prostatitis	No subjective symptoms detected either by prostate biopsy or the presence of white blood cells in EPS/semen during evaluation for other disorders

Considering the various problems around understanding prostatitis and its diagnosis [199], particularly chronic bacterial prostatitis, it is obvious that the diagnosis of an acute infection does not pose a problem for urologists. The symptoms for the acute bacterial prostatitis are quite clear; the patients are presenting with an urosepsis, fever, obstructive voiding symptoms and local pelvic pain [200]. In about 80% of acute bacterial prostatitis, *E. coli* can be identified as pathogen, while *Pseudomonas aeruginosa*, *Klebsiella* or Enterococci are the cause in the remaining patients [201]. In cases of chronic prostatitis, *C. trachomatis* (19.3%), *T. vaginalis* (10.2%), *E. coli* (8.1%) and Enterococcus (6.1%) were the cause of the disease in the majority of the cases where bacterial contamination could be detected. Other pathogens were *U. urealyticum, Proteus mirabilis, Streptococcus agalactiae, Klebsiella pneumonia* and *Pseudomonas aruginosa*, while in 42.3% of the case no pathogen was detected [202].

Nevertheless, there are major concerns differentiating the category II from category III prostatitis. Many of these patients have a history of recurrent urinary tract infections and are asymptomatic in non-infectious intervals. If the bacterial culture is positive for an established uropathogen like *E. coli* or *Klebsiella* spp., the diagnosis is unproblematic [196] (Table 32.3). However, the classic classification scheme might fail in cases where enterococci or anaerobes are identified in prostate specimens. Even more so in patients who had initially positive cultures but are negative at the time of recurrent symptoms. Apparently, in these cases, the detection is false negative because the bacterial colonization of the prostate can be veiled as bacteria can form microcolonies or aggregates, which are surrounded by a thick protective layer [196, 202].

Table 32.3 Uropathogens causing chronic prostatitis

Established pathogens	Potential pathogens
Escherichia coli	*Staphylococcus saprophyticus*
Klebsiella pneumonia	*Staphylococcus aureus*
Proteus mirabilis	*Staphylococcus epidermidis*
Pseudomonas aeruginosa	*Mycoplasma genitalium*
Enterococcus faecalis	*Ureaplasma urealyticum*
Chlamydia trachomatis	
Trichomonas vaginalis	

Based on data from Ref. [203]

32.14.4 Urethritis

Urethritis is the infectious or non-infectious inflammation of the urethra. While non-infectious causes include injuries through traumas, masturbation, manipulation by the patient or medical treatments, acute infectious urethritis may be caused by known sexually transmitted uropathogens like *C. trachomatis*, *Mycoplasms* or *N. gonorrhoeae* with incidences of 15–26%, 10–21% and 0.4–18%, respectively. In addition, among the not sexually transmitted pathogens, *Enterobacteriaceae* and staphylococci are causing the disease with frequencies between 20% and 31% [204]. Chronic urethritis is a rare condition, which is why the prevalence is not known [205].

The clinical symptoms of an acute urethritis vary considerably. While some patients present with distinct urethral discharge and dysuria, others are symptom-free or only show some pus prior to the first voiding of urine in the morning, which may be occur in 5–10% of the patients. As can be seen for the symptoms, clinical findings also vary from inflammatory stickiness, redness and swelling at the glans penis or the urethral orifice to the absence of any clinical sign. Normally, the infection remains localized to the urethra. However, ascension of gonococci may occur in about 1% of the infected patients causing epididymitis [204].

The impact of urethritis on male fertility is debatable, particularly since the inflammatory discharge present in the anterior urethra makes an ejaculate analysis impossible as the pus contaminates the ejaculate [206] and both direct effect of bacteria [20, 67, 109, 207] and leukocytes [69, 208] demonstrated detrimental effects on sperm functions. On the other hand, obstruction due to urethral stricture or as a result of lesions in the area of the seminal colliculus may result in ejaculatory disturbances [17].

32.15 Male Accessory Gland Infection

According to definition, the male accessory glands comprise the prostate, seminal vesicles and the Cowper's glands (bulbourethral glands). However, in many publications, the term *male accessory gland infection* (MAGI) describes the clinical symptoms of the inflammation as a result of canalicularily ascending infections of the male accessory glands including the deferent duct and the epididymis via the urethra as 'prostate-seminal vesiculitis', 'epididymo-prostato-vesiculitis' or 'male adnexitis' as long as urethritis or a urinary tract infection has been excluded [209]. Considering that in MAGI these organs are commonly inflamed, clear distinctions between prostatitis, epididymitis and glandulitis vesiculitis cannot be made [93]. General symptoms of MAGI are leukocytospermia (more than 10^6 peroxidase-positive leukocytes/ml), elevated seminal levels of polymorphonuclear granulocyte elastase (≥230 ng/ml), C3c complement (≥0.01 mg/ml), ROS and cytokines [210–212].

As a result of the leukocyte infiltration due to the infection as well as the elevated concentrations of pro-inflammatory cytokines like interleukin-6 (IL-6), IL-8 or tumour necrosis factor-α (TNF-α) into the genital system, sperm functions may be compromised by directly affecting sperm function and intensifying the level of oxidative stress, respectively [208, 213, 214]. As a result of the inflammatory processes in the male accessory glands, their secretory function may be impaired, consequently resulting in decreased seminal concentrations of citric acid, fructose, α-glucosidase, phosphatase and zinc [93, 215, 216].

In addition, there are concerns that patients presenting with MAGI are at a higher risk of developing sperm autoantibodies due to the inflammatory processes compromising the immune barrier [217, 218]. Furthermore, like in orchitis or epididymitis, stenosis or obstruction of the excurrent ducts may occur.

32.16 Consequences of Infections on Sperm Fertilizing Capacity

Apart from the specific effects on male fertility described above, male genital tract infections and inflammations cause general reactions that also negatively affect sperm fertilizing capacity by compromising specific sperm functions. In the light of spermatozoa being the most polarized cells in the body, the male germ cell has to maintain its extreme polarization, for which one of the most important prerequisites is a highly fluid plasma membrane. Therefore, sperm cells contain an extraordinary high amount of polyunsaturated fatty acids (PUFA), particularly docosahexaenoic acid, which has six double bonds in its molecule [219]. Since most sperm functions are dependent on membrane functionality, this high content of PUFA is also essential for normal sperm function and respective disturbances result in a loss of sperm function.

As a result of a urogenital tract infection, activated leukocytes infiltrate the infected organs releasing high amounts of ROS and cytokines such as IL-6, IL-8 or TNF-α as inflammatory mediators [220, 221]. Both ROS and cytokines have been shown to be associated not only with the impairment of sperm functions like motility but also to DNA damage and infertility by induction and stimulation of membrane lipid peroxidation through oxidative stress [212, 222–225]. Through this mechanism, male genital tract infections/inflammations do not only damage sperm DNA and reduce sperm count and seminal volume but also impair sperm functions like motility, acrosome reaction or acrosin activity [92, 208, 226–230].

32.17 Treatment of Infections

Generally, the first choice of treatment of male genital tract infections has to be antibiotic in order to eradicate pathogenic microorganisms, normalize inflammatory parameters, prevent transmission to the female partner and decrease the risk of potential complications. Considering that many urogenital tract infections are sexually transmitted, however, simultaneous treatment of the partner has to be considered, particularly in *C. trachomatis* infections. While standardized recommendations only exist for the treatment of acute bacterial epididymitis, epididymo-orchitis and specific granulomatous orchitis [17, 231], guidelines for the treatment of chronic infections and inflammations of the male genital tract have not been drawn up yet and are rather empirical and only few uncontrolled studies are available [17, 181]. For mumps orchitis, the systemic treatment with α-2β-interferon may be considered to prevent testicular atrophy and azoospermia [232].

The very same is true for acute bacterial and chronic bacterial prostatitis. While an antibiotic treatment for these conditions is mandatory, the benefit for patients with inflammatory chronic pelvic pain syndrome is questionable [193]. Still, treatment of the prostatitis syndrome with antibiotics poses the major problem of the penetrability of the agents into the prostate and its secretions, and only a few modern antibiotics like fluoroquinolones have the chemical properties to enter these compartments well [233, 234]. Yet, depending on the pathogen, an adequate treatment regime should be started soon after proper examination which should also include bacteriological testing of the semen including antibiotic resistance [235].

In order to alleviate the inflammatory lesions, therapy with both corticosteroids and non-steroidal antiphlogistic substances has shown considerable positive effects on semen quality in terms of sperm and leukocyte count and sperm motility [236–238]. Furthermore, antioxidative therapies with vitamins and/or antioxidant supplementations to reduce the oxidative stress caused by leukocytes and defective spermatozoa are currently highly debated [239, 240]. Although several studies with various antioxidants alone or in combination have shown a significant reduction in seminal ROS levels [241–243] and improvement in sperm count and motility [244–246], other studies found the opposite [247, 248]. Therefore, notwithstanding the indubitable positive effects of an antioxidative supplementation for general health purposes, no definite recommendation can be made at this point in time with regard to the treatment of male genital tract infections. Most probably, it is not only the administration of singular antioxidative substances that causes the beneficial effects but the combination of different antioxidants at very specific concentrations.

32.18 Conclusion

The purpose of this chapter was to discuss the contribution of male genital tract infections/inflammations to male infertility. Considering that many of these male genital tract infections are sexually transmitted, the knowledge of its impact on the female partner as well as treatment of the couple is mandatory. Moreover, as there is still a lack of knowledge about the impact of such infections on sperm functions, this chapter is dealing with impaired sperm functions as a result of the infection/inflammation. Since many patients suffer from asymptomatic, the so-called 'silent' infections, it is essential for the clinician to identify these conditions and also to urge the patients to continue with the treatment long enough. Appropriate treatment is particularly a problem in prostatitis as only few drugs penetrate the prostate and its secretions sufficiently. Therefore, this chapter is also to give an up-to-date overview on the impact of different male genital tract infections/inflammations on sperm functions and various treatment options.

32.19 Review Criteria

An extensive search of studies examining the impact of infections on male fertility was performed using search engines such as Google Scholar and PubMed. The searches were not limited for time. Yet, the most recent records were preferred. The overall strategy for study identification and data extraction was based on the following keywords: 'infection', 'oxidative stress', '*Schistosoma*', 'male infertility', 'HIV', '*Chlamydia*', '*E. coli*', '*Treponema*', '*Trypanosoma*', '*Neisseria*', '*Mycoplasma*', 'Zika' and '*Ureaplasma*' as well as the names of most common male genital tract infections. Articles published in languages other than English were also

considered. Data that were solely published in conference or meeting proceedings, websites or books were not included. Websites and book-chapter citations provide conceptual content only.

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Ejaculatory Dysfunction and Vasodynamics

33

Paul J. Turek

Key Points

- A thorough medical and sexual history and physical examination are fundamental to defining the various behavioral and physiological bases for human ejaculatory disorders.
- There is a growing trend to develop consensus-based, standardized definitions of ejaculatory disorders that should help to more precisely define these conditions in the future.
- The anatomical and physiological bases for ejaculatory duct obstruction and spinal cord injury are clear, but disorders of ejaculatory latency are currently the subject of intensive research to delineate their fundamental underlying pathophysiology.
- Both partial and complete ejaculatory duct obstructions are absolutely definable based on published vasodynamic criteria. Likewise, ejaculatory duct obstruction is distinguishable from seminal vesicle dysfunction.
- The psychosocial aspects of sexual and ejaculatory dysfunction are integral to human sexual behavior and, as such, will require ongoing consideration if our management of these conditions is to improve in the future.

33.1 Introduction

Ejaculation begins approximately 12 months after the onset of puberty in the male. While its importance to reproductive fitness is clear, current knowledge of the physiology of ejaculation is limited. This chapter will review the events of ejaculation, its anatomic and neuroanatomic underpinnings, the range of ejaculatory disorders, and clinical methods for evaluating and treating ejaculatory disorders. Our current understanding of reproductive tract physiology and function as well as ejaculatory duct obstruction will also be reviewed.

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33.2 Physiology of Ejaculation

33.2.1 The Events

Ejaculation is two distinct processes: emission and ejaculation [1]. Although not technically considered a separate event, pre-ejaculation, occurring during foreplay, involves closure of the bladder neck that prevents retrograde ejaculation and contractions of the prostate that lubricate the urethra. Importantly, ejaculation is also distinct from orgasm, which is a purely cerebral cortical event. Most often, these two processes are coincident.

Emission combines the transport of both seminal fluid and sperm through peristalsis from the cauda epididymis, vas deferens, seminal vesicles, and prostate into the prostatic urethra. During seminal emission, the ampullary vasa deferentia contents are transported into the prostatic urethra and mixed with prostatic fluid. The expulsion of seminal vesicle contents into the prostatic urethra completes the emission phase. Subsequently, ejaculation is the forceful expulsion of the seminal mixture from the urethra. The ejaculate is expelled from the urethra in a series of spurts, 0.8 seconds apart, caused by the rhythmic contractions of the ischiocavernosus, bulbospongiosus, and other associated periurethral muscles [2]. The entire process is governed by the autonomic and somatic nervous systems and is considered a spinal reflex.

33.2.2 Neural Control

Control of the ejaculatory reflex is mediated by the sympathetic and somatic nervous systems [3]. Control of emission involves mainly the sympathetic nervous system, while

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ejaculation is governed largely by the somatic nervous system. Efferent sympathetic nerves emerge from the thoracolumbar spine at T10–L2 and then merge to form the lumbar sympathetic ganglia that encircle the aorta. These nerves subsequently combine in the midline below the aortic bifurcation to form the superior hypogastric plexus (Fig. 33.1). Ultimately, these adrenergic nerves terminate as postganglionic fibers that innervate the bladder neck, prostate, vasa deferentia, and seminal vesicles [4]. The sympathetic outflow generated by these nerves is responsible for closure of the bladder neck and seminal emission.

The muscular expulsion of the ejaculate is mediated by somatic motor efferent nerves derived from the perineal branch of the pudendal nerve (S2–S4). Additional control is provided by relaxation of the external urethral sphincter and the urogenital diaphragm. Interruption at any point in this reflex arc may result in disordered ejaculation.

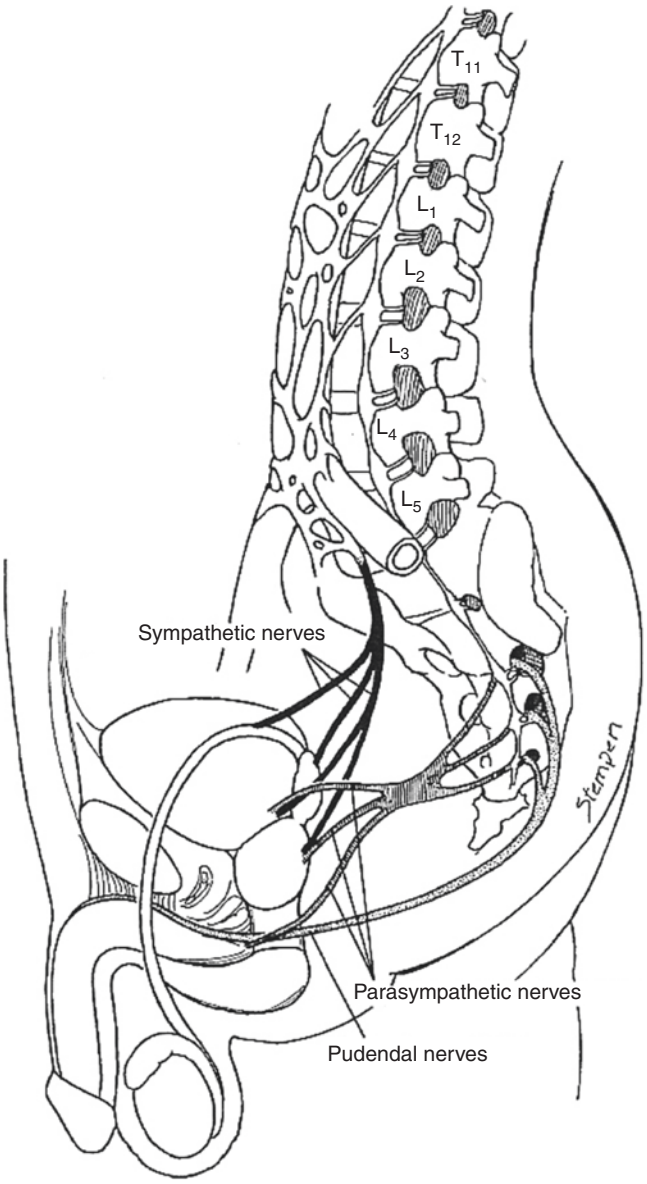

Fig. 33.1 Innervation of the male reproductive tract showing sympathetic and parasympathetic nerves and somatic innervation (Reprinted from Master and Turek [1]. With permission from Elsevier)

33.2.3 Definitions

Aspermia: Disordered ejaculation characterized by an inability to produce semen, despite the occurrence of climax.

Azoospermia: The absence of sperm in the ejaculate.

Anejaculation: The failure of ejaculation, including an absence of seminal emission and ejaculation. Climax is usually absent as well.

Premature ejaculation (*ejaculatio praecox*): Ejaculation that occurs sooner than desired, either before or shortly after penetration, causing distress to either one or both partners.

Delayed ejaculation (anorgasmia): A form of sexual dysfunction characterized by the inability to achieve climax or an extreme delay in achieving climax and ejaculation.

Retrograde ejaculation: Ejaculation of semen in reverse direction into the bladder during climax due to failure of bladder neck closure.

Congenital anorgasmia: Failure of ejaculation as primary, lifelong event.

Ejaculatory anhedonia: Ejaculation associated with a lack of pleasure.

33.3 Evaluation

33.3.1 History

The cornerstone of evaluating ejaculatory dysfunction is a detailed patient history. Critical to obtain from the history is whether or not normal ejaculation was ever present in the past. This differentiates a primary (congenital) or secondary (acquired) cause of sexual dysfunction. In addition, a thorough review of the medical and surgical history can be informative regarding possible neurologic etiologies for disordered ejaculation. Finally, a careful review of current medications such as alpha-blockers or antidepressants can suggest the presence of drug-induced ejaculatory dysfunction [1].

33.3.2 Physical Examination

A complete physical examination should include an assessment of body habitus and secondary sex characteristics, a screening neurological examination, and a thorough genital examination. Testis and epididymis size and consistency,

penile length and morphology, and genital birth defects such as hypospadias, epispadias, or surgical scars suggestive of their correction should be noted. Palpation of scrotum for masses and a check for the presence of the vas deferens should also be performed. A rectal examination noting rectal tone and any masses is also important.

33.3.3 Laboratory Evaluation

An attempt should be made to procure a semen analysis. In cases where no ejaculate is obtained, a post-ejaculate urine sample should be retrieved and assessed for the presence of sperm, suggesting retrograde ejaculation. Blood testosterone, prolactin, and serum luteinizing hormone (LH) levels should also be assessed because low ejaculate volumes may be caused by hypoandrogenism. Further diagnostic evaluation may include imaging with transrectal ultrasonography (TRUS) to define anatomical or structural abnormalities in the prostate, seminal vesicles, or ejaculatory duct complex. If indicated, formal ejaculatory duct chromotubation, seminal vesiculography, and ejaculatory duct manometry can be performed to detect subtle ejaculatory duct abnormalities [5].

33.3.4 Genetic Testing

Patients with disordered ejaculation and a history of infertility or with suspected congenital abnormalities such as congenital absence of the vas deferens or ejaculatory duct obstruction should be counseled on appropriate genetic testing for cystic fibrosis transmembrane regulatory (CFTR) gene mutations [6].

33.4 Management of Ejaculatory Disorders

33.4.1 Anatomic

33.4.1.1 Bladder Neck Incompetence

A patent bladder neck is most commonly the consequence of an incompetent internal urethral (bladder neck) sphincter. The ensuing "dry ejaculate" is due to retrograde ejaculation. It can be caused by α-blocker medication for prostate enlargement or hypertension, diabetic neuropathy, neurologic disorders such as spina bifida or multiple sclerosis, or other congenital anatomic abnormalities. It is also a common postsurgical complication of transurethral prostatectomy (TURP) [7]. Interestingly, from the patient perspective, retrograde ejaculation after TURP is often confused with anorgasmia or erectile dysfunction [8].

If retrograde ejaculation is drug induced, the offending medication should be discontinued. With neurological causes such as that associated with diabetes, alpha agonist therapy can help "close" the bladder neck and encourage antegrade ejaculation [1, 9]. Alternatives to TURP including UroLift® and Rezum® bladder neck sparing procedures should be considered in men desiring fertility to reduce the likelihood of retrograde ejaculation. Reversal after TURP is difficult. However, if fertility is sought in men after TURP, sperm in the postmasturbatory urine can be used with intrauterine insemination (IUI) or in vitro fertilization (IVF) for paternity.

33.4.1.2 Müllerian Duct Cyst

Persistence of remnants of the Müllerian ducts may exist as midline cysts associated with the prostatic utricle and ejaculatory ducts in men. If significant in size, such cysts may be occlusive and produce a low-volume ejaculate due to ejaculatory duct compression. This diagnosis is confirmed by TRUS and further investigation of ejaculatory anatomy and function with chromotubation or manometry [5, 10]. Stones, calcification, ejaculatory duct agenesis, and seminal vesiculopathy resulting in acontractile, dysfunctional seminal vesicles may present similarly [11].

In patients with confirmed obstruction, transurethral unroofing of cysts, drainage of stones, or recanalizing the ejaculatory ducts effectively treats the problem [12]. In men with functional but not obstructive disorders of the reproductive tract, surgical procedures are not indicated and have no clinical value [5].

33.4.1.3 Congenital Bilateral Absence of the Vas Deferens/Cystic Fibrosis

Among men with cystic fibrosis, 99% also have Wolffian duct abnormalities that typically cause low ejaculate volume. There may be atresia or agenesis of the vas deferens, seminal vesicles, or ejaculatory ducts with this diagnosis. Low-volume ejaculation is also associated with a forme fruste of cystic fibrosis, termed congenital absence of the vas deferens (CAVD) [13]. In this condition, there may be absence of the vas deferens but without other systemic manifestations of cystic fibrosis.

The ejaculatory disorder associated with cystic fibrosis and bilateral CAVD is currently irreversible. Parenthood, however, can be achieved with surgical sperm retrieval procedures and assisted reproduction.

33.4.1.4 Ejaculatory Duct Obstruction

The combination of low-volume ejaculate, painful ejaculation, hematospermia, and perineal or testicular pain is highly suggestive of ejaculatory duct obstruction (EDO). The diagnosis is supported by the finding of a normal physical examination and a semen analysis that shows a volume <2.0 mL, with a seminal pH < 7.2, and no sperm or fructose present. Partial EDO, a variant, is harder to diagnose, but typically

presents with low-normal ejaculate volume and disproportionately low sperm motility. Confirmatory diagnostic tests include TRUS that shows dilated seminal vesicles (>1.5 cm) or dilated ejaculatory ducts (>2.3 mm) in association with a cyst, calcification, or stones along the ducts [14, 15]. Recently, it has become clear that "static" imaging such as TRUS cannot reliably differentiate true physical obstruction from functional disorders of the reproductive tract. TRUS, although sensitive, is not specific for the diagnosis of EDO [10]. As such, adjunctive procedures such as seminal vesicle aspiration [16], seminal vesiculography, and chromotubation can further delineate the diagnosis. Such "functional" testing has been suggested before definitive surgery on the ejaculatory duct complex [10]. To this end, a prospective study of these three adjunctive techniques in EDO patients revealed that patency with chromotubation was the most accurate way to diagnose complete or incomplete ejaculatory duct obstruction [10].

With these considerations in mind and based on the concept of bladder urodynamics to assess bladder outlet obstruction, we described the technique of ejaculatory duct manometry to confirm the diagnosis of EDO (Fig. 33.2) [5]. This technique stemmed from the idea that the varying flow resistance patterns encountered with antegrade chromotubation in EDO patients could be more precisely quantified. We hypothesized that measuring ED "opening pressures," defined as the pressure above which fluid enters the prostatic urethra, could distinguish among the various forms of EDO. Indeed, in a prospective, comparative study of fertile men (vasectomy reversals) and men with confirmed EDO, ejaculatory duct opening pressures were significantly higher in untreated EDO patients (mean 116 cmH_2O) compared to fertile men (mean 33 cmH_2O) (Fig. 33.3). In addition, post-TURED duct opening pressures fell to values similar to controls. The study concluded that (1) fertile patients have consistent and low ED opening pressures with a normal pressure defined as <45 cmH_2O; (2) infertile men with EDO have significantly higher ED pressures; (3) opening pressures after EDO treatment can be lowered to that of controls; and (4) patients with suspected EDO may have other kinds of underlying pathology that will not respond to ED resection, including urethral strictures. From this analysis, ED manometry currently has the most potential to differentiate complete from partial and physical from functional forms of EDO.

Men with EDO can be treated with a transurethral resection or incision of the ejaculatory ducts, which is very effective at increasing semen volume and restoring sperm flow [15]. In cases of absence of reproductive tract organs, no remedy is currently available.

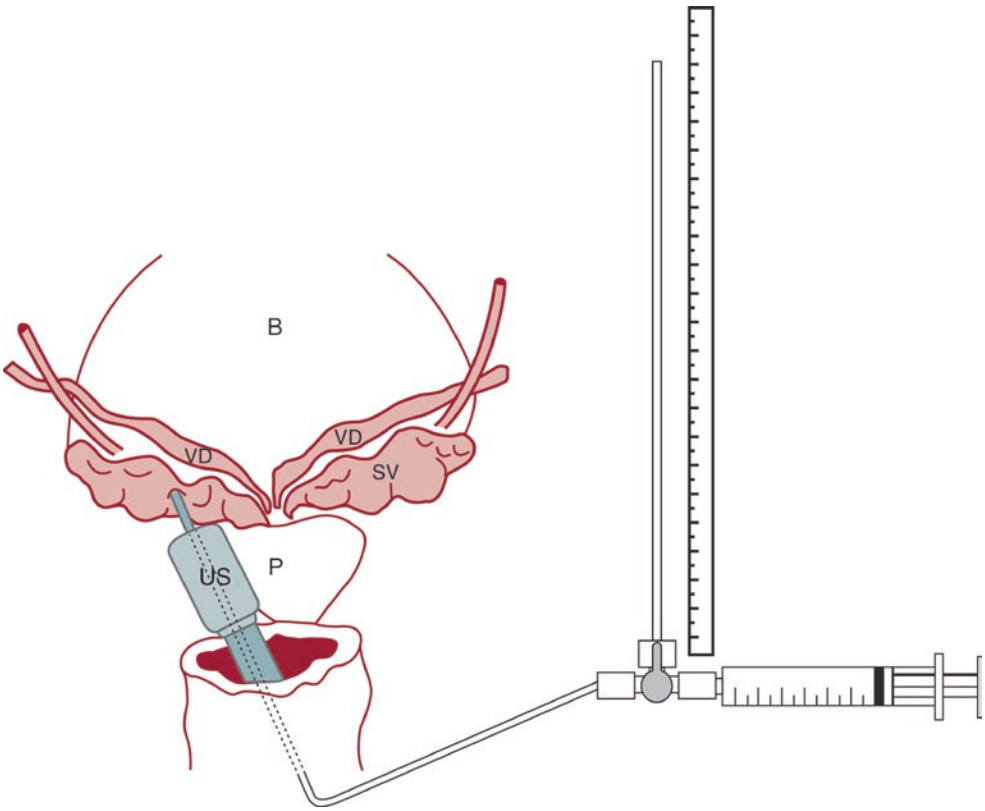

Fig. 33.2 The ejaculatory duct manometry device. Schematic representation of the intravenous tubing manometer used to measure ejaculatory duct pressure in EDO patients. After intubation of the seminal vesicle with a spinal needle attached to a three-way stopcock, the seminal vesicle is injected with saline/indigo carmine (chromotubation). The pressure at which fluid begins to traverse the ejaculatory duct orifice into the prostatic urethra cystoscopically is the "opening pressure." The pressure within the seminal vesicle is monitored by the height of the column of fluid within the IV tubing (Reprinted from Eisenberg et al. [5]. With permission from Wolters Kluwer Health, Inc.)

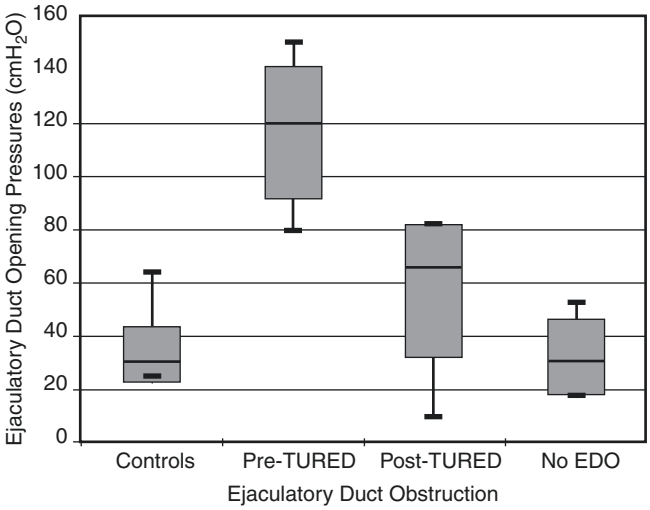

Fig. 33.3 Ejaculatory duct opening pressures in fertile men and EDO patients. Gray boxes represent the first to third interquartile range for measured values. Black horizontal lines represent the median, and whiskers represent the absolute range of values. Ejaculatory duct pressures in EDO patients were significantly higher than controls ($p < 0.001$) or post-TURED ($p < 0.001$) patients. Pressures after TURED were not significantly different from controls or the "No EDO" group. The "No EDO" group represents ejaculatory ducts evaluated and deemed clinically not to harbor EDO (Reprinted from Eisenberg et al. [5]. With permission from Wolters Kluwer Health, Inc.)

33.4.2 Neuropathic

33.4.2.1 Spinal Cord Injury

Most patients with spinal cord injury are young men who have sustained traumatic interruption of nerve pathways that modulate ejaculation. Spinal cord lesions at or below the level of T10–L2 level commonly lead to complete loss of ejaculation with preserved erections, whereas injuries above the T10 spinal level generally retain the ejaculatory reflex arc as the peripheral efferent nerves from T10–L2 and S2–S4 are intact. The integrity of this reflex arc can be confirmed by demonstrating an intact bulbocavernosus reflex and the ability to perform hip flexion, both of which predict successful ejaculation when sensory afferent input is increased to suprathreshold levels [17].

Fertility and paternity are commonly achieved in affected patients with the use of assisted reproductive technology in association with penile vibratory stimulation [18] or rectal probe electroejaculation [19–21]. Patients with spinal cord lesions above T4 also are prone to autonomic dysreflexia from penile stimulation. Symptoms of autonomic dysreflexia include hypertension, bradycardia, sweating, chills, and headache. In some cases, autonomic dysreflexia can lead to dangerously high blood pressures and can lead to stroke, seizure, or death. Pretreatment with an oral calcium channel blocker for prophylactic management of these symptoms is advised. For patients with lower spinal cord lesions (below T10) in whom penile vibratory therapy fails, rectal probe electroejaculation is an excellent alternative to achieve ejaculation [22]. For patients who fail electroejaculation, surgical sperm retrieval provides an excellent alternative for paternity [23].

33.4.2.2 Diabetes Mellitus

Long-standing diabetes is associated with genitourinary autonomic neuropathy. About 87% of type I diabetics have evidence of bladder dysfunction, and erectile dysfunction is observed in 35–75% of affected males [24]. The associated ejaculatory dysfunction may manifest as either retrograde ejaculation or total anejaculation depending on the degree of sympathetic autonomic neuropathy. This dysfunction results from incomplete closure of the bladder neck during ejaculation (retrograde ejaculation) or complete neurogenic "paralysis" of the reproductive tract smooth musculature (anejaculation). In cases of complete anejaculation, the post-masturbatory urine contains no sperm [25].

To aid in conception, sympathomimetic drugs may be taken to stimulate bladder neck closure in retrograde ejaculation and produce an antegrade ejaculate [9]. Because most sympathomimetics will exhibit tachyphylaxis with prolonged use, their administration should be limited to 5 or 7 days of timed intercourse around the window of ovulation. Unlike with retrograde ejaculation, diabetic patients with anejaculation are more difficult to treat. If there is no conversion to retrograde or antegrade ejaculation with sympathomimetics, rectal probe electroejaculation can be effective to induce a useful ejaculate [22]. Of note, patients with long-standing diabetes may have calcified vasa deferentia and seminal vesicles that are unable to contract and propel sperm. In such cases, surgical sperm retrieval is necessary to treat fertility issues [26].

33.4.2.3 Postsurgical

In general, retroperitoneal or pelvic procedures that disrupt the sympathetic nerves that course along the aorta, especially near the aortic bifurcation (hypogastric plexus), may result in ejaculatory dysfunction. The spectrum of the functional defects correlates to the degree and severity of nerve damage. Retrograde ejaculation, failure of ejaculation, and failure of ejaculation along with failure of seminal emission are all possible, depending on the degree of injury [1].

Approximately two thirds of men will have retrograde ejaculation following TURP, and one quarter to one third of patients will have a similar issue after bladder neck incision due to incomplete bladder neck closure. Major abdominopelvic surgery, such as colorectal resection for malignancy, ileoanal anastomosis for inflammatory bowel disease, repair of abdominal aortic aneurysm, aortoiliac bypass grafting, and retroperitoneal lymphadenectomy for testicular cancer may lead to some damage of the lumbar sympathetic ganglia and/

or the superior hypogastric plexus and result in retrograde ejaculation or anejaculation [27, 28].

The operative procedure that historically has resulted in the highest frequency of ejaculatory disorders is retroperitoneal lymph node dissection performed largely as curative treatment for metastatic testicular cancer. In its original form, the operation consisted of a bilateral suprahilar extended dissection of retroperitoneal nodes and almost uniformly resulted in ejaculatory dysfunction. Advances in surgical technique combined with newer limited surgical dissection templates have decreased the incidence of ejaculatory dysfunction [29–31].

Radical prostatectomy is another major procedure that results in functional anejaculation because the prostate and seminal vesicles are excised. Other operative procedures that can cause ejaculatory dysfunction include abdominoperineal operations for rectal cancer and spine surgeries performed anteriorly (transabdominally), which are associated with rates of ejaculatory disorders of approximately 14% [32].

Pediatric congenital anomalies of the pelvis are associated with anejaculation and retrograde ejaculation later in life. Ejaculatory disorders in these patients can be caused by the anatomic nature of the pelvic anomaly (cloacal exstrophy, imperforate anus) or the associated surgical procedure needed for its correction (exstrophy/epispadias repair, bladder neck reconstruction) [1].

The reversal of ejaculatory disorders from surgical sympathetic nerve disruption is difficult to reverse. In general, treatment with α-adrenergic stimulants can be attempted [9]. In a few instances, therapy can convert a failure of emission into simply retrograde ejaculation or convert retrograde into antegrade ejaculation. Success with drug therapy depends on the integrity and number of residual sympathetic nerve fibers that innervate the seminal vesicles, vasa deferentia, and bladder neck areas.

If medical therapy is unsuccessful, rectal probe electroejaculation is required to induce ejaculation and to harvest sperm for use in assisted reproductive technology. When performed under a light general anesthesia in the sensate patient, this procedure uses rhythmically applied, graded increases in voltage (0–25 V) to a rectal probe to cause contraction of the seminal vesicles and vasa ampullae directly and to induce the ejaculatory reflex. Ejaculation is obtained in virtually all men with surgically induced ejaculatory disorders through this technique [22].

33.4.2.4 Neurologic Disorders

The entire spectrum of ejaculatory disorders, ranging from premature ejaculation to anejaculation, is associated with demyelinating and inflammatory neurologic diseases including multiple sclerosis and transverse myelitis [33]. Patients with spinal dysraphism disorders (e.g., myelodysplasia, myelomeningocele, spina bifida) also harbor many of the same ejaculatory disorders. Defects above the T10–T11 cord level commonly are associated with anejaculation, whereas defects below this level allow emission without ejaculation. Patients with sacral lesions are generally spared any ejaculatory dysfunction [34].

Neurostimulatory methods may be used to induce ejaculation in men with neurogenic anejaculation. The most commonly used methods to induce ejaculation in men with neurologic disorders are penile vibratory stimulation (PVS) and rectal probe electroejaculation (EEJ) [3]. PVS involves placing a vibrator on the dorsum or frenulum of the glans penis [18]. Mechanical stimulation produced by the vibrator recruits the ejaculatory reflex to induce ejaculation [35]. This method is more effective in men with an intact ejaculatory reflex, i.e., men with a level of injury above the T10 cord level. Individuals who do not respond to PVS are often candidates for EEJ [36, 37]. EEJ is performed with the patient in the lateral decubitus position. A probe is placed in the rectum, and electrodes on the probe are oriented toward the prostate and seminal vesicles. Current delivered through the probe stimulates nerves that lead to emission of semen.

33.4.3 Pharmacologic

33.4.3.1 Antidepressants

Many common medications can cause ejaculatory dysfunction. Antidepressants, including the tricyclic antidepressants, monoamine oxidase inhibitors (MAOIs), and the newer selective serotonin reuptake inhibitors (SSRIs), are associated with sexual dysfunction and disordered ejaculation [38]. Sexual dysfunction resulting from these medications may include hypoactive sexual desire, erectile dysfunction, and delayed ejaculation. It is thought that these side effects are due to elevated central nervous system levels of serotonin (SSRIs) or catecholamines (tricyclics) [39]. In most patients, discontinuation of antidepressant therapy will restore normal sexual function.

33.4.3.2 Alpha-Adrenergic Antagonists

Both the transport of seminal fluid within the reproductive tract and bladder neck closure are controlled by α-adrenergic nerves. Therefore, α-adrenergic antagonists, given for hypertension or prostatic hypertrophy, may inhibit both seminal emission and bladder neck closure [40]. In either case, the result presents as a low-volume or dry ejaculate. In general, the sensation of orgasm is normal or nearly normal. Treatment should be directed at removal of these drugs.

33.4.3.3 Finasteride

Finasteride (1 mg) has been FDA approved for male pattern baldness since 1998. Early clinical trials showed a very low sexual side effect profile after 1 year of use, including a loss

of libido in 1.8% of men, erectile dysfunction in 1.3%, and decreased ejaculate volume in 1.2% of subjects. However, after several case reports of infertility and persistent sexual dysfunction were published [41], and after an FDA review of 421 post-marketing adverse event reports, a warning was added to the side effect profile of finasteride. The FDA warning stated that finasteride may cause loss of male fertility (low sperm counts), loss of sexual desire, inability to ejaculate, and inability to reach orgasm. It also noted that these sexual dysfunctions may continue (>3 months) after men stop using the drug. Currently, there is a recognized disorder attributed to finasteride termed post-finasteride syndrome or PFS. PFS is characterized by sexual and other symptoms experienced during use or after finasteride discontinuation. The symptoms include overall sexual dysfunction (SD), erectile dysfunction (ED), loss of libido, depression, suicidal ideation, anxiety, panic attacks, insomnia, and cognitive dysfunction. Currently, our understanding of the biological basis for PFS is entirely unclear, but it is thought to involve an effect on centrally acting neurosteroids and epigenetic changes to androgen receptor expression [42].

33.4.4 Functional

33.4.4.1 Premature or Early Ejaculation

Early ejaculation is further classified as being either a "lifelong" (primary) condition present since the onset of sexual maturity or an "acquired" (secondary) disorder that develops after an interval of normal sexual function [43]. This distinction is significant as many cases of secondary PE reflect a problem with erectile dysfunction and can be successfully treated by improving erections. The incidence of PE is high, affecting 20–35% of men between the ages of 18 and 59, and it is the most common form of male sexual dysfunction [44]. PE has not been associated with other organic diseases. Given how commonly PE is reported, it raises the question of whether it is truly an organic disorder or merely a consequence of normal sexual function associated with abnormal expectations. Implicated etiologic factors include sexual anxiety, penile skin hypersensitivity, and imbalances in the serotonergic (5-hydroxytryptamine) system that might determine ejaculatory thresholds [45, 46]. The goal of therapy for primary PE is to increase patient control over the ejaculation process by decreasing penile sensitivity and by adjusting the behavioral response. Treatments include oral medication, local anesthetic therapy, and sexual therapy. The most effective treatments are behavioral in nature as drug therapy demands high compliance rates that may not be achievable with younger men who do not otherwise take medications.

Drugs that delay ejaculation are logical choices for PE treatment, and clinical trials have shown that SSRIs can effectively prolong the time to ejaculation [47–53]. However, the increase in latency time to ejaculation varies widely depending on the medication. Although not FDA approved for this use, paroxetine, fluoxetine, sertraline, and clomipramine are the best tolerated medications for PE and have been observed to increase latency times to anywhere from 2 minutes to 10 minutes. Dapoxetine, a rapidly absorbed SSRI with a short half-life, specifically developed for the "on-demand" treatment of PE has shown an increase in intravaginal ejaculatory latency time (IELT) of 1.9 to 3.5 minutes in randomized trials [48]. For perspective, the median IELT of men in Western countries has been reported to be 6 minutes [54]. It is also the first medication to obtain approval by national health authorities in Europe, but is not FDA approved for use in the USA. Side effects of the SSRI class of agents include nausea, fatigue, headache, confusion, and diarrhea and tend to limit compliance and therefore effectiveness [45].

One of first drug classes to be used (off-label) to treat PE are the tricyclic antidepressants (clomipramine). However, since they are prescribed chronically, they are generally limited by side effects of nausea, drowsiness, and insomnia and have been associated with significant drug interactions. For these reasons, this class of drugs has fallen out of favor for PE treatment. More recently, evidence from a randomized clinical trial has emerged that "on-demand" use of clomipramine (15 mg or 30 mg) may be far more tolerable than chronic administration and also achieve the desired goal of increased IELT [55]. Tramadol, a centrally acting synthetic opioid analgesic, has shown some promise for "on-demand" treatment of PE [56], but concern about opiate dependency has limited its use.

Topical anesthetic agents such as 2% lidocaine jelly and topical 2.5% lidocaine/2.5% prilocaine cream (EMLA) [57] have been shown to decrease penile sensitivity and prolong ejaculatory latency [58]. When applied to the penile skin with a condom for 30 minutes before intercourse, EMLA cream has been observed to increase the time to ejaculation in 80% of men. Newer topical medications are now available and include a quick-acting, local anesthetic (lidocaine) spray, an SS-cream, and herbal preparations [59]. Side effects from topical agents include penile numbness, loss of erection, and burning sensation at the site of application. Condoms have also been used in selected cases of PE, as they can decrease penis sensitivity and help delay ejaculation. They are limited by patient acceptability issues.

Traditional Chinese oral herbal supplements to treat PE have also been described but have extremely limited evidence to recommend their use. They include *Epimedium* leaf extract, *Cuscuta* seed extract, *Ginkgo biloba* leaf, Asian ginseng root, saw palmetto berry (fructus serenoae), muira puama bark extract, catuaba bark extract, and hawthorn berry (fructus crataegi) [60].

Given the frequency of PE among younger, sexually active men, durable success is an important treatment goal. Therefore, medical therapy should always be combined with behavioral modification therapy [61]. The goal of sexual therapy is to provide the patient with greater control over, and satisfaction from, sexual stimulation. Typically, the sensation of ejaculatory inevitability must be explained to patients so that they can understand, observe, and ultimately control the sensations experienced and enhance sexual pleasure. Typically, patients and their partners undergo a 6- to 20-week course of therapy in which they learn systematic relaxation techniques and acquire skills to perform prolonged self- or partner-performed sexual stimulation without the demand for erection or ejaculation. Subsequently, patients are instructed in methods of passive coitus without thrusting and, eventually, coitus with pelvic thrusting. Partner participation and cooperation with such therapy is important for long-term success.

33.4.4.2 Delayed Ejaculation

Delayed or late ejaculation, also called inhibited ejaculation, is the inability to, or persistent difficulty in, achieving orgasm. The opposite of early or premature ejaculation, this disorder is characterized by a lack of orgasm or an extreme delay (30–45 minutes) despite normal sexual desire and sexual stimulation. In many cases, men with delayed ejaculation can achieve climax normally with masturbation but not with sexual intercourse. It is thought to occur in <10% of sexually active men. Its etiology is quite varied. Many medical conditions that can cause delayed ejaculation include hypogonadism and thyroid and cortisol dysfunction. Recreational drugs such as alcohol, cocaine, and marijuana have also been implicated. Medications such as SSRI antidepressants, opiates, and benzodiazepines are classic causes of late ejaculation. Additionally, pelvic surgery for prostate cancer, circumcision or condom use, and pelvic trauma or neurological disease (i.e., multiple sclerosis) that result in decreased penile sensation are possible causes. For those without these risk factors, altered psychological states due to sleep deprivation, stress and distraction, and anxiety during sex are common causes of delayed ejaculation.

Typically, treatment of delayed ejaculation involves discontinuing the offending medication or drug or stress reduction. In persistent or unexplained cases, there is some evidence to suggest that the condition is related to masturbation technique. Termed "idiosyncratic" or "traumatic" masturbatory syndrome [62], delayed ejaculation is thought to occur when sensations felt during masturbation are different than those experienced during intercourse. As masturbation tends to be a repetitive behavior in any individual, the pressure, angle, and grip on the penis during masturbation may not be compatible with that of sexual intercourse. Based on this theory, sexual therapeutic approaches to delayed ejaculation have been successful and involve stepwise masturbatory exercises in which men get accustomed to reaching orgasm with sexual stimulation regimens that more closely resemble vaginal, oral, or anal intercourse.

33.4.4.3 Seminal Megavesicles

Enlarged seminal vesicles without evidence of physical obstruction, also known as seminal megavesicles, have been reported in association with polycystic kidney disease and after surgical failure of transurethral resection of ejaculatory ducts. Dilation of the seminal vesicles may mimic obstruction of the ejaculatory ducts, and these conditions are not easily distinguishable on TRUS imaging. In a study by Hendry and co-workers, six azoospermic men with adult polycystic kidney disease had enlarged seminal vesicles. When these men were studied with seminal vesiculography, there was no obstruction found [11]. In addition, all attempts at transurethral resection of the ejaculatory ducts failed. We hypothesized that this abnormality may partly explain why 25 to 30% of men with presumed ejaculatory duct obstruction fail to improve after TURED [63]. To demonstrate this concept, we constructed an in vivo rat model and assessed the urodynamic properties of active and resting compliance within the seminal vesicle. We found that seminal vesicles, as hollow organs lined with smooth muscle, act urodynamically like urinary bladders, thus lending credence to the concept that seminal vesicle myopathies, like bladder myopathies, can exist and result in "functional" obstruction of the reproductive tract [64]. Similar to neurogenic bladders, to date, no effective treatments have been shown to correct this type of organ dysfunction.

33.4.4.4 Retrograde Ejaculation

The true incidence of retrograde ejaculation is difficult to estimate, but approximately 14% to 18% of patients with ejaculatory disorders harbor this diagnosis [65]. Among 1400 infertile couples, 0.7% of men presented with retrograde ejaculation [66]. This diagnosis is relatively straightforward and requires a history of low-volume or dry ejaculate, with a postmasturbatory voided urine sample demonstrating sperm. Systemic diseases such as diabetes mellitus; medications including alpha-blockers; neurogenic causes such as spinal cord injury, multiple sclerosis, or spina bifida; and surgical treatments such as retroperitoneal lymph node dissection and transurethral prostate resection can all cause retrograde ejaculation.

Several treatment options are available for retrograde ejaculation. If the condition is drug induced, the offending medication should be discontinued if possible. In many patients without scar tissue at the bladder neck, oral therapy can be attempted with alpha-adrenergic agonists [9]. Approximately one third of men will respond to this therapy.

Sympathomimetic agents, such as imipramine, phenylpropanolamine, or pseudoephedrine, have been used with schedules ranging from interval dosing to as needed dosing immediately prior to coitus [67]. Generally, oral medical therapy is limited by side effects, including dizziness, weakness, nausea, sweating, or palpitations. If oral therapy fails, sperm harvesting from the bladder urine obtained on post-ejaculate urination or by catheterization can be used with intrauterine insemination or in vitro fertilization to achieve family building goals.

33.4.4.5 Anejaculation

Congenital anorgasmia, also known as primary or psychogenic anejaculation, is a rare, well-described cause of conscious anejaculation. Despite the lack of willful orgasm, nocturnal emissions may occur [68]. The incidence of this condition is 0.14% in the general population and 0.39% among male patients seeking infertility care [69]. The cause is thought to be overly strict childhood upbringing. A classic setting includes parenting with intense performance demands and minimal physical affection. Secondary anejaculation is present in anejaculatory patients with a previous history of normal ejaculation and is generally due to neurologic disease or trauma (e.g., spinal cord injury).

Treatments that seek to reverse anejaculation are difficult. Often, affected individuals lack sensual awareness of their bodies. In addition, they often may seek a partner from a similar background and, as such, may accommodate to an asexual or minimally sexual lifestyle. Treatment is usually sought when the couple desires a pregnancy. Psychotherapy is often effective and is initiated with instruction in sex education, followed by cognitive behavioral treatment that includes systematic relaxation and sensate focus exercises [69]. At first, partners are taught to tolerate and become comfortable with touch. Later, when touch induces pleasure, sexual stimulation is encouraged as the shaping of the sexual response and orgasm occurs.

Fertility issues can be managed relatively efficiently in almost all cases of anejaculation with penile vibratory stimulation, rectal probe electroejaculation, and/or surgical sperm aspiration [23, 70].

33.5 Conclusion

Disordered ejaculation is a complex clinical entity that includes anatomical, psychosocial, and neurophysiological aspects of human behavior. Although ejaculation is based on a single physiological constant, a spinal cord reflex, the etiologies for primary and secondary premature ejaculation are vastly different, and the differences between "normal" ejaculatory latency and delayed ejaculation may simply reflect differences in learned behaviors. Disordered ejaculation due to anatomic causes, such as retrograde ejaculation or ejaculatory duct obstruction, is more easily defined, categorized, and treated. The complexity of ejaculation not only makes the diagnosis of these conditions difficult, but it also complicates understanding of, and ability to precisely treat, these disorders.

33.6 Review Criteria

In order of importance, randomized controlled trials, basic scientific studies, meta-analyses, case-controlled cohort studies, best practice policy recommendations, and published reviews were used to inform the work. Articles published in languages other than English were considered. Data from conference or meeting proceedings, websites, or books were not included.

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Environmental Factors

34

Kristian Leisegang and Ralf Henkel

Key Points

- Male infertility is a major issue of concern as it affects more people during their lifetime as a common disease.
- Oxidative stress is a major cause of male infertility.
- Oxidative stress can be caused by unhealthy lifestyle choices such as smoking.
- Numerous environmental pollutants, including pesticides, heavy metals and also numerous plastics can cause significant detrimental effects on male reproductive health.
- Many of these environmental pollutants are so-called endocrine disruptors, which have a detrimental effect on the following generation.

34.1 Introduction

Globally, approximately 190 million people are affected by infertility, and this number is steadily rising [1] leading to a prevalence of infertility of about 13–18% [2]. The reasons for this are manifold, but it becomes more and more evident that apart from unhealthy lifestyle choices, the exposure to environmental and occupational hazards plays a major role in this rising incidence of infertility [3]. There is also growing evidence that male infertility is a possible indicator of future development of various types of cancer, such as testicular or prostate cancer [4]. Since 1946, more than 140,000 new chemical compounds have been synthesized with an annual average of 1000–2000 [5] including, among others, solvents, plastics, or pesticides, which eventually enter the food chain and then have detrimental health effects, not only on the individual who ingested these compounds but also for the next generation. For example, a report by the US Center for Disease Control indicates that more than 116 extraneous chemicals accumulated in human bodies [6] and more than 358 chemicals were detected in umbilical cord blood of newborn babies [7]. In recent years, more and more experimental and epidemiologic evidence has indicated that certain industrial and environmental chemicals could adversely impact fertility and pregnancy [8]. This chapter intends to summarize and highlight recent findings on the impact of environmental toxicants on male reproductive health (Table 34.1).

34.2 Metals

34.2.1 Arsenic (As) (Metalloid)

Arsenic (As) is an abundantly naturally occurring metalloid element of the fifth main group (nitrogen group) in the periodic table of elements. This element rarely occurs solid but is usually combined with other metals and sulfur. Minerals containing arsenic are, for example, arsenical iron, arsenical pyrites or mispickel, tin-white cobalt or smaltite, arsenical nickel, realgar, orpiment, pharmacolite, and cobalt bloom. As a solid, its appearance is metallic grey to black, tasteless, and odorless.

Due to its toxicity and the fact that arsenic is also a psychoactive substance, arsenic was used as homicidal agent for centuries. Already in antiquity, arsenic was a popular murder poison, and in order to be at least reasonably prepared for poison killings, they protected themselves by a slow habituation to arsenic. While normally a dosage of about 0.1 g orally taken arsenic can be fatal, the continuous ingestion of much lower doses leads to a habituation of the body to the substance, which can be seen in the so-called arsenic eaters.

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Table 34.1 List of some environmental pollutants grouped into different types with some relevant pathological mechanisms

Type of pollutants	Type		Possible mechanism	Reference
Metals		Lead	Increased oxidative stress Damage to spermatogenesis	[27–31]
		Chromium	Cancerogenic Increased oxidative stress Damage to spermatogenic, Leydig, and Sertoli cells	[55–57]
Endocrine disruptors	Metals	Arsenic	Inhibition of enzymes Decrease of FSH Decrease of LH Decrease of testosterone Decrease in sperm count Decrease in motility Apoptosis in spermatogenic and Sertoli cells	[13, 16, 17]
		Cadmium	Inhibition of enzymes Decrease in sperm count Decrease in motility Decrease in normal morphology Increase in sperm DNA fragmentation	[19, 22, 23, 25]
		Mercury	Disrupting hormonal regulation of HPG axis Decrease in sperm count Decrease in motility Increase in sperm DNA fragmentation	[39–47]
		Copper	Negatively affects FSH receptor Decrease of testosterone Decrease in spermatogenesis	[60]
	Pesticides	Atrazine	Increases aromatase activity	[81]
		Carbaryl	Increase in sperm DNA fragmentation Decrease of testosterone Decrease of germ cell survival	[86–89]
		Kepone	Acts as estrogen antagonist Decrease in motility Decrease of normal morphology Decrease of sperm count Causes prostate cancer	[90–93, 96, 97]
		Dioxins	Negative effect on whole endocrine system Increase in hypospadias Increase in testicular cancer	[99–101]
		Ethyl dibromide	Decrease of sperm count	[104, 105]
		PCBs	Acts as xenoestrogen Decrease in motility Decrease in sperm count	[73–76, 106–108]
		Vinclozolin	Binds to androgen receptor Blocks action of gonadal hormones	[110–113]
	Chemical pollutants	Benzene	Cancerogenic Decrease of sperm count Decrease in motility Increase of sperm DNA fragmentation	[117, 118]
		Carbon disulfide	Induces apoptosis in Sertoli cells	[119–122]
		Glycol ether	Has testicular toxicity	[123–126]
		Methoxychlor	Negatively affects testicular development Decrease in testosterone	[131–135]
		Phthalates	Act as xenoestrogen Antiandrogenic effects Increase in cryptorchidism Causing testicular dysgenesis Causing feminization	[136–141, 149, 150]
		Bisphenol A	Estrogenic effects Antiandrogenic effects Decrease in sperm quality Increase in cryptorchidism Increase in sperm DNA fragmentation Cancerogenic	[152–156]

Table 34.1 (continued)

Type of pollutants	Type		Possible mechanism	Reference
Radiation	Ionizing	UV	Genetic changes Chromosomal changes Congenital abnormalities	[159–162]
		X-rays		
		γ-rays		
	Nonionizing	Cell phone	Increase in oxidative stress Increase in sperm DNA fragmentation	[163–175]
		3G		
		Wi-Fi		
		Microwaves		

Yet, a rapid discontinuation of the arsenic intake can then result in withdrawal symptoms like tiredness, exhaustion, and difficulty to concentrate [9].

Arsenic was and is in used in pharmaceutics to treat, e.g., syphilis or various forms of cancer, in agriculture as pesticide or herbicide, in the furniture industry as wood preservative, and in metallurgical and semiconductor industries, to name a few [9, 10]. It appears that arsenic, based on the nutritional and health condition of a person, has possibly two roles. Although arsenic is regarded as trace element in the human and appears to be desired, deficiency symptoms have thus far only been reported in animals. On the other hand, contamination can cause numerous diseases such as skin and bladder cancer [11, 12], cardiovascular diseases [12], or reproductive problems [13]. The toxicity of arsenic depends on several factors, whether the metalloid is inorganically or organically bound; its valences, solubility, physical state, and purity; and its absorption and exclusion rate [14]. Trivalent inorganic arsenic is more toxic than organic arsenic.

Inorganic arsenic can cause skin disturbances, declined resistance to infections, heart disruptions, and brain damage. Specifically, with regard to reproductive health, a very high exposure to inorganic arsenic can cause infertility and miscarriages with women [15]. Also, in the male, reports indicate significant declines in the weight of testes, epididymides, accessory sex glands, sperm count, and motility as well as follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone levels in a rat model [16]. In a total number of 117 patients exposed to more than 50 ppb arsenic in drinking water, an increased risk of erectile dysfunction with decreased testosterone levels was reported [17]. More recent studies found that environmental exposure to the substance was positively associated with reduced semen parameters in Chinese subjects at reproductive health [18, 19], hormonal imbalance, and poor sperm parameters [20].

Possible mechanisms of arsenic toxicity seem to act at different levels of the reproductive system and may be due to the inhibition or activation of enzymes. Particularly, the testosterone and gonadotrophin syntheses are negatively affected by the disruption of 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase and negative regulations of LH and FSH by increased corticosterone levels. In addition, arsenic has direct negative effects on sperm by binding to sulfur-containing proteins and inducing apoptosis in spermatogenic cells and Sertoli cells [13]. Associations with spontaneous abortion and stillbirth have been reported in more than one of these studies. Interpretation of most of these studies is complicated because study populations were exposed to multiple chemicals.

34.2.2 Cadmium (Cd)

Cadmium is a very rare metal, which makes up about 0.1 ppm of the Earth's crust. Together with zinc and mercury, it belongs to group 12 of the periodic table. The element is commonly used in batteries, paint pigments, and coatings or for electroplating to name a few. With the exception of its use in nickel-cadmium batteries and cadmium telluride solar panels, the use of cadmium is generally decreasing in its other applications, due to its toxicity and the development of alternative technologies.

Cadmium is extremely toxic even in low concentrations and accumulates in organisms and ecosystems. In the 1950s and 1960s, industrial exposure to cadmium was high, but as the toxic effects became apparent, industrial limits have been reduced in most industrialized nations. Buildup of cadmium levels in the water, air, and soil has been occurring particularly in industrial areas. Some sources of phosphate in fertilizers contain cadmium. Environmental exposure to cadmium has been particularly problematic in Japan where many people have consumed rice that was grown in cadmium-contaminated irrigation water, known under the name itai-itai disease [21]. Food and cigarettes are also a significant source of cadmium exposure.

Acute exposure to cadmium fumes may cause flu-like symptoms and kidney damage and create hypophosphatemia causing muscle weakness. As a consequence of its unpredictability in children, in the future, it can have fertility impact. With respect to male infertility, several studies showed a significant association between environmental and occupational cadmium exposure and reduced sperm count, motility, and normal morphology [19, 22, 23]. Such results were recently confirmed in a meta-analysis analyzing 11 studies including more than 1700 subjects [24]. These authors conclude that cadmium is a causative factor of male factor infertility. The

pathophysiological mechanism involved in these processes is oxidative stress as elevated cadmium concentrations can lead to increased seminal reactive oxygen species (ROS) levels and reduced seminal total antioxidant capacity [25]. In turn, oxidative stress can cause sperm nuclear DNA damage which is reflected by a significant positive correlation between seminal cadmium concentrations and 8-hydroxy-2′-deoxyguanosine levels, which is regarded as early marker of DNA damage [26]. In addition, in light of the susceptibility of calcium channels to blockage by cadmium, this can also lead to infertility as calcium channels are involved in acrosome reaction [27].

34.2.3 Lead (Pb)

Lead is a heavy metal of the fifth main group (carbon group; group 14) of the periodic table. It is a soft, malleable metal with relatively low melting point of metallic grey appearance. It is widely used in the production of batteries, metal products (solder and pipes), ammunition, and devices to shield X-rays, leading to its exposure to the people working in these industries. Due to its toxicity, the use of lead in gasoline, paint, and ceramic products, caulking, and pipe solder has been dramatically reduced in recent years.

Ingestion of contaminated food and drinking water is the most common source of lead exposure in humans. Exposure can also occur via inadvertent ingestion of contaminated soil/dust or lead-based paints. Lead has long been known to be toxic to male fertility. Since lead poisoning is asymptomatic for a long period of time, exposure may adversely also affect the hypothalamus-pituitary-gonad (HPG) axis, thus leading to significant impairment of spermatogenesis [27–30]. Exposure to toxic lead levels during boyhood is most susceptible and also irreversible as it significantly affects testosterone production [31]. In addition, the detrimental effects of lead on spermatogenesis and sperm functions may also be mediated via oxidative stress [31].

Although findings across studies and end points are not entirely consistent, the main body of evidence points to current blood lead concentrations of about 40–50 μg/dl as a threshold most likely not resulting in adverse effects. This applies to semen characteristics such as sperm count, motility, and abnormal sperm forms, as well as to fertility rate and time taken to conceive, whereas primary effects on the hormonal regulation of the male reproductive system at these exposure levels are questionable [32]. This view on male reproductive toxicity of lead is challenged by the findings on decreased fecundity among male lead workers [33, 34]. These authors observed an astonishing clear exposure-response relation between current blood lead level and time taken to conceive among male battery workers in Taiwan. The fecundability ratio in exposed compared to nonexposed subjects declined steadily from 0.9 in men with blood lead levels below 20 μg/dl to 0.4 among men with a blood level above 40 μg/dl. This is also reflected in the significant negative correlation of seminal lead levels with fertilization and pregnancy after IVF [35] and might be linked with the ability of lead to compete with the element zinc to bind to zinc-containing proteins such as human protamine-2, thereby altering the DNA-protamine binding [36]. This results in reduced stability of the chromatin, and abnormal chromatin structure is strongly related to reduced fertility in humans. It was also found that lead at environmental levels strongly interferes with the sperm acrosome reaction, which is essential for fertilization and negatively affects outcomes of artificial insemination [35]. Unlike many other metals such as zinc, chromium (Cr), manganese, copper (Cu), and iron, lead has no known essential effects for living organisms, and current exposure levels are still high compared to preindustrial populations.

34.2.4 Mercury (Hg)

Like cadmium and zinc, mercury (Hg), also known as quicksilver, is a member of the so-called zinc group (group 12) of the periodic table. It is the only metal that is liquid at standard conditions for temperature and pressure. Mercury is also an extremely rare metal, which makes up only about 0.08 ppm of the Earth's crust, where it is found as cinnabar (bright scarlet) to brick-red form of mercury (II) sulfide (HgS), corderoite (mercury sulfide chloride; $Hg_3S_2Cl_2$), livingstonite (a mercury antimony sulfosalt mineral; $HgSb_4S_8$), and other minerals. This metal has a long history of human use as cinnabar was used for ointments in ancient Greece. Ancient Egyptians and Romans used it in cosmetics, and in China and Tibet, mercury was thought to be a medicine to heal fractures or maintain good health. Despite these ancient thoughts of positive health effects of mercury, this metal has been proven to be toxic with a very long half-life in the body. Mercury is used in many products such as thermometers, dental fillings, and batteries or in its organic forms as fungicides in agriculture. Mercury salts may be used in skin creams and ointments.

Mercury, mercury salts, and elemental mercury can pollute water, due to its relatively high vapor pressure; pass into the food chain (build up in fish such as shark, tuna, or swordfish and animals that eat fish and directly cause intoxication). The nervous system is sensitive to all forms of mercury. Exposure to high levels can damage the brain and kidneys. Pregnant women can pass the mercury in their bodies to their babies. Mercury can concentrate in the kidneys, cerebellum, testes, and epididymis, leading to neurological disorders, kidney failure, and infertility, particularly on susceptible individuals and on susceptible groups such as fetuses and young children [37, 38]. Mercury is also suspected to act as

endocrine disruptors and thereby affect the endocrine system in males and females leading to sperm DNA damage, poor sperm motility and morphology and menstrual and hormonal disorders, respectively [39–41].

The endocrine-disrupting effect of mercury might be due to the negative association of this heavy metal with sex hormone-binding globulin (SHBG) [42] and its positive correlation with testosterone [43]. There appears also a positive relationship between mercury and serum inhibin levels [44, 45], which is indicative of FSH suppression and higher sperm counts [46]. However, due to small sample sizes in those studies, the available data are rather weak, and larger population studies are necessary. With regard to the effect of mercury on sperm DNA damage and poor motility, Zhou et al. [47] suggest the involvement of oxidative stress.

On the other hand, with regard to dental amalgam, some authors reviewed that existing scientific evidence does not demonstrate that mercury poses a public health hazard [48, 49], while others oppose this view and indicate that amalgam is an unsuitable material for dental restorations [50, 51].

34.2.5 Chromium

Chromium (Cr) is the first element in group 6 of the periodic table. It is a hard, brittle metal with a steely grey lustrous appearance. In the Earth's crust, it occurs quite abundant with an average concentration of about 100 ppm. In the industry, the vast majority of the chromium is used in metal alloys. Other usages are in the chemical and foundry industries as dyes, pigments, preservation of wood, leather tanning, or catalysts.

Medically, biologically beneficial effects of chromium (III) compounds are highly debated. While in the USA, Cr(III) ions are considered an essential trace element important for sugar, insulin, and lipid metabolism with recommended dietary intakes [52], there are no such recommendations by the European Food Safety Authority [53]. In contrast to Cr(III) ions, which are regarded as nontoxic, Cr(VI) ions are toxic and cancerogenic due to its oxidant properties. This ion damages kidneys and liver leading to renal and liver failure. Hexavalent chromium disrupts spermatogenesis, leading to accumulation of prematurely released spermatocytes, spermatids, and uni- and multinucleate giant cells in the lumen of seminiferous tubules [54]. Leydig, Sertoli, and spermatogonial stem cells are damaged due to the induction of mitochondria-dependent apoptosis through oxidative stress by Cr(VI) [55]. The serum concentration of hexachromium reportedly correlated with the percentage of poor sperm morphology in a study examining 61 occupationally exposed men [56]. Another study showed significantly higher FSH levels with lower sperm concentration and motility as compared to the controls [57].

34.2.6 Copper

Copper (Cu) is a red-orangey transition metal in group 11, which occurs in a proportion of about 50 ppm in the Earth's crust. Naturally, it occurs in various minerals such as chalcopyrite, covellite, chalcocite, azurite, and malachite or even as native copper. This metal is commercially used in the electrical industry as it is a good electrical conductor and building material in various alloys such as brass or bronze or is used as antimicrobial agent. Despite it is also an essential trace element, too high concentrations are toxic, and higher serum and seminal plasma levels are associated with male subfertility [58]. Copper can act on FSH receptors, interfering in spermatogenesis. In animals, the main endocrine alterations are in testosterone, LH, and FSH secretion. Significant correlations between copper concentrations in semen and sperm concentration ($P < 0.001$), percentage progressive motility ($P < 0.005$), and normal morphology ($P < 0.005$) were observed. However, semen copper concentrations of infertile men and fertile men did not differ significantly [59]. However, since copper is an important cofactor in many enzymes such as ceruloplasmin, superoxide dismutase 1 and 3, or cytochrome c oxidase, a nutritional lack of this element can have a significant negative effect on male fertility as these enzymes play essential roles in all stages in spermatogenesis as well as testicular and epididymal cells [60]. Hence, both too low and too higher copper levels seem to affect male fertility.

34.3 Endocrine-Disrupting Chemicals (EDCs)

Endocrine-disrupting chemicals (EDCs) are defined as exogenous natural or synthetic molecules that modulate and disrupt endocrine activity through receptor ligand binding [61]. There has been a significant increase in EDC production and release into the environment with industrialization in recent decades [61]. Included in this category are synthetic pollutants (PCBs, dioxins, bisphenol A (BPA), and phthalates), pesticides (organophosphates and organochlorines), and toxic metals [62, 63]. These are acquired through maternal transfer, nutritional sources, and other environmental contaminants and occupational exposures [63]. EDCs interfere with normal hormonal developmental and physiological function in utero, in children and in adults [63–65]. Evidence further strongly suggests the negative impact of EDC exposure on male reproductive system and fertility [61]. Relevant to male infertility, these predominantly include estrogen pathway modulation and testosterone pathway modulation, with poor spermatogenesis and steroidogenesis [61, 66]. Declining sperm parameters in recent decades has at least in part been attributed to environmental xenoestrogens [64, 65].

In utero EDC exposure has a negative effect on male reproductive development. Exposure is associated with increased risk of a reduced sperm concentration and increased risk of testicular cancer and cryptorchidism in the offspring [67]. EDC exposure is also known to be transmitted to the offspring through epigenetic modification, affecting development [68]. Current evidence suggests that a reduction in environmental EDC contamination is important to safeguard against male infertility [62].

34.4 Environmental Estrogens (Xenoestrogens)

Environmental estrogen (more specifically **xenoestrogens**) refers to compounds with estrogenic activity that differs molecularly to estrogenic compounds in living organisms [69]. Industrialization has resulted in the environmental contamination of numerous estrogenic compounds that negatively affect male reproduction. Natural sources include plants and fungi, with synthetic sources from organochlorine pesticides, polychlorinated biphenyls (PCBs), polychlorinated biphenyls (PCBs), phthalate esters (PEs), and phenolic compounds [70, 71]. These xenoestrogens are aromatic hydrocarbon and lipophilic compounds that are contaminants of air, water, animals (particularly fish), milk and humans (particularly adipose tissue). Concentrations in humans particularly correlate with fish consumption [64, 70]. These are the most abundant environmental contaminant, sourced predominantly through plastics. Increased exposures are associated with male developmental and reproductive dysfunction, endocrine pathology, and malignancy.

Evidence suggests a negative correlation of these compounds and male infertility; however, this is inconsistent, and mechanisms remain poorly investigated [71]. Polychlorinated biphenyls (PCB) and metabolite blood concentrations have been inversely correlated with semen parameters in fertile men [72]. Moderate but persistent exposure to 2,2′,4,4′,5,5′-hexachlorobiphenyl (CB-153) and 1,1-dichloro-2,2-bis (p-chlorophenyl)-ethylene (p,p'-DDE) in young men did not appear to negatively affect sperm concentrations, but higher exposures were reported to likely impair sperm motility [73]. PCB-153 has been suggested to cause DNA fragmentation with reduced sperm concentration and motility, which was not found to be mediated through hormonal mechanisms [74–76]. PCBs and phthalate esters (PEs) have been found to be closely associated with male infertility, and blood and semen concentrations inversely correlate with semen parameters [70]. These appear to particularly affect motility of sperm in which xenoestrogens are strongly correlated [70]. In addition to PCB exposures, other pesticides including dichlorodiphenyltrichloroethane (DDT), dichlorodiphenyldichloroethylene (DDE) and ethylene dibromide (EDB) negatively affect sperm counts and particularly sperm motility [77]. However, other reports contradict these associations. PCBs and organochlorine pesticides in a cohort of fertile and infertile men were not found to be associated with poor semen parameters, whereas obesity and sedentary lifestyle were a prominent lifestyle and environmental risk factor for infertility [78]. It is further established that concentrations of PCBs are approximately 20–40 times lower in seminal fluid compared to blood [72, 78]. In utero exposure is considered most detrimental, negatively affecting cell differentiation and organ development, including testicular dysgenesis [67]. Exposure in this fetal and perinatal period is further associated with smaller penis length and adverse sperm motility [70, 71].

Nutritional sources of EDCs are a significant contributor to human contamination, predominantly through dairy, meat, and soy [79]. Dietary estrogens from nutritional sources have also increasingly been associated with poor fertility parameters in males [79]. Nonsteroidal phytoestrogens have become increasingly prominent in Western diets, particularly soy and soy-based products [79]. Soy milk is also increasingly used as an alternative to breast and cow's milk, which plausibly affects the important pre- and neonatal periods of the developing germinal epithelium [79]. Due to intensive agriculture and milking of pregnant cows, estrogens are present in cows' milk, where only skimming removes sex steroid hormones. However, evidence suggest the concentrations of estrogens are too low to present a health risk to male reproduction potential or endocrine cancer development. However, significant concern remains on the exposure of these estrogens and xenoestrogens in the perinatal period, which requires extensive further investigation [80].

34.5 Pesticides

34.5.1 Atrazine

Atrazine is a herbicide of the chlorotriazine class used to prevent growth of broad-leaved weeds in crops. It is one of the most used herbicides in the USA and Australia. After exceedingly high concentrations of atrazine were found in groundwater, the use of atrazine was banned in the EU in 2003. Although atrazine itself is not estrogenic, it induces the expression of aromatase [81] and therefore facilitates an increased production of estrogen, which in turn results in estrogenized phenotypes such as feminized males across all vertebrate classes [82, 83]. This herbicide has also been associated with lower semen quality and infertility in men from mid-Missouri [84]. However, its role in inducing obesity and hypogonadism is not clear.

34.5.2 Carbaryl

Carbaryl (1-naphthyl methylcarbamate) is an insecticide of the carbamate family. It acts as an inhibitor of acetylcholinesterase. As pesticide, carbaryl is not specific as it kills pest insects (mosquitoes) and beneficial insects (e.g., honeybees) as well as crustaceans. Therefore, it is illegal in the EU but is used for an excess of 100 crops in the USA.

In contrast to other pesticides and chemical compounds, the literature on the effects of carbaryl on male reproductive health is rather scarce. In contrast to chlorinated pesticides, carbamates are nonpersistent. Although, in vertebrates, carbaryl is rapidly detoxified and eliminated via the urine, Baranski [85] lists carbaryl among those compounds that may affect the male genital system. About a decade later, Meeker and coworkers reported that carbaryl may be associated with increased sperm DNA damage, and its urinary metabolite 1-naphthol may be negatively associated with serum estradiol levels [86, 87]. An animal study using rats indicates that exposure to carbaryl leads to a significant decline in the number of germ cells, spermatocytes, spermatids, and Leydig cells. As a result, serum testosterone levels declined with a simultaneous increase in LH and FSH levels [88]. A recent study by Dziewirska et al. [89] found that the serum 1-naphthol concentration was negatively correlated with normal sperm morphology and positively with sperm curvilinear velocity. In addition, the results also suggest a negative effect on sperm DNA integrity.

34.5.3 Chlordecone (Kepone)

Chlordecone (decachloropentacyclo[5.3.0.$0^{2,6}$.$0^{3,9}$.$0^{4,8}$] decan-5-one), also known as Kepone, is a very stable and persistent organochlorine pesticide, which is globally banned since 2011. Occupational exposure to Kepone was found to be associated with oligozoospermia, poor motility, and normal sperm morphology [90, 91]. These effects might be caused by the estrogen-agonistic action of Kepone [92, 93]. Apart from its reproductive toxicity, chlordecone has been shown to be neurotoxic and carcinogenic in rats and mice [94, 95] and, because of its high endocrine-disrupting potential, causes therefore a significantly increased risk for prostate cancer [96, 97].

34.5.4 Dioxins

Generally, dioxins are two groups of chemically similar chlorinated organic compounds, polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs). Both are derivatives of oxygen-containing derivatives of halocarbons. Except for research and analytics, dioxins are not specifically produced but are rather by-products of the production of organochlorides or the incineration of PVC [98]. Major sources of dioxin production are combustion in municipal or medical waste incinerators, metal smelting, refining, or production of pesticides. Dioxins are highly persistent, lipophilic pollutants that can hardly be degraded in the environment, where they enrich in the food chain and will eventually be taken up by the human via fish, meat, or eggs.

Dioxins are so-called endocrine disruptors, and exposure affects the whole endocrine system in both genders. Specifically for male reproductive health, the Endocrine Society is reporting male sexual development, hypospadias, cryptorchidism, testicular cancer, poor semen quality, and negative effects on the prostate [99]. There are also indications that dioxins can change the sex ratio by decreasing the number of male births [100, 101].

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is the most toxic dioxin and has an estimated half-life time in humans of 7–11 years. It was a by-product of the herbicide Agent Orange, which was used in the Vietnam War. TCDD is causing soft-tissue sarcomas, lymphomas, and stomach carcinomas. Chloracne is also the major effect seen from long-term exposure to TCDD in humans. Animal studies have reported hair loss, loss of body weight, and a weakened immune system from oral exposure to TCDD. The results of available reproductive and developmental studies in humans are inconclusive. Reproductive effects, including altered levels of sex hormones, reduced production of sperm, and increased rates of miscarriages, have been seen in animals exposed to TCDD [102, 103].

34.5.5 Ethylene Dibromide

Ethylene dibromide (EDB) is a toxic chemical that serves as raw material for the production of drugs (e.g., tetramisole or theodrenaline), herbicides (e.g., diquatdibromide), and dyes. While the usage of EDB as pesticide for various crops has been stopped, it is still used as fumigant for termites, beetles, and moths. EDB is highly cancerogenic, and it appears that inhalation has effects on the brain. On the other hand, early studies by Ratcliffe and coworkers [104] and Schrader et al. [105] clearly indicated decreased sperm counts, viability, and motility after occupational exposure.

34.5.6 Polychlorinated Biphenyls

Polychlorinated biphenyls (PCBs) are a group of highly persistent industrial chemicals that were widely used because of their chemical stability and low flammability not only as transformer oils, hydraulic fluids, or cutting oils but also as

plasticizers in paints, stabilizers in PVC, and carbonless copy paper. As a result of their extensive use and persistence, PCBs remain ubiquitous environmental contaminants. They are distributed worldwide and have been measured in air, water, aquatic and marine sediments, fish, and wildlife. Furthermore, they are biologically concentrated and stored in human adipose tissue. Due to their bioaccumulation, persistence, high toxicity, and ability to travel long distances in water and air, PCBs are among the so-called dirty dozen, which were prohibited by the Stockholm Convention on Persistent Organic Pollutants on May 22, 2001. The treaty became effective on May 17, 2004. Despite some contrary results [106], epidemiologic data support an inverse association of PCBs with reduced semen quality, specifically reduced sperm motility [107, 108]. The associations found were generally consistent across studies despite a range of PCB levels [109].

34.5.7 Vinclozolin

Vinclozolin [3-(3–5-dichlorophenyl)-5-methyl-oxazolidine-2,4-dione] is a common dicarboximide fungicide used to control various diseases on raspberries, chicory grown, lettuce, kiwi, canola, snap beans, dry bulb onions, ornamentals, and turf. Vinclozolin is formulated as a dry flowable and extruded granule, which may be applied with aerial, chemigation, or ground equipment (broadcast, band, or soil drench); as a dip treatment on ornamental bulbs and corms, cut flowers, rosebud wood, or nursery stock; and with thermal fogger in greenhouses. It has been registered since 1981 in the USA for use as fungicide but is regulated by the Environmental Protection Agency. In Germany, this fungicide was prohibited as from 2004. As of 2006, Scandinavian countries prohibited the use of vinclozolin. Meanwhile, it is prohibited in the whole EU.

Vinclozolin is a known environmental endocrine disrupter with antiandrogenic properties [110]. Vinclozolin binds with high affinity to the androgen receptor and blocks the action of gonadal hormones on male reproductive organs [111]. Transgenerational epigenetic effects of this fungicide have also been shown by various groups [112, 113] indicating the inheritance of defects set by the fungicide to the offspring continuing through multiple subsequent generations.

34.6 Synthetic and Industrial Chemical Pollutants

Currently, it is difficult to investigate the impact of environmental pollutants on male infertility. Exposure studies that are cross-sectional are limited in power and causation determination, as are longitudinal studies. Causation is dependent on animal and laboratory experiments, which cannot be directly translated into human effects. Outcomes of the studies are also variable, making results difficult to be causative rather than correlations with more limited validity [114]. There is a significant scarcity of research into various pollutants that are postulated to have a negative impact on male fertility and reproductive systems. A variety of chemicals have postulated negative effects in male reproduction and may explain increasing cases of idiopathic male infertility [115]. This is associated with germ cell neoplasia, acrosomal dysgenesis, and testicular dysgenesis, in animals and humans [115]. Studies are increasingly suggesting the negative impact of numerous chemicals on male reproductive health, although many findings remain inconsistent and with relatively low-level evidence [116].

34.6.1 Benzene

Low-level benzene exposure in urban pollution levels is inversely correlated with testosterone levels in men [117]. Exposure has further ben associated with sperm count and motility, as well as DNA fragmentation, compared to males not exposed [118]. Benzene, a known carcinogen in humans, may also cause chromosomal abnormalities in sperm, even with low-level exposure. These chromosomal deletions are known to impact the offspring, increasing infertility, neurological disorders, and other congenital pathology in the offspring [116].

34.6.2 Carbon Disulfide

Carbon disulfide (CS_2) is a synthetic compound used in synthetic fibers, dry cleaners, and rubber, among other industries [119]. It is established that CS_2 has a negative effect on all tissues, including neurological, cardiovascular, and reproductive systems [119]. Exposure causes impaired seminal parameters [120–122]. Long-term exposure can negatively impact sexual function and semen quality. In experimental animals, CS_2 induces apoptosis of Sertoli cells via the estrogen receptor [119]. However, human studies are lacking, and further experimental evidence is warranted.

34.6.3 Glycol Ether

A number of common chemicals make up the broader ethylene glycol ether family, which are used in industry including plasticizers, circuit boards, inks, and other coatings and dyes [123]. These chemicals are known to cause toxicity to the central nervous system and sites of erythropoiesis, as well as blood cells, liver, and kidneys [123]. Early studies suggested

testicular toxicity through occupational exposure [124]. Infertile males who worked with organic solvents, particularly glycol ether, for a minimum of three months are suggested to reduce sperm motility in males attending an infertility clinic, where exposure may be detrimental to male fertility outcomes [125, 126]. However, personal habits and lifestyle had a significantly more prominent effect on poor motility than glycol ether. An earlier study first proposed glycol ether and other organic solvents as a potential risk to male fertility, in which men with poor sperm count were more likely to have been exposed to glycol ether [127]. Experimental studies subsequently established that ethylene glycol ethers (EGEs) can cause testicular toxicity, particularly monomethyl (EGME) and monoethyl (EGEE) ethers [128]. In experimental EGME-induced testicular toxicity, there is a negative effect on primary spermatocytes and spermatogonia, alongside inducing chromosomal abnormalities in experimental mice [129, 130].

34.6.4 Methoxychlor

Methoxychlor (MXC) is a synthetic organochloride used as an agricultural pesticide (insecticide), with mild estrogenic activity, as well as antiandrogenic properties. This has a similar structure to DDT, in which both substances have been banned in some countries, including the USA and Europe, although still used in other countries such as China [131]. Although MXC is >1000 weaker than 17-β-estradiol, its metabolite hydroxyphenyl trichloroethane is significantly more potent [132]. Evidence available suggests that exposure exerts endocrine disruption in males, with atrophy of sexual organs and negative effect on steroidogenesis and spermatogenesis [132]. MXC exposure, as an endocrine disruptor, has been shown to negatively modulate imprinting and methylation in epigenetic modification in sperm cells, exerting detrimental effects on the developing gamete [133, 134]. Interestingly, through male developmental exposure, low-dose experimental animals suggest an increase in fetal Leydig cell number and expressions of steroidogenesis-related enzymes; however, higher doses result in reduced steroidogenesis and Leydig cell numbers [131]. However, significant evidence suggests environmental exposure to decrease testosterone through downregulated biosynthetic pathways [131]. Perinatal exposure has been established to impair testicular development in animal experiments and adult fertility impairments as a consequence [135].

34.6.5 Phthalates

Phthalates are a group of esters of benzene-1,2,dicarboxylic acid (phthalic acid), used prominently in plastic industry, alongside cosmetics, personal care products, paints, toys, and pharmaceuticals, with widespread contamination in the modern environment [136]. Exposure to humans is through dietary consumption, containers, consumer products, and medical and pharmacological agents, among other common sources. These have further been associated with a negative impact on male reproductive health and fertility parameters in animal studies and are hormonally active [137, 138]. The impact of these chemicals on male reproduction is important, as they have been found in blood and urine of adults and children in the general population, as well as in utero and in human amniotic fluid [139].

Most evidence associated with phthalate exposure and male infertility are through animal studies, and human studies remain limited [140]. These show antiandrogenic effects, reduced sperm parameters, DNA damage, and chromatin defects in germ cells. This is more prominent in chronic exposure and fetal and perinatal exposures and developmental and adult offspring pathology [140]. In utero and lactational exposure to di(2-ethylhexyl) phthalate (DEHP) reduces sperm concentration, induces reproductive tract histopathology, and increases the risk of cryptorchidism in male offspring in laboratory rat experiments [141]. This further acts as an antiandrogen and negatively affects reproductive organ development through fetal exposure [142]. Perinatal animal exposure of DEHP further establishes the hypothesis of antiandrogenic effects and mild genital dysgenesis, causing concern for human fetal exposures [143]. Evidence strongly suggests fetal and perinatal exposure produces a variety of reproductive syndromes in males, affecting the testes (testicular dysgenesis syndrome), epididymis, seminal vesicles, and prostate, alongside feminization syndromes including smaller penis size, retention of nipples, and reduced anogenital distance [139]. This is mediated by a significant reduction in testosterone due to a downregulation of steroidogenesis enzyme cascades in Leydig cells [139]. Phthalate exposure in mice results in spermatogenesis dysfunction through microRNA alterations translating into mRNA transcription dysregulation mediating germ cell apoptosis [144].

Human evidence increasingly establishes phthalates modulating hormone levels, spermatogenesis, DNA integrity, and epigenetic modifications [145]. Moderate concentrations of phthalate exposure reduce sperm quality [140]. Monobutyl phthalate was found to negatively affect sperm concentration and motility, and monobenzyl phthalate affected only sperm concentration in a dose-dependent manner based on urine concentrations. However, no association was found for other phthalates [137]. Urinary monoethyl phthalate (MEP) was negatively associated with sperm concentration and normal morphology, where di(2-ethylhexyl) phthalate (DEHP) and mono-3-carboxypropyl phthalate correlated with morphology only [146]. In men exposed to phthalates as children, a

negative correlation between phthalate levels and sperm quality concentration, motility, and morphology has been presented [138].

Numerous phthalate metabolites in a male infertile cohort are correlated with poor semen parameters alongside endocrine dysfunction, including reduced insulin-like growth factor-3 (INS3), testosterone, free androgen index, LH, and sperm DNA integrity. This suggests that phthalates have diverse negative effects on male reproduction at environmental exposure level [147]. Urinary mono-n-butyl phthalate, mono-(2-ethylhexyl) phthalate, and mono-2-ethyl-5-carboxypentyl phthalate have been associated with infertile men, mediated independently through reduced testosterone and INS3 concentrations and associated Leydig cell dysfunction [136]. Further evidence reports various phthalates to be associated with reduced testosterone, FSH, and prolactin and have positive associations with estradiol in men presenting at an IVF clinic [148]. Furthermore, urinary phthalate concentrations have been associated with sperm DNA fragmentation in humans, and paternal environmental exposures influence epigenetic modification and negatively influence offspring development [149]. Further mechanisms in humans may include apoptosis-related gene polymorphisms caused by phthalate exposure, decreasing sperm concentration and sperm count in humans [150]. However, the evidence for a significant impact of current exposures in humans remains week, and the impact particularly in the fetal and perinatal phases is particularly critical.

34.6.6 Bisphenol A

Bisphenol (BPA) is an organic compound with two phenol functional groups and is a dysfunctional building block of several important plastic additives. BPA is an industrial chemical used to produce a hard, clear plastic known as polycarbonate, which has been used in many consumer products, including reusable water bottles and baby bottles, and is also found in epoxy resins, which act as a protective lining on the inside of metal-based food and beverage cans. On the other hand, BPA is a suspected endocrine disruptor with weak estrogenic, antiandrogenic, and antithyroid activities.

Endocrine disruptors can cause tumors, birth defects, and other developmental disorders, which may lead to testicular dysfunction and infertility. As such, BPA has been shown to be associated with elevated prolactin, estradiol, and SHBG levels in a study investigating 592 workers including 165 men from exposed factories [151]. Other studies found that urinary BPA levels are negatively correlated with semen quality and seminal antioxidant levels and positively associated with sperm DNA damage [152]. Although BPA significantly affects fertility-related sperm proteins such as the downregulation of β-actin and upregulated peroxiredoxin-5, glutathione peroxidase 4, or glyceraldehyde-3-phosphate dehydrogenase [153], the exact mechanism of action of how BPA disrupts spermatogenesis appears to remain unclear [154]. However, it seems that one of the targets for BPA action are the Sertoli cells [155]. There is also increasing evidence that BPA may be responsible for reproductive pathologies, e.g., testicular dysgenesis syndrome, cryptorchidism, cancers, and decreased male fertility [156].

34.7 Radiation

There are different types of radiations, which may have negative effects on male fertility. Generally, radiations can be differentiated into ionizing and nonionizing radiation. Among the latter radiations, one distinguishes extremely low frequency (ELF) and radio frequency (RF) electromagnetic fields, which are produced by wireless radio wave/microwave products. Among the ionizing radiations, there is ultraviolet radiation and X-rays and γ-rays. While ultraviolet radiation rather plays a significant role as a cause for melanoma, its effect on male fertility seems rather negligible.

34.7.1 Ionizing Radiation

34.7.1.1 Ultraviolet Radiation

Folate (vitamin B9) is a light-sensitive vitamin which is important for normal embryonal development [157], birth defects, and male infertility. A Norwegian study indicated a possible association between folate deficiency and autism [158]. Juzeniene et al. [159] report that 5-methyltetrahydrofolate is photooxidized under UVB radiation. In the presence of riboflavin or uroporphyrin, it is oxidized even in the blood stream. Therefore, high UV exposure might have health implications, which might also be relevant to fertility.

34.7.1.2 X-Rays and Gamma Rays

Exposure to radiation in the line of a medical treatment, even small amounts of ionizing, particularly that used for medical therapy, may destroy sperm-forming cells. Spermatogonia are particularly radiosensitive and may further impair testicular function [160]. Depending on the radiation dose, and whether or not stem cells were killed during the treatment, recovery for normal ejaculatory volume and sperm count is reportedly between 9 and 18 months if the radiation dose is below 1 Gy. At higher doses of 2–3 Gy exposure, one can expect about 2.5 years and more than 5 years at doses of 4–6 Gy [161, 162]. Radiation induces chromosomal changes, which lead to congenital abnormalities.

34.7.2 Nonionizing Radiation

34.7.2.1 Cell Phone Signals, 3G, Wi-Fi, and Microwave

In our modern world where cellular phones, Wi-Fi laptops, and microwave ovens are becoming more and more important, especially for the young generation, a negative impact of the electromagnetic waves generated by these devices has been reported [163, 164]. This widespread use of devices emitting electromagnetic waves is causing increasing exposure to electromagnetic fields, and this is recognized as "electro-smog" or "electro-pollution" [165]. Radio frequencies classified in group 2B are regarded as possibly carcinogenic for humans [166]. Currently, the legally recommended maximum specific absorption rates of cell phones are limited to 2.0 W/kg [167]. Yet, depending on the frequency and duration of talking, the position of the phone close to the ear, or keeping it in the pocket, the specific absorption rate can be even higher than this recommended maximum value [168, 169]. In this context, it has been shown that cell phone usage is a cause of decreases in motile sperm counts and sperm viability [168, 170, 171] and an increase in seminal oxidative stress due to elevated levels of reactive oxygen species [172].

As a result of this oxidative stress, sperm DNA damage [173] has been reported and may also be the cause of cancer [174, 175]. Similarly, Wi-Fi devices that are used in laptops to connect wireless to the internet caused significantly decreased sperm count, motility, and normal morphology [176–178]. It also appears that wireless devices not only have a significant effect on the development of male infertility but also cause brain tumors, hearing impairment, and heart diseases. In a survey including 300 medical doctors investigating their experience with their patients, 96% of the doctors indicated effects of cell phones, whereas for laptop 54%, Bluetooth devices 32%, tablet PC 14%, and wireless router 20% of the doctors indicated detrimental effects. Male infertility was at fourth rank with 36%, following brain tumors (84%), hearing problems (82%), and heart disease (46%) [179].

34.8 Tobacco

Tobacco is a generally socially acceptable habit, with reports of 30% of males globally who regularly smoke [180]. Tobacco consumption exposes humans to more than 4700 chemicals, including heavy metals, polycyclic aromatic hydrocarbons, and mutagenic chemicals [181]. Although the negative effects on human health of smoking have been well-established, the impact on male reproduction remains poorly reported and considered [180, 182].

It is increasingly well-accepted that tobacco consumption significantly impairs male fertility parameters [180, 183, 184]. This includes sperm concentration, motility, viability, morphology and DNA integrity, capacitation, and acrosome reactions [185–187]. The negative impact of smoking is mediated predominantly through OS in the male reproductive tract. This induces DNA damage, impairs chromatin condensation and oocyte binding, and induced epigenetic modifications with detrimental impact on the offspring [63, 184, 188]. Mechanisms of smoking-induced infertility include OS, hypoxia, inhibition of sperm creatine kinase activity, impaired energy regulation, and motility dysfunction, as well as increased hepatic of testosterone alongside impaired Leydig cell function [183, 189]. In addition, there is an improvement in male fertility parameters if he stops smoking [190, 191].

34.9 Air Pollution

There has been a significant increase in air pollution in industrialized countries over the past few decades. The negative effect on health is well-documented, including cardiorespiratory diseases, adverse perinatal effect, neurodevelopmental impairment and air pollution is an established human carcinogen [192, 193]. Exposure to increased air pollution includes a variety of different potentially toxic molecules. This includes polycyclic aromatic hydrocarbons (PAH), nitrates, sulfates, ammonium, carbon metals, lead (Pb), cadmium (Cd), nitric oxide (NO), nitrogen dioxide (NO_2), carbon monoxide (CO), carbon dioxide (CO_2), and ozone (O_3). Biomarkers established for exposure have been established in blood, urine, and seminal plasma, specifically for PAH, NO_2, Pb, and Cd [192, 194].

Animal studies have established a negative effect on male fertility, but human studies are less clear [192, 193]. Although causation remains generally poorly established, current evidence suggests a negative impact on poor sperm morphological forms and motility, with less evidence associated with other standard semen parameters and DNA fragmentation [192, 193]. Conflicting conclusions report that air pollution only affected sperm motility and no other sperm parameters [194]. DNA fragmentation due to air pollution exposure has been demonstrated in only a few studies [195–197], with contradicting results [198–200]. Air pollution is further suggested to cause polymorphisms in sperm DNA [201].

PAH, a prominent family of air pollutants, are considered important endocrine disruptors, affecting steroidogenesis and spermatogenesis [193]. This is further associated with sperm alterations [193] and mediated through acryl hydrocarbon, estrogen and androgen receptors, and inhibition of gap junctional intracellular communication, the latter of which is critical in steroidogenesis [202]. PAH have been well-established with impaired sperm DNA fragmentation, mediated through the production of PAH-DNA adducts [203]. However, clear evidence of association and any causal relationships remain unclear and require further investigation, as human studies generally lack consistency currently [192, 193].

34.10 Conclusion

Increasing amounts of evidence indicate the detrimental effect of unhealthy lifestyle choices and environmental pollution on male reproductive health. Not only fertility is increasingly compromised by poor sperm counts or poor sperm functionality through the incorporation of toxic chemicals either voluntary by an unhealthy lifestyle or involuntary by ingestion via contaminated foods. The molecular mechanisms of these detrimental effects are mainly orchestrated by direct oxidative stress on the male germ cells or via endocrine disruption. The latter can then affect the entire endocrine system and thereby seriously negatively affect embryonal and fetal development in utero. This can then lead to malformations and shifts in the gender ratio and also lead to various types of cancers including testicular and prostate cancer. Therefore, more education is needed for consumers to avoid products very prone to contain toxic substances, live a healthy lifestyle, and increase legislative pressure on the industry in order not to produce toxic substance with such far-reaching effects on environmental and human well-being. These health precautions have to include nonionizing radiations from microwave ovens, cell phones, and Wi-Fi devices.

34.11 Review Criteria

An extensive search of studies examining the relationship between environmental toxin exposure and male infertility will be performed using the following search engines: PubMed, MEDLINE, ScienceDirect, and Google Scholar. The start and end dates for these searches will be recorded within the final review criteria. The overall strategy for study identification and data extraction will be based the following keywords: “male infertility,” “infertility,” and “environmental,” as well as the names of specific environmental toxins described in this chapter. Articles published in languages other than English will be considered based on the availability of an English title and abstract. Criteria will prioritize studies within the last 10 years, published in accredited peer-reviewed journals. Data solely published in conference or meeting proceedings, websites, or books will not be included.

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Effect of Exogenous Medications and Anabolic Steroids on Male Reproductive and Sexual Health

35

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Key Points

- Many medications commonly prescribed in men of reproductive age can have effects on male reproduction. Studies in humans are limited. Physicians must know these effects to properly counsel their patients.
- Mechanisms involved in male subfertility/infertility derived from exogenous medications include: direct effects on the hypothalamic–pituitary–gonadal axis, modification in sperm production, impairment of sperm function and reduction in sexual function.
- Generally, most reproductive adverse effects are reversible and can be solved by simple cessation or switching to another drug with less or no effect on the reproductive system.
- Some drugs, such as chemotherapeutic agents, cannot be discontinued and, in such cases, semen cryopreservation before treatment is mandatory for preservation of reproductive profile.
- Infertility following anabolic steroids abuse commonly presents as oligo- or azoospermia with abnormalities in sperm motility and morphology. Sperm quality tends to spontaneously recover within 4–12 months after drug discontinuation.
- Clearly, more studies on key sperm functional tests are urgently necessary to establish the real impact of all these spermatotoxic and gonadotoxic substances in the sperm cell as well as all other cellular compartments of the testis, the Seminiferous tubules, the Sertoli cell and the Leydig cell. Modern Andrology translated in the environment of a High-Complexity Andrology Laboratory has the tools to unravel these doubts. For example, oxidative stress, reactive oxygen species, 8-OHdG, DNA fragmentation, antisperm antiboides, to name a few.

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35.1 Introduction

Males are responsible for half or even more of all the primary causes for infertility and in a significant number of them, the exact etiology remains unknown. Medical treatment of the most prevalent diseases in contemporary societies, such as diabetes mellitus, obesity and psychiatric/psychological conditions, might also impair reproductive potential. These medications and other substances provoke subfertility/infertility and sperm dysfunction by four mechanisms: (a) affecting the hypothalamic–pituitary–gonadal (HPG) axis, (b) altering sperm production, (c) impairing sperm function or (d) causing a reduction in main sexual functions (libido, erections and ejaculation). Unfortunately, most physicians do not inform patients and some do not even know about the potential deleterious effects for infertility associated with the medications they prescribe. This chapter aims to highlight the adverse

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effects mainly on male reproductive and secondarily on sexual health of frequently prescribed/used exogenous medications as well as anabolic steroids.

35.2 5α-Reductase Inhibitors

This class of medication blocks the conversion of testosterone (T) to dihydrotestosterone (DHT). In most men, spermatogenesis will be minimally affected by taking 5α-reductase inhibitors (5ARIs). A double-blinded, randomized clinical trial in normospemic males compared the use of two different 5ARIs (finasteride 5 mg and dutasteride 0.5 mg) and placebo for 1 year. In both 5ARIs groups, total sperm count decreased temporarily during treatment, but a subgroup of approximately 5% of men demonstrated a huge decrease in total sperm count to less than 10% of total baseline levels, suggesting a possible association between infertility and 5ARIs [1].

Furthermore, men taking 5ARIs have an increased risk of developing sexual disorders, such as ejaculatory dysfunction, diminished libido and erectile dysfunction (ED) [2]. Finasteride 1 mg (for male pattern hair loss) and 5 mg (for benign prostatic hyperplasia treatment) daily have been associated with sexual adverse events [3, 4]. In a 5-year study of 1553 men with androgenetic alopecia, taking finasteride 1 mg daily as treatment, the most commonly reported adverse effects were in the sexual domain (<2%), mainly reported in the first year of use. Libido decreased in 1.8% versus 1.3% in the placebo group, ED in 1.3% versus 0.7% and 1.2% versus 0.7% had ejaculatory dysfunctions [3]. In a 4-year, double-blinded, randomized, placebo-controlled clinical trial (Proscar Long-term Efficacy and Safety Study – PLESS), men with benign prostatic hyperplasia (BPH) ($n = 3040$) taking finasteride 5 mg daily reported sexual adverse effects up to 15%, with a rate of persistent sexual dysfunction among 50% of affected patients following cessation of this 5ARI use [4]. Dutasteride has the same sexual adverse effect profile as that of finasteride [2, 5]. Although these data suggest little to moderate influence of 5ARIs in main sexual domains, it is quite common to see patients with persistent refractory loss of libido after chronic use of 5ARIs in clinical practice, so one might argue that these numbers are clearly sub notified.

35.3 α-Blockers

Impairment of male fertility by α-blockers is caused mainly by ejaculatory dysfunctions, expressed by decreased ejaculatory volume, retrograde ejaculation and/or weak force of seminal emission. This class of medication is usually used to treat lower urinary tract symptoms (LUTS) from BPH. In a randomized clinical trial with healthy men, decreased ejaculatory volume caused by inhibition of seminal emission was found in two groups taking α-blockers: tamsulosin (89.6%) and alfuzosin (20.8%). Moreover, 35% of men taking tamsulosin had complete anejaculation, and these effects on ejaculate volume are reversible upon drug discontinuation [6]. A recent meta-analysis demonstrated that ejaculatory dysfunction was more common in men treated with α blockers when compared with placebo (odds ratio, OR = 5.88), treatment with silodosin had an OR = 32.5 ($p < 0.0001$) and tamsulosin an OR = 8.58($p = 0.006$) [7].

Tamsulosin can result in ED (3.8% of users) and reduced libido (1–2%) [8, 9] and can also change semen parameters in some healthy men, by decreasing sperm count and motility [10]. No studies associating α-blockers and sperm functional tests, such as reactive oxygen species (ROS) and DNA damage, have, so far, been published.

35.4 Phosphodiesterase 5 Inhibitors

The advent of phosphodiesterase 5 inhibitors (PDE5i) marked a new era in the treatment of ED, and nowadays they are undoubtedly one of the most prescribed and studied class of medications worldwide. However, conflicting data on male fertility still remain.

Prospective, randomized controlled trials in humans using sildenafil have different results. Some demonstrated an increase in total sperm motility and sperm linear velocity [11, 12], while others pointed out no changes in basic semen parameters [13, 14]. Moreover, Vardenafil achieves basal seminal levels of 71% in relation to the maximum plasma concentrations of its primary metabolite. However, and unfortunately, there are no reports on its effect in key sperm functional tests (ROS, DNA fragmentation, etc.). The limited data available does not clearly demonstrate that there is an influence on basic semen parameters or on reproductive hormones in men taking PDE5i, 20 mg once a day [15]. Thus, more studies are urgently necessary.

Tadalafil inhibits phosphodiesterases 5 and 11 and has a longer activity time than sildenafil and vardenafil, which theoretically could increase the likelihood of adverse effects. Phosphodiesterase 11 is found in the pituitary gland, prostate and testes and is involved in sperm capacitation [16, 17]. In a randomized, placebo-controlled clinical trial, healthy men with normal semen parameters were given tadalafil 10 mg or 20 mg or placebo daily for 6 months and no significant difference in reproductive hormones or basic semen parameters were seen among the groups [18]. Nevertheless, a randomized, double-blinded, cross-over clinical trial with 18 infertile men who received a single dose of tadalafil 20 mg or sildenafil 50 mg, demonstrated that tadalafil treatment resulted in a decrease in total sperm motility (median 21.5% versus 28.5%), whereas sildenafil resulted in an increase in

total sperm progressive motility (median 37.0% versus 28.5%) [19]. Again, we face an absence of more reliable and meaningful sperm functional tests.

35.5 Psychotropic Medications

Psychotropic drugs are known to affect male sexual life, especially libido, erectile and ejaculatory function. The classes of psychotropic medications that commonly exert effects on male fertility are selective 5-hydroxytryptamine reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), monoamine oxidase inhibitors, tricyclic antidepressants and lithium.

Treatment with SSRIs increases prolactin secretion and the continued use of these medications can cause ED, up to 80% of incidence when using fluvoxamine [20], and a variable degree of ejaculatory dysfunction, with greatest effects related to the use of paroxetine and fluvoxamine [20, 21]. Since 2007, studies report that chronic use of SSRI increases sperm DNA fragmentation, although the mechanisms underlying this phenomenon have not yet been clarified [22–24].

SNRIs, another class of serotonergic antidepressants, are associated with a high frequency of sexual dysfunction (up to 80% frequency of moderate to severe sexual dysfunction). A Spanish cross-sectional study with psychiatric outpatients has reported frequency of ED (57.7% for Venlafaxine and 58.1% for duloxetine) and decreased libido (63.8% for Venlafaxine and 62.8% for duloxetine) [20].

Monoamino oxidase inhibitors can cause anejaculation [25]. Tricyclic antidepressants are associated with reduced sperm motility, decreased seminal volume, decreased libido and ED [20, 26]. Lithium carbonate reduces dopamine levels in the central nervous system and can also reduce libido and cause ED [27]. In vitro studies in humans demonstrated that lithium reduces sperm viability [28]. Again, we feel the lack of information on sperm functional tests.

35.6 Anti-hypertensive Agents

Most anti-hypertensive medications can indirectly affect male fertility by causing sexual dysfunction. Diuretics and β-blockers affect sexual function through effects on the sympathetic vasculature, while the roles of angiotensin-converting enzyme (ACE) inhibitors and calcium channel blockers in male fertility are yet to be determined because data remain conflicting [29–32].

Spironolactone has antiandrogenic activity, can cause ED, reduced libido and gynaecomastia and decrease sperm concentration and motility. Hydrochlorothiazide decreases penile blood flow and is associated with loss of libido and erectile function [33, 34]. β-blockers are associated with an increased risk of ED [29] and in vitro studies have reported that propranolol might inhibit sperm mobility [35, 36]. Ultimately, calcium channel blockers decrease sperm density, motility and the acrosome reaction inhibiting sperm-egg binding and thus prevent the female gamete from being fertilized adequately [37–40].

35.7 Anti-infection Medications

Practically all major classes of antibiotics and certain anti-infection medications have significant adverse effects on sperm production and function, but most mechanisms underlying the spermatotoxic effects of these medications are unknown because most data are from studies that were performed at a moment when many current scientific andrology laboratory techniques were not yet available [41]. Therefore, even with some inconclusive data, the anti-infection agents discussed must be used with caution in men whose partners are trying to get pregnant.

Studies using Trimethoprim-Sulphamethoxazole show conflicting results concerning basic semen parameters. One study reported that after 1 month of treatment no modifications were seen in basic semen parameters [42], while another study in 40 infertile men treated during 14–17 days had opposite results. Also, total sperm count decreased in 37% in the first study and increased in 42% in the other [43]. Again and again it is shown we clearly need more studies to satisfactorily answer these questions.

For penicillins/cephalosporins, only a few studies have investigated their effects on male fertility, mostly in vitro. Sperm of normospermic men was cultured for 24 h with increasing doses of amoxicillin and no effect on sperm parameters were found [44].

Aminoglycosides negatively affect spermatogenesis. Administration of neomycin for treating chronic urological inflammatory diseases decreases sperm count, motility and concentration [41]. Use of gentamicin before prostate surgery increased abnormal sperm morphology [45]. Macrolide antibiotics, such as erythromycin and tetracycline, might cause asthenospermia or could even be spermicidal at high doses [44, 46].

Nitrofurantoin at usual therapeutic doses did not alter semen parameters, although it can cause spermatogenic arrest, decreased sperm count and sperm immobilization at high concentrations (10 mg/kg daily) [41, 47].

Ketoconazol reduces testosterone levels via central effects and possibly a reverse alteration in semen parameters, mainly impaired sperm motility. These modifications are not as prominent in other antimycotic drugs, such as fluconazole and itraconazole [48–51].

Ribavirin, used in the treatment of chronic hepatitis C reversibly alters sperm morphology and motility [52]. Some

studies suggest that antiretroviral drugs used in HIV treatment have a negative effect on semen parameters, mainly motility, but no conclusion has been established for the real effect on sperm parameters of these medications because of intrinsic factors of HIV infection that may also alter some semen characteristics [53–56].

There are scarce data in the literature about the adverse effect of chloroquine, an antimalarial drug, on male fertility, but an in vitro study reported that this medication irreversibly reduced sperm mobility and viability [44].

35.8 Anti-inflammatories and Salicylates

Chronic treatment (>6 months) with acetylsalicylic acid and other non-steroidal anti-inflammatories can cause a reversible and dose-related reduction in sperm count, vitality, motility and normal morphology [57, 58].

Sulphasalazine at chronic dose for more than 2 months has spermatotoxic effects and is associated with decreased sperm count, normal morphology and motility as well as a reduction in serum testosterone concentrations [59, 60].

35.9 Opioids and Analgesics

Chronic use of morphine has been associated with erectile dysfunction and diminished libido [61–63]. Treatment with tramadol can result in delayed ejaculation and decreased libido [64].

35.10 Gastrointestinal Medications

Histamine receptor blockers, such as cimetidine, ranitidine and famotidine, have a direct gonadotoxic effect in the sperm cell, causing cell death by increasing intracellular Ca^{+2} influx [65]. Cimetidine also presents an indirect toxicity by competition with testosterone receptors, decreasing serum testosterone concentrations in vivo, with consequent decreased libido and potential ED [66, 67].

Lansoprazole, a proton pump inhibitor, promotes inhibition of testosterone synthesis in lab animals [68, 69].

Metoclopramide, an antiemetic drug also considered a "hidden neuroleptic", causes ED and reduced libido, due to hyperprolactinemia [70, 71].

35.11 Dermatological Medications

Studies in animals reported that isotretinoin is associated with an increased apoptosis of germ cells, whereas no impact on human sperm parameters was reported in its usual doses for acne treatment [72–74]. Another study in lizards with acitretine demonstrated alterations in the seminiferous tubules, but similar to isotretinoin, no effects in humans were described, so far [75–77]. It is clear that more studies are necessary to testify to the safety of these medications in men's health.

35.12 Antigout Agents

In a single case series, there are reports of oligospermia, azoospermia and alterations of sperm motility [78]. These alterations on sperm parameters seem to be dependent on associated factors of the diseases (Familial Mediterranean Fever) [79].

35.13 Anti-cancer Medications

35.13.1 Chemotherapeutic Agents

These agents can cause temporary or permanent infertility, depending on the agent (or combination used), dosage (dose/m^2), dosing interval and type of cancer treated. The negative effects on male fertility can be caused by damage to spermatogonial stem cells, Leydig cells or Sertoli cells and by direct genetic damage to sperm DNA [80, 81]. Figure 35.1 summarizes the chemotherapeutic agents and their estimated risk of gonadotoxicity [82, 83].

Alkylating agents are among the most gonadotoxic agents and foremost among these is cyclophosphamide. Sperm counts drop 2–3 weeks after the initiation of the treatment, reaching their lowest levels 8–12 weeks after beginning chemotherapy, and in about half of patients, spermatogenesis recovered after an average interval of time of 31 months [84]. When administered alone, the cumulative dose of cyclophosphamide necessary to cause prolonged azoospermia is approximately 19 g/m^2 [85]. Total cyclophosphamide dose correlates with the incidence of gonadal dysfunction, and doses <200 mg/kg, 200–300 mg/kg, 300–400 mg/kg and ≥400 mg/kg are associated with ascending incidences of 20%, 50%, 80% and 100%, respectively [86].

Chlorambucil is another alkylating agent used in the treatment of leukemia, lymphoma and nephrotic syndrome. In a study that investigated safe doses for testicular function in young men with nephrotic syndrome, it has been reported that chlorambucil can be used at a dose of 0.2 mg/kg/day for 6 weeks without serious damage to the testicles [87] (Fig. 35.1).

Cisplatin promotes direct damage to the Sertoli, Leydig and germ cells. In a study with 1,191 men treated for testicular cancer followed for 11 years, the median sperm concentration in men treated with chemotherapy based on cisplatin

High risk	Moderate risk	Low risk
1) Alkylating agents (chlorambucil, cyclophosphamide, ifosfamide, mustine) 2) Chlormethine 3) MOPP (nitrogen mustard, vincristine, procarbazine, prednisone) 4) Nitrogen mustarde derivatives (busulphan, melphalan) 5) Procarbazine 6) Any high-dose regimen used before bone marrow transplantation	1) ABVD (doxorubicin, bleomycin, vinblastine, dacarbazine) 2) BEP (bleomycin, etoposide, cisplatin) 3) Carboplatin 4) Cisplatin 5) Doxorubicin	1) Antimetabolites 2) Bleomycin 3) Dacarbazine 4) Dactinomycin 5) Mercaptopurine 6) Methotrexate 7) Topoisomerase inhibitors 8) Vinca alkaloids (vinblastine, vincristine)

Fig. 35.1 Estimated risk of gonadotoxicity of chemotherapeutic drugs and regimens

at a dose ≤850 mg/m^2 was 18 × 10^6/ml and 1 × 10^6/ml in men treated with chemotherapy based on cisplatin at a dose >850 mg/m^2 versus 30 × 10^6/ml in a control group of men treated only with surgery (p = 0.001). Between 10% and 34% of testicular cancer survivors who received chemotherapy containing cisplatin had lower serum testosterone levels than men who were treated with surgery only [88]. These altered hormonal concentrations might reduce libido and alter erectile function, secondarily affecting fertility [89].

Methotrexate might cause reversible oligospermia [90]. Vincristine is associated with transient infertility by impairing spermatogenesis [91]. Dacarbazine has more serious effects and is considered a cause of prolonged azoospermia [92].

Theoretically, new chemotherapeutic regimens have fewer gonadotoxic effects than older regimens; however, paradoxically, every time a new drug is released onto the market, it does so based on a few animal studies, and time will tell how the drug will behave in humans. The MOPP regimen results in long-term azoospermia in almost all patients, while two-thirds of men who received the ABVD regimen as treatment for Hodgkin's disease recovered their sperm counts to baseline values at 1 year from the beginning of the treatment [93, 94]. When compared to the CHOP regimen (cyclophosphamide, doxorubicin, vincristine and prednisone), after 2 years >90% of patients who received the ABVD regimen would recover normal sperm count, while 61% of those who received the CHOP regimen behave the same way [94].

The standard option for preserving male fertility is clearly to promote and perform sperm cryopreservation in postpubertal men before chemotherapy. The ASCO guidelines of 2018 state that all efforts should be done to obtain sperm or testicular tissue by any means necessary from patients with cancer, and even for prepubertal boys it is strongly recommended that testicular tissue in the spermatogonial stage is harvested for potential future use. Doctors and health care professionals should discuss at the earliest the risk of fertility impairment and are prompted to refer the patient to a qualified specialist in fertility preservation, including the Andrologist [95].

35.13.2 Targeted Therapies

Studies of the effects of targeted therapies on male fertility are limited, but they have gained more importance because of the growing clinical role of these medications [96]. Thyrosine kinases inhibitors have a negative impact on spermatogenesis/sperm parameters and on sexual function. Studies in animals and in vitro with Sorafenib have demonstrated decreased sperm count, normally reversible after a 10-week treatment discontinuation [97, 98]. Sorafenib therapy is correlated with ED [97]. Imatinib might cause low testosterone levels and gynaecomastia in approximately 20% of men related to decreased Leydig cell function [99]. Sunitinib can induce gynaecomastia, but the effects on spermatogenesis or semen parameters are yet to be demonstrated [100].

Sirolimus is a mammalian target of rapamycin (mTOR) inhibitor that can cause reversible alterations on basic semen parameters, as well as decreased intratesticular testosterone levels [59, 101, 102]. Sirolimus may also act as an inhibitor of the Kiss system at the hypothalamus, blocking the pulsatile secretion of GnRH [103]. Infliximab, a TNFα inhibitor, is associated with reduced sperm motility and increased semen volume; however, no case of infertility has been associated [59, 104]. Again, more studies are clearly necessary to analyze sperm functional tests as a more reliable marker of sperm function/dysfunction.

35.14 Androgenic Anabolic Steroids

35.14.1 Background

Testosterone is by far the most important androgen synthesized in the human body. The effects of androgens are exerted in several tissues, serving distinct functions at all stages of life. These are most evident during puberty, as it elicits dramatical physiological changes in the male body. It includes the onset of secondary male characteristics, such as deepened voice, hair growth pattern, sebaceous gland activity, maturation of sperm and libido increase. These are considered the virilizing or "androgenic" effects [105].

Individual daily testosterone synthesis ranges from 2.1 to 11.0 mg (average 7 mg) among males and 1 to 4 mg (typically 10% of that observed in men) among females [106].

The normal range of plasma testosterone in males is 300 to 1000 ng/dL, progressively declining with age. Testosterone has several possible metabolic fates. First, it binds to the androgen receptor (AR) in target tissues to exert its anabolic and androgenic effects. Second, it is 5α-reduced (at scalp, liver, prostate and testicles) to dihydrotestosterone (5DHT), which also acts on the AR. Following a different path, testosterone may be aromatized to estradiol to exert estrogenic effects, typically water retention, breast tissue growth and increase in body fat deposition. Estradiol and estrone have importance in sexual drive (libido) and consequently, are important for overall sexual performance.

35.15 Anabolism Versus Androgenism

Along with the "androgenic" effects comes the "anabolic" changes. Anabolism is defined as any state in which nitrogen is differentially retained in lean body mass through the stimulation of protein synthesis and/or a reduction in protein breakdown. In the clinical setting, this state includes growth promotion, enhanced rate of protein synthesis, increases in muscle size, bone metabolism and collagen synthesis. Characteristically, the more anabolic a molecule is, the weaker its affinity for AR. On the other hand, steroids which present a higher androgenic:anabolic profile tend to bind more strongly at the AR, exerting a markedly more potent effect. In other words, AAS potency is usually linked to its potential androgenic effects but inversely related to its anabolic profile. In order to better quantify each drug's anabolic–androgenic proportion, a *myotrophic–androgenic index* was developed, more than 50 years ago. It is basically a relation between the growth of rat levator ani muscle weight (as a bioassay of protein anabolic activity) and growth of rat seminal vesicles or ventral prostate weight (as an assay of androgenicity) [107]. These assays are amenable to several criticisms, given the unsophisticated in vivo nature of them. However, the activities of many substances were assessed by these methods throughout the decades, allowing some degree of confrontation among them. Since testosterone is the basic AAS, it is standardized as a 1:1 anabolic–androgenic ratio (or *index*).

35.16 Anabolic Steroids: Beyond Testosterone

Some structural modifications have chemically been introduced into the basic testosterone molecule in order to maximize the anabolic effects and minimize the androgenic ones. However, all AASs are virilizing if administered for long enough, at sufficiently high dosages. Hence, a more accurate term for anabolic steroids would be "anabolic–androgenic steroids" (AAS); they are synthetic derivatives of testosterone and not only testosterone itself. These chemical modifications mainly tend to alter the relative anabolic–androgenic potency, slow the rate of inactivation and decrease their aromatization to estradiol. The AAS structural base is the so called "*steran nucleus*"; a polycyclic C17 steran skeleton consisting of three condensed cyclohexan rings in a nonlinear or phenanthrene junction (A, B and C) and a cyclopentane ring (D). The anabolic effects of testosterone (and AAS) are dose dependent and occur only when mean serum testosterone concentrations are found above the physiological levels (usually more than 1000 ng/dL), which generally requires doses of 300 mg per week or higher (a clear supraphysiological condition).

Traditionally, AAS are classified according to the route of administration and their carrier solvent [108]:

35.16.1 Oral AAS Preparations, or 17α-Alkylated Steroids

Oral activity can be conferred by substitution of the 17α-H on the steroid nucleus with a methyl or ethyl group to make the 17α-alkylated anabolic steroids. Alkyl substitution prevents deactivation of the steroid by first-pass metabolism by sterically hindering oxidation of the 17β-hydroxyl group. Hence, the 17α-alkylation allows these products to become orally active. Characteristically, they are mostly absorbed into the stomach or proximal small bowel and have a short half-life, making several daily doses necessary in order to maintain appropriate blood concentrations. The intake of these compounds should alert the assisting physician about liver stress assessment. This class includes the very common stanozolol and oxandrolone. Other compounds in this group are oxymetholone, methandrostenolone, fluoxymesterone, mesterolone and methyltestosterone.

35.16.2 Parenteral AAS Preparations or 17β-Esterified Steroids

Typically, the 17b-hydroxyl group is sterified in an acid moiety to prevent rapid absorption from the oily vehicle, usually arachis oil plus benzyl alcohol. The type of acid used to acidify the 17b-hydroxy group determines the duration of anabolic action. Roughly, the longer the chain length of the acid moiety, the more slowly the preparation is released into the blood stream, thus prolonging the duration of action. Once in the circulation, hydrolysis rapidly occurs to yield the active compounds. They usually have a longer half-life and slower absorption rate, bringing much less hepatic stress than the orally taken steroids. Another landmark of these products is the very common occurrence of a degree of pain at the injec-

tion site, due to their oily base. There are four basic active compounds:

(a) *testosterone*, bound to esters acetate, cypionate, decanoate, enanthate, isocaproate, phenylpropionate, propionate and undecanoate;
(b) *19-nortestosterone (or nandrolone),* bound to esters cyclohexylpropionate, decanoate, laurate and phenylpropionate. Nandrolone esters are extremely common among AAS users, due to their excellent anabolic–androgenic ratio. Opposite to testosterone, nandrolone is converted to a less potent metabolite after 5α-reduction. This, in addition to nandrolone's lesser affinity to AR, explains why this compound has a greater myotrophic–androgenic ratio when compared to testosterone.
(c) *boldenone*, bound to ester undecylenate,
(d) *trenbolone*, bound to ester acetate (Table 35.1).

AAS may also be classified considering the main activity of the most active substance:

1. *"Testosterone-like" effect*, very potent, including the greatest muscle strength gains. These compounds usually show an anabolic/androgenic ratio of 1:1, very similar to testosterone itself. This also explains the high aromatization rates, which are comparable to testosterone's. They include the testosterone esters and their blends, methyltestosterone, boldenone, methandrostenolone and fluoxymesterone.
2. *"Dihydrotestosterone-like" (DHT-like) effect*, potent but highly androgenic. Since they resemble a 5DHT molecule, they cannot be aromatized to estrogens (this is the group's landmark). It also explains the low water and salt retention of drugs of this group. They include stanozolol, oxandrolone, methenolone, mesterolone and oxymetholone.
3. *"Nandrolone-like" effect,* the less potent of all, showing the highest anabolic/androgenic ratio, though. They have some progesterone-like activity, inhibiting the hypothalamic axis. These are the drugs most used in the clinical setting, when anabolic effects are desired (reversing catabolic states, such as AIDS associated cachexia, severe burns, sarcopenias in patients receiving dialysis, wasting syndromes and chronic obstructive pulmonary disease). They include the nandrolone esters and trembolone (Fig. 35.2).

Table 35.1 Commonest AAS worldwide, according to main effect

Commonly used Anabolic–Androgenic Steroids	
Compounds and brand names (U.S., Europe and Brazil)	
Compound name	Brand name
"Testosterone-like" effect	
Testosterone Esters: Cypionate	*Deposteron®, Testex Leo®*
Testosterone Esters: Undecanoate	*Nebido®, Androxon®*
Testosterone Esters: Blends	*Durateston®, Testoviron®, Sustanon®, Omnadren®*
Methyltestosterone	*Methyltestosterone®, Metandren®*
Methandrostenolone	*Dianabol®, Anabol®, Naposim®*
Chlorodehydromethyltestosterone	*Turinabol®*
Fluoxymesterone	*Halotestin®*
Boldenone	*Equipoise®, Equilon®*
"DHT-like" effect	
Stanozolol	*Winstrol®, Stromba®*
Oxymetholone	*Anadrol®, Hemogenin®, Anapolon®*
Mesterolone	*Proviron®*
Methenolone	*Primobolan®*
"Nandrolone-like" effect	
Nandrolone decanoate	*Decadurabolin®*
Nandrolone phenylpropionate	*Durabolin®*
Trenbolone	*Finaplix®, Parabolan®*
Nandrolone undecanoate	*Dynabolon®*
Oxandrolone	*Anavar®*

35.16.3 Side-Effects

Side effects during anabolic steroids use are also well known and should always be kept in mind when one is assisting a user [109]. Acne is usually related to a strong androgenic effect, especially 5-DHT, which occurs after testosterone conversion or administration of a synthetic steroid with 5-DHT activity. This also explains the occurrence of alopecia among users of potent androgenic steroids, as far as the lower urinary tract symptoms, due to prostate enlargement. When one is taking steroids with a more anabolic profile, erectile dysfunction and libido loss tend to occur. This is especially true after discontinuation of these substances, since the hypothalamic axis is generally inhibited and endogenous testosterone is usually low. It creates a situation of hypogonadotrophic hypogonadism (including atrophic testicles), usually considered a transient state among men. Interestingly, this situation leads to a different condition in female individuals, with a more permanent and devastating picture, which includes virilization, hirsutism, increased facial hair, voice deepening, clitoral hypertrophy, menstrual irregularities and male-pattern baldness. The sustained rise in testosterone (or its analogues) levels leads to an increase in C19 androgens, androstenedione and testosterone aromatization of the C18 estrogens, estrone and estradiol, respectively, by the testicular CYP19A1 aromatase enzyme, located in the Leydig cell [110]. This increase in estrogens is responsible for the typical gynecomastia found in steroid users (its management should include antiestrogen therapy, with clomifen citrate, or aromatase inhibitors, such as anastrozole). Hepatic side effects are also described and are most related to the use of oral alkylated agents. They include the uncommon *peliosis hepatis*, with its multiple and small hemorrhagic liver cysts, and cholestatic jaundice, closely related to dose

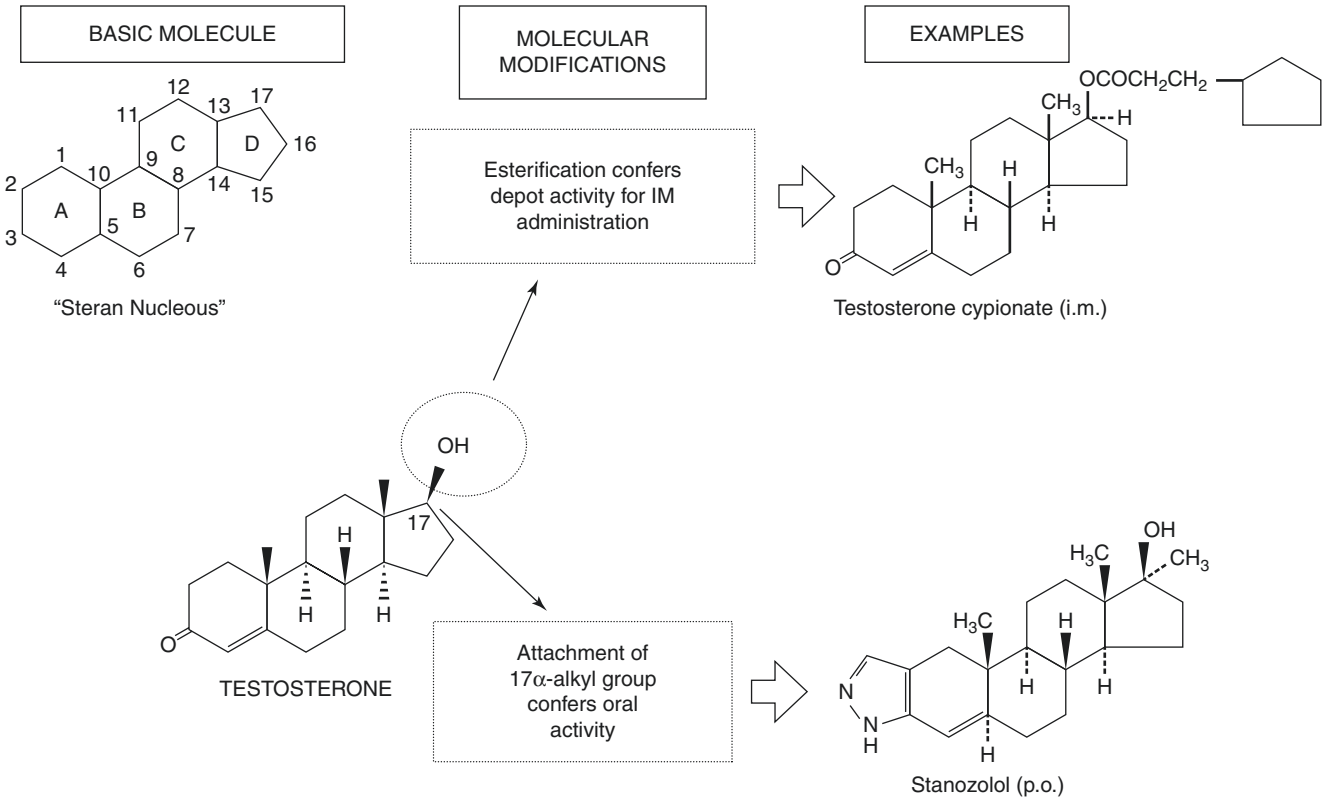

Fig. 35.2 The basic "Steran nucleous", typical structual modifications and examples of modified molecules

and duration of oral alkylated AAS intake, and therefore preventable when the recommended doses of these drugs are used. The laboratory studies reveal elevations in conjugated bilirrubin, AST and ALT. Alkaline phosphatase levels are normal or mildly elevated in most patients. Hepatic neoplasms, such as diffuse hyperplasia, nodular regenerative hyperplasia and focal nodular hyperplasia, have also been attributed to alkylated AAS. Hepatocellular carcinoma (HCC) is a more serious adverse effect associated with AAS therapy. Most reports show a clear relation between the cases of HCC and the duration of therapy (longer than 2 to 4 years) and the high dosages used. Wilm's tumor occurrence secondary to AAS intake has also been reported. There are no reports linking AAS with prostate cancer or androgen treatment with significant increases in prostate-specific antigen. The most severe consequences of long term AAS use probably rely on the cardiovascular system. Several cardiovascular side effects have been reported, including hypertension, arrhythmia, erythrocytosis and ventricular dysfunctions. Lipid profile usually shows a rise in LDL levels and decrease in HDL levels, and may contribute to the vascular damage and increase in myocardial infarction and sudden death risks seen in long-term users. It is important to emphasize the increased risk of mortality among chronic AAS users, which is reported to be 4.6 times higher than non-users. Steroid use may cause elevation in serum creatinine, blood urine nitrogen (BUN) and uric acid. The combination of AAS and creatine supplements are common and may cause renal damage. Cases of renal failure secondary to rhabdomyolysis and diffuse membranoproliferative glomerulonephritis in heavy users have been reported. Aggressive behaviour, depression, mood swings, altered libido, euphoria and even psychosis are some of the psychic patterns classically related to testosterone use [111]. The use (or stacking) of multiple substances (polypharmacy) may increase the risk of violent criminality. AAS withdrawal and dependency walk side-by-side, and the likelihood of psychiatric effects are increased with prior psychiatric history, alcohol and other drug abuse. Finally, there are complications related to AAS administration (especially parenteral ones). Systemically, anaphylaxis is certainly the most feared effect one should expect. Injection related effects may occur due to poor injection technique, repeated injection sites, sharing needles or vials and contaminated drugs. Direct muscle injection for local "best results" (biceps or pectorals) may lead to abscess formation, oil-induced granuloma, intramuscular fibrosis and dystrophic calcifications [112]. Needle stick injuries causing nerve damage may occur after misplaced injections.

35.16.4 Anabolic Steroids Impact on Male Fertility

35.16.4.1 The Classic Reversible AAS-Induced Hypogonadotrophic Hypogonadism

Infertility following AAS abuse commonly presents as oligo- or azoospermia, associated with abnormalities in sperm motility and morphology. According to most reports, sperm quality tends to spontaneously recover within 4 to 12 months after cessation of anabolic steroid abuse [113]. However, the negative effect on semen quality may persist for longer periods, along with low serum testosterone and gonadotrophins levels. Exogenous administration of synthetic testosterone may strongly affect the male pituitary–gonadal axis [110]. A hypogonadal state can be induced, being characterized by decreased serum testosterone concentrations, testicular atrophy and impaired spermatogenesis. These effects result from the negative feedback of androgens on the hypothalamic–pituitary axis and possibly from local suppressive effects of exogenous androgens on the testis. FSH and LH concentrations are also low in this steroid induced state of hypogonadotropic hypogonadism. The whole picture leads to a state of decrease in spermatogenesis and consequent low quality semen. Thus, infertility often results. In addition to it, in an AAS abuse situation, serum androgen concentrations may be supraphysiologically high, but they generally may not produce the testicular concentrations necessary to maintain spermatogenesis.

35.16.4.2 Permanent Testicular Damage

Histopathlogy

Experiments in animal models (particularly rats and stallions) report mainly Leydig cell alterations in AAS use situations [114]. Leydig cell depletion is, by far, the commonest finding among these studies, although morphology anomalies (from shape aberrations to nucleus picnosis and cellular atrophy) have also been reported. The decrease in this population of cells is accompanied by low testosterone and LH levels in all papers reviewed [114–116], especially among adult animals. Immunohistochemical analysis has also been performed, with findings suggesting decreased steroidogenesis in testicular tissue. Interesting findings show low inibin levels along with normal FSH levels, suggesting an important participation of the Leydig cell compartment on overall inibin production. Also, spermatogenesis has not been found abnormal in all experimental reports, being considered unchanged by some authors. On the other hand, one study found specific end-stage spermatogenesis impairment, with a lack of tubules at stages VII and VIII, which normally contain the most advanced forms of spermatids. Histological patterns after AAS discontinuation have also been described. Leydig cells tend to proliferate but still remain above the regular counts, even after long-term AAS withdrawal periods, up to 16 weeks (of note, previous reports mention a 2–7 week period being necessary for the re-appearance of Leydig cells after their total elimination with testosterone and estradiol implants). Clearly, long lasting, or possibly persistent effects cannot be ruled out. It is important to highlight that in all experimental studies involving testicular histology, all specimens were fixed in formalin and immediately embedded in paraffin. Bouin's liquid has not been employed in either study reviewed, which may have caused shrinkage of all germinative cell forms, leading to an underdiagnosis to some degree of spermatogenic abnormality [117].

Impact on Semen Quality

Association of hCG to steroids is a common practice among AAS users. The goal is to avoid the impact of LH suppression after long term AAS administration, which classically may lead to a persistent state of hypogonadism (with low levels of endogenous testosterone, along with all its potential effects) and low quality seminal parameters, commonly described as severe oligozoopsermia or even azoospermia (more recently also teratospermia has been identified). In an interesting study, the authors analyzed 18 concomitant AAS and hCG users. Spermatogenesis could be restored, but more abnormal and hypokinetic spermatozoa were produced, and the doses of hCG were independently correlated to the percentage of morphologically abnormal spermatozoa [118]. Again, potentially persistent alterations seem to be possible among steroids users.

Apoptosis

Germ cell apoptosis has been reported to play an important role in normal testicular physiology, and apoptotic control is important for regulating the germ cell population in the adult testis. Recently, the correlation between apoptosis and high AAS doses and exercises has been experimentally assessed [119]. TUNEL [terminal deoxynucleotidyl transferase (TdT) enzyme mediated dUTP nick end labelling], caspase-3 activity assay (C3AA) and transmission electron microscopy (TEM) were performed in order to identify apoptosis in animal models. A significant increase of apoptosis was found after nandrolone administration, clearly being amplified by physical exercise. The same authors also report evident impairment in seminal parameters among this set of individuals, in these same conditions. Testicular histopathological evaluation has also been performed, revealing low quality spermatogenesis following Johnsen's method.

Aneuploidies and Ultrastructural Changes in Spermatozoa

In order to study specific sperm malformations that cause human infertility, TEM is certainly the most valuable tool. On the other hand, evaluation of meiotic errors during

spermatogenesis requires the employment of fluorescence in situ hybridization (FISH). An elegant 2007 report made use of both these tools in an AAS user, in an innovative attempt to further enlighten the consequences of steroid abuse [120]. TEM was performed, and the three main phenotypic sperm pathologies (immaturity, necrosis and apoptosis) were searched. A high percentage of structurally normal spermatozoa were found, suggesting the absence of correlation between AAS use and ultrastructural sperm changes. Opposed to these findings, FISH sperm analysis revealed some degree of XY disomies along with higher frequencies of chromosomes 1 and 9 disomies. No significant incidence of diploidies was found. These findings indicate a segregation anomaly at the first meiotic division (the increased frequency of sex chromosome disomy), suggesting, for the first time, anomalies in the meiotic process and genetic damage among subjects that habitually use steroids.

35.16.5 Management Strategies

Management of AAS-induced male infertility has also been extensively reported. Conservative management has been previously described [121]. Simple discontinuation of AAS use may warrant fertility recovery in a certain proportion of male users. Probably, the duration of AAS abuse may interfere in the outcomes of conservative (or even non-conservative) management of this condition, even though it has also been suggested that, even after prolonged use of high doses of AAS, sperm production may return to normal levels. Also, there is little literature and considerable disagreement regarding the management of such cases (especially those involving prolonged azoospermia). On the other hand, patients may be actively treated, in a manner similar to that used for other forms of hypogonadotropic hypogonadism. In these situations there is a lack of FSH and LH production, lowering the endogenous testosterone production, similar to the findings in athletes taking anabolic steroids. Treatment for virilization in these patients involves weekly doses of intramuscular testosterone (which is clearly not part of the AAS-related male infertility). However, the induction of spermatogenesis requires treatment with gonadotropins or gonadotropin analogues, including intramuscular injections of human chorionic gonadotropin (hCG) and human menopausal gonadotropin (hMG) [122]. The goal of treating AAS-induced azoospermia focuses on resuming endocrine function. Endocrine drugs are specifically administered to ameliorate the hypothalamic–pituitary–gonadal axis. hCG alone or in combination with hMG has been reported to be successful in treating this group of patients. There is no consensus on the ideal dosage of either hCG or hMG in the treatment of this condition. Reports on hCG therapy mention dosages ranging from 2000 units 3 times weekly to 10,000 units twice weekly (for around 90 days); hMG dosages range from daily to 3 times weekly 75 units (for around 30 days). Generally, once therapy is initiated, fertility rates in hypogonadotropic hypogonadal patients are impressive. Great increases in sperm quality may occur in short periods (up to a single month), even in situations of persistent azoospermia following up to 5 years of AAS discontinuation. The papers reviewed present several subjects who were able to achieve pregnancies after seminal recovery.

AAS-associated male infertility is probably an underdiagnosed but potentially treatable form of drug-related infertility, due to its endocrine nature. Considering the increasing prevalence of AAS abuse, it is reasonable to consider it as a possible cause of male infertility and subfertility. Hormonal treatment should be considered as an efficient and suitable alternative if spermatogenesis does not show signs of potential spontaneous recovery. Obviously, conservative management is always desired and should be preferred. However, previous reviews about this issue suggest considering aggressive treatment with exogenous gonadotrophins if there is absence of improvement in sperm parameters for longer than 24 months.

35.17 Conclusions, Management Policy and Authors' Recommendations

The common thread among all available medical literature on anabolic steroids use and misuse is certainly the lack of homogeneity, not only on the subjects themselves but also on the pattern of use of these substances. The so called "performance drugs" are commonly prescribed and administered in an "unofficial" fashion, which make objective comparisons between drugs and combinations of them almost impossible. We can find some of the most known combinations ("stacks") of substances in the "underground" literature only, but never among the well-designed studies, from where proper medical conclusions should be taken.

There are several ways in which the use and abuse of anabolic steroids may impact on one's health. The impact on male fertility is one of the less reported, but certainly, one that the day-by-day urologist should know better, since reversion of the majority of the dysfunctions caused by this misuse can almost always be achieved, at least among light and moderate users. Correct medical orientation, immediate identification of users and prompt clinical management of deleterious consequences (sometimes requiring the use of hormonal therapy) are the cornerstone of good urological practice when assisting a situation of anabolic steroids use. Physical examination (with special attention to signs and symptoms of hormonal imbalances), base seminal analysis and hormonal profile should be assessed. Steroids should have their use discontinued immediately. Attention should be paid to "steroid-free" vitamin or protein supplements, especially in countries where their formulas are patent-protected or where regulatory instruments are not properly working. These supplements may con-

tain traces of hormonal substances of up to 25% in some countries. In case of doubt, their use should also be discontinued. Most of the series reviewed show recovery of seminal and hormonal levels to a normality state in periods around 6 months. In some cases, when this state is not achieved, patients' testicular function should be boosted with hormonal induction, with gonadotrophin administration. Classical reports support this practice, showing recovery from azoospermia in AAS users with up to 5 years of steroid abuse.

On the other hand, experimental studies and clinical series suggest some grade of definite impairment on testicular function, which should lead us to conclude that the impact of steroids use on male fertility is not only a transitory condition. As far as we can see, the best policy is to strongly discourage the use of this kind of substance, and thoroughly follow-up those who insist on their abuse, warranting suitable and ethical clinical and urological support.

35.18 Review Criteria

A search of studies examining medications commonly prescribed to men of reproductive age and their effect on male fertility and sexual function (including anabolic steroids) was performed using search engines, such as PubMed, MEDLINE and Web of Science. The overall strategy for study identification and data extraction was based on including the name or class of a drug in combination with "male infertility" or "male fertility", "sperm" or "semen parameters", and "sexual function" or "sexual dysfunction". Search terms included "anabolic steroids", "5α-reductase inhibitors", "α-blockers", "phosphodiesterase 5 inhibitors", "psychotropic medication", "antihypertensive agents", "antibiotics", "antimycotic drugs", "anti-inflamatory", "analgesics", "opioids", "gastrointestinal medications", "dermatological medications", "antigout agents", "chemotherapeutic agents" and "targeted therapies". References discovered during the review procedure were also included. After the exclusion of articles that were not applicable or contained duplicate information, 123 papers remained.

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Male Age and Andropause

36

Marwan Ali and Neel Parekh

Key Points

- Advanced male age is associated with decreased androgen levels.
- Decreased androgen levels impact male fertility, the genetics of future offspring, physiologic health, and overall quality of life.
- The mechanisms by which androgen levels decrease with age are multifactorial, and research elucidating these processes is ongoing.
- Research on this topic has shed light on methods that clinicians may use to better diagnose and treat this age-related process.

36.1 Introduction

Approximately 15% of couples of reproductive age experience infertility, and approximately 30–50% of infertility cases may be attributed to male factors [1]. While the role that maternal age plays in fertility success has been established, various age-related paternal factors that can also be quite significant have only recently been investigated. This topic is particularly pertinent at the present. Easier accessibility and advancements in reproductive technologies, rising life expectancy, and increasing paternal age all highlight the importance of understanding the mechanism by which aging impacts fertility, overall health, and quality of life.

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Women develop progressive decrease in fertility due to both quantitative and qualitative loss of oocytes, eventually ending in menopause. With these changes, an age-dependent increase of miscarriages, obstetric morbidities, and chromosomal anomalies of the fetus may occur [2]. Given these established age-related changes in women, this begs the question of the impact on fertility that age plays in men. In contrast to females, male reproductive functions do not abruptly cease with age. Although spermatogenesis and androgen synthesis are continuously ongoing in males, the quality and quantity of their production changes with age which has prompted increased research into the mechanisms behind these changes.

However, there can be obstacles to investigating this population. In particular, semen quality in the aging male population can be difficult to assess. Population-based studies typically recruit at least 20% of young men willing to provide semen samples [3] constituting an inevitable participation bias in such studies [4, 5]. Despite these challenges, increasing amounts of data have emerged which have suggested a clear association between advanced paternal age and decreased semen volume, sperm motility, and percent normal sperm morphology [6–9]. The basis for altered semen parameters with advanced age is still not completely understood; however, several mechanisms have been suggested including both age-related morphologic changes and molecular changes influenced by oxidative stress.

Ford et al. determined that, after adjustments for other factors, the probability that a fertile couple will take >12 months to conceive nearly doubles from 8% when the man is <25 years to 15% when he is >35 years [10]. This data is telling, yet may also be understated given its reflection of a conditional probability of conception based on couples who achieved success in a 6–12-month period, lending to a lack of generalizability. This further highlights the importance of paternal age as a notable factor when predicting the prognosis for infertile couples. The fertility implications associated with advanced male age has not only been seen in natural conceptions. Emerging data has suggested the age-related

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impact on fertility extends to conception utilizing assisted reproductive technology (ART), which has seen a threefold increase over the past 20 years [11].

In addition to altered semen parameters affecting age-related infertility, endocrine changes have been found to exhibit a hormone profile similar to a state of primary testicular dysfunction [12]. These endocrine changes result in decreased levels of free testosterone which not only can impact fertility and libido but also have been shown to have a multitude of systemic effects on a man's overall health [13–15]. This chapter will explore the mechanisms and impact of advanced male age on fertility, development of future offspring, and the endocrine changes that result in an age-related decrease in androgens over time.

36.2 Aging

36.2.1 Cellular Changes

It is well established that various physiologic events occur as the human body ages. Senescence is a general term used to describe the condition or process of deterioration that comes with age. On a cellular level, this process that arises over time may occur in various genes in the body, resulting in accumulated cellular damage that ultimately leads to an increased risk of pathologic conditions [16]. Multiple molecular hallmarks of aging have been identified including genomic instability, resulting in shortening of telomeres, subsequently causing stem cell exhaustion, altered nutrient sensing, alterations in RNA splicing, and disruptions in intracellular communication with loss of regulation of protein degradation [17].

A particularly significant age-related change that occurs is mitochondrial dysfunction, which has been shown to result in the accumulation of reactive oxygen species that can significantly alter normal cellular processes. Although it may be presumed that this constellation of cellular events occur on a global scale, studies have demonstrated that only a minority of genes are impacted by these age-related changes [18]. In fact, tissue samples from aging human samples have demonstrated a transcriptional upregulation of genes involved in stress response pathways to oxidative stress. Although there appears to be significant variability in the small degree of genes that are downregulated with age, the consistent trend appears to be that genes involved in splicing and mRNA processing are most commonly affected [18].

Despite controversy regarding why certain genes appear to be more susceptible to downregulation with advanced age, the data suggesting that DNA damage in aging tissues targets specific functions has lent itself to several hypotheses. One study examining the transcriptional changes that occur in the aging human brain has suggested that certain transcriptional promoter regions of genes demonstrating age-related downregulation are more vulnerable to oxidative DNA damage compared to promoters of unaffected genes [19]. This emerging research is ongoing and attempts to explain why the cellular effects of aging occur and how they may specifically impact male fertility.

36.2.2 Semen Analysis

While reports in the literature in the past demonstrated a decrease in semen volume with aging [20] and variable results in regard to sperm concentration [21–23], the literature now supports that increased paternal age has been associated with decreased semen volume, sperm motility, and sperm morphology [6–9]. As previously reported, increased paternal age is associated with a decreased probability of pregnancy and increased time to conception when controlling for maternal age [24].

Johnson et al. examined seven semen characteristics (volume, sperm concentration, total sperm count, progressive and total motility, morphology, and DNA fragmentation) in 93,839 subjects as part of a recent systematic review and meta-analysis [25]. Their results showed a statistically significant decline in all parameters except for sperm concentration, thereby demonstrating definitive changes in semen with increasing age.

Several mechanisms behind these age-related changes in semen parameters have been researched with various impacts on subsequent offspring (Fig. 36.1). One study demonstrated an association between advanced age and decreased semen volume and increased sperm aneuploidy with no significant difference in sperm morphology, motility, or DNA fragmentation [7]. This is in contrast to other studies that did demonstrate associations between advanced paternal age and sperm DNA fragmentation [26, 27].

Although the literature has been inconclusive as to why the changes in semen parameters occur with age, several mechanisms have been theorized. One proposed mechanism highlights the various anatomic and morphologic changes that occur over time. Age-related narrowing and sclerosis of the testicular tubular lumen, decreases in spermatogenic activity, increased degeneration of germ cells, and decreased numbers and function of Leydig cells have been found in autopsies of men who died from accidental causes [28]. Age-dependent alterations of the prostate are well known [29] and are detectable histologically in 50% of 50-year-old men, but in 90% of men aged >90 years [30]. Smooth muscle atrophy and a decrease in protein and water content, which occur in the prostate with aging, may contribute to decreased semen volume and sperm motility. Also, the epididymis, a hormonally sensitive tissue, may undergo age-related changes. The hormonal or epididymal senescence may lead to decreased

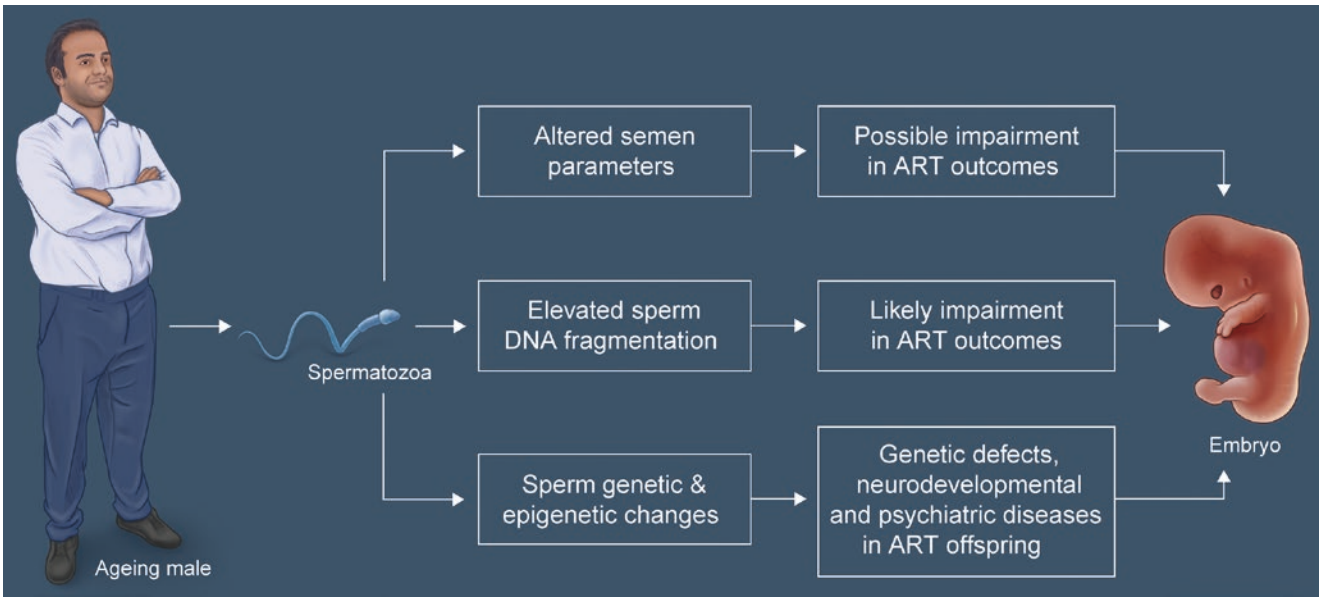

Fig. 36.1 Measures taken for better reproductive outcomes in the aging male. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2018–2019. All Rights Reserved)

sperm motility in older men. Other proposed mechanisms suggest an accumulation of oxidative damage in mature spermatozoa, with increased reactive oxygen species associated with decreases in sperm motility, increased DNA fragmentation, increased sperm mitochondrial DNA mutations, and decreased fertilizing capability [31–33].

36.2.3 Assisted Reproductive Technology

Since 1995, the Center for Disease Control (CDC) has collected data on the use of assisted reproductive technology (ART) in the United States. As of 2015, it was reported that the number of ART procedures documented to the CDC and the number of infants born to ART procedures have approximately tripled since 1995 [11]. While intrauterine insemination (IUI) was not included as an ART procedure, this demonstrates the growing use of ART, highlighting the importance of how male age-related fertility may impact pregnancy rates in this population.

While previous studies have suggested that success rates of in vitro fertilization (IVF) are not associated with male age [34–36], emerging studies have challenged this notion. A 2015 retrospective study examined 9991 IVF cycles in China, grouping samples by maternal age and then subgrouped by paternal age [37]. Analysis of this data demonstrated no difference in implantation or pregnancy rates among different paternal ages when maternal age was <30 and 35–38 years. However, in the subgroup of women aged 31–34, there was a statistically significant decrease in both implantation and pregnancy rates with increasing paternal age.

Despite these inconclusive results, more recent data has attempted to elucidate this relationship more clearly. In a 2017 retrospective study, McPherson et al. examined 4057 IVF cycles assessing the additive effects of advanced maternal and paternal age on clinical pregnancy rate, viable pregnancy rate, live birth rate, and term birth [38]. They found a statistically significant negative association between advancing paternal age and viable pregnancy and live birth, leading to a 10% decreased probability of pregnancy in women aged 35 when the male partner was older than 40 years compared to men <30 years.

This age-related impact on fertility in the area of ART has been thought to be due to DNA fragmentation in sperm. Although studies suggest that DNA fragmentation occurs with a higher frequency in sperm of older males [26], its impact on ART is significant. DNA fragmentation index (DFI) in sperm has been described in several studies examining its effect on pregnancy success rates using ART [39, 40]. They found that a DFI of >25.5% resulted in a statistically significant lower probability of pregnancy using IVF/ICSI, and with a DFI of >30%, the likelihood of pregnancy using IUI was close to zero. Consider this data with the fact that in men aged 60–80, the mean DFI of ejaculated sperm has been estimated to be 49.6%, which is approximately a fivefold increase compared to men aged 20 (mean DFI estimate of 12.9%) [26]. This emerging data highlights the importance of counseling infertile couples wishing to pursue ART on the very real impact advanced paternal age may have on pregnancy outcomes.

There are a variety of measures aging men may perform prior to utilizing ART to preserve normal semen parameters

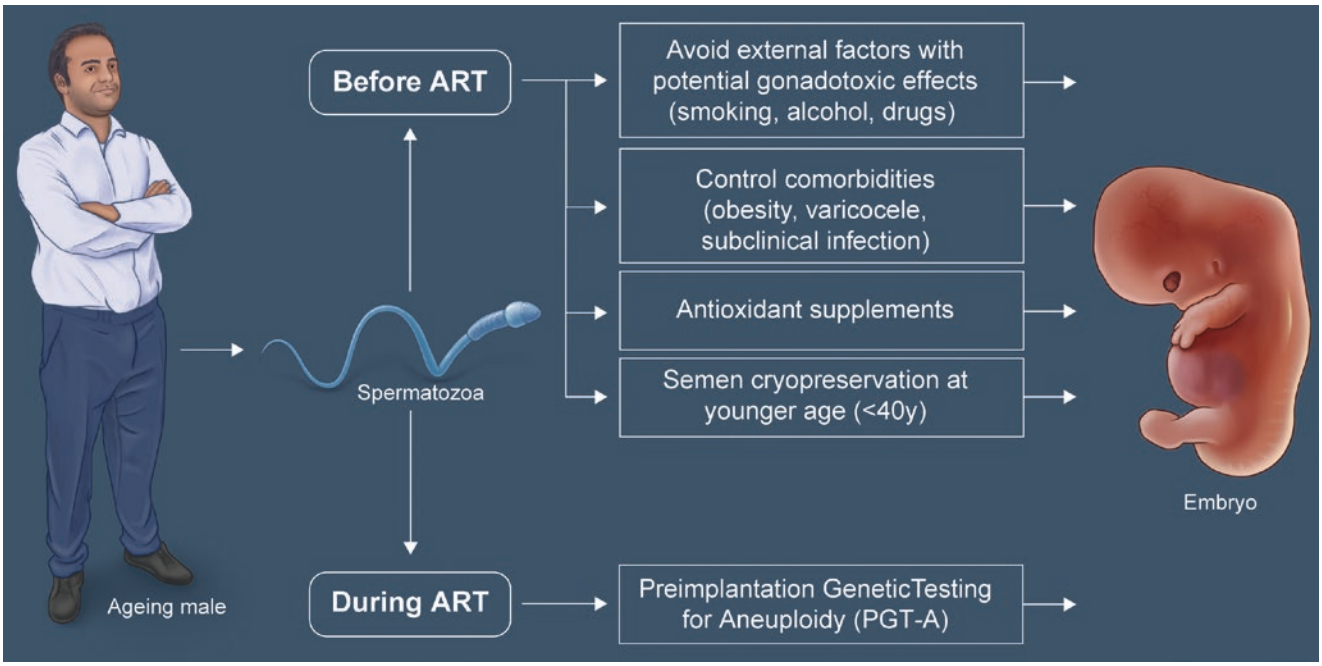

Fig. 36.2 Effect of advanced paternal age on semen quality and sperm genetic/epigenetic status and its outcomes on embryo. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2018–2019. All Rights Reserved)

(Fig. 36.2). A measure utilized during ART is preimplantation genetic testing for aneuploidy (PGT-A), which seeks to identify and exclude aneuploidies within preimplantation embryos, improving pregnancy success. Early versions of PGT-A were limited in their capacity to analyze a fraction of chromosomes and had the potential to result in pregnancy loss due to biopsy-induced trauma. However, emerging technology has led to the capability of simultaneous analysis of all chromosomes, and biopsy techniques have evolved to become less invasive though blastocyst sampling. Select studies have demonstrated the potential for improved pregnancy rates; however, large randomized controlled trials are lacking [41].

36.3 Effects of Aging on Genes of Offspring

As previously discussed, DNA mutations in sperm can impact semen parameters in men with advanced age. These mutations can also impact future offspring. Several mechanisms have been proposed regarding sperm DNA damage in older men and the impact on offspring, including higher levels of double-stranded DNA breaks [42]. This has been thought to likely be due to increasing levels of oxidative stress that increase with age [43, 44]. In addition, structural chromosomal abnormalities have also been studied, as de novo structural aberrations are of paternal origin in 84% of cases [45]. However, these changes do not appear to have an association specific to advanced paternal age.

Although multiple genomic changes have been studied, single-gene mutations appear to be the mode of disease inheritance most strongly associated with advanced paternal age [46]. Single-gene mutations occur as a result of mutated DNA sequences which ultimately lead to defects in proteins that they code for and have been found to be associated with musculoskeletal disorders and increased risk of cancers in offspring [47]. To illustrate the effects these mutations may have, consider that roughly 30 spermatogonial cell divisions occur before puberty. After puberty, spermatogonial stem cells divide every 16 days (~23 times per year). If the average age of male puberty is 15, sperm produced by a 70-year-old male have formed after ~1300 mitotic divisions. DNA replication preceded each cell division and mutations often arise as a result of uncorrected errors in DNA replication [48]. Therefore, the significantly higher number of cell divisions seen in older males compared to their younger counterparts are presumably much more likely to be susceptible to de novo gene mutations [49].

In fact, in 2012, a study by Kong et al. demonstrated that a significant relationship does indeed exist between advanced paternal age and inheritance patterns of de novo gene mutations [49]. After studying the genomic patterns of 78 Icelandic parent–offspring trios, the average de novo mutation rate per nucleotide per generation was measured via genome sequencing. The results revealed that the variability in mutation rate is most heavily impacted by the age of the father at conception of the child, translating to a rate of two new mutations per year of advancing paternal age and a dou-

bling of paternally inherited mutations every 16.5 years [49]. This breakthrough study provides significant evidence for how advanced paternal age may genetically impact future offspring.

Chromosomal aneuploidies can have significant impacts on pregnancies with the majority resulting in miscarriages while offspring reaching birth may be affected by trisomies 13, 81, and 21. Although studies do not definitively demonstrate a significant relationship between trisomies and advanced paternal age [50], others have established a relationship between advanced maternal age (>35 years) and increased risk of trisomy 21 [51]. Despite lack of evidence for paternal age playing a significant role, research has demonstrated paternal age as a possible cofactor. McIntosh et al. reported an increased risk of trisomy 21 of up to twofold for paternal age > 50 years compared to paternal age between 25 and 29 years [52].

Another method of genetic modifications with effects on offspring includes the alteration of individual nucleotides in DNA via methylation/demethylation or histones via acetylation/deacetylation. This results in altered transcription activity which in turn will ultimately affect the translated proteins. This process is termed epigenetics and has been a proposed mechanism for increased risk of psychiatric conditions in offspring born to fathers of advanced paternal age [53].

36.4 Effects of Aging on Offspring Syndromes

There are multiple conditions that have been linked to advanced paternal age. These conditions range from psychiatric/neurodevelopment disorders, various forms of cancer, as well as musculoskeletal disorders. As previously highlighted, some studies have demonstrated an increased risk of fetal trisomy 21 in fathers of advanced paternal age when compared to fathers between 25 and 29 years [52]. In addition, there have been multiple studies which have associated advanced paternal age to the development of autism spectrum disorder.

A 2006 study by Reichenberg et al. [54] examined Israeli births over a consecutive 6-year period and concluded that offspring of men 40 years or older were 5.75 times more likely to develop autism spectrum disorder than those men younger than 30 years. Five years later, Buizer-Voskamp et al. reported that a 40-year-old father is 3.3 times more likely to have a child with autism spectrum disorder than a 20-year-old father [55].

As previously mentioned, advanced paternal age has also been associated with an increased incidence of psychiatric disorders in offspring including schizophrenia and bipolar disorder [55, 56]. An important study validating this association with schizophrenia by Malaspina et al. reported advanced paternal age as a significant predictor of the development of schizophrenia in offspring, with a twofold increased risk in fathers aged >50 years compared to those younger than 25, despite no family history [56]. This association was also demonstrated in a study 6 years later by Frans et al. where the offspring of fathers aged >50 years were found to be 1.37 times more likely to be diagnosed with bipolar disorder compared to fathers aged 20–24 years [57].

The increased incidence of various cancers in offspring has also been found to be linked to advanced paternal age. According to the Swedish Family Cancer Database, there is a link between paternal age and the incidence of sporadic breast and nervous system cancer in offspring [58]. Interestingly, an association between paternal age and the male offspring's risk of prostate cancer was also found [59]. The association of paternal age with early-onset prostate cancer (<65 years) was greater than that with late onset. Single-gene mutations which have been established to occur with a higher incidence in sperm of fathers of advanced age have shown to occur in oncogenes including RET [60]. This oncogene is associated with multiple endocrine neoplasia syndromes inherited in an autosomal dominant manner and are associated with the increased risk of the formation of various endocrine system cancers. Other autosomal dominant disorders found to be associated with advanced paternal age include retinoblastoma, neurofibromatosis type 1, and osteogenesis imperfecta. However, there appears to be a more heterogenous relationship between advanced paternal age and the development of these syndromes with less consistent data supporting the association [61].

One syndrome with a clearer association is achondroplasia. The most common form of dwarfism, achondroplasia, is the first genetic disorder that was hypothesized to be linked to advanced paternal age [46]. Like some of the disorders previously mentioned, it is inherited in an autosomal dominant fashion and is caused by mutations to the FGFR 3 gene [62, 63]. Other autosomal dominant diseases that may result in cranial malformations include Crouzon syndrome, Apert syndrome, and Pfeiffer syndrome, which occur as a result of mutations in the FGFR2 gene [64]. Apert syndrome and achondroplasia have been amenable to direct sperm DNA mutation analysis noting a direct association to advanced paternal age [65]. However, while there does appear to be an association between advanced paternal age and the development of Crouzon and Pfeiffer syndrome, sporadic cases appear to have increased heterogeneity of mutations in the FGFR2 gene [66].

Ongoing research continues to support the association between advanced paternal age and various disorders that may impact the health of offspring. This information may be used to counsel older men considering new fatherhood.

36.5 Effects of Aging on Androgen Levels

It has long been presumed that testosterone levels decrease with age, leading to a constellation of symptoms including fatigue, decreased libido, and erectile dysfunction (ED). This collection of symptoms encompasses a process termed andropause, akin to the age-related process of menopause in women. However, unlike the dramatic changes that occur with menopause in women, the onset of andropause in men is much more insidious with greater symptom heterogeneity [67]. There are multiple mechanisms by which androgen levels in men decrease with age, one of which entails changes in the hypothalamic–pituitary–gonadal axis (HPG axis).

In studies examining rats, aging was found to be associated with an overall reduction in gonadotropin-releasing hormone (GnRH) secretion, as well as decreases in LH pulse interval, amplitude, and area [68]. When examining males in particular, the hypothalamic GnRH pulse generator has been recognized to decline with age. Due to the ongoing negative feedback mechanisms within the HPG axis, this ultimately results in increased basal levels of gonadotropins in response to decreased circulating levels of testosterone [69]. In 2002, an effort was made to expand the validity of these findings to men in the form of the Massachusetts Male Aging Study. Data regarding male hormone levels were established in a population-based study of 1156 men aged 40–70 years who were followed for 7–10 years. The study demonstrated a longitudinal annual increase in basal LH and FSH by 1.1% and 3.5%, respectively, and an overall hormonal pattern of testicular dysfunction with decreasing free serum testosterone and elevated LH [12].

The findings of the study were validated 6 years later in a prospective review of hormone levels in 3200 men 40–79 years old in eight European nations [70]. The results revealed a clear increase in basal LH levels and decrease in free testosterone with increasing age independent of other modifiable factors such as smoking, comorbidity, and obesity [70]. Although these consistently established hormone patterns are likely initiated by an age-related decline in GnRH pulsatility, the cycle is exacerbated by a decline in circulating androgens, which makes it prudent to explore some of the other factors that contribute to decreased androgen levels with age.

Other mechanisms by which androgen levels are thought to decrease with age include decreases in Leydig cell population in the testis and decreases in testicular perfusion in association with cardiovascular comorbidity [71]. Studies comparing levels of testosterone/testosterone precursors and progesterone in testicular tissue and spermatic vein plasma in young and old men revealed consistently decreased concentrations of testosterone/testosterone precursors and increased levels of progesterone [72]. This is thought to be due to impaired oxygen perfusion in aging testes [72]. Other age-related factors include increases in body fat, which is known to contain aromatase. This enzyme promotes the conversion of testosterone to estrogen, resulting in decreased levels of circulating testosterone and compounding the aforementioned regulatory changes that occur in the HPG axis [73].

In addition to the above, one of the most important factors impacting serum concentrations of bioavailable testosterone levels is sex hormone-binding globulin (SHBG). This glycoprotein is secreted into the blood by the liver and binds to testosterone in the blood. Testosterone levels in the blood may split into three categories: free testosterone that is unbound, testosterone bound to albumin, and testosterone bound to SHBG. Since the bond between testosterone to albumin is weak, both free testosterone and that bound to albumin are considered bioavailable. This is in contrast to testosterone that is bound to SHBG which is not considered bioavailable.

It can be presumed that increased levels of SHBG may then result in increased andropausal symptoms in men. Indeed, long-term prospective cohort studies have demonstrated that the proportion of biologically inactive levels of serum testosterone bound to SHBG is directly proportional to levels of serum SHBG and have established that these levels of SHBG increase with age [74]. This not only contributes to the ever-increasing data supporting age-related factors playing a direct role in decreased androgen levels but also may serve as a tool to practitioners. Specifically, measuring serum SHBG levels in andropausal men that are considering androgen replacement therapy may help provide prognostic information for treatment efficacy. After exploring the many different mechanisms by which age may directly influence androgen levels, it is important to then assess how these declining levels may impact the health and lives of older men.

36.6 Systemic Effects of Decreased Androgens

Given that symptoms of andropause may include a decline in erectile function, the process of how this occurs has been of recent interest. As previously discussed, data from the Massachusetts Male Aging Study demonstrated longitudinal increases in basal serum levels of LH and decreases in serum free testosterone with increased age [12]. Their data also revealed an association between hypogonadal men with increased serum levels of LH and reduced erectile function. However, this reduction was only noted with increased serum levels of LH [75]. This finding has been expanded upon, with the data from recent literature suggesting that a minimum threshold level of serum testosterone is critical for erectile function and may also impact the efficacy of medications used to treat ED [76].

In addition to highlighting the direct impact that androgen levels have on sexual function and subsequent andropausal symptoms, it is important to address the significant consequences these levels may have on men's health. Testosterone plays a significant role in the regulation of a number of bodily functions including bone mineralization, cardiac health, muscle development, lipid metabolism, and hematopoiesis [77]. In 2010, the Framingham Offspring Study examined free testosterone levels in community-dwelling men and found a correlation between the increased risk of impaired mobility and progression of impairment with low serum testosterone levels [78]. This correlates with ongoing research suggesting androgen levels playing a role in cognition and motor function [79], with one longitudinal study observing low free testosterone levels to be associated with an increased risk of falls in elderly men after adjusting for physical performance [80]. One related factor to consider is the effect low testosterone levels have on bone health. In fact, studies have demonstrated that men aged >65 who are testosterone deficient are twice as likely to suffer from osteoporosis than similarly aged men with normal testosterone levels [81]. This data may better serve clinicians in treating this patient population, as clinical trials have revealed improvements in bone density following testosterone therapy in older men.

Testosterone has also been shown to mediate increases in lipid metabolism, and its age-related decline may result in a number of long-term health sequelae [82]. Testosterone is known to inhibit lipoprotein lipase, an enzyme that mediates the uptake of free fatty acids into adipocytes. In the setting of low androgen levels, this causes an increase in triglycerides within adipocytes results in increased adipocyte proliferation and body fat. As we have established, body fat harbors the enzyme aromatase, which ultimately leads to a cycle exacerbating decreased levels of testosterone [15]. This increase in visceral fat in turn elevates the risk of developing metabolic syndromes including insulin resistance and diabetes mellitus [83]. Indeed, studies examining men with iatrogenic and non-iatrogenic hypogonadism have demonstrated associations with an increased risk of diabetes and poor glycemic control [84]. These associations are validated when observing the metabolic effects of patients receiving testosterone replacement therapy (TRT), which has been found to improve insulin sensitivity in both healthy and diabetic men in addition to decreasing triglyceride uptake into adipocytes [14, 15].

36.7 Conclusion

The impact of aging on men's health is significant and the notion that age may play a role in affecting androgen levels has been ascertained. However, continued research is necessary to better understand the age-related cellular changes as well as the subsequent impact these changes have on men's health. Symptoms such as fatigue and sexual dysfunction occurring with advanced male age have been termed andropause which has been shown to impact fertility, health of future offspring, and overall men's health. This has culminated in valuable information for clinicians to guide and treat this ever-growing patient population.

36.8 Review Criteria

An extensive literature search examining the effect of advanced male age on androgen levels and subsequent effects was performed. This was completed via search engines including PubMed, MEDLINE, Clinical Key, and Google Scholar. Date range filters were used starting at 2012 and ending at the present. Keywords and phrases utilized within the search criteria included "andropause," "testosterone and aging," "age and infertility," and "DNA fragmentation with age." Additional references were utilized from the chapter's previous edition. The 2018 American Urological Association Update Series on the Reproductive Effects of Male Aging was also used to identify landmark studies pertaining to this topic within the literature since the previous edition. Data that were solely published in conference or meeting proceedings, websites, or books were not included.

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Apoptosis and Male Infertility

37

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Key Points

- Increasing evidence from epidemiological and clinical studies suggests that male reproductive health has been deteriorating due to several factors including exposure to environmental contaminants and changing lifestyle, but the mechanistic pathway has not yet been fully elucidated.
- Recent findings have shown that the incidence of male infertility has increased due to genetic disorders and pathological apoptosis.
- Apoptosis is a highly regulated process characterized by distinct changes in the cell morphology including membrane blebbing, shrinkage of cell volume, cytoplasmic vacuolization, nuclear condensation, and DNA fragmentation, followed by the disassembly of the cell into membrane-bound apoptotic bodies. The biochemical features of apoptosis include phosphatidylserine exposure to the external leaflet of the plasma membrane, activation of caspase cascades, DNA cleavage, and DNA laddering.
- Environmental contaminants possessing endocrine-disrupting properties are known to induce oxidative stress and cause pathological apoptosis, thereby affecting the male reproductive health.

37.1 Introduction

Infertility is a global health issue and is usually defined as the failure to achieve clinical pregnancy after regular and unprotected sex for at least a year or more. Even though infertility affects 15–20% of couples worldwide [1], male factor infertility is contributing approximately 50% of cases, with sole responsibility in 30% and co-contributing with the female in 20% of cases. According to WHO, the overall prevalence of primary infertility in India is between 3.9% and 16.8% [2]. In some African countries, the problem even exceeds where one-third of the couples are infertile [3, 4]. Increasing evidence from epidemiological and clinical studies suggests that male reproductive health has been deteriorating due to several factors including exposure to environmental contaminants and changing lifestyle. The pathogenesis of male infertility can be reflected by defective spermatogenesis due to pituitary disorders, testicular cancer, germ cell aplasia, varicocele, and environmental factors or due to defective sperm transport resulting from congenital abnormalities or immunological or neurological factors. In 30–40% of male infertility cases, no cause is identified (idiopathic male infertility). Recent findings have shown that the incidence of male infertility has increased due to genetic disorders and pathological apoptosis. Of these, apoptosis has been identified as a major factor contributing to male infertility and has been studied extensively in recent years.

Apoptosis, also known as programmed cell death (PCD), is required for normal spermatogenesis in mammals and is believed to ensure cellular homeostasis, and an adequate amount of germ cells is eliminated via the process of apoptosis in order to maintain a precise number of germ cell population in compliance with the supportive capacity of the Sertoli cells. Apoptosis is a highly regulated process characterized by distinct changes in the cell morphology including membrane blebbing, shrinkage of cell volume, cytoplasmic vacuolization, nuclear condensation, and DNA fragmentation, followed by the disassembly of the cell into membrane-bound apoptotic bodies. The biochemical features of

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apoptosis include phosphatidylserine exposure to the external leaflet of the plasma membrane, activation of caspase cascades, DNA cleavage, and DNA laddering. This chapter briefs both physiological and pathological events that trigger apoptosis and their effects on the male reproductive system.

37.2 Physiological Role of Apoptosis in Male Reproduction

Testes accomplish one of the complex events called spermatogenesis, which is necessary for the propagation of germplasm. Spermatogenesis is a highly dynamic and synchronized process of germ cell maturation from diploid spermatogonia to mature haploid spermatozoa that takes place in the seminiferous epithelium of the testis. This highly intricate cellular development is fostered by the somatic cells, the Sertoli cells, which envelope the germ cells [5]. During testicular development, the Sertoli cell number increases gradually and thereafter their proliferative capacity declines to produce a stable population of nondividing Sertoli cells [6]. On the other hand, the germ cells continuously proliferate and differentiate to become mature spermatozoa. Under normal condition, overproliferation of germ cells is tempered by selective apoptosis of their progeny in order to maintain a precise germ cell population in compliance with the supportive capacity of the Sertoli cells [6]. Apoptosis also occurs as a defense mechanism, such as in immune reactions or when cells are damaged by disease or environmental agents. Necrosis and apoptosis are the two major mechanisms of cell death. Necrosis occurs in cells that are damaged by external injury, whereas apoptosis occurs in cells that are induced to commit programmed death from internal or external stimuli. Apoptosis is broadly divided into an initiation phase followed by a signaling phase and an execution phase in which cells rapidly execute a death program. Apoptosis consists of highly intricate, sophisticated, and energy-dependent cascade mechanisms and occurs through two main pathways (Fig. 37.1). The first, referred to as the extrinsic or cytoplasmic pathway, is triggered through the Fas death receptor, a member of the tumor necrosis factor (TNF) receptor superfamily [7]. The second pathway is the intrinsic or mitochondrial pathway that when stimulated leads to the release of cytochrome c from the mitochondria and activation of downstream death signal [8]. Both the pathways converge into a final common pathway involving the activation of a cascade

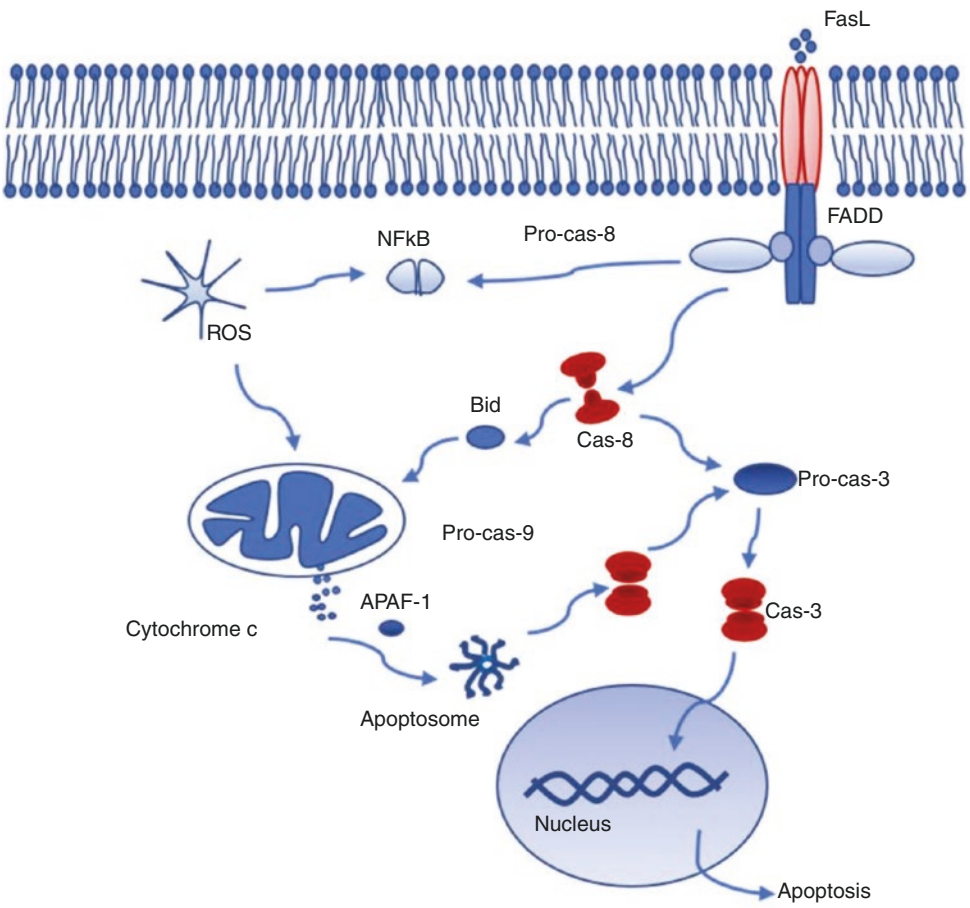

Fig. 37.1 Major pathways of apoptosis: Components involved in the mitochondrial and cell death-mediated pathways are shown in the figure

of proteases called caspases that cleave regulatory and structural molecules, culminating in the death of the cell. The pathways are linked; thus, the distinction between the two pathways is simplistic. Overexpression of antiapoptotic protein Bcl-2 in the intrinsic pathway may lead to the inhibition of extrinsic-mediated apoptosis [9]; conversely, TNFα may increase the expression of NFκB and stimulates antiapoptotic members of the Bcl-2 family proteins. Lindane, an organochlorine pesticide, is known to impair rat testicular functions and fertility through modulation of NFκB and FasL [10].

37.3 Extrinsic Pathway

Extrinsic pathway comprises several protein members including the death receptors, the membrane-bound Fas ligand, the Fas complexes, the Fas-associated death domain, and caspases 8 and 10, which ultimately activate the rest of the downstream caspases leading to apoptosis. Activation of the extrinsic pathway is initiated with the ligation of cell surface receptors called death receptors (DRs). Fas is a member of the TNF receptor superfamily and is also called Apo-1. Fas signaling plays an important role in apoptosis. The Fas ligand (FasL)-Fas system is mainly recognized for its death-related functions. When a death stimulus triggers the pathway, the membrane-bound FasL interacts with the inactive Fas complexes and forms the death-inducing signaling complex. The Fas death-inducing signaling complex contains the adaptor protein Fas-associated death domain protein and caspases 8 and 10 and leads to activation of caspase 8, which, in turn, can activate the rest of the downstream caspases. Caspase 8 interacts with the intrinsic apoptotic pathway by cleaving Bid (a proapoptotic member of the Bcl-2 family), leading to the subsequent release of cytochrome c [11].

37.4 Intrinsic Pathway

One of the most important regulators of this pathway is the Bcl-2 family of proteins. The Bcl-2 family includes proapoptotic members such as Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim, and Hrk and antiapoptotic members. Bax is a multidomain proapoptotic member of the Bcl-2 family and its deficiency results in a large accumulation of premeiotic germ cells in mature animals and a near complete absence of spermatocytes and mature sperm [12]. Antiapoptotic Bcl-2 members act as repressors of apoptosis by blocking the release of cytochrome c, whereas proapoptotic members act as promoters. Following a death signal, proapoptotic proteins undergo posttranslational modifications that include dephosphorylation and cleavage resulting in their activation and translocation to the mitochondria leading to apoptosis [8]. In response to apoptotic stimuli, the outer mitochondrial membrane becomes permeable, leading to the release of cytochrome c. Once cytochrome c is released into the cytosol, it interacts with Apaf-1, leading to the activation of caspase-9 proenzymes. Active caspase 9 then activates caspase 3, which subsequently activates the rest of the caspase cascade and leads to apoptosis [9].

37.5 Fas/FasL

Fas is a type I transmembrane receptor protein that belongs to the TNF/nerve growth factor family [13, 14], and Fas ligand (FasL) has been identified as a TNF-related type II transmembrane protein [15]. The binding of the surface protein FasL to the Fas receptor triggers apoptosis in Fas-bearing cells by the activation of various caspases. It is widely accepted that FasL is normally expressed in Sertoli cells, whereas Fas antigen is expressed in the germ cells of rodents and humans. Caspase 8 is involved in the upstream of the apoptosis process [16, 17]. Activation of caspase 8 is followed by activation of caspase 3, which is known as executioner protease in this process [18]. In the rodent testis, apoptosis, induced by FasL, has been suggested to be one of the mechanisms that limits the number of germ cells during normal spermatogenesis or after testicular injuries. Recent studies have also shown that, in the human testis, apoptosis is a conspicuous event during spermatogenesis; Fas–FasL interaction is reportedly involved in the regulation of this event [19, 20].

37.6 Caspase and Calpain Families

Caspases (cysteinyl aspartate-specific proteases) are aspartic acid-directed cysteine proteases. These proteases are synthesized as precursors that have insignificant catalytic activity. The precursor caspase is converted to the active enzyme by proteolytic processing either by another protease or by autocatalysis and triggered by the binding of cofactors or removal of inhibitors. Caspases share similarities in amino acid sequence, structure, and substrate specificity. They are all expressed as proenzymes (30–50 kDa). They contain three domains and they are the amino terminal domain, a large subunit (~20 kDa), and a small subunit (~10 kDa). During the caspase activation, proteolytic processing occurs between domains, followed by association of the large and small subunits to form a heterodimer [21]. Although the majority of caspases are situated within the cytoplasm, some of the members can be found in the Golgi apparatus (caspase 12) or in association with the mitochondria (caspases 2, 3, and 9) [22, 23]. Caspase 3 is the most important effector caspase. Its activation is important in PCD signaling [24]. Calpains are a

superfamily of related proteins, some of which have been shown to function as calcium-dependent cysteine proteases. Calpain also plays an important role in apoptosis and necrosis, and Rojas et al. [25] demonstrated the presence of a calpain–calpastatin system in human spermatozoa.

37.7 Cytochrome c

Different proapoptotic proteins, such as cytochrome c and Smac/Diablo, that are normally present in the intermembrane space of mitochondria, are released during the early stages of apoptosis. Suppression of the antiapoptotic members or activation of the proapoptotic members of the Bcl-2 family leads to altered mitochondrial membrane permeability resulting in release of cytochrome c into the cytosol. Binding of cytochrome c to Apaf-1 triggers the activation of caspase 9, which then accelerates apoptosis by activating other caspases. In the cytosol, cytochrome c participates in the formation of the apoptosome complex together with its adaptor molecule, Apaf-1, resulting in the recruitment, processing, and activation of procaspase 9 in the presence of adenosine triphosphate [26]. Subsequently, caspase 9 cleaves and activates procaspases 3 and 7; these effector caspases are responsible for the cleavage of various proteins leading to the biochemical and morphological features characteristic of apoptosis [27]. The release of cytochrome c is, therefore, considered a key initiative step in the apoptotic process.

37.8 Nuclear Factor Kappa B

The classical NFκB transcriptional factors are composed of homodimers or heterodimers of Rel protein, of which p65/p50 heterodimer is the predominant complex, in testicular germ cells [28]. In unstimulated cells, NFκB dimers are sequestered in the cytoplasm by inhibitory kappa B (IκB) protein. Upon exposure to various extracellular signals that leads to phosphorylation and degradation of IκB, free NFκB dimers rapidly translocate to the nucleus, wherein they activate transcription of target genes [29].

37.9 Spermatogenesis

Spermatogenesis is a dynamic and well-regulated process that involves multiplication, maturation, and differentiation of germ cells resulting in the formation of mature spermatozoa. The process is subdivided into spermatogoniogenesis known as mitotic multiplication of spermatogonia, maturation of spermatocytes, spermiogenesis, and spermiation. Germ cells undergo mitosis to produce primary spermatocytes. The primary spermatocytes enter meiosis to form secondary spermatocytes and proceed through meiosis to produce haploid spermatids. These in turn undergo a complex process of morphological and functional differentiation resulting in the production of mature spermatozoa, which is known as spermiogenesis. In mammalian species, to maintain proper germ cell numbers, apoptosis takes place in the testis [17]. Loss of spermatogenic cells is incurred mostly during maturity of spermatogonia and to a lesser extent during maturation of spermatocytes and spermatid in adult rat testis. The sign of spermatogenesis is started when germ cells differentiate into spermatogonia. While some spermatogonia become self-renewing spermatogonial stem cells, most differentiate into spermatocytes and, at ~10 days after birth in mice and at puberty in man, initiate meiosis and are accompanied by extensive germ cell apoptosis [17].

37.10 Steroidogenesis

Leydig cells are the principal cells involved in the process of steroidogenesis. Leydig cells secrete androgens, particularly testosterone, which is extremely essential for the initiation and maintenance of spermatogenesis [30]. Any factor affecting the Leydig cell viability, in turn, can interrupt the endocrine regulation of spermatogenesis and consequently affect the reproductive performance. Aroclor 1254, a commercial mixture of polychlorinated biphenyls, brought about a state of oxidative stress in cultured Leydig cells characterized by decline in the levels of enzymatic and nonenzymatic antioxidants accompanied by an elevation in the levels of lipid peroxidation and reactive oxygen species (ROS). In addition, the activities of steroidogenic enzymes were inhibited at the level of gene expression causing diminished testosterone production [31]. Exposure of rats to a single dose of cadmium (0.20 mg/100 g body weight) inhibited the activities of testicular 3β and 17β-hydroxysteroid dehydrogenase along with reduced expression of StAR protein resulting in lowered serum testosterone levels. The observed effects have been attributed to the excess generation of ROS in the testis resulting from depletion of antioxidant enzymes like superoxide dismutase (SOD) and glutathione peroxidase. Exposure of primary cultured Leydig cells to cadmium at the concentrations of 10 mm caused increased oxidative DNA damage resulting in decreased viability of cells and testosterone secretion [32]. The high levels of corticosterone associated with stress are known to induce apoptosis in Leydig cells. The activation of Fas system, cleavage of procaspase 3, loss of mitochondrial membrane potential, and increased ROS generation are reported to be the possible mechanisms involved in corticosterone-induced Leydig cell death [33]. The decline in testosterone production following toxicant exposure may be in part due to apoptosis of Leydig cells caused by induction of stress by corticosterone.

37.11 Effect of Environmental Contaminants

Apoptosis of spermatogenic cells is essential for the maintenance of testicular homeostasis, although increased cell death can result in defective spermatogenesis leading to infertility [34]. In the testis, apoptotic death is a common programmed event that reduces 75% of germ cells [35]. However, excessive or inadequate apoptosis of testicular cells results in abnormal spermatogenesis or testicular tumors [36]. Various testicular toxicants have been reported to induce massive germ cell apoptosis indicating that the seminiferous epithelium responds to most of the adverse stimuli by eliminating germ cells through PCD [37]. Recent studies showed that dichlorodiphenyltrichloroethane (DDT) and its metabolite induced apoptosis through either in vitro or in vivo experiments [38, 39]. Song et al. demonstrated that exposure to *p*, *p*'-dichlorodiphenyldichloroethylene (DDE), a metabolite of DDT, at over 30 μM dose level showed induction of apoptotic cell death in cultured rat Sertoli cells by inducing mitochondria-mediated apoptotic changes including elevation in reactive oxygen species (ROS) generation, decrease in mitochondrial membrane potential, and release of cytochrome c into the cytosol which could be blocked by N-acetylcysteine, an antioxidant with an elevated ratios of Bax/Bcl-w and Bak/Bcl-w, and cleavages of procaspases 3 and 9 were induced by *p*, *p*'-DDE [32]. Metabolite of DDT (*p*, *p'*-DDE) at a dose of 30 μM for 24-h exposure could induce apoptosis of Sertoli cells through a FasL-dependent pathway including nuclear translocation of NFκB, increase of the FasL mRNA, and protein expression, which could be blocked by an antioxidant agent N-acetylcysteine. In addition, caspases 3 and 8 were activated by *p*, *p'*-DDE treatment in these cells [40]. Ichimura et al. demonstrated the expression and localization of FasL, Fas, and caspase 3 proteins in mouse testis 12 h after the exposure to 4-0.004 mg/g of di(2ethylhexyl) phthalate (DEHP) and correlated the expression of these proteins with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) of the DNA-fragmented nucleus [41]. Immunocytochemical examination of the DEHP-exposed (4 mg/g) mouse revealed a distribution of FasL in Sertoli cell and Fas in nearby spermatocyte and Fas and caspase 3 in the same spermatocyte. Exposure to mono(2-ethylhexyl) phthalate (MEHP), a Sertoli cell-specific toxicant, induced massive germ cell apoptosis associated with increased expression of both the Fas and FasL genes in rat testis. Mono(2-ethylhexyl) phthalate (MEHP), a well-known Sertoli cell toxicant, could decrease the levels of procaspase 8 and increase the levels of procaspase-8 cleavage products in mice testis [42]. β-Benzene hexachloride (BHC), a major metabolite of benzene hexachloride, induced apoptosis by activation of c-Jun N-terminal kinases (JNKs), translocation of NF-κB, expression of FasL, and further activation of caspase cascade [43]. Spontaneous germ cell apoptosis has been observed in several species of mammalian testis. Vaithinathan et al. demonstrated a significant increase in the levels of cytosolic cytochrome c and procaspase 9 as early as 6 h following exposure to a single dose of methoxychlor at 50 mg/kg bodyweight. Time-dependent elevations in the levels of Fas, FasL, and pro- and cleaved caspase 3 demonstrate induction of testicular apoptosis in adult rats following a single dose of methoxychlor [44]. Recent findings reveal that methoxychlor exposure to pregnant female rats from embryonic days 8–15 at the dosage of 100 and 200 mg/kg/day showed an increase in spermatogenic cell apoptosis and decreased sperm number and motility in adult animals of F1 and F2 generation [45, 46]. In another study, oral administration of bisphenol A 480 and 960 mg/kg/day induces apoptosis of Leydig and germ cells in the mouse testis through the Fas signaling pathway [47]. Lindane, a well-known endocrine disruptor, could induce apoptosis of testicular cells by stimulating the mitochondrion-dependent pathway by elevating the levels of cytochrome c with a parallel increase in procaspase 9. A time-dependent elevation of Fas, FasL, and caspase 3 in peritubular germ cells illustrates induction of testicular apoptosis in adult rats following exposure to a single dose of lindane [10].

37.12 Oxidative Stress

Control of apoptosis may involve various pathways such as the mitochondria-mediated Bcl-2 family, Fas/FasL system that can be engaged by oxidative stress. Reactive oxygen species (ROS) is considered a potential signal for apoptosis. Elevated levels of ROS can cause oxidation of the mitochondrial pores, thereby disrupting the mitochondrial membrane potential and releasing cytochrome c and activating the mitochondria-mediated pathway of apoptosis. In addition, ROS have been shown to induce the expression of Fas receptor and ligand stimulating the Fas/FasL-mediated apoptotic signal transduction pathway. Several environmental disruptors are known to inappropriately activate apoptosis in testicular locale by increasing the levels of ROS [10, 48]. During the transit from undifferentiated germ cells to mature spermatozoa, the sperms are vulnerable to multitudinous threats which are counteracted by the powerful antioxidant defense system of the testis [49]. Many toxicants have been shown to damage this protective shield, thus increasing the susceptibility of this organ to oxidative stress [50, 51]. Experimental studies have demonstrated that exposure to hexachlorocyclohexane (i.p., 20 mg/kg/day) during the critical stages of testicular development induces elevation in the levels of lipid peroxidation and hydrogen peroxide (H_2O_2), along with reduction in the levels of superoxide dismutase (SOD), catalase, and ascorbic acid [52]. Doreswamy et al., have demonstrated the induction of oxidative stress, DNA

damage, and apoptosis in testis following exposure to multiple doses of nickel chloride [53]. Our earlier studies on various toxicants in rodent models have exemplified the role of oxidative stress in mediating its effects on testis. Oral exposure to lindane (5 mg/kg bodyweight/day) for 30 days resulted in elevated levels of hydrogen peroxide and lipid peroxidation with concomitant decline in the activities of antioxidant and steroidogenic enzymes in testis [54]. Similar impairment of antioxidant system and mitochondria-dependent apoptosis of rat testis has been observed with lindane following single dose of it [10, 55]. Methoxychlor, at dose levels of 50 mg/kg body weight, caused significant diminution in testicular antioxidant enzymes along with Fas–FasL and mitochondria-mediated apoptosis in a time-dependent manner [44]. Compilation of these studies indicates the generation of free radicals and associated oxidative stress as the pathological mechanism underpinning the adverse effects of testicular toxicants. Innumerable studies have disclosed the involvement of oxidative stress in carrying out the malicious role of apoptosis in testis. Most of the toxicants have been reported to perturb the testicular locale either directly or indirectly targeting pivotal constituents of the testis—the germ cells, Sertoli cells, and Leydig cells. A study on the exposure of testis to a single dose (2 g/kg body weight) of di(2-ethylhexyl) phthalate revealed an augmented generation of ROS with simultaneous decrement in the concentrations of glutathione and ascorbic acid leading to selective apoptosis of spermatocytes [56]. Further exploration revealed the accrual of mono(2-ethylhexyl) phthalate, a toxic metabolite of DEHP, in testis causing mitochondrial respiratory damage and release of cytochrome c inciting apoptosis [56]. Exposure of spermatogenic cells to synthetic organic chemical, methyl tert-butylether (MTBE), enervated cell viability and induced generation of ROS and enhanced lipid peroxidation [57]. Similar damaging effects of oxidative stress followed by apoptosis in maturing germ cells have been observed with multifarious array of toxicants including metals. The uninterrupted close association of germ cells with Sertoli cells is yet another obligatory factor in spermatogenesis. Apart from its fostering role, Sertoli cells play a highly remarkable phagocytic role in eliminating spermatogenic cells undergoing apoptosis in response to chemical insult [58]. Consequently, any agent that confronts Sertoli cells may have a profound effect on spermatogenesis. In vitro exposure to β-BHC can enhance ROS and oxidative stress and then induce activation of JNKs and NF-kB, expression of FasL in rat Sertoli cells. Upon ligation of FasL to Fas, an FasL-mediated apoptotic death is stimulated in a target cell leading to the activation of caspase 8. Finally, apoptosis of Sertoli cells is mediated by executioner caspase 3, thereby disturbing the spermatogenic process [43]. Sertoli cells on exposure to an environmental contaminant, nonylphenol (10–40 mm), caused accumulation of ROS within 2 h of exposure which subsequently resulted in the loss of mitochondrial membrane potential and enhanced lipid peroxidation at 12 h posttreatment [59]. In vitro studies on the effects of 4-tert-octylphenol, a degradation product of alkylphenol-polyethoxylate, at a concentration of 30–60 mm for 6–24 h showed a decrease in the viability of Sertoli cells and increased apoptosis via caspase-3 pathway in a concentration- and time-dependent manner [60]. Diverse studies have cumulated over time, which accentuates the feasible role of oxidative stress in Sertoli cell apoptosis in response to toxicants.

37.13 Mechanisms Involved in Inducing Apoptosis

The possible mechanisms involved in the action of various factors in mediating testicular apoptosis are summed up. Most of the studies involving toxicants illustrate the undoubted role of ROS in executing its detrimental effects [31, 54, 59, 61]. These elevated levels of ROS can cause oxidation of the mitochondrial pores, thereby disrupting the mitochondrial membrane potential and releasing cytochrome c [56, 62]. Once free of the mitochondrial membrane, cytochrome c rapidly assembles a multi-protein complex involving Apaf-1 and procaspase 9 leading to the activation of the caspase 9, which subsequently triggers the effector caspase 3, 6, and/or 7 [26, 27]. These caspases, in turn, activate endonucleases and proteases resulting in DNA fragmentation and degradation of nuclear and cytoskeletal proteins [63, 64]. Apart from ROS, Bax, a member of pro-apoptotic Bcl-2 family, can directly influence the release of cytochrome c from mitochondria [65]. It is interesting to note that the Bcl-2 protein family is itself regulated by ROS [66]; however, whether this regulation has any role in toxicant-mediated apoptosis is not known. ROS have been shown to induce the expression of Fas receptor and ligand stimulating the Fas/FasL-mediated apoptotic signal transduction pathway [67]. Interaction of Fas with FasL leads to a cascade of events which begins with the proteolytic cleavage of procaspase 8 to its active form, which consequently activates downstream effector caspase 3, 6, or 7 [68–70]. These caspases execute the cells by degrading the constituent proteins [71]. Elimination of apoptotic action of Fas by antioxidants further emphasizes the role of ROS in Fas-mediated death process [72, 73]. Therefore, deprivation of antioxidants and/or generation of free radicals by toxicants is capable of reducing the Fas pathway. In addition, they impair steroidogenesis and may deprive germ cell of the essential growth factor, testosterone, and increase their susceptibility to ROS attack [74].

37.14 Conclusion

The purpose of this chapter was to evaluate the role of various factors influencing apoptosis in male infertility. Mounting evidence suggests that apoptosis occurs as the predominant cell death mechanism in testis in response to several diseases and toxic injuries. Research implies that reactive oxygen species and other secondary free radicals such as nitric oxide and hydroperoxides could be inducers or mediators of apoptosis in testis through downregulation of antioxidant defense system or increased expression of apoptosis-related proteins. However, the exact mechanism of action of apoptosis in inducing male infertility remains a mystery. Further studies are warranted to evaluate the adverse effects of apoptosis on testis.

37.15 Review Criteria

An extensive literature search probing "the apoptosis and male infertility" was performed using search engines such as ScienceDirect, OVID, Google Scholar, PubMed, and MEDLINE. The start and end dates for these searches were January 2014 to December 2018. The following keywords were used to retrieve the information and the data extraction: "male reproductive health," "apoptosis," "extrinsic apoptotic pathway in testis," "intrinsic apoptotic pathway in testis," "oxidative stress," "environmental contaminants," and "endocrine disruptors." Articles published in English language were considered and the data that were solely published in conference or meeting proceedings, websites, or books were excluded in writing this review. Websites and book-chapter citations provide conceptual content only.

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Impact of Spinal Cord Injury

38

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Key Points

1. The majority of men with spinal cord injury are infertile due to a combination of erectile dysfunction, ejaculatory dysfunction, and poor semen quality.
2. There are treatments for erectile dysfunction and ejaculatory dysfunction; however, there currently is no treatment for their abnormal semen quality, the cause of which has not been conclusively established.
3. Poor semen quality in men with spinal cord injury is not due to endocrinopathy, scrotal hyperthermia, methods of bladder management, or infrequent ejaculation.
4. Inflammatory factors have been demonstrated to contribute to abnormal semen quality in men with spinal cord injury.
5. Treating abnormal inflammatory processes holds promise for improving semen quality in men with spinal cord injury.

38.1 Introduction

Spinal cord injury occurs to an estimated 10,000–12,000 people every year in the USA alone [1]. More than a quarter of a million Americans are currently living with spinal cord injury [1], with many millions more worldwide. The cost of managing the care of patients with spinal cord injury is approximately $4 billion per year. Car accidents are the most common cause of spinal cord injury, followed by violent encounters, sporting and work-related accidents, and falls [1]. Medical advances have greatly improved the prognosis for people who sustain spinal cord injury; however, it remains a major social and health-care problem.

The majority of spinal cord injury victims are young adults. Of them, more than 80% are men. As a result, young males constitute the largest part of this patient population. Reproductive function is essential for men with spinal cord injury, but unfortunately, less than 10% of them can father children without medical assistance [2]. Infertility in male patients with spinal cord injury results from a combination of erectile dysfunction, ejaculatory dysfunction, and poor semen quality [3].

As a result of advancements in assisted ejaculation techniques including electroejaculation and high-amplitude penile vibratory stimulation, semen can be safely obtained from nearly all men with spinal cord injury without resorting to surgical procedures [4]; however, semen quality is poor in the majority of cases [4]. This chapter will describe hypothesized causes of poor semen quality in men with spinal cord injury and discuss the research investigating potential treatments for this problem.

38.2 Semen Abnormalities in Men with Spinal Cord Injury

The origin and/or cause of low sperm quality in men with spinal cord injury has not been clearly defined. Several possible etiologies have been postulated, including hormonal dysfunction, elevated scrotal temperature, methods of bladder management, and alterations in sperm transport and storage due to reproductive tract stasis, but none of these causes has been conclusively proven [5].

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38.3 Role of Hormonal Alterations

Alterations in the hypothalamic-pituitary-gonadal axis may result in the disruption of spermatogenesis. The endocrine status of men with spinal cord injury has been examined in several studies that provided contradictory results. A review by Ibrahim et al. found little consensus regarding changes in serum levels of testosterone, LH, FSH, or prolactin in men with spinal cord injury [2]. For example, four studies of men with chronic spinal cord injury showed no difference in prolactin concentration, [2] whereas a study by Sanchez-Ramos found elevated serum concentrations of prolactin that increased with time (1, 3, and 6 months) postinjury [6]. We studied this problem in a group of 66 men with spinal cord injury and found no association between semen quality and serum levels of luteinizing hormone, follicle-stimulating hormone, testosterone, or prolactin [7]. The only exception was in a subgroup of subjects who had elevated levels of follicle-stimulating hormone. In each case, the patient was azoospermic, even patients with only small elevations of follicle-stimulating hormone. Hormonal alterations are unlikely to be a major contributor to poor semen quality in men with spinal cord injury.

38.4 Role of Scrotal Temperature

Elevated scrotal/testicular temperature was one of the first hypotheses to explain the origin of semen abnormalities in men with spinal cord injury. It is common knowledge that spermatogenesis is temperature-sensitive and proceeds optimally at 35 °C. Higher scrotal temperatures could have detrimental effects on sperm production [8]. It was assumed that men with spinal cord injury could have scrotal hyperthermia as a result of generalized scrotal thermoregulatory dysfunction or because of sitting in a wheelchair for prolonged periods [9]. Some studies showed that men with spinal cord injury sitting in wheelchairs had higher scrotal temperatures compared to able-bodied men, sitting in armchairs [10, 11]. Brindley reported an inverse correlation between scrotal temperatures and motile sperm counts in men with spinal cord injury [11]. However, we did not find any difference between oral temperature and scrotal temperature in control subjects and spinal cord-injured subjects or the difference between these two parameters [12]. Furthermore, men with spinal cord injury who were ambulatory (i.e., not in wheelchairs) still had impaired semen quality [12], indicating that some aspect of spinal cord injury, other than the simple act of sitting in a wheelchair, contributes to abnormal semen quality in these men. Supporting this idea is the fact that no study has found improvement in semen quality by cooling the scrotum of men with spinal cord injury.

Studies of scrotal temperature in noninjured men have suggested that short-term versus long-term exposure to elevated temperature causes reversible versus irreversible changes in the seminiferous tubules [13, 14]. In men with spinal cord injury, however, both cross-sectional [15] and longitudinal [16] studies have shown that semen parameters were not significantly related to the duration of the postinjury period, suggesting a stable (and null) pattern for the measures across time. In light of these facts, it appears that no strong evidence exists to support the role of elevated scrotal temperature as a leading etiologic factor of the semen abnormalities in men with spinal cord injury.

38.5 Role of Bladder Management

Motility can be impaired in sperm exposed to the contents of the urinary bladder [17]; however, no bladder management regime has been associated with normal semen quality in men with spinal cord injury. Some studies have shown that the use of intermittent catheterization is associated with better sperm motility than the use of indwelling urethral catheters, suprapubic catheters, or spontaneous voiding [18, 19]. Although semen quality is improved with intermittent catheterization versus the other methods mentioned, it does not become normalized. Bladder management, then, does not seem to be a significant cause of impaired semen quality in men with spinal cord injury.

38.6 Role of Ejaculation Frequency

The majority of men with spinal cord injury cannot ejaculate without medical assistance. It has been hypothesized that long periods between ejaculations may result in reproductive tract stasis which can negatively affect sperm. However, most studies investigating the effect of repeated ejaculation on semen quality in men with spinal cord injury found no improvement in semen parameters [20–24]. Only one group reported a moderate increase in sperm motility and sperm morphology after 3 months of weekly ejaculations with penile vibratory stimulation [25]. These findings indicate that frequency of ejaculation is not the sole factor causing abnormal semen quality in men with spinal cord injury.

Interesting data were presented by Ohl et al., suggesting that spinal cord injury could result in fundamental changes in sperm transport and storage [26]. In eight patients with spinal cord injury, bilateral seminal vesicle aspiration was performed immediately before electroejaculation or penile vibratory stimulation. The seminal vesicle aspirates contained large numbers of poor-quality sperm. It should be noted that normal men do not have large numbers of sperm in seminal vesicles. Duration of abstinence did not correlate with the number of seminal vesicle sperm. Furthermore, the semen parameters in samples obtained immediately after seminal vesicle aspiration were significantly better

compared to historical ejaculated parameters [26]. The authors concluded that altered transport with stagnation of sperm in the seminal vesicles could be a primary source of semen with poor quality in men with spinal cord injury. It was also shown that factors within the seminal plasma contribute to poor semen abnormalities in men with spinal cord injury [27]. This issue will be discussed in more detail later in this chapter.

38.7 Studies of Oxidative Stress in Men with Spinal Cord Injury

In addition to the aforementioned putative causes that have been investigated, there is increasing evidence that oxidative stress is an important mechanism contributing to sperm damage in this group of patients. The generation of reactive oxygen species and their relation to semen quality in men with spinal cord injury has been investigated in several studies.

38.8 Reactive Oxygen Species in Whole Semen Versus Washed Sperm

The purpose of a study performed by de Lamirande et al. was to determine whether whole semen samples versus washed spermatozoa obtained from men with spinal cord injury produced excessive amounts of reactive oxygen species [28]. This study included three groups of men: healthy volunteers ($n=20$), infertile able-bodied men ($n=166$), and subjects with spinal cord injury ($n=21$). In the latter group, semen was obtained by masturbation after butylbromide and physostigmine injections in 19 patients and by electroejaculation in the remaining two men. Formation of reactive oxygen species was measured in neat semen and in Percoll-washed spermatozoa of all subjects.

The presence of reactive oxygen species in whole semen was detected in 97% of subjects with spinal cord injury compared to 40% and 15% in infertile able-bodied men and volunteers, respectively. Compared to a threshold value of 10 mV/s/10^9, reactive oxygen species production was elevated in 81% of patients with spinal cord injury, 25% of infertile able-bodied men, and 10% of healthy controls. In healthy controls and in infertile able-bodied subjects, the levels of reactive oxygen species measured in semen were, respectively, 40 and 14 times lower than those detected in semen from subjects with spinal cord injury. No correlation was found between reactive oxygen species production and level or duration of injury.

After centrifugation on Percoll gradients, sperm from men with spinal cord injury continued to generate large amounts of reactive oxygen species. High reactive oxygen species production by Percoll-washed spermatozoa was found in 75% of men with spinal cord injury, 20% of infertile men, and 5% of healthy controls. The mean reactive oxygen species levels in washed spermatozoa from men with spinal cord injury were sixfold higher than that of infertile patients and 140-fold higher than that of normal volunteers. There was a significant inverse relationship between levels of reactive oxygen species and percentage of motile sperm in Percoll-washed specimens from patients with spinal cord injury.

Results of this study showed that semen samples and Percoll-washed sperm samples from subjects with spinal cord injury produced reactive oxygen species at a higher frequency and at higher levels than equivalent samples from normal men or infertile men. In men with spinal cord injury, levels of reactive oxygen species correlated negatively with sperm motility [28]. These data suggest that the role of reactive oxygen species as a mechanism of sperm damage leading to infertility could be more important in men with spinal cord injury compared to the general population or to the infertile population.

38.9 Reactive Oxygen Species and Sperm Characteristics

The generation of reactive oxygen species and its relation to semen characteristics in men with spinal cord injury was investigated by our group [29]. This study included 24 men with spinal cord injury and 19 able-bodied controls. In the spinal cord-injured patients, semen was obtained by penile vibratory stimulation ($n=15$), electroejaculation ($n=8$), and masturbation ($n=1$). Measurements of reactive oxygen species formation were performed before and after stimulation with *N*-formyl-methionyl-leucyl-phenylalanine and l2-myristate-13-acetate phorbol ester. These two substances trigger the generation of reactive oxygen species by leukocytes and spermatozoa, respectively.

The study showed that mean levels of reactive oxygen species in unstimulated and stimulated specimens were significantly higher in spinal cord-injured men compared to controls. The actual values reflecting the reactive oxygen species activity in the spinal cord-injured group were from 250 to 2000 times higher than that of the control group. The incidence of samples positive for reactive oxygen species specimens in unstimulated controls and men with spinal cord injury was 47.3% versus 100%, respectively. It was also found that the levels of reactive oxygen species in semen from men with spinal cord injury correlated negatively with sperm motility and positively with white blood cell concentrations. Interestingly, the levels of reactive oxygen species did not differ between antegrade and retrograde samples or between different methods of ejaculation (penile vibratory stimulation and electroejaculation). Therefore, the high levels of reactive oxygen species in semen specimens obtained

by electroejaculation and vibratory stimulation may not be due exclusively to the effects of electrical current, as was suggested by Rajasekaran et al. [30].

As can be seen from the above studies, reactive oxygen species production is elevated in semen from patients with spinal cord injury, and increased oxidative stress may be an important mechanism of impaired sperm quality in this group of men. Human ejaculate consists of several types of cells including mature and immature spermatozoa, germ cells from different stages of the spermatogenic process, epithelial cells, and leukocytes. Of these different cell types, leukocytes and spermatozoa have been shown to be the two principal sources of production of free radicals [31].

38.10 Effect of Leukocytes

Ejaculates from men with spinal cord injury are known to have increased leukocyte counts (Fig. 38.1) [32, 33]. The main sources of leukocytes in human ejaculate are the prostate gland, seminal vesicles, and epididymis [34]. Our studies showed no evidence of chronic or acute prostate gland inflammation in leukocytospermic patients with spinal cord injury [35], and we did not find any white blood cells in the vasal aspirates from these subjects [36]. These data indicate that the seminal vesicles are the most likely origin of leukocytospermia in men with spinal cord injury.

Leukocytes can produce large amounts of reactive oxygen species. A positive correlation has been reported between seminal leukocyte counts and reactive oxygen species production [37, 38]. Of different leukocyte subtypes, peroxidase-positive cells, namely, neutrophils and macrophages, are predominant sources of reactive oxygen species production [39]. In ejaculates obtained by electroejaculation, these two leukocyte subpopulations, as identified by immunohistochemical staining, were the predominant contributors to leukocytospermia in men with spinal cord injury [40]. Lymphocytes were also found to be significant contributors to leukocytospermia in men with spinal cord injury. Immunophenotypic analysis by flow cytometry showed that the greater fraction were T cells, many of which coexpressed the human leukocyte antigen HLA-DR and CD25, suggesting they were in an activated state. No significant B-cell population was evident [32].

The activation state of leukocytes plays a crucial role in determining reactive oxygen species output because activated white blood cells can produce up to 100-fold increases in reactive oxygen species compared with nonactivated cells [41]. This effect is mediated by an increase in reduced nicotinamide adenine dinucleotide phosphate production via the hexose monophosphate shunt [42]. The myeloperoxidase system of neutrophils and macrophages is also activated, resulting in a respiratory burst and production of large amounts of superoxide and other reactive oxygen species.

38.11 Effect of Cytokines

Elevated concentrations of the proinflammatory cytokines interleukin 1 beta, interleukin 6, and tumor necrosis factor-α have been detected in the semen of men with spinal cord injury [43], reflecting activation of T-lymphocytes [32] (Fig. 38.2). Inactivation of these cytokines, by adding monoclonal antibodies or receptor blockers to semen from men with spinal cord injury, improves sperm motility [44, 45]. Interleukins are important mediators of free radical generation in many tissues, and the role of cytokines as mediators of oxidative stress is well known. Supporting this notion is the observation of a positive correlation between seminal

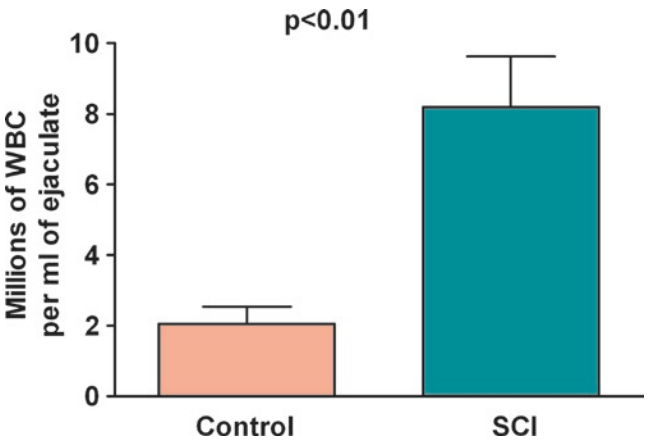

Fig. 38.1 Studies have shown that semen of men with spinal cord injury (SCI) contains higher concentrations of white blood cells (WBC) compared with semen of able-bodied, healthy control subjects

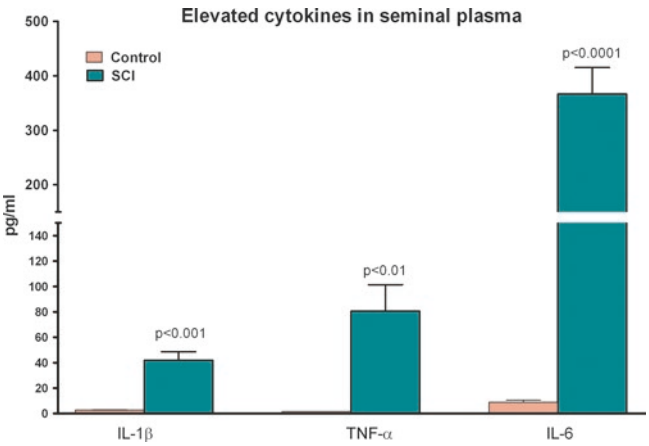

Fig. 38.2 Cytokines can be detrimental to sperm cells. Concentrations of the proinflammatory cytokines, interleukin 1β (1IL-1β), tumor necrosis factor-α (TNF-α), and interleukin 6 (IL-6) were significantly elevated in semen of men with spinal cord injury (SCI) compared with semen of control subjects. pg/ml = picograms per milliliter

reactive oxygen species production and seminal plasma concentrations of cytokines such as interleukin 6 [46, 47], interleukin 1, and tumor necrosis factor-α [48, 49] in infertile able-bodied men. Interleukin 1 and tumor necrosis factor-α were also shown to stimulate reactive oxygen species production in fertile donor semen [50]. Thus, activated seminal leukocytes have the potential to cause oxidative stress elevation in men with spinal cord injury.

38.12 The Inflammasome

In 2007, Martinon published two papers describing the role of "NALP inflammasomes: a central role in innate immunity" and "Inflammatory caspases and inflammasomes: master switches of inflammation" [51, 52]. Over the next few years, the molecular mechanisms involved in their activation, as well as the role they play in immunity, inflammatory diseases, and infection, were elucidated [53–56]. The inflammasome, briefly, is a protein platform which, when activated, regulates/initiates the inflammatory cytokine cascade of the type thought to be causally related to the poor sperm motility seen in men with spinal cord injury. The intracellular form consists of a group of pattern recognition receptors (PRRs) described as members of the NOD-like receptor (NLR) family and which contain various domains which either activate pro-caspase-1 directly via a CARD (caspase activation and recruitment domain) or through recruitment of an adapter protein such as ASC (apoptosis-associated speck-like protein containing a CARD). The pro-caspase-1 is converted to active caspase-1 which, in turn, converts pro-IL-1β and pro-IL-18 into their active forms, initiating the inflammatory cascade [53–56]. The NLRP3 inflammasome is one of the largest and has been widely studied in a number of disease states. It is found in many tissues including the urinary tract and is known to be activated by a variety of molecules, bacterial products, endogenous crystals, and inorganic environmental materials [51–53, 57]. One of the methods of activating the inflammasome is through activation of the pannexin-1 channel. Our group studied the seminal plasma of men with spinal cord injury and found significantly increased levels of ASC and caspase-1 (compared to controls) [58]. In another study, the semen of 32 men with spinal cord injury was treated with a polyclonal antibody to ASC (IgG was used as a control agent). This treatment resulted in a significant improvement in the percent of progressive motility [59].

38.13 Pannexin-1

The pannexins are a family of three channel-forming glycoproteins. Pannexin-1 is the most ubiquitous and most studied of the three. Previous studies have found pannexin-1 in the urinary tract [60, 61]. Our group has demonstrated its activity in the seminal plasma associated with changes in sperm motility [62]. Among the pannexins, pannexin-1 is most often found involved in the activation of inflammasome processes. It has been linked to many normal and essential physiologic processes as well as pathologic processes. Briefly, pannexin-1 is a cell wall glycoprotein that regulates channels or pores allowing a variety of extracellular substances to enter the cell. It is known to be a mediator of the NLRP3 inflammasome. In the innate immune responses in which it is involved, the activation of the pannexin channel allows initiation of the inflammasome processes, resulting in the release of caspase-1 and the activation of IL-1β and IL-18, in turn resulting in a cascade of inflammatory cytokines [60, 63]. Probenecid improves sperm motility in men with spinal cord injury.

There are many oral medicines known to be pannexin channel inhibitors, some of them with serious side effects and limited applications. However, a few like quinine, glyburide, colchicine, and probenecid are in common usage. A review of pannexin research pointed out that probenecid, a pannexin-1 inhibitor, has been used safely in the treatment of gouty arthritis with few side effects [60]. Our group performed a study in 20 men with spinal cord injury who were treated with a standard therapeutic dose of probenecid for 4 weeks (i.e., the standard dose for the treatment of gout). Our study found that sperm motility improved *in each subject* after 4 weeks of oral probenecid. The mean percent of sperm with progressive motility increased from 19% to 26% ($p < 0.05$). A more striking increase was seen in the mean percent of sperm with rapid linear motility, from 5% to 17% ($p < 0.001$). Table 38.1 shows that this improvement continued into the four-week follow-up period. Similar improvements were seen in the total motile sperm count. Sperm concentration was not significantly different at pretreatment, posttreatment, and follow-up [62]. This initial study holds

Table 38.1 Oral probenecid was administered to 20 men with spinal cord injury. Sperm motility was assessed prior to the first dose (pretreatment), after a four-week course of probenecid (posttreatment), and four weeks after the last pill was ingested (follow-up)

Sperm parameter	Pretreatment	Posttreatment	Follow-up
Progressive motility (%)	19.0 ± 2.8	26.0 ± 3.7	23.0 ± 4.1
Compared to pretreatment:		$p < 0.05$	NS
Rapid linear motility (%)	5.7 ± 1.5	17.0 ± 3.3	17.0 ± 3.7
Compared to pretreatment:		$p < 0.001$	$p < 0.001$
Sperm concentration (10^6/cc)	52.0 ± 8.5	53.0 ± 9.3	53.0 ± 10.0
Compared to pretreatment:		NS	NS
Total motile sperm count (10^6/ejaculate)	15.4 ± 4.4	28.0 ± 6.5	27.0 ± 10.0
Compared to pretreatment:		$p < 0.05$	NS

Values are presented as mean ± SEM
NS not significant
Sperm parameters after oral administration of probenecid

promise for developing a simple and effective treatment for improving sperm motility in men with spinal cord injury with the goal of broadening options for assisted conception in these couples.

38.14 Consequences of Oxidative Stress in Semen of Men with Spinal Cord Injury

Decreased sperm motility and decreased sperm viability are characteristic features of semen from men with spinal cord injury [9]. In contrast to able-bodied men, most immotile spermatozoa in the semen from men with spinal cord injury are dead. It was shown that the dead-to-live immotile sperm ratio in spinal cord-injured subjects was more than double that in able-bodied subjects (7:3 versus 3:7) [64]. Apoptosis may play an important role in these changes. Experimental data shows that spinal cord injury in rats is associated with decreased sperm mitochondrial transmembrane potential and decreased sperm viability, suggesting excessive apoptosis [65, 66].

Mitochondrial dysfunction has been implicated in abnormal sperm motility in men with spinal cord injury [67]. Donor sperm were co-incubated with seminal plasma from ejaculates of men with spinal cord injury. Seminal plasma from men with spinal cord injury, which induced a disruption of mitochondrial membrane potential, was associated with a loss of sperm motility and an enhanced mitochondrial reactive oxygen species generation and caspase activation. The enhanced mitochondrial reactive oxygen species generation was associated with a late induction of lipid peroxidation as assessed by boron-dipyrromethene (BODIPY) C_{11}. This effect was produced only by seminal plasma from ejaculates of men with spinal cord injury and was observed only when evaluated at 6 h but not 1 h after washing donor sperm from seminal plasma of men with spinal cord injury [67]. These findings suggest that peroxidative damage could only be induced once the production of reactive oxygen species in the mitochondrial matrix had overwhelmed the intramitochondrial antioxidant dense enzymes [68].

Increased levels of reactive oxygen species can also negatively affect the integrity of DNA in the sperm nucleus. Several forms of sperm DNA damage could be caused by reactive oxygen species, including chromatin cross-linking, chromosome deletion, DNA single- and double-stranded breaks, and base oxidation [69, 70].

Overproduction of reactive oxygen species or reduced antioxidant capacity can lead to increased DNA fragmentation [69]. In addition, aberrant histone/protamine and epigenetic anomalies render DNA sensitive to reactive oxygen species and prone to fragmentation. Significantly higher mean values for sperm DNA damage, lipid peroxidation, and reduced telomere length were found in spermatozoa of infertile men with previous failed/low fertilization compared with fertile individuals [71]. With the use of flow cytometry, it was shown that sperm from men with spinal cord injury have a high degree of abnormal chromatin condensation and reduced binding [72].

DNA fragmentation in sperm from men with spinal cord injury was also investigated by our group [73]. The study consisted of three experiments. In experiment 1, we compared the DNA fragmentation index in sperm from men with spinal cord injury to that of able-bodied controls. This experiment showed that the mean DNA fragmentation index was fourfold higher in the spinal cord injury group compared with the control group, and there was no overlap in the DNA fragmentation index between these two groups. As was discussed earlier, chronic anejaculation is considered to be one of the possible explanations of semen abnormalities seen in men with spinal cord injury. To examine this possibility, we performed experiment 2 in which we compared the sperm DNA fragmentation index in two semen specimens obtained 3 days apart from the same spinal cord-injured subjects. No significant difference was found in the sperm DNA fragmentation between the two specimens. The purpose of experiment 3 was to determine if necrospermia, leukocytospermia, or semen processing in men with spinal cord injury contributes to their sperm DNA fragmentation index. In this experiment, the DNA fragmentation index in unprocessed semen samples was compared with that of semen samples processed on a gradient (i.e., free of dead sperm and leukocytes). The results of experiment 3 found no significant difference between mean DNA fragmentation indices in aliquots of neat versus processed semen in spinal cord-injured subjects. Although removal of leukocytes did not result in a change in the DNA fragmentation index, it is possible that their negative effects on sperm were exerted prior to sperm processing. Thus, it appears that men with spinal cord injury have significantly greater sperm DNA damage, which may be related to high levels of oxidative stress in semen.

Complex relationships exist between apoptosis and DNA damage. Induction of apoptosis by reactive oxygen species results in a high frequency of single- and double-stranded DNA strand breaks in a process referred to as karyorrhexis. Severe DNA damage can initiate the apoptosis pathway. Agarwal and Said have suggested that in the context of male infertility, there may be an interaction between seminal reactive oxygen species, sperm DNA damage, and apoptosis, and this interaction may constitute a unified pathogenic molecular mechanism [74].

Semen from men with spinal cord injury is typically highly viscous. Hyperviscous seminal plasma has been reported to be associated with elevated levels of malondialdehyde, an unsaturated carbonyl product of oxidative stress indicating excessive lipid peroxidation [75]. Hyperviscosity has also been shown to be linked to reduced seminal plasma

antioxidant capacity [76]. The mechanism of change in seminal viscosity due to oxidative stress could be related to altered interactions between oxidized proteins in the seminal plasma [77]. Quantitative proteomic analysis suggests that, in patients with spinal cord injury, the liquefaction cascade can potentially be delayed because of inhibition of kallikrein activities by different protease inhibitors. Therefore, after ejaculation, sperm cells can remain immotile, entrapped in the gel clot formed by semenogelins [78].

Increased levels of reactive oxygen species can impair sperm function. This phenomenon could be attributed to changes in membrane fluidity and acrosome integrity, resulting in decreased capacity for sperm-oocyte fusion [79]. Acrosin is a sperm acrosomal proteinase with trypsin-like substrate specificity, located in the acrosomal matrix as an enzymatically inactive zymogen. Evidence suggests that its active form, acrosin, is necessary for normal fertilization in humans. If acrosin is reduced, absent, or inhibited, sperm binding to, and penetration of, the zona pellucida is severely impaired [80]. Our group showed that sperm from men with spinal cord injury is characterized by lower acrosin activity compared to healthy men [81]. These findings indicate that sperm from men with spinal cord injury could have functional defects in sperm-oocyte fusion, resulting from oxidative damage. Sperm from men with spinal cord injury was also reported to have a high degree of acrosomal abnormalities [72, 82].

It is known that seminal plasma is a major contributor to semen abnormalities in men with spinal cord injury (Fig. 38.3). For example, seminal plasma of spinal cord-injured men rapidly inhibits motility of sperm from normal men. Similarly, seminal plasma from normal men improves the motility of sperm from spinal cord-injured men [27]. Further evidence that an abnormal seminal plasma environment impairs sperm of men with spinal cord injury comes from a study measuring sperm motility and sperm viability in ejaculates versus vas deferens aspirates of the same group of spinal cord-injured subjects [36]. Because sperm from the vas deferens has not yet been subjected to the effects of the seminal plasma, a direct comparison of these sperm sources provided information about the effects of seminal plasma on sperm function in men with spinal cord injury. The results of this study showed that in patients with spinal cord injury, but not in able-bodied controls, sperm motility and sperm viability were significantly lower in ejaculated specimens (Fig. 38.4). These data provided evidence that, in men with spinal cord injury, seminal plasma is toxic to the sperm.

Interestingly, sperm obtained from men with spinal cord injury not only lose motility more rapidly than sperm from normal men, but this deterioration is also exacerbated when semen is stored at body temperature compared to room temperature. In normal men, this correlation was not found [83]. The possible explanation of this discrepancy is higher reac-

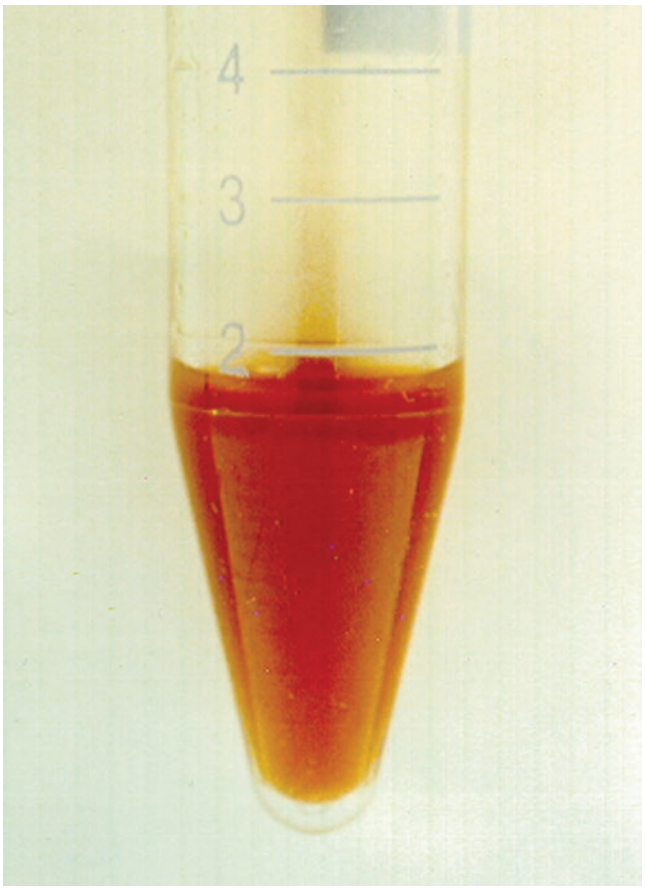

Fig. 38.3 Twenty-seven percent of men with spinal cord injury have brown-colored semen. The cause of the brown color is unknown and may be related to the presence of abnormal constituents in the seminal plasma. Evidence suggests that an abnormal seminal plasma environment contributes to sperm impairments in men with spinal cord injury

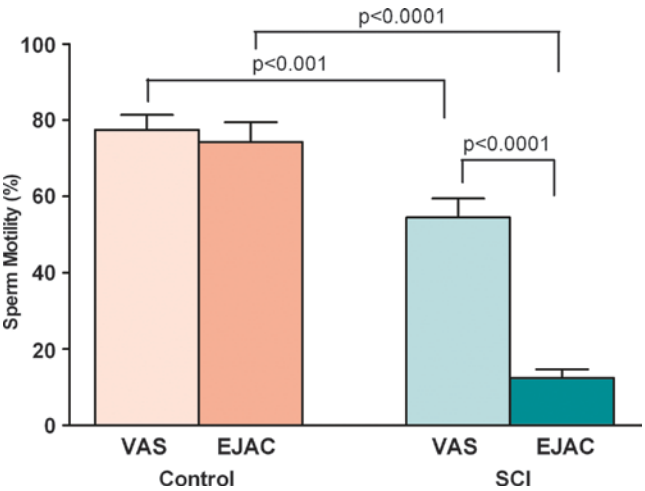

Fig. 38.4 In men with spinal cord injury (SCI), sperm motility was significantly higher when obtained from the vas deferens (VAS) than from the ejaculate (EJAC). In contrast, in control subjects, there was little difference in sperm motility between the two sites. This study provided definitive evidence that seminal plasma was a major contributor to low sperm motility in men with spinal cord injury

Table 38.2 Characteristics of semen from men with spinal cord injury suggesting increased oxidative stress

Leukocytospermia
Teratozoospermia, increased numbers of immature sperm
Low sperm motility
Necrospermia
Increased DNA fragmentation
Semen hyperviscosity

tive oxygen species production by activated leukocytes at body temperature in the semen from men with spinal cord injury. Based on the information presented above, it is reasonable to suggest that elevated levels of reactive oxygen species and/or decreased antioxidant capacity of seminal plasma could be, at least in part, responsible for its detrimental effects on sperm motility and viability. Table 38.2 summarizes characteristics of the semen in men with spinal cord injury, suggesting the presence of increased oxidative stress.

38.15 Conclusions

Numerous studies have shown that men with spinal cord injury have poor semen quality. No single factor has been convincingly proven to be the root cause, and the etiology is likely multifactorial. Among the factors, oxidative stress is a pathogenic mechanism leading to sperm damage and subsequent infertility resulting from spinal cord injury. In these patients, evidence-based research has established a relationship between inflammation and poor semen quality, typically characterized by elevated concentrations of leukocytes and inflammatory cytokines. Treating abnormal inflammatory processes may hold promise for improving semen quality in men with spinal cord injury.

38.16 Review Criteria

An extensive search of studies examining the relationship between spinal cord injury and infertility was performed using search engines such as ScienceDirect, OVID, PubMed, and MEDLINE. The searches were performed between February and March 2019. The overall strategy for study identification and data extraction was based on the following keywords: “spinal cord,” “spinal cord injury,” “paraplegia,” “infertility,” “spermatozoa,” “semen,” “ejaculation,” “oxidative stress,” “endocrinopathy,” “scrotal temperature,” “bladder management,” “reactive oxygen species,” “inflammation,” “inflammasome,” “cytokines,” “DNA fragmentation,” “pannexin,” and “probenecid.” Articles published in languages other than English were also considered. Data that were solely published in conference or meeting proceedings, websites, or books were not included. Websites and book-chapter citations provide conceptual content only.

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Obesity

39

Ashok Agarwal and Sulagna Dutta

Key Points

- Worldwide obesity pandemic may be a potential reason of the concurrent global decline in male fertility.
- Obesity may influence hypothalamic–pituitary–gonadal axis and increase in estrogen- and adipose tissue-derived hormones, such as leptin, along with reduction in testosterone and inhibin B.
- Obesity can cause sperm genetic and epigenetic changes, increased scrotal temperature, erectile dysfunctions, and other physical effects.
- Male obesity has deleterious impact on the outcomes of assisted reproductive techniques (ARTs).
- Lifestyle modifications may help to manage obesity-related disorders besides specific medications and surgical interventions.

39.1 Introduction

Men possess greater morbidity and mortality rates as compared to their female counterparts, with global men's health deteriorating gradually [1]. Male fertility is following a declining trend as well [2–4], with male factor infertility contributing to 40–50% of worldwide infertility [5]. Most of the male infertility cases are idiopathic, for which the exact cause remains unidentified [6]. Research suggests a potential role of modifiable and preventable lifestyle adoptions in the improvement of male fecundity [7]. As a result of improper lifestyle and dietary habits, the global prevalence of obesity is accelerating at a high pace, leading to a worldwide obesity pandemic. Obesity includes increased visceral (abdominal) adiposity and is clinically defined as a body mass index (BMI) that is equal to or greater than 30 kg/m^2 [8]. An increasing BMI correlates with higher morbidity and mortality. According to a 2016 WHO report, 39% of the world population of adults have a BMI of above the normal range (BMI between 18.5 and 24.9) [9]. The global decline in male fertility parameters and the concurrent increase in obesity prevalence have led to several research interventions to find the association between obesity and male infertility.

Obesity triggers an array of disorders and renders the body susceptible to various chronic diseases, including diabetes, cardiovascular disease, malignancies, early aging, and neurodegenerative diseases. Obesity-induced disorders are associated with comorbidities such as hyperleptinemia, hyperinsulinemia, hypertension, dyslipidemia, hyperglycemia, Th1 dominant chronic inflammation, as well as disrupted reproductive functions [10, 11]. The pathogenesis induced by obesity is mainly based upon the state of lipotoxicity that causes cellular injury and tissue dysfunctions [12].

A substantial percentage of infertile men are being assessed and treated for obesity [13]. Moreover, a well-defined J-shaped relationship showing increase in BMI with a decline in semen quality has been put forth [14]. Studies have also found increased rates of azoospermia and oligozoospermia in obese men compared to males with normal weight [15]. With every 3 kg/m^2 increase in BMI of the male partner, an estimated 12% reduction in achieving successful pregnancy is proposed [16]. Obesity reportedly affects sperm count, morphology, vitality, motility, and sperm DNA integrity, although further research in this direction is required for better understanding of their associations [13, 15]. Obesity may modulate the genetic and epigenetic constitution of spermatozoa or influence the endocrine regulation of the male reproductive system [13, 17–19]. This includes reduced levels of testosterone (total and free), progesterone, and sex hormone-binding globulin (SHBG), along with increased levels of estrogen, insulin, leptin, follicle-stimulating hormone

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(FSH), luteinizing hormone (LH), and prolactin [20]. In addition, physical mechanisms in obesity-induced male reproductive dysfunction include erectile dysfunction and increased scrotal temperature [13].

Obesity in male partners of the couple undergoing assisted reproductive techniques (ARTs) reportedly increases the risk for decreased impregnation rates and live birth rates [21]. Paternal obesity has also been suggested to have an adverse impact upon the offspring's health, particularly affecting metabolic and reproductive functions. The effect of paternal obesity on the health of the offspring may be mediated via DNA damage and several epigenetic modifications borne by the sperm [22]. The present chapter emphasizes the possible mechanisms by which obesity in men affects male reproductive functions, influencing the complex endocrine regulations and surge of adipose tissue-derived substances, modulating the genetic or epigenetic constitution of sperm, or directly perturbing testicular functions. Besides providing a concise understanding of the link between obesity and male fecundity, the chapter also puts forth the management and treatment strategies to combat obesity-induced male infertility.

39.2 Obesity: Metabolic Syndrome and Male Infertility

Metabolic syndrome refers to disordered energy production, usage, and storage. It is diagnosed if any three out of the following five conditions co-occurs: hypertension, obesity, high serum triglycerides, low high-density cholesterol (HDL) levels, and high fasting level of blood glucose. Metabolic syndrome renders the body susceptible to develop cardiovascular disease, diabetes, as well as reproductive disorders. It has long been shown to have an association with male reproductive dysfunctions such as hypogonadism and erectile dysfunction (ED) [23]. Studies have presented an estimated prevalence of obesity alone in 35% of all adult population and almost 50% of the aging population in the USA [24]. The rudiments of this state are observed with regard to the detrimental effects of the syndrome on male fecundity [25]. Hyperglycemia and hyperinsulinemia are almost obvious occurrences in obese men that can be considered as the confounding factors of male obesity [26]. Metabolic syndrome and its detrimental bodily consequences impair sperm quantity and quality and, therefore, are potent contributors to the reduced fertility seen in obese men [26].

The physiological mechanisms that fine-tune metabolic energy balance with reproductive functions rely upon the cross talks among metabolic hormones, hypothalamic–pituitary–gonadal (HPG) axis, and neuronal control. The neural apparatus regulating metabolic rate and energy homeostasis is the body "metabolic sensor" that transforms hormonal signals into neuronal impulses, dictating the hypothalamic gonadotropin-releasing hormone (GnRH) pulse generator. Hypothalamic GnRH is the prime regulatory hormone that mediates the orchestration of pituitary gonadotropins and subsequent testicular sex hormones to control spermatogenesis and other reproductive functions [27]. Metabolic syndrome and the related array of bodily disorders have garnered focus owing to the established association among obesity, diabetes mellitus (DM), hyperleptinemia, and infertility. Metabolic indicator hormones, such as insulin-like growth factor-I (IGF-I), insulin, leptin, ghrelin, resistin, obestatin, and growth hormone (GH), have been reported to transmit signals of nutritional status to the hypothalamic centers. This may suggest a route by which they communicate and interfere with the HPG axis milieu in the regulation of male reproductive functions [27, 28].

39.3 Obesity and Semen Quality

Certain male factors are crucial in determining male fecundity, the most conventional ones being adequate sperm volume, distinct morphology, and robust sperm motility, among others. In men, seminal fluid characteristics are reliant upon their overall health as well as environmental cues. Semen parameters are susceptible to be jeopardized even at slightest deviation from homeostatic conditions. Conditions such as trauma, systemic illnesses, hectic lifestyle, poor nutritional status, environmental conditions, and obesity-related alterations can drastically affect semen parameters [15]. The counteractions among BMI, steroidogenesis, spermatogenesis, and male infertility have been elaborately studied but still lack complete understanding [13].

Obese men have three times more probability to possess a sperm count of less than 20 million/ml than do men with normal weight. This condition is referred to as oligozoospermia [29]. Chavarro et al. [30] had put forth that men with higher BMI (>25 kg/m^2) displayed poorer total sperm count than those with normal weight. The volume of ejaculate also declined with elevation in BMI. A broad-spectrum study including 1558 Danish military men also showed a negative correlation of increased BMI with total sperm count and concentration [31]. Obesity also impairs sperm motility and morphology, but the exact mechanism of this has not been established yet [25]. Nevertheless, numerous studies have corroborated these findings to strongly suggest the disrupting impact of obesity upon male fertility [32, 33].

Human semen quality has always been a reliable predictor of male fertility status, and it is showing a global declining trend [3, 34, 35]. An all-inclusive, evidence-based review showcased an overall 32.5% decrease in sperm concentration among European population in the past 50 years [3]. Obesity and overweight along with the related allostatic load have widely been reported to be closely associated with an ele-

vated occurrence of oligozoospermia and azoospermia [36]. Proper management and disciplined weight loss showed impressive improvement in testosterone levels and semen parameters [37].

39.4 Altered Spermatogenesis in Obese Men

The seminiferous tubules sustain a dynamic yet steady balance between cellular regeneration and their death [38]. To mediate this purpose, just after the first wave of spermatogenesis, there is a phase of germ cell differentiation under tight regulations of a distinctive hormonal microenvironment. If the production of cells in this phase exceeds the physiological need, they undergo apoptosis via the Bcl-xL and Bax systems [39, 40]. Spermatogonial apoptosis may be stimulated under specific physiological or pathological conditions and is monitored by various genes. The AI spermatozoa have been shown to undergo a significantly increased rate of apoptosis in conditions of obesity. According to recent research interventions, immoderate induction of apoptosis in spermatogenic cells contributes to a majority of male subfertility or infertility [41]. Spermatogonial apoptosis is mediated and controlled by the conventional Bax and Bcl-2 homeostasis. Obesity may induce apoptosis by disrupting the ratio of Bcl-2/Bax in the testis, with increased Bax and reduced Bcl-2 expressions, thereby activating the downstream signaling caspases, especially triggering caspase 3 [42]. Moreover, obesity incurs hyperlipidemia and lipid metabolic disorders elevating the stress upon the endoplasmic reticulum, which further leads to spermatogenic cell apoptosis through high GRP78 mRNA and protein expressions [43, 44].

39.5 Obesity and Sperm DNA Integrity

The association of BMI with male infertility in terms of impaired sperm quality has been highlighted in many studies [14, 30, 36, 45–48]. The effects of obesity on the functional aspects of sperm, especially in consideration of its DNA integrity, should be more extensively studied. Sperm DNA integrity represents the major nuclear component of spermatozoa that is vital for normal fertilization, implantation process, pregnancy sustenance, as well as fetal development [49]. Thus, besides conventional semen parameters, determination of sperm DNA fragmentation (SDF(can serve as an advanced sperm function test to assess the male fertility status. The American Center of Reproductive Medicine (ACRM) through an array of studies has put forth the relevant concepts regarding SDF and several potential laboratory methods to determine the clinical value for proper assessment of SDF in male infertility [17, 50–52]. The vitality of the SDF assay for assessment of male infertility had also been recognized by the American Urological Association (AUA) and European Association of Urology (EAU) guidelines [53].

Sperm DNA integrity is a major factor that is adversely affected in obese men. The possible mechanism by which obesity impairs sperm DNA integrity or causes SDF is by inducing oxidative stress. Although there aren't a significant number of studies that have assessed the influence of obesity on sperm DNA integrity, a few studies have shown a disparity in their findings that may be due to technical issues [30, 54]. However, it is essential to assess the potential impact of obesity on sperm DNA integrity, as much reduced pregnancy rates have been portrayed in correspondence to increased SDF [52, 55]. Kort et al. [46] showed an increase in SDF rates in obese men, as assessed through Sperm Chromatin Structure Assay (SCSA). This concept was also supported by the findings of Chavarro et al. [30] and Farriello et al. [56] who determined sperm DNA integrity by the single-cell gel electrophoresis assay method (comet assay). LaVignera et al. [57] also observed using the TUNEL assay with flow cytometry that obesity negatively affects sperm DNA integrity. Yet another extensive, 3-year multicenter study explored further the association of increased BMI with sperm DNA integrity and showed that obesity is indeed responsible for increased SDF [58]. In contrary, very few studies failed to find any significant relationship between BMI and sperm DNA integrity [45, 54].

39.6 Obesity and Hormones

39.6.1 Hypothalamic–Pituitary–Gonadal Axis (HPG) and Sex Hormones

The mechanisms justifying the association of obesity with male infertility are mostly ambiguous. The most acceptable mechanism may be the dysregulation of the HPG axis by obesity-related allostatic load. HPG axis is the prime endocrine regulator of male reproductive functions along with the pituitary gonadotropins, LH, and FSH, being regulated by pulsatile GnRH from hypothalamus. The LH and FSH regulate steroidogenesis via Leydig cells and spermatogenesis by acting upon the Sertoli cells, respectively. Obese couples have an increased number and size of adipocytes, which emanate abnormal levels of various hormones and regulatory molecules. These adipose tissue-derived substances interfere with the delicate orchestration of the HPG axis, and this may explain in part the mechanism by which obesity affects male fertility (Fig. 39.1). All the obesity-related parameters such as BMI, total body fat, subcutaneous fat, and intra-abdominal fat are associated with reduced levels of testosterone and higher estrogen levels [59]. This phenomenon can be justified by

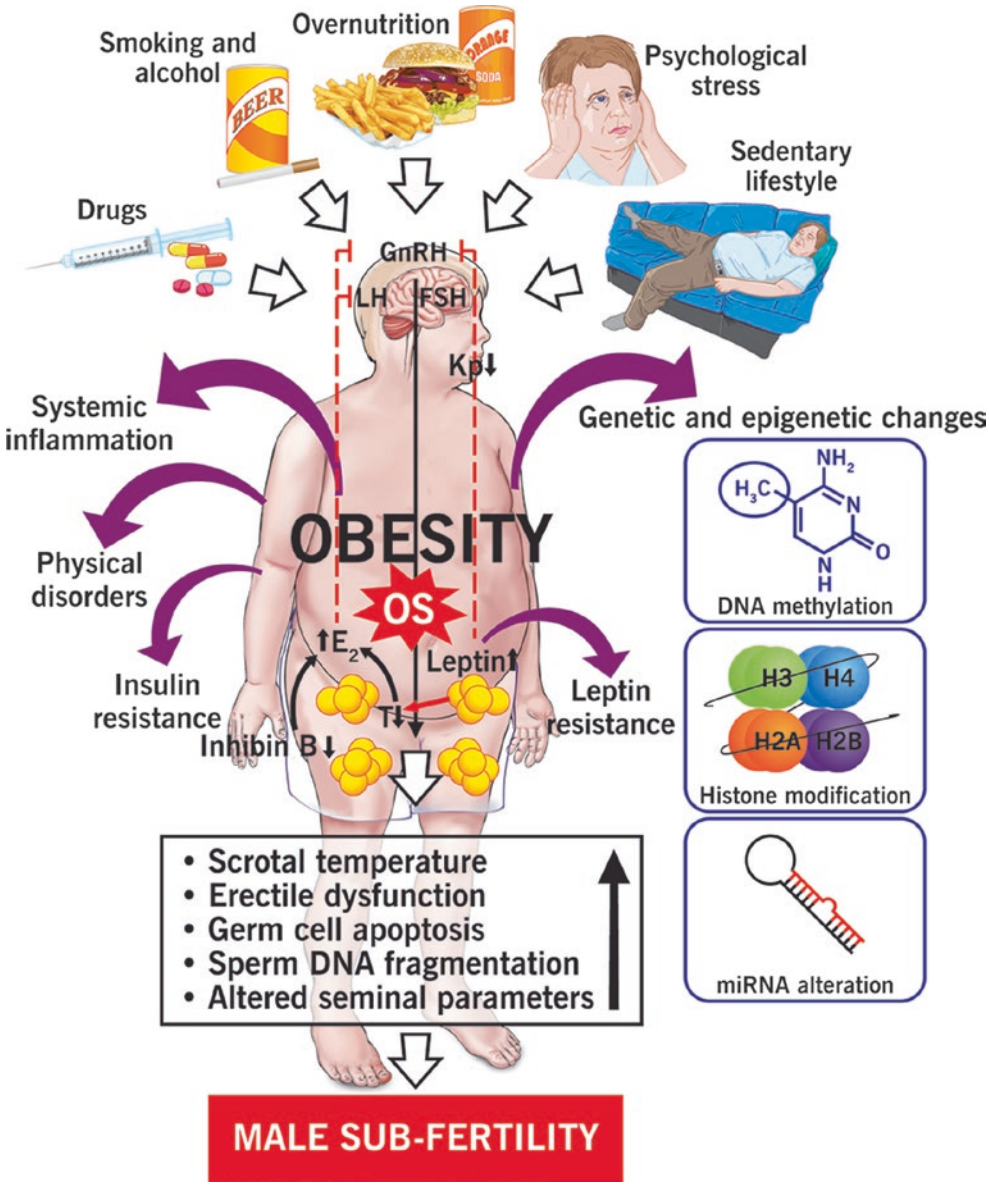

Fig. 39.1 Obesity-induced endocrine disruptions and its association with male infertility. *GnRH* gonadotropin-releasing hormone, *LH* luteinizing hormone, *FSH* follicle-stimulating hormone, *T* testosterone, E_2 estradiol, *Kp* kisspeptin, *OS* oxidative stress. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2019. All Rights Reserved)

overactivity of the aromatase cytochrome P450 enzyme, which is produced in excess in the white adipose tissue of obese men in addition to that produced by the Leydig cells. Thus the high estrogen level seen in obese men is due to elevated conversion of androgens to estrogens [60]. This impairment in sex hormone levels induces adverse alterations in spermatogenesis and other male reproductive functions. Estrogen is more biologically active as compared to testosterone. As such, even a very minute increase in its level may elicit substantial downstream impacts that could disrupt testicular functions [29]. On the other hand, complete reduction of estrogen level in the testes also affects normal steroidogenesis and spermatogenesis [61]. The presence of estrogen receptors in the male hypothalamus suggest that higher estrogen levels in obese men lead to low testosterone levels also via a negative feedback mechanism inhibiting the pulsatile GnRH release and subsequent release of LH and FSH [62]. This mechanism ultimately leads to insufficient gonadotropins for androgen production and spermatogenesis.

Inhibin B is a growth-like factor that is secreted by the Sertoli cells and mainly functions to inhibit FSH production. It also stimulates testosterone synthesis by the Leydig cells. Suppressed inhibin B production in obese men may be due to high estrogen level or any other mechanism indicating a direct disruptive effect of obesity on Sertoli cells [63].

Thus, infertile obese men exhibit hormonal alterations that differ from those demonstrated in men with either obesity or infertility alone.

39.6.2 Adipose Tissue and Metabolic Hormones

The working hypothesis used to explain the association of obesity with male reproductive dysfunctions is that white adipose depositions in obese men are responsible for elevated estrogen levels and the surge of adipose tissue hormones, which directly or indirectly affect steroidogenesis and spermatogenesis. The elevated estrogen level is due to increased aromatase enzyme activities that convert testosterone to estrogen. This is the result of excess aromatase cytochrome P450 enzyme production by the white adipose tissues in obese men in addition to that produced by the Leydig cells.

Obesity presents with complex disorders that greatly impairs hormonal regulation [64]. Obese men have a large deposition of adipose tissues, which, besides being a site of toxin depot, also emanate different hormones and inflammatory markers called adipokines. Obesity leads to alterations in adipose tissue hormonal levels in serum, such as that of ghrelin [65], leptin [66], orexin [67], adiponectin [68], obestatin [69], and other metabolic hormones [64]. Reportedly, leptin correlates positively with body fat mass [70, 71].

Leptin, a regulatory adipose tissue hormone, balances food intake and energy utilization through effects upon hypothalamic control. Leptin reportedly possesses both metabolic and neuroendocrine functions. Besides its well-known role in glucose metabolism, it can also modulate male sexual maturation and reproductive functions. Research has conveyed that the ob/ob mouse, devoid of a functional leptin gene, demonstrated reduced gonadotropin secretion which leads to infertility, while exogenous leptin treatment successfully restored fertility [72]. Moreover, chronic administration of anti-leptin antibody to rats proved detrimental to LH secretion and reproductive functions. Leptin also plays regulatory roles to mediate normal spermatogenesis as leptin-deficient mice showed disrupted spermatogenesis and elevated expressions of testicular pro-apoptotic genes, thus inducing germ cell apoptosis [73]. There are a few reports that contradict the ameliorating effects of leptin on male fertility, which shows that it also has inhibitory effects on testicular functions at levels exceeding the physiological limit [74]. Leptin induces reactive oxygen species (ROS) generation in human endothelial cells by increased mitochondrial fatty acid oxidation [70, 71]. Leptin may also stimulate the HPG axis by increasing the release of GnRH, FSH, and LH [75] (Fig. 39.1). It can impose its direct effect upon the gonads as its receptor isoforms are present in abundance in the gonadal tissue [75]. Serum adiponectin levels show an inverse relationship with both testosterone [76] and ROS levels [77].

Leptin may also regulate hypothalamic GnRH release through its influence on kisspeptin. The role of kisspeptin in regulation of reproduction is widely accepted. Located in the arcuate nucleus of hypothalamus, these peptides establish a metabolic and reproductive cross talk [78]. Kisspeptin have been reported to suppress lipogenesis and increase lipolysis [79]. In metabolic syndrome like obesity, there is reduced expression of kisspeptin mRNA, *KISS1* in hypothalamus as well as in the adipose tissues [78]. Since kisspeptin stimulates the pulsatile hypothalamic GnRH release, its deficiency in obesity may result in hypothalamic hypogonadism (Fig. 39.1) [78, 79].

Orexin (hypocretin) is another emerging adipose tissue hormone that reportedly stimulates testosterone production via inducing steroidogenic enzymes activities in Leydig cells [80]. Orexin also seems to attenuate oxidative cell damage [81].

The secretion of resistin, another adipose tissue factor, increases due to high adipocyte numbers in obese men. Resistin reportedly induces insulin resistance in obese men rendering them susceptible to type 2 diabetes [82, 83]. As per the regulations set by the Endocrine Society Clinical Practice Guidelines (2010), men with type 2 diabetes should be screened for low levels of testosterone [84]. This is justified as obese men with type 2 diabetes may possess secondary hypogonadism due to central or peripheral insulin resistance. This effect is aggravated by the deleterious actions of the associated pro-inflammatory cytokines (interleukin 6 (IL-6) and tumor necrosis factor alpha (TNFα)) upon the HPG axis [60]. Increased insulin levels in obese men lead to reduced SHBG levels, which may explain the decreased action of testosterone required to mediate normal spermatogenesis. However, compensation of low SHBG levels does not improve the low testosterone levels in conditions of insulin resistance in obesity, reflecting an autonomous direct impact of insulin resistance on the production of testosterone by the Leydig cells [13, 60].

Ghrelin, the "hunger hormone," is a neuropeptide produced by ghrelinergic cells in the gastrointestinal tract, whose association with altered serum testosterone levels has been suggested but is still contentious [85–87]. Ghrelin receptors are found in the testis, and it has a role in steroidogenesis. However, it has not yet been reported to have direct effects upon spermatogenesis [85]. Oxidative stress and ROS levels seem to positively correlate with levels of ghrelin, which trigger further obesity-related complications and in turn generate more ROS [88].

The complex network of such adipose tissue hormones, including adipokines, metabolic hormones, and classical hormones, comprising the HPG axis, mediate the pristine functioning of the male reproductive system. If any of these cross talks get jeopardized as in the case of obese men, reproductive functions are disrupted leading to male subfertility or infertility.

39.7 Obesity-Induced Genetic and Epigenetic Modifications

Obesity and male infertility can be linked through their root causes arising from genetic and epigenetic alterations. Few such common genetic mutations that find association of obesity with male infertility are conditions like Prader–Willi, Laurence–Moon–Bardet–Biedl, and Klinefelter syndromes [19, 89, 90]. Prader–Willi syndrome, characterized by abnormalities in chromosome 15, presents symptoms of both infertility and obesity. The human *ALMS1* gene mutation, causing Alström syndrome, presents metabolic and endocrinological modulations inducing childhood-onset obesity and infertility among other complications [91]. Moreover, an aromatase polymorphism has been reported to influence weight-mediated estradiol levels in obese men [92, 93]. This can be a potential reason why certain obese men have high estradiol levels followed by subfertility or infertility, while others face no such issues. Further interventions are required to find yet a wider genetic link between obesity and male infertility.

As discussed earlier, a wide range of environmental influences, including dietary and lifestyle factors, can cause obesity. These causatives can modify epigenetic arrangements which may elevate the risk of chronic systemic diseases not only in the affected individual but also his future generations.

In this context, a pioneering study by Ng et al. reported that high-fat diet in male rats results in β-cell dysfunction [94]. Early onset of disrupted insulin secretion as well as glucose tolerance was noted in the female offspring from these male rats. In another report by the same group, it was revealed that the transcriptome of retroperitoneal white adipose tissue of rat offspring was also concurrently affected [95]. Nevertheless, male germ cells were not analyzed for the same. Nonetheless, the developing germ cells in the male offspring may also carry several epigenetic changes, such as methylation, which are the essential cause of the transgenerational effects. The report from Fullston et al. revealed that diet-induced paternal obesity may affect molecular profiles of offspring spermatozoa. They have reported that mice fed with high-fat diet showed altered spermatozoa microRNA content and a 25% decline in sperm DNA methylation [96]. Palmer et al. reported that mice fed with high-fat diet presented a decrease in the level of sirtuin-6 (SIRT6), a histone deacetylase, in spermatozoa with increased DNA fragmentation [97].

Studies on the effects of obesity on human sperm epigenetics are scarce. To the best of our knowledge to date, no report has come out in human subjects revealing the function of RNA fragments in the transgeneration transmission of dietary intake. In 2014, Consales et al. examined the effect of lifestyle factors on human sperm DNA methylation in repetitive DNA sequences (LINE-1, Satα, and Alu). However, no significant correlation was found between BMI and sperm DNA methylation. Smoking, one of the causatives for obesity, showed a significant positive association with the LINE-1 methylation level [98]. There are very few studies that reported DNA methylation of individual gene or in genome of obese men. It has been reported that the DNA methylation percentages in obese men are significantly different from that of normal men [99]. Donkin et al. reported an interesting observation that loss of weight following bariatric surgery in morbidly obese men caused significant alterations in their sperm epigenetics [100].

Epigenetic modifications persevere for generations, with altered methylation patterns and molecular programming seen in the offsprings [101–103]. Children born to obese parents have been found to have altered sperm DNA methylation profiles compared to the children from non-obese parents [104]. Successively, another study has reported altered sperm DNA methylation at several differentially methylated regions, signifying that the male obesity status is perceptible from the spermatozoa epigenome [99].

39.8 Obesity-Related Disorders and Male Infertility

39.8.1 Increased Scrotal Temperature

Obesity may potentially affect sperm production/parameters by increasing gonadal heat as a result of high scrotal adiposity. Spermatogenesis is an extremely heat-sensitive process, with the human testes having an optimal temperature of 34–35 °C [60]. Testicular temperature may alter also due to several other conditions, such as varicoceles, sedentary lifestyle, using a laptop computer, sauna, warm baths, etc. [105]. In obese men, raised scrotal temperatures due to high scrotal adiposity and increased suprapubic and thigh fat also lead to sperm oxidative stress besides directly affecting spermatogenesis [13, 105]. This could damage sperm cells, reduce sperm motility, and increase SDF leading to subfertility or infertility [13].

39.8.2 Erectile Dysfunction

Infertility in obese men can also be related with decreased coital frequency. It is evident through various survey-based studies that obese men have almost one and a half times more chance of acquiring erectile dysfunction [106]. Erectile dysfunction positively correlates with male infertility [13, 107]. The mechanism by which obesity may be related to erectile dysfunction can be explained by a marked reduction in testosterone levels and the surge of potential pro-

inflammatory cytokines in obese men [108]. Such pro-inflammatory mediators are responsible for severe endothelial dysfunction that can directly lead to male erectile dysfunction acting via the nitric oxide pathway [109]. Obesity associates with several systemic pathogenesis, such as hypertension, diabetes, and dyslipidemia, which possess independent mechanisms in causing erectile dysfunction [110]. It would be beneficial if there are more research interventions to establish a consensus whether decreased coital frequency in obese men is the consequence of erectile dysfunction or if they relate to endocrine or psychological disorders [111].

39.8.3 Oxidative Stress

Reactive oxygen species are immensely reactive and unstable molecules that, when produced in excess exceeding the antioxidant defense of the tissues, lead to oxidative stress (OS). OS can induce severe cellular impairments throughout the body [112, 113]. Numerous reports have associated obesity and its related complications with increased OS [13, 33, 58, 64, 66]. Obesity marks an increase in serum free fatty acids as well as unsaturated fatty acids. These fatty acids are vulnerable to oxidative attack by ROS and subsequently undergo peroxidation, reduction in antioxidant enzyme levels, and malondialdehyde (MDA) accumulation, reflected by a state of OS in obese men [114]. As discussed above, adipocytes emanate a surge of adipokines such as IL-6, TNFα, plasminogen activator inhibitor-1 (PAI-1), and tissue factors [60, 68]. Increased levels of these adipokines owing to high white adipose tissue deposition in obese men lead to inflammatory conditions that impose toxic effects on sperm. These effects are mediated via induction of excess ROS and reactive nitrogen species (RNS) generation in testis [115]. The increased metabolic rates to sustain normal biological processes in obese men as well as high levels of stress in the immediate testicular environment trigger yet more ROS production. The local influences of pro-inflammatory mediator molecules contributed by activated leukocytes in response to inflammatory signals in obesity also aggravate the damage to the sperm and inhibit spermatogenesis. ROS is an independent causative and an emerging marker of male infertility that evidently incurs an array of disruptions in sperm production, morphology, and functions and may also curb hormonal regulations of male reproductive functions [17, 51, 107, 112, 113].

39.8.4 Sleep Apnea

Sleep apnea (SA) refers to a particular sleep disorder in which breathing pauses frequently or there is shallow or infrequent breathing during sleep. This pause in breathing leads to hypoxemia, which is very common in obese people. There is scarcity of evidence to come to a rigid conclusion that sleep apnea in obese men causes infertility, but the association cannot be ruled out. Sleep apnea affects the HPG axis and may also decrease gonadal function [13, 107]. Moreover, it is reported that sleep apnea in obese men decreases morning testosterone concentrations [116]. This can be explained by the hypothesis that fragmented sleep resulting from sleep apnea may be responsible for disruption of the nocturnal testosterone rhythm. Moreover, it is being put forth that reduction in total testosterone levels in obese men is proportional to the severity of sleep apnea, thus also disrupting spermatogenesis and inciting other sexual dysfunctions. The combination of these deleterious effects of sleep apnea associated with obesity may have a compounding effect on male fertility [60].

39.9 Consequences of Male Obesity on ART Outcomes

An increasing body of evidence claims that the non-genetic impact of a prolonged health issue of paternal origin can be transmitted to the offspring via the male gamete [96, 104]. Among the factors that can influence sperm health, obesity is of immense importance. In light of the ever-growing prevalence of obesity, it is essential to intensify the understanding of the clinical consequences of the male partner being obese. Influence of male factors upon ARTs or sperm viability needs extensive research.

39.9.1 Obesity and Pregnancy Onset from ART

There are only few studies focusing upon the impact of male obesity on achieving clinical pregnancy for the couple undergoing ART (in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI)). The assessment of clinical pregnancy following ART was done by either of the following methods: fetal heartbeat detection per embryo transfer (ET) [21], intrauterine gestational sac on transvaginal sonogram per cycle [117], ultrasound confirmation (giving no further details) per embryo transfer cycle [118], and heartbeat detected per ICSI cycle [119, 120]. Reports suggest that there is a decline in clinical pregnancy rates in couples undergoing ART with the male partner being obese.

39.9.2 Obesity and Pregnancy Outcome After ART

Significant decrease in live birth rate from ART has been reported for obese men compared to men with normal weight. Live birth rate following pregnancy through ART has

been reported by various studies through embryo transfer cycle [118], oocyte retrieval [21], treatment cycle [121], cycles of ICSI [119], and IVF cycle [120].

39.9.3 Paternal Obesity and Infant Development Following ART

Paternal obesity has also been found to affect offspring development in terms of alterations in the infant BMI growth curves from birth till the age of 3.5 years as compared with normal weight fathers [122].

With obese men being more susceptible to acquire subfertlity or infertility, the ART outcomes of couples with an obese male partner display poorer impregnation rate and live birth rate as well as compromised health of the infant. This may be explained by disruption of normal sperm morphology, increased SDF, and low mitochondrial membrane potential (MMP) in obese men.

39.10 Management of Obesity-Induced Male Infertility

39.10.1 Lifestyle Modifications

Lifestyle modifications to trigger weight loss include alterations in dietary habits such as reduction in meal portions and restricting certain high calorie food, along with adequate exercise, so as to restore a normal energy balance. It is evident that natural weight loss via strict diet and/or proper exercise improves the levels of androgen, SHBG, and inhibin B while reducing the levels of insulin and leptin. This in turn ameliorates semen parameters in obese men [30, 89]. Moreover, weight loss leads to substantial reduction in the adipose tissue mass, thus decreasing the concentrations of inflammatory mediators such as TNFα, IL-6, and other cytokines that are associated with infertility [123]. A sensible diet plan and conscious effort to exercise lead to a gradual weight loss which should be maintained for a long time. This can be stimulated through self-determination, tenacity to exercise, cognitive behavior therapy, and association with supportive groups. These lifestyle adoptions form the primary healthcare in the treatment of obesity-induced infertility [18, 124].

39.10.2 Prescription Medicine

Obesity-induced male infertility can be clinically dealt via two ways, either by providing medications for weight loss or directly addressing the male reproductive dysfunction. There are a couple of anti-obesity medications that can be used for a long period as per approval by the Food and Drug Administration (FDA). Orlistat (Xenical) is used to decrease intestinal fat absorption hindering pancreatic lipase activity. Another drug is sibutramine (Meridia) that works by inhibiting neurotransmitter deactivation, namely, norepinephrine, dopamine, and serotonin. This leads to decreased appetite and hence a modest weight loss [18, 125]. Short-term medications include a broad arena of treatment options including noradrenergic receptor activation, gastrointestinal lipase inhibition, serotonin receptor activation, and combination therapies [125].

Obese men with secondary infertility may be treated via GnRH pump or injection of human chorionic gonadotropin (hCG) that mediates the action of LH on the Leydig cells to stimulate testosterone secretion. This is effective in restoring normal spermatogenesis in obese men to some extent [126]. Aromatase inhibitors (testolactone or anastrozole) are also a potent medication option that works by inhibiting the conversion of testosterone to estrogen. These are shown to improve testosterone levels and in turn male fecundity in obese subjects [126–129]. The new era treatment approach for obesity-induced male infertility attempts to directly mitigate two simultaneous concerns: one being testosterone insufficiency and the other being excess adipose tissue-derived factors. These are attempted via testosterone replacement therapy along with regulation strategies to inhibit adipose tissue hormones, especially leptin. Reduction in leptin level in obese men may be beneficial to improve reproductive functions because leptin indirectly influences GnRH, LH, and FSH and also impose direct testicular effects [130, 131]. Modulation of some prime metabolic hormones associated with obesity such as ghrelin may lead to further insights in the development of new medications in obesity-induced health disorders as well as in male infertility [13, 17, 18].

39.10.3 Surgical Interventions

Obese men with infertility may conveniently opt for IVF. Although there are reported cases of unfavorable outcomes in IVF/ICSI cycles among the morbidly obese [132], male partner obesity does not appear to affect the results of their female healthy partners in IVF or ET [13, 18].

Subject-specific surgical interventions for obese men with heavy fat accumulation around the scrotum can be scrotal lipectomy. In such obese men, the accumulated fat results in increased scrotal temperature or toxin buildup. This procedure evidently leads to restoration of the male fertility status as it is claimed that one-fifth of obese men undergoing scrotal lipectomy were able to successfully impregnate their partners [133]. Severely obese men (BMI > 40) should go through rigorous dietary and behavioral modifications, together with surgery to decrease or bypass parts of their stomach or small intestine called the bariatric surgery ("weight loss surgery") [133]. Studies report that these sur-

geries lead to significant decline in the estrogen/testosterone ratio and aid in the restoration of other hormones and adipokines. However, bariatric surgery should be avoided if the severity of obesity-linked male infertility is lesser, until it is confirmed to have no deleterious long-term effects.

39.11 Conclusion

Obesity, characterized by BMI value that is equal to or greater than 30 kg/m^2, is a metabolic syndrome that renders the body susceptible to various pathological conditions. The simultaneous global decline in male fertility, and increase in obesity prevalence, supports an association of obesity with male infertility.

Obese men have substantial deposition of adipose tissues, which, besides being a site of toxin depot, also are sources of hormones (ghrelin, leptin, orexin, adiponectin, obestatin, etc.) and *adipokines*. Leptin acts both at HPG regulatory axis and directly on testicular cells to modulate male reproductive functions. It may trigger excess ROS to induce OS in testicular tissue, while orexin has been reported to attenuate oxidative damage. Orexin also serves to stimulate testosterone production in Leydig cells. Resistin is another important adipose tissue hormone that induces insulin resistance in obese men. In addition, obese men possess higher estrogen than testosterone owing to increased aromatase activities or by negative feedback mechanism of estrogen to inhibit the pulsatile GnRH release and subsequent release of LH and FSH. Obesity leads to reduced inhibin B production, which may also attribute to high estrogen level.

Besides indirect effects of obesity on male reproduction via hormonal cross talks, the chapter also has highlighted the direct effects of obesity on gonadal functions. Obesity may disrupt spermatogenesis by immoderate induction of germ cell apoptosis. It impairs semen quality through various mechanisms such as elevated scrotal temperature, ROS production, and increased sperm DNA fragmentation. The chapter has put forth the possible link between obesity in male partner of couple undergoing ART and its undesirable outcomes. However, there are promising strategies to prevent as well as manage obesity and its related disorders. The concise concept of obesity and its association with male infertility presented in this chapter will aid better understanding of the subject and encourage researchers to explore new therapeutic interventions.

39.12 Review Criteria

An extensive literature search has been performed to find the relationship between obesity and male infertility using search engines such as Science Direct, OVID, Google Scholar, PubMed, and MEDLINE. The overall strategy for study identification and data extraction was based on the following keywords "obesity," "metabolic syndrome," "infertile men," "infertility," "semen parameters," and "assisted reproduction" and the names of specific obesity and male infertility markers. Articles published in languages other than English were also considered. Data that were solely published in conference or meeting proceedings, websites, or books were not included. Websites and book chapter citations provide conceptual content only.

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Smoking Effects on Male Fertility

40

Jenna Meyer and Avi Harlev

Key Points

- There is no consensus across the literature regarding just how smoking contributes to male infertility.
- Smoking has detrimental impacts across nearly all organ systems, impairing hormonal signaling in the hypothalamic pituitary axis, disrupting spermatogenesis, and directly damaging sperm morphology and genetic contents.
- Some studies have shown various sperm parameters to be influenced by cigarette by-product exposure such as impaired sperm morphology, decreased sperm motility, and decreased sperm volume.
- More research is needed to determine just exactly how smoke impairs normal male reproductive processes at the molecular level.
- Treatment of male infertility in smokers depends on reversing or managing the damage caused by the toxins and chemical assailants of cigarette smoke.

40.1 Introduction

The association between smoking and male fertility has not been fully established. Conflicting studies reporting the effects of smoking and nicotine on male reproductive physiology make it difficult to conclude how exactly male fertility is affected by this habit (Table 40.1). Lifestyle modifications have become the focus of many topics in modern medicine as achievable solutions to a wide variety of health concerns and disorders. The goal of this chapter is to provide a thorough review of the literature describing how smoking may impact male fertility.

40.2 Overview of Smoking

Tobacco use, specifically cigarette smoking, is the most common culprit behind preventable causes of mortality across the world. Diseases beyond obvious lung involvement have become increasingly connected to a current or even past medical history of smoking, in addition to worsening prognoses. There are over 7000 ingredients in cigarettes including carbon monoxide, toluene, cadmium, methane, and polycyclic aromatic hydrocarbons (PAHs), each of which has adverse effects on normal physiologic function at the microscopic and macroscopic levels [13, 22]. There are two parts to smoke discharge, the first of which releases carbon monoxide gas; the second particulate phase contains nicotine and tar [6]. The addictive nature of cigarettes is due to the nicotine component. This is then metabolized into cotinine and then continued to be processed into trans-2′-hydroxycotinine [6]. The initial ingredients in addition to the metabolized end products have devastating effects on male reproductive physiology [15].

Though the prevalence of male daily smokers has decreased from 28.4% to 25.0%, it still proves to be an important contributor to adverse health effects across all body systems [28]. Consistently, smoking has been linked to cancers of the oropharynx, larynx, esophagus, trachea, bronchus, lung, stomach, liver, pancreas, cervix, and bladder. Smoking has also been shown to cause stroke, blindness, coronary heart disease, pneumonia, COPD, diabetes, ectopic pregnancy, rheumatoid arthritis, and immune disorders.

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Table 40.1 Overview of published literature findings on the effects of smoking on sperm analysis parameters

Author	Smoking effects on semen parameters			
	Concentration	Motility	Morphology	Other findings
Stillman et al. [1]	↓	↓	↓	
Dikshit et al. [2]	–	–	–	
Klaiber et al. [3]	↓			
Osser et al. [4]	–	–	–	
Dunphy et al. [5]	–	–	–	
Pacifici et al. [6]				
Sofikitis et al. [7]			↓	
Vine et al. [8]	↓	↓	↓	
Chia et al. [9]	↓	↓	↓	
Horak et al. [10]	↓	↓		Increased bulky DNA adducts in smoker sperm
Künzle et al. [11]	↓	↓	↓	
Pasqualotto et al. [12]	–	–	–	
Colagar et al. [13]	↓			
Tremellen et al. [14]		↓	↓	Increased DNA damage, membrane damage
Calogero et al. [15]		↓	↓	Increased DNA damage
Oyeyipo et al. [16]	↓		↓	
Taha et al. [17]	↓	↓	↓	
Sharma et al. [18]	↓	↓	↓	
Zhang et al. [19]	↓	↓	↓	
Kumar et al. [20]	↓	↓	↓	
Dai et al. [21]		↑		
Harlev et al. [22]	↓	↓	↓	
Cui et al. [23]	↓	↓	↓/–	Significance of sperm damage differs based on smoking amount
Esakky et al. [24]	↓			Increased DNA damage
Jenkins et al. [25]	–	–	–	
Sharma et al. [26]	–	↓		Decreased sperm count
Al Khaled et al. [27]	–	–	–	No significant difference in sperm DNA

↓ decreased from normal parameter; – no effect; ↑ increased from normal parameter

Smoking as a burden to public health has been extensively analyzed across a wide array of domains. Consumption has dramatically contributed to global burden of health in over 195 countries [29]. While the dispersal of this is weighed heavily toward low socioeconomic areas and in populations with lower education levels, smoking is a universal public health risk and does not discriminate in its ability to devastate the human body [28]. Economically, the costs associated with smoking are staggering, amounting to 8.7% of annual healthcare costs [30]. In the United States, smoking-related illnesses have cost over 300 million dollars in healthcare spending [30]. This includes 176 billion dollars in medical expenditures due to associated morbidities, as well as over 156 million dollars lost in expected productivity due to exposure to secondhand smoke [31]. Since the iconic Surgeon General's report on the economic effects of smoking, various energies have been made to stall tobacco sales, and 8 million deaths and 175 million years of life have been saved due to this emphasis [31]. Yet, the tobacco industry continues to flourish, and still smoking remains the principal source of preventable mortality across both the developing and developed worlds.

The impact of smoking on the reproductive system is not fully established. In males, smoking has been shown to contribute to impaired sperm motility and quantity, in addition to reduced ability to produce and maintain an erection (Fig. 40.1). In women, cigarette usage is complicit in increased rates of ectopic pregnancy and developmental disabilities in the growing fetus.

40.3 Overview of Male Reproductive Physiology

40.3.1 Hormonal Axis

The male hormonal axis is but another feedback loop of the hypothalamic pituitary axis. The pathway begins by the pulsatile secretion of gonadotropin-releasing hormone (GnRH) by the hypothalamus which stimulates the anterior pituitary to release luteinizing hormone (LH) and follicular stimulating hormones (FSH) that are secreted into systemic circulation [32]. Upon reaching the testes, LH induces Leydig cells to produce 5–7 grams of testosterone daily. The active metabolite 5-alpha-dihydrotestosterone (DHT) is crucial for regular male genital tract development. FSH works upon Sertoli cells, stimulating their production of proteins that support spermatogenesis as well as inhibin B [32].

The production and secretions of the hormones in this axis are highly dependent upon and regulated by negative feedback

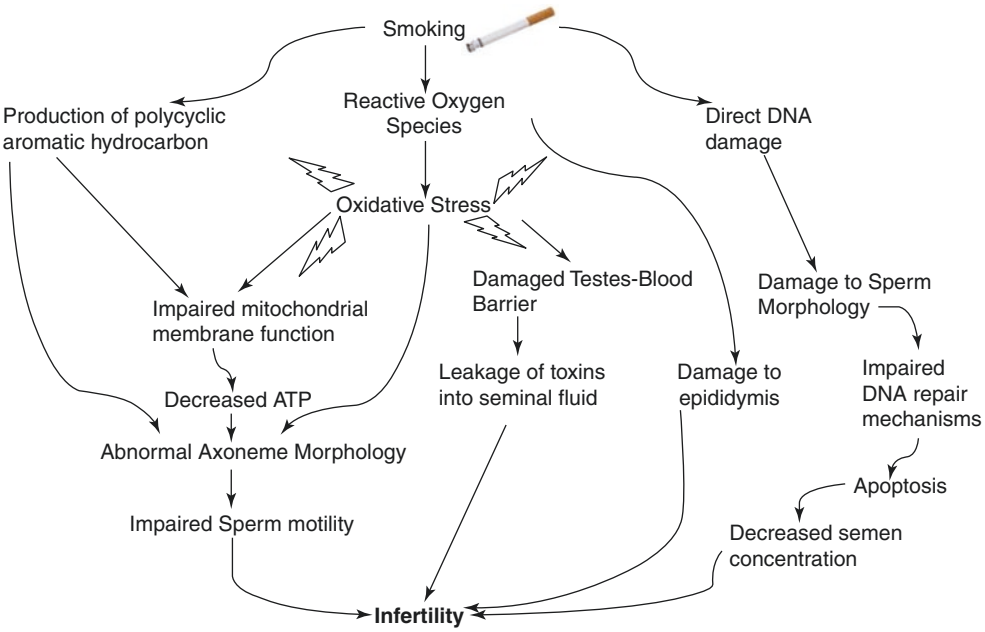

Fig. 40.1 Suggested mechanisms for how smoking impairs sperm physiology and function

principles. Increased circulating levels of testosterone and DHT inhibit the production of GnRH and LH levels by the hypothalamus and anterior pituitary, respectively. This then downregulates the testis production of hormones and proteins. FSH secretion is blocked by inhibin B from Sertoli cells. This pathway is extremely sensitive to stress, both endogenous and environmental. Smoking directly impacts the normal functioning of this exquisitely designed hormonal relay.

40.3.2 Normal Reproductive Pathway

The normal reproductive pathway of males cannot be addressed without introduction of relevant anatomical structures. The testes, as aforementioned, are a principal reproductive organ responsible for male fertility. They are divided into two anatomical compartments separated by the blood-testis barrier. The interstitial compartment contains the Leydig cells that are responsible for producing testosterone as discussed. The seminiferous tubule compartment is significantly larger, composing the majority of the testis organ. Sertoli cells reside here that secrete proteins and nutritional components required for regulating spermatogenesis. Undifferentiated spermatogonia are also occupants of the seminiferous tubule system. The damage caused by smoking to these components of male reproductive anatomy and their associated physiological processes has deleterious effects on male fecundity.

40.3.3 Spermatogenesis

The process of spermatogenesis begins with puberty, as GnRH pulsations reach the levels necessary to stimulate LH and FSH secretion, and is divided into three stages [33]. The first is the mitotic or proliferative stage where undifferentiated immature stem cells go through mitosis. Part of this group is destined to fill the stem cell reserve; the others will continue to complete spermatogenesis and become mature spermatocytes [34]. The second phase is where these spermatogonia push through blood-testis barrier and undergo meiosis to become two distinct primary spermatocytes. These then complete two further rounds of meiosis, resulting in two secondary spermatocytes and then finally four spermatids. The third and final phase of spermatogenesis is when these four spermatids differentiate into mature spermatozoa [33, 34]. This entire process yields around 100 million mature sperm products per day [35]. This process has been evolutionarily tailored to progress very specifically and can be interrupted by the toxins introduced into the body by smoking, thereby contributing to male infertility (Fig. 40.1).

40.3.4 Erectile Physiology

An erection is achieved by arteriolar dilation of vessels in the corpus cavernosum. When a man is sexually aroused, neurotransmitters are released from nerve terminals that then causes blood flow into the penis to increase. This results in compression of subtunical venous outflow, trapping blood within the penile vessels, thus causing in an erection.

The innervation of the penis is complex and is dependent upon sympathetic, parasympathetic, sensory, and motor synapsing. This combination of excitatory and inhibitory neuronal signaling pathways is crucial for obtaining and maintaining an erection. Sympathetic signaling originates through the sacral and caudal sympathetic chain ganglia before projecting to the penis and is responsible for ejaculation [36]. Parasympathetic innervation to the penis stems

from the pelvic plexus; its projections are responsible for erection [36].

Somatic and motor innervations begin at sensory receptors located in the skin of the penis, glans penis, and urethra within the corpus cavernosum. These small free nerve endings join to form the dorsal nerve of the penis, which projects medially to form the pudendal nerve with other nerve fibers. Motor innervation is crucial for ejaculation. Fibers projecting to the bulbocavernosus muscle are responsible for the rhythmic contraction necessary for expelling sperm products through the urethra [36].

40.4 Male Infertility

Each component contributing to normal, healthy male reproductive anatomy and physiology can be damaged by the toxins in smoke products. When a couple experiences difficulty in conception, both partners must be evaluated. Approximately 30% of infertility can be credited exclusively to male issues [37].

Evaluation of male infertility begins during the history taking and physical exam. Timing of intercourse, use of lubricants, or childhood illnesses such as mumps orchitis, undescended testes, or testicular torsion have all been shown to contribute to infertility in adult life [37]. Exposures to environmental toxins have become prominent in modern research into this subject.

40.4.1 Factors Affecting Male Fertility

In humans, male infertility accounts for 40–50% of all cases [38] and affects approximately 7% of all males in reproductive age. In 2010, the WHO reassessed their sperm study standards and made changes to the requirements for assessment of volume, motility, and morphology [26]. Since these changes, volume is measured by weight; motility is divided into two categories, progressive and nonprogressive; and morphology is evaluated using the Tygerberg criteria [26]. The consequences of these changes include disqualifying males once deemed infertile, thus calling for a re-examination of sperm parameters among males struggling with fertility issues.

40.4.2 Testicular Factors

Varicocele is the circumstance of swollen testicular veins prevalent in 40% of men, 15% of which have been deemed infertile [39]. Left-sided varicoceles are ten times more likely to occur because the left spermatic vein empties at a right angle into the left renal vein, whereas the right spermatic vein empties directly into the inferior vena cava. Men with varicocele are more likely to have abnormal semen quality and quantity as compared to men without. While it is well documented that varicocele is more prevalent in infertile men, the pathophysiology behind this etiology is not well understood. However, as blood pools in the pampiniform plexus, this causes relative hyperthermia of the groin, which is well known to be harmful to developing spermatozoa [40].

Comparison of smokers and nonsmokers with varicocele showed that there was a ten times greater risk of oligozoospermia in the smoking men, suggesting potentially a compounded effect of smoking and varicocele on male fecundity [3]. A study conducted by Agarwal et al. showed that men with varicocele had reduced sperm count and motility [41]. Furthermore, it seems that there is an inverse association between varicocele grade and measured sperm motility and concentration [42].

40.4.3 Genetic Contribution

Genetic abnormalities affecting hormones and receptors of the hypothalamic pituitary gonadal axis can cause infertility [37]. Kallmann syndrome is an X-linked disorder that causes idiopathic hypogonadotropic hypogonadism (IHH). A mutation in the Ka1 gene results in reduced GnRH secretion by the hypothalamus. This lack of GnRH leads to reduced FSH and LH production and secretion by the pituitary, thereby leading to decreased stimulation of the testes. Low testosterone and spermatoprotective growth factors are produced as a result [43]. Congenital adrenal hyperplasia has also been shown to cause IHH due to a mutation in the Dax1 gene associated with maintenance of testis epithelium and spermatogenesis [43]. The Prader-Willi syndrome is a congenital disorder of maternal imprinting of chromosome 15, or deletion of the short arm of paternal chromosome 15, that causes obesity, cryptorchidism, and IHH [37]. Klinefelter's syndrome is the most common genetic cause of male infertility. This sex chromosome disorder has a range of karyotype abnormalities, the most common of which is 46XXY. Yet despite this heterogeneity in chromosomal count and components, all clinically present with some variation of compromised male fertility [43]. In adolescence and adulthood, men diagnosed with Klinefelter's syndrome present with small, firm testes and some amount of androgen deficiency [43]. Others will have one testis, which may be cryptorchid or descended, and one streak gonad [37]. If the testis completed its descent into the scrotal sac, normal concentrations of Leydig and Sertoli cells are usually present. However, lost are the germ cells within the seminiferous tubules. In one study, 70% of men above the age of 25 complained of decreased libido [43].

In 46,XX men, there is a deletion of the sex-determining region (SRY) on the short arm of the Y chromosome [37]. On the long arm of the Y chromosome is the azoospermia factor region (AZF), which is integral in normal spermatogenesis.

Any disruption in hormonal signaling facilitating sexual maturity, sex hormone release, or sex hormone biosynthesis is the etiology behind male infertility. Mutations affecting the hormone, or its receptor, impede regular signaling and secondary messenger activation, leading to adverse effects ranging from moderate hypogonadism to complete failure of virilization and impaired male sexual function [37].

Other mutations that impair correct maintenance and utility of male reproductive organs include mutations of the steroidogenic acute regulatory protein (StAR) [44]. This protein serves as the rate-limiting step in steroid hormone biosynthesis by regulating transport of cholesterol into the mitochondria [44]. Mutations in enzymes responsible for conversion of cholesterol into androgens can fully halt normal male sexual development and obviously thereby male fertility. For example, 5α-reductase is essential for the complete development of external male genitalia by converting testosterone to its active metabolite DHT [37]. This results in impaired sperm delivery upon entrance into the female reproductive tract and thereby compromised fertilization.

At the DNA level, mutations in androgen nuclear receptors result in androgen insensitivity syndromes that also clinically manifest as various levels of male infertility [37].

40.4.4 Immune

Antisperm antibodies (ASAs) have been implicated as causing infertility in approximately 10–30% of infertile couples [45]. Abnormally elevated amounts have been found in men suffering from testicular torsion, orchitis, and testicular cancer [45]. These antibodies target antigens on the sperm surface and can be IgG, IgA, and IgM in nature [45]. Studies have shown that these different kinds of ASAs bind to specific regions on the sperm, be it acrosomal binding, on the sperm body, or in the tail region. This hinders the sperm's ability to navigate through the female reproductive tract and successfully penetrate the zona pellucida of the ovum. ASAs have also been documented to cause a release of spermatocytic cytokines, in addition to increasing sperm phagocytosis [45]. Yet other studies have shown healthy, fertile men to have marked levels of ASA, so it is difficult to definitely associate these antibodies with infertility [45].

The testicular blood barrier also has an immunoregulatory role in protecting sperm from immune cell destruction. This barrier develops throughout puberty as the cell-cell junctions between neighboring Sertoli cells, thus preventing passage of large immunoglobulins and lymphocytes into the tubular lumen.

Further defensive measures are found in the seminal plasma. Components of this impede the lymphocyte-antigen interaction, thereby preventing antigenic activation of the immune system's NK cells and T-lymphocytes. Prostaglandin H2 synthase, also found in seminal plasma in the rete testis and epididymis, prohibits prostaglandin synthesis and thus impairs lymphocyte extravasation through the tubular epithelium.

Sertoli and Leydig cells also have a direct protective effect on paracrine secretion or spermatoprotective substances that facilitate healthy normal spermatogenesis. Anti-inflammatory cytokines including IL-10, IL-13, IL-14, and TGF-β are immunosuppressive and prevent the antagonistic response of the immune system against sperm.

40.5 Effects of Smoking on Male Fertility

As aforementioned, the extensive toxic components of cigarettes make it difficult to assess their individual effects on the body. Regardless, there is indubitable evidence that marks cigarettes' implicit negative effects on human health.

40.5.1 Adverse Effects on General Homeostasis

The 2014 Surgeon General's report on cigarette smoking was paradigm in changing the approach to cigarette consumption and anti-smoking measures. It concluded that if smoking rates continued as projected, 5.6 million young adults under the age of 18 would prematurely die secondary to cigarette effects [46]. Many included in this estimate were subject to environmental secondhand smoke, bringing to light a public health burden that extends beyond the direct cigarette consumer. This report continued to break down causes of death due to cigarettes. Popularly known is cigarette's link to cancer. Between 1965 and 2014, smoking-related cancers claimed 6,587,000 lives, trumped only by 7,787,000 deaths due to cardiovascular and metabolic disorders. Pulmonary diseases, diseases related to birth and pregnancy, lung cancer, and coronary heart disease are all documented morbidities due to cigarette smoking.

Chronic diseases have also been attributed to cigarette smoking. Diabetes mellitus, rheumatoid arthritis, general immune impairment, as well as peripheral vascular disease have all been studied as linked to smoking [46, 47].

40.5.2 Adverse Effects on Male Reproductive System as a Whole

Cigarette smoking introduces harmful free radicals into a homeostatic system [20]. Cigarette usage consistently bar-

rages this delicate balance, resulting in a cascade of stressors that dramatically affects normal reproductive physiology at the molecular and macroscopic levels [48, 49] (Table 40.1). Reduced semen quality, compromised auxiliary gland functions, tubular obstruction, and dysfunctional spermatogenesis all result from the increased oxidative stress introduced by cigarette smoking [50, 51]. The main product of all these male reproductive processes is sperm, which under these great oxidative stressors can be fatally damaged.

Such oxidative stress also leads to increased inflammatory reactions in the male genitourinary tract [51]. This damages the local tissues, further escalating the inflammatory response [51]. Reactive oxygen species are released spurring immune system defenses. Local release of provocative inflammatory mediators, such as proteases and proinflammatory cytokines, further incites the local immune reactions [49].

40.5.3 Mechanism of Smoking Effects on Male Fertility

Depending on the mode of smoke exposure, different effects on male fertility can be found. Firsthand smoke exposure through personal cigarette usage, inhalation, and exhalation of cigarette by-products has been shown to impact sperm motility, morphology, and overall sperm quantity [1]. Active cigarette smoking is called mainstream smoking and also yields sidestream smoke, the smoke that is produced by the burning end of the cigarette. A study conducted by Polyzos et al. compared the effects of sidestream and mainstream smoke sources on murine germ cell cultures. Mainstream smoke increased sperm DNA fragmentation, while sidestream smoke impaired normal sperm motility mechanisms [52].

Secondhand smoke has been a popular topic of discussion in advocating for smoking law reformation. This refers to the passive inhalation of cigarette by-products by nonsmokers that are released into the environment by smokers [53]. This mechanism of cigarette smoke consumption has also been shown to have adverse effects on reproductive health parameters [53]. However, this is more difficult to study due the variety in secondhand smoke exposure, as well as various other confounding variables. Regardless, secondhand smoke has higher levels of reactant oxygen species as compared to the smoke actively inhaled that has been documented to impair sperm motility [14].

Evermore common, especially in younger populations, are electronic nicotine delivery systems such as electronic cigarettes [54, 55]. Though the user is inhaling vapor and not smoke, this vapor includes many chemicals such as propylene glycol, glycerol, and concentrated flavorings and is available containing a variety of nicotine concentrations [54]. Due to their relatively recent debut into the commercial marketplace, their exact effects on human biology have not yet been established [54]. Specific research focusing on their effects on reproductive physiology is required.

40.5.4 Risks to Spermatogenesis and Sperm Function

Smoking has adverse effects on spermatogenic processes and mature sperm function. Introducing a toxic environment at any point along this pathway results in abnormal spermatozoa and mature sperm production [26]. Paternal subjection to cigarette chemicals like polyaromatic hydrocarbons increases the rate of sperm death throughout spermatogenesis [24]. Aryl hydrocarbon receptor, a cytoplasmic transcription factor, has an important regulatory factor across the entire spermatogenic timeline [24]. When activated by polyaromatic hydrocarbons, this receptor was shown to impede antioxidant protective processes. Furthermore, this transcription factor was implicated in increased spermatic apoptosis [24].

40.5.5 Sperm Morphology

Well documented is how sperm morphology was impaired in smokers. Active firsthand smoking directly affects sperm morphology, but surprisingly secondhand smoke exposure has also been shown to lead to impaired sperm morphology [7, 52]. Animal studies attempted to further elucidate to what extent sperm quality was affected by smoke exposure, regardless of route [52]. However, further research is needed to completely clarify these conclusions (Table 40.1). Ultrasound evaluation of the microtubule axoneme array showed 99% of smokers to have aberrant construction of this apparatus, while 24% of nonsmokers showed altered microtubule organization [22].

40.5.6 Motility

Sharma et al. found sperm motility to be dramatically impaired in moderate and severe smokers as compared to mild and nonsmokers [18]. Sperm gain motility through the microtubules of the axoneme that compose the cytoskeleton of its tail. They are connected by the protein dynein. Much like skeletal muscle contraction, mitochondrial ATP hydrolysis generates the energy necessary for sperm motility. Any malfunctioning of this, be in protein insufficiency or defective ATPase activity, can render sperm immotile [18].

Additionally, insufficient anti-oxidative measures lead to the peroxidation of sperm membrane components and faulty

membrane stability and reliability [56]. Increased amount of lipid peroxidation results in conceded ability of sperm to maintain mobility, as compared to sperm with lower measured levels of peroxidative by-products [56].

Spermatozoa obtained from healthy males and exposed to cigarette smoke extract were shown to have reduced motility, and increased the number of spermatozoa with lower mitochondrial membrane potential [15]. Remember, this is where the sperm obtains most of its energy utilized for motility. As such, these sperm with lower potentials across the mitochondrial membrane therefore had less energy to dedicate to motility [15]. Vine et al. described a negative correlation between the number of cigarettes smoked per day, pack-years, and sperm motility [8]. Here, it is well documented this parameter of sperm quality is clearly and diversely impacted by the contents of cigarette smoke.

Another study conducted by Taha et al. explored the effects of smoking on sperm parameters in addition to the zinc concentration in seminal fluid. Zinc has proven antioxidant and antibacterial roles making it integral to the development of mature sperm [17]. When looking at both fertile and infertile males, this research showed there to be decreased sperm motility, decreased sperm concentration, decreased sperm viability, and decreased semen concentration. Seminal zinc concentration was also decreased in both fertile and infertile smokers, thus suggesting these sperm samples to be more susceptible to bacterial infection, as well as ROS damage.

Yet not all studies corroborate these findings. Work performed by Dai et al. used gel electrophoresis to isolate various proteins in the testes of mice that had daily exposure to nicotine [21]. Fifteen proteins found to be directly involved in the tricarboxylic acid cycle and cytoskeleton regulation, both integral in maintaining sperm motility, were uniquely expressed in these smoking mice. Additionally, profilin 1, a protein with critical value in cytoskeleton management, was found to be overexpressed in the nicotine-exposed cohort and revealed increased sperm motility [21].

The variation in results calls for increased research efforts to elucidate the effects of smoking on sperm at the molecular level.

40.5.7 Sperm Concentration and Volume

Sperm count was significantly reduced in smokers as compared to their nonsmoker counterparts [1, 18, 23, 57]. A meta-analysis conducted by Vine et al. measured that the sperm density in smokers was on average 13–17% lower than the sperm density of the nonsmoker cohort [57]. A separate study showed that smoking greater than or equal to ten cigarettes each day led to a dramatic decrease in sperm concentration [1]. Ramlau-Hansen et al. reported that the sperm concentration in heavy smokers was 19% reduced from healthy males.

While it seems that there is a consensus on smoking and sperm fluid concentration, Sharma et al. did not find a significant difference in seminal fluid volume between cigarette smokers and nonsmokers [18]. Again, further research is needed to untangle just how smoking disturbs seminal fluid balance and what measures can be taken to reverse this.

40.6 Impairment of Genetic Environment

40.6.1 Gene Methylation

Tobacco- and cigarette-specific carcinogens have also been linked to increased gene methylation. This process typically suppresses gene transcription. More research has been dedicated to elucidating the epigenetic causes of male factor infertility. However, studies have been inconclusive and contradictory. Work performed by Jenkins et al. found regions with increased methylation and other foci with decreased methylation in the infertile cohort as compared to the DNA methylation sites in healthy couples [25].

Santi et al. evaluated specific sites along the genome where aberrant methylation was suspected to impact sperm quality. They concluded that anomalous methylation levels of the cell cycle-associated genes of H19, MEST, and SNRPN resulted in impaired male fecundity [58].

An opposing study conducted by Al Khaled et al. also compared CpG alterations between smoker and nonsmoker sperm samples. Yet, out of 485,000 CpG sites analyzed, only seven sites were shown to have significant differences between the smokers and nonsmokers [27]. Yet six of these were found in single-nucleotide polymorphism regions, where variation is already increased across the population. The last site was found in an intron region. Their work could not determine any causative negative effects on biologically active regions of DNA between smoker and nonsmoker spermatocytes.

40.6.2 DNA Damage

While it is well documented that aging leads to DNA breakage, and therefore impaired sperm quality, smoking has also been linked to DNA damage [23]. Heavy smoking has been linked to abnormal spermatozoa and therefore male infertility [23]. In the cell cycle, there are various checkpoints at which DNA quality is assessed. If the DNA is damaged, by mutation, incorrect base pairing, the cell cycle is halted. Specifically, the checkpoint kinase 1 (Chk1) is activated, which then prevents the cell cycle from continuing through the S phase, where DNA is replicated, in addition to halting

progression past G2, thus stalling cell growth [23]. A study conducted by Cui et al. showed spermatocytes in smoking males to have markedly decreased expression of Chk1 as compared to nonsmoking men [23]. Reduced checkpoint proteins thus allow sperm with damaged DNA to continue through the cell cycle. As such, these sperm undergo less repair of impaired DNA and consequently increased amounts of apoptosis, thereby reducing sperm quality.

Sperm isolated from healthy males and exposed to cigarette smoke extract were shown to have faulty chromatin condensation as well as more early signs of apoptosis [15]. Phosphatidylserine externalization, marking the beginning of an apoptotic cell, was increased, alongside DNA fragmentation, another late presenting sign of apoptosis [15].

DNA repair mechanisms have also been reported as effected by smoking. Mismatch repair pathways are integral in maintaining DNA integrity [59]. Polymorphisms in genes involved in mismatch repair have been linked to male factor infertility [59].

Another study conducted by Horak et al. showed there to be a statistically significant increase bulky DNA accumulations in smokers, as compared to their nonsmoking counterparts [10].

40.7 Male Fertility Rescue

40.7.1 Does Cessation of Smoking Increase Male Fecundity?

The difficult and intricate nature of smoking's effects of various parameters contributing to male fertility makes it even more difficult to assess whether cessation of smoking can allow sperm and male physiology to return to a healthy state. Work performed by Oyeyipo et al. in animal models documented, as concluded previously, that nicotine and smoke contents negatively affect semen quality by all the parameters aforementioned [16]. Yet when rats were removed from the smoking environment, there was a marked increase in normal spermatozoa and male reproductive processes [16]. Thus, perhaps semen could recover from the toxins and deleterious effects of smoke exposure, essentially curing these causes of male infertility [16]. However, further research is needed in both animal and human subjects to conclude whether sperm quality and male reproductive physiology can be rejuvenated after exposure to cigarette smoke.

Further confounding any conclusions is the variety in smoke exposure. However, studies have shown a dose-dependent relationship between the level of cigarette by-product consumption and the degree of negative effects that were documented in the sperm. Ramlau-Hansen et al. exhibited an inverse relationship between the amount of smoke exposure and sperm volume, sperm quantity, and sperm quality [60]. Those with high cigarette consumption were shown to have 19% lower sperm concentration as compared to the nonsmoker cohort [60]. As such, decreasing cigarette usage and smoke exposure may reduce these adverse effects.

40.8 Conclusions

There is no question that cigarettes and their smoke contents are destructive to normal human anatomy physiology across all body systems. While the effects of smoking on female reproductive processes have been well documented, evidence has been inconclusive regarding the effects of smoking on male reproductive physiology. Environmental factors become difficult to untangle from a wide variety of confounding factors; however, it is important to provide conclusive evidence regarding just how normal male fertility can be impaired. This chapter aimed to provide an array of information regarding the multitude of avenues through which reproductive anatomy, male reproductive physiology, as well as spermatozoa end products can be impaired by cigarette smoke. Research into these subjects is also complicated by the immense difficulty in the methodology required to accurately assess spermatic fluid contents and quality. New WHO guidelines with specific protocols on how exactly to navigate this have not been globally implicated. As such, some studies may not have complied with the most recent standards and therefore may have reached conclusions that are no longer applicable. Regardless, further research is needed to better elucidate just how male fertility is impacted by cigarette smoke and whether male infertility secondary to these toxins can be cured by smoking cessation.

40.9 Review Criteria

An extensive search was conducted using the PubMed search engine to examine the effects of smoking and cigarette by-products on male infertility and sperm parameters. The search began in October 2018 and continued through January 2019. Keywords of "male infertility," "cigarette smoking," "spermatogenesis," "sperm quality," "male germ cell DNA methylation," "infertility," "semen parameters," "sperm motility," "sperm morphology," and "WHO guidelines," as well as the names of specific enzymes, were used to help formulate our conclusions. Published peer-reviewed articles were the mainstay of our information, supplemented by book chapters and some online bulletins specifying WHO parameters.

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Recreational Drugs

41

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Key Points

- Lifestyle factors, such as the use of recreational drug, are likely contributing to the steady decline of sperm concentration worldwide.
- Recreational drugs primarily exert their negative effects on fertility through either inhibition of the HPG axis or direct impairment of spermatogenesis within the testis.
- Cigarette smoking significantly impairs sperm kinetics and assisted reproductive technology (ART) success rates. Smoking cessation can reverse these negative effects.
- Marijuana is the most commonly utilized recreational drug; its impact on sex hormones is controversial. However, the negative impact on sperm kinetics is well established.
- For patients diagnosed with hypogonadotropic hypogonadism, the clinician should be aware of the association with opioid abuse.

41.1 Introduction

Male infertility affects approximately 7% of all men and is the underlying cause in at least half of the 15% of couples who have been unable to conceive after one year of unprotected intercourse [1–3]. Since the early 1990s, a number of studies have reported declines in sperm concentration, yet the impact of such findings was not fully accepted [4]. In 2017, a meta-regression analysis by Levine et al. examined 185 studies which included semen analysis results of 42,935 male participants from 50 different countries. Over the course of the last 40 years, they report a 52.4% decline in sperm concentration and a 59.3% decline in total sperm count [5]. The results of this study are alarming with the authors pointing to potentially environmental and lifestyle factors playing a key role. The cause of male infertility is likely multifactorial, and this chapter will examine the role of recreational drug use and their consequences on male reproductive health (Fig. 41.1).

41.2 Cigarette Smoking

It is well known that smoking cigarettes plays a significant detrimental role on the overall health of our population. The World Health Organization (WHO) cites tobacco as the leading cause of preventable death in the world, with a reported mortality rate of six million per year [6]. Despite compelling data of health risk, in 2014, nearly 19% of men and 15% of women were smokers in the United States according the Centers for Disease Control [7]. Although rates of smoking are trending downward, an estimated 40 million Americans continue to smoke [7]. There has been an explosion in the use of newer delivery devices for tobacco and its derivatives, such as electronic cigarettes and vaporizers. From 2011 to 2014, electronic cigarette usage among teenagers and young adults rose from 1.5% to 13.4% [7]. Unfortunately, these devices are being marketed to the reproductive age population. Overall cigarette smoking rates remain the highest in the 25–44 reproductive age range at an estimated prevalence of 37% worldwide [6].

Investigators have identified approximately 5000 chemical by-products of tobacco, including N-nitrosamines, polycyclic aromatic hydrocarbons, benzene, cotinine, lead, cadmium, and other carcinogenic compounds [8–10]. Nicotine is the primary substrate responsible for tobacco addiction. In humans, nicotine is initially metabolized to cotinine and ultimately to trans-3′3hydroxycontine (3HC) [11]. These carcinogenic compounds can also be found at elevated levels in secondhand smokers

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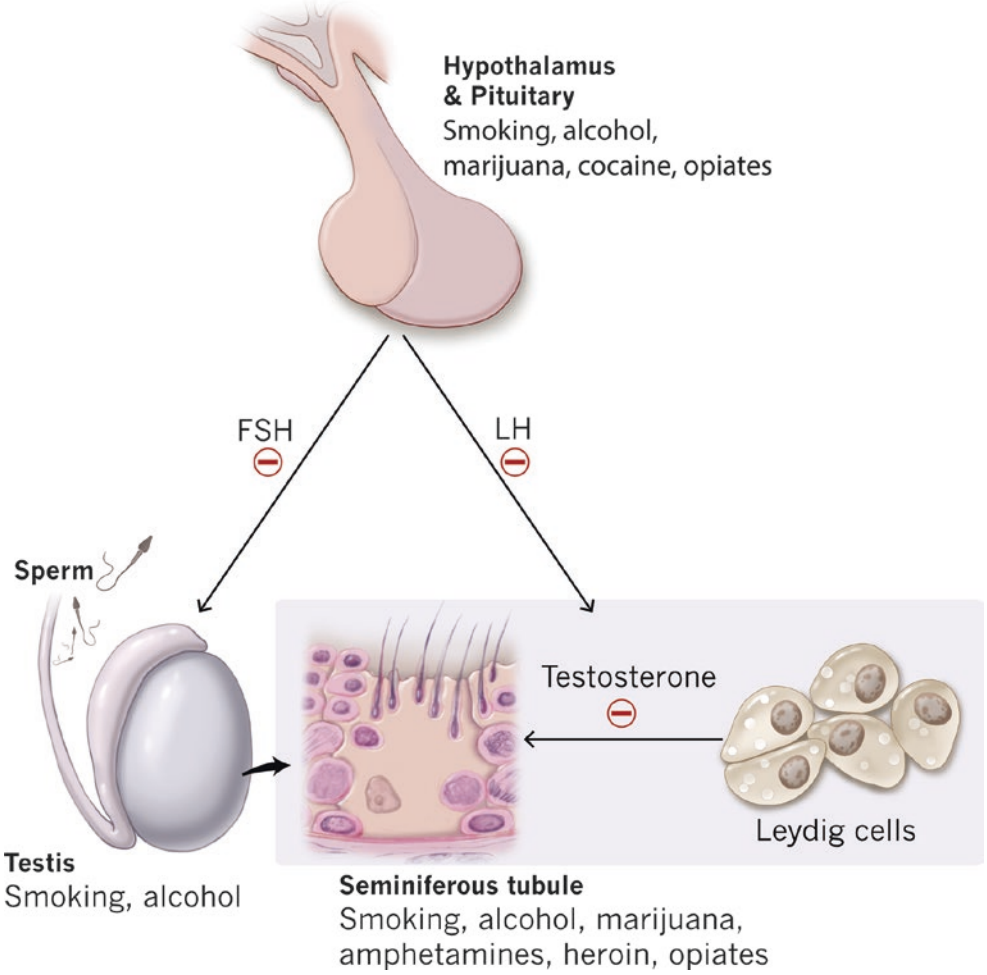

Fig. 41.1 Recreational drugs and the levels at which they may affect male fertility. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2018–2019. All Rights Reserved)

[12]. From a reproductive perspective, the by-products of smoking can detrimentally impair the integrity of human gametes; however, the mechanism by which this occurs is not clearly elucidated [13]. One theory involves elevated seminal plasma levels of lead and cadmium in smokers, which have been shown to impair sperm parameters and spermatogenesis [14–16]. However, further investigation into the direct relationship between cigarette smoking and fecundity is ongoing.

A recent meta-analysis of 20 studies by Sharma et al. highlights that cigarette smoking is a significant risk factor for impairing semen kinetics based on the latest 2010 WHO laboratory standards [17]. Cigarette smoking was associated with reduced sperm count, motility, and morphology. There was no definitive effect on semen volume. The detrimental effect of cigarette smoking was noted to be dose dependent, with moderate (10–20 cigarettes/day) and heavy smokers (>20 cigarettes/day) having the poorest semen quality. When the authors looked specifically at the subgroup of men that were both smokers and infertile, the deleterious effects on semen parameters were more pronounced. They proposed that smoking mutagenic compounds, coupled with the already vulnerable spermatozoa of infertile men, may synergistically worsen sperm kinetics. This has been also shown to be the case in smokers with a concomitant clinical varicocele, where semen parameters are markedly abnormal when compared to non-smoking varicocele patients [18]. The aforementioned findings are consistent with previous large meta-analysis studies that have demonstrated a decrease in multiple semen analysis indices in cigarette smokers [19]. Li et al. identified 29,914 patients in 57 cross-sectional studies and compared semen volume, concentration, motility, and morphology to smoking and a variety of other risk factors. Smoking, older age, psychological stress, and alcohol consumption were independent risk factors for poor-quality semen parameters. Smoking was the only risk factor to negatively impact all of the semen parameters that were measured [19].

With mounting evidence in support of smoking's deleterious effects on basic semen analysis, investigators have utilized advanced semen testing to further elucidate the underlying mechanisms of smoking's harmful effect. Taha et al. analyzed the direct impact of smoking cigarettes by specifically assessing sperm DNA fragmentation percentages, seminal reactive oxygen species (ROS), and seminal zinc levels [20]. Smokers were noted to have increased levels of

sperm DNA fragmentation and seminal ROS levels, in addition to reduced sperm motility, vitality, and seminal zinc levels when compared to nonsmokers. These findings were dose dependent to the number of cigarettes smoked per day and duration of smoking. Ultimately, as ROS levels rise and form an imbalance between natural antioxidant defenses within the seminal plasma, there is an increased risk of aneuploidy, oxidative stress, and male infertility [21]. Similarly, leukocytospermia is a negative product of cigarette smoking [22, 23]. Cigarette smoke is not the sole producer of ROS in the seminal fluid as elevated leukocytes can contribute to a significant proportion of ROS, thereby reducing fertility by exposing spermatozoa to oxidative stress [24–26]. Calogero et al. demonstrated diminished mitochondrial activity in the sperm of smokers, which thereby impaired its capacity to fertilize an egg [27].

There is additional evidence that preconception paternal smoking may carry negative consequences for progeny. Specifically, investigators have evaluated epigenetic alterations in male smokers and identified smoking-induced methylation changes in sperm DNA [28]. It is well documented that maternal cigarette smoke exposure has a variety of negative consequences to fetal health. Sobinoff et al. utilized the first animal model to suggest that maternal smoking compromised spermatogenesis of male embryos and subsequently resulted in significant germ cell damage and Sertoli cell dysfunction in the adult progeny [29]. It is particularly imperative to appropriately counsel patients proceeding with assisted reproductive technology (ART) about the risks of smoking. Maternal smoking is a significant risk factor in lowering success rates with in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) [30]. Similarly, paternal smoking may alter the clinical pregnancy rate per intrauterine insemination (IUI) cycle [31]. Vanegas et al. evaluated 225 couples where 32% of the men reported a history of smoking. They determined that for every additional year after the male had quit smoking, it decreased the risk of ART failure by 4% [30].

As investigators continue to further understand the relationship between cigarette smoke and fecundity, it is imperative that clinicians strive to educate patients of the benefits of smoking cessation, which includes improvement in sperm concentration after only 3 months [23]. The positive impact is not limited to improved sperm kinetics and reproductive health but also their personal overall health and the wellbeing of their offspring.

41.3 Alcohol

Rates of alcohol consumption vary significantly worldwide. However, it is well understood that alcohol consumption in excess confers significant sexual side effects (decreased libido, erectile dysfunction, and testicular atrophy) as well as overall health risks [32, 33]. In the reproductive age population, the popularity of "binging" is of significant concern on both psychosocial and physiologic levels. Young adults aged 18–34 years are the most susceptible, with men being twice as likely than women to binge drink. The CDC reports that one in six US adults binge drinks about four times a month [34].

There is robust clinical evidence to suggest increased alcohol consumption negatively impacts sperm kinetics. Recently, Ricci et al. performed a meta-analysis of 16,395 men in 15 cross-sectional studies and analyzed the effect of alcohol consumption on sperm parameters [35]. The authors determined that semen quality was unaffected by occasional alcohol intake; however, semen volume and morphology were significantly impaired with daily alcohol intake. Several animal studies have shown that ethanol consumption clearly disrupts nuclear maturity and DNA integrity of spermatozoa [36, 37]. Talebi et al. analyzed the cauda epididymis in rats and demonstrated increased DNA fragmentation and diminished sperm motility in ethanol-consuming rats when compared to controls [36]. Similarly, apoptosis of spermatogenic cells and decreased plasma and intratesticular testosterone have been demonstrated in mice injected with ethanol intraperitoneally [37]. Imbibing alcohol has been shown to yield an increased risk of oxidative stress [38], but unlike smoking, seminal levels of oxidative stress have not been shown to clearly correlate with alcohol [39].

Investigators have long proposed that alcohol directly effects testosterone metabolism and spermatogenesis in a dose-dependent manner [33]. Studies have attributed alcohol's effect on hormonal function to elevated serum levels of beta-endorphin which may play a role in inhibition of the hypothalamus-pituitary-gonadal (HPG) axis, specifically impaired serum levels of gonadotropin-releasing hormone (GnRH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone [40, 41]. Additionally, the ratio of estradiol to testosterone appears to be increased secondary to enhanced aromatization in heavy alcohol drinkers [42]. As a result, Leydig and Sertoli cell function is downregulated, thereby inducing primary testicular failure [40]. In an autopsy study, Pajarinen and colleagues identified pathologic evidence of spermatogenetic arrest and Sertoli-cell-only syndrome in testicular specimens associated with higher levels (>40 g/day) of alcohol consumption [43].

The current literature implores clinicians to educate patients relying on ART to reduce alcohol intake. Men who partook in one additional drink per day were twice as likely to not achieve a live birth with ART [44]. It has also been associated with impaired implantation rates in IVF and ICSI [45]. Alcohol has been implicated in adversely impacting semen parameters and hormonal levels in a dose-dependent manner. While there is clear evidence to suggest limiting

alcohol intake in those that are heavy drinkers, further investigation into binge drinking and social drinkers with fecundity is needed.

41.4 Marijuana (Cannabis)

Marijuana is the most commonly used recreational drug in the United States and throughout the world. In 2013, an estimated 232 million people, or 4.9% of the world population, used cannabis [46]. In the United States, 43% of Americans reported using cannabis in 2015. As of 2016, the prevalence of cannabis usage steadily rose to 51% [47]. The ratio of male consumption of cannabis compared to females is 2:1 [47]. Furthermore, reproductive aged 18–29-year-olds are six times more likely to use cannabis than a cohort of older adults [48]. A recent survey within the United States demonstrated that 53% of Americans favor legalization of marijuana and 77% support its use for medical purposes [49]. With public perception and legislative approach about the use of marijuana changing, clinicians should be familiar with the current literature on cannabis and male reproductive health in order to appropriately counsel patients.

Marijuana is derived from the dried leaves and flowers of a plant (*Cannabis sativa*) and can be consumed through a variety of methods (e.g., smoking, vapor, food, extract, etc.). The primary psychoactive compound of marijuana is the cannabinoid delta-9-tetrahydrocannabinol (THC). Chronic cannabis use has been associated with decreased libido, erectile dysfunction, testicular germ cell tumors, and gynecomastia, but there are a limited number of human studies directly demonstrating the effect of cannabis on male fecundity [50–53]. Early investigation of THC demonstrated lower serum levels of LH through the inhibition of LH-releasing hormone from the hypothalamus [54]. Kolodny and colleagues measured serum testosterone levels and semen parameters in chronic marijuana smokers and identified significantly lower levels of testosterone when compared to age-matched controls [55]. Additionally, 35% of those men demonstrated oligospermia. The findings were dose dependent and consistent with the theorized regulatory effect of cannabinoids on the HPG axis [55]. However, more recent studies have failed to reproduce these results. Thistle and colleagues recently performed a large cross-sectional analysis of 1577 men utilizing the National Health and Nutrition Examination Survey (NHANES) [56]. The results indicated that there was no significant difference in testosterone levels between nonusers and ever users of cannabis.

A number of investigators have studied the effect of marijuana on sperm kinetics. Cannabinoid receptors have clearly been shown to be expressed on spermatozoa; therefore, cannabinoids can directly exert their effects onto sperm [57]. Cannabis has been shown to impair sperm motility and the acrosome reaction in sperm [57]. In animal models, chronic exposure to THC has been shown to detrimentally affect spermatogenesis and result in significant deterioration of sperm morphology [58]. Clinical studies in humans have echoed these findings. Hembree and colleagues reported that a high level of cannabis use was inversely proportional to sperm concentration [59]. More recently, Gunderson and colleagues studied a cohort of 1215 Danish men and reported diminished sperm concentration in regular users of marijuana [60]. Although marijuana's effect on testosterone production is inconsistent, there is robust evidence describing marijuana's deleterious effect on spermatogenesis. As the prevalence of recreational and medicinal use of marijuana continues to rise, men should be counseled on its effects on reproductive health.

41.5 Opioids

Opioids are a heavily abused and addictive class of drug that an estimated 13.5 million people consume worldwide [61]. There are various classes of opioids, but the most commonly abused are heroin, fentanyl, and prescription analgesic medications (e.g., oxycodone, hydrocodone, codeine, and morphine). The CDC reports approximately 115 deaths per day from opioid overdoses in the United States [62]. The current opioid epidemic is a significant national crisis that adversely impacts all facets of society and healthcare.

It is well recognized that opioid analgesics primarily mediate their effects on the HPG axis via negative feedback [63]. Opioids suppress LH release which subsequently decreases testosterone production [63, 64]. Additionally, animal models have identified endogenous opioid peptides and their receptors within Leydig and Sertoli cells. Leydig and Sertoli cells synthesize endogenous opioid peptides which in turn inhibit Sertoli cell function through paracrine and autocrine signaling [64]. Therefore, the result of chronic opioid use is not only hypogonadotropic hypogonadism but also impaired spermatogenesis [64, 65].

Abs et al. evaluated a cohort of patients with intractable nonmalignant pain managed with intrathecal opioids [66]. In 23 of the 24 men administered intrathecal opioids, the patients reported decreased impotency and libido. Furthermore, LH and testosterone levels were significantly lower compared to men not consuming opioids. The authors provided androgen replacement therapy for hypogonadal men in the opioid cohort and determined that supplementation significantly ameliorated sexual side effects [66]. The clinician must be cognizant of the disruption in normal spermatogenesis that may occur with exogenous testosterone therapy in reproductive aged patients.

Daniel and colleagues explored the effect of consuming commonly prescribed oral opioids on LH and testosterone

levels and found that hormone levels were significantly decreased in a dose-dependent fashion [67]. These findings are consistent with a recent systematic review and meta-analysis of 17 studies on the relationship between opioids and hormone levels [68]. Bawor and colleagues determined that testosterone levels are suppressed by nearly 50% in men regardless of opioid type, including those men managed on methadone [68]. Patients are frequently treated with methadone, a synthetic opioid, which is used to manage addiction and opioid withdrawal symptoms.

As healthcare professionals strive to improve opioid stewardship, patients being prescribed opiate analgesics must be counseled on their risk to reproductive health. For patients diagnosed with hypogonadotropic hypogonadism, the clinician should be aware of the association with opioid abuse. The 2018 American Urological Association guidelines on testosterone recommend that clinicians screen all men with a history of chronic opioid use for hypogonadism, regardless of the presence of symptoms [69]. Among healthcare professionals, there must be greater awareness of the impact of opioid use on spermatogenesis and hormone levels in couples seeking fertility counseling.

41.6 Cocaine

There are an estimated 20 million users of cocaine globally, and the United States is the largest consumer of this illicit drug [70]. It is a highly addictive compound which primarily stimulates the central nervous system to increase dopamine release affecting mood and energy. Although cocaine usage has gone down considerably since the 1950s, as of 2008, approximately 88% of cocaine users are males in the age range of 15 and 34 years old [71].

Bracken et al. have performed the only study to date on the use of cocaine and fertility in humans [72]. The authors measured semen parameters of men presenting to a Yale infertility clinic. They determined that cocaine use within the previous 2 years correlated to a twofold higher likelihood of having a sperm concentration less than 20 million per milliliter. Furthermore, men who endorsed cocaine use for 5 or more years demonstrated low sperm motility. Multiple animal models have looked to establish a biologic cause of male subfertility. George and colleagues assessed rats receiving cocaine after 100 days and determined a pregnancy rate of only 33% versus 86% for the controls [73]. The birth weight of the progeny from the rats receiving cocaine was 10% less than that of controls. Additionally, cocaine administration resulted in a significantly reduced mean diameter of seminiferous tubules.

Additional human studies are required to fully ascertain cocaine's relationship to human fecundity; however, confounding variables (concomitant use of alcohol, smoking, drugs) and reporting bias limit this opportunity. Regardless, appropriate screening for cocaine use in male fertility patients is essential, as men who report using cocaine are more likely to be associated with high-risk behaviors which may also impair fertility.

41.7 Methamphetamine and Ecstasy

Amphetamine is a potent psychostimulant that is used to treat attention-deficit hyperactivity disorder, narcolepsy, and obesity. However, once double methylated, it is converted to the highly addictive substance, methamphetamine, which is commonly used recreationally and abused. Multiple animal models have been utilized to describe its mechanism of action on male fertility. Spermatogenesis, DNA integrity, and sperm kinetics have been shown to be significantly impaired in rats exposed to methamphetamine in a dose-dependent manner [54, 74]. Multiple studies have demonstrated increased levels of oxidative stress and apoptosis in the rat testis [75, 76].

A synthetic derivative of amphetamine is 3,4-methylened ioxymethamphetamine (MDMA) or ecstasy. In 2016, an estimated about 21 million people between 15 and 64 years of age used ecstasy [77]. Commonly consumed at "raves" and music festivals, this psychoactive compound has been shown to affect the HPG axis in animal studies [78]. Dickerson and colleagues measured suppressed levels of GnRH and serum testosterone in rats administered MDMA. The authors elucidate that the primary target of MDMA is within the neurosecretory pathway of the central nervous system [78]. Barenys et al. assessed semen parameters and testicular histology in rats exposed to MDMA [79]. They identified increased levels of DNA damage in sperm and evidence of interstitial edema and seminiferous tubule degeneration in the testis. However, morphology and motility of the sperm was unaffected.

41.8 Conclusion

It is essential for healthcare providers and patients to be aware of modifiable risk factors that can be managed in order to improve male fertility. This chapter focused on commonly abused recreational drugs: smoking, marijuana, alcohol, opioids, cocaine, and methamphetamine/ecstasy. However, the strength of evidence implicating recreational drug use and infertility is variable, with additional control studies needing to be performed. Overall, recreational drugs primarily exert their negative effects on fertility through either inhibition of the HPG axis or direct impairment of spermatogenesis within the testis. The data does suggest that the cause of male infertility is likely multifactorial. Therefore, healthcare providers

must be mindful of the negative impact of recreational drug use which can be potentially overcome through appropriate education and cessation for improved reproductive health.

41.9 Review Criteria

We extensively searched Google Scholar, PubMed, Medline, Clinical Key, and ScienceDirect for articles focusing on recreational drugs, modifiable risk factors, sperm kinetics, and fertility. We began our literature search in July 2018 and completed it by November 2018. The following keywords were utilized in our search: "tobacco," "cannabis," "alcohol," "recreational drugs," "cocaine," "infertility," and "opiates." We reviewed only English language articles. Illustrations were created with assistance from an institutional based artist.

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Part IV

Nutrition, Life-Style and Antioxidants Role for Male Reproductive Health

Nutritional Pathways to Protect Male Reproductive Health

42

Tung-Chin Hsieh, Jessica Marinaro, and Paul R. Shin

Key Points

- Oxidative damage is associated with alterations in spermatogenesis and subfertility.
- Several nutrients – including arginine, zinc, selenium, vitamin C, vitamin E, and carnitine – have been identified as antioxidant compounds and implicated in male reproductive health.
- In some studies, these antioxidant compounds have been shown to improve semen parameters and fertility outcomes.
- It is unclear what role antioxidant supplementation plays on male reproductive health and fertility outcomes.
- Obesity and alcohol intake have been found to have variable effects on semen parameters and remain the subject of active investigation.

42.1 Introduction

Nutrition is an essential component of one's overall health. Many common disease processes can be alleviated or prevented by a healthy diet. First reports of antioxidant deficiency and decreased male fertility can be traced back to over 50 years ago [1]. With the understanding of oxidative damage to spermatogenesis, most of the nutritional research has focused on the role of antioxidants in improving male fertility. However, there are not any randomized controlled trials studying whole food diet in infertile male patients. Patients are often counseled based on data extrapolated from antioxidant supplement studies. This chapter is intended to give an overview of contemporary research on nutrient and male reproductive health with guidance to natural food sources that contain high levels of antioxidants.

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42.2 Nutrients and Male Reproductive Health

42.2.1 Arginine

Arginine is a semi-essential amino acid because it can be synthesized by the human body from glutamine, glutamate, and proline. It plays an important role in cell division, wound healing, immune function, hormone production, and ammonia metabolism. Arginine also serves as a precursor for nitric oxide synthesis and, therefore, has significant effects on endothelial function. It is involved in the pathophysiology of many vascular disorders including vasogenic erectile dysfunction [2].

Arginine is required for normal spermatogenesis. Researchers have found that adult men on an arginine-deficient diet have decreased sperm counts and an increased percentage of nonmotile sperm [1]. Oral administration of arginine to infertile men for 6–8 weeks has shown improvement in sperm counts, motility, and conception rates [3–6]. However, similar benefits were not observed in patients with baseline sperm concentrations of less than 10 million/mL [7]. Interestingly, in animal models, higher doses of L-arginine supplementation actually inhibit fertility compared to controls, suggesting that further research is needed to establish the optimal dose needed for enhanced fertility outcomes [8].

Since de novo biosynthesis does not produce sufficient arginine to meet bodily needs, dietary intake remains the primary determinant of plasma arginine levels. It is considered

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an essential nutrient for human children but not adults by the US Department of Agriculture [9]. Currently, there is not a consensus on the daily recommended intake of arginine; studied doses range from 1 to 15 g/day. Although no significant adverse effects have been observed in studied doses, patients with impaired renal or hepatic dysfunction might not be able to metabolize arginine properly. The effect of arginine on the airway is also unclear, and caution should be taken in asthmatic patients. Animal sources of arginine include dairy products, turkey, pork, and beef. Vegetable sources include seeds, soybeans, and nuts.

42.2.2 Zinc

Zinc is an essential micromineral. There are 2–4 g of zinc throughout the human body with the highest concentrations found in the prostate and parts of the eye [10]. It serves as a metalloprotein cofactor for DNA binding and, as part of the enzyme copper/zinc superoxide dismutase, is involved in the repair of damaged DNA. It also has an important role in testis development and sperm function. Consequently, zinc deficiency is associated with hypogonadism, testicular/seminiferous tubular atrophy, and inadequate development of secondary sexual characteristics [11].

Semen analyses of fertile and infertile men have shown a positive correlation between low zinc levels and poor sperm quality [12]. Specifically, higher levels of zinc in seminal fluid have been found to be associated with increased sperm count and normal sperm morphology [12]. Treating men with asthenozoospermia with zinc for 3 months also demonstrated an improvement in semen parameters, an increase in seminal antioxidant capacity, and a reduction of oxidative status [13]. Researchers postulate that poor zinc nutrition can impair antioxidant defenses, be a risk factor in oxidant release, and compromise the mechanism of DNA repair, making the sperm cells highly susceptible to oxidative damage [12, 13]. In order to combat this oxidative damage, there is an increasing concentration of extracellular zinc throughout the length of the reproductive tract. While the seminiferous tubules have a zinc concentration similar to that of nonreproductive organs (i.e., liver, kidney), the epididymis, vas deferens, and seminal vesicles all have a progressively greater concentration of zinc. Ultimately, the spermatozoa are ejaculated in the seminal plasma, which has a zinc concentration nearly 100 times greater than blood [14]. This increase in zinc concentration is largely secondary to the expression of zinc transporters throughout the reproductive tract. Recent studies have begun to elucidate these pathways involved in zinc transport. For example, we now know that the epithelial cells of the testis and epididymis appear to be programmed to supply zinc, while the spermatozoa are designed to rapidly take it up. This suggests that zinc trafficking may play a role in the process of sperm maturation and sperm quality [14].

Currently, there is limited data available in humans to establish a dietary dosage of zinc necessary to achieve optimal seminal plasma levels. The daily recommended dietary allowance of zinc is 8 mg/day for women and 11 mg/day for men [9]. Excess zinc absorption (>15 mg/day) can interfere with copper and iron absorption, disrupt cholesterol metabolism, and cause anosmia. Animal sources of zinc include red meat, oysters, and liver. Vegetable sources include seeds, nuts, and whole grains.

42.2.3 Selenium

Similar to zinc, selenium is an essential micronutrient. It functions as a cofactor for antioxidant enzymes, such as glutathione peroxidase. Although rare in a healthy, well-nourished adult, selenium deficiency is associated with reduced or impaired reproduction [15]. A sperm-specific selenoprotein has been identified and is suspected to play a key role in selenium deficiency-induced subfertility [16]. In fact, a number of selenoproteins have been found to protect against oxidative damage during sperm maturation, as well as act as structural components of mature spermatozoa [17]. Specifically, x-ray fluorescence microscopy has shown that selenium is concentrated within the sperm head and midpiece structures [18]. Selenoproteins are also critical for transporting this selenium from the blood to the testis, condensing chromatin during sperm maturation, and ensuring normal sperm morphology [17].

Despite selenium's many roles on the cellular level, population studies on the effect of selenium in subfertile men have yielded conflicting results [19, 20]. In a randomized, double-blinded study, treating subfertile men with selenium once a day showed no influence on sperm count but did demonstrate an improvement in sperm motility compared to placebo [21]. Ultimately, more research is needed to fully understand the impact of selenium on semen parameters.

The recommended daily allowance for selenium is 55 μg/day for men [22]. Selenosis can occur when intake reaches the level greater than 400 μg, and may result in cirrhosis, pulmonary edema, and death. In Europe there has been a documented decline in the mean intake from 60 μg/day in the 1970s to 30 μg/day in the 1990s due to a change in the source of cereals for bread making; it is unclear what impact this decline has had on male reproductive health [23]. In addition to wheat/cereals, other vegetable sources include Brazil nuts and soy products. Animal sources of selenium include meat, fish, and eggs.

42.2.4 Vitamin C

Ascorbic acid is an essential nutrient for humans and other animal species. It has been associated with fertility for many years; however, the precise mechanism of action has not been elucidated. Most consider the effect of vitamin C on fertility to be related to these three principal functions: promotion of collagen synthesis, role in hormone production, and protection or prevention against oxidation.

Early reports on the effects of vitamin C on male fertility were based on animal studies. Ascorbate deficiency was associated with poor breeding performance and degeneration of the testicular germinal epithelium [24, 25]. The gonadal growth-enhancing effects of gonadotropins were enhanced by simultaneous treatment with ascorbic acid [26]. As one recent animal model demonstrated, oral vitamin C administration was associated with a dose-dependent increase in serum FSH level and serum testosterone, as well as sperm motility and normal sperm morphology [27]. In human studies, low ascorbate level has been associated with low sperm counts, increased number of abnormal sperm, reduced motility, and agglutination [28]. Dietary treatment with vitamin C has yielded mixed data on improvement of sperm parameters [29]. In one recent randomized trial, men treated with 1000 mg of vitamin C every other day for 6 months were found to have a significant increase in sperm concentration and motility [30]. However, additional trials are needed to reproduce this improvement in the semen parameters and assess the pregnancy rates of healthy infertile men who take oral vitamin C supplements.

The recommended daily allowance of vitamin C is 90 mg/day for adult male and 75 mg/day for adult female [9]. Dietary intake should not exceed 2300 mg/day since intoxication can lead to gastrointestinal disturbances, iron poisoning, and even hemolytic anemia in patients with glucose-6-phosphate dehydrogenase deficiency. A well-balanced diet without supplementation is generally sufficient to meet the daily requirement for vitamin C, except those who are pregnant or smoke tobacco. The highest natural sources are fruits and vegetables, particularly black currant, red pepper, and guava.

42.2.5 Vitamin E

Vitamin E is a lipid-soluble antioxidant. It protects cell membranes from oxidation by reacting with the free radicals generated during lipid peroxidation. Various forms of vitamin E have been identified, but the exact role and importance of these isoforms remains unclear. It is known, however, that the motility of spermatozoa depends on the integrity of the mitochondrial sheath, which is composed of phospholipids and can be damaged by lipid peroxidation [31]. Therefore, vitamin E has been hypothesized to be an important factor for mitigating damage to this phospholipid-rich mitochondrial sheath structure and maintaining overall health of sperm.

Specifically, men with asthenozoospermia have been found to have an increased concentration of the peroxidation by-product malondialdehyde (MDA) in their seminal fluid. Treating these patients with vitamin E in a randomized, double-blinded fashion showed improvement in sperm motility and decreased MDA concentration, as well as resulted in successful pregnancies for 11 of the 52 (21%) patients [32]. However, these improvements in semen parameters were not seen in other randomized controlled trials [33, 34].

Additionally, studies of vitamin E supplementation in combination with other micronutrients have shown mixed results. For example, one randomized trial comparing daily supplementation with both vitamin E and selenium versus controls demonstrated a significant decrease in MDA concentration and improvement in sperm motility among patients in the treatment group [35]. Other studies examining the effect of both vitamin C and vitamin E supplementation, however, failed to show any improvement in semen parameters [36, 37]. Ultimately, few studies have demonstrated any significant effect on semen parameters using vitamin E as either a single treatment or in combination with other antioxidants [38].

The recommended daily intake of vitamin E is 15 mg (30 IU) per day for adults [9]. A dose greater than 1000 mg (1500 IU) per day is associated with an increased risk of hemorrhage and death. The best sources of vitamin E are nuts, seeds, and vegetable oils, along with green leafy vegetables and fortified cereals.

42.2.6 L-Carnitine

Carnitine is a semi-essential nutrient that can be biosynthesized from lysine and methionine by the liver and kidneys. Two stereoisoforms exist, with L-carnitine as the bioactive form. It is involved in the metabolism of long-chain fatty acids and serves as an antioxidant by removing acetyl-CoA, which is responsible for mitochondrial lipid peroxidation [39]. This antioxidant effect is so significant that, in animal models, L-carnitine supplementation has been shown to protect against gamma-irradiation-induced testicular injuries [40]. The highest concentration of carnitine occurs in the epididymis, with epididymal concentrations being 2000-fold higher than plasma [41].

Low seminal levels of L-carnitine have been associated with subfertility. In one single-center study of 61 men all azoospermic, asthenospermic, and oligoasthenoteratospermic men were found to have significantly lower levels of

seminal free L-carnitine compared to fertile controls, with a lowest value of seminal free L-carnitine being found in the azoospermic group [42]. Given these findings, it has been hypothesized that L-carnitine supplementation may improve semen parameters. A multicenter, uncontrolled trial showed that oral administration of L-carnitine (3 g/day) for 4 months in asthenozoospermic patients resulted in an improvement in sperm motility, linearity index, rapid linear progression, and mean velocity [43]. In a randomized, double-blinded, placebo-controlled trial, L-carnitine therapy (2 g/day) for 4 months in infertile men showed improvements in sperm concentration and motility [44]. However, L-carnitine monotherapy may not be as beneficial as combination therapy. In one prospective, nonrandomized study of men with at least 1 year of subfertility and one abnormal semen analysis, participants were treated with either 3 months of L-carnitine or with a combined compound which included L-carnitine as well as several other micronutrients. While all semen parameters (volume, density, overall progressive motility, percent normal morphology) improved significantly from baseline in both groups, the relative change in sperm density and overall progressive motility was greater for the combined micronutrient treatment group compared to the L-carnitine monotherapy group [45]. Despite the observed improvement of semen parameters with carnitine supplementation in these studies, other randomized controlled studies have not been able to replicate similar results [46].

Seventy-five percent of the carnitine that is present in humans is derived from diet [47]. Currently, there is not a recommended daily allowance of carnitine intake or any detrimental reports of carnitine overdose. Oral intake greater than 1 g/day did not show any advantage, since absorption studies indicate saturation at this level. The highest concentration of carnitine is found in red meat and dairy products. Vegetable sources include nuts, seeds, and asparagus.

42.3 Factors Contributing to Subfertility

42.3.1 Obesity

Multiple population-based studies suggest an increased risk of subfertility among obese couples [48]. In women, there has been extensive research on the effects of body composition on altered menstrual function and fertility [49]. Epidemiologic studies have also observed a higher incidence of male factor infertility in obese males [50, 51]. Obese men often exhibit an altered reproductive hormone profile, including decreased androgen levels, decreased sex hormone-binding globulin (SHBG) levels, and decreased inhibin B levels along with elevated estrogen levels [52, 53]. Obesity has also been associated with sexual dysfunction, which may in turn affect fertility.

Currently, there is limited data on the reversibility of obesity-associated male infertility with weight loss. One small, randomized controlled trial demonstrated an increase in SHBG and testosterone after 10 weeks of a very low-energy diet and behavior modification program [54]. Other studies on the effect of weight loss - including both surgical and diet/lifestyle modification programs - have shown mixed improvements in hormonal profile and sperm parameters [48].

There is also a growing body of literature to suggest that bariatric surgery and subsequent rapid weight loss may negatively impact semen parameters. In several case studies, male patients who underwent bariatric surgery were found to have decreased sperm concentrations, sperm motility, and normal sperm morphology [55, 56]. Even more dramatically, in one case series, six previously fertile men were found to be azoospermic with complete spermatogenic arrest after undergoing a Roux-en-Y gastric bypass [57]. As the rates of bariatric surgery continue to increase, additional research is needed to fully understand the impact of this procedure on male fertility outcomes.

42.3.2 Alcohol

Alcohol abuse has been shown to cause impaired testosterone production and testicular atrophy resulting in impotence, infertility, and reduced secondary sexual characteristics [58]. It has been shown to have a deleterious effect on all levels of the male reproductive system, including: the hypothalamic–pituitary–gonadal axis, Leydig and Sertoli cell function, and spermatogenesis, even leading to spermatogenic arrest and Sertoli cell-only syndrome in advanced cases [59, 60]. In an uncontrolled study, alcohol users were found to have decreased sperm count, normal morphology, and motility. This association was most significant with alcohol consumption of greater than 40 g/day [58]. In contrast, there are reports of alcohol consumption having beneficial effects on fertility, since some drinks (such as red wine) may exert a protective antioxidant effect [61]. In one recent prospective cohort study from Italy, moderate alcohol intake was positively associated with semen quality, compared to high- or low-intake groups [62]. Currently, the dose-dependent effects of alcohol on male factor infertility are not well understood, and remain a subject of investigation.

42.4 Conclusion

Dietary modification and nutritional supplementation are popular areas of concern for the infertile patient. Altering one's nutritional habits is usually easily done at little to no cost and empowers the patient to feel as though they are

actively modifying their condition. From our survey of the literature, it appears that several antioxidant compounds have demonstrated a positive effect on semen parameters; however, no consensus exists regarding the best pathway to maximize fertility. Moreover, basic science research does not always translate into clinical success – especially in the field of infertility – and many of the findings from animal studies may not be readily applicable to humans. However, avoidance of toxicants, proper nutrition, and stress reduction are general guidelines that all men should follow, regardless of their fertility status. For infertile men, these lifestyle modifications will not only improve their general well-being but may also positively impact their semen parameters and fertility outcomes. Further research is needed to more fully understand the biochemical mechanisms behind these behavioral modifications and establish more specific recommendations for infertile patients.

42.5 Review Criteria

An extensive search of studies examining the relationship between nutrients, obesity, and alcohol intake on male reproductive health was performed using search engines such as ScienceDirect, OVID, Google Scholar, PubMed, and MEDLINE. The start and end dates for these searches were August 2018 and May 2019, respectively. The overall strategy for study identification and data extraction were based on the following keywords: "nutrition," "oxidative stress," "male infertility," "nutritional supplements," "arginine," "zinc," "selenium," "vitamin C," "vitamin E," "carnitine," "obesity," "alcohol," and "semen parameters." Articles published in languages other than English were also considered. Websites and book-chapter citations provide conceptual content only.

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Antioxidants and Male Infertility

43

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Key Points

- Oxidant radical excess has been associated with male infertility. In particular many authors have focused on the role of reactive oxygen species (ROS).
- Spermatozoa are very susceptible to oxygen effects because their membranes are rich in polyunsaturated fatty acids that assure fluidity and flexibility; this one makes spermatozoa more vulnerable to lipid peroxidation.
- The most important sources of ROS in semen are immature spermatozoa and leucocytes, and the protective antioxidant system in semen is divided into enzymatic factors, such as superoxide dismutase, catalase, glutathione peroxidase, and peroxiredoxins, and nonenzymatic factors.
- Carnitine, coenzyme Q10, vitamin E, vitamin C, zinc, and myo-inositol are the most important nonenzymatic systems for which a seminal parameter improvement was demonstrated.
- There are emerging evidences that herbal products can also improve male reproductive functions.

43.1 Introduction

Infertility is defined as the failure to establish a clinical pregnancy after 12 months of regular and unprotected sexual intercourse. Infertility affects an estimated 15% of couples globally, especially in developed countries, with around 20–50% due to male factors [1].

Unfortunately, many causes of male infertility are unknown, and for this reason, numerous studies have been carried out, analyzing gene expression, epigenetics modifications, and mostly the role of reactive oxygen species (ROS) and antioxidants.

It is well recognized that oxidative stress is a cause of male infertility, but the use of antioxidants as a treatment is still debated, and it is considered as a supportive therapy, rather than etiological or physiopathological, on the real effect of oral supplementation. Many models have been introduced to explore the role of different antioxidants in vitro, and some differences can be discovered regarding the protective effects exerted by specific enzymatic or nonenzymatic molecules.

43.2 The Role of Reactive Oxygen Species

An oxidant radical excess has been associated with male infertility. In particular many authors have focused on the role of ROS [2, 3, 4].

In the last 20 years, an assay called "TOSC" (total oxyradical scavenging capacity) was developed to measure the total capacity of the biologic fluids or cellular antioxidants that neutralize oxygen radical toxicity. This assay has been used by our group in andrology, and it helped us to show a reduced antioxidant capacity in infertile men's seminal fluid and a correlation between the scavenging action versus oxygen radicals and sperm cell parameters, in particular the motility [5, 6, 7]. Another parameter of antioxidant evaluation is the determination of total antioxidant capacity (TAC), the method that we employed in our laboratory [8], which is

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a modification of the method of Rice-Evans and Miller [9]. It is a kinetic assay, based on the reaction of H_2O_2-metmyoglobin with a chromogen (ABTS), whose radical species is spectroscopically detected. The latency time before the appearance of radical ABTS (lag phase or LAG, measured in sec) is proportional to the amount of small-chain-breaking molecules, which interrupt the propagation of oxidative reactions, mainly urate, ascorbate, and glutathione, and moreover, the rapid increase of ABTS is followed by a more gradual increase, in which antioxidant properties of proteins are involved. The interest of these parameters is based on its modulation by hormonal milieu: in fact we have shown that plasma LAG is lower in hypogonadism [10] and hypoadrenalism [11] and is influenced by growth hormone [12], another hormone involved in the control of fertility [13]. Even in seminal plasma, TAC exhibits a modulation by endocrine factors, since it is inversely related to systemic thyroid hormone levels [14].

Oxidative stress is determined when there is an imbalance between the production of ROS and the neutralizing activity of antioxidant system. It leads to a pathological and often irremediable cell damage. In particular spermatozoa are very susceptible to oxygen effects because their membranes are rich in polyunsaturated fatty acids that assure fluidity and flexibility; this one makes spermatozoa more vulnerable to lipid peroxidation [15]; in addition their cytoplasm is poor in scavenging systems. On the other hand, ROS are important for some processes like the sperm chromatin condensation, capacitation, acrosome reaction, cell signaling, and sperm motility [16].

The most important sources of ROS in semen are immature spermatozoa [17] and leucocytes [18], and the protective antioxidant system in semen is divided into enzymatic factors, such as superoxide dismutase, catalase, glutathione peroxidase, and peroxiredoxins [19], and nonenzymatic factors.

43.2.1 Nonenzymatic Systems

The first line of studies about antioxidants and fertility concerns dietary influences; in fact it is known that fertility is related to lifestyle [20, 21] and is an index of general health of men [22]. We have shown that modification of the diet in patients with metabolic syndrome, with enrichment of antioxidant by seasonal vegetable and fruits, has a synergy with metformin in reducing insulin resistance [23]; preliminary data shows that, in patients with metabolic syndrome during treatment with metformin, such kind of diet can influence testosterone levels (Fig. 43.1). Even if fertility has not been investigated in this pilot study, hormonal effects could be obviously important [24]. Moreover, a recent review of the literature about the influence of diet in male fertility indicated that a healthy diet improves at least one measure of semen quality, while diets high in lipophilic foods, soy isoflavones, and sweets lower semen quality [25]. However, the more diffuse approach was the effect of exogenous administration of antioxidants. We focused our attention on the main natural antioxidants, the efficacy of which has been supported by clinical trials. In particular our group focused on the study of coenzyme Q_{10} and carnitine, but we will also review the other main antioxidants.

43.2.1.1 Carnitine

L-carnitine (LC) or 3-aminobutyric acid is involved in intermediary metabolism being a shuttle of the activated long-chain fatty acids (acyl-Coa) into the mitochondria. The high levels found in epididymal fluid (2000 times higher than blood concentration) suggest the central role in sperm cell metabolism. There are evidences that show a positive correlation between an increase of L-carnitine in the epididymal lume and L-acetyl-carnitine (LAC) in sperm cells and initial sperm movement [26, 27]. Our group performed a 6-month double-blind randomized placebo-controlled trial using LC or LAC or combined LC and LAC treatment in infertile males affected by idiopathic asthenozoospermia [28]. The evaluation of the effectiveness of these treatments in improving semen kinetic parameters and the variation of TOSC in semen after treatment was the end points of the study. Sixty patients (mean age, 30 years) affected by idiopathic asthenozoospermia have been enrolled in the study, and all subjects underwent medical screening, including history and clinical examination, and presented a clinical history of primary infertility >2 years. The selected patients were submitted to a double-blind therapy of LC (10 ml phials containing 3 g/day orally of Carnitene – Sigma Tau, Italy, n. 15 patients), LAC (tablets containing 3 g/day orally of Zibren – Sigma Tau, n. 15 patients), and a combination of LC (10 ml phials containing 2 g/day orally of Carnitene) and LAC (tablets containing 1 g/day orally of Zibren) (n. 15 patients) or a seemingly identical placebo (each 10 ml placebo phial contains malic acid, sodium benzoate, sodium saccharinate dihydrate, anhydrous sodium citrate, pineapple flavoring, and demineralized water; each placebo tablet contains a core with l-hydro lactose, magnesium stearate, polyvinylpyrrolidone, and cornstarch and a coating with cellulose acetophtalate, dimethicone, and ethyl phthalate, Sigma Tau, Pomezia, Rome, Italy). All patients assumed a total of one phial and two tablets three times a day. The study design was 1-month run-in, 6 months of therapy (45 patients) or placebo (15 patients), and further 3 months of follow-up (controls at months T − 1, T0, T + 3, T + 6, and T + 9). Our results demonstrated that patients treated with the combination of the two molecules improved significantly during the first 3-month period of the administration. A significant improvement in total sperm motility was found in patients to whom

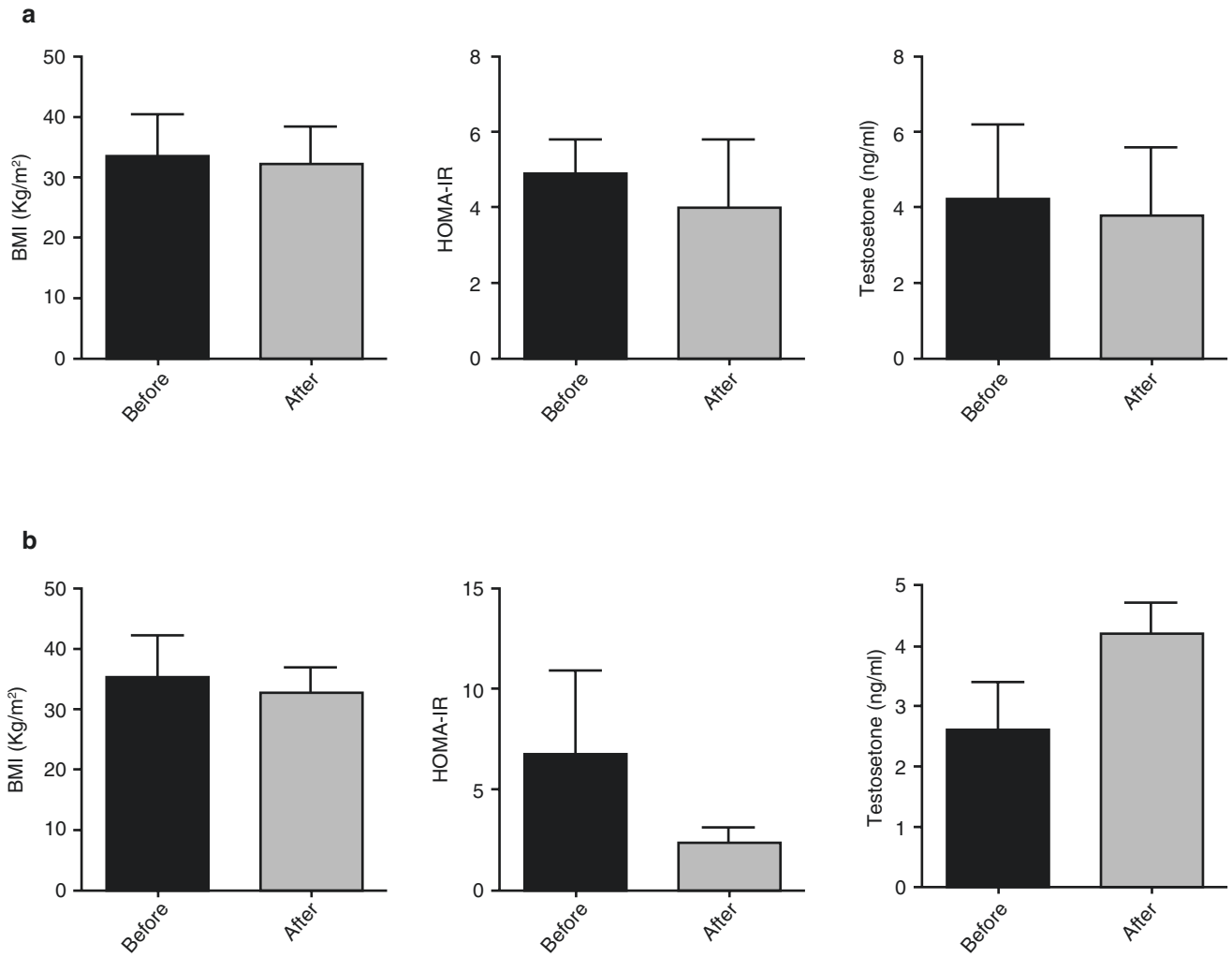

Fig. 43.1 Mean ± SEM levels of body mass index, HOMA-IR index, and testosterone levels in men with metabolic syndrome during metformin treatment: group A, hypocaloric diet; group B, hypocaloric diet enriched with natural antioxidants

LAC was administered, either alone or combined with LC. The analysis of forward sperm cell motility showed the same results. An improvement of forward motility was found when combined LC–LAC was compared with LC or LAC therapy alone, although the variations of kinetics sperm parameters were not significant. No significant modifications were found in placebo group. In all carnitine therapy groups, a significant dependence of the total and forward motility variations on the baseline values was found, and patients with lower baseline values of motility had a significantly higher probability to be responders to the treatment. Several controlled and uncontrolled studies support a potential positive effect of therapy with LC and its acyl derivatives in selected forms of oligo-astheno-teratozoospermia [29]. Lenzi et al. [30] demonstrated, in a controlled study, the efficacy of LC and LAC combined treatment in improving sperm motility, especially in patients with lower baseline levels. However, new evidence is needed to define the effective role and mechanisms of action of carnitine as an antioxidant.

43.2.1.2 Coenzyme Q10

Coenzyme Q10, also called ubiquinone, is an important component of the mitochondrial oxidative phosphorylation process because of its role of redox link between flavoproteins and cytochromes in the inner mitochondrial membrane [31]. CoQ10 also plays an import role as antioxidant; it contributes to membrane fluidity and can participate in many aspect of redox control of the cellular signaling origin and transmission; it has a probable involvement in cellular proliferation [32]. Clinically the significance of antioxidant action of ubiquinone has been clarified by many studies on lipoproteins. In fact LDL proteins are very susceptible to oxidative stress, and reduced CoQ10 present in LDL is oxidized before vitamin E, and the appearance of fatty acid hydroperoxides occurs after the oxidation of ubiquinol [33]. It can be suggested a possible

role in male infertility; in fact spermatozoa are rich in mitochondria, and they are strongly subject to oxidative stress. The previous coenzyme Q10 studies were performed with the administration of this antioxidant in cohort of unselected infertile patients, and the endogenous levels of CoQ10 were not measured [34, 35]. The first analytical data are represented by two of our works. The first one shows that the levels of CoQ10 in seminal plasma correlated with sperm count and motility except in the varicocele population. We also studied the varicocele patients after surgical repair, and the correlation between cellular CoQ10 and motility was no more detectable in postoperative VAR patients.

We conducted two different studies that showed an increase in spermatozoa motility in idiopathic asthenospermic patients undergoing CoQ10 therapy [36, 37].

We investigated the potential therapeutic role of CoQ10 by administering CoQ10 to a group of 22 idiopathic asthenozoospermic infertile patients [37], classified according to the WHO 1999 criteria [38] as having <50% forward motile forms at two distinct sperm analyses and normal sperm morphology >30%.

An increase of CoQ10 was found in seminal plasma after treatment, the mean value rising significantly from 42.0 ± 5.1 at baseline to 127.1 ± 1.9 ng/ml after 6 months of exogenous CoQ10 administration ($p < 0.005$). A significant increase of CoQ10 content was also detected in sperm cells (from 3.1 ± 0.4 to 6.5 ± 0.3 ng/106 cells; $p < 0.05$). Similarly, PC levels increased significantly both in seminal plasma and sperm cells after treatment (from 1.49 ± 0.50 to 5.84 ± 1.15 μM, $p < 0.05$, and from 6.83 ± 0.98 to 9.67 ± 1.23 nmoles/106 cells, $p < 0.05$, respectively).

Regarding semen, a significant difference was found in forward motility of sperm cells after 6 months of CoQ10 dietary implementation (from 9.13 ± 2.50 to 16.34 ± 3.43%, $p < 0.05$). The improvement of motility was also confirmed by means of computer-assisted determination of kinetic parameters. A significant increase of VCL (from 26.31 ± 1.50 to 46.43 ± 2.28 μm/s, $p < 0.05$) and VSL (from 15.20 ± 1.30 to 20.40 ± 2.17 μm/s, $p < 0.05$) was found after treatment. No significant differences were found in sperm cell concentration and morphology.

This study indicates a significant improvement of kinetic features of sperm cells after 6 months of administration of CoQ10, both on the basis of manual and computer-assisted evaluation. Moreover, these results constitute the first demonstration that exogenous administration of CoQ10 increases its levels in seminal plasma and in spermatozoa.

Similar increases of CoQ10 concentration (two to three times higher than baseline value) are commonly found in blood plasma after chronic administration of the quinine [39]. Statistical analysis did not reveal any significant functional relationship among the therapy-induced variations of CoQ10 and kinetic parameters of spermatozoa, probably due to the low number of samples. Nevertheless, the good degree of association among these variables, according to Cramer's V index of association, supports the hypothesis of a pathogenetic role of CoQ10 in asthenozoospermia, according to previously reported data [40]. Improvement of the spontaneous pregnancy rate also suggests that this therapeutic approach is beneficial.

These results were confirmed by a double-blind, placebo-controlled clinical trial, also from our group [36]. The selected patients underwent a double-blind therapy with CoQ10 (Q-Absorb softgels, Jarrow Formulas LA, USA), containing 100 mg of CoQ10, lecithin, and medium-chain glycerides. Placebo had the same composition but the softgels did not contain any CoQ10. All patients were given a total of two soft capsules in two separate daily administrations, with meals. The CoQ10 dose was similar to that used in our previous open trial on male infertility.

The study design was 1-month run-in, 6 months of therapy (30 patients) or placebo (30 patients), and further 3 months of follow-up (controls at months T − 1, T0, T + 3, T + 6, and T + 9).

CoQ10 levels increased in seminal plasma after treatment, and a significant increase of CoQ10 content was also detected in sperm cells (from 2.44 ± 0.97 to 4.57 ± 2.46 ng/106 cells, $p < 0.0001$). Similarly, QH2 levels increased significantly both in seminal plasma and sperm cells after treatment (from 31.54 ± 10.05 to 51.93 ± 16.44 ng/ml, $p < 0.0001$, and from 0.95 ± 0.46 to 1.84 ± 1.03 ng/106 cells, $p < 0.0001$, respectively). No statistically significant modifications were found in the placebo group.

A significant improvement of sperm cell total motility (from 33.14 ± 7.12 to 39.41 ± 6.80%, $p < 0.0001$) and forward motility (from 10.43 ± 3.52 to 15.11 ± 7.34%, $p = 0.0003$) was observed in the treated group after 6 months (T + 6) of CoQ10 administration. No statistically significant modifications in kinetic parameters were found in placebo group.

A significant inverse correlation between baseline (T0) and T + 6 relative variations of seminal plasma or intracellular CoQ10 or QH2 content and kinetic parameters was also found in treated group. In fact, patients with lower baseline value of motility and levels of CoQ10 had a statistically significant higher probability to be responders to the treatment.

After washout (T + 9), sperm cell kinetic features (total and forward motility, VSL) resulted to be significantly reduced in treatment groups when compared with month T + 6.

Also the group of Safarinejad confirmed a positive effect of CoQ10 treatment on sperm motility [41], in particular in improving sperm count, motility, and morphology. Our more recent work investigated the protective effects of coenzyme Q10 and aspartic acid on oxidative stress and DNA damage in subjects affected by idiopathic asthenozoospermia [42].

We found that only CoQ10 seemed to play a protective role against oxidative stress and DNA damage, thus contradicting some previous findings, which suggested these effects also for D-Asp.

43.2.2 Vitamin E

Vitamin E (alpha-tocopherol) is a fat-soluble antioxidant vitamin that can neutralize free radicals and protect cell membranes against ROS. Vitamin E can also protect against DNA damage, as demonstrated by Greco et al. in a placebo-controlled study of infertile men [43]. In this study vitamin E didn't improve seminal parameters (motility and concentration). Other studies, instead, reported an improvement in motility or morphology or both with a combination of vitamin E and selenium [44].

43.2.3 Vitamin C

Vitamin C, known as ascorbic acid, is a water-soluble vitamin that plays a role as cofactor in several hydroxylation and amidation processes, and it can be found in seminal plasma. There are several studies that demonstrated that a vitamin C oral integration can improve sperm motility and count in men with idiopathic oligozoospermia [45]. Cyrus et al. demonstrated, in a double-blind randomized controlled trial, that ascorbic acid improved sperm motility but not sperm count [46]. Other studies investigated the effect of administration of vitamin C with other antioxidants with good results in terms of sperm concentration and motility [47].

43.2.4 Zinc

It has been hypothesized that even a zinc deficiency may contribute to unexplained forms of infertility. In fact zinc plays an important role in testicular development as well as in sperm maturation [48, 49]. A recent review demonstrated that seminal zinc levels were lower in infertile men and that zinc supplementation increased semen volume, sperm morphology, and motility [50]. Some studies have demonstrated that oral supplementation of zinc can improve sperm motility in men with idiopathic oligozoospermia and/or asthenozoospermia [51]. Low seminal plasma zinc levels have been associated with a decrease in fertility, and in oligospermic patients, there are lower zinc levels than in normozoospermic men [52]. Also in this case, the data are contradictory as numerous studies have not shown a statistically significant association between the zinc levels and the seminal parameters [53, 54].

43.2.5 Myo-inositol

Inositol is a polyalcohol and in nature is present in nine isoforms, and myo-inositol (MYO) is the most abundant. Recently it was studied the important role of MYO on male reproduction. Its concentrations are higher in seminiferous tubules than in serum, and it is secreted in response to FSH.

It has been suggested that MYO could be involved in some processes such as the regulation of motility, capacitation, and acrosome reaction of spermatozoa [55, 56].

MYO probably has a role in the osmoregulation of seminal fluid contributing to reduce viscosity and improve the progressive motility and velocities [57, 58].

Few studies have investigated the role of MYO as a possible antioxidant agent for the treatment of male infertility and to improve the quality of sperm used to medically assisted reproductive procedures. Colone et al. [59] demonstrated that MYO reduces the presence of amorphous material. At a functional level, MYO seems to increase the membrane potential [60] and improve the synthesis of DNA, tRNA, and proteins [61].

43.2.6 Herbal Remedy

There are emerging evidences that herbal products can also improve male reproductive functions. Recently it was demonstrated that *Eurycoma longifolia* Jack extract has, in an in vivo study, androgenic and pro-fertility effect [62, 63].

Cardiospermum halicacabum has been found to increase testosterone levels and sperm count and motility [64]. Also the *Tribulus terrestris* has long been identified for treating male infertility in Asia and Europe [65].

"MARJORAM essential oil" has demonstrated faculty to increase spermatogenic and sperm cells in a study in which degenerative changes in seminiferous tubules were determined by fatty diet [65].

43.3 Perspectives

Although several specific genetic causes of male infertility have been acknowledged, the etiology of many forms of infertility remains unknown. It would be desirable to deepen the study of the exogenous antioxidants in order to hypothesize new therapeutic horizons, especially for those patients who do not benefit from conventional therapies, and we will hope to standardize the dosage to produce new evidences.

43.4 Conclusion

Oxidant radical excess has been associated with male infertility; in fact spermatozoa are very susceptible to oxygen effects. Also healthy diet and antioxidant agents (such as carnitine, coenzyme Q10, vitamin E, vitamin C, zinc, and myo-inositol) are the most important systems for which it was demonstrated a seminal parameter improvement. There are emerging evidences that herbal products can also improve male reproductive functions. It needs more studies to confirm and refresh data from literature and to standardize new therapy for infertility.

43.5 Review Criteria

We performed an extensive research of reviews examining the relationship between antioxidants and male infertility using PubMed and MEDLINE. We considered the reviews published in the last 5 years, including the articles researched by the following keywords: "nutraceutical," "coenzyme Q10," "nutrition," "reactive oxygen species," "diet," "male infertility," "semen parameters," and "reproduction." Articles published in languages other than English were not considered.

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Synthetic Antioxidants

44

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Key Points

- Infertility remains a major global health concern and novel treatments continue to garner research interest.
- Reactive oxidative species play a major role in inflammatory processes within semen.
- The link between leukocytospermia and poor semen quality remains up for debate within the literature.
- Synthetic antioxidants are generally safe for human use and have a similar bioavailability profile to naturally obtained antioxidants, although exceptions exist.
- Although our understanding of the interaction between ROS and male infertility continues to improve, further study is required.

44.1 Introduction

Environmental factors such as oxidative stress have been implicated broadly across multiple domains of health in both positive and negative light. The realm of male infertility is no exception, where oxidative stress has been associated with poor semen quality. Reactive oxidative species (ROS) have been shown to cause cellular injury by multiple mechanisms. Antioxidants which are shown to inactivate ROS may protect cells from this damage. Causes of increased ROS in men with less than optimal sperm quality are an active area of investigation. Furthermore therapeutic modalities to promote antioxidant activity and thus promote improvement of semen parameters are also an area of investigation. Antioxidants may be found to be active within cell signaling pathways as well as consumed in food or dietary supplements. With the largely unregulated nature of the food supplement market, it is difficult for the average consumer to find validated information on the efficacy of various synthetic antioxidants. In this chapter we aim to provide the most up-to-date definition of synthetic antioxidants, their mechanisms, and how they may ameliorate the effects of oxidative stress levels and semen quality in men with male factor infertility.

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44.2 Synthetic Antioxidants

Infertility is a major clinical concern globally. Worldwide infertility rates from available areas range from 12.5% to 20%. Of these the male factor accounts for between 4.5% and 12% based on the geographical location [1]. Many men with male factor infertility have suboptimal semen quality, the etiology of which is poorly understood. Many lifestyle, environmental, genetic, and physiological factors, including oxidative stress induced by ROS, have been implicated [2–5]. Although oxidative stress is in and of itself a risk factor for male infertility, it is also the final common result of multiple environmental and modifiable risk factors [6]. Oxidative stress induces significant damage to sperm, through decreased motility, increased DNA damage [7–9], lipid peroxidation [10–12], and decreased oocyte-sperm fusion [13]. While excessive levels of ROS can negatively impact sperm quality, low levels of ROS have been shown to be required for sperm capacitation, hyperactivation, sperm-oocyte fusion, and other critical cellular processes [14, 15].

44.3 Reactive Oxygen Species and Antioxidants

The majority of aerobic metabolism utilizes oxidative phosphorylation within mitochondria. Similar to other cells in the body, spermatozoa also produce reactive oxygen species as a natural consequence of the electron transport chain in the

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mitochondria [16]. Free radicals are defined as oxygen molecules containing one or more unpaired electrons. Free radicals induce cellular damage when they pass this unpaired electron onto nearby structures [17]. Oxidative stress has detrimental effects at the cellular level via various mechanisms. DNA damage occurs when ROS lead to DNA breaks, mutations, deletions, breakdown of bases, and protein cross-linking. Cell membrane disruption may occur when ROS disturbs the lipid membrane bilayer via peroxidation and thus may increase tissue permeability. ROS can also negatively affect proteins via alterations of peptide chains. Furthermore, ROS may negatively impact signal transduction within the cell via alteration of genetic expression [18].

Oxygen is especially susceptible to free radical formation, as it normally has two unpaired electrons. For example, the addition of one electron to molecular oxygen (O2) forms a superoxide anion radical (O2•−), the primary form of ROS. Superoxide is then directly or indirectly converted to secondary ROS, including hydroxyl radical (•OH), peroxyl radical (ROO•), or hydrogen peroxide (H2O2) [19].

In cell mitochondria, adenosine triphosphate (ATP) is produced when oxygen is reduced to water via proton and electron transfer reactions. However this process does leave a minor proportion of oxygen molecules that are not reduced [18]. ROS are also formed during the normal enzymatic reactions of inter- and intracellular signaling [20]. ROS generation by leukocytes as a cytotoxic mechanism of host defense, hypoxic states, and a wide array of drugs with oxidizing effects all contribute to oxidative stress. Significant cellular damage by oxidative stress is prevented through enzymatic and nonenzymatic antioxidant pathways which scavenge excess ROS. This oxidant-antioxidant system allows a critically important balance to be achieved, allowing beneficial oxidant generation for proper cell function, while preventing damaging oxidative stress.

Under normal conditions an overall low level of oxidative stress in the semen is maintained largely due to the relatively short-lived duration of ROS activity [16]. Thus this system allows for normal cell signaling processes and normal spermatic function while avoiding oxidant-induced cell damage. In contrast, the pathological effects of oxidative stress arise under conditions where levels of unscavenged ROS increase, thus disrupting the delicate oxidant/antioxidant balance, significantly impacting both sperm quality and function [13, 17, 20]. ROS-induced sperm damage may be a significant contributing factor in 30–80% of all cases of male infertility [17, 20].

Free radicals are scavenged most commonly by one tripeptide, glutathione, and three enzymes important for cellular metabolism. Glutathione, which contains a sulfhydryl group that directly scavenges free radicals, is the most important intracellular defense against ROS. Once oxidized, glutathione is then regenerated/reduced by glutathione reductase and NADPH to complete the cycle [13]. Of the three antioxidant enzymes, superoxide dismutase (SOD) is a metal-containing enzyme that catalyzes two superoxides into oxygen and hydrogen peroxide, which is less toxic than superoxide [21]. Catalase, an enzyme found in peroxisomes, then degrades hydrogen peroxide to water and oxygen, thereby completing the reaction started by SOD. Glutathione peroxidase, as well as other enzymes such as glutathione transferase, ceruloplasmin, or heme oxygenase, also acts to degrade hydrogen peroxide.

Pyruvate and vitamins E and C also play critical roles as nonenzymatic antioxidants [13]. Vitamin E protects cell membranes from oxidative damage by scavenging free radicals within the cellular membrane. Vitamin C is a water-soluble antioxidant that reduces a variety of free radicals and also recycles oxidized vitamin E.

44.4 Oxidative Damage to Sperm Cells

Spermatozoa are particularly sensitive to oxidative stress. The majority of the antioxidant enzymatic buffering capacity (i.e., SOD, glutathione peroxidase and catalase, vitamins E and C) is contained in the seminal fluid [13]. The polyunsaturated fatty acids found in sperm cell membranes are exquisitely sensitive to peroxidation, making sperm more susceptible to lipid membrane damage than other non-germ cells [10, 11]. Maintaining a healthy antioxidant level is further exacerbated by the ROS production by spermatozoa during its lifespan. Sharma et al. prospectively enrolled 32 infertile men compared with 20 healthy donors and assessed their seminal fluid for parameters of oxidative stress. The fluid was subjected to proteomic analysis, and subjects were categorized based on their degree of oxidative stress. Those with less markers of ROS were found to have unique proteins in their seminal plasma potentially protecting against oxidative stress [22]. Conversely, levels of these antioxidants in the sperm cytoplasm are minimal due to the extremely low volume of spermatic cytoplasm as well as the fact that most of these cytoplasmic antioxidants are lost in spermiogenesis [23].

There are multiple sources of ROS in the seminal fluid. The prime offenders are leukocytes and spermatozoa. Activated leukocytes are a considerable source of ROS in the semen. Most semen specimens contain variable numbers of leukocytes, with neutrophils noted as the predominant type [24–26]. Neutrophils function by generating and releasing high concentrations of ROS to form cytotoxic reactions against nearby cells and pathogens. Prior to the initial publication of this chapter, many studies had investigated the correlation between leukocytospermia and oxidative stress injury to sperm; however, the relationship between leukocytes in the semen and male infertility remained

unclear [19, 24, 27–35]. Today controversy still remains. Aggarwal and associates investigated 88 men with leukocytospermia and found significant positive correlation with oxidative stress [36]. On the other hand, Micillo and colleagues demonstrated correlation between oxidative DNA damage and poor semen quality but not to leukocyte subpopulation [37].

Although controversy exists regarding the link between the presence of seminal leukocytes and sperm quality, the preponderance of recent literature points to a more definitive link. The prevalence of leukocytospermia is reported with significant diversity. Lackner and colleagues reviewed available literature and noted rates from 16.1% to 60.7% [38]. Leukocytospermia has long been associated with decreased sperm concentration, motility, and morphology, as well as decreased hyperactivation and defective fertilization. Sandoval and colleagues noted consistent links between leukocytospermia and derangements of sperm motility and morphology [39]. Moskovtsev et al. also analyzed the relationship between leukocytospermia and sperm DNA damage in 1230 unselected non-azoospermic infertility patients [40]. While the authors found no significant relationship between leukocytospermia and DNA integrity, a significant negative effect was again noted between the presence of leukocytospermia and corresponding sperm concentration, motility, and morphology. The link between leukocytospermia and morphologic abnormalities was more recently supported by Singh et al. The authors found leukocytospermia in 65.5% of sperm with abnormal morphologic characteristics compared with 17% of cases with normal morphologic features, a difference which reached statistical significance [41]. The presence of seminal leukocytes, although not always considered leukocytospermia by World Health Organization (WHO) guidelines, has been shown to be associated with DNA fragmentation even in low levels [42]. While the significance of leukocytospermia on the fertility potential of the individual patient remains difficult to quantify, it can nonetheless be considered a marker of urological or systemic inflammation and possible sperm dysfunction.

44.5 Synthetic Antioxidants

Due to the cost and difficulty of chemically extracting and isolating vitamins and other dietary antioxidants from their food source, a broad number of antioxidants are chemically synthesized and packaged in a pill form as an isolate compound. Much of the safety profile of synthetic antioxidants has been addressed in studies investigating their use as food preservatives. The European Food Safety Authority has evaluated data on the most commonly used antioxidant food preservatives and noted exposure below certain thresholds is safe [43]. Several studies have suggested that these synthetic antioxidants offer suboptimal antioxidant properties due to their chemical composition and the fact that they are isolated from other synergistic compounds present in normal food sources [44, 45].

Several large clinical studies have been performed investigating the efficacy of these antioxidant isolates, used alone or in combination with one other antioxidant supplement. Pasechnikov et al. published a review of the current literature regarding antioxidant prevention of gastric cancer noting that ascorbic acid, carotenoid, and beta-carotene have been shown to have anti-oncotic effects. However the authors noted that multiple double-blind, placebo-controlled trials showed conflicting results and were limited by loss to follow up [46]. Bjelakovic et al. postulated that antioxidant supplements have not been proven to have a distinct benefit and may be overall harmful [47, 48]. They investigated the role of synthetic vitamin A, vitamin E, and beta-carotene supplements on overall morbidity and mortality in patients with gastrointestinal cancers. The authors concluded that antioxidant supplements, with the potential exception of selenium, were without significant effects on gastrointestinal cancers and, most concerning, were noted to increase all-cause mortality.

This same group went further and performed a meta-analysis of 68 randomized trials with 232,606 participants to assess the effect of antioxidant supplements on all-cause mortality in randomized primary and secondary prevention trials [49]. All included trials used beta-carotene, vitamin A, vitamin C (ascorbic acid), vitamin E, and selenium, either alone or in combination, and compared these groups to placebo or no intervention. In 47 trials with 180,938 participants, the antioxidant supplements were overall shown to significantly increase mortality (RR, 1.05; 95% CI, 1.02–1.08). After exclusion of the trials involving selenium, patients taking synthetic beta-carotene, vitamin A, and vitamin E, either singly or combined, had significantly increased overall mortality, whereas patients taking synthetic vitamin C and selenium had no significant effect on mortality.

These authors and others have postulated that natural antioxidants obtained from food sources contribute multiple antioxidants and other cofactors that have been shown to act in synergy with each other, potentially increasing the overall antioxidant capabilities of these agents. This theory has also been put forward by Liu who hypothesized that the anti-oncotic effects of antioxidants are a result of the synergistic effect of fruit and vegetable phytochemicals rather than their use in isolation [50]. A single antioxidant in high doses will donate a single electron to scavenge free radicals. However, if these "spent" or oxidized antioxidants are present in higher concentrations than the enzymes and cofactors that "recycle" or reduce the antioxidants through reduction-oxidation pathways, these high-dose synthetic antioxidants may actually increase the oxidative stress environment within the relevant physiological system. These theories offer a possible

explanation for the broad number of antioxidant trials noting no improvement in a disease process or showing a detrimental effect [51].

Here, we review the mechanism of action of these vitamins and further delineate the potential toxicities and side effects of these vitamins and dietary supplements. Overall, it should be noted that most of these dietary supplements can have a detrimental effect on patient health, and our center counsels all patients regarding the potential for toxicity with misuse of these supplements.

44.6 Mechanisms of Action

44.6.1 Vitamin E

Synthetic vitamin E is a general term used to describe a group of tocopherols, of which α-tocopherol has the highest biological activity. It is interesting to note that vitamin E has been recognized as an essential nutrient for reproduction since its discovery in 1922 [52]. Recent identification of the precise mechanisms of vitamin E catabolism has shown that it is metabolized via a cytochrome p450-mediated system [53]. Four vitamin E isoforms, α-, β-, γ-, and δ-tocopherol, are produced and stored in leaves and seeds. They are differentiated on the basis of the location of methyl groups on a chromanol ring. While α-tocopherol has been successfully used therapeutically, the remaining isoforms have not been validated [54].

Multiple mechanisms by which α-tocopherol acts as an antioxidant have been shown. In vitro and in vivo documentation of peroxyl radical scavenging has been previously elucidated [54, 55]. Multiple studies investigating measures of reduction of DNA damage have also demonstrated objective reductions in the setting of vitamin E; however the data is mixed [54]. Additionally, α-tocopherol has also been shown to have antioxidant functions apart from free radical scavenging such as inhibition of protein kinase C and subsequently monocyte NADPH oxidase [56, 57]. It is important to note that pro-oxidative functions of α-tocopherol have also been noted in healthy volunteers [58]. While anti-inflammatory roles have been found for β- and γ-tocopherol, they exist in much lower serum concentrations than their α counterpart [54]. In contrast to the other isoforms, δ-tocopherol has been shown to have a pro-inflammatory role promoted by ROS as well as anti-oncotic and antiangiogenic functions [54, 59].

Additional unsaturated isoforms of vitamin E, the tocotrienols, have more recently come into investigational interest as an antioxidant. Like their saturated counterparts, tocotrienols consist of α-, β-, γ-, and δ-isomers and have been shown to have effective cellular antioxidant and anti-inflammatory roles [58].

In regard to maintaining proper male fertility, optimal function of vitamin E has been linked to optimal levels of selenium. It has been shown that vitamin E scavenges free radicals generated from lipid peroxidation and that the by-products of these scavenged radicals, hydrogen peroxide molecules, are in turn reduced by glutathione peroxidase, a selenium-dependent enzyme [60]. Furthermore it has been shown that supplementation of antioxidants, including vitamin E, has been shown to increase pregnancy rates in the setting of assisted reproduction [61].

Synthetic vitamin E may therefore indeed play an injurious role in cellular function. Since the optimal role of vitamin E has been linked to the presence of optimal concentrations of other micronutrients and vitamin E has been shown to have pro-oxidant properties, supplementing with high levels of synthetic vitamin E may in fact lead to a pro-oxidant state due to a buildup of hydrogen peroxides or oxidized tocopherols. These data should be considered when prescribing vitamin E and when discussing dietary supplements with patients.

44.6.2 Vitamin A

Vitamin A is a fat-soluble vitamin that is required for proper vision. In clinical trials, vitamin A was shown to improve semen parameters, most significantly by improving the oxidant stress levels of the semen [62–64]. In its pro-oxidant role, vitamin A has been shown to act by neutralizing peroxyl radicals prior to their oxidative effect on lipids [43]. One caveat in these trials is that vitamin A was given in conjunction with other antioxidants, which could have induced the changes in parameters.

44.6.3 Vitamin C

Vitamin C, or ascorbic acid, is considered a vitamin because humans cannot synthesize it enzymatically and must instead obtain it through dietary intake. Vitamin C is a monosaccharide catalyst of oxidation–reduction (redox) reactions in human physiology. Vitamin C is also required for the conversion of procollagen to collagen through the oxidation of proline residues to hydroxyproline, where vitamin C deficiency leads to scurvy [65].

Ascorbic acid is capable of donating two electrons, thus preventing oxidation. An ascorbyl radical, also known as semidehydroascorbate, is formed after giving away one electron. In this configuration the ascorbyl group is very stable and is an excellent scavenger of free radicals. Upon donating its second electron, a compound known as dehydroascorbate is formed [66]. When vitamin C is oxidized, it is recycled back to an antioxidant form via the NADPH pathway, as well as by the glutathione pathways [65]. It is therefore not

surprising then that synthetic vitamin C in very high doses has been shown to have a detrimental clinical effect. Multiple studies have shown that synthetic and food derived sources of vitamin C have similar bioavailability [67]. Regular intake of high doses of synthetic vitamin C as a single dietary supplement may overwhelm the recycling pathways, leading to excessive levels of oxidized vitamin C, which would then act as an oxidant on the cellular system.

44.7 Safety, Dosing, and Side Effects of Toxicity

Adverse events due to vitamin toxicity are almost exclusively seen due to overconsumption of synthetic vitamin supplements [68]. Adverse events associated with vitamin use can be of similar magnitude as conventional pharmaceuticals, and significant toxicity can occur with high intake of most vitamins [68]. For some vitamins, such as the B vitamins, vitamin C, and vitamin K, adverse reactions are minor and usually reversible. Other vitamins, such as vitamin A or vitamin E, have been shown to cause serious, irreversible adverse events. We will review these significant adverse events and safety profiles of potentially dangerous antioxidant vitamins.

44.7.1 Vitamin E

Vitamin E is a lipid-soluble vitamin, capable of reaching toxic tissue concentrations through storage in the liver and fatty tissues of the body. In healthy adults, 200–800 mg/day may cause gastrointestinal distress, and 800–1200 mg/day may induce antiplatelet effects and bleeding disturbances, thrombophlebitis, elevated creatinine, and gonadal dysfunction [69, 70]. In several large, multicenter, randomized trials investigating the potential benefits of vitamin E on prevention of cardiac events, many of these studies described a significant increased risk of congestive heart failure. In the multicenter GISSI-Prevenzione trial studying 11,000 patients with previous myocardial infarction, vitamin E showed no benefit for all study endpoints but a 20% increased risk of developing congestive heart failure [71, 72]. In over 9500 patients studied under the multicenter Heart Outcomes Prevention Evaluation randomized trial, treatment with vitamin E did not prevent cancer or cardiovascular events but did increase the risk of heart failure [73, 74]. High-dose vitamin E can also synergistically interact with vitamin K to exacerbate bleeding diatheses, especially in patients already taking anticoagulation or antiplatelet therapy [75, 76]. Among 30,000 male smokers, a higher incidence of hemorrhagic stroke was noted in men taking vitamin E [77].

The current recommended intake for vitamin E in the USA is 15 mg α-tocopherol in adults with a minimum age of 14 years [78]. High-dose vitamin E has been shown to increase the overall cancer and mortality risk. The largest study, a meta-analysis of 19 clinical trials investigating over 135,000 patients, demonstrated that 400 IU/day or higher may increase all-cause mortality [79]. In the Women's Health Study, multivariate analysis demonstrated that vitamin E serum levels were associated with increased risk of both invasive and noninvasive breast cancers [80]. In men, supplementation with vitamin E has been demonstrated to increase risk of prostate cancer, especially in men taking vitamin E in conjunction with other supplements [81].

44.7.2 Vitamin A

Vitamin A has been associated with nausea, blurred vision, anorexia, and mental status changes, as well as electrolyte disorders [82]. Vitamin A is mainly stored in the liver. Vitamin A toxicity ranges from elevated liver function tests to cirrhosis, hepatic fibrosis, and death due to liver failure. In over 2000 men aged 49–51 years old, risk of fracture was seven times higher in men with elevated serum retinol compared to men with the lowest retinol levels [83]. Retinoic acid, metabolite of vitamin A, has been shown to intensify graft versus host disease after allogenic hematopoietic stem cell transplantation [84].

In a large, randomized, multicenter, double-blind, placebo-controlled trial of more than 29,000 male smokers receiving beta-carotene in Finland, as well as in a study of 18,000 men in the USA, the incidence of lung cancer in these men was 18% higher in men taking beta-carotene supplementation compared to the placebo group [77]. The Japan Public Health Center-based prospective study investigated the association of dietary antioxidant vitamins such as retinol, vitamin C, vitamin E, α-carotene, and β-carotene and lung cancer risk. While null associations were observed for other antioxidant vitamins, higher consumption of retinol may be associated with an increased risk of lung cancer in men [85].

In women, two large studies demonstrated conflicting results in female smokers taking beta-carotene supplementation. In one study, the Women's Health Study, no benefit or harm was noted in the incidence of lung cancer and cardiovascular events, whereas a similar study design in another trial also showed no benefit with a significant increased incidence of mortality from both lung cancer and cardiovascular disease [86, 87].

44.7.3 Vitamin C: No Updated Data on Toxicity of Vitamin C

Vitamin C is generally well tolerated, but in large doses, acute adverse effects can include nausea, vomiting, esophagitis, heartburn, fatigue, insomnia, and diarrhea [88].

Long-term vitamin C intake can induce crystallization of urate, oxalate, cysteine, and other drugs in the urinary tract [89]. In a prospective cohort study of over 45,000 men without history of nephrolithiasis, vitamin C intake may increase risk of stones, and multivariate analysis in men consuming 1000 mg/day versus those consuming less than the recommended daily allowance showed a significant risk of stone formation [90]. However, the comparison of men taking below the recommended amount versus men taking doses measured in grams could be questioned.

In a study of over 1900 postmenopausal diabetic women, vitamin C was associated with a dose-related increased risk of coronary artery disease, stroke, and overall cardiovascular mortality [91]. More significantly, in the Los Angeles Atherosclerosis Study, investigating the impact of vitamin C supplementation on more than 500 men and women without symptomatic cardiovascular disease, carotid inner wall thickening was noted in males taking 500 mg of vitamin C per day [92].

While these studies demonstrate a significant increased risk of morbidity and mortality in vitamin C supplementation, Lee et al. directly demonstrated that vitamin C can break down lipids in cell membranes into compounds that act as genotoxins, leading to increased levels of DNA damage [91]. This study is one of the few to directly demonstrate the potential mechanisms by which vitamin C may induce cell injury and decreased cellular function.

44.8 Management of Oxidative Stress in Male Infertility

44.8.1 Vitamin/Antioxidant Supplementation

The antioxidants α-tocopherol (vitamin E), ascorbic acid (vitamin C), and the retinoids (vitamin A) are all potent scavengers of ROS. Many studies have investigated the role of these and other antioxidants on improvements in sperm parameters. However, the majority of these studies are uncontrolled, focus on healthy men without infertility, or have indirect endpoints of success. Several other studies are noted due to the quality of their study design and demonstrate compelling evidence regarding efficacy of antioxidants toward improving semen parameters.

Silver et al. surveyed 97 healthy nonsmoking men aged 20–80 years old regarding antioxidant intake using a dietary questionnaire and subsequently examined semen samples [93]. Those with high daily intake of antioxidants were noted to have improved semen quality compared to men with low or moderate intake, thereby demonstrating some correlation between increased dietary antioxidant intake and improved semen parameters.

Keskes-Ammar et al. examined the therapeutic efficacy of increased antioxidant intake on semen parameters [94]. They randomized 54 men to either vitamin E and selenium or vitamin B for 3 months, with examination of semen samples quantifying the lipid peroxidation marker, malondialdehyde (MDA), as well as measurement of serum vitamin E levels. Although only 20 patients completed the study protocol, results indicated that vitamin E and selenium supplementation produced a significant decrease in MDA concentrations with improved sperm motility, whereas vitamin B showed no impact. Suleiman et al. randomized their cohort of asthenozoospermic men with normal female partners to vitamin E or placebo for 6 months, noting decreased MDA levels and increased motility, as well as increased pregnancy rates in the vitamin E arm [95].

Conversely, Rolf et al. randomized 31 men with asthenospermia to either 2 months of high-dose oral treatment with vitamins C and E or placebo and investigated semen parameters [96]. The authors found no changes in semen parameters during treatment, and no pregnancies were initiated during this period. Stenqvist et al. randomized 77 men from infertile couples with normal testosterone, LH, and FSH levels to receive combined antioxidant treatment or placebo to evaluate the efficacy in subfertile men. An increase in sperm concentration was more pronounced in the antioxidant group however not statistically significant [97].

The best designed trial by Greco et al. examined the impact of increased antioxidant intake in a randomized, prospective manner [78]. A group of 64 infertile men with >15% DNA-fragmented spermatozoa were randomized into two groups to receive either 1 g of vitamin C and E daily or placebo for 2 months. While no differences in basic sperm parameters were noted, the antioxidant cohort demonstrated a significantly reduced percentage of DNA-fragmented spermatozoa. The authors further went on to demonstrate that supplementation with vitamins E and C significantly increased rates of clinical pregnancy and implantation following ICSI [98].

44.9 Conclusion

In conclusion, infertility is a major global health concern with rates quoted as high as 20% and male factor accounting for up to 12% based on geographic location. Understanding the complex interplay between ROS and antioxidants is key to furthering the knowledge and treatment of this disease. ROS are not completely negative in their impact; however, low levels have been shown to be required for sperm capacitation and other critical cellular processes. Antioxidants are critical in mitigating the sometimes detrimental effects of ROS in semen. Natural antioxidants come in many forms including

glutathione, pyruvate, as well as endogenous vitamins E and C in nonenzymatic interactions. Synthetic antioxidants have come into popularity due to the difficulty of extracting dietary antioxidants from their food source. Despite their potential positive effects, the literature is mixed on the benefit from ingesting synthetic antioxidants which when misused may have detrimental health effects. Vitamin E has been shown to have positive effects in multiple diseases; however high doses are associated with increased cancer mortality. Data on vitamin A remains similarly mixed with large public health studies demonstrating conflicting results on incidence of cardiovascular events. Vitamin C is generally well tolerated; however, some data shows detrimental DNA damage. Multiple trials have examined the clinical efficacy of using synthetic antioxidants to improve semen parameters; although data is mixed, improvements in levels of DNA damage have been shown in well-designed trials.

44.10 Review Criteria

An extensive search of studies examining the relationship was performed using search engines such as ScienceDirect, OVID, Google Scholar, PubMed, and MEDLINE. The start and end dates for these searches were January 2011 and January 2019, respectively. The overall strategy for study identification and data extraction was based on the following keywords: "male factor infertility," "reactive oxygen species," "oxidative stress," "natural antioxidants," "synthetic antioxidants," "leukocytospermia," and "vitamin toxicity" as well as the names of specific oxidative stress markers. Articles published in languages other than English were also considered. Data that were solely published in conference or meeting proceedings, websites, or books were not included. Websites and book-chapter citations provide conceptual content only.

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New Developments for the Enhancement of Male Reproductive Health Using Antioxidant Therapy: A Critical Review of the Literature

45

Kelton Tremellen

Key Points

- Oxidative stress is a significant cause of impaired sperm function resulting in infertility, miscarriage and possibly even long-term health consequences for the next generation.
- Optimal prevention of sperm oxidative stress should focus on reduction of leukocyte ROS production using anti-inflammatory therapies (NSAID, probiotics, omega-3 fish oils), neutralization of ROS by traditional antioxidants (vitamins C and E, lycopene, coenzyme Q10 and the like) and fortification of sperm against ROS-mediated damage (DNA protamination using zinc/selenium).
- The evidence for treatment of male infertility with antioxidants is difficult to critically interpret because of underpowered studies using a large number of different types and dosages of antioxidant, failing to screen for oxidative stress at enrolment, selective reporting on sperm quality rather than pregnancy as the primary endpoint and a lack of concurrent placebo controls.
- Direct antioxidants such as vitamin E, vitamin C, selenium, lycopene, coenzyme Q_{10} and astaxanthin all appear to improve sperm health by reducing seminal ROS levels and decreasing sperm membrane peroxidation and oxidative DNA damage; in addition, some evidence show that these antioxidants may improve natural and in vitro conception.
- Anti-inflammatory therapies such as omega-3 fish oil and probiotics/prebiotics have been shown in RCT to improve sperm function, but no study to date has analysed their ability to enhance pregnancy rates.

45.1 Introduction

Infertility is a condition that affects one in six couples, with impaired sperm quality playing a role in at least half of all cases of infertility. Of even more concern is the evidence suggesting that sperm quality has actually been decreasing over the past 50 years [1], leading to more and more couples requiring expensive fertility treatments such as in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI). This trend has prompted researchers to focus more on identifying the underlying causes of male infertility, allowing treatments to be tailored to pathology, rather than a reliance on generic "mechanical" solutions such as ICSI.

While the identifiable causes of male infertility are many and varied (reviewed in Chap. 1), oxidative stress has been identified as a very significant cause.

Infertile men's semen contains higher levels of reactive oxygen species (ROS) and lower levels of protective antioxidants than fertile men, thereby placing these men's sperm at increased risk of oxidative damage. It has been estimated that between 30% and 80% of infertile men have some evidence of oxidative stress damage to their sperm, even when routine semen analysis results (concentration, motility and morphology) are within the normal WHO prescribed range [2]. Secondly, in vitro studies have confirmed that the direct application of ROS to sperm or the stimulation of sperm's own production of ROS can reduce sperm motility, membrane integrity and DNA quality, all linked with reduced male reproductive capacity. Finally, direct application of antioxidants in vitro can block the harmful effects of ROS on sperm motility and DNA integrity, confirming a causal association between oxidative stress and impaired male reproductive function. In summary, there appears to be a very sound scientific rationale to the use of antioxidants to treat male infertility.

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45.2 "Taming the Flames": A Holistic Path to Preventing Sperm Oxidative Stress

> "An ounce of prevention is better than a pound of cure"—Benjamin Franklin (American statesman, 1736)

While this famous axiom was made by Franklin in reference to fire prevention, it is equally true when applied to "taming the flames" of ROS damage to sperm. Oxidative stress is the net result between the production of potentially destructive ROS by sperm and seminal leukocytes and their neutralization by protective antioxidants [2]. As such, the optimal medical treatment of sperm oxidative stress should address both sides of this potentially destructive process. However, while a multitude of studies have examined the impact of antioxidant preparations that directly neutralize ROS, very little attention has been paid to therapies that may actually reduce the initial production of ROS or alternatively fortify the sperm against ROS attack through non-antioxidant mechanisms. The first part of this chapter will focus on the traditional antioxidant "ROS neutralization" approach, where the later parts will discuss some of the newer and less well-known therapies for reducing the production of ROS through anti-inflammatory action, plus fortifying sperm DNA against ROS attack.

45.3 Antioxidant Therapy for the Treatment of Male Infertility

This critical review of the literature will primarily focus on well-conducted randomized controlled trials (RCT) using various antioxidant and anti-inflammatory (indirect antioxidant action) agents in the setting of male infertility. The principal outcomes of interest are sperm quality, biochemical evidence of antioxidant effect and pregnancy outcome. Where appropriate, important non-randomized therapeutic and observational studies, plus supportive animal work, will also be outlined. Trials with poorly defined treatments such as the use of "general multivitamins" or studies that employed botanical preparations with poorly defined antioxidant action were excluded from this review.

Table 45.1 outlines the existing RCT evidence for the use of antioxidants to improve sperm function and treat infertility. With the existence of such a large body of evidence, one would expect that definitive conclusions on the value of antioxidant supplements in the treatment of male infertility would be able to be made. Unfortunately, this is not the case for several reasons. Firstly, there is a huge variation in the different types and dosages of antioxidants used in the published studies. Secondly, most of the studies are small and therefore underpowered, making meaningful analysis of differences in pregnancy outcomes with antioxidant therapy very difficult. Thirdly, many trials lacked appropriate inclusion criteria such as screening men for the presence of oxidative stress before enrolment. Finally, the paucity of clinically relevant endpoints such as live birth, rather than mere improvement in sperm quality, hampers the drawing of firm conclusions on the benefit of antioxidant preparations as a treatment for infertility [3].

The overview in Table 45.1 summarizes the methodological strengths and weaknesses of the available placebo-controlled antioxidant trials published up to January 2019. The finer detail of the various medications and trials is outlined below, thereby allowing the reader to make their own conclusions on the merits of antioxidant supplement therapy for the treatment of male infertility.

45.4 Vitamin E

Vitamin E is an essential fat-soluble vitamin, with α-tocopherol being the most common form of vitamin E available in food. Vitamin E is the major chain breaking antioxidant that directly neutralizes superoxide anions, hydrogen peroxide and the hydroxyl radical. As sperm membranes contain abundant phospholipids which are prone to oxidative damage, it is believed that vitamin E plays a critical role in protecting cellular structures from damage caused by free radicals and reactive products of lipid peroxidation. Furthermore, vitamin E exhibits some anti-inflammatory activity and, therefore, may also reduce leukocyte-initiated sperm oxidative stress.

The recommended dietary allowance (RDA) for vitamin E is suggested to be 15 mg (equivalent to 22.4 IU) of α-tocopherol per day for adult men, with the tolerable upper intake being suggested as 1000 mg (1500 IU) by the US National Institute of Health [4]. However, a meta-analysis of 19 clinical trials using long-term vitamin E supplementation in patients with chronic disease has reported that at dosages of 400 IU or greater per day, vitamin E may actually increase overall mortality compared to placebo [5]. Furthermore, it is known to inhibit platelet aggregation and has been linked with an increased risk of haemorrhagic stroke. Therefore, its use in infertile men on anticoagulants or at risk of serious haemorrhagic illness is probably contraindicated.

Two studies have analysed the ability of vitamin E to decrease sperm membrane oxidative damage by measurement of sperm malondialdehyde (MDA) levels before and after vitamin E supplementation. Geva et al. [6] reported that 200 mg a day of vitamin E was able to significantly reduce MDA levels within 1 month of therapy, while Suleiman et al. [7] found that the use of 300 mg of vitamin E per day for 6 months also produced a significant drop in MDA levels. Consistent with a reduction in sperm membrane oxidative damage, two studies have also reported an improvement in

Table 45.1 Randomized controlled studies examining the effect of direct antioxidant therapy on male reproductive health

Study reference	Therapy used per day	Duration of therapy (months)	Positive changes in semen quality	Positive changes in sperm OS endpoints	Positive changes in reproductive outcomes
[7]	Vitamin E 300 mg	6	↑ motility	↓ MDA	Pregnancy 17% active group vs. 0% placebo
[8]	Vitamin E 300 mg	3	Nil	Nil	Improved sperm-zona binding
[14]	Vitamin C (200 or 1000 mg)	1	↑ motility, morphology and viability	Not tested	Not reported
[16]	Vitamin C 500 mg	3	↑ motility, morphology	Not tested	Not reported
[17]	Vitamin E 800 mg, vitamin C 1000 mg	2	Nil	Not tested	None
[18]	Vitamins E and C (1000 mg each)	2	Nil	↓ sperm DNA damage	Not reported
[22]	Vitamin E 10 mg, vitamin C 5 mg, zinc 200 mg	3	Nil	Trend for ↓ MDA	Not reported
[24]	Coenzyme Q10 300 mg	6	↑ concentration and motility	Not tested	No difference in pregnancy rates
[25]	Coenzyme Q10 200 mg	3	Nil	↓ MDA	Not reported
[27]	Ubiquinol 200 mg	6	↑ concentration and motility and morphology	Not tested	Not reported
[31]	Sn 100 mg, vitamin A 1 mg, vitamin C 10 mg, vitamin E 15 mg	3	↑ motility	Not tested	No difference in pregnancy rates (11% vs. 0% placebo)
[32]	Sn 200 mg, NAC 600 mg	6	↑ concentration, motility and morphology	Not tested	Not reported
[33]	Glutathione 600 mg	2	↑ motility and morphology	Not tested	Not reported
[36]	L-Carnitine 2 g	2	Nil (raw data analysis)	No change in MDA	No difference
[38]	L-Carnitine 1 g	3	↑ concentration and motility	Not tested	Not reported
[39]	NAC 600 mg	3	↑ sperm concentration	Not tested	Not reported
[40]	NAC 600 mg	3	↑ motility	Not tested	Not reported
[41]	Astaxanthin 16 mg	3	↑ motility	↓ semen ROS ↓ MDA	↑ natural + IUI conceptions
[43]	Alpha-lipoic acid (ALA) 600 mg	3	↑ concentration and motility	↑ TAC	Not reported
[44]	Vitamin E 400 mg, Sn 225 mg	3	↑ motility	↓ MDA	Not reported
[45]	Vitamin C 30 mg, vitamin E 5 mg, β-glucan 20 mg, papaya 50 mg, lactoferrin 97 mg	3	↑ motility and morphology	No change in DNA quality	Not reported
[48]	Zinc sulphate 220 mg, folate 5 mg	4	Nil	Not tested	Not reported
[49]	Zinc sulphate 66 mg, folate 5 mg	6	↑ concentration and morphology (subfertile men only)	Not tested	Not reported
[51]	Menevit® (vitamins C and E, Sn, lycopene, folate, zinc and garlic oil)	3	Not reported	Not tested	↑ IVF-ICSI conceptions on active antioxidant (38.5% vs. 16% placebo)

OS oxidative stress, *MDA* malondialdehyde, *ROS* reactive oxygen species, *TAC* total antioxidant capacity, *Sn* selenium, *NAC N*-acetyl cysteine, *IUI* intrauterine insemination

in vitro sperm fertilization capacity assessed by routine insemination IVF or the use of sperm-zona binding assays [6, 8]. No study to date has analysed the ability of vitamin E monotherapy to improve sperm DNA quality.

Two small non-controlled studies of vitamin E supplementation have reported no effect on sperm count, motility or morphology [9, 10]. In addition, two well-conducted placebo-controlled trials of 3–12 months therapy using 600 mg of vitamin E per day also reported no significant effect on sperm concentration, motility or morphology [8, 11]. Conversely, another randomized controlled trial (RCT) using 6 months of vitamin E (300 mg/day) reported

a statistically significant improvement in sperm motility but no change in concentration or morphology [7]. However, in this last trial, the "dropout" rate for patients in the placebo arm was significantly greater than that seen in the active treatment arm (20 vs. 3 patients, respectively, from a starting number of 55 patients in each study arm). This selective "dropout" from placebo raises the possibility that patients or their treating physicians became unblinded to treatment allocation during the trial, biasing the final results.

No study to date using vitamin E monotherapy for the treatment of male infertility has been adequately powered to analyse pregnancy outcomes. While some studies have reported pregnancies [7, 8], the small number of pregnancies makes clear conclusions impossible. In the Suleiman study, 17% of patients allocated to vitamin E therapy achieved a live birth, compared to none in the placebo [7]. However, as previously outlined, the large "dropout" rate in the placebo arm suggests the potential for significant bias, thereby making it impossible to make firm conclusions on the value of vitamin E to assist pregnancy in the setting of male infertility.

45.5 Vitamin C

Vitamin C (ascorbic acid) is an important water-soluble antioxidant that competitively protects lipoproteins from peroxyl radical attack, while also enhancing the antioxidant activity of vitamin E by assisting in its recycling. Seminal plasma vitamin C levels are tenfold higher than serum [12], suggesting a very important protective role for vitamin C in the male reproductive tract. The RDA for vitamin C in the adult male is 75 mg, with the tolerable upper intake limit being suggested as 2000 mg/day [4]. However, the use of high dosages of vitamin C (≥1 g/day) may be harmful since at these high concentrations vitamin C can act as a pro-oxidant and may predispose to kidney stone formation [4, 13].

A small placebo-controlled randomized study of 30 infertile men allocated an equal number of participants to placebo, 200 mg or 1000 mg of vitamin C per day for a total of 4 weeks [14]. Both dosages of vitamin C were able to significantly increase seminal plasma vitamin C levels, with the magnitude of the increase being more significant in the group treated with 1000 mg. Sperm motility, morphology and viability all significantly improved within 1 week of vitamin C therapy. While the average sperm concentration doubled on vitamin C, this did not reach statistical significance. More critical analysis of the baseline characteristics in this study suggests that randomization may not have been successful in creating study groups that were equal. For example, at entry, the percentage of abnormal sperm morphology was 45, 64 and 62% for the placebo, 200 and 1000 mg vitamin C groups, respectively. At the end of the study, abnormal sperm morphology was 41% in the placebo group and 35 and 36% in the vitamin C groups, which was statistically significant. Critical analysis of these results suggests that the two vitamin C groups' morphology results were significantly inferior to the placebo at study entry, and that following 4 weeks of vitamin C treatment, these poor morphology results simply returned to levels equivalent to that seen in the placebo. This raises the possibility of selection bias or at least a "regression to the mean" spontaneous improvement in sperm morphology. Pregnancy outcomes were not reported in this trial, although in the introduction, the authors comment that a prior unpublished pilot study had achieved a 100% pregnancy rate with vitamin C therapy ($n = 20$ patients).

A larger study that randomly allocated 75 smokers to a placebo, 200 or 1000 mg vitamin C per day reported significant improvements in sperm morphology in the 1000 mg subgroup but no significant changes in the 200-mg-treated group [15]. As these men were not infertile and were not trying for pregnancy, the implications of this study for the infertile population are uncertain.

Finally, an RCT of 115 infertile men with probable oxidative stress (varicocele) to receive either vitamin C (250 mg/day) or placebo did report the ability of this antioxidant therapy to significantly boost sperm motility and morphology over a 3-month period but had no impact on sperm concentration [16]. Therefore, the overall impression on vitamin C monotherapy is that it may enhance sperm function (motility) but is unlikely to enhance sperm production (count).

45.6 Combined Vitamin C and Vitamin E Therapy

Two excellent placebo-controlled randomized studies have examined the ability of a combination of vitamins C and E to alter sperm quality. Rolf et al. [17] reported a small RCT in which infertile patients were allocated to either placebo ($n = 16$) or 2 months of treatment with 800 mg vitamin E and 1000 mg vitamin C ($n = 15$). The inclusion criteria for this study were impaired motility, not the presence of confirmed oxidative stress. No significant difference in sperm concentration, motility or morphology was observed during therapy, and no direct assessment of oxidative damage was made. Furthermore, no pregnancies were seen in either study group during the treatment period. A similar RCT using 2 months of therapy with 1000 mg of both vitamins C and E daily or placebo also found no significant changes in sperm count, motility or morphology [18]. However, this group did observe a very significant drop in sperm DNA damage. Unfortunately, pregnancy outcomes were not reported in this study, but were reported for a non-controlled study using the same treatment protocol by the same clinical group [19].

In this later report, patients who had failed to have a successful pregnancy after at least one cycle of IVF and who had documented elevated levels of sperm DNA damage were given 2 months of vitamins C and E combination therapy before a further cycle of IVF treatment. A total of 76.3% of participants experienced a normalization of their sperm DNA damage, and this "improved" subgroup achieved an implantation rate of 19.6%. As the study did not include a concurrent placebo control, firm conclusions on pregnancy effect are not possible.

Several small non-placebo-controlled trials have also examined the effect of vitamins C and E combinations on sperm quality. Kodama et al. [20] was able to show a significant drop in sperm DNA oxidative damage (8-OHdG) and MDA with 2 months of therapy (200 mg vitamin C, 200 mg vitamin E and 400 mg glutathione). They also reported a small but significant increase in sperm concentration but no effect of antioxidant supplementation on sperm motility or morphology. Menezo [21] observed a significant drop in sperm DNA fragmentation with 2 months of antioxidant therapy (400 mg vitamins C and E per day, plus low dosages of vitamin A, zinc and selenium) but no change in sperm concentration, motility or morphology. Interestingly, these investigators also noted a significant increase in sperm DNA decondensation. They believed that this was due to the high redox potential of vitamin C interfering with the reduction of cystine to two cysteine moieties, thereby opening protamine disulphide bridges. Decondensation of the sperm DNA may make the DNA more susceptible to ROS attack and may interfere with proper embryo development. Menezo, therefore, cautions against the use of antioxidant preparations containing high dosages of vitamin C in infertile men with sperm decondensation levels exceeding 20% at baseline. Finally, a small placebo-controlled study of 45 infertile men allocated to placebo, vitamin C 5 mg/vitamin E 10 mg/200 mg zinc or zinc alone observed a non-significant trend in improvement in sperm motility and a decrease in MDA in each treatment group [22]. The magnitude of improvement in MDA and motility was similar in the zinc-alone group compared to those treated with zinc and vitamins C and E. This observation suggests that the low dosages of vitamins C and E used in this study are likely to be subtherapeutic, with any improvement in sperm quality more likely to reflect the action of zinc.

Overall, the high-quality placebo-controlled studies suggest that vitamins C and E do not produce large improvements in sperm concentration, motility or morphology. The significant drop in sperm DNA damage seen in two trials [18, 21], together with observations of a drop in 8-OHdG and MDA in a non-placebo-controlled study [20], suggests that the combination of vitamins C and E can still have positive reproductive effects by enhancing sperm function even if they do not alter routine sperm parameters. The ability of vitamins C and E to improve pregnancy rates is still debatable until future adequately powered studies are conducted in this area.

45.7 Coenzyme Q_{10}

Coenzyme Q_{10} is primarily concentrated in the mitochondria of the sperm midpiece and plays an important antioxidant and energy production role in sperm. Coenzyme Q_{10} transports electrons from complexes I and II to complex III in the mitochondrial respiratory chain, leading to ATP synthesis in the mitochondrial membrane. In its reduced form (ubiquinol), coenzyme Q_{10} acts as a strong antioxidant preventing lipid peroxidation in biological membranes.

A small non-controlled study involving 38 men with male factor infertility and previous poor fertilization during IVF–ICSI therapy reported on the use of 60 mg of coenzyme Q_{10} per day for a period of 3 months [23]. This study found that coenzyme Q_{10} produced no significant changes in sperm concentration, motility or morphology, yet IVF–ICSI fertilization rates did improved significantly. This study did not measure sperm lipid peroxidation or DNA damage and offered no explanation on how coenzyme Q_{10} supplementation may boost fertilization without altering routine sperm parameters.

A large and very well-conducted placebo RCT recently reported on the use of 300 mg coenzyme Q_{10} or placebo per day for a period of 6 months in 212 men with male factor infertility [24]. At this dose of supplementation, a significant increase in seminal plasma coenzyme Q_{10} concentration was observed. Furthermore, significant improvements in sperm count and motility were also observed, together with an increase in serum inhibin B levels and a corresponding fall in FSH concentration. This would suggest that coenzyme Q_{10} therapy can enhance Sertoli cell function, not just sperm function. This study did not report on oxidative endpoints but did report a significant increase in the calcium ionophore-induced acrosome reaction, suggesting some improvement in sperm membrane function. Unfortunately, no significant difference in pregnancy rates was observed between the placebo and coenzyme Q_{10} supplement groups over a 12-month period of observation. This is not surprising when one recognizes that the magnitude of the statistically significant changes in sperm parameters observed were very small and unlikely to be of clinical significance. For example, total sperm motility after 6 months of coenzyme Q_{10} therapy was 27.6%, compared to 23.1% in the placebo group. While for the coenzyme Q_{10} group this was a statistically significant increase in sperm motility from baseline (22.2%), the final motility result was not significantly different from the placebo and highly unlikely to be of any clinical significance. As only men, who had partners with no evidence for female

factor infertility, were enrolled in the study, the lack of differences in pregnancy outcomes in such a large study suggests that coenzyme Q_{10} monotherapy is not of major benefit in assisting in vivo conception.

More recently an RCT using 200 mg of coenzyme Q_{10} or placebo over a 3-month period reported no significant change in routine sperm quality parameters but a significant decline in the lipid peroxidation marker MDA, confirming a biologically relevant antioxidant effect [25]. Another study of 300 mg of coenzyme Q10 daily did report some improvements in sperm parameters and a 34.1% pregnancy rate over the next 12 months [26]. However, as this later study had no concurrent placebo control, it is difficult to objectively assess coenzyme Q_{10}'s ability to boost natural conception.

Ubiquinol, the reduced more biologically active form of coenzyme Q_{10}, has been shown in an RCT (200 mg daily) to significantly increase sperm concentration, motility and morphology, while also boosting Sertoli cell health (reduction in FSH) [27]. These improvements in sperm quality with ubiquinol were greater than those observed by the same group using coenzyme Q_{10} [24], suggesting the former may be the optimal biologically active form of coenzyme Q_{10} therapy. Unfortunately, this RCT of ubiquinol did not assess pregnancy rate. Furthermore, a recent meta-analysis concluded that there is insufficient evidence that coenzyme Q_{10} boost the chances of successful conception [28].

45.8 Selenium

Selenium is an essential trace element required for normal male reproductive function. The antioxidant glutathione peroxidase 4 (GPX-4) is present within sperm and requires the presence of selenium to function. Not only does GPX-4 play an antioxidant role, it is also involved in augmenting sperm chromatin stability by acting as a protein thiol peroxidase. The adult male RDA for selenium is 55 μg/day, with the upper tolerable limit being 400 μg/day [4]. An individual's dietary intake of selenium depends on the selenium content in the local soil where food is grown. Men living in countries such as China where the soil is commonly selenium deficient are more likely to benefit from selenium supplementation. Conversely, excess supplementation of selenium may lead to toxicity and have detrimental effects on sperm quality [29].

Iwanier et al. [30] gave 200 μg of selenium per day to a group of men (33 infertile and 9 fertile) for a period of 2 months and measured sperm quality before and after treatment. The investigators observed a significant increase in seminal plasma selenium concentration and GPX activity during the trial but no significant improvement in sperm concentration, motility or morphology. In a small placebo RCT (n = 18 placebo, 46 active treatment), the supplementation of infertile men exhibiting low sperm motility with 100 μg of selenium (± very low dosages of vitamins A, C and E) produced no significant change in sperm concentration but a small significant improvement in motility (20.6–28.2%) [31]. The clinical significance of this improvement is questionable, as no significant difference in pregnancy rates was observed (no pregnancies in the placebo vs. 11% selenium group).

A very large placebo-controlled study randomized 468 infertile men to either placebo, 200 μg/day of selenium, with or without *N*-acetyl-cysteine, for a period of 6 months [32]. Sperm quality and male reproductive hormones were then assessed during supplementation and for a further 6 months. This study observed statistically significant increases in sperm concentration, motility and morphology in all treatment arms, together with an increase in serum inhibin B and testosterone. However, the magnitude of these improvements was again very small and unlikely to be of any clinical significance. Unfortunately, pregnancy outcomes were not reported for this study, making it impossible to draw any firm conclusions on the benefits of selenium supplementation to boost pregnancy rates.

45.9 Glutathione

Glutathione is an antioxidant released in large amounts by the epididymis that in turn can neutralize the damaging effects of superoxide anions, thereby preventing lipid peroxidation. Two trials by a single group of investigators have examined the effect of glutathione supplementation (600 μg intramuscular alternate days for 2 months) on two separate groups of infertile men. The first trial involved 20 infertile men with likely oxidative stress (past genitourinary tract infection with residual inflammation, varicocele) in a placebo crossover trial design [33]. This study observed no significant changes in sperm concentration, but significant improvements in sperm motility and morphology. These improvements were observed within 1 month of supplementation, suggesting an epididymal rather than a testicular mode of action. A second smaller non-controlled study using identical inclusion criteria examined changes in sperm lipid peroxidation with glutathione treatment [34]. This study observed improvement in all routine sperm parameters and a significant decrease in sperm MDA concentration, confirming an antioxidant effect. Neither study reported pregnancy outcome, making conclusions about the fertility promoting effect of glutathione treatment impossible. However, the requirement for intramuscular administration of glutathione therapy is certainly likely to limit its clinical application.

45.10 L-Carnitine

Carnitine is produced in the liver and then passes via the circulation to the epididymis, where it is taken up by the epididymal epithelium and actively transported into the luminal

fluid bathing sperm. In the epididymis carnitine is taken up by sperm, where it involves in energy metabolism by transporting fatty acids from the cytosolic compartment to the mitochondrial matrix.

Costa et al. [35] were the first to examine the effects of L-carnitine supplementation in the setting of male infertility. Their study group of 100 infertile men with unexplained impaired motility were given L-carnitine (3 g/day) for a period of 4 months, while measuring changes in sperm function. They reported small but statistically significant improvements in sperm concentration and motility but no changes in sperm morphology. Lenzi et al. [36] used an active medication/washout/placebo study design to determine if 2 months of L-carnitine therapy (2 g/day) could alter sperm quality. Analysis of the raw outcome data indicated no significant difference in sperm quality after L-carnitine therapy. However, when the researchers excluded several "outliers" from the analysis, a borderline statistically significant increase in sperm concentration and motility was reported. The subjective removal of "outliers" to create statistical significance, plus the failure of L-carnitine therapy to either improve epididymal function (alpha-glucosidase) or reduce levels of sperm lipid peroxidation, casts significant doubt on whether L-carnitine therapy has any beneficial effect on male reproductive performance.

Vicari et al. [37] studied the ability of L-carnitine in combination with non-steroidal inflammatory medication (NSAID) to alter sperm function in a group of 98 infertile men with confirmed oxidative stress. Two months of pretreatment with NSAIDs, followed by 2 months of L-carnitine (2 g/day), produced a significant reduction in seminal ROS production and an improvement in sperm motility and viability. A total of 23% of patients on NSAID/L-carnitine therapies achieved pregnancy, but the absence of a control group makes firm conclusions on these therapies effect on pregnancy rates impossible.

Finally, the most recent RCT of L-carnitine therapy (500 mg bd) over a 3-month period reported a significant improvement in sperm count and motility but no change in morphology [38]. This study did not report on pregnancy outcome, as was the case for all the previous L-carnitine studies. As such there is no convincing evidence that L-carnitine therapy actually translates into an improvement in chances of conception – the most clinically relevant endpoint.

45.11 *N*-Acetyl Cysteine

N-Acetyl cysteine (NAC) is believed to act as a precursor to glutathione, increasing the tissue concentration of this potent antioxidant. Recently, several good-quality placebo-controlled studies have examined the ability of NAC to alter sperm quality in infertile men with presumed oxidative pathology. Galatioto et al. [39] conducted a RCT in which 42 men with oligospermia were allocated to receive either 600 mg NAC a day plus a vitamin-mineral supplement for 3 months or no therapy at all. This small study reported a significant increase in sperm concentration but no change in sperm motility or morphology. A larger placebo-controlled study using 600 mg/day of NAC for a period of 3 months reported no change in sperm concentration or morphology but a small improvement in motility [40]. Finally, one arm of a multi-therapy RCT compared sperm quality between men with idiopathic male factor infertility on 600 mg NAC per day with placebo [32]. This study reported very minor, although statistically significant, improvements in sperm concentration and morphology but no changes in sperm motility.

The conflicting sperm quality outcomes for these three trials using an identical dose of NAC and the failure to report pregnancy outcomes make it impossible to conclude that NAC therapy has any clinically meaningful effect on male reproductive performance.

45.12 Miscellaneous Antioxidant Monotherapies

Astaxanthin is a carotenoid extract from the algae *Haematococcus pluvialis* with reported potent antioxidant qualities. A small placebo-controlled RCT reported on the effect of 3 months of therapy with this antioxidant in men with idiopathic male factor infertility [41]. Astaxanthin produced no change in sperm concentration or morphology but did produce a significant reduction in seminal ROS levels and improvement in sperm motility. Furthermore, the researchers observed a significant increase in natural or intrauterine insemination-assisted conceptions in the antioxidant-treated group, suggesting that the small improvement in sperm motility was of clinical significance.

Lycopene, an antioxidant found in high concentrations in fruits such as tomatoes and watermelon, is a powerful natural antioxidant. A non-controlled trial of 30 men with male factor infertility reported a significant improvement in sperm quality with 3 months of lycopene therapy at a dose of 4 mg/day [42]. However, upon further analysis of this study, it appears that the researchers only analysed sperm outcomes for the 14–20 men who showed an improvement in either sperm concentration, motility or morphology. Such an analysis is obviously flawed since excluding half the study participants who did not respond to treatment is clearly going to result in a significant difference being concluded. Therefore, this study provides no scientific support for the use of lycopene in male factor infertility.

Alpha-lipoic acid (ALA) is a sulphur-containing antioxidant involved in mitochondrial oxidative metabolism. A recent RCT using 600 mg of ALA per day reported a significant

increase in sperm count and motility, plus a reduction in biochemical markers of oxidative damage (MDA), but no improvements in sperm motility [43]. Unfortunately, pregnancy outcomes were again not reported.

45.13 Combination Therapies

The combination of vitamins C and E would appear to be the most commonly studied combinational antioxidant therapies for male factor infertility. However, other unique combinations have been trialed in the hope that using several different antioxidants with different modes of action may be more beneficial than antioxidant monotherapy.

The combination of vitamin E (400 mg/day) and selenium (225 μg/day) has been trialled in a placebo-controlled study of 54 men with male factor infertility [44]. Antioxidant therapy produced a small increase in sperm mobility and a drop in sperm MDA levels, confirming an antioxidant effect. No changes in sperm concentration or morphology were observed, and pregnancy outcomes were not reported. A significant weakness in this study was that out of a total of 54 initial participants, only 20 completed the study. This raises the possibility of bias and makes firm conclusions difficult.

Piomboni et al. [45] performed a controlled study comparing 3 months of therapy with an antioxidant combination (β-glucan 20 mg, papaya 50 mg, lactoferrin 97 mg, vitamin C 30 mg, vitamin E 5 mg) or no therapy. They observed a significant improvement in sperm motility, viability and morphology but no change in sperm DNA quality. Pregnancy outcomes were not reported in this study.

A small uncontrolled study of 33 men reported on the use of a combination of 600 mg NAC, 30 mg β-carotene, 180 mg vitamin E and a mixture of essential fatty acids for a period of 6 months as treatment for male factor infertility [46]. This combination produced no change in sperm concentration, motility or morphology, but a drop in seminal ROS levels, and sperm DNA oxidative damage (8-OHdG) was observed, together with an increase in the ionophore-induced acrosome reaction. A total of 22.2% of couples who completed the 6-month therapy did successfully conceive, but the absence of a control arm makes it impossible to determine if this is a clinical improvement above nontreatment levels.

A small case series reported on the success of using a combinational antioxidant (β-carotene 5000 IU, vitamin C 60 mg, vitamin E 30 IU, zinc 15 mg) for the treatment of early embryo loss related to sperm oxidative damage [47]. Out of the 17 men screened, 9 men were confirmed to have oxidative stress-related sperm pathology which could be amenable to antioxidant therapy. In six of these nine cases, the partners subsequently fell pregnant. When antioxidants had been taken by the male before conception, all pregnancies were viable (n = 4), whereas all the pregnancies conceived by men who refused antioxidant therapy miscarried. Such a small case series precludes definitive conclusions yet does suggest that oxidative pathology may be a significant cause of early pregnancy wastage.

Two RCTs of combinational zinc and folate therapy have been conducted, with one reporting no effect on sperm quality [48]. However, the earlier larger study involving 107 fertile and 103 subfertile men did report a significant increase in sperm concentration with a combination of 5 mg of folate and 66 mg of zinc sulphate for 6 months and a small (4%) increase in normal morphology, but only in the subfertile cohort [49]. Pregnancy outcomes were not reported.

One of the most widely studied combinational antioxidants in the field of male infertility is Menevit® (Bayer). This preparation consists of a combination of several natural antioxidants (vitamin C 100 mg, vitamin E 400 IU, lycopene 6 mg, selenium 26 μg, garlic oil 333 μg), anti-inflammatory action (garlic oil) and other ingredients involved in sperm DNA synthesis and packaging (zinc 25 mg, folate 500 mg). Three-month therapy with the Menevit® antioxidant has been reported to produce no significant change in sperm concentration, motility or morphology but did produce a significant reduction in seminal ROS levels and sperm DNA fragmentation [50]. Interestingly, while a dose of 400 mg of vitamin C has been shown to produce sperm chromatin decondensation by interfering with protamine disulphide bonds [21], the Menevit® antioxidant containing one-quarter dose of vitamin C has been reported to significantly increase sperm DNA protamination [50]. The Menevit® antioxidant has also been shown to improve pregnancy outcomes when compared with placebo in an RCT of 60 patients undergoing IVF-ICSI treatment [51]. Finally, recent preliminary studies have linked male infertility and sperm oxidative stress with impaired sperm DNA methylation, a possible risk factor for epigenetic disease in the next generation [52]. The treatment of infertile men with 3 months of Menevit® resulted in an improvement in the levels of sperm global DNA methylation [52]. This pilot study will require replication, and large epidemiological studies will need to confirm the link between sperm DNA methylation defects and childhood illness before definitive conclusions can be made regarding the utility of antioxidant supplements to prevent epigenetic disease in the next generation.

45.14 Therapies to Reduce Production of ROS Within the Male Reproductive Tract

While sperm numerically outnumber leukocytes by at least two orders of magnitude in the majority of men's semen, these leukocytes often play the dominant role in seminal ROS production [2, 53]. Activated leukocytes are professional

"oxidative killers" using ROS to destroy invading pathogens and clear damaged cells. In fact, on a per cell basis, leukocytes produce 1000-fold more ROS than sperm [2, 53], underlying the relative importance of inflammation in the male reproductive tract as a cause of sperm oxidative damage. Furthermore, studies have suggested that "extrinsic" oxidative stress initiated by leukocyte production of ROS can also increase the sperm's own "intrinsic" mitochondrial production of ROS – further potentiating oxidative damage to the sperm nucleus [54–57].

When in direct contact with Sertoli cells behind the protective "blood-testis" immunological barrier, sperm are relatively well protected from the ravages of leukocyte ROS production [58, 59]. However, once they pass to the epididymis, a non-immunologically privileged site [60], they can be damaged by leukocyte ROS production triggered by low-grade infection, sexually acquired or otherwise [61, 62]. Similarly, when sperm are expelled at the time of ejaculation, they may encounter leukocytes originating from the male accessory glands (MAG), with inflammation of these glands occurring with both infectious [62, 63] and non-infectious pathology (e.g. NIH class 3 nonbacterial chronic prostatitis) [64] – both leading to sperm oxidative stress [65–67].

Aside from infection, obesity has also been reported to produce a systemic state of chronic low-grade inflammation that extends to the male reproductive tract and can result in sperm oxidative stress [68, 69]. With two-thirds of the adult male population in the developed world now being overweight or obese [70], this is an increasing problem and may underline the decline in sperm health that has been observed in the past five decades [1]. The pathophysiology of obesity-related inflammation is multifactorial including the production of pro-inflammatory cytokines by adipose tissue [71] and disruption of the intestinal mucosal barrier with passage of gut bacteria into the circulation that triggers inflammation – the so-called metabolic endotoxemia [72, 73]. Furthermore, obesity is associated with lower serum testosterone and higher oestrogen levels compared with lean individuals. Given that testosterone is immunosuppressive and oestrogen pro-inflammatory [74, 75], it is not surprising that obese men's low serum testosterone to oestrogen ratio is associated with male accessory gland inflammation [76] and activated leukocyte-mediated sperm oxidative stress, even in the absence of infection.

The optimal approach to reducing leukocyte production of ROS and its associated sperm damage is fourfold.

45.14.1 Accurately Identify the Presence of Leukocytes in Semen

The traditional semen analysis often relies on identification of large number of round cells and altered semen viscosity and pH as potential signs of infection and leukospermia. However, definitive identification of pathological leukospermia requires additional testing with peroxidase staining or CD 45 immunohistochemistry [77] or alternatively chemical detection of neutrophil activation by quantification of seminal plasma elastase [78, 79]. Unfortunately, in the authors' experience, the majority of commercial pathology laboratories does not have the capacity or inclination to perform these confirmatory tests for leukocytospermia – resulting in a missed diagnostic opportunity. However, the increased adoptions of automated semen analysis platforms such as SQA-Vision, which routinely test for the presence of leukospermia using sensitive chemical test strips [80, 81], will result in better identification of pathological leukospermia in the future, with the hope for more informed treatment of this important trigger of sperm oxidative stress.

45.14.2 Treat Infectious Causes of Leukospermia

The presence of leukospermia should precipitate semen culture and screening for sexually transmitted diseases (STD) [82, 83]. Proof of effective antibiotic treatment should be sorted with repeat semen analysis and culture a few weeks after therapy, as many male accessory gland infections are quite resistant to antibiotic cure [63]. Failure to achieve cure should precipitate radiological assessment of the male accessory glands and referral to a urologist/andrologist experienced in the treatment of such matters [84].

45.14.3 General Anti-inflammatory Agents in the Absence of Active Infection

In many cases of leukospermia, no pathogenic bacteria are identified on repeated culture, or there is uncertainty whether the cultured bacteria indicate true infection or just skin contaminants, making directive antimicrobial therapy difficult. Altered semen viscosity or symptoms of perineal discomfort, pain on ejaculation and semen discolouration may all point to the presence of past MAGI and ongoing inflammation [63]. These men may also have increased MAG or epididymal blood flow on colour Doppler, altered prostate morphology or thickening of the seminal vesicle walls on ultrasound [84], plus biochemical signs of inflammation such as raised PSA levels [63]. Despite the absence of ongoing infection, the presence of activated leukocytes within prostatic or seminal vesicle secretions can still mediate oxidative damage to sperm [56, 65, 67]. However, this may be negated using anti-inflammatory agents such as NSAID [37], corticosteroids [85] or over the counter agents such as omega-3 fish oils [86]. None of these anti-inflammatory agents should be

Table 45.2 Randomized controlled studies examining the effect of anti-inflammatory therapies with antioxidant activity on male reproductive health

Study reference	Therapy used per day	Duration of therapy (months)	Positive changes in semen quality	Assessment sperm OS endpoints	Positive changes in reproductive outcomes
[37]	NSAID (nimesulide)	4	↑ sperm vitality ↓ seminal WBC count	↓ ROS (luminol)	Not assessed
[87]	Omega-3 (EPA/DHA) 1.8 gm	8	↑ sperm concentration, motility and morphology	↑ SOD ↑ catalase	Not assessed
[88]	Omega-3 DHA-enriched supplement (0, 0.5, 1, 2 gm)	3	↑ sperm motility and morphology	No change In LPO	Not accessed
[89]	Omega-3 DHA-enriched supplement (1.5 g)	2.5	↓ sperm DNA damage	↑ TAC	Not reported
[98]	Symbiotic prebiotic/ probiotic (Flortec)	6	↑ sperm concentration, motility and morphology ↓ sperm DNA damage	Not tested	25% pregnancy active arm, 0% placebo arm

NSAID non-steroidal anti-inflammatory drug, *EPA* eicosapentaenoic acid, *DHA* docosahexaenoic acid, *ROS* reactive oxygen species, *SOD* superoxide dismutase, *LPO* lipid peroxidation

initiated without first ruling out active infection, as suppression of the immune system with active infection could exacerbate the infection and aggravate ROS damage to sperm.

A 2–4-month course of NSAID after successful treatment of MAGI has been shown to boost sperm function and fertility [37]. Furthermore, a short course of prednisone (5–25 mg/day) is reported to boost sperm count and motility [85]. Finally, three recent RCT using 1.5–2 g of omega-3 fish oil per day have been shown to reduce oxidative stress and improve sperm quality [87, 88], including a significant reduction in sperm DNA damage [89]. It is well established that docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) contained within fish oil have powerful anti-inflammatory capacity [86], plus documented systemic antioxidant capacity [90]. Furthermore, two RCT of omega-3 oils have confirmed enhanced antioxidant capacity in seminal plasma after therapy [87, 89], with the third showing no changes in a lipid peroxidation marker of oxidative stress [88] (Table 45.2).

45.14.4 Symbiotics: A New Potential Therapy for Male Oxidative Stress

A new development in the field of andrology is the recognition of the importance that the gut microbiome may play in testicular function and fertility, principally mediated by inflammation initiated by systemic exposure to gut bacterial endotoxin – the so-called GELDING theory (Gut Endotoxin Leading to a Decline IN Gonadal function) [75]. The human intestine contains 100 trillion bacteria, with these bacteria playing essential roles in digestion of dietary fibre and production of vitamins and short chain fatty acids that keep the bowel healthy [75]. A healthy intestine permits the selective passage of water and nutrients across the mucosal lining to sustain life, while excluding the passage of intestinal bacteria into the circulation. However, with adverse lifestyle choices such as obesity, a poor "western" high-fat low-fibre diet and excess alcohol consumption, there is a breakdown in this intestinal mucosal barrier function, allowing gut bacteria into the circulation where they initiate chronic low-grade inflammation – the so-called metabolic endotoxemia [72, 73, 91]. This metabolic endotoxemia state has now been linked with a reduction in testosterone production [92, 93] and an increase in sperm oxidative DNA damage [94] and is likely to be a significant cause of sperm oxidative stress seen in obese men. Therapies targeting "leaky gut"/metabolic endotoxemia include a reduction in dietary fat and alcohol intake [72] and the consumption of symbiotic therapies [95, 96].

Symbiotics are a combination of probiotic "beneficial bacteria" that enhance gut health, together with prebiotic fibre food to help sustain the growth of these prebiotic bacteria within the individual's intestine [96]. Probiotic bacteria include *Lactobacillus* and *Bifidobacterium*, two helpful strains of bacteria that keep pathogenic bacterial growth at bay and produce chemicals which enhance the intestinal barrier function, thereby reducing metabolic endotoxemia, inflammation and its associated production of ROS [95, 96]. Furthermore, probiotics have reported to have widespread antioxidant activity and benefits in many disease states [96, 97].

While still a relatively new emerging field, a recent RCT has shown that ingestion of a symbiotic product (Flortec; Bracco, Italy) for 3 months produced an improvement in sperm quality and quantity, plus an increase in testosterone production [98]. Furthermore, a small non-randomized study has recently reported that probiotic therapy was able to improve sperm motility and DNA integrity and reduce intracellular H_2O_2 levels, implying a probiotic antioxidant advantage [99]. Importantly, animal studies have also confirmed this beneficial effect of probiotics on sperm quality via an anti-inflammatory/antioxidant mechanism [100, 101]. The

additional benefit of this probiotic/symbiotic approach to management of infertility is that it is relatively inexpensive and free of side effects but may also treat other medical conditions known to be associated with metabolic endotoxemia in obese men such as improvement in insulin resistance [102]. However, further large RCT of probiotics/prebiotics will need to be conducted before they become a widespread treatment of inflammatory oxidative stress-mediated male infertility.

45.15 Therapies That Fortify Sperm Against ROS Damage

One of the primary mechanisms that oxidative stress results in infertility is oxidative damage to the sperm DNA, with the resulting fragmented DNA producing poor-quality embryos that either fail to develop to a blastocyst [103] or implant and miscarry [104]. Furthermore, oxidative damage to sperm has been implicated in alteration in sperm DNA methylation [52, 105], with both sperm DNA fragmentation and epigenetic modification of the paternal genome being associated with impaired health of the next generation [105]. While this adverse effect can be limited by reducing initial ROS production and neutralizing their action once produced using antioxidant therapy [50], the sperm has also developed a last resort defence mechanism to preserve its DNA integrity – protamination.

In somatic cells the strands of DNA are loosely intercoiled by histone packaging, thereby allowing the cellular machinery responsible for translating the genetic code into proteins to easily gain access to the DNA and allow gene transcription. However, as sperm are generally transcriptionally silent during the later stages of maturation, this loose DNA packaging is not necessary. Instead the paternal DNA is very tightly packaged by almost complete replacement of histones with protamines, leading to a sperm nucleus in which the DNA density is significantly greater than somatic cells [106]. This is a vital protective response as the surface area of DNA in direct contact with ROS is thereby minimized, reducing the amount of paternal DNA vulnerable to ROS attack [106].

Unfortunately, many infertile men have defective protamination of their sperm, resulting in decondensed sperm DNA that is vulnerable to oxidative attack [106, 107]. While it is presently uncertain why these infertile men have defective protamination packaging of their sperm, it is known that the micronutrients zinc and selenium play a vital role in forming these protamination cross links [108, 109]. Fortunately, studies using zinc and selenium supplements have been shown to improve protamination and reduce sperm DNA damage [50, 108, 109]. Conversely, other studies have shown free radicals to play an important role in maintaining the disulphide cross links between protamine bridges, with high-dose antioxidant therapy causing decondensation of the sperm and possibly increasing sperm oxidative damage vulnerability [21]. This is a salient cautionary tale emphasizing the centuries old therapeutic maxim that "the dose differentiates a poison from a remedy" (Paracelsus, sixteenth-century Swiss physician). While moderate doses of antioxidants are likely beneficial to sperm health, excessive doses may impair sperm health and functional activities such as capacitation, an ROS-dependent process vital to natural fertilization. Therefore, care should be exercised in all antioxidant therapies, with treatments backed up by well-conducted randomized controlled trials showing a positive risk/benefit profile (Fig. 45.1).

45.16 Conclusion

The current evidence clearly identifies oxidative stress as a major cause of impaired sperm function and male infertility. While many studies have been conducted examining the ability of various antioxidants to improve male reproductive function, it is still uncertain if many male preconception antioxidant therapies can actually improve a couple's chances of becoming parents. However, critical analysis of the higher-quality RCT trials suggests that combinational therapies using vitamin C, vitamin E, lycopene, coenzyme Q10, zinc, selenium and astaxanthin may be of benefit in improving sperm health and the chances of conception. Evidence supporting the use of other antioxidants such as glutathione, L-carnitine and NAC as effective therapies for male infertility is relatively weak. More recent novel approaches to managing oxidative stress by reducing leukocyte production of ROS include the use of omega-3 fish oil and probiotics/prebiotic therapy. These treatments have been shown in RCT to boost sperm health, but unfortunately no study to date has confirmed that this translates into a better chance of natural or IVF-assisted conception. The optimal future therapy for preventing sperm oxidative stress is most likely to combine these anti-inflammatory treatments and direct antioxidant agents (Fig. 45.1). Given the low cost and positive safety profile of these agents, we hope that large RCT targeting men with documented sperm oxidative stress are conducted, with the primary endpoint being the birth of a healthy baby, not improvements in semen analysis. Until these trials occur, physicians caring for infertile couples will remain sceptical regarding the benefits of male antioxidant therapy.

45.17 Review Criteria

An extensive search of studies examining the impact of antioxidant agents (direct antioxidants and indirect antioxidant action via anti-inflammatory action) on sperm health was per-

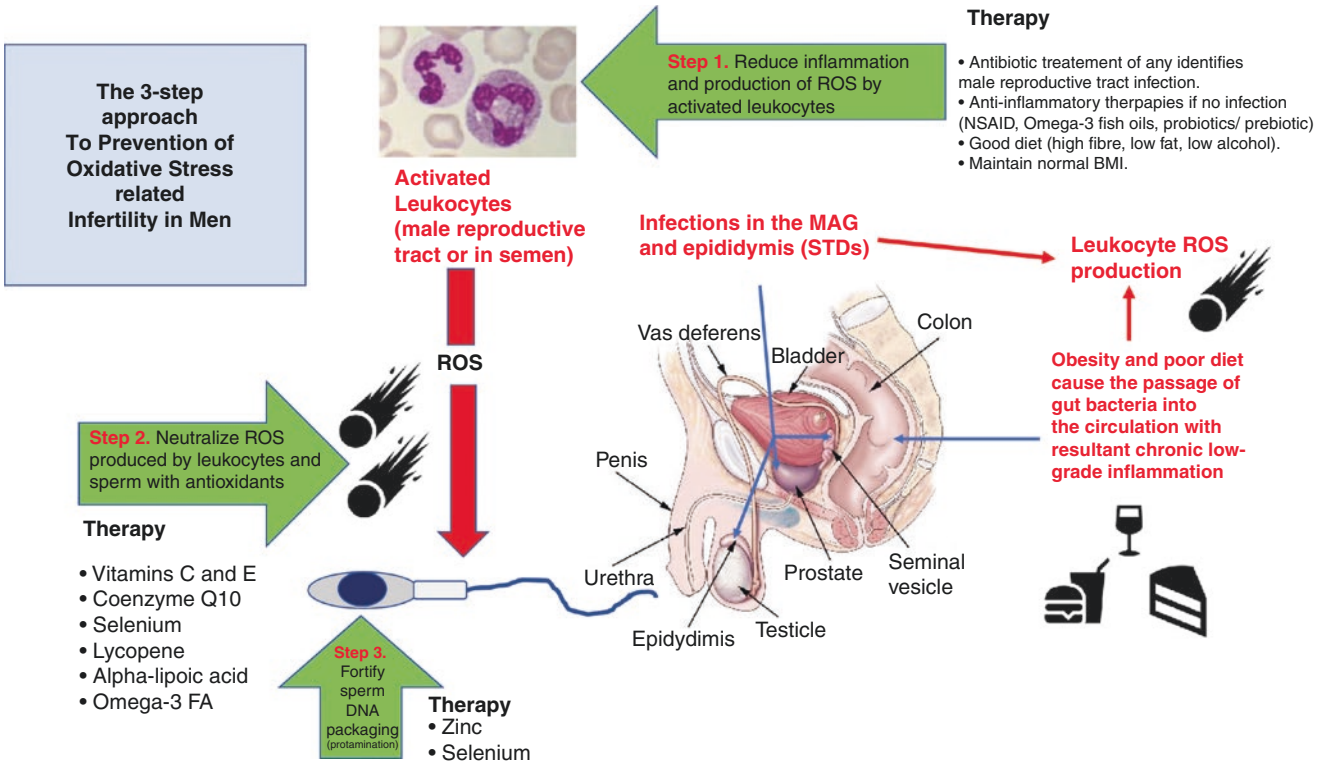

Fig. 45.1 Overview of the clinical management of male oxidative stress-related infertility. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2016–2019. All Rights Reserved)

formed using PubMed and Google Scholar. The completion date for this search was January 2019. The overall strategy for study identification was based on the keywords: "antioxidant", "oxidative stress", "reactive oxygen species", "infertility", "semen", "sperm", "anti-inflammatory", "pregnancy" and "randomized control trial (RCT)". Only peer-reviewed full-length articles published in English were considered, with a primary focus on randomized controlled studies conducted on men. Trials using "general multivitamins" and studies that employed botanical preparations with poorly defined direct antioxidant action were all excluded. Furthermore, studies which combined an antioxidant with an established endocrine therapy known to influence sperm production (clomiphene, aromatase inhibitors) were also excluded.

Findings published at conferences or on web sites that had not been later published in peer-reviewed journals were not considered.

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In Vitro Studies of Antioxidants for Male Reproductive Health

46

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Key Points

- During semen processing, spermatozoa are particularly vulnerable to oxidative stress because the seminal plasma (rich in antioxidants) has been removed.
- Manipulation of sperm in vitro may result in the generation of ROS with subsequent sperm dysfunction.
- Antioxidants can protect immature sperm from endogenous ROS and from gentle semen processing.
- Antioxidants can be used to protect sperm from exogenous ROS and from the process of cryopreservation with subsequent thawing.
- There is limited evidence supporting the use of antioxidants to protect normal spermatozoa from endogenous ROS or gentle sperm processing.
- Additional studies are needed to determine the optimal antioxidant preparation to protect spermatozoa from oxidative stress in vitro.

46.1 Introduction

The detrimental effects of oxidants on spermatozoa were suggested close to 70 years ago with the demonstration that oxygen is sperm-toxic. Later studies confirmed the susceptibility of spermatozoa to oxidative stress and the fact that human spermatozoa and semen leukocytes can generate reactive oxygen species (ROS). These observations have led to studies on the role of antioxidants in protecting spermatozoa from oxidative stress in vitro. Furthermore, the inherent susceptibility of human spermatozoa to oxidative stress is particularly relevant during semen processing as a result of the removal of seminal plasma, a natural antioxidant.

The purpose of this chapter is to discuss the rationale for antioxidant therapy in male infertility and evaluate the data on the efficacy of in vitro antioxidant preparations on sperm function. A review of the literature demonstrates a beneficial effect of in vitro antioxidants in protecting spermatozoa from exogenous oxidants and from cryopreservation (and subsequent thawing). However, the protective effect of in vitro antioxidants on sperm preparations subjected to endogenous ROS and gentle sperm processing has not been established.

46.2 ROS and Male Infertility

The relationship between seminal ROS and male infertility is the basis for proposing treatment with antioxidants in these men [1, 2]. High levels of ROS have been detected in the semen of 25% of infertile men but not in the semen of fertile men [3, 4]. Semen ROS levels are inversely related to the probability of achieving a spontaneous pregnancy [5]. Moreover, the levels of sperm DNA oxidation (a marker of oxidative stress) are higher in infertile compared to fertile men [6, 7]. Semen ROS are generated by spermatozoa (especially, defective or immature) and semen leukocytes [8–12].

In contrast to the pathologic effects of excess ROS production, small amounts of ROS may be necessary for the initiation of critical sperm functions, including capacitation and

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the acrosome reaction [13–15]. Therefore, there is a finely tuned balance between ROS scavenging and the low, physiologic levels of ROS that are necessary for normal sperm function and maturation.

The susceptibility of human spermatozoa to oxidative stress stems primarily from the abundance of unsaturated fatty acids localized within the sperm plasma membrane. These fatty acids provide the fluidity necessary for membrane fusion events, such as the acrosome reaction and sperm-egg interaction, and for sperm motility. However, the unsaturated nature of these fatty acids predisposes them to oxidative stress and lipid peroxidation. Once the lipid peroxidation cascade has been initiated, sperm dysfunction (e.g. loss of motility) ensues as a result of accumulation of lipid peroxides on the sperm membrane, depletion of ATP and oxidative damage to the DNA [16–19]. It has been shown that ROS can cause damage to the sperm DNA, directly or indirectly, via production and subsequent translocation of lipid peroxides [19–22].

46.3 Semen Antioxidants and Sperm Function

Seminal fluid is an important source of antioxidants (both enzymatic and non-enzymatic) that can protect spermatozoa from oxidative injury [4, 23, 24]. This feature of seminal plasma is of critical importance in view of the inherent susceptibility of spermatozoa to oxidative stress and also because spermatozoa themselves have little cytoplasmic fluid and minimal antioxidant capacity [4]. There are several endogenous antioxidant enzymes (superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX) in the male reproductive tract and in seminal fluid [4, 23, 25–28]. Moreover, there are several small, non-enzymatic antioxidants (e.g. vitamins C and E, hypotaurine, taurine, L-carnitine, lycopene) in semen and, in fact, this non-enzymatic fraction represents most of the total seminal antioxidant activity [4, 29].

A number of investigators have proposed that oxidative sperm dysfunction may be secondary to reduced semen antioxidant capacity. However, clinical studies have reported conflicting results in this respect. Several studies have found that seminal antioxidant activity is reduced in infertile men with high levels of seminal ROS (relative to those with normal levels of ROS), whereas others have not shown this [4, 30–32]. Studies have also reported that a deficiency in semen antioxidants is related to sperm dysfunction (including DNA damage), whereas other studies have not observed this relationship [17, 33–37].

There is no evidence to suggest that male infertility is caused by systemic antioxidant or vitamin deficiency. Silver et al. evaluated a group of fertile men and did not identify any relationships between dietary antioxidant intake (vitamins C, E or ß-carotene) and sperm DNA damage [38]. Nonetheless, it is very likely that a subgroup of infertile men may have specific antioxidant deficiency, particularly, vitamin C deficiency [35, 39, 40]. Moreover, infertile men with various lifestyles (smoking, excessive alcohol intake, dieting) may also be at high risk for antioxidant or vitamin deficiency [41, 42].

46.4 In Vitro Antioxidants in Male Infertility

Several studies have examined the role of in vitro antioxidant supplementation in protecting spermatozoa from oxidative injury and resulting sperm dysfunction (i.e. loss of motility and viability). This is clinically relevant as sperm washing is routinely performed prior to assisted reproductive technology (ART< (e.g. intrauterine insemination and in vitro fertilization) and the process may result in the generation of ROS with ensuing sperm dysfunction [43]. During semen processing, spermatozoa are particularly vulnerable to oxidative stress because seminal plasma (rich in antioxidants) has been removed in the process [44, 45]. For assisted reproductive techniques that require use of spermatozoa with progressive motility (e.g. IUI and IVF), minimizing sperm dysfunction during semen processing is critical for fertilization and subsequent pregnancy.

Recently, a number of studies have examined the role of in vitro antioxidant supplementation in protecting the sperm DNA from oxidative damage because of the concern that unrepaired oxidative sperm DNA damage may be transmitted to the offspring when used in the context of ARTs [46]. However, it is important to note that subpopulations of spermatozoa will exhibit variable susceptibility to oxidative stress: the DNA of normal spermatozoa is reportedly less susceptible to gentle processing techniques than is the DNA of abnormal or immature spermatozoa [10, 47]. It is likely that the susceptibility of the sperm DNA to oxidative injury is related to the degree of sperm chromatin compaction (i.e. level of protamination) [48, 49]. Experimental (animal) studies suggest that the spermatozoa of infertile men may be more susceptible to oxidative injury in vitro but benefit more so from antioxidants than the spermatozoa of fertile men [50]. Clinical studies of in vitro antioxidants support the use of antioxidants in protecting spermatozoa (particularly abnormal spermatozoa) from exogenous ROS and cryopreservation. However, the optimal antioxidant and concentration has not been established.

46.5 Role of In Vitro Antioxidants in Protecting Spermatozoa from Exogenous ROS

This is of clinical relevance as many of the semen samples contain leukocytes and these cells have the potential to generate exogenous ROS [51]. Antioxidants, such as vitamin E,

Table 46.1 Role of in vitro antioxidants in protecting spermatozoa from the loss of motility and DNA damage due to exogenous ROS

Study	Exogenous ROS	Antioxidant supplement and results
Sperm motility		
de Lamirande (1992)	X + XO	Catalase protects spz from X + XO-induced loss of motility
		SOD, DTT, or GSH less effective in protecting spz motility from ROS
Griveau (1995)	X + XO	Catalase protects spz from X + XO-induced loss of motility
		SOD or mannitol ineffective in protecting spz motility from ROS
Sperm DNA		
Lopes (1998)	X + XO	GSH + hypotaurine protect spz from X + XO-induced DD
		Catalase protects spz from X + XO-induced DD
		n-Acetylcysteine protects spz from X + XO-induced DD
Potts (2000)	H_2O_2 + Fe + ADP	S. plasma (>60%v/v) lowers oxidative spz damage (↓DD, LPO)
Sierens (2002)	H_2O_2	Isoflavones, vit C and E protect spz from H_2O_2-induced DD (Isoflavones: genistein, equol). Dose effect noted
Russo (2006)	1. H_2O_2	Propolis lowers oxidative spz damage (↓LPO, DD, LDH) (Propolis—a natural resinous hive product)
	2. Benzopyrene	
	3. H_2O_2 + Fe + ADP	

ADP adenosine diphosphate, *COMET* single-cell gel electrophoresis, *DD* DNA damage, *DFI* DNA fragmentation index, *Fe* iron, *GSH* glutathione, *LDH* lactate dehydrogenase, *LPO* lipid peroxidation, *S. plasma* seminal plasma, *Spz* sperm, *TUNEL* terminal deoxynucleotidyl transferase dUTP nick end labelling, *X* xanthine, *XO* xanthine oxidase

catalase and glutathione, have been shown to protect sperm motility from the effects of exogenous ROS (see Table 46.1) [19, 52]. In contrast, superoxide dismutase is less effective in preventing the loss of motility due to exogenous oxidants [19, 52]. Altogether, these data suggest that H_2O_2 is the most sperm-toxic exogenous ROS.

Antioxidants have also been shown to protect the sperm DNA from the effects of exogenous ROS (see Table 46.1) [44, 53–55]. This is of clinical relevance as sperm DNA damage may impact on reproductive outcomes after ARTs [56]. Indeed, sperm DNA damage has been associated with reduced pregnancy rates with IUI, and, to a lesser extent with conventional IVF.

46.6 Role of In Vitro Antioxidants in Protecting Spermatozoa from Endogenous ROS

Spermatozoa can be stimulated to generate ROS using a variety of agents (e.g. NADPH, estrogens) and this ROS production can impair sperm function [57]. In contrast to the beneficial effect of antioxidants in protecting spermatozoa from exogenous ROS, antioxidants appear to be of limited value in protecting spermatozoa from endogenous ROS production [58]. Twigg et al. demonstrated that SOD, catalase or both are ineffective, whereas albumin is effective in protecting spermatozoa from loss of motility due to endogenous ROS generation [58]. These findings stress the importance of using gentle (brief, little centrifugation) semen processing protocols so as to minimize the production and adverse impact of low levels of endogenous ROS.

Table 46.2 Role of in vitro antioxidant supplements in protecting sperm DNA from stimulated endogenous ROS generation

Study	Assay	ROS stimulant	Antioxidant supplement and results
Twigg (1998)	IS NTL	NADPH	Vit E, SOD, catalase, hypotaurine, albumin all ineffective in protecting spz DNA from endogenous ROS
Anderson (2003)	COMET	Estrogens	Catalase protects spz from estrogen-induced oxidative DD SOD and vit C less effective (Estrogens: equol, daidzein, genistein, DES, E2)
Cemeli (2004)	COMET	Estrogens (1 h 37 C)	Flavonoid (Kaempferol) protects sperm from estrogen-induced oxidative DD
Dobrzynska (2004)	COMET	DES, T3, T4, NA (1 h 37 C)	Flavonoids and catalase protect spz from stimulant-induced oxidative DD (Flavonoids: Kaempferol, Quercetin)

COMET alkaline single-cell gel electrophoresis, *DD* DNA damage, *ISNTL* in situ nick translation assay, *LPO* lipid peroxidation, *NA* noradrenaline, *ROS* reactive oxygen species, *SOD* superoxide dismutase, *Spz* sperm, *T3* triiodothyronine, *T4* thyroxine, *vit* vitamin

Similarly, antioxidants appear to be of limited value in protecting the DNA of normal spermatozoa (with normal chromatin compaction) from endogenous ROS production (e.g. NADPH-induced or centrifugation-induced) (see Table 46.2) [58–61]. In samples with poor morphology and poor sperm chromatin compaction, antioxidants may protect the sperm DNA from endogenous ROS production, as these samples are more vulnerable to oxidative stress [10, 47].

46.7 Role of In Vitro Antioxidants in Protecting Spermatozoa from Semen Processing

Several studies have reported on the effects of antioxidants in preventing the decline in sperm motility after semen processing and incubation (Table 46.3). These studies have clinical relevance because it is important to maximize sperm motility prior to assisted reproductive techniques, such as IUI and standard IVF. The available studies report conflicting results regarding the effects of antioxidants in preventing the loss of sperm motility during sperm processing, such as centrifugation

Table 46.3 The effect of in vitro antioxidants on sperm motility during semen processing

Study	Parameter	Semen processing	Antioxidant supplement and results
Griveau (1994)	Motility	1. CF at 400 *g* × 2	DTT, Catalase, SOD or GSH improve motility
		2. Swim-up	
		3. 24-h incubation	
Zheng (1997)	Motility	2- and 3-h incubation (fertile and infertile)	Ferulic acid improves sperm motility and reduces LPO
	LPO		Ferulic acid increases sperm cAMP and cGMP
Oeda (1997)	Motility	2-h incubation	NAC lowers semen ROS levels
	ROS		NAC improves sperm motility
Verma (1999)	Motility	6-h incubation	Vitamin E lowers sperm LPO and protects spermatozoa from loss of motility
	LPO		
Donnelly (2000)	Motility	Percoll DGC + 4 h incubation	GSH or hypotaurine do not protect spermatozoa from loss of motility
Calamera (2001)	Motility	2–47-h incubation	Catalase did not protect spermatozoa from loss of motility
	ROS		
Chi (2008)	Motility	Centrifugation (1000 rpm × 2) + 1 h incubation	EDTA or catalase lower CF-induced sperm ROS
	ROS		EDTA (but not catalase) protects spermatozoa from CF-induced loss sperm motility

CF centrifugation, *COMET* alkaline single-cell gel electrophoresis, *DD* DNA damage, *DGC* density-gradient centrifugation, *DTT* dithiotreitol, *GSH* glutathione, *LPO* lipid peroxidation, *NAC* *N*-acetyl-l-cysteine, *ROS* reactive oxygen species, *SOD* superoxide dismutase

Table 46.4 Role of in vitro antioxidant supplements in protecting sperm DNA from semen processing

Study	Assay	Semen processing	Antioxidant supplement and results
Hughes (1998)	COMET	Percoll DGC	Vits C, E, or urate lower sperm DD after DGC
			Vits C + E or AC increase sperm DD after DGC
Donnelly (1999)	COMET	Percoll DGC	Vit C or E does not lower baseline sperm ROS and DD
			Vit C or E protect sperm from H_2O_2-induced ROS and DD
			Vits C + E induce sperm DD and increase H_2O_2-induced DD
Donnelly (2000)	COMET	Percoll DGC ± H_2O_2	GSH, hypotaurine or both do not alter baseline sperm DD
			GSH, hypotaurine or both do not alter sperm motility at 4 h
			GSH and/or hypotaurine lower H_2O_2-induced sperm DD
Chi (2008)	COMET	Centrifugation (1000 rpm × 2) + 1 h incubation	EDTA or catalase lower centrifugation-induced sperm ROS
			EDTA or catalase lower centrifugation-induced sperm DD
			EDTA or datalase have no protective effect on LPO

AC Acetyl cysteine, *COMET* alkaline single-cell gel electrophoresis, *DD* DNA damage, *DGC* density-gradient centrifugation, *GSH* glutathione, *LPO* lipid peroxidation, *ROS* reactive oxygen species, *vit* vitamin

and incubation. Some studies have shown that antioxidants (e.g. vitamin E, glutathione, n-acetyl cysteine, catalase, ferulic acid) are effective in reducing ROS levels and in preventing the decline in sperm motility during sperm processing [62–65]. In contrast, other studies have reported that antioxidants (e.g. glutathione, catalase) are ineffective in protecting spermatozoa from the loss of motility during sperm processing [66–68]. It is important to note that sperm samples from infertile men may be more susceptible to oxidative injury (from semen processing) and be afforded greater protection by antioxidants than samples from fertile men [50].

Older studies have shown a limited value of antioxidants in protecting sperm DNA from gentle semen processing (e.g. incubation or density-gradient centrifugation) (see Table 46.4) [67–70]. In some cases, antioxidants supplementation in vitro (e.g. combination of vitamins C and E) may cause sperm DNA damage [68, 70]. More recently, several studies have suggested that in vitro treatment with vitamin C, hydroxy-tyrosol, zinc, D-Asp, CoQ10, selenium and CAPE may have a protective effect against DNA damage [71–76].

46.8 Role of In Vitro Antioxidants in Protecting Spermatozoa from Cryopreservation and Thawing

Several studies have evaluated the role of antioxidants in protecting spermatozoa from the loss of motility that occurs following cryopreservation and thawing. Most studies have reported on the use of pentoxyfilline (an antioxidant and phosphodiesterase inhibitor). Some studies have shown that pentoxyfilline improves post-thaw sperm motility and/or sperm function [77–80], whereas others have demonstrated that this antioxidant does not have a beneficial effect [81]. Other antioxidants (vitamins E and C and rebamipide) have been used to enhance post-thaw motility; however, the results have been modest [82, 83].

Several studies have also evaluated the role of antioxidants in protecting sperm DNA from injury following cryopreservation and thawing. Most studies have shown that antioxidants (vitamin C, vitamin E, quercetin, tempol, catalase, resveratrol, genistein) can protect the sperm DNA from oxidative injury during cryopreservation and subsequent thawing [84–91] (Table 46.5). Moreover, four recent studies have shown that the addition of vitamin E, quercetin, tempol or catalase during cryopreservation improves post-thaw motility [84–87]. Interestingly, the combination of quercetin and tempol did not have an additive effect, but the beneficial effect was noted when they were used alone [84]. In contrast, Taylor et al. reported that the antioxidant vitamin E does not protect sperm DNA during cryopreservation [92].

Table 46.5 Role of in vitro antioxidants in protecting human sperm DNA from injury caused by cryopreservation and thawing

Study	Assay	Antioxidant	Effect of antioxidant on cryopreservation and thawing
Taylor ‘09	TUNEL	Vitamin E	No effect on sperm DNA integrity
			Improved post-thaw motility
Li ‘09	COMET	Catalase or ascorbic acid	Improved sperm DNA integrity
			Reduced ROS production
Branco ‘09	COMET	Resveratrol or ascorbic acid	Improved sperm DNA integrity
Martinez-Soto ‘09	TUNEL	Genistein	Improved sperm DNA integrity
			Reduced ROS production, improved post-thaw motility
Thompson ‘09	8-OHdG TUNEL	Genistein	Improved sperm DNA integrity (reduced oxidative damage)

8-OHdG 8-hydroxy-2-deoxyguanosine, *COMET* alkaline single-cell gel electrophoresis, *ROS* reactive oxygen species, *TUNEL* terminal deoxynucleotidyl transferase dUTP nick end labelling

Taken together, the data suggest that antioxidants are generally effective in protecting spermatozoa from the effects of cryopreservation and thawing. However, the technique of cryopreservation and type of cryoprotectant are also important in improving post-thaw sperm function [93].

46.9 Conclusion

Oxidative stress plays an important role in the pathophysiology of male infertility. The study of in vitro antioxidants is highly relevant in the era of assisted reproduction because of the susceptibility of human spermatozoa to oxidative injury and the vulnerability of these cells during semen processing. Most studies have demonstrated a beneficial effect of in vitro antioxidant supplements in protecting spermatozoa from exogenous oxidants and cryopreservation (with subsequent thawing). In contrast, the effect of these antioxidants in protecting normal spermatozoa from endogenous ROS and gentle sperm processing has not been established conclusively. Additional studies are needed to determine the optimal antioxidant preparation to protect spermatozoa from oxidative stress in vitro.

46.10 Review Criteria

An extensive search was performed including articles from 1987 to 2018.

The PubMed & MEDLINE search terms included: “sperm; sperm DNA fragmentation; oxidative stress; semen; antioxidants; sperm washing; cryopreservation; male infertility”.

Articles published in languages other than English were not considered.

The main focus was on studies discussing the role of antioxidant therapy in male infertility and the efficacy of in vitro antioxidant preparations on sperm function.

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Antioxidants Use and Sperm DNA Damage

47

Marlon P. Martinez, Ahmad Majzoub, and Ashok Agarwal

Key Points

- Sperm DNA is prone to damage from insults of intratesticular, posttesticular and external factors which are known to cause deleterious effects on quality and function of spermatozoa leading to poor reproductive outcome.
- The vulnerability of compacted DNA to oxidative stress after lipid peroxidation of plasma membrane of spermatozoa established the positive relationship between high ROS production and subsequent SDF.
- Despite no strong recommendation for its routine use for their initial evaluation, there are still clear indications in requesting SDF testing to infertile men.
- High SDF is considered to play an important role in the outcomes of natural pregnancy, IUI, IVF, ICSI in additional to possible risk of pregnancy loss and birth defects.
- Antioxidant use is a reasonable option to improve SDF in infertile men, however, well-designed, large randomized placebo controlled trials should be conducted in order to prove these definitive effects.

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47.1 Introduction

In a recent consensus-based and evidence-driven set of terminologies set by the International Committee for Monitoring Assisted Reproductive Technologies (ICMART), infertility is defined as a "disease characterized by the failure to establish a clinical pregnancy after 12 months of regular, unprotected sexual intercourse or due to an impairment of a person's capacity to reproduce either as an individual or with his/her partner" [1]. About 80 million people worldwide are believed to suffer from infertility [2] with the male factor accounting for approximately 50% of these infertility cases [3].

During the initial evaluation of an infertile man, a conventional semen analysis is typically the initial screening tool for diagnosis. Despite its value in providing an overview of the male fertility potential [4], semen analysis does not necessarily correlate with reproductive outcome. It does not equate with fertility and it cannot differentiate infertile from fertile men. Furthermore, the functional physiology of the sperm is not accurately portrayed with conventional semen analysis making it as an imperfect tool [5].

Oxidative stress (OS) is a key pathophysiologic process believed to account for 30–80% of male factor infertility [6]. ROS are highly reactive free radicals containing one or more unpaired electrons and are capable of independent existence. These products of cellular metabolism are involved in normal sperm physiological processes especially when in low or moderate concentrations. However, in high concentrations, ROS can yield detrimental effects such as aggravated peroxidation of membrane lipids, DNA damage and abortive apoptosis. Lipid peroxidation is considered the most innocuous effect of high ROS as it entails impairment of normal cellular functions. The end products of lipid peroxidation are commonly used as biomarkers for OS. One of these is malondialdehyde (MDA) which is commonly used in many studies [7].

As early as 1953, Leuchtenberger [8] discovered that male infertility did not only involve deranged conventional

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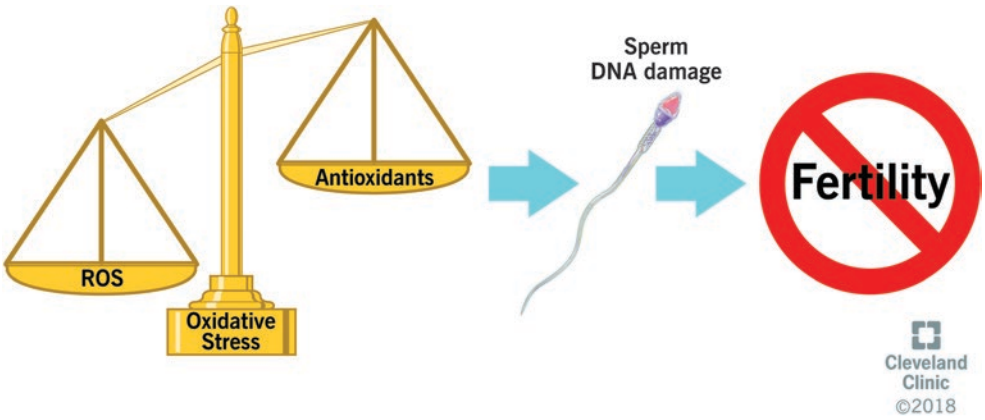

Fig. 47.1 High level of ROS can shift the balance towards the pathologic effects of oxidative stress. As a result, SDF can develop leading to male infertility with poor reproductive outcomes. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2018. All Rights Reserved)

semen parameters. He found significant variability in sperm DNA content between infertile and fertile men. Poorly compacted nuclei are vulnerable to OS, resulting in SDF, which is potentially lethal to spermatozoa. Furthermore, the plasma membrane of the spermatozoa contains abundant polyunsaturated fatty acids which make it susceptible to attack by ROS. Therefore, the positive relationship between high levels of ROS production and SDF seems to be predictable (Fig. 47.1). High levels of SDF is mediated by an overabundance of ROS [9].

Evidence suggests that oral antioxidant supplementation may improve semen quality and functions by reducing ROS and SDF [10]. Antioxidants are readily available medications that are routinely offered to infertile men. They are relatively inexpensive and can be purchased over the counter. It is prudent enough for infertile men taking oral antioxidant supplementation to have access to high quality studies and evidences on the risks and benefits of these medications because some may consider such treatment as the only hope to improve their fertility problems. Samplaski et al. [11], reviewed the discrepancy between the internet and academic literature on the use of vitamins for infertility. A total of 2.94 million results were generated from searching the terms "vitamins and sperm". There are many advertisements for vitamin supplements, particularly on the internet, claiming to have beneficial effects on semen parameters and pregnancy outcomes. Patients should be cautious in believing these claims as many are not supported by medical evidence.

In 2017, the World Health Organization (WHO) [12] described the consensus guideline methodology for global applicability for the diagnosis of male infertility. They reported that a low level of evidence exists, with insufficient data to recommend antioxidant use for treatment of infertile men with abnormal semen parameters. Despite these findings, use of antioxidants for the treatment of male infertility is widely accepted. This chapter will primarily focus on the effects of antioxidant treatment in infertile men with SDF.

47.2 Sperm DNA Damage

47.2.1 Pathophysiology and Etiology of SDF

Human sperm DNA is a complex structure that is susceptible to damage through a variety of mechanisms. The process of spermatogenesis is complicated process, comprised by an orchestrated machinery of complicated proliferation and differentiation. Due to its complex nature, it is prone to alterations. A single point defect can lead to deleterious effects to spermatozoa causing infertility. During spermiogenesis, nuclear histones are replaced by protamines. It is believed that protamines are responsible for chromatin condensation necessary for fertilization. Disulfide bonds account for the chromatin stability during the transport of spermatozoa from the testis to epididymis. That is why it is important that at this molecular level, no damage should ensue [13].

Causes (Fig. 47.2) of SDF can be from both internal (intratesticular or posttesticular) and external factors [14]. There are different mechanisms that induce SDF. These include induction of apoptosis during spermatogenesis, DNA breaks during spermiogenesis and activation of caspases and endonucleases. These effects can be caused by chemotherapy and radiotherapy, environmental toxicants and lifestyle exposures [15].

Sertoli cells are responsible for induction of apoptosis during spermatogenesis. These cells through their screening mechanism phagocytise and eliminate defective germ cells. However, some of these defective germ cells may still enter sperm remodelling during spermiogenesis [16]. During this process, nicks formation may occur as the spermatozoa transit through the epididymis where caspases and endonucleases activation can cause SDF [17]. Esteves et al. [18] noted a five fold higher SDF in ejaculated sperm compared to testicular sperm. Additional evidence is needed to explain if intratesticular insults during spermatogenesis predispose the spermatozoa to damage after their transport from the testicular level. It has been noted that low levels of disulfide cross linking in sperm chromatin existed during maturation

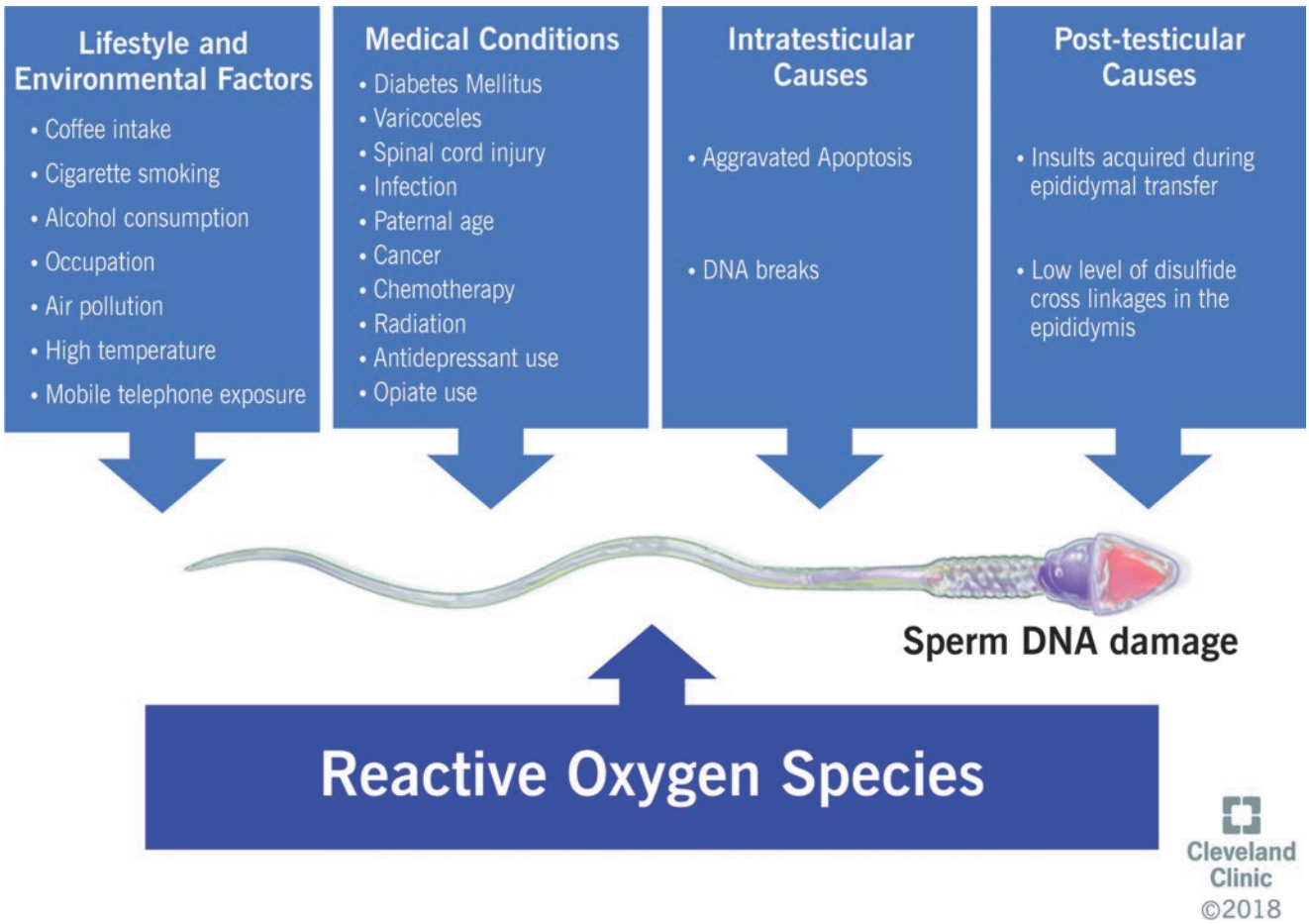

Fig. 47.2 Etiologies of SDF. Intratesticular and post testicular insults can cause SDF. External factors including medical conditions, lifestyle and environmental exposures can contribute to its development. Most of the causes of SDF are ROS-induced. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2018. All Rights Reserved)

particularly at the level of corpus and caput of the epididymis which are prone to SDF. Similar to Sertoli cells, there is a screening mechanism that discards defective spermatozoa at the level of epididymis [19]. Its aberration will lead to SDF and subsequent infertility.

Historically, the presence of free radicals in spermatozoa has been implicated as the possible cause of infertility in men [20]. ROS can originate from immature sperm and can illicit sperm DNA damage of a nearby mature sperm. A correlational study between SDF and seminal plasma ROS was done by Xie et al. [21]. They investigated 80 infertile and 20 fertile men and found that infertile men had significantly higher DNA fragmentation index (DFI) (36.5 ± 3.87% vs 16.23 ± 2.65%, $p = 0.008$) and higher ROS level (548.9 ± 108.2 vs 416.3 ± 95.5, $p < 0.05$) compared to fertile men. Semen of infertile men had enormous intracellular ROS levels resulting in high SDF. In a recently published metaanalysis [10] of 65 studies, the authors showed that levels of seminal plasma nitric oxide ($p = 0.001$), carbonyl protein ($p < 0.00001$) and MDA ($p < 0.00001$) were significantly higher in infertile men. On the other hand, reduced concentrations of gluthathione ($p < 0.00001$), vitamin C ($p < 0.00001$), vitamin E ($p = 0.003$), catalase ($p < 0.0001$), glutathione peroxidase ($p = 0.0002$) and glutathione-S-transferase ($p = 0.009$) among infertile men were also noted. It was proven that these several markers of OS were abnormal among infertile men. The association between SDF and ROS can be best described in patients with varicoceles [22]. Varicoceles can be seen in 15% of the general population and between 19–40% of infertile men [23]. Varicocelectomy has shown a positive impact on the reduction of SDF and ROS, thereby providing improved pregnancy rate outcomes. Several mechanisms are known to take place in men with varicoceles altering their reproductive outcomes. These include higher sperm apoptosis [24], increased sensitivity of sperm DNA to denaturation due to high intratesticular temperature [25], high ROS impairing chromatin packaging [26] and abnormal sperm chromatin condensation [27]. However, the exact mechanism of elevated ROS production by varicoceles requires further evaluation. Association of SDF and ROS is also demonstrated in patients with leukocytospermia. One study determined that men with low levels

of leukocytospermia (0.1–1.0 × 106 WBC/mL) had significantly elevated ROS levels (944.8 vs 116.7, $p < 0.001$) and SDF (19.89 vs 26.47, $p = 0.05$) compared to men without leukocytospermia [28].

Intratesticular and posttesticular insults are not the sole causes of SDF. External factors can also contribute to these undesirable effects [29]. It was shown that diabetic men had more SDF compared to nondiabetic men undergoing assisted reproductive technology (ART). DFI of diabetic men was significantly higher (37.05 ± 12.68% vs 21.03 ± 10.13%, $p < 0.001$) compared to nondiabetic men. In addition, 8-oxo-2-deoxyguanosine (8OhdG) which is one of the products of DNA oxidation was significantly increased [30, 31]. In earlier studies, these products are likewise seen to be elevated in infertile men [32, 33]. Men with spinal cord injuries (SCI) are not exempted from SDF. Most of these men are in their reproductive years. It was reported that men with SCI had higher DFI compared to fertile men [34, 35]. Infection is associated with increased ROS production, thereby, leading to membrane lipid peroxidation and subsequent SDF [36]. Alcohol consumption is also associated with negative reproductive health outcomes. Komiya et al. [37], reported an increased DFI (49.6 ± 23.3% vs 33.9 ± 18.0%) among alcoholics compared to non-drinkers. Several studies have shown an association between increased paternal age and SDF. It is believed that ROS production increases with paternal age and ROS is the major contributory to SDF. Advanced paternal age is also associated with protamine deficiency and deficient chromatin packaging [38, 39].

Daily activities and dietary consumption of men can contribute to the lifestyle and environmental factors causing SDF. In a study by Schmid et al. [40], men who consumed more than 3 cups of coffee per day had approximately 20% higher SDF compared to those who do not drink coffee. There is an impact of male nutrition on male infertility and obesity can lead to SDF. In a study of 333 infertile men, it was found that obese men had an increased DFI (OR = 2.5, 95% CI 1.2–5.1) [41]. Dietary factors will be discussed extensively in another part of this chapter. Numerous investigators have assessed for the association between cigarette smoking and male infertility. Pasqualotto et al. [42] showed that cigarette smoking has a significant inverse correlation with superoxide dismutase level, a known enzymatic antioxidant ($p = 0.01$). Smoking causes ROS production leading to defective protamination and subsequent SDF [43]. In a meta-analysis [44] of 11 studies on the effect of mobile telephones on conventional semen parameters, mobile phone exposure was found to carry equivocal effects on sperm concentration but was associated with decreased motility and viability. More studies are needed to link mobile phone exposure to SDF. It is known that exposure to radiotherapy and chemotherapy has a negative impact on male fertility. Such treatment can cause alteration in the processes that are involved in sperm DNA integrity and compaction [45].

Other potential etiologies of SDF are antidepressant drugs [46], opiate use [47], occupation [48], air pollution. [49], and high temperature [50]. These findings warrant additional studies in order to find a definitive correlation between these factors and SDF.

47.2.2 Impact of SDF on Male Infertility

SDF has an impact on reproductive outcomes including natural pregnancy, intrauterine insemination (IUI), in vitro fertilization (IVF) with intracytoplasmic sperm injection (ICSI), risk of pregnancy loss and possible birth defects (Fig. 47.3). With the rapid advancement in ART, a strong understanding of the effects of SDF is warranted.

Two hundred fifteen Danish first pregnancy planners were included in a study by Spano et al. Chromatin susceptibility was measured by sperm chromatin structure assay (SCSA) and time to pregnancy was followed for 2 years. The authors demonstrated that fecundability started to decline if there was abnormal chromatin of more than 20%. No pregnancy occurred when abnormal chromatin was more than 40% [51]. In another study, DFI measured by SCSA was compared between 127 infertile men and 137 proven fertile men. Men with a DFI 10% to 20% had an increased risk for non fecundability (OR 2.5, 95% CI: 1.0–6.1) compared with men with DFI < 10%. Infertility is more prevalent in men with a DFI >20% (OR 8.4; 95% CI: 3.0–23) [52].

A systematic review on the role of SDF on male fertility has been conducted by Cho et al. [53]. They reported a more discernible impact for high SDF on pregnancy loss compared to its relation to IVF and ICSI outcomes. For natural conception and IUI, high SDF was also inversely associated with poor reproductive outcomes. DFI can be utilized as an independent predictor of reproductive outcome in couples undergoing IUI. In a study by Bungum et al. [54], a total of 387 IUIs from 998 cycles were performed. Couples with DFI < 30% (measured by SCSA) had significantly higher biochemical pregnancy (24.0% vs 3.0%, $p < 0.05$), clinical pregnancy (23.7% vs 3.0%, $p < 0.05$) and deliveries (19.0% vs 1.5%, $p < 0.05$) compared to those with DFI > 30%. In another study [55] of 119 patients (154 cycles of IUI), no pregnancy resulted from DFI > 12% using Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labeling (TUNEL) assay and acridine orange (AO) tests. The relationship between DFI and IUI outcomes in 76 women (48 autologous; 28 donor sperm insemination) were analyzed showing significant correlations between DFI and pregnancy rate ($p < 0.05$). In this study, SDF was evaluated by flow cytometry after staining with AO. A DFI < 26.9% showed a clinical pregnancy rate of 64.29% while DFI > 27% revealed no reproductive outcome [56].

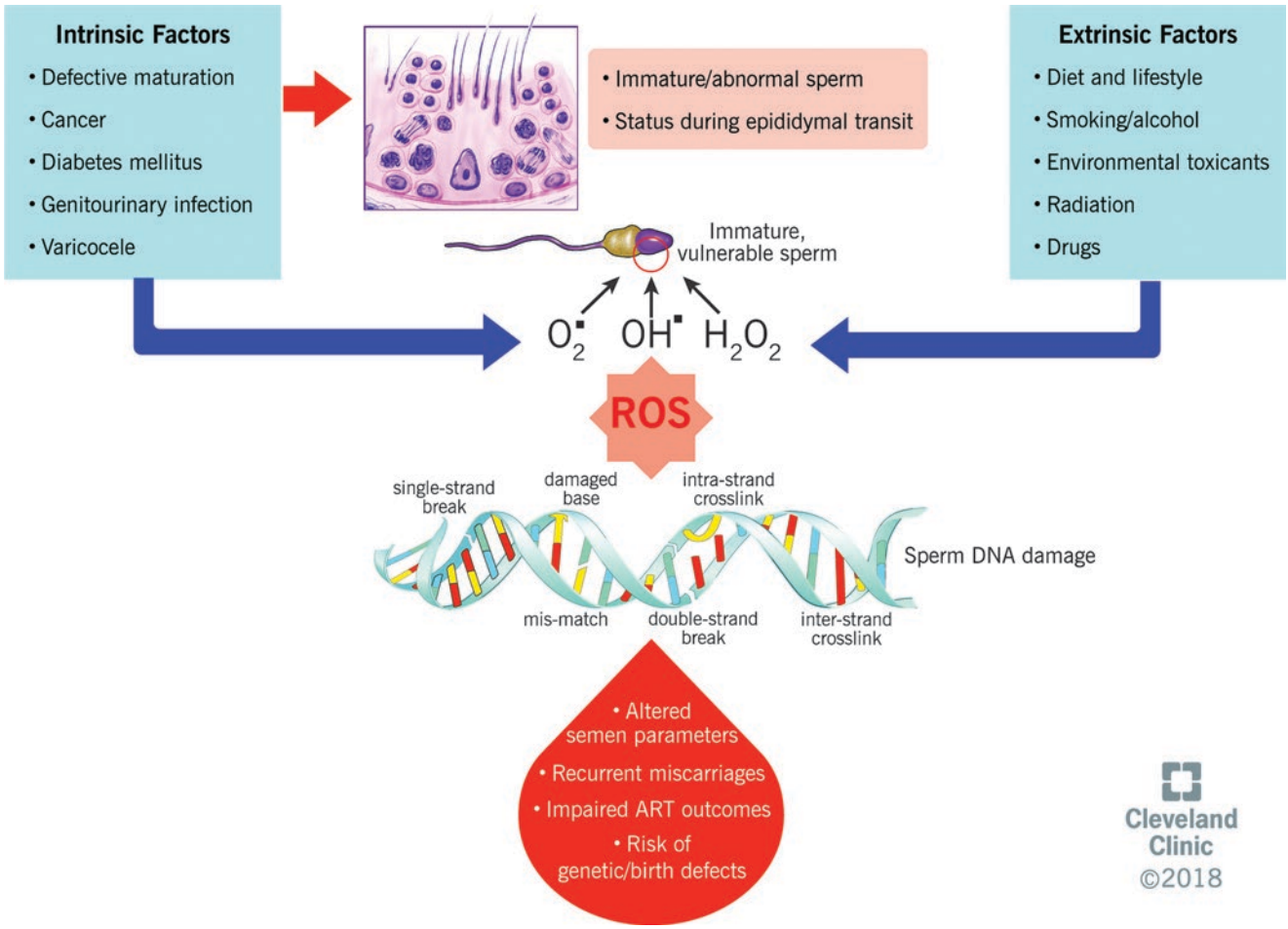

Fig. 47.3 Oxidative stress induced sperm DNA Damage and consequences on male fertility. (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2018. All Rights Reserved)

On the other hand, studies on the effects of SDF on IVF/ICSI showed conflicting results. Evenson et al. [57] reported a significant predictive ability for SDF on the reproductive outcomes of IUI and IVF with no significant effect on ICSI using SCSA. With a DFI <30%, couples achieved pregnancy 7.3 times higher in IUI ($p = 0.001$), 2.0 times higher in routine IVF ($p = 0.03$) and 1.6 times higher in ICSI ($p = 0.06$) compared with couples with a DFI > 30%. In another study [58] using TUNEL assay, SDF levels showed no significant effect on IVF fertilization rate ($p = 0.23$) but significant effect on clinical pregnancy rate ($p = 0.006$). IVF outcome demonstrated both non significance in clinical pregnancy rate ($p = 0.09$) and fertilization rate ($p = 0.70$) using SCSA.

Two recent meta-analysis studies [59, 60] reported little or no difference in SDF predictive value on ICSI and IVF outcomes. The authors concluded that there is insufficient evidence to recommend routine use of SDF testing in ART. On the other hand, Simon et al. identified 120 studies demonstrating the association of sperm DNA abnormalities with male infertility and ART outcomes [61]. Fertilization rate showed significant inverse relationship with SDF. This was observed more commonly in microdrop insemination cases than ICSI and mixed IVF plus ICSI cases. Overall, SDF was significantly associated with clinical pregnancy outcomes following ART (OR 1.15, CI 1.08–1.23; $p < 0.0001$). Compared to those with low SDF, a significant spontaneous pregnancy loss was observed in patients with high SDF (RR 2.16, CI: 1.54–3.03; $p < 0.0001$). A differential association between SDF and embryo quality was shown in this study

47.2.3 Impact of Sperm DNA Damage on Semen Parameters

Several studies have investigated the relationship between SDF and conventional semen parameters with equivocal results. Even with normal conventional semen parameters, about 25–40% of these men suffer infertility due to a DFI of more than 20–30% [62].

Xie et al. [10] failed to find a correlation between SDF and semen volume ($p = 0.106$), density ($p = 0.533$), motility ($p = 0.068$), and morphology ($p = 0.093$). Additionally, Evgeni et al. [63], studied a total of 770 Greek men who were divided into 3 groups [Group 1: fertile men, Group 2: subfertile men (achieved pregnancy but not resulting to live

birth) and Group 3: infertile men]. DFI was measured by sperm chromatin dispersion assay (SCD). Both subfertile and infertile groups showed significant association between SDF and most of the semen parameters ($p < 0.001$) including sperm concentration, total count, rapid progressive motility, progressive motility and morphology but not with semen volume (subfertile group, $p = 0.762$ and fertile group, $p = 0.307$). The SDF value showed no correlation with all the semen parameters of the fertile group. Another study [64] included 373 patients and 28 fertile sperm donors looking for correlations between semen parameters and SDF by AO. For the donor group, there was no significant correlation between all semen parameters and SDF. Conversely, significant differences in SDF were detected when normozoospermic samples were compared with asthenozoospermic (82.7 ± 16.0, $p = 0.002$) and oligoasthenozoospermic (79.6 ± 18.5, $p = 0.001$) samples. Subanalysis was done on oligoasthenozoospermic men revealing a significant increase in DNA normality for motility >25% (89.72 ± 9.4 vs 73.82 ± 20.0, $p = 0.0013$) compared to motility <25%. DNA normality for the whole patient group was likewise significantly increased if morphology is >30% (89.18 ± 12.6 vs 82.46 ± 18.2, $p = 0.011$) compared to morphology <30%. The authors concluded that SDF testing is a reliable tool to show correlation with these conventional semen parameters.

47.2.4 Diagnosis and Treatment

Many of the known medical societies do not recommend the routine use of SDF testing during male fertility evaluation. The American Society for Reproductive Medicine (ASRM) [65] does not advise its routine use in the evaluation and treatment of the infertile couples due to lack of sufficient evidence. Likewise, the American Urological Association (AUA) [66] and European Association of Urology (EAU) [67] have similar recommendations. However, they acknowledged the importance of its use in male infertility and recently emerging studies should stimulate changes to these recommendations in the future.

In 2016, a clinical practice guideline [68] was created by a panel composed of 5 urologists and 1 andrologist with expertise in their respective fields. Different testing methods were discussed including the AO test, aniline blue (AB) staining, chromomycinA3 (CMA3) staining, toulidine blue (TB) staining, TUNEL assay, SCSA, sperm chromatin dispersion (SCD) or halo test and single cell gel electrophoresis (SCGE) or comet assay.

The panel recommended the following indications (Table 47.1) for SDF testing: varicoceles (grade 2/3 varicoceles with normal conventional semen parameters and grade 1 varicoceles with borderline/abnormal conventional semen parameters), unexplained infertility, recurrent pregnancy loss, recurrent IUI failure, IVF and ICSI failure and lifestyle risk factors.

Table 47.1 Indications for SDF testing

Varicoceles
Unexplained infertility
Recurrent pregnancy loss
Recurrent IUI failure
IVF and ICSI failure
Lifestyle risk factors

Treatment options to reduce SDF include short abstinence interval, oral antioxidant therapy, varicocelectomy when indicated, sperm selection and testicular sperm use in ART [69].

Abstinence time has an impact not only on basic semen parameters but on advanced sperm function tests as well. SDF was noted to be increased if the period of ejaculatory abstinence was lengthened (day 1: 9.90% vs day 11: 20.36%, $p < 0.001$) [70]. In a meta-analysis including 7 studies by Wang et al. [71], infertile men with varicocele had significantly higher SDF with a mean difference of 9.84% (95% CI 9.19 to 10.49; $p < 0.00001$) than controls. Patients who underwent varicocelectomy had significant reduction of SDF with a mean difference of −3.37% (95% CI -4.09 to −2.65; $p < 0.00001$). Several approaches on how to reduce the effect of SDF have been proposed to deliver better outcomes during ART. The use of testicular sperm instead of ejaculated sperm for ICSI showed promising results. Greco et al. [72] observed that SDF levels, as assessed by TUNEL, were lower in testicular sperm (4.8% ± 3.6%) than in ejaculated sperm (23.6% ± 5.1%; $P < 0.001$), and reported higher pregnancy rates by ICSI using testicular sperm (44.4% vs. 6%; $P < 0.05$) [84]. Using SCD, Esteves et al. [18] demonstrated five fold lower SDF in testicular sperm versus ejaculated sperm (40.7% ± 9.9% vs. 8.3% ± 5.3%; $P < 0.001$).

With regards to ICSI outcomes, patient who have high SDF will benefit from physiological intracytoplasmic sperm injection (PICSI) and intracytoplasmic morphologically selected sperm injection (IMSI) [73, 74]. In contrary, Bradley et al. showed no significant difference in live birth rates among PICSI patients (38.3%, $p = 0.151$) and IMSI patients (28.7%, $p = 0.680$) when compared to those with high SDF who did not undergo any interventions [75]. Prospective randomized studies are still needed to validate these findings.

47.3 Use of Antioxidants for Sperm DNA Damage

47.3.1 What Are Antioxidants

Increased concentrations of ROS and OS are both implicated in male infertility. The production of antioxidants is one of several process. The human body has to counteract OS. Antioxidants

are derived either from endogenous or exogenous sources (mainly dietary supplementation) and are the bodies' defense mechanism. An ideal antioxidant should eliminate free radicals and chelate metals at physiological levels. These can be enzymatic or non enzymatic antioxidants. Enzymatic antioxidants include catalase, superoxide dismutase and gluthathione peroxidase [76]. This chapter will discuss deeply nonenzymatic antioxidants rather the enzymatic antioxidants and will limit its discussion to antioxidants used for SDF.

There are many antioxidants used for male infertility and the following are commonly used antioxidants for improvement of SDF.

47.3.1.1 Vitamin B12

This vitamin is necessary for growth and development of many organs in human body. It plays an integral part in the conversion of homocysteine to methionine. Its antioxidative effect is shown to reduce the destructing effect of hydrogen peroxide on sensitive cell lines [77].

47.3.1.2 Vitamin C (Ascorbic Acid)

This water soluble antioxidant is a reducing agent capable of neutralizing ROS. Together with tocopherols, it can raise the level of gluthathione providing proteins with protection against oxidation [78].

47.3.1.3 Vitamin E

Vitamin E, which is mainly obtained from diet, is the major lipid soluble integral component in the cell antioxidant defense system. It is primarily situated in the cell and organelle membranes. Even though its concentration is outnumbered by phospholipid molecules, it can still exert it most potential protective effect in that location. Alpha-tocopherol is the major form of this vitamin which is known to inhibit the formation of new free radicals [79].

47.3.1.4 Folic Acid

Folic acid exhibits its role in reaction with oxidizing radicals by inhibiting the oxidation of hypoxanthine into uric acid and its hydroxyl group. It can inhibit lipid peroxidation in spite of it being a water soluble molecule [80].

47.3.1.5 Carotenoids

These are lipid soluble antioxidants that play a role in lipid peroxidation by efficient scavenging of singlet oxygen and trapping peroxyl radicals at low oxygen pressure. Beta-carotene has the highest pro – vitamin A activity [81].

47.3.1.6 L-carnitine

L-carnitine is an essential nutrient obtained from dietary supplementation, although it can be synthesized endogenously. Its antioxidant effect is known to neutralize free radicals and to provide source of energy for cellular process [82].

47.3.1.7 Zinc

This important trace element exerts its protective property by retardation of oxidative process through inducing metalothioneins expression which are required for maintaining zinc induced cell homeostasis. As an antioxidant, zinc stabilizes protein sulfhydryls against oxidation and antogonizes transition metal catalyze reactions [83].

47.3.1.8 Coenzyme Q10

CoQ10 is known to be the only lipid soluble antioxidant that is formed endogenously. It can hamper DNA oxidation and lipid peroxidation [84].

47.3.1.9 Selenium

This is a micronutrient essential in the development and processes of spermatogenesis. The exact mechanism by which it reduces oxidative stress is still under debate. However, its mechanism of action is believed to be mediated by glutathione peroxidase [85].

47.3.1.10 N-acetyl-L-cysteine (NAC)

This thiol containing antioxidant has the ability to stimulate glutathione (GSH) synthesis thereby scavenging ROS. Its thiol group decreases free radical and serves as chelating site for metals [86].

It is true that antioxidants can have a favorable effect on spermatozoa [85, 87]. However, their unregulated and excessive use, can provoke a pathological effect. The term "antioxidant paradox"raised from the report of harmful "reductive state"effects from the unphysiological and uncontrolled use of antioxidants has been reported [88].

Despite this risk, the sensible use of antioxidants for infertile men with SDF is still acknowledged.

47.3.2 Role of Antioxidants in Male Infertility: Overview

In a 2015 Cochrane review [89], positive effects for antioxidants use in infertile men have been reported. These include increased live birth rate (OR 4.21, 95% CI 2.08 to 8.51, $p < 0.0001$) and increased pregnancy rate (OR 3.43, 95% CI 1.92 to 6.11, $P < 0.0001$). There is limited evidence to determine the difference in effect of the use of antioxidants and placebo with regards to miscarriage rate (OR 1.74, 95% CI 0.40 to 7.60, $p = 0.46$). Gastrointestinal adverse events showed no significance with antioxidant use compared to placebo (OR = 1.60, 95% CI 0.47–5.50, $p = 0.46$). This review concluded that there was a low quality of evidence suggesting that antioxidants use can deliver significant improvement on live birth and clinical pregnancy rates. No evident findings on increased risk of miscarriage and adverse events were noted. The body suggested the conduction of large controlled studies to further investigate these results.

In general, antioxidant use showed beneficial effects on male fertility despite the low level of evidence that has been reported in literature.

47.3.3 Evidences Regarding Utility of Antioxidants for SDF

Multiple studies (Table 47.2) have been conducted to determine the effect of the use of antioxidants to reduce SDF. Although beneficial effects on semen parameters and reproductive outcomes were reported, evidence is still inconclusive due to the low quality of methodologies used in different studies. Most of the authors explored the use of antioxidants in combination therapy rather than monotherapy.

Forty eight randomized controlled clinical trials were included in the recent Cochrane review [89] determining the effect of antioxidant use on male factor infertility. In this review, there were 2 trials which studied the effect of antioxidants on SDF. Both trials showed a statistically

Table 47.2 Results of studies on antioxidant use on sperm DNA damage

Study	Population size	Antioxidants use	Methodology	Results
Stenqvist et. al. [105]	77 infertile men with DFI >25% Treatment group: $n = 37$ Placebo group: $n = 40$	Vitamin C 30 mg, vitamin E 5 mg, vitamin B12 0.5 ug, l-carnitine 750 mg, coenzyme Q10 10 mg, folic acid 100 ug, zinc 5 mg, selenium 25 ug (twice a day for 6 months)	Prospective placebo-controlled, double-blind, randomized study	No significant reduction in DFI (SCSA) in treatment group when compared to placebo (30.0% vs 34.5%, $p = 0.27$) after 3 months (34.0% vs 29.5%, $p = 0.18$) and 6 months of treatment No significant increase in sperm concentration after 3 and 6 months of treatment
Martinez-sotto et. al. [92]	74 infertile men Treatment group: $n = 42$ Placebo group: $n = 32$	DHA 1500 mg (daily intake for 10 weeks)	Prospective placebo-controlled, double-blind, randomized parallel-group study	Significant reduction in SDF (TUNEL) (22.0 ± 2.1% vs 9.3 ± 1.3%, $p < 0.01$) before and after treatment No significant difference on semen parameters Placebo group no significant changes
Amar et. al. [102]	304 infertile men for 3 years with previous IVF/ICSI failed attempts Group 1: Treated with Fertibiol for 5 weeks followed by Condensyl for 4 months; $n = 151$ Group 2: Treated with Condensyl for 4 months; $n = 69$ Group 3: No supplement given; $n = 84$	Fertibiol: Coenzyme Q10, vitamin E 12 mg, NAC 200 mg, carnitine tartrate 134 mg, asthaxanthine 4.3 mg, vitamin B, chelated zinc (15 mg) Condensyl: Vitamin B, zinc, betalains quercetin, NAC (daily intake)	Prospective comparative study	Group 1 DFI (TUNEL) significantly reduced (from 30.0% to 20.9%, $p = 0.001$) SDI (AB) significantly reduced (from 39% to 35%, $p < 0.01$) Clinical pregnancy significantly improved (50.7%, $p < 0.001$) Spontaneous pregnancy before ART (22%) Group 2 DFI significantly reduced (from 24.6% to 20%, $p = 0.003$) SDI significantly reduced (from 42% to 35%, $p = 0.001$) Clinical pregnancy significantly improved (50.7%, $p < 0.003$) Spontaneous pregnancy before ART (28%) Control group DFI and SDI showed non significance
Gual-Frau et. al. [104]	20 infertile men with grade 1 varicoceles	L-carnitine 1500 mg, vitamin C 60 mg, coenzyme Q10 20 mg, vitamin E 10 mg, vitamin B9 200 ug, vitamin B12 1ug, zinc 10 mg, selenium 50 ug (daily intake for 3 months)	Prospective observational study	Significant reduction in DFI (SCD) (22.1%, $p = 0.02$) Reduction in highly degraded sperm cells (31.3%, $p = 0.07$) Increased total numbers of sperm cells ($p = 0.04$) Unaffected other semen parameters unaffected

Table 47.2 (continued)

Study	Population size	Antioxidants use	Methodology	Results
Abad et. al. [93]	20 asthenoteratozoospemic men	L-carnitine 1500 mg, vitamin C 60 mg, coenzyme Q10 20 mg, vitamin E 10 mg, zinc 10 mg, vitamin B9 200 1 g, selenium 50 1 g, vitamin B12 1 g (daily intake for 3 months)	Prospective observational study	Significant reduction in DNA degraded sperm (7.32 ± 4.12 vs 5.66 ± 3.2, p = 0.04) Significant reduction in DFI (SCD) at all different experimental time point after treatment at 0 h (28.5 ± 14.97 vs 20.12 ± 8.26, $p = 0.004$) at 2 h (28.77 ± 13.45 vs 20.7 ± 8.42, $p = 0.003$) at 6 h (31.65 ± 12.44 vs 23.07 ± 11.63, $p = 0.004$) at 8 h (34.9 ± 12.92 vs 25.87 ± 10.13, $p = 0.006$) at 24 h (53.97 ± 21.94 vs 33.02 ± 13.35, $p = 0.0002$)
Vani et. al. [98]	240 men 120 men exposed to lead 120 healthy men	Vitamin C 1 g (daily intake 5x/week for 3 months)	Prospective comparative study	Significant reduction in alkaline-labile sites and in the mean tail length of the comet ($p < 0.001$) compared to the control group
Martinez-Soto et. al. [91]	36 infertile men Treatment group: $n = 21$ Placebo group: $n = 15$	DHA 1500 mg (daily intake for 10 weeks	Prospective placebo-controlled, double-blind, randomized study	Significant reduction in DFI (TUNEL) (0 week 25.98 ± 4.73, 5 weeks 15.60 ± 2.46, 10 weeks 8.79 ± 1.92, $p < 0.01$) compared to placebo group ($p = 0.25$)
Tunc et. al. [94]	50 infertile men with high ROS	Menevit: Lycopene 6 mg, vitamin E 400 IU, vitamin C 100 mg, zinc 25 mg, selenium 26 g, folate 500 g, garlic oil 333 g (daily intake for 3 months)	Prospective observational study	Significant improvement in SDF (from 22.2% to 18.2%, $p = 0.002$) Significant change in median level of sperm DNA protamination (69.0% vs 73.6%, $p < 0.001$), reduction of early apoptosis (27.3% vs 22.5%, $p = 0.004$) and ROS production (66.4% vs 44.4%, $p = 0.027$)
Gil-Villa et. al. [103]	17 infertile men whose spouses had history of >2 embryo loss before 12 weeks of gestation treated	B-carotene 5000 IU, vitamin C 60 mg, vitamin E 30 IU, zinc 15 mg (daily intake for 3 months)	Prospective observational study	9/17 men had increased DFI (SCSA) 6/9 of spouses became pregnant and achieved successful pregnancy
Piomboni et. al. [96]	36 men with leukocytospermia 15 control men	Beta-glucan 20 mg, fermented papaya 50 mg, lactoferrin 97 mg, vitamin C 30 mg, vitamin E 5 mg (daily intake for 3 months)	Prospective observational study	No significant difference on DFI (AO) between the treated group (16.7 ± 8.0 vs 14.4 ± 6.0, $p > 0.05$) and untreated group (15.8 ± 6.7 16.1 ± 5.4, $p > 0.05$)
Omu et. al. [97]	45 asthenozoospermic men Group 1: Zinc sulphate 200 mg Group 2: Zinc sulphate 200 mg + vitamin E 10 mg Group 3: Zinc sulphate 200 mg + vitamin E 10 mg + vitamin C 5 mg Group 4: Control	Zinc sulphate 200 mg BID, vitamin E 10 mg BID, vitamin E 10 mg, vitamin C 5 mg BID (2x/day intake for 3 months)	Prospective placebo-controlled, double-blind, randomized study	Increased expression of superoxide dismutase and anti-apoptotic Bcl-2 in the treatment group Decreased expression of Bax and ASA titers in the treatment group Zinc deficiency was significantly associated with increased level of MAL ($p < 0.01$) and increased DFI (SCSA) ($p < 0.01$)
Menezo et. al. [101]	88 infertile men whose spouses had at least 2 previous failure of IVF or ICSI treated	Vitamins C 400 mg, vitamin E 400 mg, B- carotene 18 mg, zinc 500 mg, selenium 1 mg (daily intake for 90 days	Prospective observational study	Significant reduction in DFI (SCSA) (from 32.4% to 26.2%, $p < 0.004$) Significant increase in SDI (from 17.5% to 21.5%, p < 0.001)

(continued)

Table 47.2 (continued)

Study	Population size	Antioxidants use	Methodology	Results
Tremellen et. al. [95]	60 infertile men	Menevit: Lycopene 6 mg, vitamin E 400 IU, vitamin C 100 mg, zinc 25 mg, selenium 26 ugm, folate 0.5 mg, garlic 1000 mg, palm oil (daily intake for 3 months)	Prospective placebo-controlled, double-blind, randomized study	No significant difference on DFI (TUNEL) (37.9 ± 11.9 vs 40.3 ± 15.3, $p > 0.05$) No significant difference in pregnancy rate (63.9% vs 37.5%, $p = 0.077$) and implantation rate 946.2% VS 24%, $p = 0.062$) Significant difference in viable pregnancy rate (38.5% vs 16%, $p = 0.04$)
Greco et al. [91]	64 infertile men with elevated DFI >15%	Vitamin C 1 g, vitamin E 1 g (daily intake for 2 months)	Prospective placebo-controlled, double-blind, randomized study	Significant reduction in DFI (TUNEL) in the treatment group (22.1 ± 7.7 vs 9.1 ± 7.2, $p < 0.001$) No significant difference in DFI in placebo group (22.4 ± 7.8 vs 22.9 ± 7.9, $p > 0.05$)
Greco et al. [100]	38 infertile men with spouses had a history of 1 failed ICSI	Vitamin C 1 g, vitamin E 1 g (daily intake for 2 months)	Prospective observational study	Significant improvement in DFI (by TUNEL) (24.0 ± 7.9 vs 8.2 ± 4.3, $p < 0.001$) Significant improvement in implantation rate (2.2% vs 19.6%, $p < 0.01$) Significant improvement in pregnancy rate (6.9% vs 48.3%, $p < 0.05$) No difference in fertilization and cleavage rates or in embryo morphology
Kodama et al. [32]	19 infertile men 17 control patients	Vitamin E 200 mg, vitamin C 200 mg, glutathione 400 mg (daily intake for 2 months)	Prospective observational study	Significant increase in the level of 8-hydroxy-2′-deoxyguanosine in infertile men (1.5 ± 0.2 vs 1.0 5 ± 0.1, $p < 0.05$)
Fraga et al. [33]	10 men	Vitamin C 250 mg (daily intake for 15 weeks)	Prospective observational study	Level of oxo8dG in sperm DNA increased to 91% when dietary ascorbic acid was decreased from 250 mg to 5 mg Repletion caused decreased level of oxo8dG by 36%

significant lower DNA fragmentation rate when compared to placebo after treatment with antioxidants (MD -13.85, 95% CI -17.28 to −10.41, $p < 0.00001$). In one of the studies included, Greco et al. [90] investigated 64 men with infertility who were randomized into 2 groups (those with oral treatment of vitamin C 1 g once a day and vitamin E 500 mg twice a day for 2 months; and those who received placebo). SDF was measured by TUNEL. There was a statistically significant difference in reduction of SDF (58.4 ± 27.8 vs 6.6 ± 12.1, $p < 0.001$) in patients taking the medication compared to those who were into placebo group. In the other study [91], 46 men were assigned to Docosahexaenoic acid (DHA) 1050 mg/day as a treatment group and sunflower oil 1050 mg/day as a placebo group for 10 weeks. The treatment group showed a significant reduction of SDF measured by TUNEL that was directly proportional to the number of weeks of treatment (0 week 25.98 ± 4.73, 5 weeks 15.60 ± 2.46, 10 weeks 8.79 ± 1.92, $p < 0.01$) compared to placebo group ($p = 0.25$).

Although not included in this Cochrane review, another randomized, double blind, placebo-controlled, parallel study for DHA was done by Martinez-Soto et al. [92]. No difference in semen parameters nor the composition of the sperm membrane was noted after 10 weeks of treatment with three DHA 500 mg capsules daily. However, the study showed significant reduction of SDF (22.0 ± 2.1% vs 9.3 ± 1.3%, $p < 0.01$) before and after treatment. The placebo group did not show any changes in sperm DNA damage. This was in congruent with the findings of their initial study on DHA and SDF.

Abad et al. [93] determined in a cohort of 20 infertile patients diagnosed with asthenoteratozoospermia, the effect of oral antioxidant treatment using L-carnitine 1500 mg, vitamin C 60 mg, coenzyme Q10 20 mg, vitamin E 10 mg, zinc 10 mg, vitamin B9 200 ug, selenium 50 ug and vitamin B12 1 g for 3 months. The authors primarily determined the dynamics of SDF using the SCD test following varying periods of sperm storage (0 hour, 2 hours, 6 hours, 8 hours and

24 hours) at 37 °C before and after treatment. In addition, they examined the antioxidant effect on highly DNA degraded sperm. Overall, there was a statistical significant reduction of sperm DNA damage at all different experimental time periods following antioxidant treatment ($p < 0.05$). Likewise, degraded sperm with high levels of DNA damage were decreased significantly after treatment (7.32 ± 4.12 vs 5.66 ± 3.21, $p = 0.04$).

A total of 50 infertile men with ROS were asked to take 1 capsule Menevit (lycopene 6 mg, vitamin E 400 IU, vitamin C 100 mg, zinc 25 mg, selenium 26 g, folate 500 g and garlic oil 333 g) [94]. After 3 months of intake, SDF as assessed by TUNEL significantly improved (from 22.2% to 18.2%, $p = 0.002$). This was associated with corresponding significant change in median level of sperm DNA protamination (69.0% vs 73.6%, $p < 0.001$), reduction of early apoptosis (27.3% vs 22.5%, $p = 0.004$) and ROS production (66.4% vs 44.4%, $p = 0.027$) after treatment. This showed a compelling improvement in sperm DNA structure after 3 months of antioxidant therapy. Another study on Menevit capsule was done by Tremellen et al. [95]. This was a prospective randomized double-blind placebo-controlled trial on 60 couples with severe male infertility aiming to determine the effect of antioxidants use on outcome during ART. Patients were randomly assigned to receive 1 capsule of Menevit or placebo (palm Oil) for 3 months. Although the Menevit group showed numerically higher pregnancy rate (63.9% vs 37.5%, $p = 0.077$) and implantation rate (46.2% vs 24%, $p = 0.062$), the differences were not statistically significant compared to placebo group. Same is true with DFI levels measured by TUNEL which were lower in the Menevit group compared to placebo group (37.9 ± 11.9 vs 40.3 ± 15.3, $p > 0.05$) without achieving statistical significance. Nonetheless, significant effects were observed in the viable pregnancy rate (38.5% vs 16%, $p = 0.04$) with the Menevit group. In a study [96] of 36 asthenoteratozoospermic men with leukocytospermia, no significant reduction in DFI (16.7 ± 8.0 vs 14.4 ± 6.0, $p > 0.05$) was seen after 3 months of treatment with beta-glucan 20 mg, fermented papaya 50 mg, lactoferrin 97 mg, vitamin C 30 mg and vitamin E 5 mg.

Both monotherapy and combination therapy with Zinc [97] were used to improve semen parameters in asthenozoospermic men through different mechanisms not only in prevention of OS and apoptosis but most importantly for lowering SDF levels. Forty five asthenozoospemic men were randomized into 4 groups (Group 1: zinc sulphate 200 mg BID, Group 2: zinc sulphate 200 mg + vitamin E 10 mg BID, Group 3: zinc sulphate 200 mg + vitamin E 10 mg + vitamin C 5 mg BID and Group 4: control). Semen analysis was done 6 weeks after 3 months of treatment with the medications. Low level of zinc was significantly associated with increased level of MAD ($p < 0.01$) and increased DFI ($p < 0.01$) as measured by SCSA. This was accompanied by increased expression of OS biomarkers like superoxide dismutase and anti-apoptotic Bcl-2 and decreased expression of Bax and antisperm antibody titers. This only proves the role of ROS in inducing SDF. Monotherapy with vitamin C 1 g for 3 months showed significant reduction in alkaline-labile sites and in the mean tail length of the comet ($p < 0.001$) compared to the control group [98].

In the recently published systematic review by Majzoub et al. [99] of 19 randomised clinical trials and 10 prospective studies commonly investigating compounds of vitamin E 400 mg, vitamin C 500–1000 mg, carnitines 500–1000 mg, NAC 600 mg, CoQ10 100–300 mg, zinc 25–400 mg, selenium 200 mg, folic acid 0.5 mg and lycopene 6–8 mg, 26 studies showed positive effects with the use of antioxidants on advanced sperm function tests consisting of SDF and ROS as well as the basic semen parameters, ART outcomes or live birth rate. This highlights the promising effects of antioxidants particularly due to the lack of high quality research studies. The effects of oral antioxidant use in ICSI cases has also been investigated. Thirty eight men with >15% DFI in ejaculate after one failed ICSI attempt were treated with daily intake of vitamin C 1 g and vitamin E 1 g for 1 month [100]. DFI as measured by TUNEL was significantly improved before and after the treatment (24.0 ± 7.9 vs 8.2 ± 4.3, $p < 0.001$). Likewise, there was a significant improvement in implantation rate (2.2% vs 19.6%, $p < 0.01$) and pregnancy rate (6.9% vs 48.3%, $p < 0.05$) on the second ICSI attempt. However, there was no difference in fertilization and cleavage rates or in embryo morphology. This observation makes the use of antioxidants after failed ICSI a reasonable and relatively less expensive and non-invasive option compared to other treatment modalities like use of testicular sperm. Menezo et al. [101] performed a study on 88 patients who had at least 2 previous IVF or ICSI failures using SCSA to measure DFI. The authors included patients who were prescribed vitamin C 400 mg, vitamin E 400 mg, B- carotene 18 mg, zinc 500 mg and selenium 1 mg for 90 days. A significant decrease in DFI (from 32.4% to 26.2%, $p < 0.004$) after treatment with antioxidants was noted. However, sperm decondensation was noted to significantly increase (from 17.5% to 21.5%, $p < 0.001$). The authors recommended against the use of antioxidants on patients with a degree of decondensation more than 20%. In another study by Amar et al. [102], they grouped patients into Group 1 [daily dose of Fertibiol containing coenzyme Q10, Vitamin E (12 mg), N-acetyl cysteine (200 mg), Carnitine tartrate (134 mg), asthaxanthine (4.3 mg), vitamin B, and chelated Zinc (15 mg) which was followed by treatment with Condensyl containing vitamin B, zinc, betalains quercetin and N-acetylcysteine], Group 2 [Condensyl only] and Group 3 [no supplements]. Included male partners had primary infertility for >3 years with previous IVF/ICSI attempts. Group 1 patients were treated with daily doses of Fertibiol

for 5 weeks followed by 2 daily capsules of Condensyl for 4 months while Group 2 patients took 2 daily capsules of Condensyl only for 4 months. They measured the DFI using TUNEL and sperm nuclear decondensation index (SDI) using microscopy of 200 cells and aniline blue staining. DFI was reduced significantly in Group 1 (from 30.0% to 20.9%, p = 0.001) and in Group 2 (from 24.6% to 20%, $p = 0.003$). SDI was likewise improved in Group 1 (from 39% to 35%, $p < 0.01$) and in Group 2 (from 42% to 35%, $p = 0.001$). Control group did not show any changes in both DFI and SDI. Both Group 1 (50.7%, $p < 0.001$) and Group 2 (50.7%, $p < 0.003$) reported significant improvement in clinical pregnancy rates compared to control group. In addition, spontaneous pregnancies occurred in Group 1 (22%) and in Group 2 (28%) before the planned ART cycles.

There is also a proven effect of antioxidants use in early recurrent pregnancy loss. Seventeen infertile men with spouses having history of >2 early embryo losses (before 12 weeks of gestation) were included in the study [103]. Fifty three percent (9 out of 17 men) had an increased DFI determined by SCSA and deranged measure of lipid peroxidation as determined by high thiobarbituric acid reactive substances (TBARS). Patients were advised to take multivitamins (B-carotene 5000 IU, vitamin C 60 mg, vitamin E 30 IU, and zinc 15 mg) for at least 3 months. Eventually, 5 spouses successfully conceived while the remaining had embryo demise. This showed an improvement in gestational outcome in couples with history of embryo losses. Although, additional large volume studies are needed to derive this possible beneficial effect on embryo development.

Antioxidants can also reduce the SDF levels in patients with varicocele, even in lower grades of disease. Gual-Frau et al. [104] investigated 20 infertile patients with grade 1 varicoceles. Multivitamins (L-carnitine 1500 mg, vitamin C 60 mg, Coenzyme Q10 20 mg, vitamin E 10 mg, vitamin B9 200 ug, vitamin B12 1ug, zinc 10 mg and selenium 50 ug) were given for 3 months. There was a significant reduction in SDF (22.1%, $p = 0.02$) determined by SCD after treatment. Sperm concentration increased significantly ($p = 0.04$). Further studies examining the effect of antioxidant use on patients with varicocele are needed.

In the most recently published study by Stenqvist et al. [105], the use of vitamin C 30 mg, vitamin E 5 mg, vitamin B12 0.5 ug, l-carnitine 750 mg, coenzyme Q10 10 mg, folic acid 100 ug, zinc 5 mg, selenium 25 ug did not show significant difference in DFI compared to placebo after 3 months (30.0% vs 34.5%, $p = 0.27$) and 6 months (34.0% vs 29.5%, $p = 0.18$) of treatment.

Despite the beneficial effects of antioxidants use on semen parameters, advanced sperm functions and pregnancy outcomes, most of the studies presented have low methodological qualities. They suffer from high heterogeneity, lack of standardization of the SDF testing methods used, varied small population, variable composition and dosages of antioxidants and non-reporting of adverse events. This should be addressed by conducting well-designed, large randomized placebo controlled trials. Despite these limitations, antioxidant use can help reduce the level of SDF.

47.4 Safety of Long-Term Antioxidant Use

The benefits and risks of antioxidants have been studied on overall health and well-being [106]. In a randomized, double-blind, placebo-controlled study of 8112 male and female participants on long term antioxidant supplementation of vitamin C 120 mg, vitamin E 30 mg, beta-carotene 6 mg, selenium 100 mg and zinc 20 mg, no significant effect on global health was noted throughout the median follow up of 77 months [107]. This proved that antioxidants are relatively safe. However, safety on the long-term use of antioxidants for male infertility has not been studied extensively and for appropriate duration.

Excessive use of antioxidants, in high dosages, can paradoxically alter sperm parameters as noted in two in vitro studies [108, 109]. In a study by Verma et al. [110], sperm viability and motility ($p < 0.001$) was severely affected if supplemented with more than 1000 uM of ascorbic acid. A concentration of 4000 uM rendered the spermatozoa completely immotile. In another study, a selenium level between 50 to 69 ng/ml showed maximal sperm motility, higher pregnancy rate and low abortion rate. However, a higher incidence of asthenozoospermia was noticed outside this level [111].

The previously reported Cochrane review by Showell et al. [89] noted the adverse events conveyed in its included trials. These were gastrointestinal upsets, euphoria, miscarriage and ectopic pregnancy. Side effects after 3-month intake of the Menevit antioxidant were reported by Tremellen et al. [95]. Out of the 37 men included in his study, 2 experienced mild gastroesophageal reflux and the other 1 became constipated. In another study, 1 case of severe diarrhea and 2 cases of transient diarrhea were reported in 10 men with idiopathic infertility taking magnesium 3000 mg for 90 days. Cavallani et al [112]. reported 2 cases of mild epigastric pain and nausea and 5 cases of mild euphoria out of 39 men with oligoasthenozoospermia treated with L-carnitine 2 g and acetyl-L-carnitine 1 g for 6 months. However, further analysis failed to show a significant association between the use of antioxidants and gastrointestinal upsets (OR 1.60, 95% CI 0.47–5.50, $p = 0.46$) or euphoria (OR 1.21, 95% CI 0.16–9.01, $p = 0.85$) when compared to placebo [89].

Negative effects on reproductive outcome have been also linked to antioxidants use. In a study of of 11 women whose partners took zinc sulphate 200 mg for 3 months, one woman had spontaneous abortion at about 8 weeks of gestation

[113]. In another study by Suleiman et al. [114], 2 cases of abortion were reported in women whose partners took vitamin E 100 mg for 6 months. Tremellen et al. [95] reported 2 clinical miscarriages and 1 ectopic pregnancy on Menevit group. Similarly, statistical analysis of these results failed to find a significant association between the use of antioxidants and miscarriage (OR 1.74, 95% CI 0.40–7.60, $p = 0.46$) or ectopic pregnancy (OR 4.48, 95% CI 0.07–286.49, $p = 0.48$) when compared to no treatment [89].

Currently, there is insufficient evidence showing a significant association between adverse events and antioxidant use. Larger population and longer follow up studies are needed to determine the safety of long term use of these antioxidants.

47.5 Conclusion

ROS play an important role in the generation of SDF. These advanced tests of sperm function provide valuable information during male infertility evaluation. A growing body of evidence regarding the beneficial effects of antioxidant use in infertile men with SDF is widely available. However, due to high heterogeneity and low quality of methodological techniques used in these studies, we cannot conclusively determine the definitive effects of these compounds. A well-designed, large randomized placebo controlled trial should be conducted to strongly establish its use for male infertility in order to promote better reproductive outcome. Despite the lack of strong evidence, use of antioxidants is still considered a valuable option for treatment of infertile men with SDF.

47.6 Review Criteria

A thorough search of medical literature was done on antioxidant use and sperm DNA damage using the following search engines: PubMed, Google Scholar, MEDLINE and Science Direct. The keywords “antioxidants”, “sperm DNA fragmentation”, “sperm DNA damage”, “infertile men”, “male infertility” “semen parameters” and “reactive oxygen species” were used for study identification and data extraction. Only articles published in English language were included.

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Yoga, Meditation, and Acupuncture for Male Reproductive Health

48

Vidhu Dhawan and Rima Dada

Key Points

- Male factor infertility is a complex disorder and an etiology is not known in about 70% of the cases; 15–20% of men with non obstructive azoopsermia and severe oligozoopsermia harbor genetic abnormalities.
- Unexplained infertility is on the increase. Such cases have high seminal oxidative stress and DNA damage. DNA damage is the major cause of defective sperm function. Oxidative damage to DNA and accumulation of oxidized DNA adducts like 8-OHdG are mutagenic and also adversely impact sperm epigenome and thus increase genetic and epigenetic disease burden in the offspring.
- Acupuncture, has been shown to mitigate any imbalances in the flow of vital energy or blockage of Qi energy and circulation of blood flow essential for health.
- Yoga is a profound science of inner well-being. It effectively decreases both oxidative stress and nuclear and mitochondrial DNA damage. It upregulates expression of DNA repair, cell cycle control, anti-inflammatory genes and modulates the immune response. It promotes neuroplasticity by upregulating expression of BDNF, DHEA, serotonin, melatonin and thus reduces severity of depression, stress and anxiety. It upregulates activity and levels of telomerase and slows the rate of testicular aging. It may thus reduce the number of couples who need assisted conception.
- Yoga thus improves mitochondrial and nuclear genomic integrity and thus the health trajectory of the offspring and may also reduce the risk of infertile men developing gonadal and extragonadal tumors.

48.1 Introduction

Health, the optimal goal for all people, is particularly challenging as it not only demands due concern early in life but is also sustained across an individual's lifespan. It has been largely recognized in recent years that there is a complex interplay between the occurrences of chronic diseases among reproductive-age individuals. The association between health and the fertility spectrum has gained significant speculation. The exact pathophysiology of the association between the chronic complex lifestyle disorders and fecundity, defined as biological capacity to reproduce regardless of pregnancy intention, has not been delineated yet. It has been observed that reproductive-age individuals who are affected by chronic diseases may experience impairments in fecundity, such as decline in semen quality, infertility, pregnancy loss, and increased incidence of congenital malformations, adversely affecting the health of the future progeny [13, 15, 34]. Derangements in fertility have been associated with a strong psychosomatic component and have been associated with a social stigma in certain societies. An enigma sill remains of whether the occurrence of chronic diseases and impaired fecundity share any similar or distinct etiology and what the effects are on the pregnancy outcomes and offspring health.

48.2 Background

There has been an increasing interest in the wide arena of research exploring the association between infertility and the development of complex chronic diseases and mortality. Infertility is found to be negatively associated with the interpersonal, sexual and psychosocial well-being of an individual. Diagnosis of infertility not only culminates in the distressing symptoms of anxiety and depression but also exerts detrimental effects on the patient's quality of life. To add to the complexity, it is now established that the impairments in fecundity (decline in semen quality, infertility, endometriosis, polycystic ovarian disease) have been greatly

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linked with diseases originating later in the life, such as adult onset carcinomas, cardiovascular diseases, and impending morbidity and mortality [15, 32, 33, 74]. The evolving body of clinical and epidemiologic data supporting an association between fecundity and life course health has prompted a call to incorporate fecundity into general health screening [14, 15, 69, 88]. Infertility has stepped up the ladder to be considered as a complex chronic lifestyle condition. There is growing evidence that lifestyle choices account for the overall quality of health and life (QoL) reflecting many potential lifestyle risks widely associated with alterations of the reproductive function up to infertility. Lifestyle factors can be modified to enhance overall wellbeing, and they are ultimately under one's own control [70]. It has been witnessed that there has been a phenomenal increase in the incidence of complex lifestyle diseases, such as depression, cardiovascular diseases (CVD), diabetes mellitus (DM), cancer, arthritis, infertility, recurrent spontaneous, and implantation failures in the past decade [26–29, 31, 41, 85].

These lifestyle disorders have become fast growing epidemics and a bane of modern society. The surge of these various lifestyle disorders demands an active interplay of various lifestyle modifications as well as adoption of various complementary and alternative medicine (CAM) approaches as an adjunct to modern medicine therapies [51, 53]. In industrialized nations, decreasing the number of people affected by infertility has become a top priority for many health organizations [48]. Furthermore, various recent studies and reviews have suggested that a male's reproductive and general health are largely intertwined. These disorders have a strong psychosomatic component and need to be approached and managed using a mind body intervention. Thus, a holistic approach to male factor infertility and in management of infertile couples is the need of the hour. This chapter intends to portray a descriptive outline of the interventions adopted for the management of male factor infertility. We aim to highlight the adoption of integrative medicine (IM) approaches, which represent a cusp of both CAM and conventional modern medicine.

48.3 Mind Body Interventions (MBI) and Integrative Health for Male Infertility

As infertility has a strong psychosomatic component, an integral component in its management should include the adoption of a holistic and complementary approach. Also, a majority of couples with a long duration of infertility experience high levels of stress and anxiety as compared to their fertile counterparts. Advent of mind–body practices along with various lifestyle interventions and adoption of a healthy and holistic lifestyle has been shown to improve a healthy biological profile of an individual, along with positive alterations in the stress-related physiological and psychosomatic processes implicated in the occurrence of disease.

Mind–body interventions (MBIs), widely known as mind–body medicine, have been mainly centered to "focus on the interactions among the brain, mind, body, and behavior, and on the powerful ways in which emotional, mental, social, spiritual, and behavioral factors can directly affect health" as described by the National Center for Complementary and Alternative Medicine. Diverse techniques have been ascribed to the MBIs, which include yoga, meditation, tai chi, qigong, bio-feedback, progressive muscle relaxation, guided imagery, hypnosis, and deep breathing exercises. The percentage of people now adopting MBI has increased substantially around the globe and a growing body of research has now resulted in evidence based integration into clinical practice [9]. Though all the practices may vary, they do share similarities, especially in the health sector as an integral component of integrative medicine. While many MBIs, such as hypnosis and muscle relaxation, have long standing legacies, many MBIs, such as yoga, tai chi and qigong, have spiritual traditions associated with them.

MBIs involve regulation of the mind's attention processes to impact the body's physiology [9]. These interventions have proven to be effective in reducing the symptoms and improving quality of life, and research has begun to examine the impact of these therapies on biological processes, including inflammation, oxidative stress, gene expression, and epigenetic modifications [8, 9, 30].

48.4 Yoga Based Lifestyle Intervention

There is a growing interest worldwide for modification of lifestyle factors, as well as holistic, complementary, and alternative approaches to treat male infertility. Yoga, essentially described as a psychosomatic–spiritual discipline, is aimed at achieving union and harmony between our mind, body, and soul and brings balance to all aspects of one's being from physical, mental, emotional to the spiritual spectrum. This ancient Indian discipline includes all aspects of an individual from health to self-realization. It is described as the ultimate union of one's own consciousness with the universal consciousness for attaining a super conscious state "*Samadhi*". It caters to self-management of life, and includes regulation of diet, mental attitude, and the practice of specific techniques, such as asanas (postures), breathing practices (pranayamas), and meditation, to attain the highest level of consciousness. Therapeutic yoga is defined as the application of these yoga postures and practice to the treatment of health conditions and involves instruction in yogic practices and teachings to prevent, reduce or alleviate pain, suffering, and limitations of a disease. Yoga was described as

"meditation in motion" by Khalsa et al., in 2009 [55]. Yogic practices have been seen to enhance flexibility, muscular strength, stamina, and endurance, improve respiratory and cardiovascular function, reduce stress, anxiety, depression, and chronic pain, hasten recovery from addictions, improve sleep pattern, and enhance overall well-being and quality of life. Various randomized controlled trials have been previously conducted citing the significant positive impact of yoga in the management of several diseases, such as bronchial asthma, cardiovascular disorders, diabetes mellitus, attention deficit hyperactivity disorders, depression [86], aging [85] primary open angle glaucoma [24, 66], infertility [23], implantation failure [27], autoimmune arthritis, and rheumatoid arthritis [41].

48.4.1 Yoga: The Historic Outlook

Yoga, originating as early as 3000 BC with roots in Indian philosophy, is now regarded in the Western world as a form of Complementary and Alternative Medicine (CAM) by the National Institutes of health. The word "yoga" is derived from a Sanskrit root "yuj" meaning "yoking" or "joining" and acts as means or techniques that transform one's consciousness to attain liberation (*moksha*) from karma [7]. Yoga philosophy and practice was systemized by Patanjali in the classic text "*Yoga Sutras"* (300–200 BC). It is considered to be the most authoritative text on yoga and defines the purpose of yoga as knowledge of the true "Self" (self-realization) and outlined an eightfold path to awareness and enlightenment of self, called *"ashtanga"* [59, 63].

Yoga (*ashtanga*), depicted metaphorically as a tree, comprises eight aspects or "limbs" representing the ethical principles of leading a purposeful life [62] (Fig. 48.1). They channelize an individual to ethical conduct, self-discipline, and attention toward one's health. The yogic limbs are connected to the whole similar to the way bodily limbs are connected to each other. On a whole, these eight limbs can be conceptualized as methods that aid in the regulation of thoughts, emotions, and behaviors, thus increasing an individual's well-being. If one yogic limb is pulled off, the others will naturally come in the same way as when someone pulls the body by a leg and no stages are achieved in succession [49].

Though any of the eight limbs may be used separately, the physical postures and breathing exercises prepare the mind and body for meditation and spiritual development [19]. Different yogic disciplines based on Patanjali's eight limbs have been developed, with their own technique for preventing and treating disease [90].

48.4.2 Physiological and Psychological Effects of Yoga

There is a dearth of studies which have employed an integrated yoga course (yoga of transformation) or practices of hatha, sahaj, and sudarshan kriya yoga with asanas, pranayama, and meditation, which included dhyana, transcendental, omkar, and amrita meditation. Transcendental meditation (TM) involves the mind doing well on a series of words (called a mantra) [11].

The coordinative interactions between the hypothalamo–pituitary–adrenal (HPA) axis, central nervous system (CNS), autonomic nervous system (ANS), limbic system, and immune system maintain hormonal homeostasis [10, 37]. Increasing research in this naïve field has suggested numerous beneficial effects of yoga interventions on a myriad of changes in psychological health. Two pathways can be hypothesized for the mechanism of action of yoga: (i) vagal stimulation and (ii) parasympathetic activation and HPA axis modification [71]. Yoga and meditation are a package of mind–body based practices, which exert their beneficial effects through both '*Top down'* and '*Bottom up*' approaches in contrast to the classical psychotherapeutic approaches involving cognitive strategies [39, 82]. Yoga encompassing meditation affects an individual's affective/cognitive state affecting the activity of brain regions, including the orbitofrontal cortex, amygdala, hippocampus, and somatosensory cortex. This exerts beneficial effects in

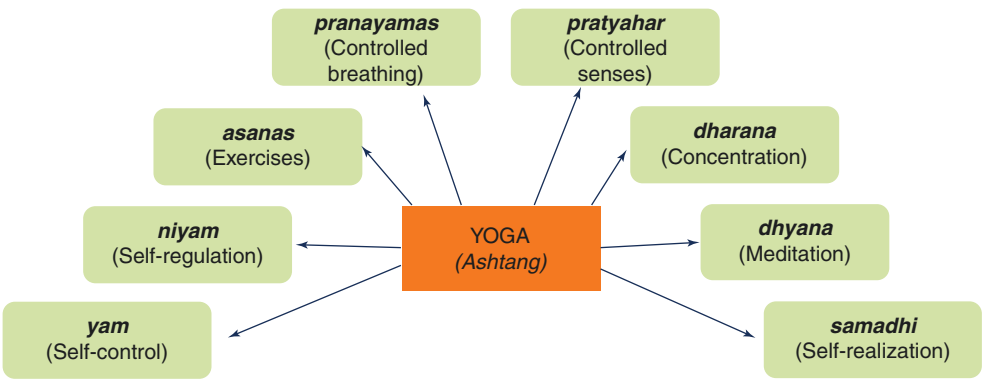

Fig. 48.1 The Yoga "Ashtang"

reducing psychological stress, decreased sympathetic activity, increased parasympathetic autonomic nervous system (ANS) tone, decreased production of inflammatory cytokines, and enhanced sensitivity to glucocorticoids produced via HPA signaling [17, 39]. Studies now support the belief that yoga techniques improve physical and mental health through downregulation of the HPA axis and the sympathetic nervous system, stress reduction, and immune modulation. Controlled breathing and various physical postures and asanas exert influences via '*Bottom Up*' mechanisms. It is seen to directly affect physiology by causing musculoskeletal exertion, improving cardiovagal tone causing downstream effects on HPA activity, maintaining sympathetic and parasympathetic balance, immune functions, and alleviating the mood. Yogic practices condition the limbic system, which regulates the homeostatic mechanisms through the ANS-endocrine modulation [44]. Higher cortical centers of the brain responsible for cognition and emotion directly affect the hypothalamo–pituitary–gonadal (HPG) axis and reproduction. Spermatogenesis, a highly regulated process encompassing a transition of the primordial germ cells to differentiate into the mature sperm, is tightly regulated by the HPG axis, which is further influenced by higher cortical centers, including the limbic system. These are also seen to be influenced by changes taking place in other systems of the body, including disturbances in metabolic and immune responses [26]. Optimum functioning and regulation of the higher cortical centers of the brain are thus fundamental for the regulation of the HPA, HPG axis, as well as autonomic and immune systems [39, 68]. Any perturbation in the mind, body, and psychological states causes imbalances in reproductive organs resulting in disordered spermatogenesis, poor spermatozoal structure and function. Reduction in stress and improvement in immune functions has been observed with regular practice of sudarshan kriya and rhythmic breathing processes in pranayam [56].

Thus, owing to the ability of yoga and meditation to address both mind and body, it works through a well-defined psychoneuroendocrine pathway that then affects a wide range of processes from basic metabolism, epigenetics, DNA repair, oxidative bioprocesses to aging, maintenance of vital organ systems, subjective well-being, and reproductive health.

Overwhelming psychological stress and anxiety have contributed to the pathogenesis of many chronic diseases and to decreased quality of life. The advent of non-pharmacologic treatment modalities like yoga and meditation are one of the promising modalities for opting to relieve stress and anxiety. Various biochemical markers can be used to quantify psychological stress, such as cortisol, β-endorphins, IL-6, and TNF-α. Regular adoption of yoga and meditation in our lifestyle has been seen to improve various cardinal biomarkers, facilitate immunomodulation, regulate the activity of various neurotransmitters, neuromodulators, and gene expression [17, 22, 52, 56, 81, 85]; (Fig. 48.2).

Tolahunase et al. [86] reported a decrease in clinical severity in major depressive disorder patients along with an associated increase in neuroplasticity with a brief yoga and

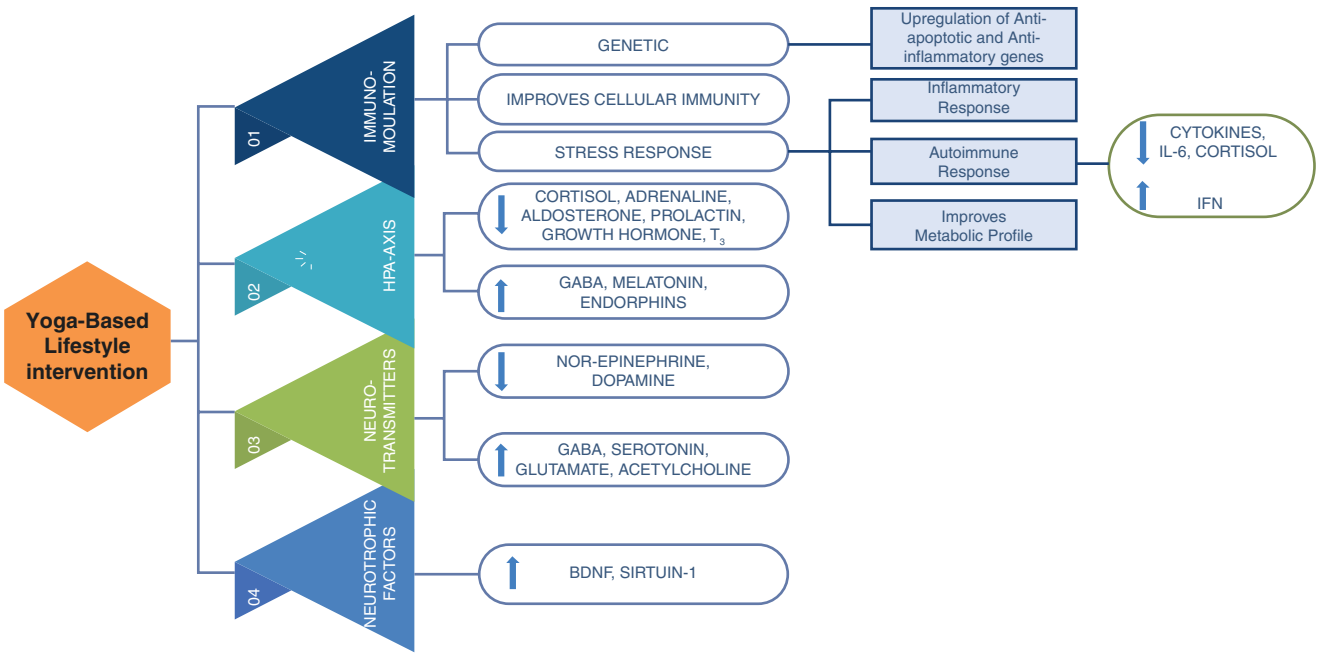

Fig. 48.2 The physiological basis of Yoga: effects of Yoga on regulation of hormones and neurotransmitters which effect physiological and psychological functions. HPA hypothalamo-pitutitary adrenal axis, T3 triiodothyronine, GABA gamma amino butyric acid, IL-6 interleukin-6, IFN interferon, BDNF brain derived neurotrophic factor

meditation based lifestyle intervention. This randomized controlled trial conducted in our laboratory documented an increase in the levels of DHEAS, sirtuin 1, telomerase activity, and a decrease in the levels of cortisol and interleukin 6 in addition to a significant decline in ODD and maintenance of oxidative eustress. Yoga thus acts as an important component of mind–body medicine that helps to improve overall health and has significant beneficial effects in male factor infertility.

48.4.3 Effect of Yoga on Oxidative Stress, Genomic Integrity, and Telomere Dynamics

Regular practice and adoption of yoga and meditation training as a part of one's lifestyle has been seen to exert a range of salubrious effects, including decline in oxidative stress, improved genomic integrity and semen quality, telomere regulation, and sperm transcript normalization [28, 30, 31, 57, 74]. Oxidative stress in the male germ line, thought to be produced by a host of intrinsic and extrinsic factors, affects male fertility and exerts a detrimental impact upon normal embryonic development. Spermatozoa are vulnerable to oxidative attack due to an abundance of polyunsaturated fatty acids in the sperm plasma membrane. Impending oxidative attack on the sperm is detrimental to the sperm nuclear and mitochondrial genomes [4, 5]. ROS metabolites attack the DNA bases (particularly guanine) and phosphodiester backbones, thus destabilizing the structure and inducing DNA fragmentation [5, 27, 67]. The accumulation of the harmful mutagenic base adduct due to the self-perpetuating lipid peroxidation cascade affects sperm plasma membrane fluidity, resulting in loss of sperm motility and ensues oxidative DNA damage ([2], Aitken & De Iuliis. 2010, [3, 5, 12]).

Previous studies from our laboratory have reported a rapid, significant decline in seminal oxidative stress even within 10 days of practice of yoga and meditation [28, 31, 57]. A minimal nonsignificant improvement in DNA fragmentation index (DFI) was observed by [28, 31] in male partners of couples with recurrent implantation failure in IVF cycles with a brief yoga-based lifestyle intervention for 21 days. A significant improvement in progressive motility and sperm count (taken at two separate intervals) has been found in primary infertility patients who experienced failed implantation in IVF cycles [28]. Significant improvement in DNA integrity was observed in another study on male infertility patients after following yoga-based intervention for 6 months. Sperm DNA damage is not only an underlying etiology of poor implantation and pregnancy rates but also affects the health of offspring and may also result in *denovo* mutations in the male germ line and post fertilization and a higher rate of post-zygotic mutations. A decline in DFI was accompanied by a decline in the levels of oxidative DNA base adduct 8-OHdG, which induces mutations and epimutations [23, 57, 74] and an increase in total antioxidant capacity (TAC) levels in seminal plasma [58, 74]. The decline in levels of this oxidative DNA adduct is important to assess the levels of oxidative DNA damage, and as this product is highly mutagenic, it can be the cause of infertility, post implantation losses, congenital malformations, and even childhood cancers.

Various antioxidant formulations are being prescribed for the current alleviation of the burden of overwhelming oxidative stress and its detrimental effects on genomic integrity and telomere dynamics [75]. Antioxidants cause improvement in sperm motility or concentration, but only a few have been implicated to impact the nuclear DNA damage at therapeutic doses [1, 64, 77]. Furthermore, the ideal balance of the redox system necessary for optimal sperm function is not known, and overconsumption of antioxidants may result in 'reductive stress' that could cause detrimental effects on human health and wellbeing [12, 28, 30, 31, 45]. Apart from causing untoward reductive stress, unregulated and unsupervised administration of the antioxidants has also been associated with impairment of mitochondrial activity, decreased permeability of the blood–brain barrier, and suppression of endothelial cell proliferation. Yoga and meditation interventions significantly impact free radical levels and also cause a collateral increase in antioxidant levels and decrease in inflammatory cytokines [28, 31, 40, 43, 74] as documented by an increase in total antioxidant capacity [58, 74]. They have been documented to regulate rather than simply scavenge reactive oxygen species. Indiscriminate use of antioxidants causes very low levels of free radicals, resulting in impaired sperm function and several redox sensitive metabolic reactions. Yoga and meditation mediated ROS regulation alleviates and reverses stress-causing processes.

Some of the important biomarkers of cellular and testicular aging affected by a yoga-based lifestyle are the telomeres [6, 46], activity of the related telomerase enzyme [60, 61], and the expression of telomere-related genes [20, 21, 35]. Telomere is a complex trait highly affected by the OS, which is the prime cause of rapid telomere attrition, genomic instability, and testicular aging. OS is associated with shorter telomeres predisposing to genome instability as well as genome wide hypomethylation and unmasking of repetitive elements [12, 27]. Telomere length is seen to shorten incrementally during cellular division and in response to cellular damage associated with various stressors. Thus, telomere length is counted as a crucial indicator of cellular aging, physiological and psychological stress ([20, 21, 87]; Conklin et al. [21]). Shorter telomeres are potentially associated with an increasing number of degenerative and age-associated disorders, infertility, cancers, etc. [73, 83, 92]. The regulation and maintenance of these highly conserved hexameric

repeats and optimum telomere length is done by a complex network of molecular components referred to as the '*telomere interactome'*. The telomerase enzyme is the key component of this interactome [20, 21, 76, 93]. Telomere length and telomerase activity have been shown to be highly sensitive to a range of psychosocial and behavioral factors. Telomerase activity is not only seen to predict cell survival but also to facilitate the actions of BDNF [38]. Studies from our laboratory have shown a significant increase in telomerase activity and a serotonin DHEA, BDNF mediated decrease in severity of clinical depression [86]. An increase in melatonin levels regulates the circadian rhythm and improves mitochondrial integrity, as the highest subcelluar concentration of melatonin is in the mitochondria. This improves mitochondrial integrity and reduces free radical production and increases ATP production. Though mild OS exerts beneficial effects in the maintenance of telomere length, low and high OS is associated with shorter telomeres and genomic instability [65]. YBLI has been shown to exercise beneficial effects in decline in OS and ODD as well as upregulation in the telomerase activity. An increase in telomerase activity with decline in OS and ODD and an increase in total antioxidant capacity were observed by Kumar et al. [58] following a brief yoga intervention for 21 days. There is significant decline in OS and ODD mainly due to improvement in mitochondrial integrity as seen by increased COX II activity [85]. This thus suggests that though psychosocial stressors and their biochemical consequences have the potential to cause telomere erosion, the resilience and adoption of yoga and meditation might offer some level of protection against such degradation. Yoga can aid in reducing the rate of biological as well as testicular aging. Age associated accumulation of mitochondrial mutations and associated high free radical levels predispose to genome hypermutability and genomic instability and might predispose to cancer [25].

The male factor infertility phenotype includes focusing on the next generation as it is associated with attention to the offspring's wellbeing and childhood mortality. Understanding the link between health and disease and infant outcomes is highly relevant for children conceived with infertility treatment received in the course of management of an infertile couple. Establishing the pathophysiology between the disease and fertility impairments remains an enigma and needs to be elucidated. In a collaborative yet unpublished study on the impact of yoga on sperm epigenome in infertile men between our group and Dr. R K Mishra of CCMB, Hyderabad, we found differentially methylated regions on the sperm genome [the technique employed was reduced representation bisulfite sequencing (RRBS)], an upregulation in expression levels of genes of folate membrane transport and folic acid transport, genes for DNA repair and cell cycle control, intrinsic apoptotic signaling pathway in response to oxidative stress, and anti-inflammatory genes. Thus, improving genomic DNA integrity can reduce the incidence of male factor infertility, idiopathic recurrent spontaneous abortions, idiopathic recurrent congenital malformations, and even reduce incidence of denovo germ line mutations, which set the stage for childhood cancers and numerous autosomal dominant disorders. Yoga thus not only promotes health but may prevent onset of age associated complex diseases and also exert a rehabilitative and curative potential.

48.5 Traditional Chinese Medicine: Role of Acupuncture

The use of acupuncture as another component of complementary/alternative medicine (CAM) in health care has been increasing and is also being adopted for the treatment of infertility. The World Health Organization (WHO) thus released a global policy in 2002 to assist the countries practicing these methods in regulating traditional medicine to facilitate safety and effectiveness, improve standardization, and also preserve ancient knowledge and safeguard the rich cultural heritage [50]. Acupuncture was adopted from traditional Chinese medicine (TCM), dating back almost 3000 years, and has become an integral component. Acupuncture practices have now gained immense popularity in the Western world as well [91]. The term acupuncture was essentially coined by Jesuit missionaries from the Latin word 'acus' meaning needle and 'punctura' that refers to pricking [91]. Theories of this ancient Chinese medicine state that there are certain patterns of energy flow (Qi) throughout the body that are vital for the maintenance of optimum health. TCM was greatly influenced by Chinese philosophic systems, such as Confucianism and Taoism. It was in those ancient times when the concepts of channels (meridians) through which the flow of the vital Qi energy flows for the regulation of harmony of the body status emerged [54]. A complete set of 365 acupoints has been described in relation to the respective meridians [47, 89]. In the sixth century, acupuncture spread to neighboring countries, such as Japan and Korea.

A National Institutes of Health interview survey conducted in the United States in 2002 stated that lifetime use of acupuncture had been reported in 4.1% of the respondents. On average, 2.13 million (1.1%) Americans were stated to have recently adopted the use of acupuncture [16]. Seven percent of the adult population was estimated to visit an acupuncture practitioner in the UK [84]. Acupuncture has now become immensely popular in Western medicine in the past decade, while the attempts to merge traditional Chinese and Western medicine have not largely been successful.

The use of acupuncture to treat reproductive disorders has gained significant attention. Various studies of acupuncture treatment have been conducted on male infertility patients.

Significant positive reports from uncontrolled trials on infertile men have shown a positive impact on sperm concentration and motility [72]. Regulations of endocrine status in male infertility by an increase in testosterone and improvement in luteinizing hormone (LH) level have also been reported [42]. These studies have also shown an increase of normally shaped sperm and a significant decrease in the percentage of morphologically abnormal sperm. Some studies also have shown that acupuncture did not trigger subjective behavior alterations or influence sexual behavior [72].

48.6 Physiologic Basis of Acupuncture in Treating Subfertility

The occurrence of infertility as described by theories of TCM is due to disruptions in the vital balance and blockage in the Qi energy and circulation of blood flow essential for health. These disruptions in the flow of energy and imbalances and disturbances, organ deficiencies or any excesses have been cited to be responsible for various disease states in human reproduction, such as infertility, polycystic ovarian diseases, and dysmenorrhea. Acupuncture has been proposed to mitigate any such imbalances in the flow of life force along meridians as a cure to specific diseases.

When the free flow of the energy is blocked, it can cause deficiency, stagnancy or heat syndrome [94]. Deficiency syndrome blocks and disrupts the sexual and reproductive function both in men and women. The stagnancy syndrome disables the free flow of the energy and blood and restricts it from circulation to the tissues in the reproductive organs. The heat syndrome is connected to inflammation processes that have an impact on semen quality and gynecological infections [94]. The insertion of intramuscular needles and stimulation causes afferent activity in the peripheral nerves. The acupuncture needles are then either stimulated by manual manipulation and/or by electrical stimulation, i.e., electro-acupuncture after insertion at the specific acupoints [80]. The stimulation of these acupuncture points in muscle tissue causes local release of neuropeptides—including neuropeptide Y, substance P, vasointestinal peptide, and calcitonin gene-related peptide—from the peripheral nerve endings. The muscle afferents modulate transmission of signals in the spinal cord and central nervous system [78]. The regulation of the pituitary gland in the CNS may thus modulate the endocrine system [79].

Recent advancements in modern scientific principles led to a better understanding of the underlying physiologic mechanisms of acupuncture. The beneficial effects of acupuncture in the management of infertility may be related to the central sympathetic inhibition modulated by the endorphin system and decrease in the levels of psychological stress. Nevertheless, the underlying mechanism of body acupuncture is still elusive. Most of the patients of male infertility, especially those who are undergoing IVF treatment, are under great levels of psychological stress, which is detrimental for fertility outcomes. Acupuncture exerts both physiologic and psychologic effects, and it may provide an excellent alternative for stress reduction in patients undergoing subfertility treatment ([18]; Yu Ng et al. [91]). Feelings of relaxation were reported by as many as 86% of patients following acupuncture [36]. Acupuncture thus aids in the maintenance of endogenous regulatory systems, including the endocrine system, sympathetic nervous system, and neuroendocrine system. The current data on the use of acupuncture as a component of TCM in Western medicine for the treatment of reproductive dysfunctions has not been well investigated. With sparse literature, only a few clinical studies have been reported. The studies also lack reliability in terms of flaws in poor design and a lack of diagnostic criteria and valid study outcomes measures, thus making the results difficult to interpret [80].

Though acupuncture has been shown to be beneficial for improving semen quality, the evidence in favor of acupuncture is still not compelling. Discrepancies in the effectiveness of acupuncture among the studies conducted so far is one of the factors that make it difficult to draw definite conclusions. Acupuncture treatment has been shown to be a simple, non-invasive modality for male infertile patients opting for either natural fertility or assisted conception to improve semen quality. Further research needs to be conducted to elucidate what stages and times in spermatogenesis are affected by acupuncture and what physiological changes are caused by acupuncture in spermatogenesis.

48.7 Conclusion

Infertility might be the first health crisis faced by an otherwise healthy couple, but they might learn of a lifelong, non-reproductive condition from their clinician. However, less is known about the extent to which fertility status can impact, or act as a marker for, future overall health. Infertility is not necessarily a unique disease of the reproductive axis but is often physiologically or genetically linked with other diseases and conditions. Recent epidemiologic studies demonstrate links between fertility status in both males and females and various somatic diseases and disorders.

With increased attention to infertility, there is increased potential to reach people during their reproductive years; when they are highly motivated to protect their current and future health yet young enough to begin to make changes to their lifestyle/health, which may mitigate later disease risk. Management of infertility with the adoption of lifestyle modifications can be a potential clinical ‘game changer’ and would aim to provide new insights for the diagnosis of chronic diseases and a window into future health.

48.8 Review Criteria

An extensive search of studies was performed to examine the impact of various complementary and alternative therapies in male reproductive health using search engines, such as PubMed, Google Scholar, and Science Direct, for full-text English-language articles. The search was performed between June 2017 and December 2018. Various combinations of the following search items: 'male infertility', 'integrative medicine', 'mind–body interventions', 'yoga', 'meditation', 'acupuncture', 'oxidative stress', 'DNA damage', 'testicular aging', 'testicular cancers', 'ART', 'ICSI', 'antioxidants', 'hypothalamic–pituitary axis', 'hypothalamic–pituitary–gonadal axis', 'psychological stress' as well as the names of specific oxidative stress biomarkers, neuromodulators, and neurotrophic factors. The reference lists of selected articles were searched for further relevant publications. Relevant primary research papers, reviews, and meta-analyses were then classified and analyzed for coherent theoretical explanations. All relevant literature reports were taken into consideration when writing the manuscript.

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Part V

Assisted Reproductive Technology in Male Fertility

The Role of Interventions to Reduce Oxidative Stress and Improve Sperm DNA Integrity Before ICSI

49

Chak-Lam Cho

Key Points

- Interventions alleviating oxidative stress (OS) and sperm DNA fragmentation (SDF) may improve ICSI outcomes and reduce the chance of injecting DNA-damaged sperm into oocyte.
- The use of oral antioxidant therapy represents a simple noninvasive approach to reduce OS and SDF though a solid conclusion cannot be made in view of heterogeneity of current studies.
- Various advanced sperm selection techniques and the use of sperm with short ejaculatory abstinence are proposed with promising effect on reduction of SDF. However, current evidence on the clinical application of the techniques remains scarce.
- Varicocelectomy in patients with concomitant clinical varicocele and high SDF is a rational treatment option as supported by current evidence.
- Sperm retrieval and the use testicular sperm in ICSI for patients with high SDF and previous failed ICSI attempt may potentially improve ICSI outcomes.

49.1 Introduction

Assisted reproductive technologies (ART) are the treatment of choice for many couples facing infertility issues. It is reflected by the increasing number of couples seeking ART by 4% each year. The advent of intracytoplasmic sperm injection (ICSI) has revolutionized the management of infertile couples since 1992 [1]. The technology of ICSI is applied to couples with the most severe form of male and/or female factors or idiopathic infertility by injecting a single spermatozoon directly into the cytoplasm of oocyte. It potentially promises the couple a baby without exploring the detailed cause of underlying infertility which may be reversible. Tremendous effort has been invested to ART in improving embryo quality and pregnancy outcome by workup of female partner over the last few decades. However, the live birth rate utilizing ICSI as the treatment of infertility, particularly in face of severe male factor, has stagnated at 30% which warrants further improvements [2].

The male gamete contributes half of the DNA content of the offspring, and male factors are responsible for approximately half of all infertility cases [3]. The importance of paternal DNA on ART outcomes is being increasingly recognized [4]. Although sperm concentration and motility after sperm preparation were widely accepted in determination of the choice of ART method, conventional semen parameters were not considered predictive of ART outcome either alone or in combination [5]. Recently, the central role of oxidative stress (OS) in the pathogenesis of testicular damage has been identified as the common pathway mediating male subfertility and various clinical conditions [6]. Sperm DNA fragmentation (SDF) is considered one of the major consequences of OS. Emerging evidence supports the negative impact of high SDF on pregnancy outcome in ICSI cycles. More importantly, it has been reported that around 50% of injected sperm during ICSI contain damaged DNA [7]. The unclear and potentially hazardous consequences of injecting DNA-damaged sperm into the oocyte in ICSI cycles during treatment of couples with severe male factor infertility should not be overlooked [8].

In this chapter, we first illustrate the deleterious effect of OS and high SDF on ICSI outcomes. Then, available treatment strategies for OS and high SDF are highlighted and reviewed.

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49.2 Oxidative Stress, Sperm DNA Fragmentation, and ICSI Outcome

The inadequacy of semen analysis for the assessment of male infertility has led to a search for better predictors. Current evidence supports OS as a key element in male subfertility. OS exerts its negative impact on male fertility via various mechanisms, and SDF is one of the important mediators [6]. Numerous studies have attempted to assess the association between elevated SDF and ART outcomes in parallel with the wider clinical application of various SDF assays. On the other hand, the relationship between OS and ART outcomes is less reported.

The clear association between high SDF and decreased pregnancy rates in natural conception and intrauterine insemination (IUI) provides strong support for the clinical utilization of SDF in male fertility evaluation [8]. However, the impact of SDF on pregnancy outcomes in ICSI cycles is less clear. Earlier studies suggested that SDF has a greater clinical relevance on outcome of in vitro fertilization (IVF) cycles than in ICSI. As a result, ICSI had been advocated as a potential therapeutic option for men with elevated SDF [4, 9]. The negative impact of SDF on ICSI became more clear recently with the knowledge of high SDF on miscarriage rate. A systematic review and meta-analysis including 16 studies and 3106 couples found a significant increase in miscarriage rates for men with high SDF undergoing ICSI without significant decrease in pregnancy rate, in contrary to couples undergoing IVF [10]. Although the exact pathophysiology is not fully understood, the result provided insight into possible mechanism linking high SDF and poor ICSI outcomes. Currently, most of the studies pooled data from both IVF and ICSI patients, and the number of studies assessing ICSI outcomes alone is scarce.

It is suggested that OS is a significant contributor to sperm damage in 30–80% of infertile men. The relationship between OS and male infertility is illustrated by the significantly higher level of reactive oxygen species (ROS) and significantly lower levels of antioxidants in semen of infertile men compared to their fertile counterparts [11]. The association was further supported by the deleterious effect of oxidants and protective effect of antioxidants on sperm motility in vitro [12, 13]. Moreover, significant negative correlations between OS and semen parameters and fertilization rate have been reported [11, 14]. Unfortunately, studies directly correlating OS and ICSI outcomes in human are lacking at the moment.

Despite the expanding body of evidence, the clinical application of SDF and OS assays in directing management decisions in infertile couples is still under debate. Alongside the often heard criticism of nonstandardized laboratory assays, the lack of effective treatment in alleviating SDF and OS represents another major hurdle. However, the situation is changing, and various treatment strategies have proven their efficacy in the treatment of high SDF and/or OS.

49.3 Interventions to Reduce Oxidative Stress and Sperm DNA Fragmentation Before ICSI

Regardless of the questions on the clinical validity of SDF and OS assays, management of patients with high SDF and OS is of paramount importance in optimizing ICSI outcomes. Shortening of ejaculatory abstinence and oral antioxidant therapy have been shown to reduce SDF and OS. Varicocelectomy may be an effective treatment option in patients with concomitant high SDF and/or OS and clinical varicocele. Advances in laboratory strategies by sperm processing and selection are another technique in reducing the risk of injecting DNA-damaged sperm into oocyte. Recently, the use of testicular sperm in ICSI has been proposed and represents a novel approach in the alleviation of SDF and its deleterious effect on ICSI outcomes.

49.3.1 Short Ejaculatory Abstinence

Ejaculatory abstinence (EA) of 3–7 days prior to ART is usually recommended based on the finding of higher sperm concentration with longer EA [15]. However, the effect of lengthy EA may increase SDF by extended exposure of sperm to the damaging effect of OS in the epididymis [16].

The impact of EA on basic and advanced semen parameters in normozoospermic men has been reported. While ROS level remained static with different EA periods, SDF was significantly increased with prolonged EA [17]. More importantly, reduced EA is not detrimental to conventional semen parameters which remained within the established reference ranges [17]. It is of note that prolonged incubation should be avoided with the use of short abstinence ejaculates which may significantly increase SDF in vitro [18]. It is also suggested that the combination with sperm selection by density gradient centrifugation may protect sperm from further damage by ROS after collection [19].

Although the utilization of short-term recurrent ejaculation may be a valuable noninvasive maneuver to improve SDF before ICSI, its beneficial effect has been demonstrated only in normozoospermic men in current literature. The application of the technique to men with impaired conventional semen parameters remains doubtful. In addition,

there is only a single report on higher pregnancy rate following IUI in association with 1–2 days of EA [20]. Data on direct correlation between short EA and ICSI outcome is lacking.

49.3.2 Oral Antioxidant Therapy

A significantly higher levels of ROS are present in up to 25% of infertile men when compared to their fertile counterpart [21]. Antioxidants are capable to counterbalance the action of ROS and maintain the equilibrium of redox potential which is essential for optimal sperm function [22]. The alleviation of OS by oral antioxidant therapy appears to be an easily accessible and relatively inexpensive treatment approach in reducing OS and SDF.

A large number of compounds with antioxidant properties have been investigated including vitamin E, vitamin C, coenzyme Q10, carnitines, selenium, zinc, lycopene, folate, and N-acetylcysteine. These compounds have demonstrated promising ability in scavenging free radicals in vitro [23]. The efficacy of oral antioxidant in reducing OS and SDF in vivo has also been summarized in a review [24]. However, the effect of antioxidant therapy in the optimization of ART outcomes is still controversial, and a clear consensus is lacking. Despite the increasing number of studies supporting the role of antioxidant supplementation on reducing SDF/OS and improving natural pregnancy rate, data on the implication of antioxidants to ART outcomes is relatively scarce (Table 49.1). The considerable heterogeneity in the dosage and combination of antioxidants and outcomes measures among different studies further complicates the debate.

Despite the skepticism over routine application of SDF and OS assays in the evaluation of infertile men, the assays are widely adopted among andrology laboratories worldwide [25]. Together with the wide acceptance of oral antioxidant therapy in the management of infertile men, the role of antioxidant supplementation before ART in couples with male factor or idiopathic infertility has been explored. The use of oral vitamin E (200 mg for 3 months) has been shown to increase oocyte fertilization rate per IVF cycle in 15 normozoospermic men who initially had low fertilization rates during a previous IVF attempt in an early prospective study. The improved fertilization rate was associated with reduced lipid peroxidation potential in their spermatozoa [26]. The toxic effect of OS on IVF and ICSI outcomes was demonstrated in another study. A significant negative correlation between ROS in seminal plasma and fertilization rate in IVF/ICSI program has been reported [27].

Few studies further looked into the effect of oral antioxidant therapy on the clinical pregnancy and live birth rates after ICSI. Seventeen patients with low fertilization rates during prior ICSI for male factor infertility were treated with oral coenzyme Q10 (60 mg/day) for a mean of 103 days before subsequent ICSI trial. While no significant change was observed in most semen parameters, a significant improvement was found in fertilization rates from a mean of 10.3% in their previous cycle to 26.3% after coenzyme Q10 therapy [28]. Another study of similar design assessed the effect of vitamin C and vitamin E (1000 mg each for 2 months) on 38 men who had at least one unsuccessful previous ICSI cycle and elevated DNA-fragmented spermatozoa. The study revealed no differences in fertilization and cleavage rates or in embryo morphology. However, a marked improvement in implantation rates from 2% to 19% and pregnancy rates from 7% to 48% was noted in association with decrease in percentage of DNA-fragmented spermatozoa in the majority of patients after treatment [29]. Similarly, a randomized double-blind placebo-controlled trial examined the beneficial effect of oral antioxidant treatment with Menevit by enrolling 60 couples in ICSI program with high SDF. The viable pregnancy rate was significantly higher in antioxidant-treated group (38.5%) compared with placebo (16.0%) despite a similar oocyte fertilization rate and embryo quality [30].

The evidence on antioxidant use in male subfertility has been summarized in a Cochrane review including 48 randomized controlled trials and 4179 subfertile men. Albeit a considerable variability in the reported antioxidant effect on semen parameters, a significant improvement in clinical pregnancy rate (odds ratio [OR] 3.43, 95% confidence interval [CI] 1.92–6.11, $p < 0.001$) and live birth rate (OR 4.21, 95% CI 2.08–8.51, $p < 0.001$) after antioxidant therapy was concluded [31]. A systematic review concurred with the findings by analyzing 17 randomized studies including 1665 infertile men in whom oral antioxidant therapy was compared to placebo or no treatment. The benefit of treatment on sperm quality and pregnancy rates was illustrated in the majority of the studies. However, the authors suggested that adequately powered trials are needed in guiding clinical practice in view of methodological and clinical heterogeneity of the included studies [32].

On the other hand, the potentially serious consequences associated with exaggerated use of antioxidants should be avoided. The reductive stress associated with overuse of oral antioxidants was associated with increased sperm decondensation which may be deleterious to ICSI success [33, 34].

Table 49.1 Summary of study outcomes involving oxidative stress/sperm DNA fragmentation and the use of oral antioxidants in men

Study	Antioxidant(s)	Patient	Intervention regimen (daily dose)	Control	Outcomes
Suleiman, 1996 [76]	Vitamin E	Men with asthenozoospermia or oligoasthenozoospermia	300 mg vitamin E for 6 months	Placebo	Less LPO in spermatozoa
Kodama, 1997 [77]	Vitamin E + vitamin C + glutathione	Infertile men	200 mg vitamin E and 200 mg vitamin C and 400 mg glutathione for 2 months	None	Decreased biomarker of LPO
Lewin, 1997 [28]	Coenzyme Q10	Infertile men with low fertilization rate on ICSI	60 mg coenzyme Q10 for a mean of 103 days	None	Significant improvement in fertilization rate on ICSI compared to previous cycle
Comhaire, 2000 [78]	Acetylcysteine/ vitamin E + vitamin A	Infertile men	600 mg acetylcysteine or 30 mg vitamin A + 180 mg vitamin E for 6 months	None	Decreased biomarker of LPO Reduced ROS
Keskes-Ammar, 2003 [79]	Vitamin E + selenium	Volunteers and infertile men	400 mg vitamin E and 225 ug selenium for 3 months	4.5 g vitamin B for 3 months	Less LPO in spermatozoa
Greco, 2005 [80]	Vitamin E + vitamin C	Men with elevated SDF who have unexplained infertility	1000 mg vitamin C and 1000 mg vitamin E for 2 months	Placebo	Reduced percentage of DNA fragmented sperm
Greco, 2005 [29]	Vitamin E + vitamin C	Men with elevated SDF who failed first ICSI attempt	1000 mg vitamin C and 1000 mg vitamin E for 2 months	None	Reduced percentage of DNA fragmented sperm Marked improvement in implantation and clinical pregnancy rates in the second ICSI attempt
Tremellen, 2007 [30]	Menevit	Men recruited into ICSI treatment with high SDF	400 IU vitamin E and 100 mg vitamin C and 25 mg zinc and 26 ugm selenium and 6 mg lycopene and 0.5 mg folate and 1000 mg garlic for 3 months	Placebo	Higher viable pregnancy rate at 13 weeks gestation No difference in oocyte fertilization rate and embryo quality
Omu, 2008 [81]	Zinc/zinc + vitamin E/zinc + vitamin E + vitamin C	Men with asthenozoospermia	400 mg zinc sulfate +/− 20 mg vitamin E +/− 10 mg vitamin C for 3 months	No therapy	Reduced SDF Decreased biomarker of LPO Increased total antioxidant capacity
Ciftci, 2009 [82]	N-acetylcysteine	Men with idiopathic infertility	600 mg N-acetylcysteine	Placebo	Increased total antioxidant capacity Reduced ROS levels
Nadjarzadeh, 2011 [83]	Coenzyme Q10	Men with idiopathic oligoasthenoteratozoospermia	200 mg coenzyme Q10 for 12 weeks	Placebo	Increased total antioxidant capacity in seminal plasma Reduced plasma MDA levels
Nadjarzadeh, 2014 [84]	Coenzyme Q10	Men with idiopathic oligoteratozoospermia	200 mg coenzyme Q10 for 3 months	Placebo	Decreased biomarker of LPO Increased enzymatic antioxidant activity Increased levels of coenzyme Q10 in seminal plasma
Gvozdjakova, 2015 [85]	Carni-Q-Nol	Infertile men	440 mg L-carnitine and 30 mg ubiquinol and 75 IU vitamin E and 12 mg vitamin C for 6 months	None	Less LPO in spermatozoa

ICSI intracytoplasmic sperm injection, *LPO* lipid peroxidation, *ROS* reactive oxygen species, *SDF* sperm DNA fragmentation

49.3.3 Varicocele Repair

Varicoceles are considered the most common surgically correctable cause of male factor subfertility. Over the last few decades, the pivotal role of OS and SDF in the pathophysiology of varicocele-associated infertility has been increasingly recognized and provides insight into the highly controversial topic [6]. The association between OS and varicocele has been summarized by a review. A number of studies have consistently reported elevated levels of reproductive tract ROS in infertile men with and those without varicocele compared to their fertile counterparts. However, a higher level is observed in both fertile and infertile patients with varicocele signifying the implication of varicocele in exacerbation of ROS generation. The positive correlation between varicocele grade and OS has also been demonstrated [35]. A systematic review and meta-analysis have illustrated the close relationship between high SDF and presence of clinical varicocele irrespective of fertility status [36]. Another meta-analysis echoed by reporting a significantly higher SDF of 9.84% in patients with varicocele than normal healthy controls without varicocele from seven studies [37]. Moreover, the efficacy of varicocele repair in alleviating OS and SDF has been widely reported. In addition to the reduction of OS markers in spermatozoa of infertile men, varicocelectomy improves levels of seminal and peripheral blood plasma total antioxidant capacity [35, 38]. The effectiveness of varicocele repair in alleviating oxidatively induced SDF has been summarized in a meta-analysis with a mean decrease of 3.37% [37]. More recent studies further assessed the impact of OS/SDF reduction on pregnancy outcomes. Lower postoperative SDF was associated with a higher chance of pregnancy, both naturally and with ART [39, 40]. Table 49.2 summarizes studies evaluating the effect of varicocelectomy on SDF and/or OS.

The benefit of varicocelectomy in infertile men with clinical varicocele prior to ICSI only becomes more clear in recent decade after publication of a few studies. In a retrospective study including 242 infertile men with clinical varicocele who underwent ICSI, the authors reported a significantly higher clinical pregnancy (60.0% vs 45.0%; $p = 0.04$) and live birth (46.2% vs 31.4%; $p = 0.03$) rates in patients with microsurgical subinguinal varicocelectomy prior to ICSI. It was also noted that the chance of miscarriage (22.9% vs 30.1%) was decreased in patients with treated varicocele. The fertilization rate was also improved after varicocelectomy in addition to increased total number of motile sperm [41]. Following this initial report, another retrospective study of similar design did not showed significant difference in pregnancy rates (31.1% vs 30.9%), implantation rates (22.1% vs 17.3%), and miscarriage rates (21.7% vs 23.9%) between treated and untreated patients. Although the fertilization rate (73.2% vs 64.9%; $p = 0.04$) was higher in patients with prior varicocelectomy before ICSI, there was no difference in semen parameters detected between the groups [42]. More recently, two retrospective studies added supporting evidence in the role of varicocelectomy before ICSI. Despite a similar fertilization rate, clinical pregnancy (61.9% vs 28.3%; $p = 0.02$) and live birth (52.3% vs 24.5%; $p = 0.02$) rates were statistically higher in 21 patients with varicocelectomy performed compared to 53 patients without [43]. The largest study involving 306 infertile men with clinical varicocele demonstrated significantly higher pregnancy (62.5% vs 47.1%; $p = 0.001$) and live birth (47.6% vs 29.0%; $p = 0.0002$) rates in the varicocelectomy group. The difference in miscarriage rates did not reach statistical significance, though the finding was in favor of treated male (14.9% vs 18.1%; $p = 0.057$) [44]. The findings on studies evaluating the outcome of ICSI in men with treated and untreated varicocele have been reviewed in a meta-analysis. Outcomes of 870 ICSI cycles were analyzed by comparing 438 men with prior varicocelectomy to 432 men without. There was a significant increase in pregnancy rate (OR 1.59, 95% CI 1.19–2.12) and live birth rate (OR 2.17, 95% CI 1.55–3.06) associated with varicocelectomy [45]. Although all current studies are retrospective, the findings substantiate the potential benefit of varicocele repair before ICSI in men with clinical varicocele and high SDF and provide a rationale for further prospective research. Table 49.3 summarizes the outcome of ICSI in infertile men with treated and untreated varicocele.

49.3.4 Sperm Processing/Selection Techniques

Conventional techniques of sperm selection for ICSI circumvent the strict natural sperm selection process that occurs in the female genital tract. Currently employed techniques primarily select sperm based upon motility and morphology while neglecting other potentially important parameters including sperm DNA integrity and ROS generation. The risk of injecting DNA-damaged sperm during ICSI cannot be completely eliminated, and its potential consequences should not be overlooked [8].

While the procedure of ICSI attempts to mimic as closely as possible the environment of natural fertilization, the exact conditions cannot be recreated precisely in the laboratory setting. Indeed, the gametes and embryos are exposed to various potential ROS-inducing factors during ICSI including visible light, pH, temperature, culture media, and oxygen concentration of partial pressure. The risk of in vitro OS development is greater than in vivo. Therefore, meticulous handling of gametes in the laboratory is of utmost importance

Table 49.2 Summary of studies evaluating the effect of varicocelectomy on sperm DNA fragmentation and/or oxidative stress

Study	Design	Patients	Controls	SDF assay	OS markers	Results
Zini, 2005 [86]	Retrospective cohort	37 patients with varicocele who had microsurgical subinguinal varicocelectomy performed	N/A	SCSA	N/A	Mean SDF decreased after varicocelectomy (pre: 27.7%; post: 24.6%; $P = 0.04$).
Sakamoto, 2008 [87]	Retrospective cohort	30 infertile men with grade 2 or 3 varicocele (15 oligozoospermic and 15 normozoospermic) who had microsurgical subinguinal varicocelectomy performed	N/A (TUNEL result of controls was not provided)	TUNEL	NO, 8-OHdG, HEL, SOD activity in seminal plasma	TUNEL-positive sperm decreased significantly 6 months after treatment (pre: 79.6%; post: 27.5%; $P < 0.001$). Significant reduction in the levels of NO, 8-OHdG, HEL and SOD activity after varicocelectomy.
Werthman, 2008 [88]	Retrospective cohort	11 patients with clinical varicocele and DFI >27% who had microsurgical subinguinal varicocelectomy performed	N/A	SCSA	N/A	10 of the 11 patients showed a significant decrease in SDF 3 to 6 months after varicocelectomy. 7 of 11 patients showed decrease in DFI to normal level and the mean percent change in DFI was 24%.
Moskovtsev, 2009 [89]	Retrospective cohort	Patients with clinical varicocele was treated with oral antioxidants alone (37 men), or subjected to both microsurgical subinguinal varicocelectomy and oral antioxidants (9 men)	N/A	SCSA	N/A	SDF decreased in 78% of patients subjected to both varicocelectomy and oral antioxidants (pre: 44.7%; post: 28.4%; $P < 0.03$). No improvement in SDF was observed in patients on oral antioxidants alone (pre: 45.3%; post: 42.5%).
Smit, 2010 [39]	Prospective cohort	49 patients with clinical varicocele and oligozoospermia who had high inguinal ligation (36 men) or microsurgical varicocelectomy (8 men) performed	N/A	SCSA	N/A	Improvement in SDF was observed after treatment (pre: 35.2%; post: 30.2%; $P = 0.019$). 37% of couples conceived naturally and 24% achieved pregnancy with assisted reproduction after treatment. Mean postoperative DFI was significantly lower in couples who conceive naturally or with assisted reproduction than those who did not (spontaneous pregnancy: 30.1% vs 37.5%; assisted reproduction: 21.3% vs 36.9%).
Zini, 2011 [36]	Prospective cohort	25 patients with clinical varicocele and abnormal semen parameters who had microsurgical subinguinal varicocelectomy performed	N/A	SCSA	N/A	Improvement in SDF was observed at 4 and 6 months after varicocelectomy (pre: 18%; 4 months: 10%; 6 months: 7%).
Lacerda, 2011 [90]	Prospective cohort	21 adolescents (age 15–19) with grades 2 or 3 varicocele who had microsurgical subinguinal varicocelectomy performed	N/A	Comet	TBARS levels	Sperm with intact nuclear DNA (comet class I) increased after varicocelectomy (49.6% to 64.5%; $P = 0.011$). TBARS level remained unaltered after varicocelectomy.

Table 49.2 (continued)

Study	Design	Patients	Controls	SDF assay	OS markers	Results
La Vignera, 2012 [91]	Not specified	30 patients with grade 3 left varicocele and oligoasthenoteratozoospermia who had microsurgical subinguinal varicocelectomy performed	30 normozoospermic controls without varicocele	TUNEL	N/A	Significant reduction in SDF at 4 months after varicocelectomy (5.0% to 2.1%; $P < 0.05$), and postoperative results were similar to that of healthy controls (2.0%).
Li, 2012 [92]	Not specified	19 patients with clinical varicocele who had microsurgical subinguinal varicocelectomy performed	19 normozoospermic men	SCSA	N/A	SDF was higher in men with varicocele than controls (28.4% vs 17.4%; $P = 0.007$). DFI decreased 3 months after operation (28.4% to 22.4%; $P = 0.018$), and postoperative results were similar to that of controls.
Baker, 2013 [93]	Retrospective cohort	24 patients with clinical varicocele who had microsurgical subinguinal varicocelectomy performed	N/A	TUNEL	ROS and TAC levels	SDF decreased after varicocelectomy (40.8% to 24.5%). A higher preoperative SDF was associated with a larger improvement postoperatively. Postoperative SDF in pregnant and non-pregnant couples showed no difference (22.2% vs 25.7%). Mean TAC decreased after varicocelectomy. No statistically significant change in ROS levels after varicocelectomy.
Kadioglu, 2014 [94]	Retrospective cohort	92 infertile patients with clinical left varicocele and abnormal semen analysis who had microsurgical subinguinal varicocelectomy performed	N/A	TUNEL	N/A	SDF decreased 6 months after varicocelectomy (42.6% to 20.5%; $P < 0.001$). A higher preoperative SDF was associated with a larger improvement postoperatively.
Ni, 2014 [95]	Prospective cohort	42 infertile men with clinical left varicocele and abnormal semen parameters who had microsurgical varicocelectomy performed	10 normozoospermic fertile controls	SCSA	N/A	Higher DFI was observed in preoperative group compared to controls (27.4% vs 11.5%; $P < 0.01$). DFI in patients who achieved pregnancy (20.6%) were lower than preoperative value (27.4%) and those of non-pregnant patients (24.7%). DFI in patients who achieved pregnancy after varicocelectomy were not significantly different from controls (20.6% vs 11.5%).
Pourmand, 2014 [96]	Randomized controlled trial	100 infertile patients with clinical left varicocele or subclinical varicocele who had varicocelectomy alone (group 1) or varicocelectomy plus oral L-carnitine for 6 months (group 2)	N/A	TUNEL	N/A	Improvement in SDF was observed in both groups after varicocelectomy (group 1: 14.0% to 9.5%; group 2: 13.9% to 8.5%). The results were not different between groups.

(continued)

Table 49.2 (continued)

Study	Design	Patients	Controls	SDF assay	OS markers	Results
Telli, 2015 [97]	Prospective cohort	72 infertile patients with clinical varicocele and oligozoospermia who had macroscopic inguinal varicocelectomy performed	N/A	Acridine orange assay	N/A	SDF decreased after varicocelectomy (34.5% to 28.2%) with a mean follow up of 6.2 months.
Tavalaee, 2015 [98]	Not specified	23 infertile patients with grades 2 or 3 left varicocele who had varicocelectomy performed	N/A	TUNEL	DCFH-DA staining	SDF improved 3 months after varicocelectomy (15.9% to 10.8%; $P < 0.001$). Percentage of sperm with OS improved 3 months after operation (47.6% vs 36.6%; $P = 0.03$).
Mohammed, 2015 [40]	Prospective cohort	75 infertile patients with clinical varicocele and altered semen parameters who had subinguinal varicocelectomy performed with loop magnification	40 healthy fertile volunteers without varicocele	Acridine orange	Sperm chromatin decondensation	Higher DFI was observed in preoperative patients than controls (32.4% vs 18.2%; $P = 0.003$). DFI deceased significantly after varicocelectomy (32.4% to 20.0%; $P = 0.05$). DFI in patients who achieved pregnancy at 1 year were significantly lower than those who did not (16.4% vs 24.2%; $P = 0.04$). No significant changes were detected regarding DNA chromatin decondensation (25.4% vs 22.0%)
Alhathal, 2016 [99]	Prospective cohort	29 infertile patients with clinical varicocele and abnormal semen parameters who had microsurgical subinguinal varicocelectomy performed	6 healthy sperm donor with normal semen parameters	SCSA	Sperm DNA decondensation	DFI was significantly higher in preoperative patients than controls (20.0% vs 7.4%; $P = 0.01$). DFI improved significantly after varicocelectomy (20.0% to 12.0%; $P = 0.001$). Sperm DNA decondensation of infertile men with varicocele were significantly higher than controls which decreased after operation.
Ni, 2016 [100]	Not specified	51 patients with clinical varicocele and abnormal semen analysis who had microsurgical retroperitoneal high ligation performed	15 men with subclinical varicocele, 22 men with clinical varicocele and normozoospermia, and 25 healthy fertile donors	SCSA	Seminal MDA	SDF was higher in patients with clinical varicocele (range: 20.6% to 30.0%) compared to patients with subclinical varicocele (14.9%) and controls (12.0%). SDF reduced in patients with clinical varicocele and altered semen parameters, irrespective of clinical grade of varicocele. Seminal MDA reduced in men with clinical varicocele after varicocele repair. SDF and MDA were lower in patients who achieved pregnancy than non-pregnant patients.

Table 49.2 (continued)

Study	Design	Patients	Controls	SDF assay	OS markers	Results
Abdelbaki, 2017 [101]	Prospective controlled cohort	60 infertile patients with clinical varicocele and abnormal semen parameters who had inguinal varicocelectomy performed with loop magnification	20 normozoospermic healthy fertile men	SCSA	ROS and TAC levels	A higher DFI was observed in patients with varicocele than controls (29.9% vs 7.6%). DFI improved 3 months after varicocelectomy (29.9% to 18.8%; $P < 0.001$). ROS levels decreased and TAC levels increased at 3 months after operation
Zaazaa, 2018 [102]	Randomized controlled trial	80 infertile patients with clinical grade 2 or 3 varicocele and DFI >30% who had varicocelectomy (group 1), or varicocelectomy followed by ketotifen 1 mg twice daily for 3 months (group 2) 40 men with clinical grade 2 or 3 varicocele and DFI >30% treated with oral ketotifen 1 mg twice daily for 3 months (group 3)	N/A	SCD	N/A	DFI improved after 3 months of treatment in all groups. DFI improvement was the the highest in patients with varicocelectomy followed by mast cell stabilizer (26.8%) compared with varicocelectomy alone (18.2%; $P = 0.04$) and mast cell stabilizer alone (16.8%; $P = 0.02$). Improvement in DFI was higher in infertile patients with grade 3 varicocele compared to those with grade 2.
Sun, 2018 [103]	Randomized controlled trial	358 infertile patients with left clinical and right subclinical varicocele who had either unilateral left or bilateral microsurgical subinguinal varicocelectomy performed	N/A	SCSA	N/A	DFI was significantly reduced in both groups 1 year after varicocelectomy (unilateral: 21.6% to 11.8%; bilateral: 23.0% to 12.1%). No difference in preoperative and postoperative DFI between the two groups was observed, despite greater improvement in semen parameters in the bilateral group.

8-OHdG 8-hydroxy-2′-deoxyguanosine, *DCFH-DA* 2′,7′-dichlorodihydrofluorescein diacetate, *DFI* DNA fragmentation index, *HEL* hexanoyl-lysine, *MDA* malondialdehyde, *N/A* not applicable, *NO* nitric oxide, *OS* oxidative stress, *ROS* reactive oxygen species, *SCD* sperm chromatin dispersion, *SCSA* sperm chromatin structure assay, *SDF* sperm DNA fragmentation, *SOD* superoxide dismutase, *TAC* total antioxidant capacity, *TBARS* thiobarbituic acid-reactive substances, *TUNEL* terminal deoxynucleotidyl transferase dUTP nick end labeling

Table 49.3 ICSI outcome in infertile couples in whom the male partner had treated or untreated clinical varicocele

Study	Outcome	ICSI with prior varicocelectomy	ICSI without varicocelectomy	P-value
Esteves, 2010 [41]	Fertilization rate (%)	78.0	66.0	0.04
	Clinical pregnancy rate (%)	60.0	45.0	0.04
	Miscarriage rate (%)	22.9	30.1	0.46
	Live birth rate (%)	46.3	31.5	0.03
Pasqualotto, 2012 [42]	Fertilization rate (%)	64.9	73.2	0.04
	Clinical pregnancy rate (%)	30.9	31.1	0.98
	Implantation rate (%)	22.1	17.3	0.59
	Miscarriage rate (%)	21.7	23.9	0.84
Shiraishi, 2012 [43]	Fertilization rate (%)	70.3	68.8	0.93
	Clinical pregnancy rate (%)	61.9	28.3	0.02
	Live birth rate (%)	52.3	24.5	0.04
Gokce, 2013 [44]	Clinical pregnancy rate (%)	62.5	47.1	0.001
	Live birth rate (%)	47.6	29.0	0.0002
	Miscarriage rate (%)	14.9	18.1	0.057

ICSI intracytoplasmic sperm injection

[46]. Density gradient centrifugation (DGC) and swim-up are commonly used sperm preparation methods for ART which often utilized various centrifugation protocols. Centrifugation increased the chance of identifying rare better-quality sperm particularly in patients with severe oligospermia which are often ICSI candidates. In addition, the separation of seminal plasma, which contains leukocytes and represents a source of ROS, may potentially protect sperm from OS attack in vitro. On the other hand, generation of ROS during the centrifugation steps is a noticeable drawback. Moreover, sperm with high SDF may be more vulnerable to the deleterious effect of centrifugation [47]. The effects of reduction in the proportion of sperm with damaged DNA after DGC and swim-up have been reported by a number of studies. However, the results were inconclusive and the implication on ICSI outcomes remains unclear [48]. Supplementation of culture media with antioxidant may improve ICSI outcomes by scavenging excessive production of ROS. It was supported by the negative correlation between ROS levels in the culture media and pregnancy rate after ICSI [49]. However, the antioxidant regimen is unknown, and the literature presents conflicting reports regarding the validity of using any one specific antioxidant therapy in culture media to improve ICSI outcomes. In view of pitfalls of the current practice, advanced sperm selection techniques are drawing a growing interest from fertility specialists. By isolating mature, structurally intact, and nonapoptotic sperm with DNA integrity, it is supposed that ICSI outcome would be improved.

Mature spermatozoa which have completed the spermatogenetic process of plasma membrane remodeling, cytoplasmic extrusion, and nuclear maturity contain a high density of hyaluronic acid (HA) receptors [50]. It was shown that sperm bound to HA in vitro has less DNA fragmentation and chromosomal aneuploidy, and sperm selection based on HA binding seems a reasonable approach to improve ICSI outcomes in patients with high SDF [51]. However, conflicting results have been reported on the utilization of HA-bound sperm in ICSI in the literature. A recent meta-analysis has summarized the data from 7 studies including 1437 ICSI cycles. It showed improvement in embryo quality without difference in fertilization and pregnancy rates. The authors suggested further study to identify selective patient groups that might benefit from the technique [52]. Indeed, two studies observed improvement in ART outcomes and reduction in pregnancy loss only in patients with low HA-binding score, meaning that they had a higher percentage of abnormal sperm [53, 54].

Electrophoresis-based sperm sorting depends on the negative charge of normal mature sperm and separates functionally active spermatozoa from immature sperm and leukocytes. An obvious benefit of the procedure is the decrease in exposure to ROS by eliminating the centrifuge steps. Sperm sorted by electrophoresis had been shown to have significantly lower SDF [55] and establish pregnancy from semen sample with high SDF by ICSI [56]. However, the number of studies on electrophoresis-based sperm sorting and ART outcome is scarce, and further clinical trials are needed in order to better assess its efficacy.

Based on the presence of phosphatidylserine on the surface of plasma membrane of apoptotic sperm, the high affinity of annexin V to phosphatidylserine is used as a biomarker for apoptotic cells in magnetic-activated cell sorting (MACS). The activated magnetic field retained apoptotic sperm which bind to micromagnetic bead-coated annexin V, while healthy sperm flow through the selection column and are collected. MACS-selected sperm had less SDF and normal protamine content [57]. Embryo cleavage and pregnancy rates were higher in ICSI utilizing MACS-selected sperm compared to DGC-selected ones [58].

Motile sperm organelle morphology examination (MSOME) studies morphology of ultrastructural components of live spermatozoa by a digitally enhanced light microscope using Nomarski optics which allows magnification up to 6300× [59]. Sperm with more than 50% vacuolated nuclei has been associated with SDF [60]. The technique is now often used in standard ICSI, which is called intracytoplasmic morphologically selected sperm injection (IMSI). Studies have suggested a higher implantation, pregnancy, and birth rates associated with IMSI, together with lower miscarriage rate compared to ICSI particularly in men with severe oligoasthenoteratozoospermia [61]. However, two meta-analyses on the implication of IMSI yielded contrasting findings. While increase in pregnancy and reduction in miscarriage rates were concluded in one meta-analysis [62], another study did not showed significant difference between IMSI and ICSI [63].

There is a study that compared interventions aimed at selecting sperm populations with better DNA integrity in men with high SDF for use with ICSI [64]. In the report, ICSI cycles from couples whose male partners had high levels of SDF were retrospectively reviewed. Interventions to reduce SDF including PICSI (physiological ICSI, one of HA binding selection technique) and IMSI have been applied to ejaculated sperm. The outcomes were compared to a control group with no intervention. Live birth rates were 38.3% and 28.7% in PICSI and IMSI groups, respectively. No statistical significance could be demonstrated in comparison with the control group with a live birth rate of 24.2% [64]. Moreover, a recent review indicated no significant differences in ICSI outcomes could be demonstrated with the use of samples prepared with or without PICSI, MACS, and IMSI [65]. Notably, many of the studies applied advanced sperm selection techniques to an unselected population of men regard-

less of SDF rates. Further clinical studies on the impact of various sperm selection techniques on ICSI outcomes have to be conducted.

49.3.5 Use of Testicular Sperm

Retrieval of sperm directly from the testis for ICSI (Testi-ICSI) is an attractive strategy to circumvent infertility associated with high SDF in the ejaculate. The intervention allows sperm acquisition prior to transit through the epididymis and male excurrent ductal system, where SDF is thought to be acquired. The finding of a threefold lower SDF in testicular sperm obtained by testicular sperm extraction (TESE) compared to ejaculated sperm obtained on the day of ICSI from 12 men with persistently elevated SDF despite oral antioxidant therapy for 3 months supported the notion [66].

The first report on the use of testicular sperm for ICSI was published in 2005. The study included 18 couples who had at least two unsuccessful ICSI cycles with ejaculated sperm and high SDF. A higher pregnancy and lower miscarriage rates were observed in ICSI cycles carried out with testicular sperm in addition to a statistically lower SDF [67]. Following the initial report, several case series and retrospective cohorts supported the findings and demonstrated better outcomes associated with the use of testicular sperm in ICSI in couples with high SDF and previous ART failure [68–70]. In a recent prospective study, ICSI outcomes between ejaculated and testicular sperm in 172 men with mild-to-moderate (5–15 × 10^6 spermatozoa per mL) idiopathic oligozoospermia and persistently elevated SDF after oral antioxidant therapy were evaluated. Testicular sperm retrieved by testicular sperm extraction or aspiration showed a fivefold lower SDF. The miscarriage rates were lower (relative risk [RR] 0.29, 95% confidence interval [CI] 0.10–0.82; $p = 0.019$), and live birth rates were higher (RR 1.76, 95% CI 1.15–2.70; p- = 0.008) in couple subjected to sperm injection with testicular sperm [71]. To our knowledge, there is only one study that compared interventions for patients with high SDF undergoing ICSI. The study found higher live birth rates with Testi-ICSI compared to sperm selection with PICSI and IMSI and no intervention groups [64] (Table 49.4). A meta-analysis had summarized the published literature and revealed that overall clinical pregnancy (odds ratio [OR] 2.42, 95% CI 1.57–3.73) and live birth (OR 2.58, 95% CI 1.54–4.35) rates are higher when Testi-ICSI is performed as compared with ICSI using ejaculated sperm in men with high SDF. Moreover, a significant 72% reduced likelihood of miscarriage in the Testi-ICSI cohort was reported [72]. However, another systematic review highlighted the lack of well-designed prospective studies and concluded that there is only limited low-quality evidence to suggest the use of testicular sperm in men with high SDF and oligozoospermia, but not in men with high SDF only [73].

The current evidence favoring the use of testicular sperm for ICSI in patients with high SDF is limited. The studies are predominantly small, retrospective, and nonrandomized. Variability in SDF assays without established threshold is another critique. The previously reported higher aneuploidy rates observed in testicular sperm may represent a threat to offspring health [74]. It is of note that certain specific defect in ejaculated sperm is associated with SDF in seminiferous tubules or defects in chromatin remodeling during spermatogenesis which cannot be bypassed by testicular sperm retrieval [68]. Possible complications associated with sperm retrieval and uncertain cost-effectiveness of Testi-ICSI are other obstacles in the generalization of technique currently. However, more recent study adopting advanced sequencing molecular karyotype revealed that total aneuploidy of surgically retrieved sperm is comparable to that of ejaculated sperm. The preliminary data appear promising and support the safety in using testicular sperm [75]. While awaiting further evidence, the decision to pull the trigger on surgically retrieved testicular sperm for a cycle of ICSI in infertile couples experiencing high SDF and/or impaired semen parameters and/or prior ART failure should be made carefully.

49.4 Conclusion

The central role of SDF and OS in male infertility is being increasingly recognized. There is also emerging evidence revealing the negative impact of high SDF on ART, including ICSI, outcomes. In addition, the alleviation of SDF and OS also reduces the chance of injecting DNA-damaged sperm into oocytes during ICSI and potentially improves ICSI outcomes. A number of interventions have demonstrated the potential to decrease SDF and/or OS prior to ICSI including short ejaculatory abstinence, oral antioxidant therapy, varicocele repair, advanced sperm selection techniques, and use of testicular sperm. Most of these measures have promising results in reducing SDF/OS significantly. However, the data on clinical utilization of the techniques remain limited, and most of the clinical trials are nonrandomized. Ample opportunities exist to further clarify the role of different interventions or their combination in improving ICSI outcomes in patients with high SDF.

Table 49.4 Summary of studies comparing ICSI outcomes by using testicular and ejaculated sperm in men with high sperm DNA fragmentation

Study	Patient	SDF assay	Sperm retrieval	Outcomes
Greco, 2005 [67]	18 couples with at least 2 unsuccessful ICSI attempts using ejaculated sperm Male partners have ≥15% ejaculated sperm with damaged DNA All couples subjected to consecutive ICSI cycles with ejaculated and testicular sperm	TUNEL	Open testicular biopsy or fine needle aspiration	SDF is lower in testicular than ejaculated sperm (4.8% vs 23.6%; $p < 0.001$) No difference in fertilization rate (74.9% vs 70.8%) and cleavage rate (95.0% vs 94.7%) No difference in proportion of embryos with good morphology (51.1% vs 47.6%) Use of testicular sperm was associated with higher pregnancy rates (44.4% vs 5.6%; $p < 0.05$)
Sakkas, 2010 [68]	72 couples with repeated IVF failures using ejaculated sperm 30 and 42 couples used testicular and ejaculated sperm during ICSI cycle, respectively Male partners have >20% SDF	TUNEL	NR	Higher clinical pregnancy rate in Testi-ICSI group (40.0% vs 13.8%; $p = 0.035$) Higher implantation rate in Testi-ICSI group (28.1% vs 6.6%; $p = 0.021$) Lower miscarriage rate in Testi-ICSI group (6.3% vs 75.0%; $p = 0.017$)
Mehta, 2015 [69]	24 couples with previous failed IVF or ICSI using ejaculated sperm Male partners with oligozoospermia ($<5 \times 10^6$/mL) and high SDF (>7%)	TUNEL	Testicular sperm extraction	Testicular sperm has lower TUNEL-positive score than ejaculated sperm on the same day of sperm retrieval (5% vs 24%; $p = 0.01$) Clinical pregnancy was achieved in 50% of the couples. No miscarriage was observed and all pregnancies resulted in live births
Esteves, 2015 [71]	172 couples on first ICSI attempts 81 and 91 couples used testicular and ejaculated sperm respectively Male partners with moderate oligozoospermia ($5 \times 10^6 - 15 \times 10^6$/mL) and persistently elevated SDF (>30%) after oral antioxidant therapy	SCD	Testicular sperm extraction or testicular sperm aspiration	SDF rates were lower in testicular than ejaculated sperm (8.3% vs 40.7%; $p < 0.001$) Use of testicular sperm was associated with lower two pronuclei fertilization rate (56.1% vs 69.4%; $p = 0.0001$). Difference in clinical pregnancy rate was not statistically significant (51.9% vs 40.2%; $p = 0.131$) Miscarriage rate was significantly lower in Testi-ICSI group (10.0% vs 34.4%; $p = 0.012$) Live birth rate was significantly higher in Testi-ICSI group (46.7% vs 26.4%; $p = 0.007$)
Bradley, 2016 [64]	448 ICSI cycles in couples whose male partner had high SDF (≥29%) 146 cycles of Testi-ICSI was compared to 80 cycles of conventional ICSI	SCIT	Testicular sperm extraction or testicular sperm aspiration	Lower two pronuclei fertilization rate was observed in Testi-ICSI group (57.0% vs 66.0%; $p < 0.001$) No difference in miscarriage rate between the groups (13.2% vs 10.2%) Implantation rate was higher in Testi-ICSI group (41.1% vs 24.0%; $p < 0.05$) Clinical pregnancy rate was higher in Testi-ICSI group (49.5% vs 27.5%; $p < 0.05$) Live birth rate was higher in Testi-ICSI group (43.7% vs 24.9%; $p < 0.05$)
Pabuccu, 2017 [70]	71 couples with at least 2 unsuccessful ICSI attempts using ejaculated sperm 31 and 40 couples used testicular and ejaculated sperm, respectively Male partners with normozoospermia and high SDF (>30%)	TUNEL	Testicular sperm aspiration	Higher clinical pregnancy rate was reported in Testi-ICSI group (41.9% vs 20.0%; $p = 0.045$) Higher ongoing pregnancy rate was reported in Testi-ICSI group (38.7% vs 15%; $p = 0.023$) No statistically significant difference in two pronuclei fertilization and implantation rates was observed

ICSI intracytoplasmic sperm injection, *NR* not reported, *SCD* sperm chromatin dispersion, *SCIT* sperm chromatin integrity test, *SDF* sperm DNA fragmentation, *Testi-ICSI* testicular sperm used for sperm injections, *TUNEL* terminal deoxyribonucleotide transferase-mediated dUTP nick-end labeling assay

49.5 Review Criteria

An extensive search investigating the relationship between oxidative stress/sperm DNA integrity and assisted reproduction with ICSI was performed using search engines including ScienceDirect, OVID, PubMed, and MEDLINE. The study identification was based on the following keywords: "oxidative stress," "reactive oxygen species," "sperm DNA damage," "sperm DNA fragmentation," and "ICSI." The start and end dates for the searches were January 2000 to October 2018, respectively. Only articles published in English were considered. Data that were solely published in conference or meeting proceedings, websites, or books were not included.

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Sperm Retrieval Techniques

50

Ricardo Miyaoka and Sandro C. Esteves

Key Points

- Percutaneous epididymal sperm aspiration (PESA) and microsurgical epididymal sperm aspiration (MESA) are the most commonly used methods to harvest epididymal sperm.
- Testicular sperm aspiration (TESA) and open testicular sperm extraction with or without the aid of microsurgery (micro-TESE and TESE, respectively) are the methods used to retrieve testicular sperm.
- Surgical sperm retrieval can be performed on an outpatient basis with the intention to cryopreserve sperm for future use or in association with oocyte retrieval and immediate sperm injection.
- In men with obstructive azoospermia, spermatogenesis is normal, and sperm can be easily retrieved from the epididymis or testis.
- In obstructive azoospermia, the sperm retrieval technique and the cause of obstruction seem to have little effect on sperm retrieval rates and ICSI outcomes. Likewise, the sperm source (epididymis or testis) and the sperm status (fresh or frozen-thawed) do not seem to impact ICSI outcomes. However, MESA yields a higher number of motile sperm than does PESA, thereby offering the possibility of cryopreserving larger quantities of sperm that might enable multiple ICSI cycles without the need for repeat sperm retrieval.
- In men with nonobstructive azoospermia, success in harvesting sperm is higher, and complication rates are lower with micro-TESE than with conventional TESE. Nevertheless, the likelihood of harvesting sperm with both approaches is related to testicular histology. Men with hypospermatogenesis and maturation arrest have a more favorable outcome than those with Sertoli cell-only syndrome.
- Testicular parenchyma or epididymal aspirates should be carefully handled in the IVF laboratory, as these specimens might be more fragile than ejaculated counterparts.
- The classification of azoospermia into obstructive azoospermia and nonobstructive azoospermia has a significant influence on sperm retrieval rates and ICSI success. Results are less favorable among men with nonobstructive azoospermia than with obstructive azoospermia.
- In non-azoospermic men with high sperm DNA fragmentation in the semen, ICSI with sperm harvested from the seminiferous tubules seems to yield better ICSI outcomes and higher live birth rates.
- The underlying parental infertility seems to have a significant effect on the health of ICSI offspring. While the risks of congenital malformations, epigenetic disorders, chromosomal abnormalities, subfertility, cancer, delayed psychological and neurological development, and impaired cardiometabolic profile are reported to be greater in infants born as a result of ICSI than in naturally conceived children, it remains to be determined to what extent the observed adverse outcomes might be aggravated by using surgically retrieved sperm.

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50.1 Introduction

Overall, 3–12% of men at reproductive age present fertility-related issues, making male infertility the sole responsible for approximately one-third of all infertility cases [1].

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Azoospermia – a complete absence of spermatozoa in the ejaculate – affects approximately 1% of the general male population and 10% of men with infertility. This condition relates to the complete absence of any sperm in the ejaculate even after centrifugation [2].

Sperm retrieval techniques were initially developed to overcome obstructive causes of male infertility associated with azoospermia, either acquired (e.g., vasectomy) or congenital (e.g., congenital bilateral absence of the vas deferens (CBAVD)). Its first successful report resulting in a pregnancy was published in 1985 when Temple-Smith and colleagues described the microsurgical epididymal sperm aspiration (MESA) [3].

Later, in 1994, Devroey and colleagues described the use of testicular sperm extracted by open biopsy (testicular sperm extraction (TESE)) to treat men who had previously failed epididymal sperm aspiration (percutaneous epididymal sperm aspiration (PESA)) [4]. Nonetheless, the first series to describe successful pregnancies using aspirated epididymal sperm was reported by Craft and Shrivastav in the same year [5]. In 1996, Lewin et al. were able to achieve pregnancy using aspirated testicular spermatozoa (TESA) to treat a nonobstructive azoospermic patient with a histological diagnosis of maturation arrest [6]. More recently, in 1999, Schlegel described the microdissection testicular sperm extraction technique (micro-TESE) which consists of direct identification of active spermatogenic regions within the testis under microscopic magnification [7]. This approach is the method of choice for sperm extraction in cases of nonobstructive azoospermia (NOA) as it offers significantly higher rates for sperm yield when compared with open TESE.

The reproductive urologist who performs sperm extraction should be familiar with all methods to recommend the best technique for each case scenario and to provide the in vitro fertilization (IVF) laboratory the best specimen for use with assisted reproductive technology (ART). One must also be able to both execute and foresee the need for a rescue procedure such as in cases of failed PESA in obstructive azoospermia (OA) which may require a rescue TESA or TESE or in cases of a misdiagnosed NOA, which may require immediate micro-TESE instead of PESA/MESA.

Knowing the surgical complications, the IVF outcomes, and the predictors of success will help the urologist to adequately counsel the patient regarding the procedure itself and its expectations toward the treatment as a whole. This knowledge will likely enhance the patient's confidence and engagement in the therapeutic proposal.

50.2 Sperm Retrieval Techniques

50.2.1 Percutaneous

50.2.1.1 Epididymal Sperm Aspiration (PESA)

Percutaneous epididymal sperm aspiration (PESA) is used to harvest epididymal sperm in cases of OA [8]. PESA is a noncomplex and straightforward method that can be performed under intravenous (IV) sedation with propofol without the need for locoregional anesthetic blockade (Fig. 50.1). A 10-mL solution of 2% lidocaine without epinephrine is injected outside the outer ring to block the spermatic cord. We use loupe magnification to avoid injury to small scrotal vessels seen through the skin [9]. A 29–33-Gauge butterfly needle was initially used to retrieve sperm from the epididymis [5]. Alternatively, nowadays it is more common to use a 23-Gauge needle connected to a 1-mL tuberculin syringe that is inserted through the skin into the epididymis with negative pressure [10]. A gentle movement in and out of the epididymis allows the aspiration of a small quantity of fluid while holding the epididymis firmly with the other hand.

PESA may be repeated at a different portion from the tail/body toward the caput epididymis until enough motile sperm is recovered for intracytoplasmic sperm injection (ICSI) or cryopreservation. TESA should be done as a rescue procedure when no motile sperm is available for ICSI [11]. A short movie depicting the main steps of the procedure can be found at http://www.brazjurol.com.br/videos/july_august_2015/Esteves_817_818video.htm [9].

50.2.1.2 Testicular Sperm Aspiration (TESA)

TESA is performed on an outpatient basis, either concomitantly with oocyte retrieval to allow immediate use of sperm for ICSI or to freeze sperm for future use (Fig. 50.2) [10]. TESA has been used to retrieve sperm from men with OA, selected cases of NOA, and, more recently, nonazoospermic men with excessive elevated DNA fragmentation [11–13]. Occasionally, the procedure is also used to obtain additional sperm in cases of cryptozoospermia when ejaculated semen is inadequate or insufficient for ICSI [14].

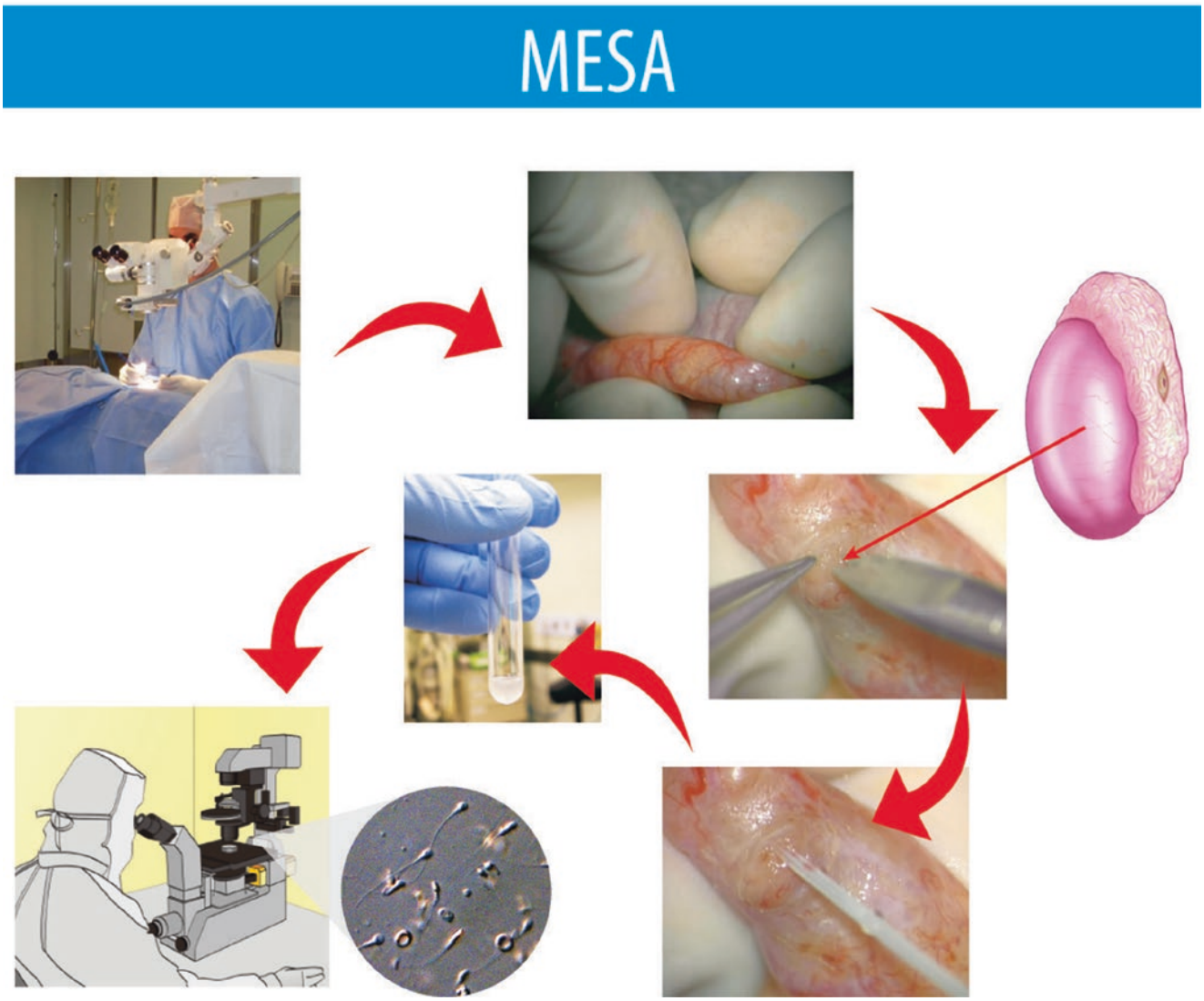

Fig. 50.1 Microsurgical epididymal sperm aspiration (MESA). A dilated epididymal tubule is dissected and cut open under surgical microscopy and microsurgical technique. Seminal fluid is aspirated, diluted with sperm medium, and sent to the laboratory for analysis (Reprinted with permission from ANDROFERT. All Rights Reserved)

TESA can be carried out under intravenous sedation or general anesthesia combined with local anesthesia applied to the spermatic cord. The testicle is held firmly and punctured using an 18-Gauge needle attached to a 20-mL syringe. Negative pressure is applied using a syringe holder (e.g., Cameco syringe holder) that aids in removal of seminiferous tubules. Like in PESA, loupe magnification may be used during puncture to avoid scrotal skin vascular injury. The needle is moved back and forth to disrupt tubules, so they can be adequately aspirated. Ideally, all testicular regions should be sampled during aspiration; the needle is inserted in an oblique angle in its anterior aspect of the upper pole. The sample is immediately analyzed in the IVF laboratory, and if inadequate, the contralateral testis is punctured at the same operative time [15]. A short movie depicting the main steps of the procedure can be found at https://www.youtube.com/watch?v=o9MgknYEzN0.

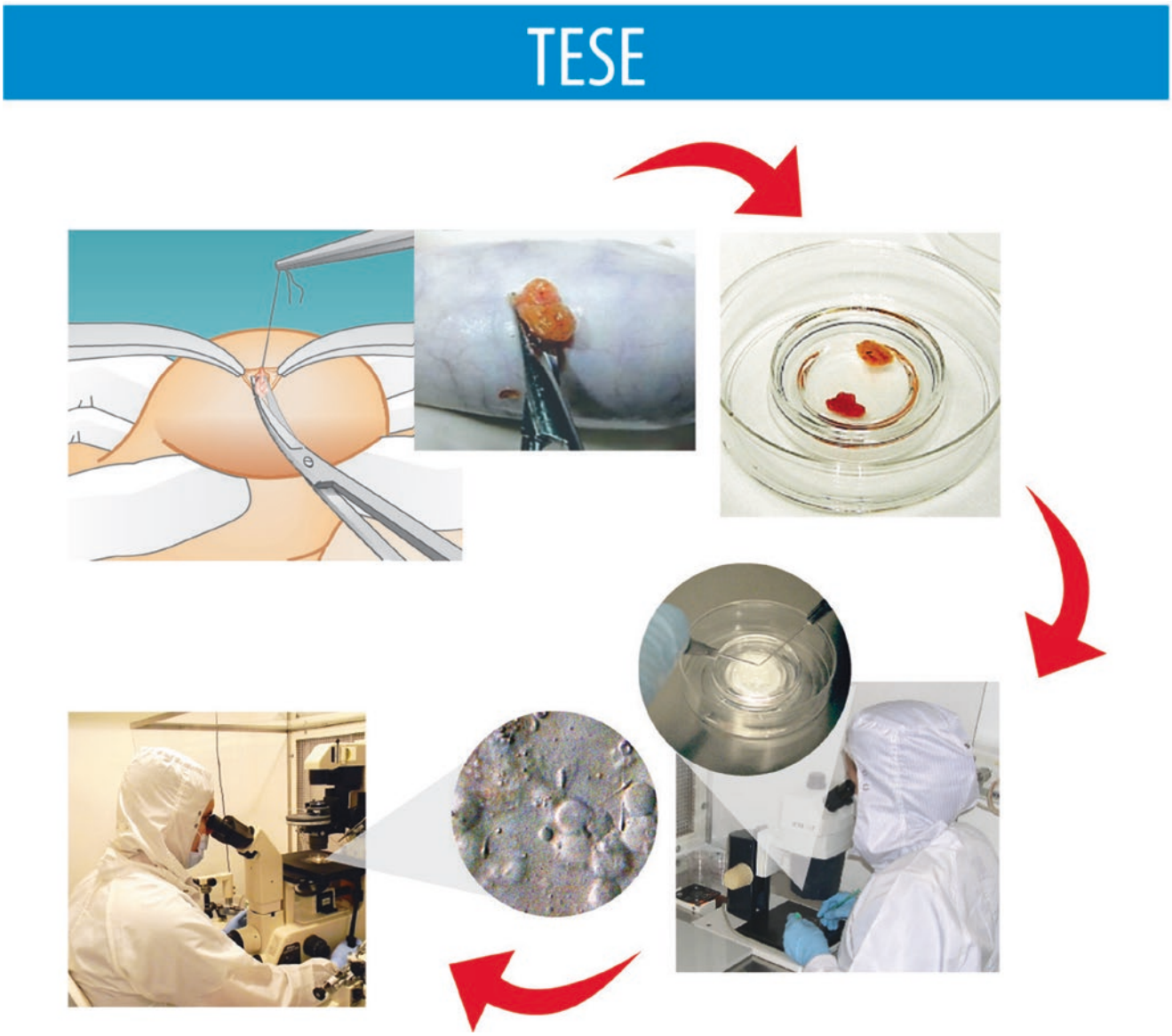

Fig. 50.2 Testicular sperm extraction (TESE). A single or multiple incisions are made on the tunica albuginea, and one or several testicular biopsies are taken. Specimens are sent to the laboratory for mechanical mincing and examination under the inverted microscope for sperm search (Reprinted with permission from ANDROFERT. All Rights Reserved)

50.2.2 Open Non-microsurgical

50.2.2.1 Testicular Sperm Extraction (TESE)

Open TESE can be performed under intravenous sedation associated with locoregional anesthesia, local anesthesia only, or spinal block. It can be performed with or without testis delivered. The skin and subjacent layers are incised transversally to expose the tunica albuginea, which is opened with the knife. Usually, a small transversal albuginea opening (0.5–1.0-cm incision) is made at the mid-testicular pole, and a small sample of the parenchyma is cut off with scissors. The tunica is closed with a non-absorbable 5–0 running suture. The tunica vaginalis, dartos, and skin are sutured with absorbable suture [12, 15] (Fig. 50.3).

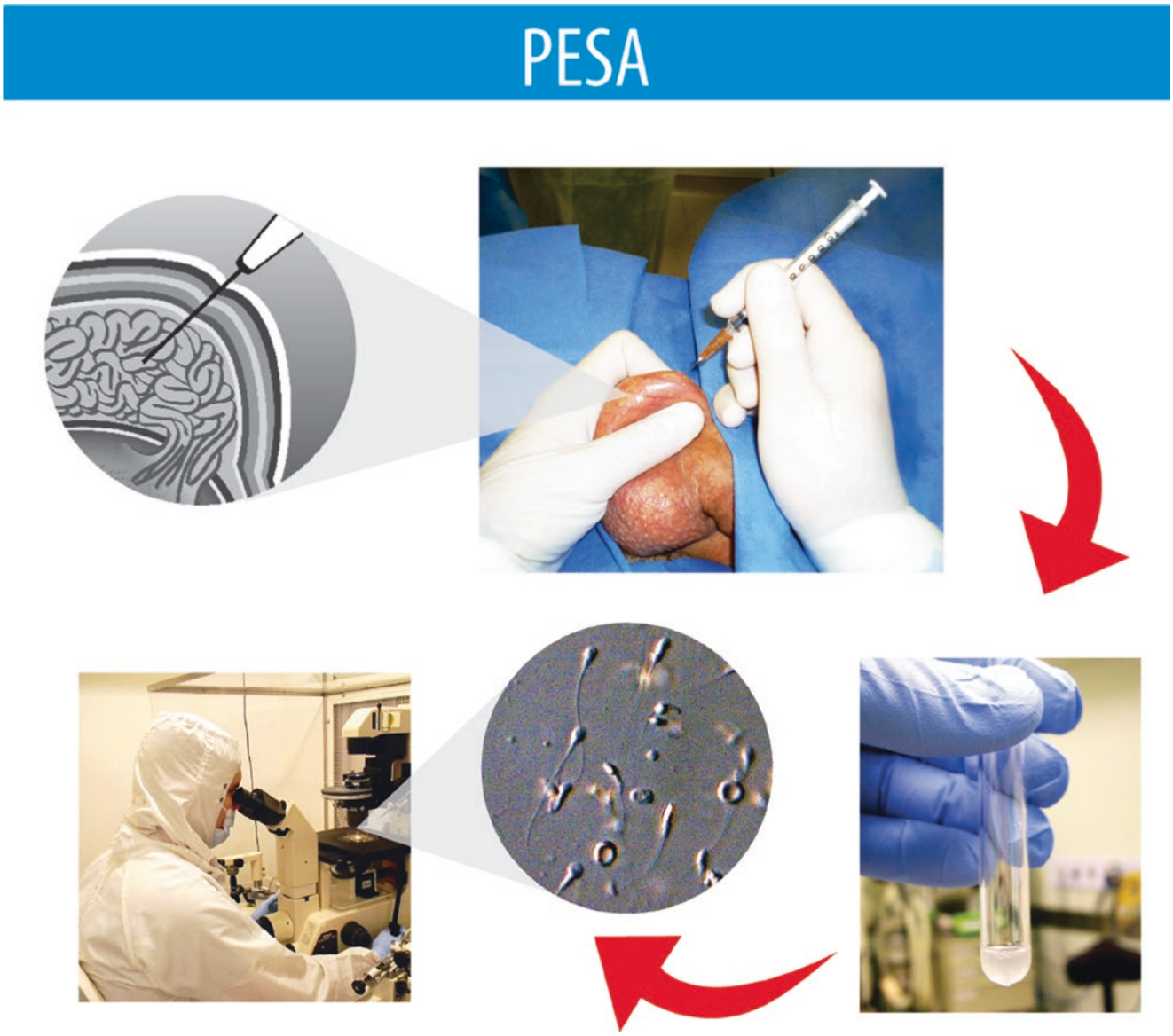

Fig. 50.3 Percutaneous epididymal sperm aspiration (PESA). Percutaneous aspiration of the epididymis is made using a thin needle connected to a syringe filled with sperm medium. Aspirate is sent for laboratory examination under the inverted microscope for sperm search (Reprinted with permission from ANDROFERT. All Rights Reserved)

50.2.3 Open Microsurgical

50.2.3.1 Epididymal Sperm Aspiration (MESA)

MESA is usually performed through a scrotal incision large enough to allow testis delivery. Since some degree of spermatic cord traction is expected unconsciousness is desirable for the procedure. General anesthesia under controlled ventilation may be used, but it is also possible to obtain sufficient analgesia with spermatic cord blockade using local anesthetics, associated with intravenous sedation with propofol.

An operating microscope and ×16–25 magnification are used to dissect the epididymal tunica and to open an opaque enlarged tubule with a microscopic knife or micro-scissors (Fig. 50.4). Culture media is added drop by drop over the

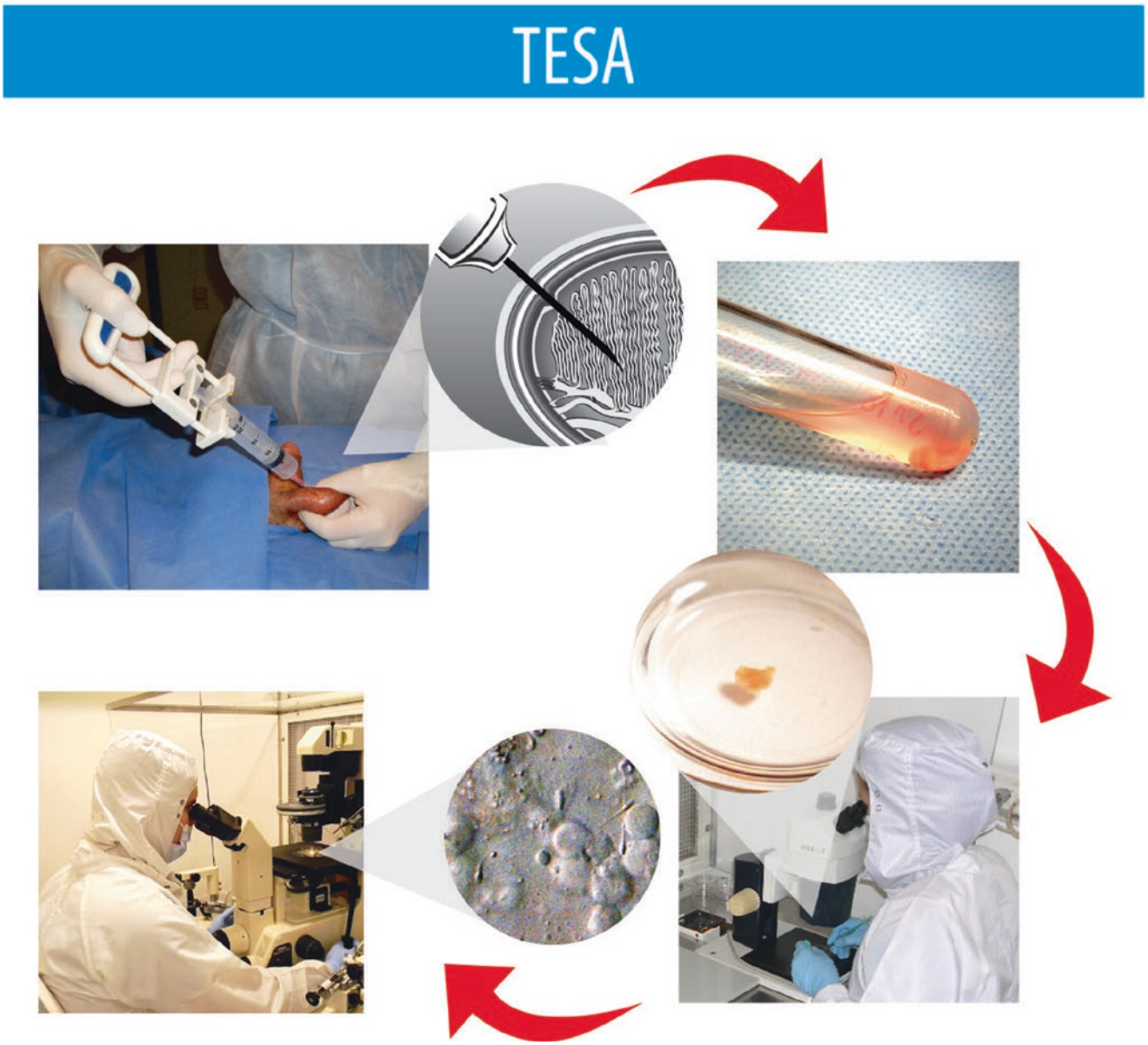

Fig. 50.4 Testicular sperm aspiration (TESA). Percutaneous aspiration of the testicle is carried out by inserting a 40 × 12-mm needle connected to a 20-mL syringe mounted on a syringe holder (e.g., Cameco syringe holder). The testis is firmly held, and the needle is moved in and out on various directions under negative pressure to disrupt and facilitate extraction of seminiferous tubules. Specimens are sent to the laboratory for mechanical mincing and examination under the inverted microscope for sperm search (Reprinted with permission from ANDROFERT. All Rights Reserved)

incised tubule to allow fluid aspiration and sperm recovery. If adequate sampling is not possible, another aspiration should be made more proximally than the first one toward the head of the epididymis in a different tubule. The proximal epididymal aspirate tends to have better quality than the distal ones; the latter is often more abundant in senescent sperm with reduced chromatin integrity [16]. If MESA fails to retrieve adequate numbers of motile sperm, TESA or TESE can be performed at the same side and operating time. However, MESA often permits the recovery of large numbers of high-quality sperm that can be used for immediate ICSI or cryopreserved for subsequent attempts avoiding additional surgical interventions [17].

50.2.3.2 Microdissection Testicular Sperm Extraction (Micro-TESE)

Micro-TESE is performed under intravenous sedation combined with local anesthesia or under general anesthesia or

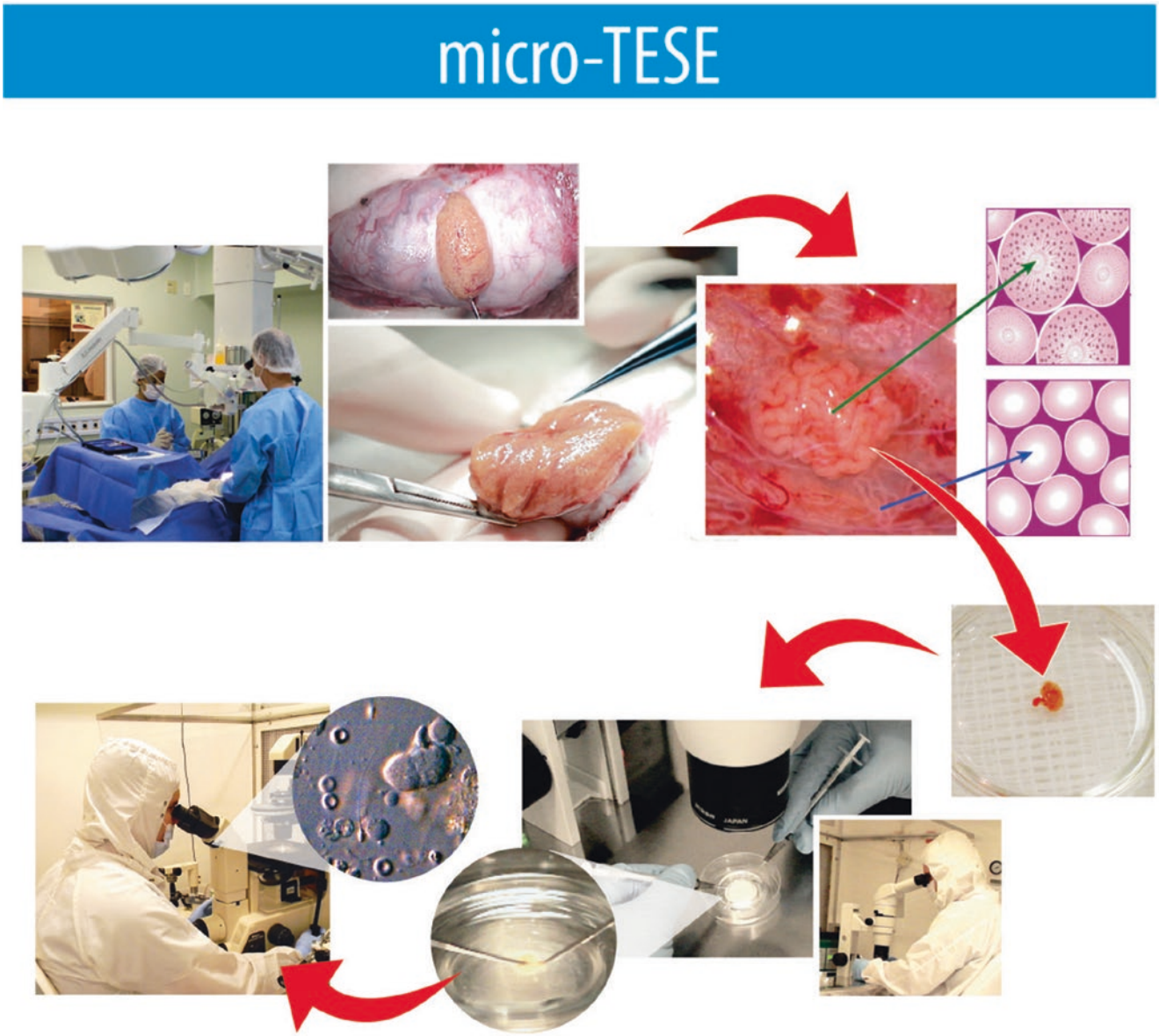

Fig. 50.5 Microdissection testicular sperm extraction (Micro-TESE). After testis delivery, a large equatorial incision is made in an avascular area of the tunica albuginea, and the testicular parenchyma is widely exposed. The seminiferous tubules are dissected at ×16 to ×25 magnification to enable identification of enlarged seminiferous tubules (see picture on the top right-hand side). Optical magnification reduces the chances of vascular injury by proper visual identification of testicular blood supply. Enlarged tubules are more likely to contain germ cells and therefore sperm production (green arrow shows the histological representation of an enlarged tubule exhibiting full spermatogenesis). Thin tubules are usually devoid of germ cells (blue arrow shows the histological representation of a thin tubule exhibiting Sertoli cells only). Microsurgical-guided biopsies are performed to extract enlarged tubules which are sent to the laboratory for examination. The initial laboratory step involves mechanical mincing of the seminiferous tubules and examination of specimens under the inverted microscope for sperm identification (Reprinted with permission from ANDROFERT. All Rights Reserved)

even spinal block [18]. After skin incision, the testis is delivered outside the scrotum. The tunica albuginea is then widely incised transversally, and the parenchyma is fully exposed (Fig. 50.5). Under microscope magnification of 16–25×, each testicular region is screened for enlarged seminiferous tubules as these have a higher chance to harbor germ cells and therefore sperm production [19]. If all tubules are identical, then random micro-biopsies (3–6 at each testicular pole) are recommended. Blood supply is actively avoided to preserve the remaining testicular parenchyma. Collected samples are immediately searched for sperm by the IVF lab. Given the initial sperm quantity and quality, the surgeon can

decide upon either ending the procedure or extending exploration to the contralateral side [12]. A short movie depicting the main steps of the procedure can be found at http://www.brazjurol.com.br/videos/may_june_2013/Esteves_440_441video.htm [20].

50.3 Prognostic Factors for Successful Sperm Retrieval

50.3.1 Obstructive Azoospermia

In patients with OA, spermatogenesis is preserved, and retrieval rates are virtually 100% regardless of the cause of obstruction [21]. However, sperm retrieved from the various parts of the seminal tract may vary in quality. The distal epididymis, for example, contains a high number of sperm fragments and macrophages [22]. The number of macrophages progressively decreases toward the proximal epididymis and testis, while the quantity of motile sperm gradually increases.

In one study, Esteves and colleagues evaluated sperm retrieval outcomes in 146 OA patients treated by ICSI [8]. The authors compared the results according to the cause of obstruction (congenital, vasectomy, or post-infection). In their study, epididymal sperm retrieval reached 78.0% of success, and testicular retrieval was able to rescue almost all failed epididymal attempts. Epididymal sperm retrieval was successful in all congenital cases, whereas in the other etiology groups (vasectomy, post-infectious obstruction), approximately 1/3 of patients required TESE. In the latter, the cumulative sperm retrieval rate (SRR) was 97.3% and did not differ among groups: CBAVD (100%), vasectomy (96.6%), and post-infection (96.3%) [8].

50.3.2 Nonobstructive Azoospermia

In NOA, few studies have looked into predictive factors for successful sperm retrieval. A recent systematic review showed that micro-TESE is more effective than conventional TESE as it offers higher SRR and lower surgical complications such as hematoma formation, fibrosis, and testicular atrophy [23]. Overall, SRR for conventional TESE and micro-TESE range from 17–45% and 25–63%, respectively. In the paper mentioned above, five out of seven included studies reported a significant difference regarding SR favoring micro-TESE ($p < 0.05$) [24–28].

Nonetheless, the main factor determining the odds of success in harvesting viable sperm with both techniques relates to testicular histology. Hypospermatogenesis (HS) and maturation arrest (MA) are associated with higher chances of harvesting sperm from the seminiferous tubules than Sertoli cell only (SCO) [8, 29, 30]. In a study involving 365 NOA patients undergoing micro-TESE, the SRR was higher in patients with MA (40.3%) than SCO (19.5%) ($P = 0.007$). Both groups did worse when compared with hypospermatogenesis (SRR = 100.0%; $p < 0.001$) [31]. Patients with SCO histopathology seem to benefit the most from micro-TESE. In SCO, success rates range from 22.5% to 41% using micro-TESE compared to 6.3% to 29% with conventional TESE [25, 28]. In patients with maturation arrest (MA), success rates are highly variable with some studies reporting SRR of 36.4% to 75% with the use of micro-TESE and 0% to 37.5% with conventional TESE [23]. In one study, however, there was a clear advantage of using micro-TESE in preference over conventional TESE in patients with MA [26]. The SRR with the former and latter approaches were 81–100% and 50–84%, respectively.

Other clinical predictors concerning SR success are serum follicle-stimulating hormone (FSH), inhibin levels, testicular volume, and testosterone levels. The studies by Okada et al. [25], Colpi et al. [27], and Ghalayini et al. [28] found FSH to be a predictive factor for successful sperm retrieval. Although no definitive cutoff value has been established, the authors reported that increased FSH levels were associated with significantly more failures in both TESE and micro-TESE [27, 28]. By contrast, Ramasamy et al. in a micro-TESE study involving men with NOA demonstrated that FSH levels have poor predictive value concerning success in sperm acquisition. In their study, patients with FSH levels >15 IU/mL had higher SRR than those with FSH levels <15 IU/mL [31]. In general, FSH levels correlate inversely with the spermatogenic status. However, despite reflecting the predominant testicular histology, FSH levels cannot be used to predict whether sperm-producing areas exist within the testis of a man with NOA. For instance, patients with failed SR might have normal FSH levels and normal-sized testes. This condition is explained by the presence of numerous Sertoli and germ cells (arrested at a specific spermatogenic stage); the former secrete adequate amount of inhibin that negatively feedback the FSH production [31].

Some studies suggest inhibin B to be more sensitive than FSH as an index of the spermatogenesis status [32, 33]. However, inhibin B, either alone or in combination with serum FSH, also fails to predict TESE outcomes in NOA patients and should not be used as a criterion to contraindicate the procedure [34]. Colpi et al. and Ghalayini et al. also evaluated testicular volume as a predictive factor for successful sperm retrieval but found that the data is equivocal [27, 28]. Along the same lines, a 2017 study reviewing the data of over 400 NOA patients found no significant difference in serum total testosterone levels in patients with successful and failed SR [35].

As for the location of the biopsy, Hauser et al. [36] could not demonstrate the advantage of performing the biopsy in any particular region of the testicle as a means to improve SR

success. However, Witt et al. suggested that the midline testicular area might provide the highest chance of harvesting sperm [37].

In one study including patients with Klinefelter syndrome (KS), the overall SRR was 51% (26/51) [38]. However, the authors could not find any predictive factor of success when analyzing FSH, LH, and testosterone levels, as well as testicular volume. By contrast, another study involving patients with non-mosaic KS found that advanced paternal age (>35 years old) adversely affected the SRR [39].

Testing for Y chromosome microdeletions is essential for counseling the affected men concerning the likelihood of SR success and risk of infertility in resulting male offspring [18, 40]. Among men diagnosed with complete AZFa and AZFb microdeletions, micro-TESE offers virtually zero chance of sperm recovery and therefore should not be encouraged [41].

Lastly, in the context of medication, Ramasamy et al. assessed the impact of preoperative hormonal therapy on SRR of KS patients. They concluded that patients who received hormonal therapy and responded with a resulting total testosterone level above 250 ng/dL (8.7 nmol/L) reached a SRR of 77% versus 55% in those who remained under this level [42]. In patients already receiving exogenous testosterone replacement, the pituitary is suppressed, and therefore cessation is recommended for at least 6 months before microdissection to allow gonadal axis reestablishment [43].

Enhancing intratesticular testosterone production and correcting abnormalities in the ratio of testosterone to estrogen have been advocated to optimize SR success. In a multicenter nonrandomized study involving 442 subjects diagnosed with NOA, SR was higher in the hormonal optimization group than in the group that underwent SR without previous hormonal treatment (57% versus 34%; $p < 0.05$) [44]. Nevertheless, the quality of evidence currently available is very low to recommend routine hormonal optimization therapy [18, 39].

50.4 Complications

Postoperative complications following SR include persistent pain, swelling, infection, hydrocele, and hematoma [26, 45, 46]. Ultrasound scans performed 3 months after single or multiple biopsy TESE reveal the presence of intratesticular hematoma in approximately 80% of patients, which tend to resolve spontaneously without compromising testicular function [46, 47].

In large-volume conventional TESE, however, temporary or definitive testicular damage (such as complete devascularization) might decrease serum T levels [26, 48]. TESA and micro-TESE minimize the risk of complications and long-term adverse consequences, including hypogonadism [7, 18, 26, 46, 48, 49].

In micro-TESE, subalbuginea vessels are spared during the testicular opening [20]. The use of optical magnification and microsurgical technique not only allow preservation of intratesticular blood supply but also increase the chances of identifying sperm-producing tubules [18, 20, 26]. Hence, SR efficacy is optimized since the risk of complications, and the quantity of tissue removed is reduced. The smaller amount of tissue extracted – compared to conventional TESE – speeds up tissue processing and sperm search [20, 50]. In a large cohort study involving 435 NOA patients subjected to micro-TESE or conventional TESE, postoperative ultrasound examination confirmed that micro-TESE caused fewer acute and chronic testicular changes than TESE [26]. The authors of the study mentioned above reported that although there is an initial reduction in T levels after micro-TESE, such levels return to 95% of their preoperative values in an 18-month follow-up period. These findings have been corroborated by others [51].

Nevertheless, men with severely hypotrophic testes and low serum T levels (e.g., Klinefelter syndrome) might have a more significant reduction in T levels, thus being at a higher risk of requiring permanent T replacement therapy [52]. In one report involving KS men, serum T levels significantly declined by 30–35% ($p < 0.01$) after micro-TESE over a 1- to 12- month period, but returned to 75% of the preoperative levels after 18 months [43]. Given the potential risk for severe adverse effects, it is critical that sperm retrieval in NOA men be performed by well-trained surgeons [48].

50.5 Assisted Reproductive Technology

50.5.1 Role of IVF Laboratory

In general, sperm processing techniques are needed to remove cellular debris, microorganisms, and red blood cells that might contaminate the extracted specimens. These methods should be mastered to avoid deteriorating the sperm fertilizing potential further since the quality of surgically retrieved sperm is often lower than ejaculated counterparts [52]. Processed sperm can be either used for immediate ICSI or cryopreserved for future use.

From the surgeon's perspective, all efforts should be made to deliver specimens with minimal or no contaminants to the IVF laboratory. Lab personnel, on its turn, should minimize iatrogenic cellular damage during sperm preparation. Controlling centrifugation force and duration, limiting exposure to ultraviolet light and temperature variation, optimizing laboratory air quality conditions, and using high-quality reagents, culture media, and disposable materials are critical elements [53]. Whenever possible, techniques aimed at improving the sperm fertilizing potential should be applied, including the use of chemical stimulants and/or methods to

Table 50.1 Laboratory strategies to handle surgically extracted sperm

Process	Procedures	Techniques	Main goal
Testicular tissue processing	Micro-TESE	Extraction of minimum volume of tissue	Speed up tissue processing and search efficiency in men with NOA
Testicular tissue processing	Mechanical tissue mincing	Disruption of seminiferous tubules using fine needles or micro-scissors and forced pass through small-diameter catheters	Tubular breakdown and cellular content loss
Testicular tissue processing	Enzymatic tissue digestion	Incubation of testicular suspensions with collagenase and/or DNAse	Tubular breakdown and cellular content loss in men with NOA
Testicular tissue or epididymal fluid processing	Red blood cell lysis	Incubation of testicular or epididymal suspensions with erythrocyte lysing buffer solution	Removal of excessive blood cells from testicular or epididymal specimens
Testicular tissue of epididymal fluid processing	Motility enhancement	Incubation of testicular specimens of epididymal fluid with pentoxifylline	Selection of viable sperm for ICSI
Laboratory environment and laboratory practices	Air quality control	Air particulate and volatile organic compound filtration	Secure optimal safety conditions for gamete handling, sperm injection, and embryo culture
	Maintenance of temperature and pH stability	Quality control and quality assurance of instruments, equipment, and reagents	Avoid iatrogenic cellular damage
	Centrifugation	Simple washing with buffered medium or mini-gradient centrifugation using low centrifugation forces	Avoid iatrogenic cellular damage
	Sterile techniques	Manipulation of gametes and embryos in laminar flow cabinets or inside controlled environments	Secure optimal safety conditions for gamete handling, sperm injection, and embryo culture
Intracytoplasmic sperm injection	Sperm selection	Hypoosmotic swelling test, mechanical touch technique, and laser-assisted sperm selection	Selection of viable immotile sperm for ICSI
Sperm storage	Cryopreservation	Sperm freezing using low-volume carriers	Improve post-thaw sperm survival

ICSI intracytoplasmic sperm injection, *NOA* nonobstructive azoospermia

select viable sperm for ICSI. The latter is particularly important when only immotile spermatozoa are harvested [54]. An overview of the laboratory aspects concerning the processing of surgically extracted specimens is provided in Table 50.1. A detailed laboratory procedure for processing such specimens can be found elsewhere [12, 17].

50.5.2 Influence of Type of Azoospermia

Although spermatogenesis is normal in cases of OA, ICSI rather than conventional IVF should be the fertilization method to be used with sperm retrieved from both the epididymis and testicle due to the low fertilizing capacity of such gametes in conventional IVF [54, 55]. With ICSI, the use of fresh or frozen-thawed sperm harvested from the epididymis or seminiferous tubules from men with OA does not seem to affect outcomes [56, 57]. However, the evidence is not categorical as a retrospective study involving 374 men with OA reported that the likelihood of achieving a live birth was higher with epididymal than with testicular sperm (OR 1.82, 95% CI 1.05–3.67) [12].

A meta-analysis pooling 100 ICSI cycles compared ART outcomes in OA according to congenital or acquired causes [58]. Men with CBAVD achieved higher fertilization rates than those with acquired obstruction (p = 0.04). In their study, no difference was noted in clinical pregnancy rates (CPR) and LBR between groups, but miscarriage rates were higher in the congenital group (RR ~ 2.7). By contrast, Kamal et al. studied 1661 ICSI cycles in 1121 men with proven histological OA (normal spermatogenesis). Mean female partner age was 30.9 ± 5.7 years (17–45 years). Implantation rate (IR) (19.9% vs. 20.8%), CPR (43.2% vs. 42.3%), and miscarriage rate (18.4% vs. 17.6%) were not significantly different when testicular or epididymal sperm were used for ICSI, respectively. The same trend was noted concerning the cause of obstruction (CBAVD vs. acquired obstruction), thus suggesting that ICSI success is independent of the factors discussed above [59]. The 2PN fertilization rate (68.0% vs. 64.2%, p = 0.02) was the only significant parameter favoring testicular sperm.

In another study, Esteves et al. retrospectively analyzed 146 men with OA to compare ICSI results according to the cause of obstruction (congenital versus acquired). Live birth rates (LBRs) were similar among congenital (34.4%), vasectomy (32.2%), and post-infection groups (36.4%). Clinical pregnancy rates, miscarriage rates, and prematurity and low birth weight rates were also not significantly different [10].

Sukcharoen et al. studied the influence of time of obstruction on ICSI outcome. They reported on a cohort of 17

patients and 21 ICSI cycles within a period of 2 years, analyzing 3 groups according to the time elapsed since vasectomy: 0–10 years, 11–20 years, and more than 20 years. Fertilization rate, IR, and CPR per transfer were not significantly different among the groups [60]. However, this cohort was too small to allow any conclusion.

ICSI outcomes seem to favor OA over NOA, which is not surprising since spermatogenesis is considered normal in the former. In OA, the method of sperm retrieval – percutaneous or open surgery – and the site of sperm acquisition, testis or epididymis, may be chosen according to the preference and expertise of the attending urologist. There is no solid evidence that the site or method of sperm retrieval influences the outcome of ICSI for patients with OA [61, 62]. Additionally, neither the cause of obstruction nor the use of fresh or frozen-thawed epididymal/testicular sperm seems to have any significant effect on the success of ICSI regarding fertilization, pregnancy, or miscarriage rates. ICSI provides fertilization rates of 45–75% per injected oocyte when epididymal or testicular spermatozoa from men with OA are used. In such cases, CPR and LBR range from 26 to 57% and 18 to 55%, respectively [21, 59, 63–66].

By contrast, the reproductive outcomes of men with NOA subjected to ICSI with testicular sperm harvested from the seminiferous tubules are less optimal. In one study, Esteves and Agarwal compared sperm injection outcomes using fresh surgically extracted sperm from men with OA (182 cycles) and NOA (188 cycles) to those from a general population of infertile men using freshly ejaculated sperm for injections (621 cycles) [67]. The lowest LBRs were reported in men with NOA (21.4%; p = 0.003), whereas men with OA (37.5%) and those from the general male infertility population using ejaculated sperm (32.3%) had similar LBRs. In this study, ICSI outcomes were comparable between obstructive azoospermia and ejaculated sperm groups. In another report, ICSI outcomes were compared in NOA men with successful (n = 365) and failed (n = 40) SR by micro-TESE [29]. ICSI was carried out with testicular sperm and donor sperm, respectively. Live birth rates in both groups were compared with those from a group of 186 men with OA in whom epididymal or testicular sperm were used for ICSI. The adjusted OR showed that the likelihood of achieving a live birth was lower in men with NOA who had successful SR than in those with NOA in whom donor sperm was used (OR 0.377, 95% CI 0.233–0.609; p < 0.001) and to men with OA (OR 0.403, 95% CI 0.241–0.676; p = 0.001) [29]. The authors also noted that fertilization rates after ICSI (47% vs. 61–64%, p < 0.01), high-quality embryo rates (43% vs. 61–66%, p < 0.01), and CPR (28% vs. 47–50%, p < 0.01) were lower in NOA men with successful SR than in both men with NOA in whom donor sperm was used and OA [29].

50.5.3 Sperm Retrieval in Non-azoospermic Men

Sperm retrieval from epididymides or seminiferous tubules for use with ICSI is the clear strategy for overcoming untreatable azoospermia-related infertility [68]. Recently, testicular sperm retrieval has also been used in non-azoospermic men to bypass post-testicular oxidative-induced DNA fragmentation.

Indeed, current data indicate that among non-azoospermic infertile men, sperm harvested from the seminiferous tubules have threefold to fivefold lower sperm DNA fragmentation (SDF) – a marker of chromatin quality – than ejaculated sperm [69–72]. Given the importance of sperm chromatin integrity for ART success, the use of testicular in preference over ejaculated sperm for ICSI has gained increasing attention. The aim is to increase the chances of oocyte fertilization by genomically intact sperm, which might ultimately result in the development of embryos with higher implantation potential.

In one prospective cohort study, Esteves et al. compared ICSI results with the use of ejaculated and testicular sperm in a population of 172 infertile men with high SDF [70]. The authors included infertile men with mild-to-moderate idiopathic oligozoospermia (5–15 × 10^6 spermatozoa per mL) who presented with persistent high SDF (>30%) even after using oral antioxidant therapy for at least 3 months. SDF was re-assessed in both ejaculated and testicular specimens on the day of sperm collection for ICSI using the sperm chromatin dispersion test (SCD). Paired ejaculated and testicular specimens from the same men showed that SDF rates were fivefold higher (40.7% ± 9.9%) in the ejaculate than in the testis (8.3% ± 5.3%; P < 0.001). In this group, ICSI was performed with testicular sperm (Testi-ICSI). In patients subjected to ICSI with ejaculated sperm, SDF rates were 40.9% ± 10.2%. Miscarriage rates were lower, and LBRs were higher in couples treated with testicular sperm than with ejaculated sperm. The adjusted relative risks for miscarriage and live birth between testicular sperm and ejaculated sperm groups were 0.29 (95% CI 0.10–0.82; p = 0.019) and 1.76 (95% CI 1.15–2.70; p = 0.008), respectively. The authors reported that five couples needed to be treated (NNT; 95% CI 2.8–16.8) by testicular compared to ejaculated sperm to obtain an additional live birth per fresh transfer cycles [70]. These data indicate that using Testi-ICSI, it would be possible to avoid one out of five oocyte pickups [73].

In another study, Bradley et al. also compared ICSI outcomes between ejaculated and testicular sperm in non-azoospermic men with high SDF in the ejaculated sperm [74]. Among patients in the ejaculated sperm group, the authors applied interventions such as IMSI (intracytoplasmic morphologically selected sperm injection) and HA sperm selection ICSI (physiological ICSI (PICSI)) to select sperm

with less DNA fragmentation and compared outcomes with a control group in which no particular intervention was used to select sperm with an intact chromatin. The authors evaluated the results of ICSI using ejaculated sperm – with (228 cycles) and without such interventions (80 cycles) – or testicular sperm (Testi-ICSI; 148 cycles). Higher LBRs ($p < 0.05$) were obtained with Testi-ICSI (49.8%) than IMSI (28.7%) and PICSI (38.3%). The lowest LBRs (24.2%) were achieved when no intervention was used to avoid the use of sperm with fragmented DNA ($p = 0.020$) [74].

A 2017 systematic review followed by meta-analysis corroborated the findings of the studies mentioned above concerning (i) the lower rates of SDF in testicular sperm than in ejaculated sperm and (ii) the better ART outcomes with the use of testicular in preference over ejaculated sperm for ICSI in men with high SDF in the semen [11]. By contrast, the benefit of using testicular sperm rather than ejaculated sperm for ICSI has not been confirmed among men with cryptozoospermia. A meta-analysis pooling five small case-control and observational studies with a total of 300 cycles looked at ICSI results in this scenario [75]. The authors reported no differences in fertilization rates (RR 0.91, 95% CI 0.78–1.06) and pregnancy rates (RR 0.53; 95% CI 0.19–1.42) when testicular sperm was compared with ejaculated sperm.

Currently, the advantage of using Testi-ICSI over ejaculated sperm ICSI in non-azoospermic men seems to be restricted to men with confirmed abnormally high SDF in the semen; in these cases, a favorable outcome with testicular sperm has been found regarding clinical pregnancy, miscarriage, and live birth [69, 70, 73, 74, 76–79].

50.6 Health of Offspring

The widespread use of surgically retrieved sperm for ICSI has raised concerns about the health of resulting offspring owing to the related severe male infertility conditions and because such gametes have not completed full maturation. Concerns include possible increased risk for congenital and urogenital malformations, epigenetic alterations, chromosomal aneuploidies, infertility, childhood cancer, delayed psychological and neurological development, and impaired cardiometabolic profile.

In general, any increase in these conditions is believed to be consequential of parental sperm defects rather than the ART method [68]. In fact, the integrity of the sperm genome and epigenome is essential to assure healthy offspring [80]. Several environmental insults can damage histone-bound sperm DNA including oxidative stress. The male gamete is highly vulnerable to free radical-induced DNA damage since the majority of cytosolic antioxidants during spermiogenesis are lost. Persistent DNA damage in ejaculated sperm from subfertile men exposed to in vitro conditions can be partially explained by low levels of essential DNA repair enzymes [81, 82]. When used for ICSI, DNA-damaged sperm may lead to an increased risk for fertilization failure, poor embryo development, abortion, congenital malformations, childhood cancers, and perinatal morbidity [80, 83].

Current evidence suggests that children born through ICSI have an increased risk of congenital malformations and chromosomal abnormalities (~1.0%) when compared with naturally conceived children (~0.2%) or conventional IVF (~0.7%) [68, 84–88]. Additionally, childhood cancer and disrupted reproductive hormonal profile have been observed in offspring born from ICSI compared with naturally conceived counterparts [68]. Lastly, epigenetic disorders and impaired neurodevelopment have also been observed in infants born from ICSI compared with naturally conceived children. The underlying parental infertility seems to have a significant effect on the health of ICSI offspring [68].

Whether the risk of health issues is increased further in infants born through ICSI using surgically retrieved sperm is unknown. The literature is scanty on this matter, but the existing data from ICSI studies evaluating congenital and chromosomal abnormalities in offspring of azoospermic fathers who have used epididymal or testicular sperm for ICSI are reassuring overall [67]. The current studies indicate that congenital malformation rates (~1.6%) and short-term neonatal outcomes are comparable between infants born through ICSI from OA and NOA fathers [67, 89, 90]. Additionally, these rates seem not to differ when the overall population of children born from azoospermic fathers is compared to that born from non-azoospermic fathers subjected to ICSI with ejaculated sperm [91, 92]. Some evidence does, however, suggest that autistic disorder and mental retardation might increase in children born after ICSI and TESE to treat azoospermia compared to conventional IVF. In a prospective cohort study involving 30,959 children born after ART and 2,541,155 children conceived naturally, autistic disorder (adjusted RR 4.60, 95% CI 2.14–9.88) and mental retardation (adjusted RR 2.35, 95% CI 1.01–5.45) were higher after ICSI using surgically extracted sperm than in IVF, although the association was not evident among singletons (RR 0.70, 95% CI 0.10–5.16) [93]. As the published data lack a strong level of evidence, these associations demand further investigation.

50.7 Conclusion

Sperm retrieval techniques are widely used to harvest sperm from the epididymis or seminiferous tubules, in particular, in men with azoospermia. Surgically retrieved gametes are used for intracytoplasmic sperm injection (ICSI). In men with obstructive azoospermia, both percutaneous and open sperm retrieval methods are highly effective to retrieve sperm

from the epididymis or testes. In nonobstructive azoospermia, open testicular sperm retrieval is the method of choice, preferably using a microsurgical approach. Lately, testicular sperm retrieval has been used successfully to retrieve sperm with better chromatin integrity from non-azoospermic men. Overall, sperm retrieval methods have low complication rates. ICSI outcomes mainly depend on the type of azoospermia rather than the method used to harvest sperm, with less favorable results in men with nonobstructive azoospermia. The health of offspring from ICSI using surgically retrieved gametes is overall reassuring. However, a call for continuing monitoring is warranted as the underlying parental infertility might increase the risk of congenital malformations, epigenetic disorders, chromosomal abnormalities, subfertility, cancer, delayed psychological and neurological development, and impaired cardiometabolic profile. It remains to be determined to what extent the observed adverse outcomes might be aggravated by using surgically retrieved sperm.

50.8 Review Criteria

A search of studies examining the use of surgical techniques to retrieve sperm from the testes and epididymides from infertile men for intracytoplasmic sperm injection was performed using PubMed and MEDLINE. The start date for the search was not specified, and the end date was November 2018. The overall strategy for study identification and data extraction was based on the following keywords: "male infertility"; "sperm retrieval"; "epididymal sperm"; "testicular sperm"; "azoospermia"; "reproductive techniques, assisted"; "ICSI"; "in vitro fertilization"; "sperm injections, intracytoplasmic"; and "IVF," with the filters "humans" and "English language." Our search did not include the use of surgical and non-surgical sperm retrieval techniques in patients with ejaculatory dysfunctions as the matter concerned was out of the scope of this chapter. Citations from book chapters and grey literature were only included if provided with conceptual contents.

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Microdissection Testicular Sperm Extraction

51

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Key Points

- Microdissection testicular sperm extraction (microdissection TESE) is considered as a gold standard technique for sperm retrieval in the patients with non-obstructive azoospermia (NOA).
- Non-obstructive azoospermia can typically be diagnosed by careful clinical examination, laboratory testing, and imaging necessary in patient with previous intervention and difficult examination or for those who suspected to have obstructive azoospermia.
- Most non-obstructive azoospermia patients with identifiable causes have a high success rate of retrieval by microdissection testicular extraction except for patients with risk factors such as AZFa and AZFb deletion.
- No definitive laboratory tests currently exist to reliably predict microdissection TESE outcome success; to date, the most reasonable predictive factor is a histological analysis when available. Even that evaluation is inadequate to recommend biopsy in routine workup.
- Up to 50% of the NOA patients are considered idiopathic, most likely related to genetic conditions that cannot be currently identified. Many of these men can still be treated with microdissection TESE.
- For men not currently treatable with microdissection TESE, the future foci are on improved tissue processing and gene and stem cell therapies.

51.1 Introduction

NOA is a major cause of male infertility, with prevalence of about 1% of the entire male population and up to 10% of men who have male-related infertility [1]. Patients with NOA have no spermatozoa in their semen analysis because spermatogenesis is severely impaired. Despite overall impairment, focal sperm production can be detected in the testis of nearly 60% of men with NOA [2, 3]. As recently as two decades ago, the use of donor sperm or adoption was the only option offering a chance of parenthood for couples affected by NOA. The approach to azoospermic patients has dramatically changed with the various sperm retrieval techniques and assisted reproductive technologies. Novel various techniques have been used to retrieve sperm from these patients, including fine-needle aspiration (FNA), testicular sperm extraction (TESE) also called conventional TESE, and microdissection testicular sperm extraction (microdissection TESE), and these techniques are used in combination with in vitro fertilization and intracytoplasmic sperm injection (IVF/ICSI).

In this chapter we focus on considerations and techniques related to microdissection TESE. Microdissection TESE for NOA yields superior sperm retrieval rates and requires removal of a minimal amount of testicular tissue compared with other methods of sperm extraction.

51.2 Diagnosis of Non-obstructive Azoospermia

To determine if a semen sample is truly azoospermic, centrifugation of the semen sample with meticulous microscopic examination of the pellet is necessary. Although this might seem obvious, at least one study reported that sperm was found on extended sperm analysis of a centrifuged semen specimen in up to 35% of men who were thought to have non-obstructive azoospermia [4]. In addition, we have found that up to 10% of men who have inadequate sperm on standard

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evaluation preoperatively will actually have sperm usable for ICSI from the ejaculate on the day of oocyte retrieval. Therefore, we always repeat a semen analysis on the day of planned sperm retrieval for men with non-obstructive azoospermia. The definitive diagnosis of NOA involves histological diagnosis, but a preoperative biopsy is not indicated prior to microdissection TESE. A clinical diagnosis of NOA can be established with reasonable certainty based on the combined basis of history/physical examination, small testes <15 cc, flat/empty epididymides, azoospermia on semen analysis, and elevated serum FSH levels with normal or nearly normal testosterone and estradiol level. Definitive diagnosis can then be made by histological analysis of tissue extracted at the time of the sperm retrieval procedure. Successfully retrieved sperm may be cryopreserved for future use or can be immediately used for ICSI. Each technique has associated advantages and disadvantages. The author's experience strongly suggests that use of fresh spermatozoa provides substantial advantages over intentional cryoTESE due to loss of spermatozoa during processing, freezing, and the lower pregnancy rates with frozen sperm. The published literature is less definitive, but this likely reflects reporting biases that would be obvious in a high-quality "intention-to-treat" analysis.

51.3 Surgical Approaches

In a recent systematic review of surgical techniques of sperm retrieval for men with non-obstructive azoospermia, 15 studies with a total of 1890 patients were identified. The conventional TESE procedure was 2-fold more effective at sperm retrieval than testicular sperm aspiration/fine-needle aspiration (TESA/FNA), and microdissection TESE was 1.5-fold more effective at retrieving sperm than conventional TESE [5]. Microdissection TESE is now accepted by many experts as the gold standard method for surgical sperm retrieval in NOA.

51.4 Conventional TESE

This procedure involves single or multiple testicular biopsies performed in an open fashion under local or general anesthesia. A scrotal incision is made, and tunica albuginea incisions are performed. Random samples of testicular parenchyma are excised and may be evaluated for the presence of sperm by an embryologist. Conventional TESE has the advantage that it takes less time and does not require microsurgical training. However, these blind open tunical incisions have a greater risk of interrupting the vascular supply and devascularizing testicular tissue. Conventional TESE does not allow dissection of most areas of the testis, increasing the possibility of missing foci of spermatogenesis compared with microdissection TESE. Many studies show a strong correlation between the presence of tubules with spermatozoa on histopathologic evaluation and success of open TESE [6]. In contrast, since microdissection TESE identifies small focal areas of sperm, the chance of sperm retrieval is not well predicted by histology. A large study attempted to address the issue of single or multiple TESE. They split their cohort into single and multiple biopsy arms and demonstrated a higher success rate with multiple site biopsy (38% vs. 49%) [7]. However, complications can occur more frequently with open TESE, including either intra-/extratunical hematoma or rarely testicular atrophy resulting from devascularization with the multiple site approach to biopsy.

51.5 Fine-Needle Aspiration/Testicular Mapping

FNA was first described by Obrant in 1965 in the human testis [8], and the technique has been modified by many to optimize its efficacy. FNA is usually performed under local anesthesia in the office setting, requires less time compared to other techniques, is generally well tolerated by patients, and does not required advanced training. There are technical variations in the needle caliber and the number of samples taken. Usually it is performed by inserting a 19–23-gauge butterfly needle directly into the testis and aspirating contents into plastic tubing that is connected to the needle. Multiple needle punctures are usually directed into various parts of the testis. A major disadvantage of FNA is the potential risk of disruption of testicular vascular supply with this blind technique. The risk of injury may be higher in patients with a history of previous scrotal surgery and pre-existing scar tissue; another disadvantage of the FNA approach is the chance of failure due to sampling error. To address this issue in a systematic manner, Turek et al. described fine-needle aspiration mapping [9]. This technique purports to identify the site of sperm production within the testis to guide future biopsy. Although the safety of TESA/FNA has not been investigated systematically, one series showed no hematoma on follow-up ultrasound [9, 10]. Anecdotal experience, however, suggests a high frequency of intratesticular scar after FNA mapping or other percutaneous sperm retrieval procedures.

51.6 Microdissection TESE

Microdissection TESE is the most sophisticated method for retrieving spermatozoa in men with NOA. Initially described by Schlegel [11], he postulated that bivalving the testis (Fig. 51.1) and using optical magnification would allow the surgeon to minimize testicular trauma, maximize exposure for a complete exploration and dissection of the seminiferous tubules, and identify specific regions that might harbor preserved spermatogenesis.

Microdissection TESE requires advanced microsurgical training, as testicular tissues are sensitive to damage, despite

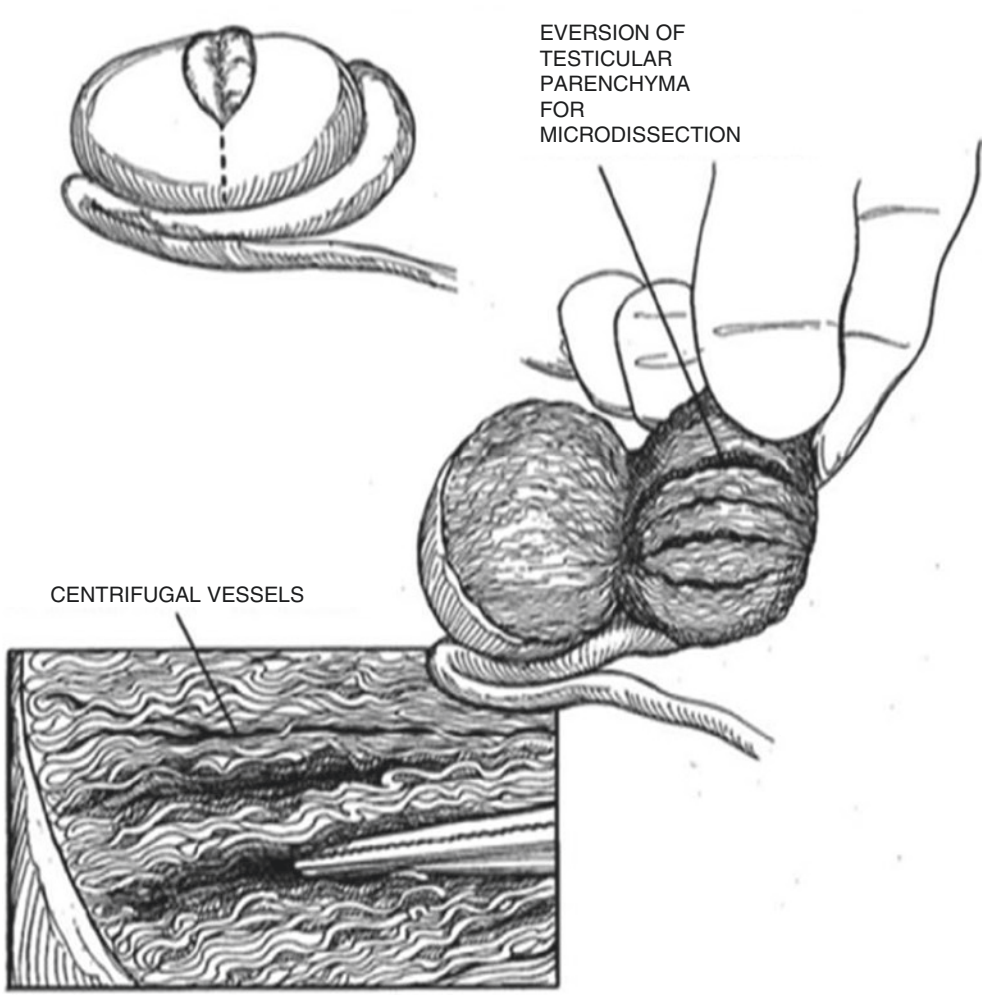

Fig. 51.1 An area of the tunica albuginea is incised and microdissected (Copyright 2019, Brady Urology Foundation)

the anatomical approach used with this procedure. An embryologist should optimally be available in the operating room to evaluate processed samples. By evaluating testicular samples in real time, the surgeon can stop the procedure as soon as the presence of sperm is confirmed, thereby limiting the extent of the procedure and minimizing operative time and removal of testicular tissue. Microscopic dissection of tubules can require up to 2 h per testis if sperm is not identified during the case. In an early prospective study from our institution, we reported a sperm retrieval rate of 45% (10/22) for patients undergoing conventional TESE compared with 63% (17/27) of patients undergoing microdissection TESE ($p < 0.05$). We further showed that the average of 64,000 spermatozoa yielded by conventional TESE biopsy samples were obtained in samples averaging 722 mg each, compared with an average of 164,000 spermatozoa in the microdissected samples that averaged only 9.4 mg ($p < 0.05$ for all comparisons) [11]. In another prospective study, Amer et al. performed conventional TESE on one testis and microdissection TESE on the other in 100 patients with NOA. The authors reported a significantly higher sperm retrieval of 47% for the microdissection TESE side versus the 30% obtained on the conventional TESE side ($p < 0.05$). Follow-up ultrasound testing demonstrated significantly fewer acute and chronic complications on the testes that underwent microdissection TESE, presumably due to improved vascular control [12]. Another systematic review compared cTESE to microdissection TESE in the final analysis of patients with NOA. Overall SRR was significantly higher in the microdissection TESE group in comparison with conventional TESE, and overall sperm retrieval (SRR) ranged from 16.7 to 45% in the conventional TESE vs. 42.9 to 63% in the microdissection TESE group [13].

51.7 Preoperative Preparation and Optimization Prior to Microdissection TESE

51.7.1 Genetics Screening

Genetic male infertility disorders include chromosomal alterations, Y chromosome microdeletions (YCMD), gene mutations, and epigenetic disorders. The use of advanced reproductive techniques, such as microdissection TESE combined with IVF techniques, preimplantation genetic

diagnosis (PGD), and screening (PGS), may overcome some of these conditions [14]. Evaluation of a sequential series of 190 men with NOA who were candidates for TESE at Weill Cornell Medical College revealed 17% (33/190) had definable genetic abnormalities on Y chromosome microdeletion and/or karyotypic anomalies [15]. Given the high rate of genetic abnormalities and the effect of anomalies on sperm retrieval, we believe that genetic screening should be conducted on all TESE candidates prior to attempts at assisted reproduction. Of note, even men with a defined genetic cause for abnormal sperm production (e.g., Klinefelter syndrome) have a very low risk of having a child with a definable genetic problem. Karyotype evaluation and Y chromosome microdeletion (YCMD) analysis are indicated for men with sperm concentrations lower than 5–10 × 10^6/cc. Men with abnormalities detected on screening should receive genetic counseling for an effective discussion of the potential implications of their condition prior to undergoing attempts at sperm retrieval. Depending on the likelihood of passing infertility traits or other genetic defects to offspring, some couples may elect to use donor spermatozoa or consider preimplantation genetic diagnosis. Genetic screening of TESE candidates facilitates specific genetic testing and subsequent selection of embryos prior to implantation.

51.8 Karyotype Evaluation

Common abnormalities seen in men with NOA include Klinefelter syndrome (47, XXY), Robertsonian translocations, chromosomal inversion, and other sex chromosome anomalies (i.e., 46, XX). Of 190 sequential TESE candidates who underwent genetic evaluation at Weill Cornell, 33 (17%) were found to have genetic abnormalities. Of the 183 patients who underwent molecular analysis for Y chromosome partial deletion, defects were detected in 17 (9%). Of the 101 patients who underwent karyotype testing, 21 (21%) had cytogenetic abnormalities, including 13 with KS. Five men with sex chromosome anomalies detected on karyotyping also had Y chromosome deletions. Of the 33 men in whom genetic anomalies were discovered, 31 underwent counseling with their partners. Knowledge of the specific genetic defect affected the choice of clinical treatment in 7 of the 33 couples (21%), including change of treatment choice to donor insemination, adoption, or delaying treatment. The remainder of couples elected to proceed with TESE/ICSI after genetic counseling [15].

51.9 Endocrine Evaluation and Treatment

Medical optimization strategies have been investigated for patients with azoospermia due to testicular dysfunction, with the goal of increasing intratesticular testosterone and circulating FSH levels to stimulate spermatogenesis. A multi-institutional study [16] evaluated the effectiveness of using a combination of clomiphene citrate (CC), hCG, and/or hMG to increase serum testosterone to 600–800 ng/dL and serum FSH to 1.5 times baseline in 612 men with NOA. In this study, the intervention group received hormonal optimization according to a defined protocol, while the control group received no hormonal optimization, with a significantly higher sperm retrieval rate (SRR) for the intervention group (SRR 57% vs. 34% for intervention group vs. control group). While this study supports the medical hormonal optimization of FSH and endogenous testicular testosterone levels to improve SRR, it is important to note that the control group had a lower SRR than previous reports in the literature. Furthermore, a large retrospective study of 1054 men did not find benefit of hormonal therapy in men undergoing microdissection TESE. Here, no difference in SRR was identified among men with baseline testosterone >300 ng/dL, compared to men with baseline testosterone <300 ng/dL and not receiving medical hormonal treatment or men receiving aromatase inhibitors, selective estrogen receptor modulators, or any combination with hCG [17]. The use of aromatase inhibitors such as anastrozole and letrozole, to optimize hormonal parameters in NOA and cryptozoospermia patients, is supported by data suggesting that men with severely impaired spermatogenesis often have excess estrogen relative to testosterone levels. Although most studies were performed using anastrozole, which appears to have fewer side effects, one study comparing letrozole to placebo in cryptozoospermia and NOA patients suggested that letrozole may help enhance spermatogenesis with sperm return to the ejaculate in some NOA patients with observed decreased libido in a substantial proportion of treated men [18]. Candidates for management with aromatase inhibitor include men with low serum testosterone (<300 ng/dL) and low testosterone/estradiol (measured in pg/mL) ratios (<10), in whom aromatase therapy has been suggested to enhance intratesticular testosterone levels and improve spermatogenesis [19, 20] at our institution, we now routinely obtain testosterone and estradiol levels in men with NOA or severe oligozoospermia. Men who have low testosterone and low T/E2 ratios are commonly treated with anastrozole, 1 mg per day at our center [21].

51.10 Microdissection TESE Technique

51.10.1 Surgical Approach

Microdissection TESE is typically performed under a general or local anesthetic. An incision is made through the median raphe to allow easy access to both testes. The larger of two testicles is delivered first by incising through the dartos and subsequently the tunica vaginalis. Under the

operating microscope, the tunica albuginea of the testicle is then incised transversely using a 15-degree micro-knife, taking care to avoid equatorial testicular vessels. Mosquito clamps are placed on each respective side of the tunical incision including the edge of seminiferous tubules to prevent avulsion of the tissue as the testicle is bivalved under gentle pressure from the surgeon's fingers. Right-handed surgeons should stand to the patient's left so that the surgeon's left hand can be in a more comfortable position between the patient's legs rather than on the abdomen. A three-finger technique is utilized with the left hand both to stabilize the testicle and to maintain exposure. Here, the third digit supports the posterior side of the testis, while the thumb and index finger provide exposure on the cut surface of the testis. The seminiferous tubules are then systematically examined for dilated, opaque tubules. Excellent visualization of the seminiferous tubules is critical and is achieved both by maintaining the seminiferous tubules within the microscope's focal distance and by ensuring adequate hemostasis using bipolar electrocautery. Once dilated, opaque tubules are identified, and the entire length of the centrifugally oriented tubule is removed with tissue micro-forceps and placed in a small petri dish with sperm transport buffer. After normal tubules are identified (or if no normal tubules are seen, the entire cranial or caudal half of the testis is searched), the tissue is prepared for handoff to an embryologist, preferably in the surgical theater, who will examine the removed testicular tissue for sperm. If sperm is identified, further dissection, including dissection of the contralateral testicle, is unnecessary, but if sperm is not identified, then continued exploration should proceed.

Tissue processing of the excised tissue is essential to optimize successful identification of sperm that are present within the tissue. Excised tubules are minced with scissors until the suspension is fine enough to be aspirated in and out of a 24-gauge angio-catheter prior to inspection by the embryologist. This technique provides a 300-fold increase in sperm recovery [22]. Once a testis has been fully dissected, a series of mosquito clamps are then used to re-approximate the tunica albuginea edges that are then closed using a 5-0 non-absorbable monofilament suture in a running fashion. This serves to mark the location of dissection if a repeat procedure is required in the future. The testicle is returned to its anatomical position within the tunica vaginalis that is then closed with an absorbable monofilament suture in a running fashion. If the contralateral testicle requires dissection, it is performed in a similar fashion. Otherwise, the tunica vaginalis is infused with local anesthetic, and the dartos layer is closed with a running absorbable suture, with care being taken to include the entire cut edge in the closure for optimal hemostasis. Prior to completing the knot, 5 mL of local anesthetic is infused into each hemi-scrotum. The knots are buried within the dartos layer. Finally, the skin can be closed with interrupted horizontal mattress stitches. Interestingly, running subcuticular closure of the scrotal skin tends to easily break down, resulting in disruption of the scrotal incision. Bacitracin ointment and appropriate dressing are applied with scrotal support [23].

51.11 Complications and Considerations After Microdissection TESE

A retrospective study reviewed 147 TESE consecutive cases for patients with azoospermia [24]. All patients underwent testicular ultrasound 1 month after TESE. Diffuse heterogenic patterns or hypoechoic areas on ultrasound were considered indicative of hematoma. Among the 47 patients who underwent conventional TESE, 24 (51%) showed evidence of hematoma. Of the 100 patients who underwent microdissection TESE, only 12 (12%) had ultrasound findings consistent with hematoma. On follow-up ultrasound at 6 months, hematoma was identified in 3/40 (7.5%) of conventional TESE patients and 2/80 (2.5%) of microdissection patients. A retrospective review from our institution involved a series of patients who underwent either conventional (83 attempts) or microdissection TESE (460 attempts) were followed with serial color Doppler scrotal ultrasounds at 3 and 6 months following surgery. Acute and chronic changes were both significantly lower for the patients who underwent microdissection TESE than conventional TESE. When compared to conventional TESE, microdissection TESE results in lower complication rates, with fewer hematomas, less testicular fibrosis, and less frequent testicular atrophy with higher SRRs [13]. We have not experienced testicular loss following microdissection TESE, although some anecdotal reports exist for men with low baseline testosterone levels and small testes. Studies have found serum testosterone levels following microdissection TESE decrease from 316 to 251 ng/dL, but return to 95% of baseline at 18 months; of these 5–10% of men will have a decrease in testosterone significant enough to warrant subsequent androgen replacement. Since time is required for recovery of the limited sperm production that is present in men with NOA, at least 6–12 months should be allowed after microdissection TESE, before considering repeat microdissection TESE procedures if additional attempts are required [25, 26].

51.12 Predictors of Microdissection TESE Success

Microdissection TESE is commonly carried out in conjunction with a programmed IVF cycle. Microdissection TESE–IVF/ICSI cycles are financially and emotionally burdensome for infertile couples, but simultaneous sperm retrieval and

IVF appear to provide optimal treatment results. It is important to discuss appropriate expectations for reproductive outcome.

51.13 Effect of Prior Biopsy or Conventional TESE Procedure

Successful sperm retrieval is often possible with microdissection TESE in men with NOA in the context of multiple prior negative biopsies. Study reported that half of these patients required multiple (range 2–14) biopsies to retrieve sperm [23]. The effect of prior negative biopsies or conventional TESE procedures on the rate of sperm retrieval with microdissection TESE in men with NOA was evaluated at Weill Cornell. Successful sperm retrieval in patients who underwent no prior biopsies (56%) was higher than for those who had underwent 1–2 biopsies per testis (51%) or 3–4 biopsies per testis (23%) ($p = 0.04$) [27]. In contrast, repeat microdissection TESE following prior successful retrieval has been shown to yield a 60–80% success rate. However, if sperm are not found on prior microdissection TESE, the SRR drops to 33% [28].

51.14 Testicular Histology on Diagnostic Biopsy

Diagnostic biopsy helps to predict the chance that a microdissection TESE procedure will obtain sperm. Since diagnostic biopsy does not sample all areas of the testis, small foci of more advanced patterns may be missed. However, four testicular histologies are associated with NOA [22]. Hypospermatogenesis, which is the least severe form of NOA, carries the highest surgical SRR of 73–100%, while late maturation arrest has a SRR of 27–86%, early maturation arrest has a SRR of 27–40%, and Sertoli cell-only syndrome (SCOS), which is the most severe form of infertility, has a SRR of 22.5–41% (3–6). These findings from a diagnostic biopsy may have counseling implications. The presence of heterogeneity within the testis is perhaps the most important finding to predict sperm retrieval in NOA. Since all men with NOA have abnormal spermatogenesis, the presence of a different or more advanced pattern of spermatogenesis, especially in men with predominant Sertoli cell only on diagnostic biopsy, is a potential predictor of sperm retrieval.

51.15 Microdissection TESE in Setting of Elevated FSH Levels

Elaboration of inhibin by Sertoli cells is decreased in the setting of testicular failure. With less negative feedback mediated by inhibin and other factors, production of FSH from the anterior pituitary increases. Elevated serum FSH levels are generally associated with impaired spermatogenesis. FSH levels are less relevant for predicting success of microdissection TESE. Even though serum FSH indirectly reflects the global histology of the testes, the chance of sperm retrieval is predicted only by the best area of spermatogenesis, which is not predicted by serum FSH levels. This hypothesis was tested in a retrospective study of nearly 800 men with NOA who underwent microdissection TESE. Patients were divided into four groups by serum FSH levels (<15, 15–30, 31–45, and >45 IU/mL) have found little association between SRRs and FSH levels, with FSH levels less than 15 IU/mL having a SRR of 51%, FSH levels between 15 and 30 IU/mL having a SRR of 60%, FSH levels 31–45 IU/mL having a SRR of 67%, and FSH levels greater than 45 IU/mL having a SRR of 60%, as well. Sperm retrieval rates were maintained as FSH levels increased, furthermore, even for several patients with FSH >90 IU/mL [29]. These findings underscore the concept that FSH levels are not predictive of sperm retrieval success with microdissection. Azoospermic patients with normal FSH may represent a distinct infertility population. Indeed, it has been reported that many patients with diffuse maturation arrest have normal FSH levels (and testicular volume) [30], and these patients may have a particularly poor chance of sperm retrieval.

51.16 AZF Deletions

PCR-based analysis of Y chromosome sequence-tagged sites is prognostically important and is routinely performed for microdissection TESE candidates. The Y chromosome microdeletions that relate to infertility are seen in part of all of the AZFa, AZFb, or AZFc regions of the DAZ (deleted in azoospermia) gene and are found in 6–18% of men presenting with NOA or severe oligozoospermia [31, 32]. In men with isolated AZFc deletions, sperm can be found in the testis at similar or better rates than for other patients with NOA. Seventy percent of men with AZFc deletions will have sperm in their ejaculate, but typically at very low concentrations less than 1 million sperm per mL [33]. The SRRs for azoospermic men with AZFc deletions range between 60 and 70%. At Weill Cornell, in a retrospective analysis of 1591 men with sperm concentrations less than 5 million sperm/mL, a total of 149 microdeletions (9.4%) were found. Of the 718 patients who underwent microdissection TESE, sperm retrieval failed in all men with AZFa, AZFb, AZFb + c, as well as complete Yq deletions (reflecting deletion of AZFa, b, and c regions). Presence of an AZFc microdeletion, in contrast, was associated with a 71% SRR. Of the 15 patients with AZFc deletions with successful sperm retrieval, 10 of them achieved clinical pregnancy. AZFc deletions were reported, for the first time, to be favorably associated with sperm retrieval in comparison

to 385 patients with idiopathic azoospermia, for whom microdissection TESE yielded a 48.8% retrieval rate [33]. Given the exceptionally poor prognosis for patients with complete AZFa or AZFb deletions, deferring surgical intervention or primary utilization of donor sperm is advised rather than microdissection TESE. Genetic counseling for the couple is important, since potential male offspring will carry the same genetic condition (AZFc deletion) as the father and are expected to eventually face similar reproductive issues.

51.17 Microdissection TESE in Patient Subpopulation

There are several patient populations that may particularly benefit from microdissection TESE, including Klinefelter syndrome patients, men post-chemotherapy, and NOA associated with cryptorchidism.

51.17.1 Klinefelter Syndrome

Klinefelter syndrome (KS) is the most common identified genetic cause of male infertility, found in 1/600 of all men, in 0.6% of severely oligospermic men, and in 11% of men with non-obstructive azoospermia (NOA) [34, 35]. Nondisjunction during meiosis I is the origin of the extra X chromosome, which has been reported to be paternally derived in 60% of cases. Association with increased paternal age is controversial, while advanced maternal age is recognized as a risk factor for having a child with KS [35]. KS patients have traditionally been considered a difficult group to retrieve sperm from because they typically have small testes, high FSH levels, and a predominant expression of tubular sclerosis on testicular histopathology. Higher retrieval rates have been demonstrated. In 2005, we at Will Cornell retrospectively reviewed a series of 42 patients with KS who underwent 54 attempted microdissection TESE procedures. Patients had mean FSH levels of 33.2 IU/L and a successful sperm retrieval rate of 72% per TESE attempt [3]. Men with Klinefelter syndrome (KS) have progressive intratesticular fibrosis and impairment in both testosterone production and spermatogenesis. The mean SRR more recently reported from our center for men with KS was 66% with clinical pregnancy rates of 50% [36]. In men with KS, predictive factors for successful sperm retrieval included LH <17.5 U/L, increase in testosterone to >250 ng/dL following medical therapy, younger post-pubertal age, and the presence of a normal baseline testosterone/estrogen ratio [37, 38]. As such, treatment with aromatase inhibitors and selective estrogen receptor modulators may be considered. Fullerton et al. [39] reviewed cases reported in the literature and identified 101 live births from Klinefelter syndrome patients who had sperm extracted via TESE or microdissection TESE. All babies born were genetically normal. Patients at Weill Cornell are screened with hormonal profiles prior to undergoing sperm retrieval procedures. We have previously shown that while normal fertile men have an average T/E2 ratio of 16 ± 3, patients with KS have an average T/E2 ratio of 4 (T measured in ng/dL, with E2 measured as pg/mL) [40]. We treat patients with low T/E2 ratios with aromatase inhibition and have found dramatic improvements in sperm concentration and motility in patients with severe oligozoospermia [21]. Other possible explanations for our better sperm retrieval rates in KS patients include use of the more effective surgical technique of microdissection TESE versus standard TESE procedures, as well as the benefits inherent with a substantial single-surgeon (PNS) experience.

51.17.2 Post-Chemotherapy Azoospermia

As screening and treatment for various types of cancer continue to improve, a growing population of cancer patients has the opportunity to focus on quality-of-life issues. Although the importance of the concept of fertility preservation has been increasingly recognized, fewer than half of oncologists in the USA routinely refer patients to a fertility specialist prior to treatment that may further threaten their patients' fertility potential [41]. Systemic chemotherapeutic agents are associated with dose-dependent toxicity to germinal epithelium and posttreatment azoospermia, especially for men treated with alkylating agents. A subset of patients with persistent post-chemotherapy azoospermia has been treated at Weill Cornell. Of 20 of our initial attempts of TESE–ICSI performed for 17 patients, 45% (9/20) resulted in successful sperm retrieval. The mean time period between chemotherapy and microdissection TESE was 16.3 years (range 6–34 years). Clinical pregnancy was established for a third (3/9) of patients with sperm retrieved with two live deliveries. Sertoli cell-only pattern was demonstrated in 76% of patients, with hypospermatogenesis as the most advanced pattern seen in the remainder of patients. Sperm was retrieved in 23% of men with Sertoli cell-only pattern [42]. In the follow-up study by Hsaio et al., men treated for testicular cancer with platinum-based chemotherapy regimens had a higher rate of sperm retrieval than men treated with alkylating agent-based chemotherapy [43]. The use of microdissection TESE/ICSI for patients with persistent post-chemotherapy azoospermia has enabled conception and delivery of healthy children. Although patients should ideally be encouraged to bank ejaculated sperm prior to treatment when possible, patients with long-standing post-chemotherapy azoospermia may now be successfully treated with advanced reproductive techniques.

51.17.3 NOA Associated with Cryptorchidism

Failure of testicular descent can be associated with impaired sperm concentration, sperm quality, and fertility rates. Cryptorchid testes are also associated with an increased risk of eventual testicular germ cell tumor. There is limited evidence that orchiopexy decreases the risk of cancer, although it facilitates testicular examination and detection of testicular cancer. In contrast, subfertility secondary to cryptorchidism appears to be a duration-dependent process. Increased age at orchiopexy, as well as bilateral cryptorchidism, has been associated with worsening fertility parameters and outcome [44]. Several mechanisms for increased risk of infertility with cryptorchidism have been proposed. The first step in postnatal spermatogenic development, maturation of gonocytes to type A spermatogonia, is believed to be deficient in infants with cryptorchidism. Gonocytes that fail to mature degenerate and consequently yield a decreased total number of germ cells. Androgen production, which is impaired in cryptorchid testes and may play an important developmental role of the testis in the few months after birth, may be either a primary or secondary defect. Poor steroid secretion may be a causative factor in deficient germ cell maturation. Finally, cryptorchid testes are subject to an increased temperature of several degrees compared with the normal scrotal temperatures [45].

Although most men with a history of cryptorchidism treated by orchiopexy will have adequate sperm production for fertility, some men with this history are azoospermic. In 2003, we reported on our experience at Weill Cornell with sperm retrieval in men with NOA associated with cryptorchidism. In our initial experience, a total of 38 men (mean age 36.7 ± 6.5 years) underwent a total of 8 conventional and 39 microdissection TESE procedures. Retrieval of sperm was achieved in 63% (5/8) of open TESE procedures and 77% (30/39) of microdissection TESE procedures for a combined rate of 74%. Spermatozoa were retrieved in all patients (9/9) with a history of unilateral cryptorchidism and 68% (26/38) patients with a history of bilateral cryptorchidism. For cases of successful sperm retrieval, couples achieved clinical pregnancy for 46% (16/35) cycles. Serum FSH was not correlated with successful sperm retrieval, but larger testicular volume ($p < 0.05$) and younger patient age at orchiopexy ($p < 0.001$) were independent predictors for spermatozoal retrieval [46].

51.18 Next Years' View

Improving fertility rates in the near future will likely be related to the development of treatment options in gene therapy, stem cell therapies, improved testicular tissue processing techniques, and possibly in vitro spermatogenesis. Approximately 50% of men with NOA have an idiopathic underlying etiology. At Weill Cornell, we are currently addressing this issue by seeking to identify novel genetic abnormalities that may underlie the lack of germ cell support seen in Sertoli cell-only regions of the testis as well as ineffective spermatogenesis in men with maturation arrest and impaired germ cell maturation, typically seen in men with idiopathic NOA.

51.19 Conclusions

The technique of microdissection TESE is an optimized approach to treatment of men with non-obstructive azoospermia, where the sperm yield is optimized and risks of the procedure, including the amount of tissue removed, are minimized. Laboratory handling is improved for men with non-obstructive azoospermia because the number of sperm is optimized, but the amount of tissue sent to the lab is limited. Treatment of men with non-obstructive azoospermia is very challenging, but the ability to retrieve sperm for use with assisted reproduction is possible for the majority of men with this condition who were previously considered sterile.

51.20 Review Criteria

A standardized search was done to study the advantages and disadvantages of microdissection testicular sperm extraction in patients with non-obstructive azoospermia using search material of PubMed, Google Scholar, Science Direct, and Medline. The start and the end date for these searches were June 1997 and December 2018, respectively. The strategy of data extraction was based on keywords "azoospermia," "non-obstructive azoospermia," "micro-testicular sperm extraction," "microdissection TESE," "male infertility," "Klinefelter syndrome," "cryptorchidism," and "hypospermatogenesis." All articles in English language are considered.

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Sperm Processing and Selection

52

Rakesh Sharma and Ashok Agarwal

Key Points

- Sperm selection is important in assisted reproductive technology to obtain good-quality sperm with high DNA integrity.
- Centrifugation-based techniques can produce reactive oxygen species, result in oxidative stress, and decrease DNA integrity.
- Non-centrifugation-based techniques reduce oxidative stress and select sperm with high DNA integrity.
- Advances tests such as magnetically activated cell sorting, hyaluronidase binding, and microfluidics are promising and warrant further investigation in ART outcomes.

52.1 Introduction

Assisted reproductive technologies (ART) such as intrauterine inseminations (IUI), in vitro fertilization (IVF), and intracytoplasmic sperm injection (ICSI) are effective treatment options that allow infertile couples the opportunity to have their families. However only a third of the ART cycles result in live births, and it is unclear why so many attempts result in non-fertilization [1]. Male infertility is a factor in 50–60% of infertility cases [2, 3]. Infertile men tend to have abnormal sperm parameters, such as low sperm concentration, poor motility, abnormal morphology, and elevated levels of sperm DNA damage [4, 5]. Additionally, about 40–88% of sperm samples from infertile men have high levels of reactive oxygen species (ROS) [6–8]. Low concentrations of ROS are required for physiological sperm functions such as capacitation, acrosome reaction, and hyperactivation, and the overproduction of ROS is usually due to the inability of antioxidants to neutralize ROS [9–12]. A high level of ROS and decreased levels of antioxidants can cause oxidative stress, which decreases sperm motility, DNA integrity, and viability and increases midpiece defects [5, 12–14]. Poor DNA integrity is correlated with lower in vitro fertilization pregnancy rates, irregular pre-implantation development, early loss of pregnancy, and increased disease rates in offspring conceived through ART [15–18].

In natural conception, only a small fraction out of the millions of sperm that are deposited in the vagina very near to the cervix reach the oocyte. This indicates the presence of a strict and efficient sperm selection process that naturally occurs in the female genital tract. Subsequently sperm are drawn through the cervix, uterus, uterotubal junction, and oviductal isthmus to reach the egg in the oviductal ampulla. The sperm are presented with different structural, fluidic, ionic, and molecular environments resulting in a complex process of sperm migration [19]. In in vitro fertilization, an oocyte is incubated with about 50,000 sperm from an initial sample containing about 100 million sperm. In intracytoplasmic sperm injection (ICSI), a single sperm is selected and directly microinjected into the oocyte. Therefore the fundamental challenge of sperm selection is dictated by the sperm biology, sample volume, sperm concentration, and lifetime in vitro. The ideal time for sperm selection process is about 10 min for 1 mL of sample containing 100 million/mL sperm. This indicates an extremely high biological sorting rate of ~100 kHZ which is extremely higher than the current cell sorting technologies [20]. The two most common sperm selection methods for sperm preparation for ART are density gradient centrifugation and swim-up based on sedimentation and migration, respectively. The density gradient technique separates about 36% of sperm from 0.5 mL raw semen in about 30 min, whereas swim-up selects about ~12% of the sperm population from 1 mL of the sperm population in about 1 h. This results in about 18–19% and 5% sperm improvement in sperm motility [21].

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The current sperm processing techniques suffer from a number of limitations:

- The methods do not resemble the natural process in vivo.
- Selected sperm population is contaminated with poor motile cells.
- Presence of leukocyte contamination.
- Iatrogenic injury due to prolonged exposure and oxidative stress due to centrifugation [22].
- Further visual inspection which is related to the embryologist specific step which can influence the success rate between the clinics [23].

Therefore, there is a need to develop and implement improved sperm sorting techniques and protocols in order to select sperm containing normal DNA and lesser ROS, improve ART success rates, and decrease birth defects. New sperm selection methods should closely mimic the natural selectivity of the female genital tract to ensure that only the healthy sperm are selected. In this paper, we review current sperm selection technologies and their respective effects on sperm morphology and function as well as ART outcomes.

52.2 Reduction of Semen Viscosity

Human semen normally liquefies within 5–20 min after ejaculation [24]. However, some ejaculates fail to liquefy, and some are viscous by nature. Semen viscosity is a problem since it can reduce sperm motility. To reduce viscosity, the semen can be mixed with a sperm wash medium. However liquefaction achieved by this method might not be adequate for highly viscous samples. In such cases, the viscosity can be reduced by forcing the viscous semen through a needle with a narrow gauge which is another option [24]. The common method to reduce viscosity is by treating the sample with 5 mg of trypsin. If the sample fails to liquefy after 20 min, trypsin powder is directly added to the sample, and after swirling, the sample is incubated for an additional 10 min. This results in complete liquefaction of the sample.

52.3 Conventional Sperm Selection Methods

A variety of selection techniques have been introduced that are based on centrifugation, filtration, or sperm migration. Among the centrifugation techniques, density gradient centrifugation has been proposed as the gold standard for sperm preparation. The latest developments in sperm selection are focused on the sperm surface combined with or without a standard preparation protocol as illustrated in advanced sperm selection section.

52.3.1 Simple Sperm Wash

Both one-step and two-step sperm washing methods involve resuspension of sperm in the culture medium after complete liquefaction. The one-step wash technique does remove or reduce any cellular component such as the number of leukocytes, immature spermatozoa, or other cellular debris. It only removes the seminal plasma. Furthermore, centrifugation causes additional harm by formation of reactive oxygen species (ROS) by abnormal spermatozoa and leukocytes [25]. Increased levels of ROS result in DNA damage in spermatozoa, decreased sperm motility, increased numbers of apoptotic spermatozoa, and decreased sperm plasma membrane integrity [26].

52.3.2 Swim-Up

It is one of the most commonly used migration techniques for sperm preparation. Sperm culture medium containing antioxidants provides the nutritional support. In the conventional swim-up technique, a prewashed pellet obtained after a soft spin is placed at the bottom of an overlying medium. In addition, after complete liquefaction, semen sample (0.5 mL) can be carefully layered at the bottom of a round bottom tube containing about 2 mL of the sperm wash medium. The tube is placed at an angle of 45° and incubated for 60 min. Depending on the original sample, multiple tubes can be prepared. At the end of incubation, using sterile technique, clear supernatant is aspirated into a separate tube (Fig. 52.1). The sample can be centrifuged at 1600 rpm for 7 min and the pellet resuspended in 0.5 mL of sperm wash medium. The swim-up procedure uses the active motion of the spermatozoa. All motile spermatozoa move out of the pellet into the clear supernatant. Highly motile, morphologically normal intact spermatozoa are enriched in the absence of other cells, proteins, and debris within the supernatant. A modified swim-up method called the direct swim-up is used for oligospermic samples [27]. In this method, sperm swim out directly from the semen rather than from the cell pellet. Round bottom tubes are used to maximize the surface area between the semen and the medium [25]. Swim-up method is inexpensive, and highly motile sperm can be obtained. The disadvantages are that the sperm recovery is relatively low. Only 5 to 10% of sperm cells are retrieved. When a pellet is used, sperm are trapped in the pellet and may not move into the clear medium. In addition, centrifugation results in the generation of ROS [28]. Furthermore if the sample is contaminated with leukocytes, the close cell-to-cell contact may further result in production of reactive oxygen species (ROS).

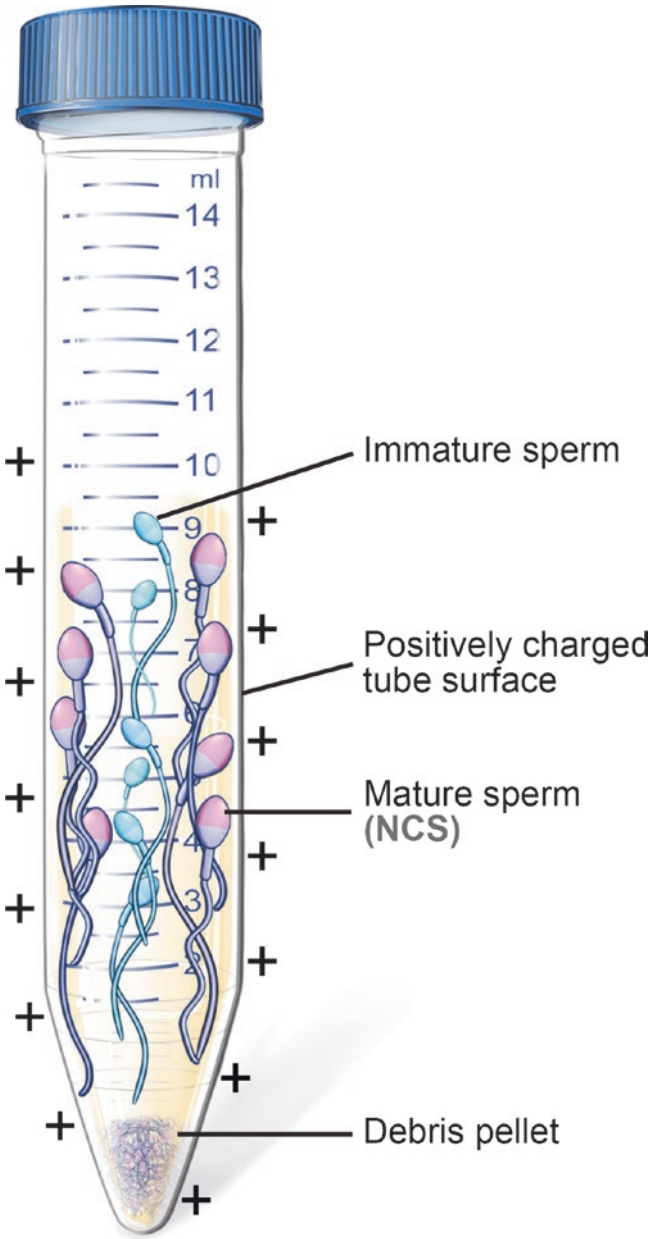

Fig. 52.1 Sperm selection by swim-up technique (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2011–2019. All Rights Reserved)

52.3.3 Density Gradient Centrifugation

Density gradient centrifugation is considered the gold standard technique for sperm preparation. It separates cells based on the density, motility, and centrifugation speed. Morphologically normal and abnormal spermatozoa have different densities. Mature morphologically normal sperm are denser (1.10 g/mL) compared to immature and morphologically abnormal sperm (1.06–1.09 g/mL) [29]. Components of the density gradient sperm separation procedure include a colloidal suspension of silica particles stabilized with covalently bonded hydrophilic silane supplied in HEPES. There are two gradients: a lower phase (80%) and an upper phase (40%). Sperm washing medium (modified HTF with 5.0 mg/mL human albumin) is used to wash and resuspend the final pellet (Fig. 52.3).

Percoll™, a colloidal suspension of silica particles coated with polyvinylpyrrolidone, was widely used by ART laboratories until it was withdrawn from the market for clinical use. Media containing silane-coated silica particles are commonly used. Isolate™ (Irvine Scientific, Santa Ana, CA), IxaPrep™, Sperm Preparation Medium™ and SupraSperm™ (Origio, MediCult, Copenhagen, Denmark), SpermGrad™ (Vitrolife, San Diego, CA), SilSelect™ (FertiPro NV, Beernem, Belgium), and PureSperm™ (NidaCon Laboratories AB, Gothenburg, Sweden) are commonly used [24]. This method allows for the enrichment of mature and motile sperm, and recovery rates of 30–80% can be achieved depending on the initial semen sample and the technical skill of the individual doing the procedure.

A gradient is prepared by carefully layering 2 mL of lower phase at the bottom of the 15 mL graduated centrifuge tube, and a 2 mL of upper layer is layered on top without mixing the two gradients [30]. Up to 2 mL of a completely liquefied semen sample is layered on top and centrifuged for 20 min (Figs. 52.2 and 52.3). During this procedure, highly motile spermatozoa move actively in the direction of the sedimentation gradient and therefore can reach lower areas quicker than poorly motile or immotile cells. The resulting interphases between seminal plasma and 40%, 40%% and 80% containing the leukocytes, cell debris, and morphologically abnormal sperm with poor motility are discarded. The highly purified motile sperm cells are enriched in the soft pellet at the bottom. The pellet is resuspended in 2 mL of the medium and centrifuged again at 1600 rpm for 7 min. The clear pellet is finally resuspended in 0.5 mL of sperm washing medium before use in intrauterine insemination. Centrifugal force and time should be kept at the lowest possible values (<300 g) in order to minimize the production of ROS by leukocytes and non-viable sperm cells [31]. Also, non-viable sperm cells and debris should be separated from viable sperm cells as soon as possible to minimize oxidative damage. Double density gradients comprise the commonly used sperm preparation protocol for ART [25].

Compared to swim-up, density gradient takes only 30 min. It is relativity easy to perform under sterile conditions. Spermatozoa from oligozoospermic patients can also be separated by this method. Density gradient eliminated the majority of the leukocytes in the ejaculates. The disadvantages are that the interphases between the layers may take some time; there are reports that sperm prepared by density gradient still have some degree of DNA fragmentation compared to spermatozoa prepared by swim-up [32].

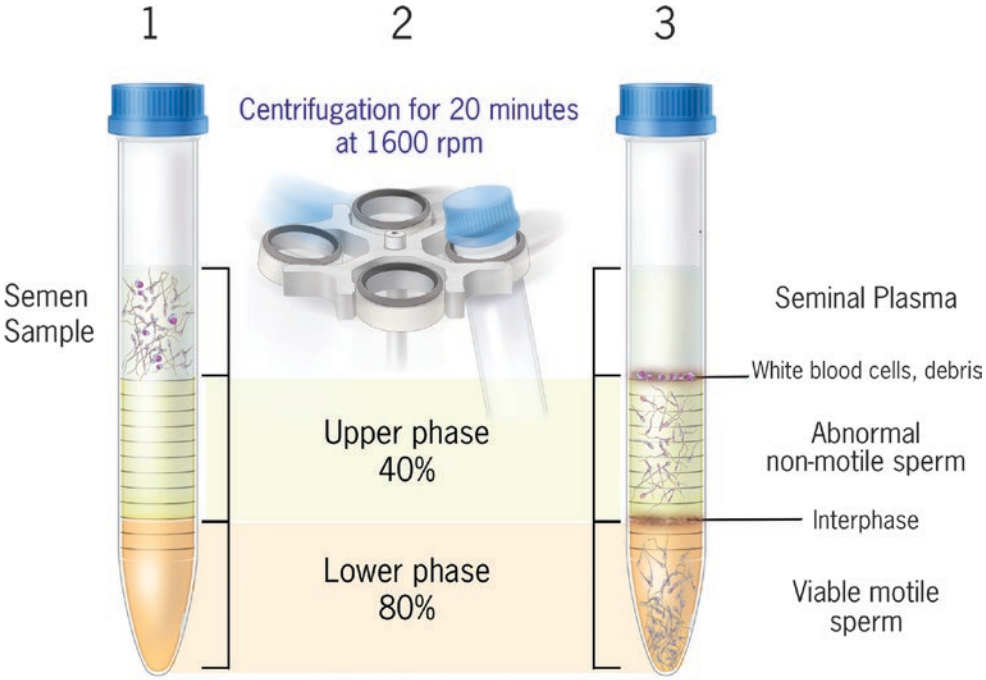

Fig. 52.3 HTF resuspended sample centrifuged to produce viable sperm pellet (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2011–2019. All Rights Reserved)

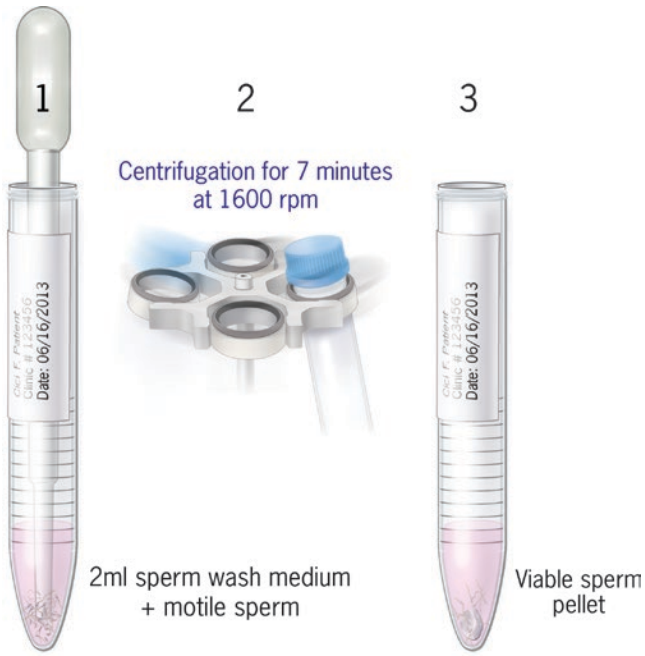

Fig. 52.2 Double density gradient wash procedure; separation of seminal plasma, abnormal nonmotile sperm, and viable motile sperm (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2011–2019. All Rights Reserved)

52.4 Preparation of Assisted Ejaculation Samples

Patients with spinal cord injury often have ejaculates with a high sperm concentration and low sperm motility [33]. These ejaculates are also contaminated with red blood cells and white blood cells. In these patients, ejaculates can be obtained by electroejaculation using direct penile vibratory stimulation or indirect rectal stimulation. These ejaculates can be effectively prepared with density gradient centrifugation [33].

52.5 Preparation of Retrograde Ejaculation Samples

Retrograde ejaculation occurs when the semen is directed into the urinary bladder during ejaculation. If there are an inadequate number of spermatozoa in the ejaculate, sperm cells in the urine need to be retrieved. The patient is first asked to urinate without entirely emptying his bladder. Then, he is asked to ejaculate and urinate again into another specimen cup containing 9 mL of warm sperm wash medium to alkalinize the urine. The urine sample volume is noted and analyzed after centrifugation. Both the concentrated retrograde specimen and the antegrade specimen are prepared with density gradient centrifugation technique to obtain motile sperm for use in ART [34].

52.6 Sperm Preparation Techniques for Cryopreserved Semen

There are many medical conditions where sperm banking is indicated to preserve fertility. Cancer patients both young adolescents and adults are the most common group of men

who are referred for sperm cryopreservation. In addition, patients with medical conditions such as lupus, multiple sclerosis, varicocele, testicular torsion, spinal cord injury, and ejaculatory dysfunction, gender reassignment, and travelling husbands can also benefit from sperm cryopreservation. Protocols for sperm freezing include conventional slow freezing [35] and vitrification [36, 37]. In the slow freezing technique, after liquefaction, a TEST-yolk buffer (TYB) aliquot equal to 25% of the original volume of specimen is added to the semen sample using sterile technique. The specimen and TYB combination are placed on a test tube rocker for 5 min to ensure that the sample is gently mixed. The process of adding a 25% aliquot of TYB followed by gentle mixing should be repeated three more times so that the total volume of TYB added over the four different aliquots equals that of the original semen sample. The samples are aliquoted into cryovials and stored in liquid nitrogen (LN_2) [35].

Vitrification is a technique based on the ultra-rapid freezing of cells by directly immersing in LN_2. In this technique, there is no formation of ice crystals. Vitrification of spermatozoa is challenging due to the unique properties of the spermatozoa. A superior preservation of motility and viability is seen in sperm preserved by vitrification when compared to standard slow freezing. Spermatozoa are osmotically fragile, and use of high concentration of permeable cryoprotectants is toxic and also potentially mutagenic [36]. Samples can be processed by swim-up or other method and vitrified samples loaded onto straws or cryoloops and immersed in LN_2. Cryoprotectant-free vitrification can be accomplished by using high cooling rates by directly plunging samples into LN_2, ~720,000 K/min, and increasing the surface area for heat exchange using extremely small sample volume [37]. Sperm can be stored during vitrification using cryoloop, droplet, open straws, and open pulled straws.

52.7 Preparation of Epididymal and Testicular Sperm

Sperm can be obtained from the epididymis or the testicular tissue in case of epididymal obstruction or complete azoospermia. A large number of sperm can be collected from the epididymis which are not contaminated with other non-germ cells such as red blood cells [38]. Sperm wash technique can be used if the number of cells obtained is low and if sufficient numbers are collected; density gradient centrifugation can be used to prepare the spermatozoa for ART. Spermatozoa obtained from the testes by open biopsy or by percutaneous needle biopsy contain large numbers of non-germ cells such as red blood cells. Spermatozoa have to be separated from non-germ cells. Sperm motility in spermatozoa is generally low. Hypoosmotic solution or pentoxifylline is occasionally used to increase the motility of epididymal and testicular spermatozoa before ICSI [24].

52.8 Advanced Sperm Preparation Methods

Advanced sperm preparation techniques allow the spermatozoa to be selected on their surface charge and morphology and overcome the limitations of classical sperm selection procedures. New insights into the molecular biology of the spermatozoa have allowed the molecular selection strategies including hyaluronic acid-mediated sperm selection, annexin V magnetic-activated cell sorting (MACS), and the latest technology of selecting sperm by microfluidics.

52.8.1 Zeta Potential and Sperm Birefringence

The electrical potential between the sperm membrane that is negatively charged and its surrounding is called zeta potential. Negative charge is due to the presence of the epididymal proteins that are present on the sperm membrane surface [39]. Zeta potential is lower in sperm with DNA damage, and this property can be used to select sperm with intact DNA [40]. The washed sperm (~100 μL) are suspended in a 15 mL of serum-free HEPES-HTF medium. The tube is rapidly pulled after rotating it a couple of times in a latex glove. This allows the negatively charged sperm to stick to the walls of a positively charged plastic tube (Fig. 52.4). Immature-abnormal sperm in the suspension are discarded. The tube is maintained at room temperature without agitation for about 1 min and centrifuged at x300g for 5 min. The sperm retaining the negative zeta potential are attached to the walls of the tube. These can be recovered in a 0.2 mL of serum-supplemented HEPES-HTF medium, thereby neutralizing the charge on the wall of the test tube [40]. Markers of apoptosis were significantly reduced in zeta selected sample [41]. Zeta selection results in a significant reduction in progressive motility and is not very helpful when used in cryopreserved sperm [41]. High-quality spermatozoa can be separated from the poor-quality sperm using a positively charged centrifuge tube. In ART, sperm sample containing superior motility, normal morphology, and intact DNA can be separated by this technique [42]. Protamine-deficient sperm are eliminated, and sperm with DNA integrity are retained resulting in high fertilization rate. Negative zeta potential sperm in IVF had a higher fertilization rate (65.79%) compared with sperm isolated with double density gradient centrifugation. Embryo cleavage and pregnancy rates were high in sperm used in

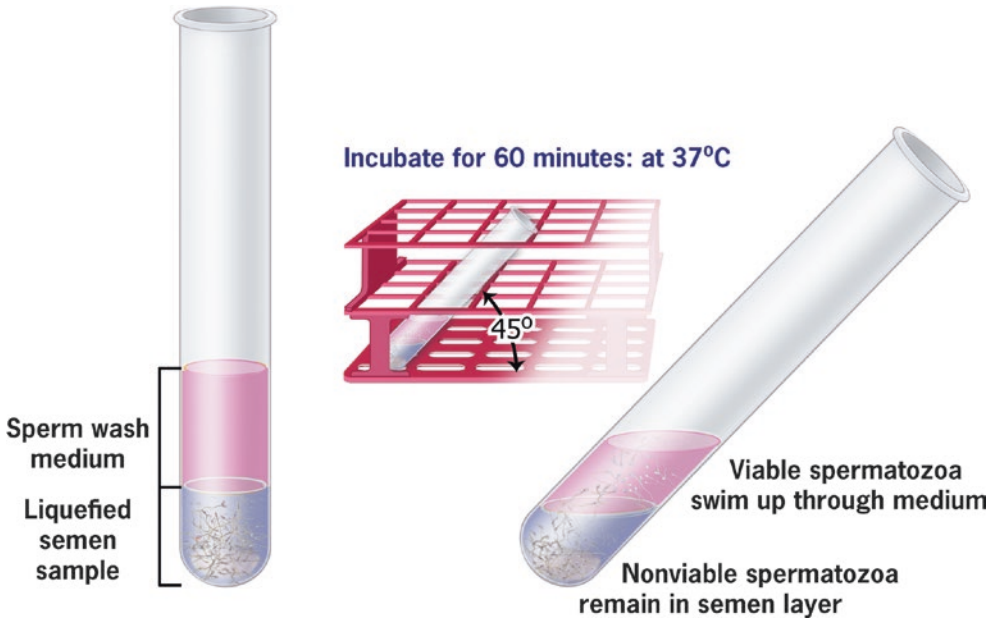

Fig. 52.4 Selection of spermatozoa using zeta potential principle. The negatively charged mature sperm sticks to the walls of the positively charged centrifuge tube, while non-mature sperm in the suspension are discarded (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2011–2019. All Rights Reserved)

ICSI selected by MACS compared with DGC in oligo-, astheno-, and teratozoospermic men [43, 44].

The subacrosomal protein filaments are longitudinally arranged in mature sperm. Therefore mature sperm nucleus exhibits higher birefringence that can be examined by polarized light microscopy. This allows the evaluation of birefringence and selection of mature sperm [45]. Acrosome intact sperm with high DNA integrity can be selected from the acrosome-reacted spermatozoa with DNA fragmentation using this technique [45, 46].

52.8.2 Ultrastructural Sperm Selection

Subtle defects in the sperm morphology in the acrosome, nucleus, mitochondria, post-acrosomal lamina, and neck can be observed using real-time inverted light microscope equipped with Nomarski optics enhanced by digital imaging. It achieves an ultra-high magnification microscopy (6300x) called motile sperm organelle morphological examination (MSOME) [47, 48]. The ultrastructural morphology of the sperm head components correlates with sperm fertilizing capacity in vitro [49]. MSOME was also shown to be positively associated with both fertilization rate and pregnancy outcome [47]. Sperm selected by strictly defined morphologically normal nuclei, especially when sperm with vacuoles were avoided, significantly improved the incidence of pregnancy in couples with previous ICSI failures [48]. A correlation was also reported between higher fraction of sperm with high DNA fragmentation and presence of large nuclear vacuoles. These results support the use of MSOME for routine selection of sperm for ICSI [50]. The procedure however is time-consuming, and the selected sperm may still be exposed to oxidative stress. In a retrospective study by Bradley et al. [51] examining the efficacy of interventions such physiological intracytoplasmic sperm injection (PICSI), MSOME or intracytoplasmic morphologically selected sperm injection (IMSI), testicular sperm, or other intervention such as frequent ejaculation alone or in combination with PICSI or IMSI, or testicular sperm in combination with IMSI with high sperm DNA fragmentation (SDF) on pregnancy rate, birth rate, miscarriage rate. High SDF patients who underwent IMSI intervention had the poorest outcomes of all intervention groups and were very similar to those of the no intervention group. Furthermore a recent meta-analysis included 9 randomized controlled trials and 2014 couples (IMSI = 1002; ICSI = 1012) compared regular ICSI for assisted reproduction. The results from this study show lack of evidence that IMSI improves clinical pregnancy rates compared to ICSI [52].

52.8.3 Hyaluronic Acid-Mediated Sperm Selection

This is a novel selection technique comparable to the earlier sperm-zona pellucida binding. Hyaluronic acid receptors are present on the plasma membrane on acrosome-intact sperm and are indicative of sperm maturity [53]. It is also the main component of the extracellular matrix of the cumulus oophorus. Mature sperm bind to the hyaluronic acid and therefore have a better chance of reaching the oocytes for fertilization. Sperm can be selected by physiological intracytoplasmic sperm injection (PICSI) which is a plastic dish containing spots of HA attached to its base. Sperm are attached to HA by the head, and the sperm can be easily

selected for microinjection. The frequency of sperm with chromosomal disomy is significantly reduced when compared with ejaculated sperm. Hyaluronic acid binding also excludes immature sperm with cytoplasmic extrusion, presence of sperm with histones, and DNA fragmentation indicating selection of sperm with reduced oxidative stress [53]. Pregnancy has been reported after the use of PICSI [54]. A viscous medium containing HA called SpermSlow slows the active sperm and allows the selection of the appropriate sperm.

52.8.4 Electrophoretic Sperm Selection

Sperm can be separated based on their size and charge on their surface by electrophoretic separation [55]. It consists of two outer chambers separated by two inner chambers by polyacrylamide restriction membranes of 15 kDa pore size. Semen is placed in the electrophoretic device, and a current is applied. The pore size allows the competent spermatozoa to move in the applied electric field [56]. Normally differentiated sperm are rapidly separated and collected at the adjacent chamber. Normally differentiated sperm are charged negatively. The resulting population shows a low incidence of DNA damage. It compares favorably with the density gradient separation technique in purity, absence of ROS, and superior viability and morphology of the isolated spermatozoa. Motility has been reported to be affected by electrophoresis [55]. Ainsworth et al. effectively used the sperm selected with high DNA integrity, to establish pregnancy from the semen sample with high DNA fragmentation by ICSI [56].

52.8.4.1 Microflow Cell

A microflow cell consists of an outer chambers connected with a platinum-coated titanium electrodes, and the inner chamber is divided into two compartments – the inoculation (loading) chamber and the collection chamber. A polycarbonate membrane 5 μm thick separates the two compartments (Fig. 52.5). The membrane filters out the good-quality sperm from the contaminating cells such as the leukocytes and germ cells. A 400 μL semen sample is loaded in the inoculation chamber and the buffer in both loading and collecting chambers, and sample is equilibrated for 5 min at 23° C. A constant current of 75 mAmps is applied with a variable voltage of 18–21 mV [55]. The highly motile good quality spermatozoa are sorted out and are ready to be used in ART.

Microelectrophoresis technique can be used to select the negatively charged spermatozoa from the seminal ejaculate as well as after density gradient selection [57]. The microelectrophoresis chamber consists of the egg inoculation and the bubble restriction chambers. Microelectrophoresis is carried out under ICSI stage. A 10–15 μL semen sample is electrophoresed in the buffer, and increasing current (6–14 mA) and variable voltage (3–100 V) are applied. The sperm are monitored under the inverted microscope under 200x, and good-quality sperm are selected for ICSI [57]. This technique allows the selection of viable, morphologically normally, motile sperm with high DNA integrity. Selected sperm are negatively charged and free from oxidative stress and exhibit normal zona pellucida binding [58, 59]. Selected sperm used in ICSI have resulted in pregnancy [56].

52.8.5 Annexin V and MACS Separation

Phosphatidylserine is a phospholipid that is present on the inner leaflet of the plasma membrane. It moves to the outer surface when the membrane is damaged. Thus externalization of the phosphatidylserine residue is a marker of apoptosis [60]. Reactive oxygen species (ROS) not only affects nuclear and mitochondrial DNA but also is involved in the activation of apoptosis signaling cascade parts [61, 62]. Annexin V is a phospholipid-binding protein. It has a strong affinity for phosphatidylserine residue. It cannot penetrate the sperm membrane, and its binding to the sperm membrane signifies that the sperm integrity is compromised and the sperm phosphatidylserine has been externalized. Therefore annexin V is used to label sperm that have a compromised membrane integrity and are less able to fertilize the egg [63].

Magnetically activated cell sorting (MACS) uses a colloidal super-paramagnetic microbeads conjugated with annexin V antibodies. A strong magnetic field is employed, and the sperm that are non-apoptotic pass through the magnetic field, whereas those that are apoptotic are tagged and retained in the magnetic field [64–66]. This allows the rapid separation and selection of non-apoptotic sperm from apoptotic sperm [64] (Fig. 52.6a–c). Selection of non-apoptotic spermatozoa for use in ART is based on the ability of phosphatidylserine residues on the external surface of the spermatozoa in early stages of apoptosis.

Higher-quality sperm have been obtained using sperm selected by density gradient and MACS than by density gradient alone [67]. While density gradient removes immature sperm cells, debris, and leukocytes, the annexin V MACS removes already damaged sperm with altered membranes, activated apoptosis signaling, and DNA fragmentation [68]. All these indirectly result in a significant reduction in oxidative stress. Similarly sperm selected by MACS before cryopreservation had a larger number of sperm with intact mitochondrial membranes, a reflection of mitochondrial survival after cryopreservation, than sperm prepared by cryopreservation alone (36.1% ± 18.9%) [67]. Hence sperm selection by MACS before cryopreservation can be used to improve motility and cryosurvival rate. The clinical significance of using annexin V MACS technique is that it allows the selection of sperm with improved motility, viability, and

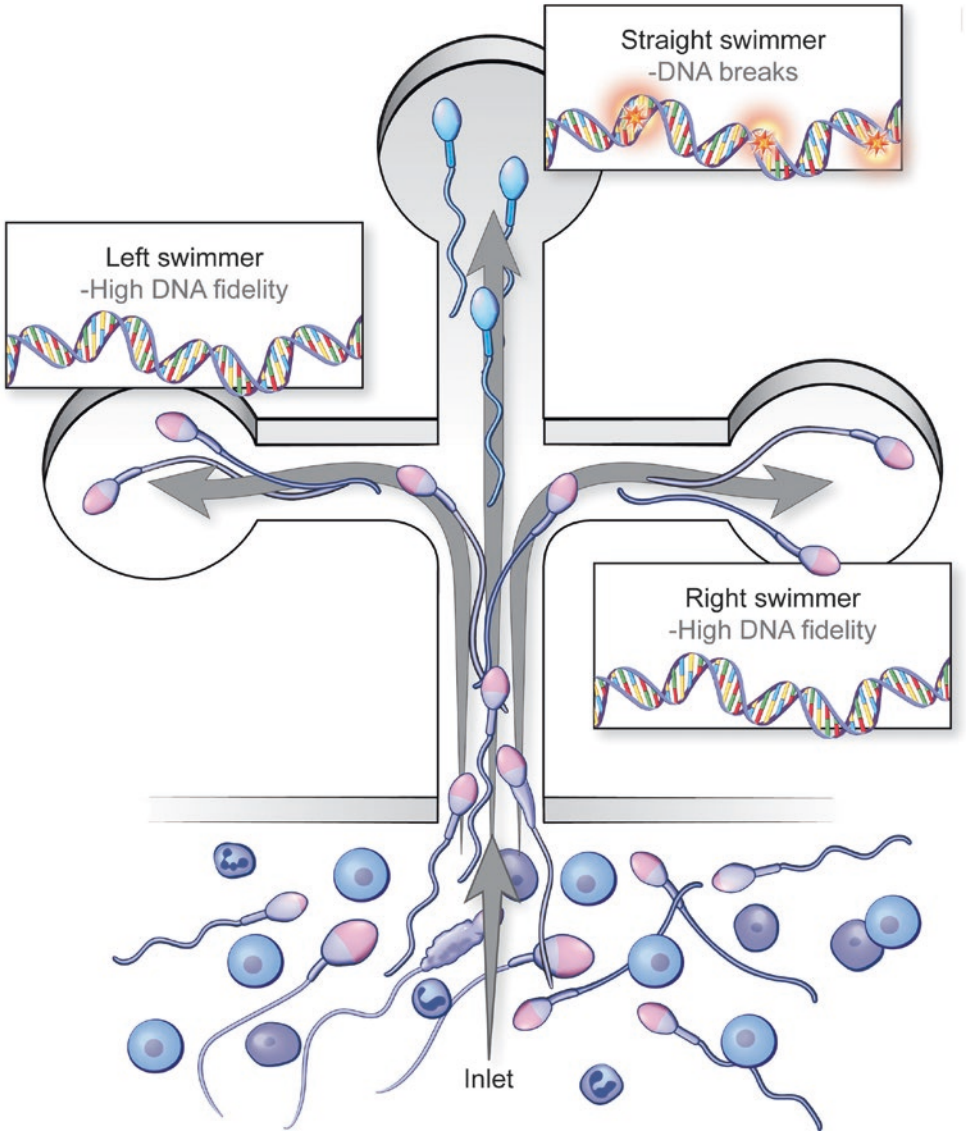

Fig. 52.5 Microflow cell separation of spermatozoa from leukocytes using polycarbonate separation membranes and sorting based on the movement in the applied electric field (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2011–2019. All Rights Reserved)

morphology and significantly improved fertilization rates and oocyte penetration [68, 69]. Improved pregnancy rates have been reported compared to sperm prepared by density gradient alone [43].

52.8.6 Microfluidic Separation of Sperm

This is the latest in sperm selection technologies. Microfluidic devices use microchannels made from polydimethylsiloxane (PDMS) silicon polymers that are nontoxic and transparent [70]. Lab-on-chip approaches have been used to select sperm based on motility [71–73], chemotaxis [73–75], optical forces [76, 77], and electrophoresis [78]. Sperm can be selected using (1) passively driven microfluidic device [71, 79], (2) chemoattractant microfluidic device [80], (3) chemotaxis device [74], (4) microfluidic fertilization device [81], (5) macro-microfluidic sperm sorter [82], (6) Zech selector [83], (7) circular microfluidic device [20], (8) microgroove and channel device [84], and (9) boundary-following behavior-based passive microfluidic device [85].

The popular passive microfluidic device selects spermatozoa based on the boundary-following behavior (Fig. 52.7). This device consists of radial network of channels (52 μ width) which separates sperm into left, right, and straight swimmers. Using a plastic syringe, an aliquot of raw semen (200 μL) is loaded into the inner ring and kept undisturbed for 15 min at 37° C. Motile sperm move and flow through the microchannel in the medium that mimics the viscosity of the reproductive tract fluid. Dead or immotile sperm are retained in the inlet, and motile sperm are collected from the microchannel outlet [85].

Sperm are selected according to the normal motility, morphology, and high DNA integrity [20, 71, 82, 83, 86, 87].

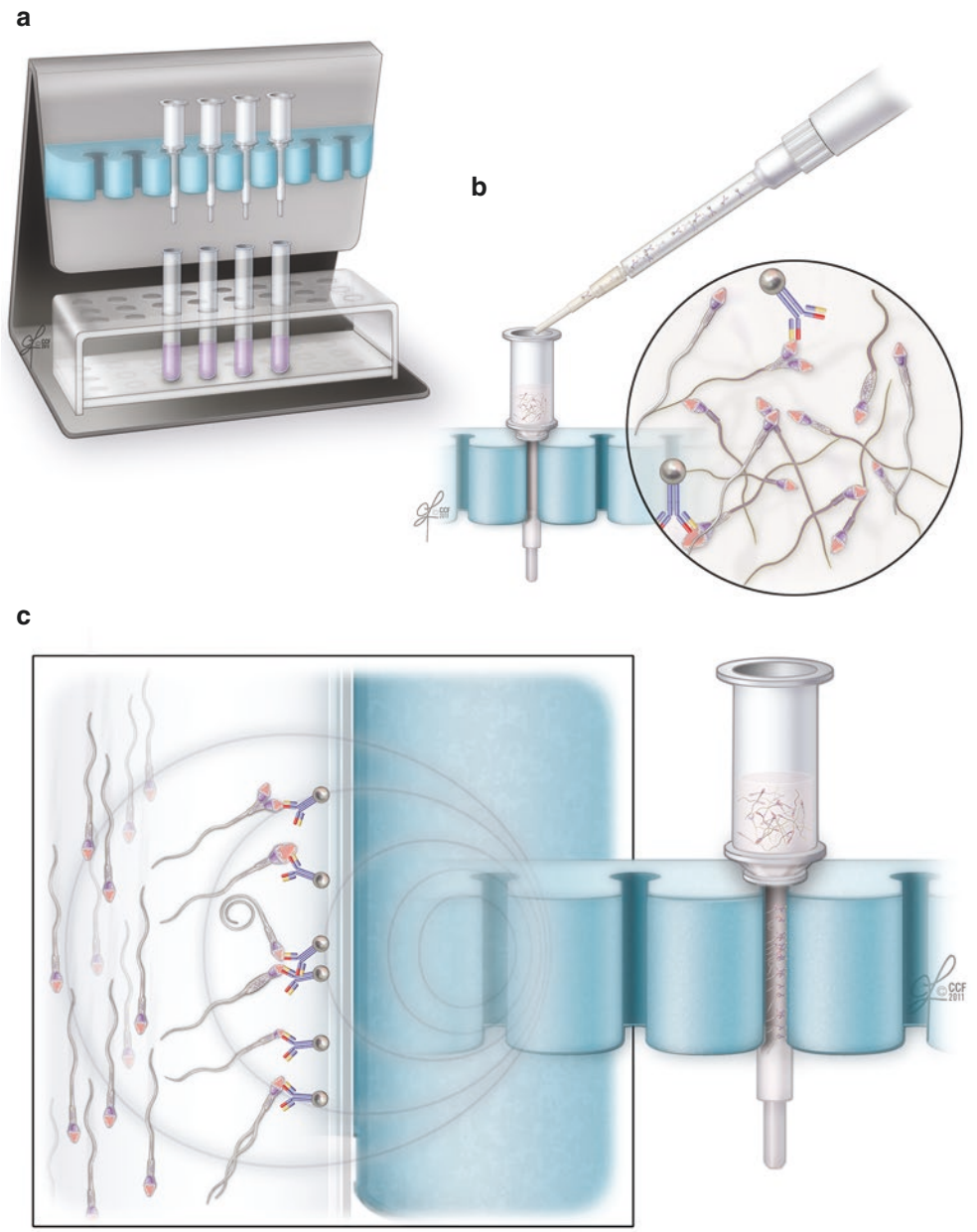

Fig. 52.6 Sperm selection by (**a**) magnetic-activated cell sorting and collection device. The MACS columns are placed on the stand surrounded by magnetic field. (**b**) Loading the MACS columns with liquefied semen (apoptotic and non-apoptotic sperm) labeled with annexin V-coated micromagnetic beads. (**c**) Activated magnetic field retains the apoptotic sperm bound to micromagnetic beads coated with annexin V in the column and allows the non-apoptotic healthy sperm cells to flow through the selection column (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2011–2019. All Rights Reserved)

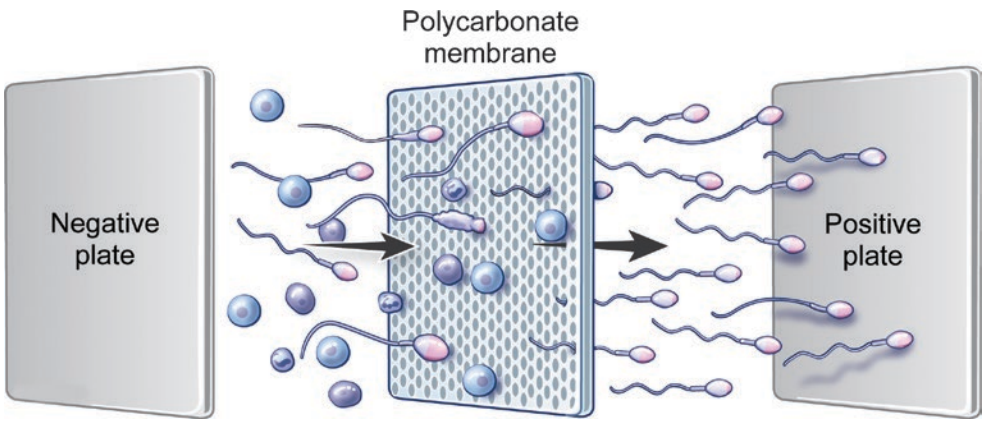

Fig. 52.7 Microfluidic device used for sorting sperm based on their swimming patterns: left-hand side (left swimmers), right-hand side (right swimmers), or straight (straight swimmers). Live sperm navigate from the inlet toward the outlet, while dead sperm and debris remain in the inlet (Reprinted with permission, Cleveland Clinic Center for Medical Art & Photography © 2011–2019. All Rights Reserved)

Selection of sperm by microfluidic technology has great potential in IVF and ICSI setting. A simple clinically applicable lab-on-a-chip method was reported for sperm selection based on progressive motility in 500 parallel microchannels [20]. In this one-step procedure, 1 mL of semen could be processed under 20 min resulting in over 80% improvement in selected sperm DNA integrity.

The major advantage of microfluidic devices over conventional selection techniques is the ability to work with small sperm sample volume, the short processing times, and the ability to manipulate single cells in a noninvasive manner [88–90]. The yield of the selected sperm by microfluidics is about 41% and comparable to recovery rates of currently used methods [70]. Another advantage is the one-step process that eliminates centrifugation and the exposure to reactive oxygen species and thereby preserves the DNA integrity [20, 82]. Fertilization of ova with preselected spermatozoa of superior quality by microfluidic technique was accomplished using a robotic-assisted platform and IVF on a chip [91].

DNA fragmentation is significantly decreased in sperm separated with the microfluidic sperm sorting system [88, 92] compared the swim-up method with a microfluidic device, resulting in a significantly lower rate of DNA damage (16.4% swim-up vs. 8.4% microfluidic). Using radial array of microchannels to select the most motile sperm, an 80% improvement in sperm DNA integrity after sorting was reported [20].

The use of a microfluidic device shortened the time in the ICSI treatment of porcine sperm and increased the number of viable embryos without reducing the in vitro production efficiency. An application in human ART is suggested [88, 93]. The technique requires only a low concentration of sperm in a murine IVF model [94]. A robotic-assisted reproduction platform was developed to carry out IVF on a chip by fertilizing the preloaded ova with superior-quality spermatozoa selected by microfluidic technique [91]. Thus, the microfluidic sperm sorting proved to have a great potential in clinical IVF and ICSI for achieving early embryo development.

52.9 Specific Indications of Sperm Selection Techniques: Clinical Implications

A variety of techniques can be used to select sperm for use in ART both intrauterine insemination and IVF and ICSI. Conventional methods such as sperm preparation by swim-up and density gradient are very popular. In addition, the introduction of newer techniques such as preparation by MACS alone or in combination with density gradient separation is also being used. The goal is to use a method that selects a highly motile sperm population with intact DNA from the raw semen. In this context, sperm selection utilizing the microfluidic techniques is important and is gaining popularity. It allows the rapid selection of sperm with intact DNA in a one-step process which replaces the previous multi-stage processes involving centrifugation steps associated with oxidative stress and iatrogenic risks.

52.10 Future Directions

Sperm separation from seminal fluid removes also the natural protective antioxidants contained in the seminal fluid. To prevent excessive oxidative stress to the sperm, antioxidants like human serum albumin must be added to sperm preparation and incubation media for assisted reproduction. Standard sperm selection techniques like density gradient centrifugation are able to reduce oxidative stress by depletion of immature sperm and leukocytes. Prolonging the selection methods, sperm selection with enhanced motility may be achieved but at the expense of DNA damage due to oxidative stress. Advanced sperm separation techniques focus rather on the depletion of already damaged sperm. In ART, procedures such as IVF and especially ICSI require fewer spermatozoa, so lower sperm recovery is not an adverse factor, and therefore microfluidic techniques are more versatile. Microfluidics offers new opportunities to better understand human sperm migration and to use this understanding to prepare sperm for intrauterine insemination, IVF, and ICSI. New sperm sorting technologies have been shown to improve DNA integrity, morphology, and motility, but whether these improvements are significant over the conventional centrifuged-based techniques is unclear [46], and these devices need thorough evaluation.

52.11 Conclusion

In summary, we have described a number of sperm preparation methods that are available to process sperm for use in ART. Each infertile couple must be carefully examined to determine the best sperm preparation method. Future research should seek to improve the efficacy and the safety of the sperm preparation techniques. Advanced sperm selection strategies include selection according to surface charge, sperm birefringence. sperm morphology under ultra-high magnification, ability to bind to hyaluronic acid, sperm apoptosis, and microfluidic separation. These techniques may improve the chances of selecting structurally intact and mature sperm with high DNA integrity for and help improve fertilization and pregnancy rates.

52.12 Review Criteria

An extensive search of studies examining the relationship between sperm selection techniques and improvement in sperm quality and ART outcome was performed using search

engines such as Google Scholar and PubMed. The start and end dates for these searches were September 1992 and September 2018, respectively. The overall strategy for study identification and data extraction was based on the following keywords: "sperm selection techniques," "centrifugation," "reactive oxygen species," "oxidative stress," "DNA fragmentation," "density gradient techniques," "electrophoretic cell separation," "hyaluronic acid binding," "magnetically activated cell sorting," "microfluidics," and "ART and pregnancy rate." Articles published in languages other than English were excluded. Data published in conference or meeting proceedings, websites, or books was also excluded.

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Microfluidic Sperm Selection

53

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Key Points

- Sperm cells show tendency to swim and accumulate close to surfaces instead of spreading in a tridimensional space. This behavior can be exploited using microchannels that increase the processing surface.
- The pattern of motility and migration of spermatozoa in viscous media differs substantially from the pattern exhibited in nonviscous media. This feature can be exploited by the use of culture media with different viscosities or even generating viscosity gradients.
- Sperm exhibit positive rheotaxis, which is the tendency in swimming against a liquid flow. This feature can be explored with the use of constant flow streams or even generating flow gradients.
- The classic configuration of a microfluidic chip consists of an inlet reservoir, a connecting channel, and an outlet reservoir. The liquid volume in each reservoir will define whether there will be a flow or not, and if so, what is the direction of flow. Scaling, orientation, and combination of these basic components can lead to a variety of systems with different selective characteristics.
- The selective approach of microfluidic devices resembles natural sperm selection processes, indirectly attesting the functionality of the male gamete.

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53.1 Introduction

Among the millions of sperm present in the ejaculate, only hundreds or even dozens can reach the ampulla or the site of fertilization [1]. The task of accomplishing its purpose is hindered to the ones without the proper attributes by selection mechanisms of the female reproductive tract.

Several studies report the existence of different populations of spermatozoa within the same ejaculate, which have different characteristics in terms of morphology, motility, chromatin integrity, mitochondrial status, and others [2–6]. This phenotypic heterogeneity might be caused by genetic variability, epigenetics, disturbed spermatogenesis, altered epididymal sperm transit time, etc. [7]. In fact, this diversification of sperm function and structure may lead to distinct performances depending on the obstacles to be overcome.

In the female reproductive tract, these challenges translate into migration through viscous media (cervical mucus), migration through confined spaces (uterine villi), and countercurrent migration (against the flow of tubal fluid) [8–10]. Among the different populations that compose the ejaculate, only the ones with specific phenotypical features will be able to overcome the various hurdles and will have the "opportunity" to fertilize the oocyte.

Of the characteristics found in spermatozoa collected in fallopian tubes after mating in various studied species, three stand out: progressive motility, normal morphology, and chromatin structure [11]. Many selection mechanisms acting together in the female reproductive tract are responsible for this selection, eliminating the spermatozoa that do not exhibit the necessary attributes to the oocyte fertilization and further embryonic development [1].

The viscosity of the cervical mucus imposes itself as an obstacle to the morphologically abnormal spermatozoa (immature sperm), presenting residual cytoplasm and poor DNA packaging. In result, such featured gametes will be eliminated right on the beginning of the path [12]. Additionally, the migratory effort throughout the female reproductive tract appears as an obstacle to those spermato-

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zoa that have some degree of mitochondrial damage due to apoptotic processes linked to senescence or due to reactive oxygen species (ROS) attack. Such gametes will not have enough resistance to complete the path, even if exhibiting normal morphology [13–18]. Therefore, in a simple way, we can infer that normal morphology attests the spermatogenesis efficiency, whereas vigorous motility attests the functional excellence of spermatozoa. Thereby, the natural sperm selection processes can be compared to the "Ironman" Championship, where different challenges are imposed (swimming, cycling, running). The ones who are able to complete the course will be apt to fertilize the oocyte. The success now will depend on whether they are in the correct uterine tube in the right moment or not. The spermatozoa remained along the way will never have this chance. It is not an infallible process, but it is an effective method to select the best characteristics from such heterogeneous semen samples.

53.2 Sperm Characteristics that Can Be Used as Selection Factors

The spermatozoon is a peculiar cell: it is the only flagellated cell in humans and carries its highly compacted genetic material confined into a volume that is typically 10% of a somatic cell nucleus [19]. This uncommon cell shows particular characteristics that can be used as key points in the selection process.

53.2.1 Migration in Space-Constricted Environment

Sperm cells show tendency to swim and accumulate close to surfaces [20]. Instead of spreading in a tridimensional space, these cells follow a channel border or the surface of a glass slide [21]. It is estimated that swimming following the surface or border is approximately 50% faster than swimming in the channel center [22]. This confers benefit in confined regions of the reproductive tract or in microchannels.

53.2.2 Migration Through Viscous Fluids

The pattern of motility and migration of spermatozoa in viscous media differs substantially from the pattern exhibited in nonviscous media, such as those used in assisted reproduction technologies (ART) [10]. In low viscous media, the swimming pattern is characterized by the rotation of the flagellum in its longitudinal axis and consequent head rolling (rolling mode) [23]. This tail movement produces a cone-shaped helical pattern with a cross section many times larger than the diameter of the sperm head [9]. That is, morphology of the head does not influence the sperm hydrodynamic behavior in low viscous media. However, in viscous media, the sperm angular speed (rotation along its longitudinal axis) decreases, resulting in a planar movement of the tail [24]. This makes the morphology of the head and midpiece much more relevant in hydrodynamic terms.

53.2.3 Positive Rheotaxis

Rheotaxis is the tendency of a cell to orient its movement against or in favor of the fluid flow that surrounds it. Positive rheotaxis is the tendency to swim against this flow of fluid [9]. Several studies describe this behavior as being the main factor that guides spermatozoa to the oocyte [25–28]. The main argument is based upon the evidence that there is an increase in fluid flow from the uterine tubes after coitus. This flow would help transporting the oocyte to the uterus and guide the spermatozoa toward the oocyte [29]. Countercurrent swimming demands vigorous and constant motility.

53.2.4 Thermotaxis

Thermotaxis is the tendency of modifying the direction of movement following a temperature gradient [30]. Like the rheotaxis, it is also considered a sperm orientating factor that manifests in long distances. Thermotaxis manifests following the temperature gradient established at the uterus-tubal junction during ovulation [31, 32]. It is believed that only spermatozoa that suffered sperm capacitation are responsive to temperature changes [33].

53.2.5 Chemotaxis

Phenomenon of chemotaxis has been observed for many years in animals presenting external fertilization. It is believed that chemoattractants secreted by oocytes are able to alter the pattern of spermatozoon tail beating orientating it toward the oocyte [34]. However, in mammals, their role in sperm orientation is still controversial. Even though the presence of chemoattractant substances in the follicular fluid was confirmed, uterine contractions and ciliary currents would be able to disrupt the gradients, which would make it not viable for long-distance orientation [35]. It is believed that chemotaxis may be relevant as a short-distance mechanism only.

53.3 Current Sperm-Sorting Technologies

The intracytoplasmic sperm injection (ICSI) revolutionized the treatment of male-factor infertility. Nevertheless, the use of this technique raises concerns about the possibility of inadvertent selection of a spermatozoon containing DNA damage [36–38]. ICSI eliminates all natural selection barriers, since the spermatozoon is directly injected into the oocyte cytoplasm [39]. In practice, it comes down to a subjective "choosing" process. Once polyvinylpyrrolidone (PVP) prevents the analysis of motility, morphological analysis is the only remaining resource to the embryologist. While visual inspection permits a trained embryologist to identify immature spermatozoa, the damage in sperm DNA itself does not result in any morphologic changes, making its identification nearly impossible [36]. The success of sperm selection will depend not only on the technique used but also on the quality of the ejaculates.

As seen before, ejaculate is a heterogeneous mixture of sperm from which we must pick some individuals. Therefore, previous seminal processing is crucial to ICSI, not only for the removal of seminal plasma but also for the removal of spermatozoa that do not show those fundamental attributes: superior morphology, vigorous motility, and intact chromatin [40]. The more efficient the selection, the lower the risk of inadvertent injection of a nonfunctional gamete [37].

Sperm wash is the simplest seminal processing technique that has been utilized in the field of assisted reproductive technologies (ART). It may not be considered a sperm selection technique, since there is no removing of inadequate spermatozoa, but seminal plasma removing only. This technique is used lone for the processing of poor seminal samples [41].

Discontinuous density gradient (DG) highlights among the most commonly used sperm selection techniques. It is based on the sedimentation capacity of the sperm through layers of culture medium with different densities, after centrifugation [40]. It is efficient for the removal of immature spermatozoa, which are less dense, but does not show the same performance in the removal of spermatozoa with DNA fragmentation. The migratory effort is minimal.

Swim-up is another technique widely used in clinical practice. In this method, motile sperm migrate from a sample deposited on the bottom of a conical tube into an overlaying medium [42]. This approach explores sperm motility, but over short distances [43]. The migration occurs mainly through the liquid column, since the small surface area does not allow sperm accumulation by the near-wall swimming effect [21]. Swim-up is efficient in the removal of spermatozoa with DNA damage; however, it does not present the same performance in the removal of abnormally chromatin-condensed spermatozoa [40]. This technique recovers relatively low numbers due to two main reasons: the small surface area for migration and the entrapment of motile spermatozoa in the lower layers of the sample. For this reason, this technique is inadequate for the processing of poor samples and is still the standard method for patients with normozoospermia and female infertility [40].

53.4 Microfluidics

A new approach to innovative techniques for the selection of functional spermatozoa is the use of microfluidics, which is the manipulation of tiny volumes of liquid. Microfluidic devices have microchannels that explore the tendency of sperm to migrate along surfaces [20, 44]. In this way, the possibility of subjecting the male gametes to several selection factors in a controlled environment is created. Recent papers describing microfluidic sperm selection devices, most of the times, use distinct classification focusing on the bioengineering characteristics of these devices [29, 41, 45]. We will be using an approach which focuses on the selection factors of each of these devices, that is, the characteristics that distinguish the sperm population meant to be isolated. Some devices use distinct mechanisms to isolate the same sperm population.

The classic configuration of a microfluidic chip consists of an inlet reservoir, a connecting channel, and an outlet reservoir. If the system works equally in volume for both reservoirs, there will be no liquid flow in the channel. If the inlet reservoir volume is higher than in the outlet reservoir, there will be a directional flow. Once the volume goes higher in the outlet reservoir than in the inlet reservoir, there will be an inverted flow (Fig. 53.1a–c). The demanded migratory effort is minimum in directional flow systems, as the flow "pushes" spermatozoa toward the outlet reservoir. In no flow systems, however, there is an important migratory effort, as spermatozoa must swim during the whole course toward the outlet reservoir. Finally, in inverted flow systems, the migratory effort is even greater, because the migration will occur against liquid flow. These basic characteristics can be multiplied or combined in the search for an efficient device.

53.4.1 Chemotaxis and Thermotaxis

Sperm capacitation is a series of structural and functional modifications that give spermatozoa the ability to fertilize the oocyte. Human capacitated spermatozoa appear to behave similarly when exposed to a temperature gradient or to a chemoattractant gradient, directing the swim toward the origin of the stimulus, without changes of direction.

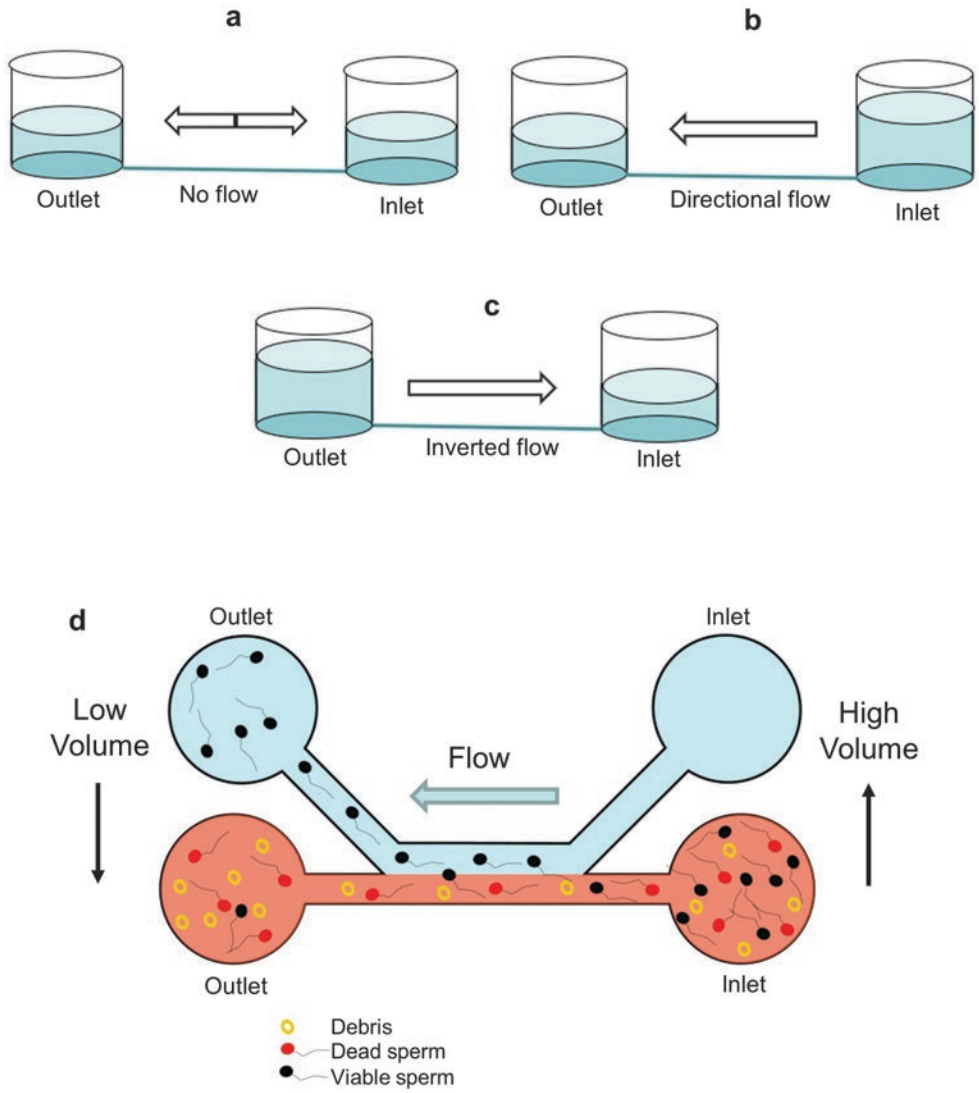

Fig. 53.1 Basic fluid dynamics in a microfluidic sperm selection chip. (**a**) No flow system. The volume of liquid is the same in both reservoirs. There is no flow in the channel. (**b**) Directional flow system. The volume of liquid in the inlet reservoir is higher than in the outlet reservoir. There is liquid flow in the channel from the inlet reservoir to the outlet reservoir. (**c**) Inverted flow system. The volume of liquid in the outlet reservoir is higher than in the inlet reservoir. There is liquid flow in the channel from the outlet reservoir to the inlet reservoir. (**d**) Microfluidic sperm selection device developed by Cho et al. [51]. It is a directional flow system containing two inlet reservoirs, two outlet reservoirs, and a common main channel. Motile sperm capable of changing from one stream to another are carried to the respective outlet reservoir. (**d**: Adapted with permission from Cho et al. [51]. Copyright (2003) American Chemical Society)

Microchannels can help guiding the gametes, increasing selection efficiency. Thus, only capacitated spermatozoa possessing the specific receptors would be able to migrate toward the origin of the stimulus, where they will accumulate. Microfluidic devices that explore chemotaxis [46–48] and thermotaxis [49, 50] are still experimental concerning to human sperm selection.

53.4.2 Short-Distance Migration

This strategy resembles that used in swim-up. The focus is on obtaining motile spermatozoa. It aims to select spermatozoa capable of swimming short distances, separating them from debris, immotile spermatozoa, and seminal plasma.

Cho et al. used a passively driven integrated microfluidic system to separate motile sperm from raw semen samples [51]. Due to a phenomenon known in the field of microfluidics as "laminar flow," the liquid from two parallel flows present in the main channel and coming from distinct reservoirs do not mix (Fig. 53.1d). This allows motile sperm to migrate from the stream containing the seminal sample to the stream of sperm-sorting medium [51, 52]. A strong flow pushes the spermatozoa to a specific reservoir, separating them from the debris and dead cells present in the original stream (Fig. 53.1d). The liquid streams are generated by a pressure difference between inlet reservoirs and outlet reservoirs. The volume of liquid in inlet reservoirs is higher than in outlet reservoirs causing the difference in pressure [53, 54]. The spermatozoa recovered show higher vitality, better morphology, and less DNA fragmentation when compared to the original sample [55].

Seo et al. developed a similar system; they used rheotaxis to the initial separation of spermatozoa, though [56]. Motile spermatozoa able to migrate outside from the inlet reservoir are directed to swim against a weak flow until reaching a

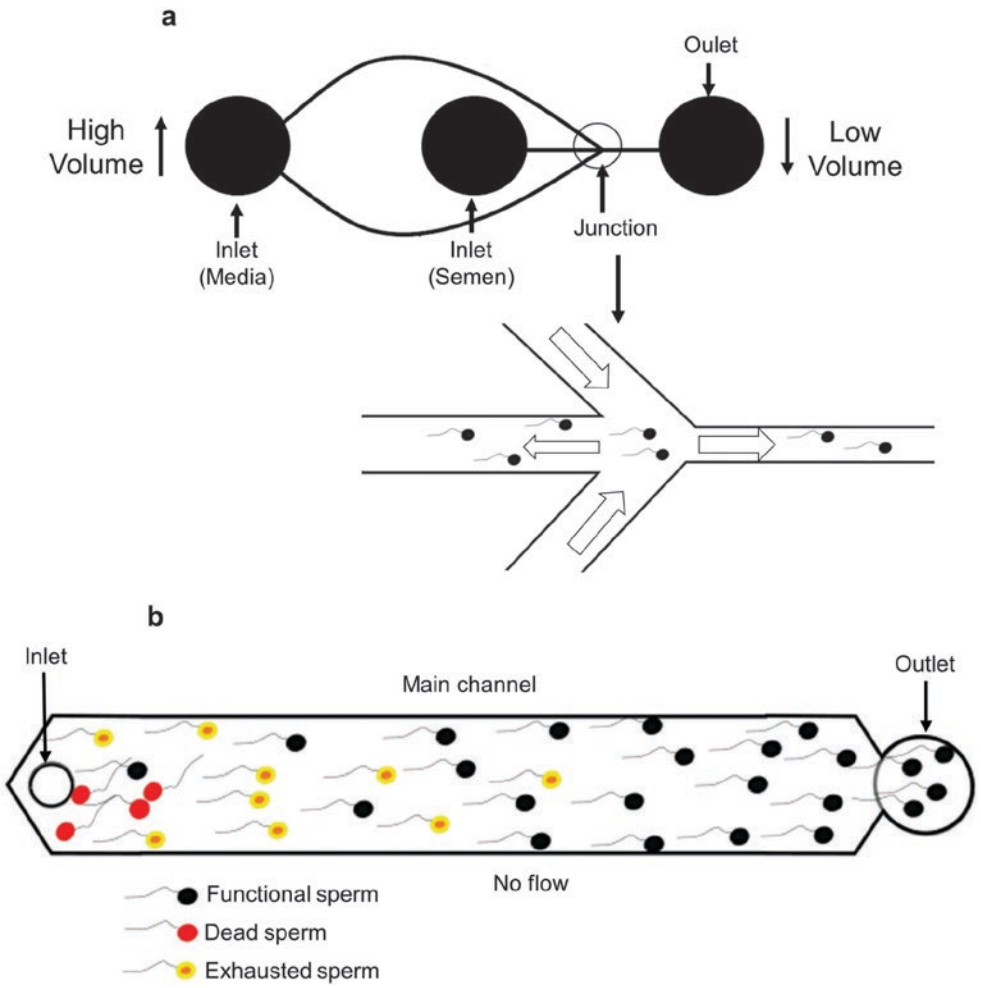

Fig. 53.2 (**a**) Microfluidic sperm selection device developed by Seo et al. [56]. It is a directional flow system containing two inlet reservoirs and one outlet reservoir. The main flow occurs between the media inlet reservoir and the outlet reservoir. There is a weak secondary flow toward the semen inlet reservoir that stimulates positive rheotaxis behavior. The spermatozoa that reach the junction are pushed toward the outlet reservoir. (**b**) Microfluidic sperm selection device developed by Tasoglu et al. [59]. This is a no flow system. Resilient sperm are able to migrate from the inlet reservoir to the outlet reservoir (functional sperm). Sperm unable to migrate for long distances due to structural or physiological impairments do not reach the outlet reservoir (exhausted sperm). (**a**: Adapted from Seo et al. [56]. With permission from Springer Nature)

junction. Spermatozoa are dragged by a strong flow to the outlet reservoir from this point (Fig. 53.2a).

The migratory effort is relatively small for both techniques, enough only to leave the semen sample. The transporting to the outlet reservoir is made by means of a strong flow of liquid.

53.4.3 Resilience

Although the role of mitochondria in sperm function is still a matter of debate, several studies report that sperm motility is closely related to mitochondrial membrane potential (MMP) [4, 18]. Besides, there MMP is correlated with DNA integrity as well [16, 17]. Therefore, we may suppose that spermatozoa able to high migratory efforts hold intact DNA [11]. Perhaps that is where the key for the success of natural processes in obtaining high-quality gametes relies on.

Spermatozoa migration in "extended drops" had already been used mainly for poor semen samples, but there was no accurate estimate of how far the gamete should go for its functionality to become attested [57, 58]. The use of microfluidic devices enabled this analysis. Comparing spermatozoa migration through channels of different lengths, Tasoglu et al. determined that a 2 cm migration is enough to obtain functional human spermatozoa [59]. According to the authors, sperm exhaustion is an important phenomenon in microfluidic sperm selection.

Based on the experimental data, a microfluidic device for clinical use was developed [60]. The device displays the classic configuration: an inlet reservoir, a connection channel, and an outlet reservoir. The system is filled with low viscosity culture medium. The spermatozoa must actively swim from the inlet reservoir through the main channel to the outlet reservoir, where they are collected. There is no flow and it is a considerable migratory effort. The spermatozoa that show low resistance to migration stand in the way and are eliminated (Fig. 53.2b). A recently published study shows that the spermatozoa recovered with this type of device exhibit greater vitality, better morphology, and less DNA damage compared to density gradient centrifugation and swim-up [61].

Nosrati et al. developed a device that expands the layout inlet/channel/outlet. It is a platform containing 500 channels

in a radial arrangement filled with a viscoelastic medium [62]. Yet again, there is no flow. The viscosity of the surrounding medium makes the migration even more difficult (Fig. 53.3). The device was tested for raw semen sample processing, showing up an 89% improvement in vitality and 80% improvement in sperm DNA integrity.

De Martin et al. developed the positive rheotaxis extended drop (PRED) which also exhibits the classical configuration. In this inverted flow system, there is a difference of hydrostatic pressure between the reservoirs, generating a flow of liquid from the outlet reservoir toward the inlet reservoir [63]. Besides that, polyvinylpyrrolidone (PVP) deposited in the distal end of outlet reservoir generates a viscosity gradient. Thereby, spermatozoa placed in the inlet reservoir must leave the semen sample (short-distance migration), swim against a fluid flow through the connecting channel (positive rheotaxis), and migrate against a viscosity gradient until reaching the outlet reservoir where they will be captured (migration through viscous media). The circuit is set manually in an ICSI dish (Fig. 53.4a, b). Spermatozoa that were able to reach the distal end of the outlet reservoir will be collected with an ICSI needle and injected in the oocyte. Therefore, the PRED dish tries to mimic natural obstacles faced by the spermatozoa such as migration in viscous media, migration in confined environments, and countercurrent migration. The device was able to reduce uncondensed chromatin spermatozoa to nearly 1%, processing raw semen samples [63].

Wu et al. described the flowing upstream sperm sorting (FUSS) that is basically a directional flow system that works

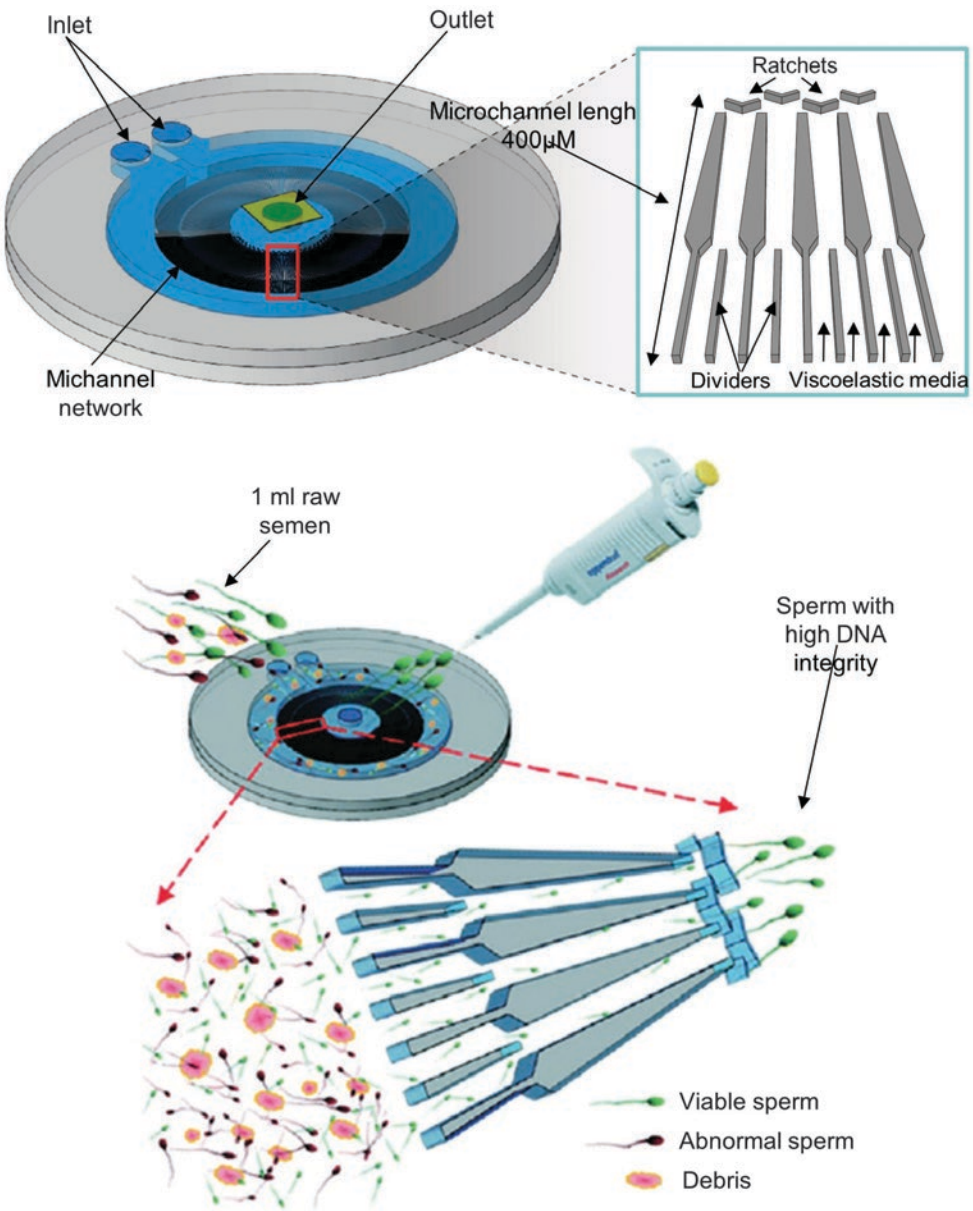

Fig. 53.3 Microfluidic sperm selection device developed by Nosrati et al. [62]. It consists of 500 parallel channels in a radial array filled with viscoelastic media. Motile sperm guided by dividers migrate through the channels toward the central outlet reservoir (viable sperm). Sperm unable to migrate for long distances due to structural or physiological impairments do not reach the outlet reservoir (abnormal sperm). (Reprinted from Nosrati et al. [29]. with permission from Springer Nature)

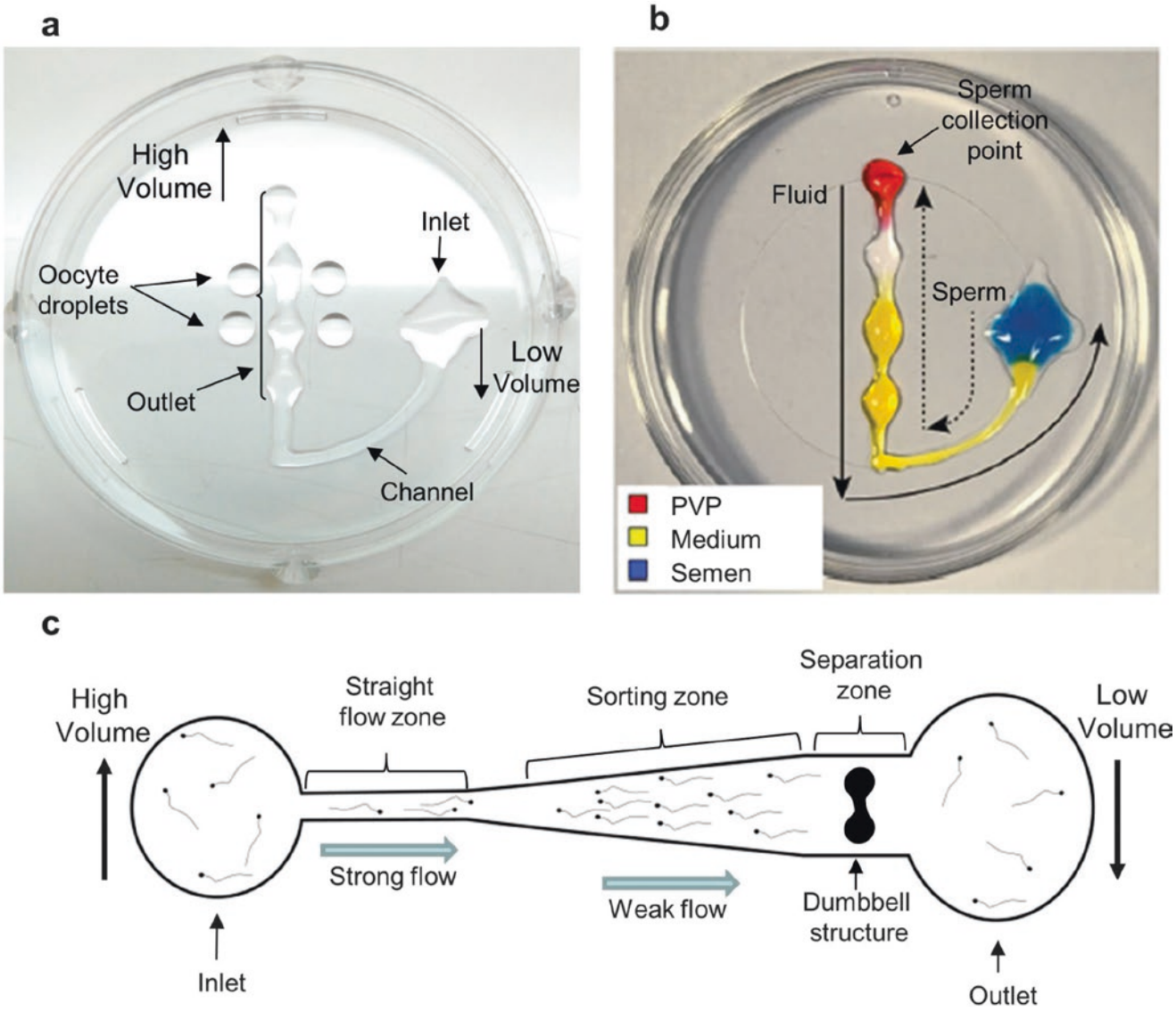

Fig. 53.4 Microfluidic sperm selection device developed by De Martin et al. [63]. (**a**) It is an inverted flow system containing one inlet reservoir, a connecting channel, and a long outlet reservoir. PVP is added at the distal end of the outlet reservoir. The system is set on an ICSI dish. (**b**) There is liquid flow through the channel from the outlet reservoir. This flow of culture medium prevents diffusion of the semen sample from the inlet reservoir. The spermatozoa deposited in the inlet reservoir should swim against the flow through the channel and against a viscosity gradient until reaching the distal end of the outlet reservoir where they will be captured. **(c)** Microfluidic sperm selection device developed by Wu et al. [64]. It is a directional flow system with a peculiar characteristic. The channel intermediate sector shows progressively higher widths which generates a velocity gradient allowing the positive rheotaxis behavior. Motile spermatozoa that remain swimming against the flow are "trapped" in this sector, while immotile sperm and debris are carried out to the outlet reservoir by the same flow. (**a**, **b**: Reprinted from De Martin et al. [63]. With permission from Springer Nature)

as an inverted flow system [64]. The system shows the classic configuration (inlet/channel/outlet) with the flow being directed from the inlet to the outlet. The spermatozoa initially deposited in the inlet are drawn out by a strong flow toward the outlet at the first segment of the circuit (straight-flow zone). The channel has an enlargement in its intermediate part (diffuser-type sperm sorter) that slows down the flow, allowing the spermatozoa to swim against the fluid stream (positive rheotaxis). As the channel increases its width, the flow velocity decreases, and this allows to differentiate sperm with different motilities. Debris, dead cells, and immotile sperm are carried by the flow to the outlet. Motile sperm are collected in the diffuser channel (Fig. 53.4c). The system was able to recover enriched samples containing 80% viable sperm, processing batches of ~ 200,000 spermatozoa, with an estimated time from 5 to 15 minutes.

53.5 Future Perspectives

The strategy of mimicking the natural selection processes is promising in obtaining functional spermatozoa. Some microfluidic devices are already available in the market, and more comprehensive clinical trials evaluating their effectiveness are on the way. One of the factors hindering the adoption of these devices in daily clinical practice is the relatively high cost. This is due to the use of materials and production processes suitable for laboratory tests but unsuitable for mass production. The adoption of industry-friendly materials and processes can help reduce manufacturing costs and consequently the prices.

Microfluidics allows unprecedented control of the environment that surrounds sperm. Thus, the viscosity, the flow velocity, the distance to be traveled, and the time spent in each step can be manipulated. With the evolution of microfluidic technology, the selection of functional spermatozoa may be more efficient than that exhibited in the female reproductive tract. It would be possible to obtain spermatozoa with intact DNA even from poor semen samples. In addition, the use of microfluidic chips in clinical practice will aid in the standardization of processes improving the treatment of male factor infertility.

53.6 Conclusion

The inadvertent injection of a spermatozoon containing DNA damage is a growing concern, and sperm selection techniques may potentially prevent this from happening. It would be ideal if we could access the chromatin status of each spermatozoon prior to injection, but this is not feasible without destroying it or compromising its functionality. Probably, the most effective approach would be to access male gamete functionality indirectly. Thus, the sperm would be tested not for what it is but for what it can do. The challenge is to find out which is the least challenge or effort able to select functional spermatozoa. Therefore, microfluidics may be the ideal tool in the aim of reaching this purpose due to its capacity of control and precision.

53.7 Review Criteria

A careful investigation of all the articles related to the use of microfluidics in the selection of functional spermatozoa evaluating articles published until October of 2018 was carried out. The search engines Google Scholar, PubMed, Science Direct, and MEDLINE were used. The search was limited to studies published in English. Searches were performed using keywords such as “microfluidics,” “microfluidic technologies,” “microfluidic chip,” “sperm sorting,” “sperm selection,” “motile sperm,” “semen,” “male,” “infertility,” “in vitro fertilization,” and “sperm DNA fragmentation.”

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Antioxidants in Sperm Cryopreservation

54

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Key Points

- Sperm damage is likely to occur during cryopreservation and thawing secondary to OS. The occurrence of OS during these processes results from increased ROS production and decreased antioxidant levels.
- Sperm damage occurring during cryopreservation and thawing is manifested by decrease in motility, viability, and DNA integrity. Such changes will ultimately affect the sperm fertilization potential.
- The use of antioxidants is proven to counteract ROS preventing their damaging effect on the sperm.
- The supplementation of cryoprotectant and post-thaw media with antioxidants prevents, to a certain extent, the damage inflicted on spermatozoa during cryopreservation and thawing.
- Further studies are still needed to identify the optimum antioxidant supplementation protocol during sperm cryopreservation and thawing.

54.1 Introduction

Cryopreservation of human spermatozoa and achieving successful fertilization via assisted reproductive techniques (ART) has been well-established [1]. The indications for sperm cryopreservation are many. The technique could be of help in many scenarios encountered in infertility management. It provides an option for storing spermatozoa while maintaining their functional capabilities. Subsequent uses of the preserved semen include intrauterine insemination, in vitro fertilization (IVF), and intracytoplasmic sperm injection (ICSI). Sperm cryopreservation is the only standardized and most feasible method for fertility preservation in men. Cancer patients often resort to cryopreservation to preserve their fertility prior to treatments such as radiation and chemotherapy. Additionally, men undergoing vasectomy procedures will use this method to maintain fertility [2].

Cryopreservation and thawing expose spermatozoa to various stresses that could eventually lead to the loss of fertilizing potential. Therefore, several improvements have been made to the process of cryopreservation [3]. Despite various advances in cryopreservation methodology, the recovery rate of functional post-thaw spermatozoa remains to be improved [4]. The use of cryoprotectants such as glycerol, ethylene glycol, dimethyl sulfoxide (DMSO), and 1,2-propanediol (PROH) marked one of the most significant advancements in cryopreservation. Cryoprotectants are low-molecular-weight, highly permeable chemicals that serve to protect spermatozoa from freeze damage induced by ice crystallization. Cryoprotectants act by decreasing the freezing point of a substance, reducing the amount of salts and solutes present in the liquid phase of the sample, and decreasing ice formation within the spermatozoa [5].

Oxidative stress (OS), resulting from an imbalance between reactive oxygen species (ROS) and antioxidants, is detrimental to human spermatozoa resulting in significant loss of function. Increased ROS production and decreased antioxidant levels are known to occur during sperm cryopreservation and thawing [6, 7]. Therefore, OS does play a role in injury sustained by spermatozoa during cryopreservation. Subsequently, antioxidants that counteract the effects of ROS could be of use in preventing OS-induced cryoinjury.

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54.2 Oxidative Stress and Male Infertility

OS has become the focus of interest as a potential cause of male infertility [8–12]. Under physiological conditions, spermatozoa produce small amounts of ROS, which are needed for capacitation, acrosome reaction, and fertilization [13]. However, excessive amounts of ROS produced by leukocytes and immature spermatozoa can cause damage to the normal spermatozoa by inducing lipid peroxidation and DNA damage [14–16]. The primary product of the spermatozoon's free radical generating system appears to be the superoxide anion, which secondarily dismutase to hydrogen peroxide (H_2O_2) through the catalytic action of superoxide dismutase (SOD) [17]. The combination of superoxide anion and H_2O_2 is potentially harmful, and in the presence of transition metals, it can precipitate the generation of hydroxyl radicals [18]. Sperm damage induced by OS includes membrane and DNA damage leading to necrozoospermia, asthenozoospermia, and DNA fragmentation [19].

Normally, an equilibrium exists between ROS production and antioxidant scavenging activities in the male reproductive tract. However, the production of excessive amounts of ROS in semen may overwhelm the antioxidant defense mechanisms of spermatozoa and seminal plasma, leading to OS [20, 21]. The OS status of an individual can be identified through direct or indirect measurement of the ROS/antioxidant levels or the inferences of such redox imbalance, respectively [22]. ROS levels can directly be measured by the chemiluminescence method [23], while total antioxidant capacity is measured by enhanced chemiluminescence assay or colorimetric assay [22, 24]. Indirect measures of OS include assessment of lipid peroxidation products (malondialdehyde), protein oxidation products (carbonyl groups), and oxidized DNA (8-hydroxy-20-deoxyguanosine [8-OHdG]) [25].

Spermatozoa are naturally surrounded by the seminal plasma, which is well-endowed with an array of antioxidants that act as free radical scavengers to protect spermatozoa against OS [26]. This defense mechanism compensates for the loss of sperm cytoplasmic enzymes occurring when the cytoplasm is extruded during spermiation, which in turn diminishes endogenous repair mechanisms and enzymatic defenses. Seminal plasma contains a number of enzymatic antioxidants such as SOD, catalase, and glutathione (GSH) peroxidase. In addition, it contains a variety of nonenzymatic antioxidants such as vitamin C (ascorbic acid), vitamin E (α-tocopherol), pyruvate, glutathione, and carnitine [27].

54.3 Oxidative Stress During Cryopreservation

OS occurring during sperm cryopreservation may be due to increased ROS production and/or decreased antioxidant scavenging activities. The process of ROS generation during cryopreservation and thawing of spermatozoa has been well-documented. Data shows that freezing and thawing of spermatozoa cause an increase in the generation of superoxide radicals. A sudden burst of nitric oxide radicals was also observed during thawing [7]. In samples with initially detectable ROS levels, these levels were further significantly increased after cryopreservation/thawing. On the other hand, when samples with no detectable ROS were subjected to cryopreservation/thawing, ROS became detected [28].

The decrease in recovery of motile viable sperm following cryopreservation/thawing may be due to damage caused by OS leading to lipid peroxidation of the sperm membrane. The increase in lipid peroxidation sometimes appears to be more significant than the increase in ROS levels when comparing fresh to cryopreserved/thawed sperm [29]. This was demonstrated by the detection of ROS-induced membrane lipid damage in frozen spermatozoa [30]. It has been also reported that the extent of lipid peroxidation is negatively correlated with post-thaw sperm motility [31]. Thus, cryopreservation does enhance lipid peroxidation in human sperm, and this enhancement may be mediated at least in part by the loss of SOD activity occurring during the process [30].

The cryopreservation process has been shown to diminish the antioxidant activity of the spermatozoa making them more susceptible to the ROS-induced damage. In bovine spermatozoa, levels of antioxidants were diminished during freeze/thaw cycles. Cryopreservation significantly reduced sperm GSH levels by 78% and SOD activity by 50% [6]. In humans, one consistent effect of cryopreservation is loss of the enzymatic activity of the peroxidation defense enzyme and SOD [32].

Recent body of evidence suggests that sperm DNA fragmentation (SDF) occurs as a result of increased OS during sperm cryopreservation [33]. The alteration in the mitochondrial membrane fluidity that occurs during cryopreservation will lead to rise in mitochondrial membrane potential and the release of ROS. Subsequently, released ROS causes DNA damage in sperm. It has been reported that ROS production by both human sperm and seminal leukocytes increases on cooling to 4 °C [34]. Thus, cryopreserved semen samples containing leukocytes may be more prone to DNA fragmentation.

The increase in sperm DNA damage during cryopreservation remains to be fully elucidated. While some studies documented it [4, 35], others found that it does not occur [36, 37]. The oxidative DNA biomarker 8-oxoguanine was recently used to assess oxidative DNA damage. Results showed that oxidative sperm DNA damage increased significantly after cryopreservation/thawing [38].

DNA damage resulting from OS is not limited to only ejaculated sperm. Aerobic incubation of testicular sperm results in a significant increase in DNA fragmentation. DNA fragmentation was noted to be higher in cryopreserved sperm than in fresh testicular sperm, and it was maximal after 4 h of incubation [39]. Therefore, care must be taken to avoid incu-

bating cryopreserved and fresh testicular sperm for prolonged periods of time before ICSI is performed.

ROS-induced DNA damage that occurs during sperm cryopreservation may be further increased by other technical procedures that are concurrently conducted. Current laboratory cryopreservation protocols include freezing of raw semen and freezing of washed sperm without seminal plasma [40]. The removal of the antioxidant-rich seminal plasma by sperm preparation prior to cryopreservation may lead to the deterioration in sperm motility post-thaw, cryosurvival rates, and DNA integrity [35, 41]. However, it is important to note that the decrease in quality in prewashed frozen-thawed sperm may not result in an actual decrease in cycle fecundity [42]. A potential source of ROS in the ART media during semen preparation is the activation of ROS production by immature spermatozoa by either centrifugation or leukocyte contamination. Longer centrifugation time exposes sperm to higher temperatures exaggerating ROS levels and negatively affecting sperm parameters [43]. Furthermore, factors such as the culture media, oxygen concentration, degree of illumination, and PH and temperature must all be controlled during sperm preparation as their alteration can significantly raise ROS levels and have a detrimental impact on sperm quality [43].

Recent studies suggest that DNA fragmentation in sperm is induced, for the most part, during sperm transport through the seminiferous tubules and the epididymis [15]. This could be mediated by ROS produced by immature sperm. A similar mechanism occurs in the pellet of centrifuged semen where sperm would be also highly packed.

54.4 Effects of Antioxidants on Spermatozoa In Vitro

Antioxidants act as the main defense against OS induced by free radicals. Thus, the concept of their integration during the cryopreservation/thawing procedure to protect sperm against OS has been extensively evaluated (Table 54.1).

54.5 Reduction of Reactive Oxygen Species Levels

In vitro supplementation with antioxidants is responsible for decreasing ROS in sperm suspensions. Significant reduction of H_2O_2 was achieved by adding different concentrations of vitamin C (300 and 600 μmol) and vitamin E (40 and 60 μmol) to the sperm preparation medium [44]. Significant reduction in the release of superoxide anion by 29–72% was also achieved following the addition of 10 μmol pentoxifylline [45, 46]. Other studies also showed that pentoxifylline is capable of reducing spermatozoal generation of ROS and subsequent lipid peroxidation in asthenozoospermic men [47, 48]. The addition of *N*-acetyl-l-cysteine (NAC) (1 mg/mL) also effectively reduced ROS levels. It may of importance to note that samples with initially high ROS showed the greatest tendency for reduction of ROS in response to antioxidant supplementation in vitro [49]. This notion is confirmed by studies examining the effect of vitamin E on post-thaw sperm integrity, revealing the greatest benefit in samples obtained from older men and samples with high prevalence of abnormal forms [50–52].

The positive impact for adding the antioxidant rebamipide was also validated in an in vitro study. Rebamipide effectively scavenged ROS during sperm processing and cryopreservation. The levels of ROS and lipid peroxidation in semen were significantly decreased in proportion to the concentrations of rebamipide both after incubation and after cryopreservation [53].

In support of using antioxidant combinations, a study was performed to evaluate the in vitro supplementation of cryoprotectant media with SOD and catalase using samples from 25 male partners of infertile couples. The authors reported significant improvement in sperm integrity only with SOD and catalase combination compared with each antioxidant alone suggesting an additive effect on superoxide anion and H_2O_2 [54].

54.6 Effects on Sperm Motility

Antioxidants counteract lipid peroxidation which has a negative effect on sperm motility [55]. In vitro exposure of spermatozoa to several antioxidants proved to be of benefit in terms of promoting sperm motility. Vitamin C (ascorbate) is one of the main antioxidants in seminal plasma; it is a chain-breaking antioxidant that protects the lipoproteins from peroxyl radicals. Data show that vitamin C can preserve sperm motility yet in a dose-dependent manner. Motility was highest after 6 h of incubation in 800 μmol vitamin C. However, motility was decreased with concentrations exceeding 1000 μmol [56]. Vitamin E is another major chain-breaking antioxidant that when added in vitro can efficiently protect sperm motility and morphology by suppressing lipid peroxidation [57]. The effects of supplementation with vitamins C and E on sperm motility are also dose dependent. Higher concentrations of vitamins C and E are not protective against H_2O_2-induced peroxidative damage of motility; instead, they increase the damage in both normozoospermic and asthenozoospermic patients [58].

Glutathione (GSH) appears also to have a protective effect on sperm motility. In samples characterized with leukocytospermia, the addition of GSH during sperm Percoll preparation and during 24-h incubation resulted in higher recovery of motile spermatozoa [59]. Similarly, a significant improvement was observed in sperm motility after 2 h of incubation with 1.0 mg/mL NAC [49]. L-Carnitine is another antioxidant that is considered as a fuel source involved in sperm

Table 54.1 Review of human studies exploring antioxidant effects in vitro/during cryopreservation

Antioxidant compound	Study	Study design	Result
Individual compounds			
Vitamin C	Verma and Kanwar [56]	In vitro supplementation with vitamin C (50–4000 μmol)	Vitamin C in concentrations below 1000 μmol protects spermatozoa from ROS as evidenced from improvement in motility and viability and reduction of MDA levels. Vitamin C at 1000 μmol concentration and above, however, is not protective, as evidenced by abrupt fall in sperm motility and viability and concomitant increase in LPO.
Vitamin E	Taylor et al. [50]	Normal (n = 23) and abnormal (n = 20) samples were divided into three aliquots prior to cryopreservation: 1. no treatment, 2. vitamin E 100 μmol, 3. vitamin E 200 μmol	Vitamin E dose was significantly associated with post-thaw motility (P = 0.041), and the pattern of response across doses was similar for normal and abnormal groups. Post-thaw motility was significantly improved by the addition of 200 μmol vitamin E (P = 0.006), but neither vitality nor SDF were altered.
	Verma and Kanwar [51]	Split seminal fractions were subjected to varied vitamin E concentrations (0.1–2 μmol/L)	Dose-dependent improvement in both motility and viability accompanied by concomitant decrease in MDA following vitamin E supplementation was noticed.
Pentoxifylline	McKinney et al. [45]	Samples from 10 asthenospermic (AS) men were divided into three aliquots: 1. control, 2. pentoxifylline 3.6 μmol, 3. pentoxifylline 7.2 μmol	ROS decreased in both pentoxifylline doses; however, the reduction was more in the 7.2 dose. MDA decreased in both pentoxifylline doses similarly.
	Gavella and Lipovac [46]	Incubated semen samples for 30 min at 37 °C in the following: 1. control, 2. pentoxifylline 3.7 μmol, 3. pentoxifylline 10 μmol	Decreased superoxide levels with treated sperm.
N-Acetyl-l-cysteine	Oeda et al. [50]	Semen samples were incubated with or without N-acetyl cysteine (NAC) (1.0 mg ml-1) at room temperature	ROS levels decreased significantly after 20 min incubation with NAC. This reduction was greater in samples with high ROS group than with low ROS. Total sperm motility improved after incubation with NAC, but no significant change was observed with respect to the acrosome reaction.
Rebamipide	Park et al. [53]	Rebamipide was added to semen samples and cryoprotectant to a final concentration of 10 μmol/L, 30 μmol/L, 100 μmol/L, or 300 μmol/L. Specimens were incubated at 37 °C for 1 h or cryopreserved at −196 °C for 3 days	The sperm motility was significantly increased after incubation with 100 μmol/L and 300 μmol/L rebamipide. After cryopreservation, the sperm motility was significantly decreased in all concentrations ($P < 0.05$), but the decrease was less with 100 μmol/L and 300 μmol/L rebamipide than that with other concentrations.
Glutathione	Parinaud et al. [59]	Thirty semen samples divided into two equal parts and incubated for 24 h in media with/without glutathione	Higher percentage of motility in the treatment group regardless of the level of leukocytospermia.
L-Carnitine	Zhang et al. [60]	Thirty-seven AS and 33 normospermic (NS) samples cryopreserved with/without L-Carnitine 1 g/L	L-Carnitine induced a significant improvement in post-thaw sperm parameters in both the AS and NS semen samples, compared with those of the control, regarding sperm fast forward motility, forward motility, total motility and VIA. L-Carnitine showed better protective effects against SDF in the AS group only.
Coenzyme Q10	Lewin and Lavon [64]	Sixteen NS and 22 AS samples divided into four equal parts and incubated for 24 h in: 1. HAM's medium alone, 2. HAM's medium with 1% DMSO, 3. HAM's with 5 μmol, 4. HAM's with 50 μmol coenzyme Q10	While no significant change in motility after incubation was observed in the samples with initial normal motility, a significant increase in motility was observed in the 50 μmol CoQ10 subgroup of sperm from AS men.
Leptin	Fontoura et al. [74]	Forty-five NS samples were frozen and thawed with/without capacitation and leptin incubation	Significant post-thaw rise in SDF compared with fresh samples. SDF was significantly reduced when sperm capacitation was performed before freezing. The addition of leptin to capacitated sperm before freezing reduced SDF ($p < 0.0001$) and enhanced SOD (p = 0.001) and glutathione peroxidase (p = 0.02) antioxidant enzyme activity.

Table 54.1 (continued)

Antioxidant compound	Study	Study design	Result
Antioxidant combinations			
Vitamin C and vitamin E	Donnelly et al. [44]	Fifteen NS and 15 AS divided into treatment and control groups	Vitamin C or vitamin E alone did not affect baseline DNA integrity but did provide sperm with complete protection against H_2O_2-induced DNA damage. Vitamins C and E in combination induced DNA damage and intensified the damage induced by H_2O_2; however, H_2O_2-induced ROS production was significantly reduced in a dose-dependent manner by supplementation with both vitamins.
	Donnelly et al. [58]	Ten NS and AS divided into treatment and control groups	The production of reactive oxygen species by sperm was reduced by supplementation in vitro with ascorbate and α-tocopherol. However, progressive motility, average path velocity, curvilinear velocity, straight-line velocity, and linearity were decreased significantly, with the greatest inhibition observed with the highest concentrations of antioxidants.
SOD and catalase	Rossi et al. [54]	NS samples ($n = 25$) were divided into four aliquots: 1. control, 2. SOD 200 U/ml, 3. catalase 200 U/ml, 4. SOD + catalase (both 100 U/ml) Each aliquot was mixed (v/v) with medium and then frozen at −196 °C	No significant variation in the recovery of progressive motility was seen in the aliquots with added SOD or catalase alone, compared to the control group. On the other hand, a significant improvement in sperm parameter recovery was seen in the aliquot with both SOD and catalase supplementation.
Vitamin C, catalase, and SOD	Li et al. [72]	Semen sample form 30 fertile men were mixed with modified cryoprotectant and divided into six groups: 1. vitamin C 300 μmol/L, 2. vitamin C 600 μmol/L, 3. catalase 200 U/ml, 4. catalase 400 U/ml, 5. SOD 200 U/ml, 6. SOD 400 U/ml	After cryopreservation, compared with the control group, progressive motility rates of the vitamin C 300 μmol/L, catalase 200 U, and catalase 400 U groups were all higher than that of the control group. ROS levels were significantly lower in vitamin C 300 μmol/L, catalase 200 U, and catalase 400 U groups than control groups. Tail SDF levels were similar in vitamin C 300 μmol /L, catalase 200 U, and catalase 400 U groups in comparison to raw semen; however, they were significantly lower than the control group.
Vitamin C, vitamin E, urate, and NAC	Hughes et al. [75]	One hundred fifty semen samples were prepared in the presence of vitamin C (300, 600 μmol), vitamin E (30, 60 μmol), urate (200, 400 μmol), or acetyl cysteine (5, 10 μmol) SDF was induced by 30 Gy X-irradiation	Sperm DNA was protected from DNA damage by vitamin C (600 μmol), vitamin E (30 and 60 μmol), and urate (400 μmol).

motility. The addition of 1 g/L of L-carnitine to cryopreservation medium at a ratio of 1:1 improved sperm fast forward motility, forward motility, and total motility in both asthenozoospermic and normozoospermic men compared with controls [60]. Albumin is an antioxidant/extender that has been extensively used in sperm preparation. Its antioxidant properties are due to its ability to react against peroxyl radicals and prevent the propagation of peroxidative damage in sperm [61, 62]. When albumin was used in sperm preparation media, it resulted in significant improvement in motility and viability compared to Percoll [63]. Other antioxidants that proved to be of benefit to the sperm motility include coenzyme Q10 (50 μmol) [64], hypotaurine, and catalase [65].

Enzymatic antioxidants such as SOD and catalase protect spermatozoa from superoxide anion and H_2O_2. Sperm suspension treated with SOD (400 U/mL) had significantly reduced motility loss and malondialdehyde concentration [66]. Similarly, the addition of catalase (0.008 mg/mL) to sperm suspension offered protection against H_2O_2-induced toxicity [67]. In support, the role of catalase and SOD against sperm intracellular (mitochondrial and plasma membrane) and extracellular (leukocytes) ROS and their beneficial effects on sperm motility have been consistently reported [68, 69]. Different concentrations of vitamin E (800 μmol, 10 μmol) can protect sperm against lipid peroxidation [56, 66]. Similarly, pentoxifylline at the dosage of 3.6 and 7.2 μmol was proven to limit lipid peroxidation in asthenozoospermic men [47].

54.7 Protection of Sperm DNA Integrity

Sperm preparation protocols that are routinely applied during ART and cryopreservation involve repeated high-speed centrifugation and the isolation of spermatozoa from the protective antioxidant environment provided by seminal plasma.

This has been shown to result in sperm DNA damage via pathways that are mediated by increased ROS generation [70]. Swim-up media supplemented with antioxidants (NAC (0.01 μmol), catalase (500 U/mL), reduced GSH (10 μmol), and hypotaurine (10 μmol)) lead to significant reduction of DNA damage induced by ROS generation [71]. Similarly, albumin in doses from 0.3% to 10% protected sperm DNA integrity by neutralizing peroxides produced during lipid peroxidation [62].

Supplementation with the antioxidants ascorbate and catalase with the cryoprotectant pre-freeze resulted in a decrease in ROS levels and sperm DNA damage during cryopreservation/thawing [72]. Similarly, the addition of GSH to the thawing medium resulted in the following: (1) a higher number of non-capacitated viable spermatozoa, (2) a reduction in ROS generation, (3) lower chromatin condensation, (4) lower DNA fragmentation, (5) higher oocyte penetration rate in vitro, and (6) higher in vitro embryo production compared with control group [73].

Fontoura et al. confirmed the presence of significant elevation in sperm DNA fragmentation following a freeze-thaw cycle compared with fresh samples from the same individuals. The authors also revealed that the addition of the antioxidant leptin to capacitated spermatozoa prior to freezing significantly increased SOD and glutathione peroxidase activity and decreased sperm DNA fragmentation [74].

It is critical to consider the approach for adding antioxidants as protective agents for the sperm DNA integrity whether single or in combination. Protection of the sperm DNA against H_2O_2-induced damage was demonstrated by vitamin E and vitamin C individually in both normozoospermic and asthenozoospermic samples [43]. However, when a combination of vitamin C and vitamin E was used for spermatozoa incubation, DNA damage was increased. This may be due to vitamin E and vitamin C acting as prooxidants [75].

54.8 Conclusion

Strong body of evidence currently supports the use of antioxidants in cryoprotectant and post-thaw media to prevent OS during cryopreservation and thawing. However, there is no consensus as to the type, combination, and concentrations of antioxidants to be added. Therefore, caution should be exercised as excessive amounts of antioxidants may act paradoxically to damage spermatozoa. Well-controlled studies are still needed to identify the ideal antioxidant combination/concentration to supplement the cryoprotectant and post-thaw media.

54.9 Review Criteria

An extensive search of studies examining the relationship between antioxidants and sperm cryopreservation was performed using search engines such as ScienceDirect, OVID, Google Scholar, PubMed, and MEDLINE. The start and end dates for these searches were December 2000 and December 2018, respectively. The overall strategy for study identification and data extraction was based on the following keywords: "antioxidants," "oxidative stress," "reactive oxygen species," "infertile men," "cryopreservation," "cryoprotectants," "semen parameters," and "assisted reproduction" and the names of specific antioxidant supplements. Articles published in languages other than English were also considered. Data that were solely published in conference or meeting proceedings, websites, or books were not included. Websites and book chapter citations provide conceptual content only.

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Antioxidants in ICSI

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Key Points

- The oxidative stress in semen is a likely cause of idiopathic male infertility, and this may be caused by internal and external factors by enhancing reactive oxygen species. Many of them can be identified through an adequate anamnesis.
- Several tests and measurements can be done to check the oxidative stress status in spermatozoa, although many bring a partial view of the whole picture.
- The consequences of oxidative stress at the molecular level can induce microscopical harm in sperm count, morphology or motility, or invisible damage to sperm cell structures and biomolecules such as DNA.
- The control exerted by antioxidants maintains reactive oxygen species within a physiological threshold, but the breakage of this homeostasis can also lead to suboptimal reproductive function.
- Therapeutic use of antioxidants to overcome these situations has been attempted for both the individuals and biological samples, but, unfortunately, due to the variety of outcomes analysed, populations studied and intervention formulas and combinations, a single conclusion cannot be drawn from them.

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55.1 Introduction: Male Factor Infertility Relevance and Causes

Infertility is defined by the failure of a couple to achieve a clinical pregnancy after at least 1 year of regular unprotected sexual intercourse [1]. It is estimated to affect 15% of the couples at reproductive age worldwide, where male factor is present as a cause in approximately half of the cases, either as the only cause of infertility or in combination with female factor [1–3]. Male fertility would then be the ability of a man to impregnate a healthy, fertile woman of reproductive age. Subsequently, infertility or subfertility will affect 5% of men globally [4].

The male partner can be evaluated for infertility or subfertility using a variety of clinical tests, but mainly with a laboratory evaluation of semen. The parameters for the evaluation of the quality of a semen sample established by the World Health Organization [5] are considered the standard for assessing male factor infertility, principally based on the volume ejaculated, spermatozoa concentration, motility and morphology. However, it fails to predict the probability to achieve a conception by natural means or by assisted reproduction techniques (ART), and it is unable to detect molecular abnormalities that may originate subfertility [1]. In addition, given the variability among ejaculates from the same man, a single sperm analysis is usually not enough to diagnose or identify a reproductive problem.

In vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are two ART that require the preparation of the semen sample in order to successfully overcome the infertility problems caused by a suboptimal count of motile progressive spermatozoa, avoiding several natural barriers [6]. In the case of ICSI, the cell to inject and fertilize is currently chosen based mainly on its morphology and motility, decided by the operator, and yet there are cases of fertilization failure, embryo blockage during development or implantation failure, indicating that there are undetected factors causing a reproductive failure at those levels. This is often called idiopathic infertility, meaning that its cause remains

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unknown and several candidate factors appear to have a deleterious combined effect. Among them, oxidative stress (OS), the imbalance between free radicals and antioxidants that in normal conditions favor and facilitate physiological processes, has proven to be very relevant, as oxidative biomarkers in the semen of idiopathic infertile men were found to be significantly higher than in fertile men [4]. It has been extensively described in the scientific literature that OS in sperm not only correlates with suboptimal values of routine sperm analysis due to an impairment of spermiogenesis but also with other features in sperm that are not routinely analysed, namely DNA fragmentation [4], epigenetics [7], mRNA transcripts dysregulation in the sperm cell [1] and apoptotic events. This chapter will discuss the causes and consequences of OS on male factor infertility and some of the different approaches described in the most recent literature for its management and treatment, focusing on the potential improvement of the reproductive outcome after ICSI with the use of antioxidants.

55.2 Oxidative Stress and Male Factor

OS is the disequilibrium between oxidative and anti-oxidative molecules in a biological system where the oxidants overwhelm the defensive systems. Those oxidants are reactive oxygen species (ROS) that can produce interferences or destruction on cell biological structures, functions and properties, the most important for spermatozoa being the superoxide anion ($O_2{\cdot}^-$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH·). The anti-oxidative molecules are also called antioxidants, enzymatic or non-enzymatic molecules that are naturally present in the cells or surrounding media within a biological environment (such as ejaculates) and whose activity can be measured together as total antioxidant capacity (TAC) [1, 2]. These two antagonistic activities, oxidative and anti-oxidative, must be balanced in order to form complex systems to maintain the oxidative homeostasis that is essential for the reproductive function, since a small and controlled level of ROS is necessary for normal cell activity, and in sperm cells they play an important role in capacitation, acrosome reaction and sperm-oocyte fusion [8].

Systemic oxidative stress in a man directly affects the process of spermiogenesis, worsening semen quality in all terms: sperm count, concentration, viability [9, 10] motility and morphology [11], DNA integrity [12], epigenetics [7], chromatin condensation, mRNA transcripts dysregulation and apoptotic events [1, 2]. Therefore, the chances of achieving a natural conception are reduced, leading to subfertility and the need of ART for reproductive purposes.

At this point, the performance of an ICSI procedure allows overcoming the very low quality of the sperm in terms of concentration, motility and morphology, as it bypasses the natural selection barrier existing during normal fertilization and conventional IVF. Nevertheless, with ICSI there is still a risk of injecting a compromised spermatozoon, as some of the mentioned consequences of OS are held at a molecular level, thus remaining occult to routine analysis and selection filters, not preventing the spermatozoa from successfully getting through the conventional sperm preparation process for ICSI, since they do not affect sperm morphology either, remaining invisible when examining the sperm under the microscope [13].

Also, an excess of ROS in a semen sample affects sperm function by oxidation of lipids, proteins and DNA [14], directly affecting sperm motility that can be identified under the microscope. Additionally, the special composition of sperm cells' membrane, which is richer in polyunsaturated fatty acids, makes it more sensible to lipid peroxidation, which occurs in a chain reaction causing extensive membrane damage and by-products of lipid peroxidation include mutagenic and genotoxic molecules, such as malondialdehyde (MDA) and 4-hydroxy-nonenal (4-HNE), which can cause DNA damage. Free radicals causing oxidative damage attack DNA, and they trigger apoptosis leading to the disruption of mitochondrial membranes, activating caspases and releasing cytochrome c, which increases ROS production, and a caspase-mediated destruction of DNA, feeding the apoptotic cycle [15].

The use of a compromised spermatozoon for ICSI may lead to fertilization failure, embryo blockage or low quality during development, implantation failure, or in the cases of live birth, the health of the offspring might be affected due to de novo mutations [16].

ROS in sperm can be classified according to their origin. Endogenous sources of ROS are other cells present in semen, which include immature spermatozoa, round cells from different initial steps of the spermatogenesis process, leukocytes and other occasional cell types as epithelial cells. Exogenous sources of ROS are external factors that introduce ROS or stimulate intrinsic ROS production [17].

55.2.1 Endogenous Sources of Free Radicals

Among the cells that are contained in semen, the main sources of ROS are immature spermatozoa and leukocytes [18]. Immaturity of sperm cells and poor sperm morphology are characterized by the presence of abnormal spermatozoa with aberrant cytoplasmic droplet retention [19], which is where ROS are produced by a cytosolic enzyme involved in the glucose metabolism, glucose-6-phosphate dehydrogenase (G6PD), through the NADPH oxidase system in sperm plasma membrane, or through the NADH-dependent oxidoreductase pathway at the mitochondrial respiratory level

[14]. Moreover, it has been confirmed that higher ROS levels correlate with a lower percentage of normal morphology (mature spermatozoa) [11].

Leukocytes on the other hand generate huge amounts of ROS, as they are essential for their immunological function, although white cell presence in normal healthy raw semen is very limited. Semen samples with leukocytospermia have a pathological excess of leukocytes within the ejaculate, frequently as a response to infection, implying high seminal ROS levels, DNA damage and lower sperm concentration and motility [1, 17]. Nevertheless, leukocyte concentrations considered normal by WHO criteria can still produce damaging ROS levels, and leukocytes can also stimulate sperm ROS production.

55.2.2 Exogenous Sources of Free Radicals

55.2.2.1 Lifestyle Factors and Medical Conditions Affecting ROS in Sperm

Several lifestyle factors are related to increased systemic OS, potentially leading to a reduced sperm quality and reproductive function. The relevance of these habits depends on an exact evaluation of their impact on the reproductive capacity, allowing clinicians to design an adequate strategy to assess their presence or risk on a patient's lifestyle survey in a preliminary consultation and anamnesis, and therefore give recommendations to modify them and then contribute to reproductive success before starting ART.

Approximately 37% of men of reproductive age smoke cigarettes [20]. *Tobacco smoking* is considered a major factor leading to male infertility since it can affect sperm development and function through the impairment of sperm maturation, chromatin compaction, capacitation and acrosome reaction [1]. The reason is that cigarettes themselves contain free radicals, toxins and mutagenic carcinogen substances such as benzo(a)pyrene, which is spermatotoxic and has been detected in semen of smoking men [21]. Additionally, smoking seems to reduce enzymatic antioxidant activity in semen, while it increases inflammatory processes, leading to an increased presence of leukocytes in sperm, which causes increased ROS production and risk of OS damaging sperm cells [22]. Among infertile men, smoking men have an estimated risk of suffering oligozoospermia 1.29 times higher than non-smokers [23], and among the general population, smokers show a mean reduction in sperm concentration of −9.72 × 106 sperm/ml, 3.48% less motile sperm and 1.37% less morphologically normal sperm, with a dose-dependent effect and a more pronounced reduction of semen quality in infertile smoker men than in fertile ones [20]. Interestingly, a more recent meta-analysis comparing smoker and non-smoker infertile men did not find a significant difference in sperm motility and pH, nor did they find any hormonal imbalance regarding the reproductive axis [23].

Alcohol is another toxic substance, sometimes excessively consumed, able to induce systemic OS, as ethanol stimulates the production of ROS by altering mitochondrial structure and respiratory function. Within the testicle, this implies a significant reduction in plasma testosterone, an increase in serum lipid peroxidation by products and a fall in antioxidants [1]. By these mechanisms, alcohol seems to cause a progressive and dose-dependent deterioration in semen quality, starting from teratospermia followed by oligoasthenoteratospermia, cryptozoospermia and finally azoospermia due to an arrest in spermatogenesis [24]. Even when semen parameters are not affected, alcohol use is associated with higher miscarriages and lower birth rates with IVF [21]. Hopefully, 3 months alcohol abstinence allows the restoration of normospermia in men with no other subjacent cause of infertility [24].

Obesity, defined as the accumulation of abnormal or excess fat that may impair health, is a serious chronic disease with several comorbidities, and it constitutes a risk factor for infertility [25]. There is a confirmed relationship between obesity and subfecundity with impairment in spermatogenesis and a negative impact on semen parameters, especially sperm count and density have been found to be negatively correlated to body mass index [19]. The mechanisms by which obesity stimulates semen abnormalities are multifactorial, the most relevant being the production of ROS leading to OS and the dysregulation of the hypothalamic–pituitary–gonadal axis showing a hormonal profile of lower levels of follicle stimulating hormone (FSH) and luteinising hormone (LH), hyperestrogenism and androgen deficiency, which may lead to secondary hypogonadism [21]. Inflammatory processes are enhanced in obese people due to adipose tissue-derived inflammatory cytokines, resulting in an increased leukocyte production of ROS and an increased risk of oxidative stress, ultimately causing male factor infertility [25]. In addition, obesity might be an endocrine disruptor given the high bioavailability of the aromatase enzyme in adipose tissue, increasing the conversion of not only testosterone to estradiol but also other hormones associated with obesity and visceral fat accumulation, potentially acting as comorbidity factors [21, 25]. It has been described in scientific literature that gradual weight loss through a slight caloric deficit and exercise, but not bariatric surgery, may help restore a normal hormonal profile and improve spermiogenesis and semen parameters increasing the fertility potential [21], suggesting that the cause of infertility is not only the disease of obesity itself but also the set of habits that lead to weight gain in the first place. Finally, it is imperative to notice the pandemic state of overweight and obesity in the modern world, where the intake of energy-dense foods very rich in fat and sugars has increased, along with sedentary

jobs and modes of transportation, while physical activity has generally decreased.

On the other hand, *extreme underweight* has also been shown to affect the hormonal profile causing infertility [21], but, additionally, *dietary deficiencies* may also be linked to OS in sperm and impaired spermatogenesis, especially when there is a lack of foods containing essential antioxidants. Dietary and exercise habits are likely influenced by the stressful lifestyle that many people follow in large cities, which has an unbalancing effect on ROS production and antioxidants activity, thus affecting spermatogenesis and male fertility. Also, *extreme exercise activity*, either in amount or intensity, leads to large ROS production because of the aerobic metabolism, and it might overwhelm the antioxidant capacity of the body, leading to oxidative stress and spermatogenesis impairment, reduced semen quality and infertility [1].

Some *medical conditions* such as varicocele are intimately related to infertility, in this case due to the increased temperature of testicles that causes heat stress and therefore oxidative stress affecting spermatogenesis [1]. Infections, autoimmunity and chronic disease are other factors indirectly causing OS in sperm [4]. Common mental health issues, such as stress, anxiety or depression, are also factors affecting sperm production, apparently through hormones and OS-related mechanisms, but their pharmacological treatment might as well be deleterious [26].

55.2.2.2 Environmental Sources of ROS

During the past decades, development of industries and enlargement of cities contributed to the rise in pollution and contamination in developed countries. The increase in the prevalence of infertility problems is one of the consequences, and it is believed to be mediated by OS mechanisms.

Most *air pollutants* act as potent stimuli for leukocyte ROS generation, leading to higher risk of oxidative damage to sperm lipids, proteins and DNA. As is the case of diesel particulate matter that has recently been classified as a carcinogen by the WHO [1].

Moreover, people in the modern world are constantly exposed to toxins, released from structural materials, industrial products or other consumables, that tend to accumulate in the human body causing chronic intoxication and long-term adverse effects [27]. For example, parabens—preservatives in cosmetic and pharmaceutical products, including vaginal lubricants—are capable of stimulating sperm generation of ROS, both from mitochondrial and cytosolic origin, with a dose-dependent deleterious impact on sperm motility and viability, although no significant effect was detected in DNA fragmentation [28]. Reproductive toxicity of bisphenol A, a component of plastic containers, was well documented several year ago, and recent studies in rats indicate an OS related mechanism, given that after the administration of this agent rats' sperm showed a reduction of TAC and a rise of ROS levels. *Phthalates* are a family of chemicals widely used in several plastics that may accumulate along the food chain and show in semen, correlating with higher ROS levels and sperm DNA damage [27].

Exposure to low energy non-ionizing *radiation* is a rising concern since the use of mobile phones has become a daily practice for most people in developed countries and sometimes even an addiction, usually carrying them in a pocket very close to the genitals. This type of radiation is present also in visual light and microwaves, and it is believed to increase ROS production, thus potentially affecting sperm quality [27].

Finally, exposure to *heavy metals* and *biological hazards* may be less frequent in the average population and more of a job-related factor, but its effects on sperm quality are dramatic, and the negligence in the use of safety measures in specific jobs is a matter of concern, as well as the reticence to adhere to the limitation of the presence of these substances in industrial wastes [19]. Contribution to ROS production is believed to be the mechanism by which these substances cause spermatogenesis impairment leading to reduced sperm viability, motility and morphology [1].

55.2.2.3 Laboratory Manipulation

It is an extended belief that the mere light of the laboratory can bring negative consequences for both gametes and embryos, but the reality is that lab protocols can be far more dangerous. For example, *centrifugation* time directly correlates with ROS levels augmentation of a semen sample, so this routine step of the sperm preparation process can seriously aggravate sperm oxidative stress [17]. *Oxygen concentration* in sperm culture has also proven to be a risk factor for OS that can be minimized using 5% O_2 instead of atmospheric 20% O_2 content, the same as the embryo culture [29]. Other culture conditions such as *pH* may also affect ROS production in sperm, so the use of buffers is needed to minimize the impact, although concern exists regarding the type of buffer to use in each step to avoid undesired consequences, as some hypothesize that during ICSI the buffer could be introduced in the oocyte and be deleterious [30]. *Temperature* can affect pH, and as well as illumination, temperature itself may induce ROS production in sperm [17]. Furthermore, it would be ideal to avoid the use of cryopreserved sperm for fertilization, since ROS production is enhanced during freezing and thawing processes, thereby decreasing sperm quality [31], although to what extent this affects reproductive results is controversial. Sperm preparation media may as well be supplemented with a variety of antioxidants to guard against oxidative stress, as will be further discussed in this chapter.

55.3 Consequences of OS in Sperm Affecting ICSI Results

It has been extensively described that oxidative stress, usually assessed detecting high ROS levels and/or low TAC, is present in sperm samples of infertile men in a higher propor-

tion than in fertile men's sperm. When performing ICSI, the consequences of OS in the spermatozoa selected for injection that can affect the reproductive outcome are epigenetic impairment, DNA oxidation, DNA fragmentation and apoptotic events.

55.3.1 Epigenetic Alterations

Several studies have recently found that the exposure of sperm to oxidative stress induces epigenetic changes altering normal gene expression that may have an impact on the early development of the embryo [7]. As is the case of the genes Igf2 and HI9 that are respectively implicated in the induction or negative regulation of cell proliferation. Researchers found in human normozoospermic samples from men in couples suffering from unexplained infertility, a correlation between high ROS levels and methylation of Igf2 and HI9 demethylation, which is the opposite of the normal methylation pattern in human cells, where both genes are imprinted so that Igf2 mRNA is transcribed from the paternal chromosome, while HI9 is silenced in it and expresses from the maternal one. This led to the speculation that an embryo generated by this ROS-affected sperm may carry epigenetic impairments, probably affecting its development and reproductive outcome [11], or even future offspring health.

This speculation might be supported by a separate experiment that was carried out using cattle model gametes in vitro, intentionally exposing some of the sperm samples to oxidative stress before fertilization. A major developmental arrest was observed at the time of embryonic genome activation in embryos generated from OS damaged sperm, due to the impairment of zygotic active DNA demethylation in paternal pronuclei, impeding its fusion with maternal pronuclei and thus the correct formation and activation of the embryo genome [7]. Separately, another study found that OS in spermatozoa affects mRNAs that play a role in fertilization and early embryo development [32]. This indicates that OS in sperm has an impact on the dynamics of epigenetic reprogramming and paternal contribution to the embryo, along with hypomethylation and shortening of telomeres, with a negative impact on embryo development and quality, thus potentially diminishing reproductive results [33].

55.3.2 DNA Oxidation

ROS and free radicals directly attack DNA purine and pyrimidine bases, leading to the formation of oxidative adducts such as 8-hydroxy-2′-deoxyguanosine (8-OHdG), which is the most common. This causes mutations in DNA, enhances shortening of telomeres and hypomethylation affecting all the genome, leading to dysregulation of transcription. Also, lipid peroxidation products produced under OS such as MDA are mutagenic. All these secondary consequences of OS in sperm DNA can have an effect on sperm motility and morphology in the worst cases, but some spermatozoa can appear normal and functional but still have oxidative DNA damage [13].

Subsequently, it could be expected that performing ICSI with a damaged spermatozoon would lead to poor embryo quality and reproductive outcome. However, studies describing ICSI results in relation with DNA oxidation are very limited, and the results do not clearly support this hypothesis. On one hand, pregnancy rate after IUI was negatively correlated to DNA oxidation and a threshold value of 11.5% 8-OHdG was proposed as a success predictor, but on the other hand no correlation was found in ICSI cycles [34]. The percentage of DNA oxidation in sperm used in ICSI cycles was not found to correlate with implantation rate either, although embryo quality was shown to be reduced, so embryo selection was proposed as a confounding factor [35].

55.3.3 DNA Fragmentation

One of the OS consequences, and probably the most studied, is a direct damage to sperm DNA integrity due to the direct attack of ROS on DNA that can cause strand breaks. Although OS is not the only cause of DNA fragmentation, it has been observed that a 25% increase in ROS levels results in a 10% increase in DNA fragmentation [36]. DNA fragmentation index (DFI) and oxidation–reduction potential (ORP) have been correlated with male infertility and suboptimal sperm parameters, finding in infertile patients a significantly lower mean sperm count (32.7 vs 58. 7×10^6 sperm/mL), total motility (50.1% vs 60.4%) and normal morphology (5.7% vs 9.9%), while those patients showed higher values of ORP and DNA fragmentation index (DFI) than men confirmed fertile. Furthermore, a threshold value of 25.5% DFI was proposed to predict ART success [37]. On the other hand, it has also been found that sperm with highly fragmented DNA can still have normal motility and morphology [12]. DNA fragmentation analysis has recently been supported as a more significant predictor of male fertility than classic sperm parameters, as it has been strongly correlated with the increase in time-to-pregnancy and reduction of the probability of natural or intrauterine insemination (IUI) conception [34].

IVF techniques (conventional or ICSI), conversely, greatly reduce the amount of sperm needed to fertilize, which makes (DFI) less relevant, to the point that some researchers have found DFI determination to be useless in the prediction of conventional IVF and ICSI results in terms of reproductive outcome [15, 34]. Interestingly, a study conducted in 2014 found DNA fragmentation affected IVF results, observing that higher DFI led to lower fertilization rate, percentage of good quality embryos and implantation rate, describing a 33.33% implantation rate when fertilization occurred with

sperm from the sample with higher DFI, while lower DFI sperm had a 65% implantation rate [16]. Other studies have described that DNA fragmentation also affects the synchrony of the nucleolar precursor bodies' pattern in pronuclei, embryo ability to achieve blastocyst stage, embryo morphological quality and blastocyst rate [1].

In general, scientific literature fails to provide solid evidence of a correlation of DFI with IVF and ICSI pregnancy outcome, which may be caused by the bias of embryo selection at the time of embryo transfer. Several researchers have attempted to compare the impact of DFI in conventional IVF and in ICSI pregnancy outcomes, some of them finding no statistically significant difference [38], and others finding the first to be modestly affected in a negative way, while the effect in ICSI was reported to be insignificant in most of them in terms of pregnancy rate, although the risk of pregnancy loss was higher than normal in both procedures [39]. A possible explanation for the difference between the two techniques might be the longer gamete culture time in IVF, prolonging OS exposure, which can cause further DNA damage and reduce the ability of oocytes to repair it. Nevertheless, ICSI was suggested to be the best approach for treating men with high DFI and previous failed reproductive attempts either by IUI or natural means [12].

Finally, it is important to notice that sperm cells cannot repair the DNA damage because they lack most of the enzymes needed for the base excision repair (BER) pathway, except for the first one, because of the extreme condensation of chromatin in mature spermatozoa, where transcription is disabled. This is why oocyte quality is so important, because that cell has the mission of repairing some of the damage in paternal pronucleus after fertilization before initiating the S-phase of the first mitotic division of the zygote, thus retarding this event [7]. This provides a scientific explanation to the results obtained in 2011 that showed in the IVF cycles performed with oocytes from the infertile woman, the probability of not achieving pregnancy increased by 1.31 for every 10% increase in sperm DNA fragmentation, while in the IVF cycles performed with donor oocytes it did not have a statistically significant effect [38].

When DNA damage cannot be repaired, mutations occur all along the embryo cell, increasing the probability of poor embryo quality, implantation failure, miscarriage or childhood problems. In fact, animal studies in mice have shown increased incidence of tumors in the offspring generated from high DFI sperm by ICSI, and premature aging and aberrant growth and behavior have also been observed [12].

55.3.4 Apoptosis

Apoptosis is a non-inflammatory response to tissue damage characterized by a series of cellular, morphological and biochemical changes resulting in cell death. In the context of male reproductive tissue, this mechanism functions as a quality control to eliminate abnormal spermatozoa, but sometimes this process is aborted before its completion, leading to the presence of apoptosis-like conditions in ejaculate [15]. This event can be assessed by surface phosphatidylserine detection using antibodies. It has been well studied and described that semen samples from infertile men have a larger incidence of apoptotic sperm, especially the ones with less concentration, and that this has a negative effect on fertilization potential. Interestingly, the exact mechanism of this apoptotic pathway is still unclear [40].

Separately, high levels of ROS may initiate apoptosis by disrupting the inner and outer mitochondrial membranes via lipid peroxidation, releasing apoptosis inducing factor (AIF) and Bax protein that also regulates apoptosis. By this mechanism, Cyt-C is released, and caspases are activated, inducing DNA fragmentation that feeds the apoptotic cycle [2].

55.4 The Control of Free Radicals: The Antioxidants

Antioxidants are molecules with the ability to scavenge free radicals and ROS, stopping the chain reaction that leads to OS in tissues [3]. Antioxidant activity within sperm cells is very limited due to the reduced cytosolic volume, and seminal (TAC) relies mainly on seminal plasma (SP) [19], since it is the extracellular environment designed for spermatozoa survival, also providing optimal pH, nutrition and adequate viscosity. SP is secreted by the prostate, the seminal vesicles and the bulbourethral glands, and it naturally contains enzymatic and non-enzymatic antioxidants, either because they are produced by the human body or because of dietary intake or pharmacological treatments, being either natural or synthesized. This can be observed when incubating sperm cells in the absence of SP: after 2 h of incubation they experience an increase in ROS levels and a significant loss of sperm motility [2]. Also, TAC has been found to be lower in SP from men with idiopathic infertility than fertile men [9].

Some important *enzymatic antioxidants* for fertility are superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX).

The **SOD** family presents three different classes, depending on the catalytic metal at the active site, SOD-1 being the most active in seminal plasma. These enzymes work by catalyzing the transformation of superoxide anion into hydrogen peroxide and oxygen (O_2) [18]. It has been described that sperm samples with higher SOD activity present lower levels of markers of oxidative stress and DNA damage [2].

Catalase enzyme on the other hand converts hydrogen peroxide into water and O_2, preventing the reaction of that H_2O_2 with remaining free radicals, which would generate

more ROS such as the extremely reactive hydroxyl radical [1]. This enzyme is implicated in the capacitation process through a complicated mechanism [18], and its activity has been found to be lower in men with asthenozoospermia compared to normospermic semen [2].

Finally, **GPX** catalyzes the reactions of H_2O_2 and superoxide anion using glutathione as an electron donor. This enzyme protects from lipid peroxidation because it can detoxify organic peroxides converting them into stable alcohols. As a by-product of the ROS scavenging reactions of GPX, the oxidized form of glutathione needs to be reduced to restore the stock available for further GPX reactions; hence, the glutathione reductase (GR) reaction is coupled to GPX [2].

Non-enzymatic antioxidants that are naturally present and act as free radical scavengers in semen include vitamins E and C, folate, zinc, selenium, carnitine and carotenoids [4]. In addition, exogenous non-enzymatic antioxidants might be used to protect sperm from OS during its in vitro manipulation during ART procedures, as will be discussed later in this chapter.

55.5 The Use of Antioxidants to Improve Reproductive Results

The identification or perception in a patient of any of the above mentioned risk factors of OS may lead to the possibility of suggesting an antioxidant treatment for the improvement of the reproductive results compromised by that situation. Two different non-exclusive approaches can be taken for the antioxidant treatment, treating the male patient with oral supplementation or treating the sample in vitro during ART procedures.

55.5.1 Treating the Male Patient

Some non-enzymatic antioxidants can be endogenously produced in cells, but others need to be incorporated from food or supplements. Dietary intake increases antioxidant presence in seminal plasma, and it may also affect other biological processes. It is believed that an excess of antioxidants could have a pathological effect within the organism due to an imbalance between oxidation and reduction, namely the antioxidant paradox [2]. Nevertheless, oral treatment with antioxidants for the patient has proven to generally ameliorate the classic parameters evaluated in the routine semen analysis, to the point that in some cases ART is not necessary anymore [41]. Furthermore, in the cases that ICSI is still needed, this improvement might make it easier to find a suitable spermatozoon to inject, especially regarding morphology.

Few studies have been conducted following an adequate design as well-powered randomized placebo-controlled trials (RPCT) with an adequate sample size and proper main outcome established. Most of the studies only use the routine sperm analysis to evaluate their results (sperm count, concentration, motility and morphology) (Table 55.1), although

Table 55.1 Studies where only classic sperm parameters were measured

Author	Sample size	Type of control and study population	Compound	Daily dose	Time (months)	Semen analysis improvement
ElSheikh (2015) [53]	$T = 30$	Baseline Infertile men with idiopathic oligoasthenozoospermia	Clomiphene citrate	25 mg	6	C +3.2 ×10⁶ spz/mL TM +10.33%
	$T = 30$		Vitamin E	400 mg	6	C +0.7 ×10⁶ spz/mL TM +6.4%
	$T = 30$		CC + Vitamin E	25 + 400 mg	6	C +4.83 ×10⁶ spz/mL TM +16.83%
Cyrus (2015) [55]	$P = 69$ $T = 22$	Placebo-controlled Infertile men with varicocele, before and after varicocelectomy+treatment	Vitamin C	500 mg	3	N +15.9 ×10⁶ spz PM +20.8% Nm +23.2%
Rafiee (2016) [56]	$T = 75$	Baseline Infertile men with suboptimal semen quality	Vitamin C	1 g	6	Vol NS C +9.63 ×10⁶ spz/mL TM +6.95% Nm NS
Lipovac (2016) [57]	$T = 143$	Baseline Subfertile men with at least one recent pathological semen analysis	Profertil®	–	3	Vol +0.7 mL PM +15.2% Nm +9.7%
	$T = 156$	Baseline Subfertile men with at least one recent pathological semen analysis	L-carnitine	500 mg	3	Vol +0.5 mL PM +9.2% Nm +9.6%
Çinar (2016) [61]	$T = 81$	Baseline Men with severe or refractory acne vulgaris	Isotretinoin[a]	0.66 mg/kg	6	C +1.66 ×10⁶ spz/mL N +1.393 ×10⁶ spz PM +3.05% Nm NS

[a]Isotretinoin is a common treatment for acne with a variety of demonstrated side effects and speculated effect on fertility

in some cases other sperm parameters, such as DNA fragmentation or ROS levels, are assessed as well (Table 55.2); however, still very few studies can be found that perform and evaluate ICSI clinical results in terms of embryo quality or pregnancy/livebirth outcome (Tables 55.3 and 55.4). Furthermore, the variability among the types and doses of antioxidants and other substances proposed in scientific literature makes it very difficult to compare results and draw valid conclusions about the benefits of each one in improving reproductive outcome.

Table 55.2 Clinical trials where any OS biomarker was measured

Reference	Sample sizes	Type of control and study population	Compound	Daily dose	Time (months)	Semen analysis improvement	OS biomarkers
Alsalman (2018) [44]	*Cg* = 60 *T* = 60	Fertile men without treatment versus asthenospermic men before and after treatment	Zinc	440 mg	3	Vol +0.56 mL C +3.6 ×10^6 spz/mL N +23 ×10^6 spz PM +18% Nm +12%	(RSH/RSSR)[a] *Cg* = 1 *T* = 0.7 at baseline *T* = 1.55 after treatment
Raigani (2014) [45]	*P* = 18 *T* = 24	Placebo-controlled Infertile OAT men	Zinc	220 mg	4	NS	TAC NS ROS NS DNA abnormality 45% → 40%
Nadjarzadeh (2014) [49]	*P* = 24 *T* = 23	Placebo-controlled Infertile OAT men	Coenzyme Q10	200 mg	3	C NS PM +3% TM +5.78% Nm NS	+catalase activity +SOD activity (no numerical data published)
Haghighian (2015) [46]	*P* = 21 *T* = 23	Placebo-controlled Infertile asthenozoospermic men, some of them OAT	Alpha-lipoic acid (ALA)	600 mg	3	Vol NS N +12.55 ×10^6 spz C +3.72 ×10^6 spz/mL PM +5.51% Nm NS	TAC +58.9% MDA −22.9%
Martinez-Soto (2016) [48]	*P* = 25 *T* = 32	Placebo-controlled Subfertile men	Docosahexaenoic acid (DHA)	1.5 g	2.5	NS	TAC +10% (+0.8% in placebo)
Alizadeh (2018) [50]	*P* = 28 *T* = 28	Placebo-controlled Infertile OAT men	Curcumin nanomicelles	80 mg	2.5	Vol NS N +10.82 ×10^6 spz C +3.21 ×10^6 spz/mL PM +4.82% TM +5.27% Nm +2.42%	TAC +57.2% MDA −23.9%
Guo (2015) [52]	*T* = 41	Baseline Oligoasthenospermic men	Indomethacin	50 mg	3	C + 4.33 ×10^6 spz/mL N + 11.59 ×10^6 spz PM + 5.56% TM + 5.05% Nm + 0.29%	ROS −4.3% TAC +0.8%
Guo (2015) [52]	*T* = 55	Baseline Oligoasthenospermic men	Tamoxifen	20 mg	3	C + 0.82 ×10^6 spz/mL N + 1.21 ×10^6 spz PM + 3.25% TM + 5.3% Nm + 0.19%	ROS −30.6% TAC +31.8%

Sample sizes are expressed as *T* for treatment group, and, when applicable, *P* for placebo group or *Cg* for control group without placebo. Semen analysis improvement is the result of comparing the study group after treatment with baseline values for volume (Vol), concentration (C), total sperm count (N), progressive motility (PM), total motility (TM) and normal morphology (Nm). Reports of not statistically significant differences have been noted as NS

[a]RSH/RSSR = Thiol oxido-reductive index value in seminal plasma

Table 55.3 ICSI procedure was included in the study and pregnancy statistics were recorded

Reference	Sample sizes	Type of control and study population	Substance	Time (months)	Fertilization rate	Good quality embryos D3	Pregnancy rate per transfer	Miscarriage risk
Korosi (2017) [51]	*Cg* = 13 *T* = 22	OAT men with or without oral antioxidant treatment	Folandrol®	2	*Cg* = 60′5% *T* = 84.8%	*Cg* = 32% *T* = 54′7%	*Cg* = 0% *T* = 50%	– *T* = 9%
Rago (2017) [47]	*Cg* = 59 *T* = 59	OAT men in previous ICSI cycles without oral treatment versus a cycle with oral treatment	Fertiplus SOD®	1	*Cg* = 52.8% *T* = 86.3%	+19.8% type A embryos −14.7% type B	*Cg* = 8.9% *T* = 44.8%	*Cg* = 100% *T* = 19%

Sample sizes are expressed as *T* for treatment group and *Cg* for control group (not on placebo)

Table 55.4 Pregnancy statistics were recorded but study included only evaluation of some classic sperm parameters of concentration (C), total sperm count (N), progressive motility (PM), total motility (TM) and normal morphology (Nm)

Reference	Sample sizes	Type of control and study population	Substance	Time (months)	Semen analysis improvement	Percentage of patients that reported pregnancies	Miscarriage risk
Canepa (2018) [41]	*T* = 100	Baseline Subfertile men with at least one altered semen parameter	Sinopol®	3	C +7.8 ×10^6 spz/mL N +22.6 ×10^6 spz PM +5.3% Nm +3%	40% = 7% IUI + 30% ICSI +3% naturally	–
Amory (2017) [62]	*T* = 19	Baseline Oligoasthenozoospermic men	Isotretinoin	5	C +1.3 ×10^6 spz/mL TM NS	31% = half spontaneous and half by ICSI	16.7%
Busetto (2018) [42]	*P* = 49 *T* = 45	Placebo-controlled Infertile men with at least one abnormal semen parameter, some OAT	–	6	C +10.6 ×10^6 spz/mL N +49.3 ×10^6 spz PM +5.2%	*P* = 4% *T* = 22%	*P* = 50% *T* = 0%

Sample sizes are expressed as *T* for treatment group and *P* for placebo group, when applicable. Reports of not statistically significant differences have been noted as NS

55.5.1.1 Trace Elements

Selenium is an essential trace element implicated in several major metabolic pathways. It takes part in the antioxidants defense systems as a cofactor for certain forms of the GPX enzyme. It is also related to the regulation of the thyroid hormone metabolism and immune function. Selenium levels in sperm have been correlated with sperm count and motility [42]. For these reasons, some combined antioxidant products include selenium in its formula (Table 55.5).

Zinc is a trace element than cannot be kept in the body, so we need to incorporate it through our diet. It has been found in infertile smokers that high ROS levels influence zinc levels, reducing them and thus affecting sperm physiology, as it may have a role in the regulation of capacitation and the acrosome reaction. Moreover, zinc is a hormone balancer for testosterone; its deficiency reduces the concentration of this hormone in serum, and this negatively affects spermatogenesis, causing sperm abnormalities. Zinc is also an antibacterial agent in the urea system of men, contributing to prostate and sexual health. Although several studies have found a significant correlation between Zn content in SP and sperm dysfunction and infertility, many studies could not find it and others found high Zn concentrations to be toxic for sperm, making it impossible to assure that its deficiency is a cause of infertility, although it may threaten sperm function. Therefore, this trace element has many potential uses in prevention, diagnosis and treatment of male infertility [43], and it also has an antioxidant quality that may provide protection against lipid oxidation [2]. A recent study found that Zn supplementation in asthenospermic men restored the oxidative stress indicator measured to almost normal values (comparing to a control group of healthy donors), apart from improving progressive sperm motility and morphology [44]. Oppositely, a recent RPCT could not find any significant improvement of semen parameters, TAC, ROS levels or zinc content in SP (Table 55.2), and only DNA integrity had a significant reduction of 5 points in the percentage of abnormal DNA after treatment when taking into account the placebo effect [45].

55.5.1.2 Alpha-Lipoic Acid

Also called thioctic acid, α-lipoic acid (ALA) is a coenzyme for two mitochondrial enzymes, α-ketoglutarate dehydrogenase and pyruvate dehydrogenase, which are key enzymes of the Krebs cycle, so ALA deficiency might be a limiting factor for energetic metabolism and ATP gen-

Table 55.5 Composition of the combined antioxidant products

Product	Folandrol ®	Sinopol®	Profertil®	–	Fertiplus SOD®
Reference	Korosi (2017) [51]	Canepa (2018) [41]	Lipovac (2016) [57]	Busetto (2018) [42]	Rago[a] (2017) [47]
Myo-inositol	1 g	1 g	–	–	–
Folic acid	200 µg	400 mg	800 µg	200 µg	–
a-lipoic-acid	–	800 mg	–	–	Yes
L-carnitine	30 mg	–	440 mg	1 g	–
Acetyl-L-carnitine	–	–	–	500 mg	–
L-arginine	30 mg	–	250 mg	–	–
Glutathione	–	–	80 mg	–	Yes
Betaine	–	100 mg	–	–	–
Fumarate	–	–	–	725 mg	–
Selenium	55 µg	–	60 µg	–	–
Zinc	–	–	40 mg	10 mg	Yes
CoQ10	–	–	15 mg	20 mg	–
Vit E	30 mg	–	120 mg	–	–
Vit C	–	–	–	90 mg	–
Vit B2	–	1.7 mg	–	–	Yes (described as B vitamins)
Vit B6	–	1.9 mg	–	–	
Vit B12	–	2.6 mg	–	1.5 µg	
Microencapsuled SOD (Extramel®)	–	–	–	–	Yes
Orisod® Complex	–	–	–	–	Yes

[a]Author did not detail the concentration of each component, only their presence

eration, thus affecting sperm motility and vitality. Furthermore, ALA and its reduced form DHLA, more frequent inside cells, have antioxidant properties, being able to scavenge ROS in both aqueous and lipid phases, chelate transition metals and prevent lipid peroxidation and protein damage via interactions with glutathione. Because of these characteristics, ALA has been suggested as an oral supplementation treatment for improving fertility by reducing oxidative stress in sperm and enhancing sperm quality. A triple-blind RPCT was conducted in 2015 on men with idiopathic asthenozoospermia, including some OAT. ALA supplementation was found to significantly increase sperm concentration, count and total motility, while volume and morphology were not significantly changed. Seminal oxidative stress biomarkers measured during the abovementioned study were MDA (stable product of lipid peroxidation) levels and TAC, finding ALA treatment to cause a significant mean augmentation of 58% seminal TAC and 22% mean decrease in MDA levels (Table 55.2). Therefore, ALA was suggested as a suitable antioxidant for medical therapy of asthenoteratospermia [46].

Both zinc and alpha-lipoic acid, together with glutathione and B vitamins, are in a product called *Fertiplus SOD*® that was offered to 59 couples in two Italian fertility clinics in 2017. All included couples had been diagnosed with tubal or idiopathic infertility and a mild idiopathic OAT male factor and had previously failed an ICSI cycle, which was used as control. The second attempt was considered as the intervention group where the antioxidant supplementation was introduced 1 month before ICSI. Results of the study compared endpoints of the first cycle to the second one, regarding fertilization rate, percentage of good-quality embryos, implantation rate per transferred embryo, clinical pregnancy rate per transfer and miscarriage relative risk (per clinical pregnancy) (Tables 55.3 and 55.4). Although negative correlation was found between female age and fertilization and clinical pregnancy rates, finally an important number of pregnancies were successfully carried to term after the supplementation treatment of the male partner. In the first attempt only five pregnancies were achieved and all of them miscarried, while after intervention 26 pregnancies occurred, and only five miscarried, which is very interesting considering that the number of embryos obtained was not statistically different. This would be partially explained by the increased fertilization rate (+33.6%) and type A embryos (+19.8%) and the decreased frequency of type B embryos (−14.7%), and the implantation rate, which in the first attempt was only 4.6%, went up to 20.3% after treatment. Interestingly, sperm parameters before and after the supplementation were not significantly changed, suggesting that the improvement in ICSI results may be due to its effect on the final stages of spermatozoa maturation [47].

55.5.1.3 Docosahexaenoic Acid

Docosahexaenoic acid (DHA) is an omega-3 polyunsaturated fatty acid found in extremely high levels in human ejaculates, associated with high sperm quality and fertility. That is why it has been considered an antioxidant with the potential to improve semen quality. An RPCT was conducted to evaluate the impact of a 10-week DHA treatment on sperm quality, finding seminal parameters not significantly changed, increased TAC and decreased DNA fragmentation. These results indicate an improvement in semen quality (Table 55.2) that may improve reproductive outcome, although those changes would not be evident in a routine semen analysis [48].

55.5.1.4 Coenzyme Q10

Coenzyme Q10 is a very powerful antioxidant and a critical intermediate of the mitochondrial electron transport chain that regulates cytoplasmic redox potential. It has been found to be present in a deficient concentration in infertile men, associated with poor sperm quality and severe conditions such as OAT. An RPCT conducted in 2012 showed a significant improvement in forward and total motility after 3 months of CoQ10 supplementation therapy, and a positive correlation between CoQ10 concentration in sperm and normal morphology, although the mean normal morphology among all subjects was not significantly improved, as well as sperm count and concentration. Although ROS levels were not measured, oxidative stress attenuation as indirectly evaluated by measuring enzymatic for SOD and catalase, finding it increased only in the treatment group with a positive correlation with CoQ10 concentration in sperm (Table 55.2). Hence, CoQ10 may attenuate oxidative stress by enhancing antioxidant enzymes activity, but it seemed not to be enough to neutralize the deleterious effects of ROS on sperm and male fertility [49].

55.5.1.5 Curcumin Nanomicelles

A recently conducted double-blind RPCT with OAT men analysed TAC and MDA levels in SP apart from performing routine semen analysis before and after 10 weeks of treatment with curcumin micelles. Sperm count, concentration and motility showed improvement in the intervention group compared to placebo and baseline values, although sperm morphology did not (Table 55.2). TAC in the intervention group was increased by 57% compared to baseline, while the placebo group showed no difference, and MDA level was almost 24% lower after treatment. This study also considered inflammatory factors and sexual hormones to be interesting parameters to evaluate the effects of the treatment, since the OAT men had abnormal baseline hormone levels that curcumin treatment was able to restore to normal values, thus offering a better approach than hormonal treatments because of the absence of side effects [50].

55.5.1.6 Myo-Inositol

Myo-inositol is an extensively studied molecule in the field of fertility, both in vitro or as an oral supplementation. It is the most biologically active form of inositol and it is synthesized in human testis from glucose-6P. Previous literature describes its implication in numerous physiological processes, among which the most interesting for reproduction are the regulation of seminal plasma osmolarity and volume, the expression of essential proteins for sperm chemotaxis, capacitation and acrosome reaction and the regulation of intracellular Ca^{2+} concentration (as an element of the signal transduction pathway), which is key for sperm function [31]. This supports its use in oral supplementation treatments for enhancing fertility, and the development of nutraceutical products including myo-inositol in its formulation, such as Sinopol® and Folandrol®, whose chemical formulations are detailed in Table 55.5.

Sinopol® was administered for 3 months to subfertile men in a prospective study comparing results with baseline values of classic sperm parameters before intervention. Significant improvement was observed on sperm concentration, count, percentage of progressive motile (+5.3%) and morphologically normal spermatozoa (+3%). Although OS biomarkers were not measured, and pregnancy was not an endpoint of the study either, patients' follow-up registered that 40% of them achieved pregnancy, most of them by ICSI (Tables 55.3 and 55.4). This could suggest a positive impact of the treatment, but there was no control group to compare with.

Folandrol® on the other hand, underwent a randomized controlled trial, although the sample size was smaller, and the control group was not on placebo. Two capsules per day were administered during 2 months to men with idiopathic oligoasthenoteratozoospermia and a history of previous failed ICSI. After the treatment was completed, the ICSI procedure was performed, and significant increases were observed for the fertilization rates (+24.3%) and the rate of good quality embryos at day 3 (+22.7%), while pregnancies were achieved only in the treated group but none in the control group (Tables 55.3 and 55.4). However, that improvement in reproductive results cannot be attributed only to the oral treatment, since it was combined with in vitro supplementation with 2 mg/mL myo-inositol for 2 h incubation before the injection [51]. Nevertheless, these results are very promising for the ART management of couples with a severe male factor such as OAT.

55.5.1.7 Antiestrogens

Tamoxifen (TAM) is a selective modulator of estrogen receptor, indirectly helping to increase testosterone synthesis and enhance spermatogenesis. This compound has been commonly used in the therapy of idiopathic male infertility under the premise that it may reduce oxidative stress and improve

mitochondrial functionality, thus improving sperm motility. A randomized clinical trial conducted in 2015 in men with idiopathic oligoasthenozoospermia compared the effects of TAM versus indomethacin administration (Table 55.2). TAM treatment showed significantly better results at improving sperm count and concentration compared to indomethacin after 3 months, although both substances slightly improved sperm motility. The relevance of this study is that measurement of ROS levels and TAC in SP were performed, finding tamoxifen to reduce ROS levels by 30.6% and increase TAC by 31.8%, while indomethacin values were −4.3% ROS and +0.8% TAC. ATP content in sperm was also measured, aiming to assess mitochondrial functionality, and an increased value was observed after both types of treatment. Although this trial was not placebo-controlled, it is safe to say that tamoxifen has far better results than indomethacin at improving the oxidative status of sperm in addition to classic sperm parameters [52].

Clomiphene citrate (CC) is a synthetic non-steroidal antiestrogen drug, such as Tamoxifen, sometimes used as an empirical medical treatment for idiopathic oligoasthenozoospermia, which is the most common cause of infertility. The aim of the use of this molecule is to stimulate spermatogenesis by blocking estrogen and testosterone receptors at the hypothalamus, thus indirectly enhancing FSH and LH secretion. A comparative randomized clinical trial was conducted to study its effects on sperm parameters in men with idiopathic oligoasthenozoospermia, finding that nearly 24% of the patients treated with CC for 6 months showed a significant improvement of both sperm concentration and motility (Table 55.1). Drawbacks of this study are the lack of placebo control group, that OS biomarkers were not measured and that further fertilization potential was not studied either. However, the aim of this study was to compare the use of CC alone and in combination with vitamin E as an antioxidant, finding the results more promising than alone, as 40% of the patients in the combined treatment experienced significant sperm concentration and motility improvement [53].

55.5.1.8 Vitamins

Vitamin E (lycopene) is a lipid-soluble vitamin with antioxidant properties able to protect essential fatty acids from oxidation. The potential of antioxidant supplementation with vitamin E, alone or in combination therapy, has been broadly examined to treat oxidative stress-induced male factor infertility in many studies during the past 20 years. Among those that had an RPCT design, without very large sample sizes, some did report an increase in sperm motility in response to vitamin E treatment and no improvement in sperm concentrations, others reported improvement in both parameters, and others did not find any significant improvement, hence leading to diverse conclusions on whether vitamin E supplementation is effective or not for treating male infertility. In the abovementioned comparative randomized clinical trial, only 10% of the patients that were treated for 6 months with vitamin E alone showed a significant improvement of sperm motility and almost no effect on improving concentration (Table 55.1), suggesting a poor response [53]. However, no study was found to report further reproductive potential tests or pregnancy outcomes after vitamin E treatment.

Ascorbic acid (vitamin C) is a water-soluble molecule that neutralizes ROS. Some have found no improvement or even a detrimental effect, while others have reported an increase in semen parameters (motility, concentration, morphology), DNA integrity and also higher pregnancy rates after this antioxidant therapy [54]. A study conducted in 2015 proved the intake of vitamin C (250 mg twice a day for 3 months) effective for improving seminal parameters in patients after varicocelectomy, especially motility and morphology, although they found no effect on sperm count [55]. Separately, a later study found that vitamin C consumption (1 g/day for 6 months) significantly improved sperm concentration and mobility but did not significantly change semen volume or the percentage of normal morphology [56].

55.5.1.9 Carnitines

L-carnitine is a hydrophile molecule present in the mitochondrial membrane as a free radical catcher and has been related to male fertility within the literature, describing an improvement of the parameters assessed via routine sperm analysis. A prospective open-labelled, nonrandomized study conducted in 2016 aimed to compare the effectiveness of a previously described carnitine supplementation treatment (500 mg twice a day) versus a combination of substances called *Profertil*® (including L-carnitine, formulation in Table 55.5) when taken once a day for 3 months. Although both treatments improved all sperm parameters when compared to baseline values, the combined treatment was found to enhance sperm density and total motility by 6% more than the mono-substance treatment, achieving 15% more motile spermatozoa (Table 55.1) [57]. Drawbacks of this study were the lack of randomization and a placebo group for control, the limited amount and variety of the analyses performed to assess sperm quality, obviating any measure of OS, DNA integrity or further reproductive function indicators, such as acrosome reaction or fertilization capacity, not to mention ICSI performance and results.

Finally, several combinations of metabolic substances, antioxidants and micronutrients (Table 55.5) have been shown to increase sperm count and concentration, total and progressive motility and morphology when comparing with placebo after 6 months of treatment in men with and without varicocele (separated groups) with oligo- and/or astheno- and/or teratozoospermia (Tables 55.3 and 55.4). Sample size was not very large but being a double blind RPCT study design gives strength to the results. Although further sperm

analysis and fertility tests were not conducted, and generation of ICSI embryos and pregnancy were not endpoints in this study, ten spontaneous pregnancies were reported in the treatment group (n = 45) and two in the placebo group (n = 49), one of them ending in miscarriage [42].

55.5.2 Treating the Sample

Laboratory manipulation of gametes and embryos during IVF procedures is known to be a source of ROS increasing the risk of OS that may lead to poor embryo quality and reduced reproductive success. Therefore, several research groups thought it would be interesting to introduce antioxidants in vitro as an attempt to restore the equilibrium avoiding the increase of ROS levels in the sample during laboratory manipulation by increasing the antioxidant capacity of the fluid where spermatozoa are contained, thus reducing the risk of oxidative stress and subsequent consequences. Several approaches can be used to choose the antioxidant and the moment to use it in the laboratory.

55.5.2.1 Embryo Culture Media

In 2014, a study was conducted to determine which of two commercial brands of embryo culture media allowed the better embryo development. They found the blastocysts developed under the culture media that contained a lower amount of ROS to be morphologically superior [58].

Following that line of thought, one could think that ROS production by the developing embryos could be used as a selection criterion for their transfer. A recent study found no significant correlation between ROS levels in culture media and embryo quality, development or arrest, or the likelihood of conception, though. However, differences in ROS levels were found between IVF and ICSI embryos, especially in the first 3 days of in vitro development, suggesting that the main source of ROS in these procedures is the sperm [59].

55.5.2.2 Sperm Treatment

Various studies have found that the addition of antioxidants in vitro helps to protect mature spermatozoa against OS due to the increased ROS production by the rest of the cells within ejaculate fluid (mainly leukocytes and immature sperm cells) [1]. It is well established that cryopreservation and thawing of sperm is a source of ROS that increases the risk for OS-derived damage to sperm quality, affecting not only the routine sperm analysis parameters but also DNA integrity and epigenetics, leading to increased chances of fertilization failure and poor embryo quality, implantation and pregnancy outcomes. Many agents have been investigated as in vitro additives to sperm culture or cryopreservation medium, aiming to ameliorate this situation [31].

One of them is *ascorbic acid* (aka vitamin C), which is a water-soluble vitamin that the human body is unable to produce but is present in fertile men's semen, acting as an antioxidant at a certain concentration that is reduced in infertile men's semen. Multiple studies have attempted to use ascorbic acid to improve sperm quality, both in vivo and in vitro, with contradictory results. As for the in vitro studies, the common approach was to incubate sperm samples in the presence of ascorbic acid, then measure semen parameters and oxidative stress. Semen analysis was manually performed in some of the studies, while computer-assisted in others, and different techniques have been used for OS assessment. One of the experiments showed morphology improvement after 1 h incubation of sperm with 600 µmol/L of ascorbic acid, while another found no improvement in morphology after 2 h and 4 h incubation (probably due to the excessive time). On the other hand, this same experiment showed that the concentration of vitamin C was able to protect sperm against heat-induced OS but not when H_2O_2 was added as a second OS inducer. A different study, however, showed decreased sperm motility after a similar incubation of asthenozoospermic samples [54].

Another promising substance is *N-acetyl-L-Cysteine (NAC)*, which has been investigated using bovine gametes to perform ICSI with cryopreserved sperm. Knowing that ROS reduce embryo quality, an experiment was conducted to compare embryo development and quality of three types of ICSI embryos: those injected with OS-induced and NAC-treated sperm, those injected with non-treated sperm after OS induction, and those injected with NAC treated sperm without OS induction. The intention was to find the NAC concentration range within which NAC alone does not diminish embryo quality (avoiding the antioxidant paradox) but is effective to avoid the deleterious effect of OS (that was induced by incubation with H_2O_2). This treatment seems to help maintain good embryo quality by protecting spermatozoa from oxidative stress, but they did not get to see further reproductive outcomes. Furthermore, NAC was proposed for testing in human sperm as a pre-ICSI treatment when using cryopreserved samples, aiming to reduce OS caused by cryopreservation and thaw processes [13].

A different case is that of **T-AT-peroxiredoxin 2**, a fusion protein investigated for its potential effect as a cryoprotectant when added to cryopreservation medium. An experiment was conducted comparing sperm quality for each sample in fresh with post-thaw quality when cryopreserved with and without the addition of this protein (three aliquots from each semen sample). Various parameters related to OS damage were assessed to evaluate sperm quality: motility, viability, mitochondrial potential, DNA fragmentation, ROS levels, lipid peroxidation, acrosome reaction and fertilization ability (zona-free hamster oocytes penetration test). They found the improvement in asthenozoospermic samples espe-

cially significant , but a certain cryoprotective effect was proved for every kind of sample with a reduction of oxidative stress indicators, thus improving the rest of the parameters [60].

The addition of *myo-inositol* to sperm in vitro is widely motivated by previous literature supporting its antioxidant properties and its possible protective effect against OS from multiple sources, although the exact mechanism is still not defined. Also, this molecule has proven to be well tolerated by spermatozoa in vitro, clearing it for the use in ART sperm preparation [31]. Some studies have found that incubation of semen samples with myo-inositol resulted in a significant increase in sperm motility and the number of spermatozoa retrieved after swim-up in both normozoospermic men and patients with abnormal sperm parameters. A study conducted in 2015 found that ICSI performance using spermatozoa prepared in media enriched with myo-inositol (2 mg/mL) resulted in increased fertilization rate and embryo quality. The same concentration was used 2 years later for OAT men, in combination with a 2-month oral antioxidant therapy including myo-inositol, showing an increase in fertilization index, embryo quality in day 3 and pregnancy rate [51]. A recent study incubated fresh and thawed human semen samples with myo-inositol for 15 minutes and found a significant increase in motility for both [31].

Another recent study aimed at using this substance as a cryoprotectant prior to the slow freezing cryopreservation protocol, incubating an aliquot of sperm for 20 minutes with 1 mg/mL myo-inositol and another aliquot without it as a control. Unfortunately, post-thaw concentration and morphology were not significantly different from before freezing in either group, and a not statistically significant improvement was found in total and progressive motility. The only parameter that showed an improvement in the supplemented samples, and only in those that were classified as abnormal, was cryo-survival rate (CSR = post-thaw TM / pre-freeze TM ×100), and hence it was suggested to be a more accurate measure for post-thaw sperm recovery. This suggests that myo-inositol could be useful for cryo-preservation of poor-quality semen samples [31].

55.6 Conclusions

The effect of OS on pregnancy rate by ICSI remains controversial, although its effect on time-to-pregnancy and on the classic sperm parameters has largely been proven. Interestingly, in the two studies discussed in which ICSI was performed, classic seminal parameters were not significantly changed by the antioxidant treatment. Factors that can appear in the routine semen analysis and that may indicate an abnormal oxidative condition are asthenozoospermia, hyperviscosity (related to MDA levels) and high levels of leukocytes, immature sperm cells or morphologically abnormal spermatozoa with residual cytoplasm, since those cells are the main producers of ROS in the ejaculate. Anamnesis in search for possible OS inducing factors in the first visit followed by lifestyle recommendations and/or oral antioxidant supplementation might be a good and economic strategy for improving reproductive probabilities by natural means. In patients already undergoing ART with a history of previous failed cycles or miscarriages, testing for oxidative stress when other causes of infertility have been discarded might be a good approach, but currently no fertility guideline recommends ROS testing systematically or antioxidant supplementation treatment depending on the results of OS found.

55.7 Review Criteria

A comprehensive research of studies examining the relationship of oxidative stress, antioxidants and ICSI outcomes was performed using search engines, such as Pubmed, Google Scholar and ScienceDirect. The search period comprised 3 months (from November 2018 until January 2019). A first revision was performed to compile the most current articles about this topic based on the keywords “sperm oxidative stress”. The articles were classified according to their content and the type of study. Review studies were specially selected to answer the first two questions in this review. While a data extraction, based on prospective studies and meta-analysis, was included to answer the last questions, using the additional following keywords: “antioxidants AND ART outcomes” and “sperm oxidative stress AND ART outcomes”, focusing the data analysis mainly in ICSI cycles.

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Role of Sperm-Hyaluronic Acid Binding in the Evaluation and Treatment of Subfertile Men with ROS-Affected Semen

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Key Points

- Oxidative stress (OS) and consequential DNA chain degradation is one of the most relevant contributors to male factor infertility, affecting paternal contribution of sperm to the embryo.
- Sperm that are damaged by OS do not bind to zona pellucida (ZP), and sperm-hyaluronic acid (HA)-binding assay assesses the proportion of spermatozoa that would bind to ZP.
- Spermatozoa selected by the HA-mediated approach are viable and devoid of DNA fragmentation, persistent histones, and apoptotic markers, and the frequencies of chromosomal disomies and diploidies are within the normal range.
- Systematic reviews and meta-analysis performed to date suggested that identification of patients that might benefit from this technique needs further studies, and well-designed trials are needed.
- Thus, HA selection may be a feasible compensatory approach for men who did not respond to antioxidant treatment yet wishes to father children.

56.1 Introduction

Reactive oxygen species (ROS) are free radicals that have one or more unpaired electrons capable of oxidizing adjacent biomolecules, and their contribution to male infertility has been widely reported. The current literature reports that ROS may be a contributing factor in 30–80% of infertile men [1]. Excessive levels of free radicals diminish the functional integrity of spermatozoa, and ROS levels negatively correlate with the quality of sperm in semen, thus fertility. In clinic, there is still a search for objective selection of healthy sperm for intracytoplasmic sperm injection (ICSI), and the potential relationship between abnormal sperm morphology and chromosomal aberrations has been of a long-term interest. Hyaluronic acid (HA) is the natural secretion of the cervical mucus and the cumulus-oophorus complex. Sperm can selectively bind to HA, and HA-bound sperm are viable, have normal range of chromosomal disomies and diploidies, and are devoid of DNA fragmentation, persistent histones, and apoptotic markers, such as caspase-3.

In this part of the review, we summarize the scientific rationale behind human sperm selection with hyaluronic acid-binding assay (HBA) and the main findings of clinical studies related to HA-binding-mediated ICSI sperm selection. The literature was reviewed for updates on the markers of oxidative stress and assessment of ROS in semen. Additionally, we discuss sperm properties associated with ROS-related DNA defects, improved cellular attributes of HA-selected sperm, and evaluation of subfertile men with ROS-affected semen. Since HA-mediated sperm selection for ICSI may optimize fertilization rates and sperm quality with respect to the various parameters, it may be suggested that HA-mediated selection of mature sperm may facilitate the avoidance of sperm affected by oxidative damage and may be a treatment opportunity for male infertility due to oxidative damage of sperm.

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56.2 Human Sperm Selection with Hyaluronic Acid-Binding Assay (HBA): The Scientific Rationale Behind a Novel Technology to Select Best Sperm in ART

During natural fertilization, a competent sperm which was selected throughout the female reproductive system – in the vagina, cervical canal via cervical mucus, uterus, and fallopian tubes – will fertilize the oocyte. Viability and motility of sperm are important aspects of sperm fertility; however, out of many millions of sperm ejaculated, a few numbers of sperm reach the fertilization site during natural conception. Understanding and clarifying in vivo sperm selection mechanisms in the female reproductive system will help to develop useful tools for mature sperm selection for in vitro fertilization (IVF) therapies by understanding the nature of a fertile sperm. Major advances have been made regarding the biochemical markers of human sperm development and function over the years. In a review by Sakkas et al. [2], characteristics of in vivo selected sperm with various in vitro sperm selection methods have been compared systematically. Although gradient-based systems that select motile and to some extent mature sperm are being used routinely in IVF laboratories, more sophisticated sperm selection technologies mostly rely on specific cellular characteristics of sperm such as sperm membrane integrity, DNA integrity, surface charge, or evaluation of sperm morphology at high magnifications. These recent techniques are not used in routine but under clinical investigation regarding their potential for selecting the best sperm for IVF treatments for conceiving healthy take-home babies. One of these sperm membrane maturity-based selection technologies is sperm binding to hyaluronic acid (HA).

Human sperm cellular maturation, which is necessary to attain zona pellucida (ZP) binding and oocyte fertilizing potential, comprises three interrelated and synchronized events: (1) extrusion of excess cytoplasm, which is a phase of normal development, facilitates the formation of normal sperm shape; (2) sperm membrane remodeling in spermiogenesis and acquisition of ZP- and HA-binding sites; and (3) sperm nuclear maturation with the milestones of histone-transition protein-protamine replacement with the associated changes in DNA packing and chromosomal status. In the past three decades, the Huszar lab has studied several key events of sperm maturation, including cytoplasmic extrusion (can be determined by low creatine kinase (CK) content) and expression of the HspA2 chaperone protein independently from the classical semen parameters where the proportional concentrations of CK and HspA2 (or HspA2 ratio) reflect the proportion of mature and immature spermatozoa in semen samples [3–12]. Spermatids with low HspA2 expression are characterized by cytoplasmic retention, abnormal head morphology, increased concentrations of reactive oxygen species (ROS), and consequential additional DNA fragmentation. Further, due to the incomplete spermatogenesis, the process of plasma membrane remodeling and diminished formation of the ZP- and HA-binding sites failed to occur. Thus, arrested-maturity spermatozoa are unable to bind to the zona pellucida or fertilize via natural or IVF conception, but only by intracytoplasmic sperm injection (ICSI). This was demonstrated in sperm-hemizona complexes immunostained with CK, in which all hemizona-bound spermatozoa were clear-headed without cytoplasmic retention [5, 9, 13]. Sperm with normal development that are able to bind to HA and to ZP show high DNA integrity and a high proportion of sperm with normal Tygerberg morphology [12, 14–16]. In experiments related to sperm function and fertilizing potential, Huszar et al. have established that, simultaneously with cytoplasmic extrusion during terminal spermiogenesis, there is a remodeling of the plasma membrane that facilitates the formation of the ZP- and HA-binding sites [13]. Studies with HA immobilized to glass slides or Petri dishes showed that sperm firmly bind to HA. However, not all sperm exhibited HA-binding ability [17, 18]. These data supported the hypothesis that the ability of sperm-HA binding is related to sperm cellular maturity. The validity of this concept was confirmed by studying the properties of both HA-binding and HA-nonbinding spermatozoa, utilizing the various biochemical markers of spermatozoa maturity and function. It has become clear that only spermatozoa that fully completed the maturation process in late spermiogenesis, along with membrane remodeling, cytoplasmic extrusion, and the steps of histone-transition protein-protamine replacement, are able to bind to HA.

Based on the presence of the HA receptors in fully developed spermatozoa, but not in spermatozoa with arrested development, and absence of plasma membrane remodeling HA selection of single mature spermatozoa with high DNA integrity and low frequencies of chromosomal aneuploidies (a fivefold decline in X, Y, and XY disomies) for ICSI [19], further attributes of HA-bound spermatozoa indicated that the HA-selected sperm fraction is devoid of cytoplasmic retention, persistent histones, DNA fragmentation, and the apoptotic marker caspase-3 [10]. These properties are very important because nuclear and cytoplasmic immaturities, particularly the presence of DNA fragmentation, are known to adversely affect the paternal contribution of sperm to the zygote [11, 12, 14, 15, 20–25].

56.3 Clinical Significance of HA-Binding-Mediated ICSI Sperm Selection: An Update

With respect to the sperm selection for ICSI, the potential relationship between abnormal sperm morphology and chromosomal aberrations has been of a long-term interest.

Examination of the same individual spermatozoa for both shape and chromosomal aneuploidy has become possible because it was established that spermatozoa preserve their shape following the decondensation and denaturation steps that are a prerequisite of fluorescence in situ hybridization analysis [22]. These experiments revealed that visual shape assessment, i.e., choosing the "best-looking" spermatozoon, is an unreliable method for ICSI selection of mature haploid spermatozoa [26, 27]. The increased rates of chromosomal aberrations and other potential consequences of using immature spermatozoa for ICSI are of major concern [28–30].

Many studies have been performed to compare the clinical outcomes of HA-selected sperm with conventional sperm selection techniques. Although a study concluded that HA sperm selection has no beneficial effects to improve fertility with regard to early stages of embryo development [31], many studies declared improvement in fertilization rate, embryo quality, and implantation rates [32–37]. In a prospective randomized study to evaluate the effect of HA sperm selection on the ICSI outcome of patients with unexplained infertility having normal semen parameters, while fertilization rates and clinical pregnancy rates between the ICSI and physiological ICSI (PICSI) (hyaluronan-treated Petri dishes) groups were similar, a higher pregnancy loss rate was observed in the ICSI group (25% vs. 12%) as compared to the PICSI group, but the difference was not statistically significant [38]. A recent study compared conventional morphology sperm selection (ICSI-PVP) and selection through PICSI, when male factor was associated, and found that PICSI had a considerably higher chance (≈fivefold) to achieve pregnancy than those who had sperm selected only by morphology assessment [39]. Teratozoospermic patients were those who benefited most with PICSI. The study suggested that the technique should be included in laboratory routine avoiding the selection of immature sperm with increased rates of peroxidation and DNA fragmentation, although closing their conclusion with caution that prospective and randomized studies should be applied to strengthen their suggestion.

Overall, to date, there is no consensus on the effect of HA-selected sperm on ART outcomes that can be drawn which has been evaluated in various systematic reviews [40, 41]. In 2014, a Cochrane Review included two RCTs where both evaluated sperm selection by HA binding for ICSI, but only one reported live birth [40]. The authors have concluded that although the evidence was insufficient to indicate sperm selected by HA binding improve live birth or pregnancy outcomes in ART, also no data on adverse effects were present. In 2016, a systematic review and meta-analysis by Beck-Fruchter et al. included seven studies and 1437 cycles where all studies showed an improvement in embryo quality and implantation rate, but the evidence did not support routine use of HA-binding assays in all ICSI cycles [39]. A systematic review in 2018 aimed to determine the efficacy of the PICSI vs. conventional ICSI in the prognosis of couples with male factor, with respect to live births, clinical pregnancy, implantation, embryo quality, fertilization, and miscarriage rates [42]. These authors have excluded twenty-one publications and included two publications out of their search and found no statistically significant difference between PICSI and ICSI, for any of the outcomes analyzed. Altogether, current systematic reviews and meta-analysis suggested that identification of patients that might benefit from this technique needs further study for use in clinical practice.

In 2018, based on the hypothesis that HA sperm selection in partial globozoospermia should help avoiding the injection of immature, DNA-fragmented spermatozoa, the success of a healthy childbirth and an ongoing pregnancy has been reported [43]. Failure of conventional ICSI (ICSI-PVP) that has been performed on sibling oocytes of the same couple suggested that HA-ICSI may be a safe, cost-effective, useful tool in cases of partial globozoospermia. The Hyaluronic Acid-Binding Sperm Selection (HABSelect) trial, being conducted in 15 centers across the UK, is designed to test the hypothesis that selection of sperm for injection using HA binding prior to ICSI has beneficial effects on clinical outcomes compared with standard ICSI and will be the largest male infertility trial undertaken to date in the UK [44]. The trial's main strength is its accommodation of clinical and basic science aspects that are fully complementary. The trial report should be available soon. Altogether, recent review of the literature on the efficacy with HA selection of human sperm reported a lack of efficacy but all with the same caution that is either inadequately powered or of low quality. Thus, well-designed further studies are needed.

56.4 Role of ROS Production in Sperm DNA Damage and Clinical Manifestations in Assisted Reproduction: ROS as a Threat for Male Fertility?

ROS have a significant role in many of the sperm physiological processes and in causing sperm damage [20, 45, 46]. Therefore, numerous reports have been published related to the impact of oxidative stress on spermatozoa with its beneficial and detrimental effects of ROS [47–51].

Low levels of free radical production by sperm may also facilitate sperm capacitation. Hydrogen peroxide stimulates the acrosome activation and hyperactivated motility, thus improving the transit of sperm through the cumulus and zona pellucida during fertilization. Low concentrations of hydrogen peroxide also cause tyrosine phosphorylation, which is associated with sperm membrane binding to the zona pellucida and sperm-oocyte interaction [52, 53]. However, excessive levels of free radicals diminish the functional integrity of spermatozoa [52–54]. Gomez et al. demonstrated that

levels of ROS produced by spermatozoa were negatively correlated with the quality of sperm in the original semen [55]. "Intrinsic," "extrinsic," and "iatrogenic" sources of ROS production were identified within semen: intrinsic sources of ROS in semen are morphologically abnormal and arrested-maturity spermatozoa and leukocytes. Poor sperm quality showing attributes of arrested sperm maturation is linked to increased ROS generation as a consequence of excess residual cytoplasm related to the arrest of cytoplasmic extrusion in terminal spermiogenesis. Simultaneously, in the Aitken and Huszar laboratories, correlations are found between sperm creatine phosphokinase content (a component of the residual cytoplasm) and lipid peroxidation in semen specimens. This suggested that both the increased ROS production and CK activity are related to increased cytoplasmic content of sperm [56, 57]. Further, sperm fractions with high and low (normal) cytoplasmic content and malondialdehyde (MDA) levels (representing ROS production) were co-incubated and co-centrifuged, to test the potential propagation of high ROS production from high to the low ROS-producing (normal) sperm fractions [57]. In these experiments, the ROS production did not increase in the combined low and high MDA sperm fractions. Thus, the conclusion was drawn that the increased ROS production (and higher cytoplasmic content) was an "inborn" rather than an acquired attribute of individual spermatozoa [3, 57–59]. Others also confirmed that sperm with excess cytoplasmic droplets were classified as immature and functionally defective cells and were one of the sources of increased ROS production.

High levels of ROS may damage the sperm membrane and DNA, leading to a decline in sperm fertilizing potential and paternal contribution to the embryo. DNA damage in sperm seems to be linked to the reduced fertilization rates, lower embryo quality and pregnancy rates, higher miscarriage rates, malformations, and childhood diseases [60]. Ghaleno et al. (2014) reported that the intracellular levels of hydrogen peroxide (H_2O_2) and superoxide ($O_2-\bullet$) correlate negatively with impaired sperm mitochondrial membrane potential leading to poor-quality pronuclear embryos [61]. Furthermore, the integrity of early embryonic development as well as implantation rates appears to be significantly reduced in patients with high paternal sperm DNA damage [62], suggesting that the second and third mitoses are the sensitive periods [63]. Currently, thiol oxidation, tyrosine nitration, and S-glutathionylation are reported as significant redox-dependent protein modifications associated with impairment of sperm function and alteration of paternal genome leading to infertility [64]. However, many unknowns remain.

The role of additional factors, such as leukocytes, particularly neutrophils and macrophages in semen, was shown to be associated with excessive ROS production and diminished sperm function. However, conflicting data are presented in the literature indicating no impact on the sperm fertilizing potential regarding relationship between male subfertility/infertility and the presence of genital tract infection and an increased number of leukocytes [65]. The seminal plasma includes natural antioxidants which act as a free radical scavenger which is crucial after normal ejaculation in vivo [66–69].

The presence of high levels of DNA damage in the spermatozoa is one of the major complications associated with male infertility. Among the lifestyle factors negatively influencing are cigarette smoking [49, 70]; drugs [71]; alcohol abuse [72]; heat exposure [73, 74]; metals, particularly transition metals such as iron and copper [75]; radiofrequency electromagnetic radiation [73]; environmental toxicants such as acrylamide [76]; air pollution; plasticizers; pesticides [77] and chloroacetanilide herbicides such as alachlor [78]; and obesity [50, 79].

Other factors that contribute to DNA damage are sperm storage and sperm separation techniques by enhancing ROS production. There are data indicating that sperm preparation during ART has the potential to aggravate sperm oxidative stress [80]. A contributing factor may arise from the removal of seminal fluid with its protective antioxidant content [81]. Cryopreservation of sperm, another commonly used technique in ART, is associated with an increase in sperm oxidative stress and an increase in DNA chain degradation. Indeed, with the method of in situ DNA nick translation, the degradation of DNA chain integrity following sperm cryopreservation [82], and particularly thawing [83], has been observed [84].

Reactive oxygen species formation was detected in 40% of the semen with spermatozoa from infertile patients with a related damage to the sperm membrane and DNA [85, 86]. Jones et al. reported that ROS-induced peroxidation of the sperm membrane diminishes its fluidity and increases membrane rigidity, thus reducing tail motion [87, 88]. Sperm membranes are vulnerable to this type of damage as they contain large amounts of unsaturated fatty acids. On the other hand, indirectly via end products, lipid peroxidation causes the formation of carbonyl-containing compounds such as MDA, various 4-hydroxy-2-alkenals such as 4-hydroxynonenal (4-HNE) [49], and 2-alkenals [89], which are known to be genotoxic and cancerogenic [49, 90]. Therefore, they affect male fertility, thereby possibly contributing to higher rates of malformations [91]. Directly or indirectly, by either mechanism, oxidative stress impairs sperm motility, their DNA integrity, and their competence for sperm-oocyte fusion [92–95]. Thus, the sperm-HA-binding assay may be an excellent test for the proportion of sperm with arrested cellular maturation, damaged sperm membrane integrity, and thus diminished fertilizing function in semen samples. Also, HA-mediated sperm binding facili-

tates the selection of individual, fully developed spermatozoa without ROS generation and with high DNA chain integrity [10, 14].

Normally, sperm DNA is tightly packaged with protamine, and the complex withstands well the attacks by free radicals. However, sperm with diminished maturity often exhibit excess histones detected by aniline blue staining [12, 96, 97], and the consequential deficient protamination leaves the sperm DNA more vulnerable to ROS attack [98]. Several investigators have now demonstrated connections between oxidative stress and sperm DNA damage [66, 94, 99, 100]. All available sperm DNA fragmentation assays are reviewed recently [101].

56.5 Sperm Properties Associated with ROS-Related DNA Defects, Improved Cellular Attributes of HA-Selected Sperm, and Evaluation of Subfertile Men with ROS-Affected Semen

Following up on the attributes of arrested sperm cellular maturation and the potential relationships between early and late spermatogenetic failures and between nuclear and cytoplasmic events, solid support was developed by studies of double-stained individual spermatozoa [12]. Testing of the same spermatozoa with aniline blue staining and CK immunostaining (persistent histones and cytoplasmic retention), aniline blue staining and caspase-3 immunostaining (persistent histones and apoptosis), and aniline blue staining and DNA nick translation (persistent histones and DNA chain degradation) demonstrated that the presence or absence of such cellular attributes shows an impressive, approximately 70% agreement [12]. Thus, there is a solid relationship between the early and late spermatogenetic events within the same sperm whether one probes the nuclear or cytoplasmic compartment. This experiment is important for sperm fertilizing potential by indicating the lack of sperm membrane remodeling, and diminished ZP or HA binding is influenced by upstream events, as the HA-bound spermatozoa do not exhibit staining with probes detecting arrested sperm development, such as aniline blue staining of retained histones, cytoplasmic retention, or presence of the apoptosis marker caspase-3 and DNA chain degradation, attributes that are collectively related and reflect elevated sperm ROS levels.

Although clinical success of HA-selected sperm has still limitations as mentioned above, it still keeps the potential for future applications. To our knowledge, no study tests the HA-binding ability of ROS-affected sperm. Moreover, it would be also interesting to compare the outcomes of physiological ICSI vs. PICSI in ROS-affected subfertile males. However, there are few recent reports in the literature regarding ROS and HA-bound sperm. In 2015, a study evaluated the correlation of HBA with conventional semen parameters, lipid peroxidation, intracellular ROS, DNA fragmentation, DNA maturity, and mitochondrial membrane potential levels in human spermatozoa and found that HBA is not a sensitive test for prediction of sperm intracellular ROS [102]. Another study in 2017 investigated the oxidation-reduction potential of human spermatozoa selected for ICSI after PVP or HA exposure and found that sperm incubation in PVP and HA yields similar outcomes with regard to oocyte activation and embryo development, whereas PVP provides more antioxidative protection [103]. A recent study in 2018 evaluated protein kinase C and tyrosine kinase participation in intracellular signaling and oxidative metabolism in HA-induced capacitation of cryopreserved bull spermatozoa and found that HA intracellular signal system may modulate capacitation with a lower oxidative metabolism than heparin in cryopreserved bull sperm [104].

56.6 Markers for Sperm Oxidative Stress

There are several potential causes of sperm oxidative stress, including various pathogenetic origins, such as idiopathic, iatrogenic, lifestyle, environmental, infection, chronic diseases, autoimmune, and testicular [105–107]. Male infertility is a multifactorial condition in which oxidative stress plays a central role [107]. As mentioned above, this eventually results in the formation of MDA, 4-HNE, and acrolein [107]. Therefore, these reactive aldehydes are known to be accepted as markers for oxidative stress. Furthermore, it was observed that they subsequently end up damaged by reacting with positive, hydrophilic amino acids in proteins causing not only ROS production but also mitochondrial dysregulation and ROS leakage from the inner mitochondrial membrane.

The routine laboratory test "sentinel signs" suggesting the possible presence of sperm oxidative stress is summarized in a review [105]. These include poor sperm motility, teratozoospermia, high number of leukocytes in semen, increased semen viscosity, poor sperm membrane integrity on hypoosmotic swelling test (HOST), poor fertilization in routine IVF, poor sperm motility after overnight incubation, and poor blastocyst development in the absence of a clear female factor (diminished paternal contribution by sperm), in addition to potential female factors (such as maternal age of >40 years or poor ovarian reserve).

Recently, oxidation-reduction potential (ORP) has been suggested as a new marker for oxidative stress by using MiOXSYS System [108], a system that has shown promise as a diagnostic tool in the evaluation of male infertility. The ORP test has advantages since it is a cost-effective and convenient option for patients. However, the interpretation of the

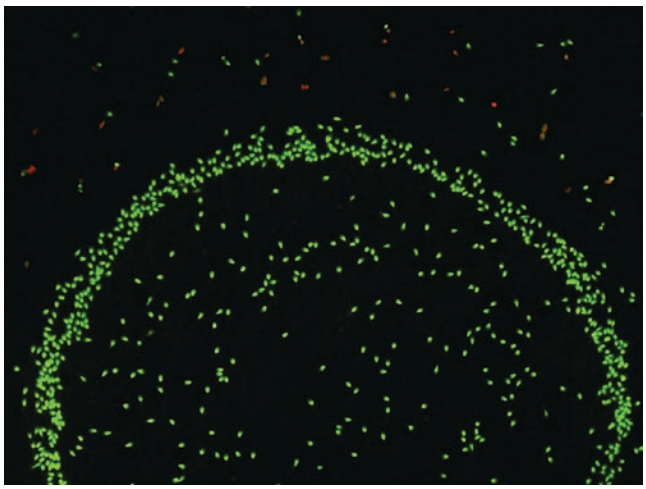

Fig. 56.1 Acridine orange-stained sperm (DNA-damaged sperm, red; high DNA chain integrity sperm, green). Perimeter of hyaluronic acid spot – washed semen sperm (red and green), middle of PICSI (inside the hyaluronic acid spot ring). Hyaluronic acid-selected sperm, green. (Reprinted from Ref. [110]. With permission from John Wiley and Sons)

test is incomplete yet across different patient groups and to analyze therapeutic effectiveness. Therefore, its measurement has not been incorporated into standard clinical applications as a stand-alone test.

Sperm with cytoplasmic retention and abnormal shape have an increased capacity for ROS generation [56, 57, 109]. For instance, men with sperm concentrations in the normal range but with high frequency of sperm with arrested development, and thus diminished fertility, may show increased ROS levels and lower antioxidant capacity compared to fertile men. These changes in semen/sperm quality and function (including zona pellucida-binding ability) are detectable by the 10-min-long test by a lower sperm-HA-binding score (<65%). In the light of the research by the Huszar, Aitken, and other laboratories, one can also consider increased sperm cytoplasmic retention, caspase-3 apoptotic activity, increased proportion of sperm with aniline blue staining, and a low HA-binding score [4, 12, 15, 21, 56, 57]. Sperm selected by the HA-mediated methods of either by the hyaluronic acid-coated glass slide or by the IVF–ICSI "PICSI" dish have high sperm DNA integrity whether tested by the DNA nick translation method or by the acridine orange fluorescence assay (Fig. 56.1) [10, 110].

56.7 Assessment of ROS in Semen: Where Are We Now?

Whereas screening for oxidative stress would be important in andrology laboratories, there are three factors that hinder this development: (a) the cost of the tests, (b) the complexity of testing, and (c) the lack of a generally accepted assessment/approach of oxidative stress measurements. Currently, there are over 30 different assays for the assessment of seminal oxidative stress [111–113].

Direct assays include chemiluminescence, nitroblue tetrazolium (NBT) test, cytochrome *c* reduction, flow cytometry measurement of hydrogen peroxide and superoxide anion, electron spin resonance, and xylenol orange-based assay [114]. Luminescence methods based on either luminol or lucigenin are used frequently to detect ROS production within semen [115]. One of the main concerns with this technique is the presence of leukocytes as a confounding factor. Therefore, fresh semen samples with high sperm count (>1 × 106/mL) are needed [116]. The concentration of reactants, sample volume, incubation time, pH, reagent injection, temperature control, instrument sensitivity, and background luminescence are the other factors that can also affect the results [117].

A variety of redox-sensitive fluorescence probes can be loaded into spermatozoa and subsequently monitored by flow cytometry or fluorescent microscopy. The advantages of fluorescent techniques can be summarized that they have a higher specificity, accuracy, sensitivity, and reproducibility compared to chemiluminescence for intracellular ROS. However, these assays are sophisticated and expensive hardware is needed, but also the results do not quantify the target ROS but simply show the percentage of cells with high level of activity with no information regarding the concentration or cellular content of the metabolites being analyzed [118]. The general introduction of these assays in clinical andrology laboratories has been slow because of the expensive equipment (luminometer) and high levels of necessary quality control, to standardize incubation times and seminal plasma contaminations [119, 120]. In addition, no clear reference values for seminal ROS in order to help predict fertility outcomes from different studies are accepted yet, probably due to the small sample size of patients studied, the involvement of patients with different pathogenesis, or the use of non-standardized assays for the assessment of ROS [121].

Nitroblue tetrazolium test is another test for assessment of seminal oxidative stress to determine the state of activation of seminal leukocytes. The NBT reduction test is commonly available, easily performed, inexpensive, and has high sensitivity [122]. Even though previous reports have established cutoff values for the amount of formazan to determine the fertility status of individual subjects, its use within clinical andrology laboratories is hampered by a lack of published normal ranges due to relatively small groups, and the results are need to be validated by other large multicenter trials. The method is reported to be relatively ineffective in samples with leukocytic contamination or low sperm concentration [123]. Early detection of novel biomarkers such as TLR2, TLR4, COX-2, and Nrf-2 is suggested to facilitate better

therapeutic interventions to analyze oxidative stress-induced infertility in semen of leukocytospermia patients [124].

Indirect methods include measurement by myeloperoxidase test, measurement of redox potential, lipid peroxidation levels, levels of chemokines, antioxidants, and antioxidant enzymes measuring levels of DNA damage and proteomic alterations [114]. The primary approach is the assessment of sperm MDA levels with the thiobarbituric acid assay. Other assessment methods of sperm membrane lipid peroxidation based on isoprostane 8-iso-PGF2a or the c11-BODIPY assays are promising but are not used widely [96, 125, 126]. MDA levels in sperm are quite low. Therefore, the use of sensitive high-pressure liquid chromatography (HPLC) equipment or iron-based promoters and spectrofluorometry measurement is required [127–129]. Another option is to assess the activity of antioxidant enzymes (SOD, catalase, glutathione peroxidase, and reductase) as well as the redox potential defined by the ratio of oxidized and reduced glutathione [130].

Since oxidative stress seems to be one of the primary causes of damaged sperm DNA [54, 99, 131, 132], sperm DNA can also be damaged by nonoxidative mechanisms, such as aberrant apoptosis and incomplete sperm protamination. Sperm DNA oxidative damage is reflected by the levels of oxidized deoxynucleoside, 8-OHdG, in sperm or seminal plasma [96, 133]. Indeed, a study has reported that chances of natural conception are inversely related with sperm 8-OHdG levels [134]. Immunohistochemistry or western blot analysis has been used to study and quantify oxidative DNA adduct 8-OHdG [135]. However, commercial kits are readily available to assess 8-OHdG directly.

Measurement of total antioxidant capacity (TAC) within semen is another approach via the inhibition of chemiluminescence generated by a constant added source of ROS (e.g., horseradish peroxidase). The total antioxidant capacity may be quantified against a vitamin E analogue and expressed as a ROS-TAC value [136]. Since the impact of ROS on fertilization and pregnancy is controversial partly because of the lack of consensus on what type of patients may be suitable for ROS testing and also what kind of assay should be used, routine ROS and TAC testing is not currently recommended as a part of a standard male infertility evaluation [113].

In order to use a molecularly healthy semen sample for insemination, a quantitative PCR (qPCR)-based technique that can be used for DNA evaluation in specific genes (PRM1, BIK, FSHB, PEG1/MEST, ADD1, ARNT, UBE3A, and SNORD116/PWSAS) is also recommended that could assist in selecting and improving cryopreservation protocols used in clinics [137].

Chemiluminescent and fluorescent methods are most commonly used in clinical settings for ROS testing. However, most of them do not identify the source of ROS (leukocytes vs. immature or abnormal spermatozoa) [138]. Due to the complexity of the abovementioned ROS measurements in human semen, a clinically useful test is needed for assessment of sperm DNA fragmentation or selection of sperm with high DNA integrity. Since sperm affected by free radicals have both DNA fragmentation and lipid peroxidation, the ability of HA binding can well discriminate the identity of mature vs. ROS-damaged immature sperm. The data powerfully indicate that diminished maturity sperm with DNA damage and arrested membrane remodeling (unable to bind to HA or the oocyte), thus handicapped in natural conception, are eliminated by HA-mediated sperm selection [10, 14].

56.8 Conclusions

Oxidative stress and consequential DNA chain degradation has been recognized as one of the most relevant contributors to male factor infertility, affecting the fertilization process and/or the paternal contribution of sperm to the embryo.

Regarding the diagnostic and therapeutic benefits of sperm-HA interaction, until the development of the sperm-HA-binding assay, there were no objective methods for the assessment of the proportion of native sperm that were affected by ROS, including plasma membrane damage and DNA chain degradation. Sperm-HA-binding assay introduced in the past 15 years for semen laboratories and for reproductive physicians is a welcome addition for four reasons:

(1) Sperm-HA-binding assay is a 10-min objective test that probes the integrity of the plasma membrane with respect to HA and ZP receptors. Thus, in addition to measuring the HA-binding score, the test reflects the proportion of sperm with full cellular maturity that are candidates for zona pellucida binding.
(2) The HA-bound sperm population was shown to lack DNA degradation by both the DNA nick translation assay and by acridine orange fluorescence [10, 110]. The HA-bound sperm also lack attributes of arrested sperm development, such as cytoplasmic retention, persistent histones detectable by aniline blue, and apoptotic processes.
(3) Further, HA-bound sperm fraction is enriched in sperm with Tygerberg normal morphology to the same extent as ZP-bound spermatozoa.
(4) The genetic integrity of the HA-selected sperm is supported by the findings that, in addition to the lack of DNA fragmentation, such sperm exhibit aneuploidy frequencies comparable to that of normozoospermic fertile man [10, 16, 19].

Beginning from 2008, various studies have been performed to compare the clinical outcomes of HA-selected

sperm to conventional sperm selection techniques. Although one study concluded that HA sperm selection has no beneficial effects to improve fertility with regard to early stages of embryo development, as assessed by the zygote score, many studies declared improvement in fertilization rate, embryo quality, and implantation rates. Altogether, systematic reviews and meta-analysis performed to date suggested that there is no consensus about routine use of HA-selected sperm in clinics; however, identification of patients that might benefit from this technique needs further studies. A successful healthy childbirth and an ongoing pregnancy in a case of partial globozoospermia by HA-sperm selection have been announced in 2018 [43]. Pregnancies from HA-selected sperm are promising to support the future applications. To date, there is no study in literature to test the HA-binding ability of ROS-affected sperm. Moreover, it would be also interesting to compare the outcomes of physiological ICSI vs. PICSI in ROS-affected infertile males.

Because sperm that are damaged by oxidative stress do not bind to the ZP, these male infertility patients are often treated with ICSI. However, following ICSI fertilization with visually selected spermatozoa, increased rates of de novo numerical and structural chromosomal aberrations and increased rates of spontaneous abortions and birth defects may occur [28, 139, 140]. HA-mediated sperm selection for ICSI may optimize fertilization rates and sperm quality with respect to the various parameters. Therefore, HA-mediated selection of mature sperm may facilitate the avoidance of sperm affected by oxidative damage. Thus, HA-mediated sperm selection may be a treatment opportunity for male infertility due to oxidative damage of sperm.

56.9 Review Criteria

An extensive search of studies examining the relationship between hyaluronic acid binding of human sperm and oxidative stress was performed using search engines such as ScienceDirect, OVID, Google Scholar, PubMed, and MEDLINE. The start and end dates for these searches were May 2018 and December 2018, respectively. The overall strategy for study identification and data extraction was based on the following keywords: "sperm-hyaluronic acid binding," "hyaluronan," "PICSI," "sperm function," "oxidative stress," "reactive oxygen species," "male infertility," and "intracytoplasmic sperm selection." Articles published in languages other than English were not considered. Data that were solely published in conference or meeting proceedings, websites, or books were not included. Websites and book chapter citations provide conceptual content only.

In Memoriam This chapter is dedicated to the memory of our wonderful colleague and mentor Dr. Gabor Huszar, a man with deep passion for andrology. He was an accomplished investigator in several areas of Reproductive Science with the ability to inspire and enthuse millions around the world. Dr. Huszar will be missed, but his memory will carry on in our hearts with our full respects.

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Male Gametes In Vivo to In Vitro: Clinical and Laboratory Management of Nonobstructive Azoospermia

57

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Key Points

- There is a considerable overlap in the diagnosis of obstructive and nonobstructive azoospermia.
- Testicular histopathology has limitations in predicting surgical sperm retrieval.
- Y-chromosome genetic testing forms the cornerstone in prognostication.
- Varicocelectomy can improve the surgical sperm retrieval as well as can downstage ART.
- Medical management may aid in correcting the altered hormonal milieu and can increase the odds of retrieving sperm.
- Monitoring the ejaculate for sperm and cryopreservation after any intervention is a good practice point.
- TESA can be offered in the diagnosis flowchart but carries the risk of damaging the only foci of spermatogenesis.
- MTESE is a more effective way of identifying and retrieving the best tubule.
- Good laboratory handling techniques of surgically retrieved sperm are essential for better outcome.

57.1 Introduction

Azoospermia is one of the most challenging clinical situations in male infertility. Though the classifications, diagnosis and management seem clear-cut in the literature, there is considerable overlap in a pragmatic setting. This chapter focuses on the clinical aspects of diagnosis and management of nonobstructive azoospermia (NOA).

57.2 Definition and Epidemiology

Azoospermia is defined as the absence of spermatozoa in the semen. If no spermatozoa are seen in the initial microscopic examination of wet film, the World Health Organization (WHO) recommends centrifugation of the sample (3000 *g* or greater for 15 minutes). If the pellet also shows absence of sperm, the semen analysis should be repeated. The presence of a small number of spermatozoa in either of the centrifuged samples is defined as cryptozoospermia, and the complete absence of spermatozoa is defined as azoospermia [1].

Incidence of azoospermia is 10–20% among infertile men and 1% in the general population [2]. Nonobstructive azoospermia includes all causes of azoospermia excluding the obstructive aetiologies and broadly involves both the pretesticular and testicular causes of azoospermia. In the pretesticular azoospermia, the hypothalamo-pituitary regulation of spermatogenesis is at fault, and in testicular azoospermia, the hypothalamo-pituitary gonadal axis is intact, but the spermatogenesis fails due to intrinsic defects in the testes. Since the reproductive ductal systems are functional and patent, these two causes fall into the category of nonobstructive azoospermia.

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57.3 Aetiology of NOA

Pre-testicular azoospermia may be due to endocrine abnormalities leading to low levels of sex steroids and gonadotropin levels. The causes can be congenital (e.g. Kallmann syndrome), acquired (e.g. acquired hypothalamic or acquired pituitary disorders) or secondary suppression (e.g. due to medication).

Testicular causes include congenital, acquired or idiopathic disorders that lead to spermatogenic failure. Congenital testicular causes consist of anorchia, testicular dysgenesis (cryptorchidism), genetic abnormalities (Y chromosome deletions), germ cell aplasia (Sertoli cell-only syndrome) and spermatogenic arrest (maturation arrest). Acquired testicular causes include trauma, torsion, infection (mumps orchitis), testicular tumours, use of gonadotoxic medication, irradiation, surgery (compromising vascularization of testis), systemic diseases (cirrhosis, renal failure) and varicocele [3].

Despite the absence of ejaculatory sperm in patients with NOA, targeted interventions and testicular sperm recovery techniques can help in achieving the pregnancy. In this chapter we reviewed the current scientific evidence and techniques in the management of NOA.

57.4 Testicular Histopathology in NOA

Testicular biopsy may aid in the diagnosis and prognostication of NOA.

Five major histological patterns of spermatogenesis have been described in testicular biopsy, namely, (i) absence of seminiferous tubules (tubular sclerosis); (ii) no germ cells within the seminiferous tubules (Sertoli cell-only syndrome); (iii) incomplete spermatogenesis, not beyond the spermatid stage (spermatogenic arrest); (iv) all germ cell stages present including spermatozoa, but with a distinct decline in the number of germ cells (hypo-spermatogenesis); and (v) majority of the times these patterns coexist giving rise to a mixed pattern [4].

Though various scoring systems including the Johnsen scoring have aimed at quantitatively classifying the histopathology, they lacked the correlation with the surgical sperm retrieval rates. The study by Esteves and Agarwal evaluated 356 patients with NOA and found that 19.5% of males with SCO and 40.3% of those with MA had sperm retrieved ($P = 0.007$). Surgical sperm retreival rates (SRRs) were significantly higher in men with hypo-spermatogenesis (SRR, 100.0%; $P < 0.001$). This study confirmed the popular notion that the main histological classification of the tubules had the best predictive accuracy for successful sperm harvesting [5].

57.5 Investigations in NOA

History and physical examination provide clues to the compartmentalization of NOA. Klinefelter syndrome patients may present with incomplete pubertal development, and body habitus may be abnormal. Small-sized testes give suspicions about defective spermatogenesis as testicular parenchyma and seminiferous tubules provide testes its volume. Hence, any defect in these leads to spermatogenic failure and small volume testes. But testes volume can be normal in spermatogenic failure due to maturation arrest [6]. Anabolic steroid abuse, history of anosmia or signs of pituitary tumours or disorders point to hypogonadotropic hypogonadism.

The confirmation of the diagnosis of NOA is very relevant as the prognosis is unfavourable when compared to obstructive azoospermia (OA). Many of the times, there would be considerable overlap between the signs and investigations of OA and NOA. Though NOA includes the pre-testicular and testicular causes, the spectrum of prognosis is in a wide range for these two conditions. The hypogonadotropic hypogonadism is characterized by follicle-stimulating hormone [FSH] and luteinizing hormone [LH] <1.2 IU ml^{-1}; testosterone [T] levels <300 ng dl^{-1} could be due to inherent hypothalamo-pituitary dysfunction or due to exogenous androgenic steroids. This category of patients show remarkable recovery to targeted therapies with exogenous gonadotropins or gonadotropin-releasing hormone agonist (GnRH-a) [7].

We perform hormonal tests in all cases of azoospermia and genetic testing for patients with suspicion of NOA due to spermatogenic failure.

57.5.1 Hormonal Testing

Serum measurements of FSH, LH, testosterone, oestradiol and prolactin are performed. Marked elevation of serum FSH (greater than two times the upper limit of normal) is a reliable indicator of abnormal spermatogenesis [8].

Highly elevated FSH and LH levels, along with low normal or below normal testosterone levels, suggest diffuse testicular failure and may have either a congenital (e.g. Klinefelter syndrome) or acquired cause. Since the feedback of FSH and LH secretions is driven by the number of spermatogonia and Leydig cells, respectively, FSH and LH values within normal range may be found in patients with hypo-spermatogenesis or maturation arrest [6].

Concomitant low levels of FSH and LH may involve hypogonadotropic hypogonadism. This condition may be congenital or secondary to a prolactin-producing pituitary tumour [9] Hyperoestrogenism secondary to a higher conversion rate of testosterone into oestradiol in Klinefelter

syndrome (KS) and obese patients inhibits testosterone production via a negative feedback pathway and may indicate the overexpression of aromatase CYP19 in the testis and in the adipose tissue [10].

57.5.2 Genetic Testing

Karyotyping and YCMD testing is routinely performed at our centre in cases of men with NOA due to testicular cause, and the aim is mainly to prognosticate surgical sperm retrieval and to assess potential risks to the offspring [11]. Patients with YCMD AZF a and b are counselled regarding the poor prognosis and are advised for donor gametes or adoption. Those with AZF c deletions are informed about the risk of 100% inheritance in male offspring.

57.6 Varicocele in NOA

Varicocele repair before ART in men with NOA and varicocele had been the subject for debate in the scientific forums. Pathophysiology of varicocele leading to azoospermia in NOA men is yet to be elucidated. In patients with good histological prognostication, varicocele repair might improve sperm retrieval rates. Varicocelectomy may also help to restore sperm production and result in appearance of sperm in the ejaculate. In a meta-analysis of three cohort studies, Esteves et al. concluded that SRRs are significantly higher in patients with NOA and clinical varicocele subjected to microsurgical varicocelectomy before sperm retrieval compared to those with no varicocele repair (OR, 2.65; 95% CI, 1.69–4.14; $P < 0.0001$) [12].

The interval between varicocelectomy and surgical sperm retrieval had been in a wide range in different studies. Since the duration of a spermatogenic cycle is around 64 ± 8 days, it is prudent to repeat semen analysis after 3 months. In a retrospective series, shorter interval (42.2 ± 8.9 months vs 80.0 ± 12.3 months) was associated with better pregnancy outcomes [13]. Since azoospermia may elapse post-varicocelectomy in men who show sperm in their ejaculates, sperm cryopreservation should be carried out as soon as viable sperm are identified in the post-operative semen analyses.

57.7 Medical Management in NOA

Though there is a lack of complete understanding of the intratesticular endocrine milieu of spermatogenesis, the intratesticular testosterone concentration through the androgen receptor is thought to be the key player. Therefore, the primary goal of the medical therapy is to optimize the hormonal levels before the surgical retrieval in the hope that in the neo background of higher testeosterone within the spermatogenic tubule, more foci of spermatogenesis would appear [14, 15].

57.8 SERM

Clomiphene and tamoxifen disrupt the negative feedback exerted by oestrogens at the hypothalamus and anterior pituitary, thus increasing the serum FSH and luteinizing hormone (LH) levels that then stimulate spermatogenesis and testosterone production. SERMs have been utilized for the management of idiopathic male infertility. The only series which have supported the effectiveness of clomiphene citrate in NOA patients have restricted their inclusion criteria to good prognosis histology patients [14]. The ongoing trial NCT02137265 is a randomized, double-blind, placebo-controlled trial of clomiphene citrate in hypoandrogenism and azoospermia.

57.8.1 Aromatase Inhibitor

The impairment of testosterone to oestradiol ratio, which results from decreased testosterone and excess conversion to oestradiol in men with NOA could be reversible and forms a potential therapeutic target. In two different trials, the aromatase inhibitor treatment (both steroidal and non-steroidal) did not yield any improvement in azoospermic men, though it improved the T/E2 ratio in all of them [16, 17].

57.8.2 Gonadotropins

The use of exogenous gonadotropins will inhibit the endogenous gonadotropin production which is hypothesized to 'reset' the gonadotropin receptors in the testes, which would eventually respond better. The literature has conflicting evidence in this aspect. Selman et al. and Efesoy et al. have reported the appearance of sperm in the ejaculate in men with nonobstructive azoospermia with spermatogenic failure after treatment with gonadotropins [18, 19]. Shiraishi et al. reviewed the data of 48 men with NOA who were second attempters for TESE [20]. Among them, 28 men received gonadotropin treatment consisting of hCG 5000 IU. Two months later, recombinant FSH (150 IU three times a week for 2 months) was added to those who had their FSH supressed, while the others continued to receive hCG until micro-TESE was repeated. While all the second attempters who did not take gonadotropin failed, 21% of the treated group had sperm retrieved by micro-TESE. Similar results

were obtained by Selman et al. by treating men with normal karyotyping and normal basal testosterone [18–21].

Hypogonadotropic hypogonadism is the best prognosis NOA. Men with these abnormalities respond to gonadotropin supplementation. The general consensus is to treat these men with FSH and hCG supplementation, and most of the existing studies support this. GnRH agonist therapy in a pulsatile manner can also be helpful, but the cost and the technical issues of injection preclude the use. It may be of use in men who fail to respond to exogenous gonadotropins [22–25].

In our centre, we treat all men with NOA who are candidates for surgical sperm retrieval with HP-HMG and hCG in a dose of 150 IU thrice in a week and 5000 IU twice weekly, respectively, for 3 months and anastrozole supplementation of 1 mg daily if the T/E2 < 10.

57.9 Monitoring and Freezing of Ejaculate Sperm in Successfully Treated NOA

Considering the length of a spermatogenic cycle, it is practised to re-analyse the semen after 3 months of medical therapy for spermatozoa. The analysis should be done in an embryology lab so that sperm if present can be cryopreserved.

57.10 TESA in NOA

Since there is a considerable overlap between the OA and NOA cases in the signs and laboratory parameters, we, in our centre, perform diagnostic TESA in all azoospermia cases except in cases of Klinefelter syndrome. In the latter, the testicular volume is very low, and the testes are firm with Leydig cell hyperplasia, which makes TESA more difficult and increases the risk of complications. Nonetheless, the disadvantage of this approach would be that we might remove the only remaining foci of spermatogenesis, thus jeopardizing success in future sperm retrievals [26]. Also, histopathology results by TESA, even if a pattern of SCO is shown, do not conclusively indicate presence or absence of spermatogenesis in a micro-TESE. If a trial TESA is done, it is mandatory that spermatozoa, if retrieved, are frozen. A part of the tissue is sent for histopathology to confirm the diagnosis of NOA.

57.11 Micro-TESE Case Selection

All trial TESA-negative cases are chosen for mTESE excluding AZF a and AZF b deletions. There is no correlation for the surgical sperm retrieval with serum FSH, testosterone and histopathology.

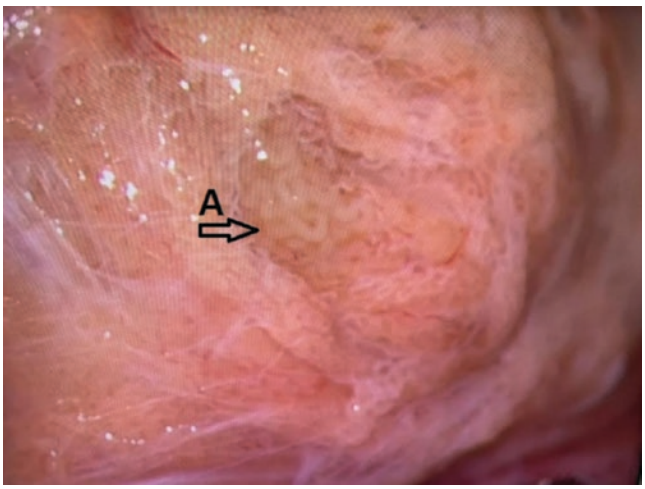

Fig. 57.1 Dilated seminiferous tubules as compared to the surrounding tubules

57.12 Micro-TESE Technique and Tubule Retrieval

All micro-TESE procedures should be performed by an andrologist with experience in microsurgery. In our centre, procedures are performed under spinal anaesthesia. The patient is positioned in a supine position. The testis is delivered and the tunica albuginea is opened to expose the seminiferous tubules. We perform microdissection TESE using a floor-standing operating microscope (OPMI Vario/S88 System, Karl Zeiss, Jena, Germany). The seminiferous tubules are dissected using up to 25× magnification. Areas with dilated tubules are identified, comparing it with the size of the tip of a 180 micrometer tissue forceps. Multiple sections of testicular tissue are obtained (Fig. 57.1), and these specimens are analysed for the presence of sperm after tweezing all the tubules. Sperm are collected and prepared for use in ICSI [27].

57.13 Micro-TESE Tissue Processing in IVF Laboratory

The main goal of the surgical team for micro-TESE should be to maximize the yield of viable and mature spermatozoa while minimizing the risk of patient risks and costs. The focus of the laboratory andrology team should be on minimizing the cell damage and maintaining the sterile environment as much as possible. Laminar flow cabinet are used during all laboratory steps to optimize sterile handling conditions. Micro-TESE fragments are handled [28] using 23 gauge needle tuberculin syringes, with needles bent parallel to the dish to remove blood clots and disperse the seminiferous tubules under a stereomicroscope. Then, the specimen

which is transferred to a dish containing fresh sperm medium is examined under inverted microscope, and the diameter of the tubules is determined using a digital imaging system (CIVA, Hamilton-Thorne, USA) attached to the inverted microscope. The images of individual seminiferous tubule at ×100 magnification are captured using the system. Measurements are taken in microns from edge to edge of the most dilated tubules, and the larger ones from each patient are considered for analysis (Fig. 57.2). Following this mechanical mincing of the tubules is done with tuberculin syringe till no tubules are seen. Homogenates are then examined on a warm staged inverted microscope at ×200–400 magnification to confirm the presence of sperm. These steps are repeated on all micro-TESE samples. A team of two laboratory technicians are involved in tissue processing – one to mince the tissue and the other to examine for sperms. The surgical team is promptly informed when the sperms are found. When the sperms are not observed after initial microscopic examination, the cell suspension is diluted with sperm medium and centrifuged at ×300 g for 7 minutes. The supernatants are discarded and the pellets are resuspended in approximately 0.2 mL of sperm culture medium. 1–5 microlitre of this testicular cell suspension is loaded onto the flat microdroplets of sperm medium prepared on petri dishes. Addition of theophylline (GM501 SpermMobil – Gynemed) eases the identification as twitching/progressive movement can be observed in most of the cases when sperm cells are present in suspension [29]. The simple in vitro incubation of fresh (overnight) or frozen retrieved sperm may help in obtaining a more viable and functionally normal sperm population that obviates the risks of using immotile unselected sperm for ICSI.

Testicular tissue removal is often 50–70-fold less in micro-TESE compared to conventional TESE [30, 31]. The small amount of tissue extracted increases the ease and speed of sperm processing as the number of contaminants are less. The larger amount of tissue removed on TESE may lead to missing of some occasional spermatozoa in the sea of contaminants.

TESE/micro-TESE may be scheduled either on the day of oocyte collection and ICSI or on the day before. In the latter, processed specimens are incubated in a closed HEPES-buffered culture system (microdrops under mineral oil) at room temperature (inside a laminar flow cabinet or in a clean room), to avoid bacterial contamination. Culture of specimens inside the incubator should often be avoided as contamination with scrotal skin-derived bacteria is seen. The data shows that the time frame should be less than 44 hours from hCG administration to microinjection in ICSI using surgically retrieved sperm for optimal fertilization [32]. Testicular tissue sperm processing, searching and selection of viable spermatozoa for ICSI may take several hours in NOA cases. We have observed that it takes around 12 minutes to handle an individual testicular spermatozoon from processing to microinjection in cases of NOA but only 5 minutes in OA. In other words, the average time required to perform ICSI in a standard NOA treatment cycle involving 8–12 metaphase II oocytes is approximately 2 hours. As such, it is recommended to carry out testicular retrievals the day before oocyte collection when a busy next day IVF laboratory workload is anticipated.

It has been suggested that testicular spermatozoa of men with severely impaired spermatogenesis have decreased fertilization potential and higher tendency to carry defects in the centrioles and genetic material, which ultimately affects the fertilization and embryo development [33]. In fact, the clinical outcomes of ICSI using surgically extracted testicular sperm in NOA are lower than those obtained with either ejaculated or epididymal/testicular sperm from men with OA [32].

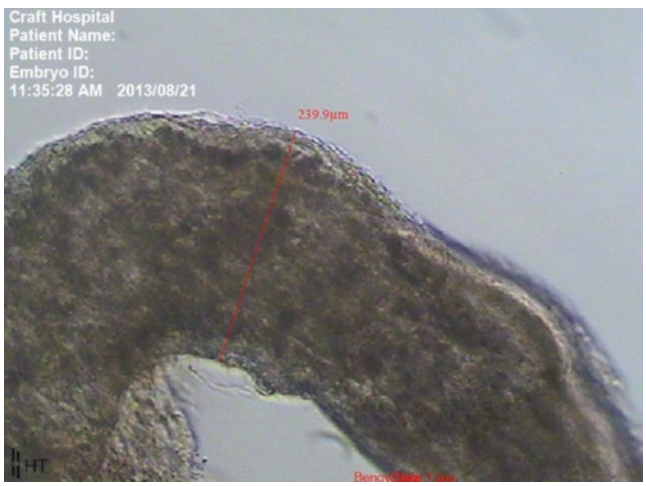

Fig. 57.2 The size of a normal seminiferous tubule

57.13.1 Methods for Selecting Viable Immotile Sperm for ICSI

It has been observed that conventional seminal parameters have little or no influence in ICSI outcomes, except when only immotile spermatozoa are available [34, 35]. In some cases after testicular sperm retrieval, only immotile spermatozoa are available for ICSI. The strategies to identify immotile live sperms are described below.

57.13.2 Hypoosmotic Swelling (HOS) Test

Firstly, pick up a single morphologically normal immotile spermatozoon from the sperm medium aspirated head first into the pipette. Immerse the pipette into the HOS microdroplet [36, 37]. Transfer the picked spermatozoon to the

hypoosmotic solution. Instead of placing the spermatozoon completely into the medium, only the sperm tail is moved out of the pipette tip into the HOS microdroplet. Keep the tail for 5–10 seconds into the solution, and observe the tail tip for swelling or non-swelling; minimal sperm tail swelling is a marker of viability in fresh specimens. However, HOS test is not suitable for cryopreserved sperm [35].

If tail swelling is present, the spermatozoon is aspirated to the fresh medium for osmotic equilibration (tail swelling often disappears in 5–20 seconds). The selected sperm populations are placed in the PVP droplet until an adequate number of viable sperm is available for ICSI.

The application of a single laser shot causes the curling of the sperm tail in viable spermatozoa, but it has not been validated in cryo-thawed specimens [38].

57.13.3 Sperm Tail Flexibility Test

The morphologically normal sperms are subjected to micromanipulation by moving the tail. The sperm tail is considered flexible when it moves independently of the sperm head. Sperm tail flexibility is considered as a marker of sperm viability [39, 40]. If the tail remains rigid upon touching and the sperm head and tail move together as a block, spermatozoon is then considered non-viable for ICSI.

57.13.4 Motility Stimulant Sperm Challenge Using Pentoxifylline

A 4 microlitre aliquot of fresh or cryopreserved testicular sperm suspension is loaded into the motility stimulant solution microdroplet. After incubation of the specimen for 20 minutes, it is examined microscopically to search for a motile sperm. A slight tail twitching is considered positive [35]. Motile sperm is picked up using the microinjection pipette and transferred to a fresh microdroplet of sperm medium. This step is repeated several times to wash out any residual pentoxifylline (PF) because it has been suggested in animal studies that PF is embryotoxic. Motile spermatozoa are kept in culture or placed into the PVP droplet for sperm selection and intracytoplasmic injection.

The use of theophylline before injection or after thawing in immotile sperms for enhancing sperm motility and increasing fertilization capacity has been suggested by some authors. Theophylline is a methylxanthine derivative that elevates cAMP and cAMP-dependent processes of sperm, including motility, capacitation and acrosome reaction. Its immediate effect allows for faster and more accurate selection of viable sperm, which in turn improves fertilization and pregnancy outcome [29]. In a study performed by Wober and colleagues, the addition of theophylline enhanced motility in 24 out of 28 samples and led to better fertilization rates, blastocyst formation, implantation and clinical pregnancy rates [41].

57.14 ICSI Dish Preparation and Sperm Fishing

An ICSI dish containing a HEPES/MOPS-buffered media drop (50–70 microL like a river) along with the oocyte microdrops and PVP drops under mineral oil is prepared for sperm pickup from a processed testicular cell suspension (Fig. 57.3a). If progressive motility is low or absent or the sample is contaminated with cellular debris, a sperm suspension aliquot of approximately 1–4 microliters is loaded in the 'river' of holding medium to facilitate search and selection of motile sperm (Fig. 57.3b). Addition of theophylline (20–25 microlitre into the 70 microlitre river) will enhance the sperm motility and make the ICSI process faster for the operator [29]. An optimal protocol that is practiced in our lab is described below:

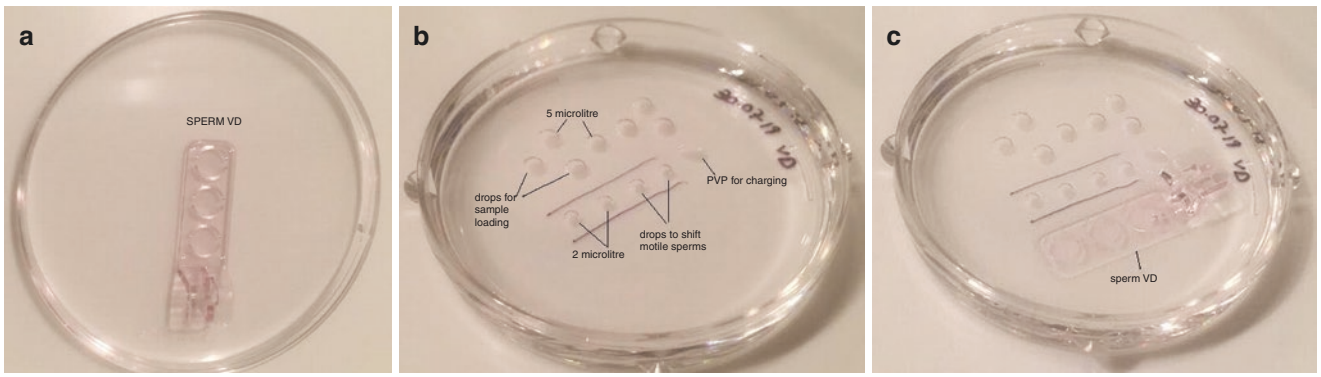

Fig. 57.3 (**a**) A novel sperm vitrification device (VD) for freezing small no. of spermatozoa; (**b**) dish preparation and sample loading before placing VD; (**c**) dish after placing sperm VD

The testicular specimens can be incubated up to 48 hours before ICSI at room temperature in an attempt to improve testicular sperm motility (Fig. 57.3c).

A slightly large diameter ICSI microinjection pipette (e.g. Humagen 9–30 with an internal diameter of 9.0 micrometer) can be used to avoid clogging of the needle while searching for the sperm. In majority of cases, moving spermatozoa through the clean shore area of the river (media-oil interface at the edge) can be easily picked up. Once the required number of spermatozoa is picked up from the river of cell suspension using the large diameter pipette, these are moved to the PVP droplet for immobilization. At this point the injection pipette is changed to a regular one; spermatozoon is immobilized, aspirated into the micropipette and injected into the cytoplasm of metaphase II oocytes. Culture media temperature during sperm handling and processing is kept in the range of 32–37 °C.

57.15 Assisted Oocyte Activation in Testicular Sperm

Failed fertilization may happen with ICSI due to defects in male or female gametes or by the injection process [27, 28]. Sperm viability issues, lack of sperm chromatin decondensation and inability of spermatozoa to initiate sperm activation may account for such failed fertilization [42].

During the failed fertilization, the harmony of the cell cycle process is deranged [43]. In mammalian oocytes, calcium oscillations precede the exponential phase of calcium concentration which is driven by the rise in inositol triphosphate (IP) [34, 35]. A soluble sperm factor phosphoinositide-specific phospholipase C (PLC-Z) is responsible for calcium oscillation which is delivered during the sperm oocyte fusion [40]. The induced calcium oscillation leads to a series of events at the cellular and molecular level: meiotic resumption, sperm nucleus decondensation, recruitment of maternal RNA, initiation of male and female pronuclei, commencement of DNA synthesis and finally the cell cleavage. Activation of the oocyte marks a series of events including extrusion of the second polar body, decondensation of a haploid set of chromosomes, nuclear membrane formation around the chromosomes and beginning of embryonic development [44, 45].

Total fertilization failure may also occur due to the total lack of motility in the short- or long-term cultured testicular samples. Sperm motility stimulants and assisted oocyte activation by calcium ionophores have been tried by many groups in such situations. Each laboratory should standardize these specialized techniques and should be used with a caution as we still don't know the molecular-level perturbations these chemicals might induce.

57.16 ICSI Outcome in MTESE

Studies have shown conflicting results. Few studies show no difference in the fertilization rates between NOA and OA, while others show decreased fertilization rates in NOA. The number of top-quality embryo and the pregnancy rates were found similar between the testicular sperms of NOA and OA in studies which could be explained by the fact that the selection of best sperm and TQE for transfer could have eliminated the difference [46–50].

57.17 Cryopreservation of MTESE Sample

The concept of surgical retrieval and cryopreservation of male gamete offers an advantage of avoiding ovarian stimulation when no sperm is obtained from testicular specimens. The cryopreservation of testicular spermatozoa can be done as per protocols which are routinely used for ejaculated sperm [3, 4].

The need to organize two operations (oocyte and sperm retrieval) at the same day can be avoided as thawing can be done at any time, once the sperm is found and frozen. If the treatment cycle does not result in pregnancy, future ICSI attempts may be carried out by the cryopreserved leftover specimen that would be usually discharged after ICSI, thus avoiding the need of repeated surgical retrievals. For men with NOA, who required multiple ICSI attempts to conceive but may not have adequate number of sperm available for repeated retrieval, testicular sperm freezing is a valid option.

After thawing, simple washing is employed for the removal of cryoprotectant. However, post-thawing, testicular sperm are often immotile or exhibit only a twitching motility. Use of immotile testicular sperm yields lower ICSI outcomes, including pregnancy rate, than using motile testicular sperms [43, 51]. Although methods for selecting immotile viable sperm for ICSI are available, results are either limited or not validated for cryopreserved specimens [34, 35, 39]. The components of sperm culture media should support normal metabolism of immotile mature retrieved spermatozoa that may become motile by incubation [40]. Thus, in vitro incubation should be limited to a maximum of 48 hours. Different strategies can be developed based on the results of each group. If surgically retrieved frozen specimens provide similar results as of fresh sperm, then the freezing specimens would be preferable. If not, fresh specimens can be used. To

date, the standard liquid nitrogen vapour method using TEST-yolk buffer and glycerol as cryoprotectants is being used for the cryopreservation of surgically retrieved sperms. Testicular sperm are freed from the testicular parenchyma, i.e. testicular homogenates are frozen. A test vial is kept to check the sperm survival test.

Either processed tissue along with the spermatozoa or the whole tube can be frozen. The freezing is done using cryovials and thawed 1 day prior to OPU. The tissue is minced properly and processed by density-gradient centrifugation (DGC). For DGC, 80/40 gradient, 1 mL each is used. Recently, it has been shown that human spermatozoa can be successfully vitrified, and this strategy may be used for preserving small quantities of surgically retrieved gametes [44]. Cell sleepers and sperm VD (vitrification device) are novel devices in which individual spermatozoa can be frozen in microdroplets. The successful use of cell sleepers for sperm freezing has been recently reported by Coetzee K et al. [45]. The study by these authors concludes that the sperm frozen in cell sleepers shows good recovery and this can avoid repeated mTESE or surgical procedure in the male on the egg collection day.

57.18 MTESE in Special Situations

MTESE can be considered in the following conditions though the evidence is still limited.

1. Non-azoospermic infertile men with high sperm DNA fragmentation or cryptozoospermia, in particular, in couples with multiple failed IVF/ICSI failures.
2. In case of hypogonadotropic hypogonadism when ejaculated sperm fail to appear after medical therapy. In these cases, TESA will be difficult to perform as the testes would be small in volume. MTESE will also prevent tubular damage which is already less in number.
3. In case of solitary testes associated with tumour and as a part of testes-sparing surgery.

57.19 Peri- and Postnatal Outcomes in NOA

Esteves et al. reported the neonatal profile of babies born with ICSI and showed similar outcomes from using NOA, OA or ejaculated donor sperm. The studies are on a very limited number; hence further studies and follow-up data are awaited.

In our centre, a total of 748 micro-TESE cases were carried out over the last 6 years. The clinical profile is shown in Table 57.1. A total of 359 patients had sperm retrieved during micro-TESE. ICSI with retrieved testicular sperm resulted in a clinical pregnancy rate of 38%. In our series, the live birth rate was 32.1%, whereas miscarriage rate was 10%.

Table 57.1 Data of 748 patients who underwent micro-TESE at CRAFT Hospital and Research Centre from 2014 to 2018

Patient characteristics	Micro-TESE; negative	Micro-TESE; positive	p value
Number	389	359	
Male age (years)	32.73 ± 5.94	33.22 ± 6.52	0.28
Follicle-stimulating hormone; FSH (IU/ml)	19.47 ± 15.42	17.32 ± 14.41	0.049
Testicular volume (mL)			
Left	8.27 ± 3.05	8.47 ± 3.16	0.34
Right	8.26 ± 3.04	8.33 ± 3.21	
Total testosterone (ng/dl)	5.49 ± 28.16	6.18 ± 23.42	0.72
Luteinizing hormone; LH (IU/ml)	7.59 ± 5.71	7.13 ± 5.27	0.22
Prolactin (IU/ml)	13.58 ± 8.10	15.31 ± 7.43	0.54
Thyroid-stimulating hormone; TSH (IU/ml)	2.85 ± 4.82	3.09 ± 5.25	0.43
Oestradiol; E2 (pg/ml)	38.86 ± 50.84	44.38 ± 105.07	0.3550
Klinefelter; N	27	11	
Kallmann; N	0	1	
Cryptorchidism; N	7	9	

Micro-TESE; negative: no sperm found
Micro-TESE; positive: presence of viable sperm for ICSI

57.20 Conclusion

The surgical retrieval and in vivo manipulation of male gametes definitely have increased the probability of genetic parenthood in men with NOA. Optimizing the results by fine-tuning the case selection strategies, pretreatment before attempting sperm retrieval, mTESE, the laboratory handling of testicular sperm can be the way forward.

57.21 Review Criteria

An extensive search of studies on nonobstructive azoospermia was performed using search engines such as ScienceDirect, OVID, Google Scholar, PubMed and MEDLINE. The overall search strategy was based on the following keywords: male infertility, nonobstructive azoospermia, spermatogenic failure, genetic testings, testicular sperm, surgical sperm retrieval, mTESE and laboratory handling. Articles published in languages other than English were not considered. Data that were solely published in conference or meeting proceedings, websites or books were not included.

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Sperm DNA Damage, ART Outcomes, and Laboratory Methods for Selecting DNA Intact Sperm for ICSI

58

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Key Points

- The sperm DNA fragmentation is a possible cause of idiopathic male infertility, and there are three mechanisms which explain how it is produced, along with external factors involved.
- The main diagnostic methods are sperm chromatin structure assay, sperm chromatin dispersion, and terminal deoxynucleotidyl transferase dUTP nick end labeling, which analyze different degrees of fragmentation in a semen sample.
- A spermatozoon with DNA fragmentation can fertilize the oocyte, and the ability to repair DNA damage by this or by the embryo has not yet been fully described.
- Intracytoplasmic sperm injection (ICSI) remains the gold standard in assisted reproduction techniques. However, a morphologically normal sperm can have the damaged DNA, be microinjected, and negatively affect the embryo development.
- Several sperm selection methods are based on the best intrinsic characteristics of the sperm to reduce the probability to choice of sperm with damaged DNA mainly in ICSI cycles.

58.1 Introduction

Conventional semen analysis as described by the World Health Organization (WHO) remained the only routine test for male infertility today [1]. Despite of this, it is usually insufficient for the diagnosis of male fertility by itself, as it does not account for sperm molecular physiological dysfunctions, such as presenting immature chromatin, oxidative stress status, and DNA damage, among others, so under the WHO criteria of 2010 it is likely to an increasing number of men classified as having unexplained male infertility [2].

Since the 1980s, the diagnostic assays of sperm DNA damage have improved the precision of the estimations of reproductive outcomes and defined or delimited thresholds to apply treatments in assisted reproduction laboratories. Nevertheless, the increasing number of papers reporting the relationship between DNA integrity and reproductive outcomes resulted in a confusing amount of literature with extremely difficult interpretation and limited clinical applicability and generalizability [3].

Sperm DNA fragmentation (SDF) is the presence of breaks with the DNA strands, and several factors are responsible of such damage. The main molecular mechanisms include defects in spermatogenesis and spermiogenesis within the testicle, defects in epididymal maturation, and a possible oxidative stress-induced damage due to an increased susceptibility or the implication of external factors [4]. External factors such as disease, life habits, lab procedures, and drugs also contribute to sperm DNA damage, the most of them via creating an oxidative stress condition [5].

The significant role of SDF in male factor infertility is supported by current evidence [6]. *Intracytoplasmic sperm injection (ICSI)*, over the rest of *assisted reproduction techniques (ART)*, seems to be the preferred method when sperm DNA fragmentation is high as well as being the technique for excellence in assisted reproduction laboratories [7]. In this way, together with the evidences found on the impact of SDF in the delay of embryonic development in microinjected donor oocytes [8] and in repeated pregnancy failures [7], the

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need to applicate both, diagnostic techniques in DNA fragmentation and sperm selection techniques in clinics, at least for selected cases, seems to be justified.

The aim of this chapter is to describe the origins and types of sperm DNA damage and the main DNA fragmentation tests employed today, evaluating its value within the context of its link with assisted reproduction techniques' outcomes, together with the strategies of sperm selection which have shown a significant reduction of sperm DNA fragmentation for ICSI treatment or identifying the spermatozoa with most intact DNA cargo and the clinical benefit contributed.

58.2 Which Are the Origins of Spermatic DNA Breakage?

Currently, there are three main theories to describe the molecular mechanisms causing DNA damage in spermatozoa: the abortive apoptosis theory, the defective maturation theory, and the oxidative stress theory [9]. In addition, several external factors are related with these processes, subsequently increasing SDF (Fig. 58.1).

According to *the abortive apoptosis theory*, spermatozoa initiate apoptosis by inducing activated endonucleases leading to DNA double-stranded breaks. However, as spermatozoa are transcriptionally inactive, they lose their capacity to undergo programmed cell death, therefore unable to complete this process. Thus, a restricted form of apoptosis leading to DNA fragmentation compatible with other sperm functions has been suggested in spermatozoa, and this phenomenon is more prevalent on infertile males [10]. Apoptosis is characterized by externalization of plasma membrane phosphatidylserine (PS) and activation of effector caspase cascade, thus leading toward DNA fragmentation [11]. Therefore, apoptosis assays by the presence of apoptotic markers measured in flow cytometry are included along with SDF tests in some cases [12]. Some studies have demonstrated that the percentage of apoptotic sperm is positively correlated with SDF in the ejaculate of infertile men with varicocele and both are higher than fertile men [12]. In addition to this, there are evidences that high levels of SDF in the ejaculates from infertile men could lead to the creation of apoptotic embryos [8].

On the other hand, the replacement of histones by protamines is a step-by-step process starting in primary spermatocytes, when part of the somatic histones is exchanged by testis-specific histone variants [9]. Subsequently, transition proteins and most of the histones are replaced by protamines during the spermiogenesis phase, and the chromatin is wound into unique supercoiled structures named toroids

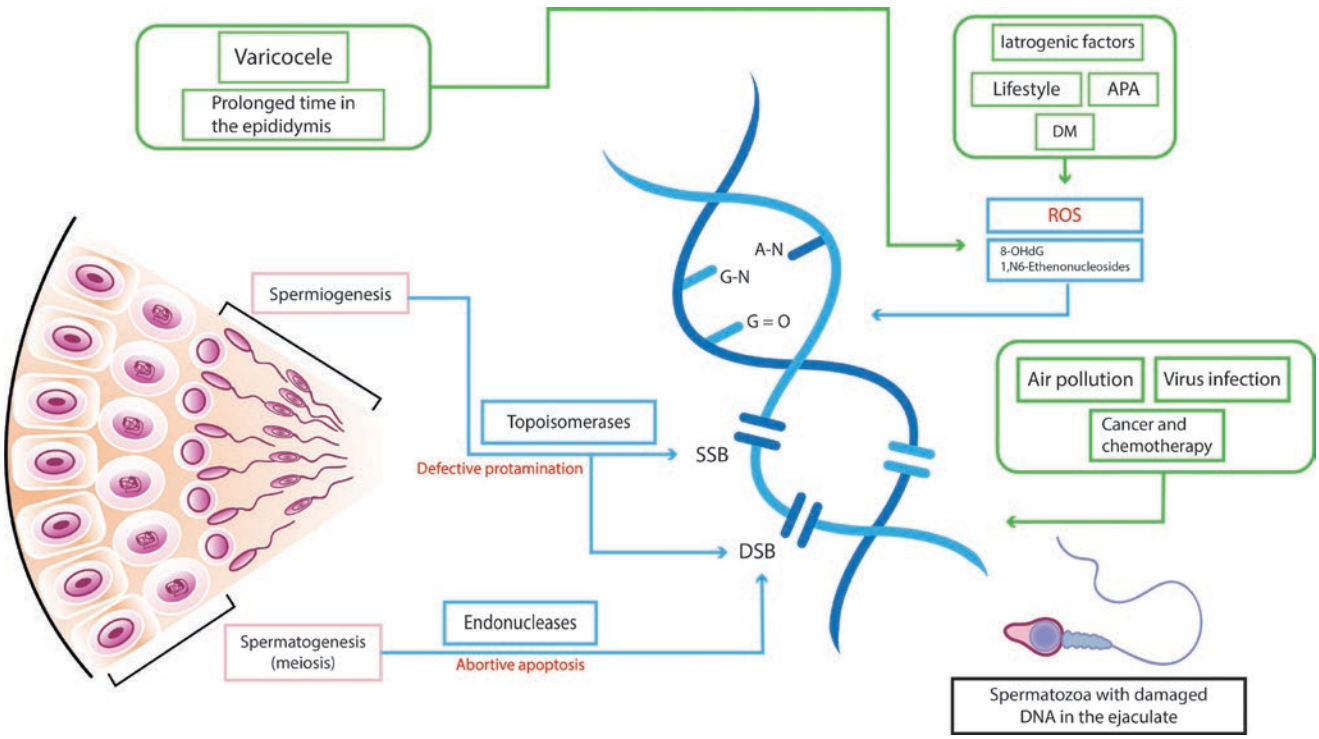

Fig. 58.1 The main factors that produce different types of sperm DNA damage. The three theories about molecular mechanisms (arrows in blue) and the input of different external factors in OS (arrows in green) are represented in the diagram. DM Diabetes mellitus; APA advanced paternal age; ROS reactive oxygen species; 8-OHdg 8-hydroxy-2′-deoxyguanosine; 1, N6-ethenonucleosides of adenosine and guanosine; SSB single-stranded breaks; DSB double-stranded breaks; OS oxidative stress

[13]. The chromatin stability is achieved by disulfide bond formation during epididymal transit of spermatozoa [14], and this dense condensation gives protection against exogenous assault and potential harm to the sperm DNA structure [15]. Today, it is estimated that only 5–15% of the chromatin in the human spermatozoa consists of histones and the major part consists of protamines [16]. The action of some enzymes as topoisomerases or other nucleases facilitates the histone-protamine replacement in elongating spermatids inducing single- and double-stranded (ss and ds) DNA breaks in order to reduce torsional stress, but if these nicks are not properly linked afterwards, they will evolve into permanent DNA fragmentation on mature sperm [15], according with *the defective maturation theory*.

The third main mechanism of DNA fragmentation described in the literature concerns *reactive oxygen species (ROS)* which are thought to represent the main cause for DNA damage [9]. The oxidative stress (OS) is caused by an imbalance between ROS production and antioxidant scavenging activities in semen [17]. Unlike other cells, spermatozoa are more vulnerable and prone to OS because of their lipid nature (rich in polyunsaturated fatty acids) [18]. For this reason, to protect spermatozoa from ROS-induced damage, seminal plasma is well equipped with natural nonenzymatic antioxidants (vitamins A, C, and E, ascorbate, and glutathione) and enzymatic antioxidants (superoxide dismutase, glutathione peroxidase, and catalase) [17, 18].

As result of ROS production imbalance, or due to diminished sperm chromatin packaging by defective spermatid protamination and disulfide bridge formation because of the inadequate oxidation of thiols during epididymal transit, sperm cells turn very vulnerable to ROS-induced DNA fragmentation [17]. Therefore, ROS react with sperm DNA leading to changes and deletions in nitrogenous bases, as oxidative base modifications like 8-hydroxy-2′-deoxyguanosine (8-OHdG) and ethenonucleosides (1, N6-ethenoadenosine and 1, N6-ethenoguanosine), markers of OS and lipid peroxidation, respectively, and consequently have high frequencies of DNA fragmentation [9, 19].

Recently, in a study of mitochondrial membrane potential, it has been observed that a high degree of the sperm mitochondria activity reduces the sperm DNA fragmentation, and on the other hand, in the presence of membrane potential dissipation, an increase in ROS has been observed [20].

In addition to this, there are several external factors which have been related recently with the molecular mechanisms of sperm DNA damage related to disease [12, 21–24], life habits [5, 25–28], laboratory procedures, and drugs [23, 29, 30], but although most of them create an oxidative stress

Table 58.1 External causes that affect sperm DNA

Causes	Consequences in sperm DNA	References
Varicocele	Varicocele generally creates a condition of OS that affects sperm functions including specially protamine deficiency and an increased DFI	[12, 21]
Infections	Men infected with HPV have been shown to have higher SDF values than those with no HPV. The possible origin of this damage is an increase susceptibility of DNA breakage due to defective repair of DNA damage of infected cells	[24, 108]
Cancer and chemotherapy	A significant increase of sperm aneuploidies and SDF has been observed in patients with testicular cancer in chemotherapy treatment. In addition, independently of the type of cancer, patients with cancer before the treatment have higher SDF values compared to fertile donors	[23, 109]
Diabetes mellitus	The DM2 caused an inflammatory condition with increased OS resulting in decreased sperm vitality and increased DFI. DM1 altered epididymal voiding causing low ejaculate volume and mitochondrial damage resulting in decreased sperm motility	[22]
Advanced paternal age	SDF is higher in APA (≥ 40 years) patients due to ROS generation increases with age, with worst IVF outcomes rates and miscarriages	[110]
Lifestyle	The relationship between high BMI with impaired sperm DNA integrity is not clear yet; recently, it has shown influence in semen parameters as concentration and motility but not with DNA fragmentation, sperm protamination, and sperm apoptosis Smoking and alcohol intake lead to increase in enzymatic antioxidant activity due to long-term unbalanced antioxidant/oxidation ratio with high OS, which cause consequently SDF and chromatin decondensation The exposure of bisphenol A (an endocrine disruptor, plastic derivate) is negatively associated with antioxidant levels and semen quality and positively correlated with SDF, especially due to defects of seminal plasma lipid peroxidation	[5, 26–28, 60]
Air pollution	There are some polymorphisms (XPD6 and XPD23) of metabolic genes, which have been associated with high levels of SDF, being more susceptible to high level of air pollution	[5, 25]
Iatrogenic factors	Laboratory procedures: DGC has been reported to result in increased SDF because of centrifugation force and duration of processing in samples of infertile men The ejaculatory abstinence >4 days in men is related to a significant higher sperm DNA fragmentation index and lower rates of fertilization, high-quality embryos on day 3, blastocyst development, implantation, and pregnancy	[29, 30]

OS oxidative stress, *DFI* sperm DNA fragmentation index, *SDF* sperm DNA fragmentation, *HPV* human papillomavirus, *DM1/DM2* type 1 and 2 of diabetes mellitus, *APA* advanced paternal age, *BMI* body mass index, *DGC* density gradient centrifugation

condition, some of them affect directly DNA strand (Table 58.1).

These evidences justify a need of an exhaustive anamnesis on infertile patients attending assisted reproduction units, which could give information about the possible harm in DNA and should be properly introduced in the routine clinical practice. From these findings, identifying males with elevated risk of exhibiting high SDF levels and then recommend them the possibility to perform a diagnostic study with the SDF test application. Furthermore, all theories shown are not mutually exclusive, since an oxidative damage in the DNA could be caused by a defective protamination in the spermiogenesis or a susceptibility of DNA strand by a defect in programmed cell death, being extremely difficult to know and isolate a unique origin of that DNA break through diagnostic techniques.

In any case, it has been demonstrated that sperm with fragmented chromatin can fertilize the oocyte and may be compatible with an evolving pregnancy mainly facilitated by good quality oocytes' capacity to repair this damage after fertilization or during embryo development [31]. The possible consequences on couple's ART outcomes and health of the offspring due to SDF have been extensively studied in the last years.

58.3 How Can We Measure the Different Types of Sperm DNA Damage?

The study of sperm DNA has evolved since the 1940s, when Pollister and Mirsky discovered that a large part of the protein complexes surrounding the sperm DNA was not composed of histones but of protamines [32]. Since then, the possible impact of sperm DNA physiological characteristics and damage on fertility has been studied resulting in the development of several tests for the analysis of sperm DNA damage, improving the technology successively by designing variations of the initial tests and approaches by new methods [4, 33].

In brief, all these tests fall into two main categories: direct and indirect methods to measure the sperm DNA integrity, depending of the kind of damage measured [4, 34]. Direct methods can directly identify and measure the broken parts of the sperm DNA, while indirect methods typically measure the susceptibility of sperm DNA to be damaged under the experimental conditions generated by the assay [12].

Within the available methods, sperm chromatin structure assay (SCSA) and sperm chromatin dispersion (SCD) are considered indirect methods, while TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling) and comet assay are direct methods, being all of them the most widely used techniques in DNA fragmentation assessments today [3, 34–36]. Each one of them has its advantages and disadvantages for its application in the clinic (Table 58.2).

In the 1970s, the *acridine orange (AO) test* [37] was employed as the first sperm DNA damage measuring tool, specifically to evaluate the stability of sperm DNA in bull semen samples during the spermiogenesis with fluorescence results revealed by microfluoriemetry. AO is a nucleic acid-selective cationic fluorescent dye that interacts with dsDNA (by intercalation) or ssDNA (by electrostatic attraction) following mild acid denaturation of sperm DNA [38], showing a green emission when bound to dsDNA and a red emission when bound to ssDNA [4]. This method has recently been discredited by Evenson (2016) because of AO staining fluo-

Table 58.2 Main assays to determine the DNA fragmentation grades

Method	Equipment	Advantages	Limitations	References
SCSA	Flow cytometer	Quick and robust analysis Standardized protocol Clinical threshold generally accepted around SDF 30% Able to detect immature spermatozoa	Equipment costs	[38]
COMET	Fluorescence microscopy	Is possible to analyze different types of DNA damage produced by ROS (adding additional enzymes in the incubation)	Laborious procedure Requires highly specialized personnel Difficult to distinguish between endogenous and induced DNA breaks The extent of DNA damage can be underestimated due to entanglement of DNA strands	[35, 38, 43]
TUNEL	Fluorescence microscopy Flow cytometer	Identify ssDNA and dsDNA breaks	Equipment costs Has limited capacity to evaluate immature cells	[33, 35]
SCD	Light microscopy Fluorescence microscopy	Quick Does not require complex instruments	Possible errors due to different position of halos in the focal plane Low chromatin density may not be distinguishable Categorize the halo degrees is occasionally subjective	[35, 38]

SCSA sperm chromatin structure assay, *TUNEL* terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling, *SCD* sperm chromatin dispersion

rescence fading and artefacts induced by glass/AO interactions [39].

The next developed method was *sperm chromatin structure assay (SCSA)*, which was established in the 1980s to set up the first relationships found between sperm DNA integrity and pregnancy outcomes [40]. In this method, the sperm DNA is denaturized by acid or heat, and the sites of DNA strand breaks are stained with fluorescent cationic dye AO [4]. After staining with AO staining solution, sperm chromatin damage can be quantified by flow cytometric measurements (under a 488 nm light source) of the metachromatic shift from green (native, dsDNA) to red (denatured, ssDNA) fluorescence [41]. SCSA also measures high DNA stainability (HDS), which is believed to be an expression of immature spermatozoa containing excess histones or other abnormal proteins [42] (Table 58.2).

Since then, hundreds of publications on the use of the SCSA test in animals and humans have validated the SCSA results as a highly useful test, more robust and reproducible than other methods later developed [39]. That is why nowadays it is considered by some as the gold standard method of DNA damage measuring in comparison with the rest of the assays [33].

In the 1980s, the *single cell gel electrophoresis (SCGE)* was also developed, also known as *Comet assay*, which permits to quantify the amount of DNA damage per spermatozoa. The lysed sperm cells are loaded onto an agarose gel under electrophoretic conditions, and the result is evaluated by fluorescence microscopy [4, 43] (Table 58.2). If the DNA contains breaks, the free small DNA fragments migrate toward the anode due to their negative charge [43]. This migration leaves a comet-like tail; being the fluorescent intensity of the tail, it forms the product of DNA fragments with different sizes and represents the amount of migrated DNA from the head, indicating different degrees of SDF [4, 38].

The comet assay also gives information about the extent of several kinds of damage (basal breaks or oxidative damage), depending the enzyme used in the incubation [43]. There are two variants of this method depending on sperm DNA damage measured. The dsDNA damage can be measured in a neutral buffer, but an alkaline buffer can detect all ssDNA and dsDNA damage [35]. These latter include apurinic and apyrimidinic sites, which arise from the loss of damaged bases [43]. So, two-tailed comet assay, a bidimensional electrophoresis which integrates both buffers, can distinguish between ssDNA and dsDNA breaks within the same sperm cell [44].

A decade later, the *TUNEL* assay became available, in which a terminal deoxynucleotidyl transferase (Tdt) labels the fluorescent dUTP nucleotides (deoxyuridine triphosphate) into the 3'-OH terminal of the ssDNA or dsDNA breaks through an enzymatic reaction creating a signal, which increases with the number of DNA breaks [33, 38]. The assay can be performed using either flow cytometry or bright/fluorescence microscopy (Table 58.2), this later normally with fluorescein 5(6)-isothiocyanate (FITC), a fluorochrome (excitation/emission 450/570 nm) with green fluorescence [4, 33, 38]. A recent study has been performed with the aim of developing standardized staining protocols of this method inter- and intralaboratory, obtaining the same results of SDF using the same model of flow cytometer and staining protocol [33]. TUNEL currently has been considered the most accurate method to predict pregnancy in ART, due to the fact that SDF values as a result of this test tend to be lower than with the indirect methods, by its own sensitivity [45]. These lower SDF values could be explained in the way that the tests measure different aspects of SDF, and while TUNEL measures the proportion of sperm with existing DNA breaks only, indirect methods assess the proportion of sperm with existing DNA breaks and potentially denaturable DNA due to the preexistence of ssDNA or dsDNA breaks [45].

In situ nick translation (ISNT) is a variant of TUNEL assessment that quantifies biotinylated dUTP that attaches to ssDNA breaks through DNA polymerase [46]. For this reason, the clinical value of ISNT is extremely limited and lacks sensitivity compared to other assessments [35].

Another method to measure direct DNA damage is *DNA breakage detection-fluorescence in situ hybridization (DBD-FISH) assay*, which was developed later. In this method, the spermatozoa are fixed in an agarose matrix, and the DNA is transformed into ssDNA by an alkaline unwinding solution. After the proteins are removed, the DNA is made accessible to hybridization. If DNA strand contains increased amount of DNA breaks, more probes will hybridize resulting in an increased fluorescence. This technique can be used for detecting DNA damage within specific sequence areas [4].

Due to its easy implementation in any laboratory given the technical and material requirements, *sperm chromatin dispersion (SCD) or Halosperm* a*ssay* [4] is probably one of the most popular tests to evaluate sperm DNA integrity. In this assay, spermatozoa are subjected to acid denaturation to remove nuclear proteins, exposed to a lysing solution and subsequently embedded in an agarose gel matrix. The relaxed DNA loops prevent dispersion into the surrounding area, indicating an unstable state of chromatin caused by a DNA damage. Conversely, spermatozoa without a DNA damage which have packed DNA loops present a dispersed halo [4, 38]. Halos can be observed by bright microscopy if the staining is done with a Diff-Quick staining or by fluorescence microscope if DNA-directed fluorochromes are used (e.g., as 6-diamino-2-phenylindole, DAPI) [47] (Table 58.2).

Because of the fluorescence or staining patterns, an SDF value or *DNA fragmentation index (DFI)* is obtained for each assay, and it could be a used as a diagnostic indicator to forecast or somehow predict the ART outcomes, then enable clinical decisions, and recommend a specific assisted repro-

duction treatment to the patients. Despite the great effort carried out in sperm DNA analysis test improvement and its implication in fertility for a half century ago, the true nature of sperm DNA is not totally understood yet and neither exactly what is that each test measures. Furthermore, SDF thresholds (or cutoffs) for many of these test have not been clearly described [4, 33, 38]. All together have made impossible any official, global, or unified recommendation about the indications of the sperm DNA fragmentation test in a routine analysis of the semen by professional bodies and experts' groups [48], and most importantly, the protocols are to follow once the results are available in order to improve patient's reproductive chances.

58.4 Which Is the SDF Implication in Male Fertility and What Indications Should Be Followed in ART?

Semen analysis is currently the most accepted test for evaluating male infertility by now, in which measured parameters are concentration, vitality, motility, and morphology [1]. However, it has been demonstrated that although useful information can be gathered from it, and a rough estimation of the male fertile potential, a guidance about the most convenient reproductive options or the need for additional tests can be drawn from their results, as conventional semen analysis does not provide information regarding all functions of sperm, nor is it sufficient for predicting male fertility potential and the likelihood of success of ART [49].

In this way, many studies have revealed a significantly increased SDF in men with *unexplained male infertility (UMI)*. Having normal semen parameters, according to traditional diagnostic methods, they showed remarkably high degrees of fragmented sperm DNA, about 17.7% of these cases; the men presented a fragmentation index greater or equal to 20% until 30% (95% CI; 10.8–24.5), and about 8.4% (95% CI; 3.40–13.4) had DFI greater or equal to 30% (CI: confidence interval) [49]. Other study also showed significant higher DFI and OS in men with UMI compared to fertile donors [50]. Despite the lack of standard methods to evaluate DNA damage, it is agreed that SDF seems to be higher in infertile men and is well known that SDF correlates with poor semen quality in terms of motility and concentration [35]. Therefore, several studies have tried to set the relationship between SDF with all types of ART and their outcomes.

First of all, the number of reports on the relationship between SDF and *natural conception* (*NC*) probabilities seems scarce compared to reports on ART outcomes [51]. The Danish First Pregnancy Planner Study Team includes in their study the *time to pregnancy* (TTP) of 215 couples during a 2-year follow-up period, and they have demonstrated that in men who had SDF value greater than 20%, the probability to achieve pregnancy was reduced (OR = 0.43; 95%CI; 0.24–0.76; $p < 0.01$), and it turns negligible when it is up to 40% (OR = 0.13; 95%CI; 0.02–0.97; $p < 0.05$)(OR: Odds ratio; CI: confidence interval) [52].

Currently, the clear impact of SDF especially in NC and *intrauterine insemination* (*IUI*) outcomes has been reported [6]. In 154 IUI cycles resulting in pregnancy, the degree of SDF after preparation was significantly higher (SDF > 12% by TUNEL) in couples who did not result in pregnancy than in those that achieved pregnancy (13.9% vs. 7.3%; $p < 0.05$). In addition, two patients who miscarried were inseminated with samples containing the highest degree of DNA fragmentation (10% and 12%) among all cases who became pregnant [53]. Some years ago, some studies provided solid evidence by illustrating the relationship between pregnancy failure in both and a DFI of >30% in population studies of around 400–600 patients (Table 58.3) [54, 55]. Therefore, it suggests that SDF testing could be indicated, by this type of infertile couples of unknown cause, in order to reduce the time to pregnancy (TTP) with possibility to apply other treatment in assisted reproduction laboratories and bring parenthood earlier [53]. Nevertheless, there has been controversy about the influence of SDF over time. The research group of Muriel et al. (2006) did not find the same relationship of SDF in IUI outcomes; despite of this, the relatively low values of SDF of the studied patients (<30 in raw semen and < 20 after swim-up procedure) should be noted, according the fractions of sperm cell without or with very small halo (measured by SCD). In addition to this, the highest SDF values, in terms of sperm cell with very small halo, were negatively correlated with sperm motility ($r = -0.22$; $p < 0.05$) in 100 couples under IUI treatment. The authors concluded that future studies using tests that measure primary dsDNA fragmentation such as TUNEL may prove to show a stronger correlation with pregnancy outcome in IUI than performing the SCD assay [56]. In general, it is accepted that the SDF directly or indirectly affects in NC and IUI outcomes today [6].

Secondly, regarding in vitro fertilization techniques, the results and messages delivered from studies evaluating the influence of SDF on the *in vitro fertilization (IVF)* and *intracytoplasmic sperm injection (ICSI)* outcomes are complex and varied (Table 58.3) [35]. Regarding fertilization rates, while there are studies showing a significant difference between higher SDF men groups and lower SDF groups in IVF/ICSI outcomes [57, 58], there are other studies in which no correlations in terms of fertilization are found [8, 59, 60]. Additionally, some of them demonstrated that males presenting significantly higher SDF values exhibited a relevant reduction in the fertilization rates (74.9% vs. 55.1%; $p < 0.001$) in ICSI cycles but not in IVF cycles [57], while other authors demonstrated a negative correlation with fertilization rates in IVF cycles ($r = -0.32$; $p < 0.01$) [58].

Table 58.3 Summary of SDF implications in male fertility

	Design of study	Study population (size)	Considered SDF thresholds (low vs. high) (%) (diagnostic assay)	Outcomes (%) and SDF influence	References
NC	Systematic review Follow-up study	616 couples 215 couples	SDF *<30 vs. ≥ 30* (measured by SCSA) SDF *<20* vs. *>20* (measured by SCSA)	*SDF ≥ 30* difficult the possibilities to achieve pregnancy (OR = 7.01; 95% CI; 3.68–13.36; $p < 0.001$) *SDF > 20* prolongs TTP and reduces the fertility potential: Reduced probability to achieve *pregnancy* (OR = 0.43; 95%CI; 0.24–0.76; $p < 0.01$). And it gets negligible when this up to 40% (OR = 0.13; 95%CI; 0.02–0.97; $p < 0.05$)	[52, 55]
IUI	Prospective cohort study Prospective cohort study Prospective cohort study	387 infertile couples 154 infertile couples 100 infertile couples	SDF *≤ 30 vs. > 30* (measured by SCSA) SDF *<12 vs. > 12* (measured by TUNEL) (Undefined) (measured by SCD)	*SDF > 30%* reduce the possibilities of: *Biochemical pregnancy rate* (3 vs. 24; OR = 0.10; 95% CI; 0.02–0.41) *Clinical pregnancy rate* (3 vs. 23.7; OR = 0.10; 95% CI; 0.02–0.42) *Live birth rate* (1.5 vs. 19; OR = 0.07; 95% CI; 0.01–0.48) Couples that not result in *pregnancy* had higher SDF than in those that did achieved pregnancy (13.9 vs. 7.3; $p < 0.05$) DNA dispersion, as measured by the SCD test, is not correlated with pregnancy outcome in IUI	[53, 54, 56]
IVF/ICSI IVF/ICSI	Prospective cohort study Retrospective study Prospective cohort study Prospective cohort study Retrospective and prospective studies Prospective cohort study Retrospective cohort study	390 IVF cycles (IVF $n = 238$, ICSI $n = 152$) 550 IVF cycles (IVF $n = 415$, ICSI $n = 135$) 605 cycles of IVF 1102 IVF cycles (IVF $n = 379$, ICSI $n = 723$) 1633 IVF cycles (IVF $n = 1117$, ICSI $n = 516$) 82 infertile patients with donated oocytes ICSI cycles (embryos $n = 187$) 79 infertile patients with donated oocytes ICSI cycles (embryos $n = 644$)	SDF *<30 vs. ≥ 30* (measured by SCD) SDF *≤22,3 vs. > 22,3* (measured by SCD) SDF *<5 vs. 5–10 vs. 10–15 vs. ≥ 15* (measured by SCD) SDF *<30 vs. ≥ 30* (measured by SCD) SDF *≤10 vs. 10–20 vs. 20–30 vs. > 30* (measured by SCSA) SDF *≥15 vs. < 15* (measured by TUNEL) SDF *< 6.5 vs. 6.5–10.7 vs. 10.7–20.1 vs. > 20.1* (measured by TUNEL)	No relationships are founded between high DFI and IVF/ICSI outcomes High SDF (>22.3) vs. low (≤22.3) is associated with a *reduced fertilization rate* in ICSI (74.9 vs. 55.1; $p < 0.001$) High SDF *(≥15)* is correlated negatively in IVF cycles with: *Rates of fertilization* ($r = -0.32$; $p < 0.01$) *Cleavage* ($r = -0.19$; $p < 0.01$) *High-quality embryo* ($r = -0.40$; $p < 0.01$) *Clinical pregnancy* ($r = -0.20$; $p < 0.01$) *Live birth rate* ($r = -0.09$; $p < 0.05$) *Clinical pregnancy rate* is higher by ICSI than IVF in groups with high SDF *(≥30)* (44.5 vs. 35; $p < 0.05$) *Live birth rate* is reduced in IVF in groups of SDF > 20 (OR = 0.61; 95% CI: 0.38–0.97; $p < 0.05$) *Live birth rate* is higher by ICSI than IVF in groups with high SDF (>20) (OR = 1.7; 95% CI; 1.2–2.9; $p < 0.05$) High value of SDF (≥15) compared to lower (<15) values has been related with: Promoting the *apoptotic pathway activation* in blastomeres in good quality oocytes (21.9 vs. 16.4; $p < 0.05$) High level of *fragmentation in blastomeres* (15.9 vs. 9.1; $p < 0.05$) compared to low value of SDF (<15) Affecting negatively to *blastulation rate* (37.5 vs. 59.2; $p < 0.01$) High value of SDF (>20) can *delay* division times of *embryo development* in terms of: *Presence of second polar body* (3.6 h vs. 3.4/3.3/3.5 h; $p < 0.01$) *The development of morula stage* (78.5 h vs. 79.5/68.6/75.1 h; $p < 0.05$)	[7, 8, 57–59, 63, 65] [7, 8, 57–59, 63, 65]

(continued)

Table 58.3 (continued)

	Design of study	Study population (size)	Considered SDF thresholds (low vs. high) (%) (diagnostic assay)	Outcomes (%) and SDF influence	References
Miscarriages	Retrospective and prospective studies Prospective cohort study Case-control study	1633 IVF cycles (IVF $n = 1117$, ICSI $n = 516$) 605 cycles of IVF 42 couples with RLP and 42 fertile men (control)	SDF *≤10 vs. 10–20 vs, 20–30 vs. > 30* (measured by SCSA) SDF *<5 vs. 5–10 vs. 10–15 vs. ≥ 15* (measured by SCD) (Undefined) (measured by SCSA/TUNEL)	The risk of *miscarriage* increase significantly in men groups with *SDF > 40%:* (OR = 3.8; 95% CI: 1.2–12; $p < 0.05$) High SDF *(≥15%)* is correlated positively with miscarriage ($r = 0.23$; $p < 0.01$) Higher SDF values are related with groups with RPL compared to fertile men as control with lower SDF (26 vs. 19.4; $p < 0.001$)/(14.5 vs. 9.7; $p < 0.0$)	[7, 41, 58]

NC natural conception; *TTP* time to pregnancy; *OD* odds ratio; *CI* confidence interval; *SDF* sperm DNA fragmentation; *SCSA* sperm chromatin structure assay; *IUI* intrauterine insemination; *TUNEL* terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling; *IVF* in vitro fertilization; *ICSI* intracytoplasmic sperm injection; *DFI* DNA fragmentation index; *SCD* sperm chromatin dispersion; *RPL* recurrent pregnancy loss; *h* hours; *n* size of study cohort

In the same way, within studies that have considered other ART outcomes in IVF/ICSI, it has shown a negative relationship between SDF embryo development and pregnancy rates, presenting different results along the fertilization rate [8, 57, 58, 60, 61] (Table 58.3).

Some authors as Bach and Schlegel (2016) suggest that the absence of an agreement between the results of IVF/ICSI could be explained due to the fact that many of the studies contain patients undergoing IVF and ICSI together, despite differences between the two techniques' outcomes and also because they have examined different clinical endpoints, such as clinical pregnancy, miscarriage, and live birth. In addition, there are several variations between SDF assay protocols, their respective thresholds, and differences in study populations, which have resulted in systematic reviews and meta-analyses filled with heterogeneity and unable to come to robust and straightforward conclusions [62].

On the other hand, regarding the effect of SDF in the embryo development, the research group of Muriel et al. (2006) studied 85 couples under IVF/ICSI treatment and shown that higher DNA fragmentation rates gave an increased proportion of zygotes showing asynchrony between the nucleolar precursor bodies of zygote pronuclei (73.8% vs. 28.8%; $p < 0.001$) compared with lower DNA fragmentation rate. In addition, the slower embryo development and worst morphology on day 6 were correlated with higher sperm DNA fragmentation (47% vs. 29.4%; $p < 0.05$). This group also observed a negative correlation between DNA fragmentation and the implantation rate ($r = -0.25$; $p < 0.05$). However, SDF values were not statistically different in cycles that resulted in a pregnancy compared with those that did not (33.2% vs. 28.2% and 32.4% vs. 34.7%; $p > 0.05$); thus, it seemed to be that SDF is not related with pregnancy rates, despite a minor increase in SDF in patients, which did not achieve pregnancy, was observed; these authors concluded that the absence of signification due to the good quality embryo selection before the transfer and the different results could be explained by the extreme complexity of interactive effects of the diversity of DNA damage along with the variable DNA repair capacity of each oocyte [61]. Probably, analyzing cumulative live birth rates per stimulation cycle after having also frozen/thawed embryos transferred afterward, instead of per transfer, may be more informative about the effect of DNA fragment analysis on the reproductive results.

In fact, some recent studies have included oocytes from young and healthy donors as model in ICSI treatments and evaluating embryo by time-lapse monitoring (TLM), to control the female factor and check the repairing capacity of oocytes [8, 63]. These have associated high SDF values in a study of 79 patients with a cell division delaying of the embryo, from second polar body appearing to morula stage, maybe due to the time taken by oocyte to repair paternally inherited DNA [63] (Table 58.3). Also, high SDF value in a study of 82 infertile patients has been related with low blastulation rates with the presence of an apoptotic pattern in most blastomeres (caspase 3 and survivin, apoptotic markers, localized by immunohistochemistry) [8]. The apparent association observed between embryo quality and sperm DNA fragmentation could explain the negative correlation with implantation rate. In any case, it seems that the SDF affects both directly and indirectly in vitro fertilization outcomes, from the fertilization to later stages of the evolving embryos.

Furthermore, a very recent meta-analysis from Simon et al. (2017) comprising 41 articles and as a consequence of a large number of IVF/ICSI cycles (8068) has demonstrated that sperm DNA damage has a negative effect on clinical pregnancy following IVF and ICSI treatment either separately (OR = 1.65; 95% CI: 1.34–2.04; $p < 0.0001$ for IVF and OR = 1.31; 95% CI: 1.08–1.59; $p < 0.01$ for ICSI) or mixed (OR = 1.68; 95% CI: 1.49–1.89; $p < 0.0001$ for both).

In this study, female factors were controlled, and all thresholds of the four main assays were included (SCSA, TUNEL, SCD, and comet). The authors concluded that there is sufficient evidence in the existing literature suggesting that sperm DNA damage has a negative effect on clinical pregnancy following IVF and/or ICSI treatment and that the effect of SDF value appears to vary according to the type of assay used to measure sperm DNA damage and their different thresholds measured due to the sensitivity of each method, having shown all of them a negative impact in IVF outcomes [64].

Despite the controversial results from IVF treatments, it has been reported from 1102 IVF cycles that the pregnancy rate of ICSI cycles was significantly higher than IVF cycles (44.8% vs. 25%; $p < 0.05$) in high SDF groups (SDF > 30%) (Table 58.3) [65]. Also it has been shown that ICSI cycles increase the probability of live birth compared to IVF cycles when SDF value was >20% (OR = 1.7; 95% CI; 1.2–2.9; $p < 0.05$) [7], leading to conclude that ICSI might be the optimal method of ART to reduce the possible impact of SDF in fertilization in cases of high DFI [7, 65]. Similar results leading to similar conclusions have been recently reported in a more compelling meta-analysis [66].

Finally, it should be noted that independently of the technique employed and ART outcomes, currently high values of SDF have been associated with *miscarriage* and *recurrent pregnancy loss (RLP) (*Table 58.3) [7, 41, 67, 68]. As a result of DFI > 40%, a significant increase of miscarriage (OR = 3.8; 95% CI: 0.38–0.97; $p < 0.05$) has been reported, in a retrospective study of 1633 IVF/ICSI cycles [7]. In addition to this, several researches that have focused their analysis especially on RPL have found a clear relationship between high value of SDF in couples with RPL compared to fertile men control without ART application (26% vs. 19.4%; $p < 0.001$), measured by different tests (Table 58.3) [41]; this negative association has also been shown in recent studies [41, 67, 68]. The most recent study also has shown a significant higher total antioxidant level (84.9% vs. 2.7%; $p < 0.001$) in men with high SDF and who suffered RPL in several cycles of ART compared with control group [41]. These evidences show that the possibility of sperm with DNA damage can fertilize an oocyte after ICSI, develop an aberrant embryo able to implant, and result in pregnancy loss that compromises health in both the offspring and the couple.

However, there are some contradictions with this later issue, from a previous study of Bellver et al. (2010), where it was demonstrated that SDF, measured by SCD, was not sufficient to be applied as a diagnostic tool in cases of RPL due to the lack of the statistical power in this analysis, but it should be noted that these couples had not attempted assisted reproduction treatments [69], unlike the other studies mentioned above and the different test performed to measure the SDF value. This later study was based on the evaluation of semen samples and SDF values of 60 patients (30 fertile donors as control and 30 couples with history of RLP) and, despite of the authors, could not demonstrate the evidence that a diagnostic test would avoid an episode of RLP, but there were significant differences in the SDF values between fertile controls and the group of patients which have suffered RLP (24% vs. 33.5%; $p < 0.05$) [69].

In this sense, the lack of a generally accepted cut-off value for SDF tests (Table 58.3) creates doubts about the wider and global clinical application of these tests [70]. There have been a number of professional bodies and experts' groups such as the European Society of Human Reproduction and Embryology (ESHRE) in 2010 and American Society for Reproductive Medicine (ASRM) in 2013 which concluded that current methods for evaluating sperm DNA integrity do not reliably predict treatment outcomes, and properly designed and controlled prospective studies are absolutely required to confirm these results [38, 48, 71]. It is important to note that this approach is not realistic for all situations, due to the different nature of SDF, and results in multiple continuous variables interacting with other confounding factors, including female endometrial receptivity, quality of oocytes, and its interaction with spermatozoa [70, 72].

Therefore, in the last years, Cho et al. (2016) have shown that a single cut-off value of SDF testing may not fit all, so it suggested to adopt multiple cut-off values according to different scenarios in clinical practice [70], just so it could set a *clinical practice guidance for SDF tests*. Researchers who specialized in the subject as Agarwal et al. (2016) have recommended that couples with clinic history as unexplained infertility, recurrent pregnancy loss (at least down ART treatments), repeated ART failures, clinical varicocele, or possible harming effects of lifestyle factors are indicated to take an SDF assay, as possible candidates may cause infertility due to high SDF values [38].

58.5 Is It Possible to Select a Sperm with the DNA Intact for ICSI?

The unclear long-term consequences of transmitting defective genes, particularly in cases of extremely high SDF treated with ICSI, should not be overlooked [6]. In addition, within a semen sample, there is a mixture of spermatozoa with different DNA integrity; so recently, several strategies to increase the probability to choose the spermatozoa with intact DNA for ICSI treatments have been purposed and are being developed, many of them already available as therapeutic tools offered to infertile patients, although most of the studies have not related ICSI outcomes with a significant reduction of SDF up to now, and the evidences supporting their use are in some cases scarce [73]. In general, their aim is to reduce the intrinsic mean SDF of the semen sample or neat ejaculate (NE) and also avoid the possible damage produced by centrifugation force of conventional methods of semen processing such as

the density gradient centrifugation (DGC) and swim up (SU), trying to enhance patient's success rates.

One of the most widely available methods *is magnetic activated cell sorting (MACS)*, a method in which annexin V, which has high affinity for PS, is conjugated with superparamagnetic microbeads for identification and selective sorting of apoptotic and nonapoptotic spermatozoa subsequently decreasing the chance of such sperm to be inseminated during ICSI [74, 75].

In this way, when a sperm sample previously incubated with annexin V conjugated magnetic microbeads is loaded into a column filter and a magnetic force is applied, apoptotic spermatozoa expressing PS are attached to the walls (annexin V positive fraction, MACS+), whereas nonapoptotic sperm (the annexin V negative fraction, MACS−) passes through the column to be collected and afterward employed in ART [74]. Currently, this sperm selection method has shown no significant adverse effects on obstetric and perinatal outcomes in children conceived when this technology was performed [76].

While some studies including MACS-DGC protocols have shown improvements in ICSI outcomes [75, 77, 78] in terms of percentage of high-quality embryos (87.8% vs. 46.3%; $p < 0.001$), clinical pregnancy rate (66.7% vs. 29.2%; $p < 0.05$), and implantation rates (50.0% vs. 19.5%; p = 0.01) which were significantly higher in the DGC-MACS group compared to DGC alone in patients with high DFI ICSI (DFI ≥20) compared to patients with lower DFI values (DFI < 20)

Table 58.4 Studies which include methods in sperm selection with SDF reduction and clinical outcomes for ICSI

Selection method for ICSI	Design of study	Study population (size)	Mechanism/criteria of sperm selection	SDF relative reduction (%) (in comparison with different methods as control)	Clinical outcomes (%) (significant)	References
PICSI	Prospective cohort studies	Volunteers ($n = 20$) Infertile patients ($n = 206$) Infertile patients ($n = 98$)	Bound spermatozoa to hyaluronic acid are more competent to fertilize to oocyte by their morphology and the membrane antigens presence	*PICSI vs. NE and vs. SU-PVP HA-bound spermatozoa* (5.3 vs. 16.5 vs. 11; p ≤ 0.001) None	*HA-ICSI vs. PVP-ICSI* Embryo quality and development improved *(35.8 vs. 24.1; p < 0.05)* *(95 vs. 84; p ≤ 0.001)* NS	[87, 88]
IMSI	Prospective cohort studies	Infertile patients ($n = 8$) N, OA, OAT ($n = 45$)	Spermatozoa with normal morphology without vacuoles in the head have the DNA intact and are able to fertilize the oocyte	*IMSI vs. 200×* Motile normal spermatozoa with vacuole-free head (4.1 vs. 18.7; $p < 0.001$) None	NS	[92, 93]
MACS	Prospective cohort studies Prospective cohort studies	Idiopathic infertile, N patients ($n = 20$) Infertile patients ($n = 216$) N ($n = 10$) Infertile couples under ICSI cycles with donor oocytes in MACS group and SU group as control ($n = 237$)	Select the non- apoptotic sperm fraction which theoretically has the intact DNA (MACS -)	*MACS vs. NE* in patients with SDF <30 and SDF >30 (8.6 vs. 18.3 and 7.1 vs. 41.4; $p < 0.001$) *DGC-MACS vs. DGC* (55 vs. 65; $p > 0.05$) *SU-MACS vs. SU* (10.3 vs. 21.4; $p > 0.05$) *SU-MACS vs. SU* (ns)	NS *Live birth and miscarriage rates do not improve*(42.9 vs. 34,8; $p > 0.05$) and (8.7 vs. 0; $p > 0.05$) NS ICSI outcomes were not significantly different in the MACS vs. control group in terms of; *fertilization rates* (75.3% vs. 72.1%), *percentage of good-quality embryos on day 3* (54.2% vs. 48.9%), *implantation rates* (42.2% vs. 40.1%), *clinical pregnancy* (63.2% vs. 68.6%), and live birth rates (48.4% vs. 56.4%) ($p > 0.05$)	[74, 79–81]
TESE	Meta-analysis Prospective cohort study	Infertile patients TS ($n = 278$), ES ($n = 229$) N patients TS ($n = 31$), ES ($n = 40$)	Testicular biopsy to select spermatozoa avoid the possible exposition of ROS to spermatozoa during their transport through sperm pathways	*TS vs. ES* (8.9 vs. 33.4; $p < 0.0001$) (44 vs. 44.5; $p > 0.05$)*	*Clinical/ongoing pregnancy in TS vs. ES* (50 vs. 29.4; $p < 0.001$) (41.9 vs. 20; $p < 0.05$)/(38.7 vs. 15; $p < 0.05$) *Live birth rates in TS vs. ES* (46.9 vs. 25.5; $p < 0.001$) *Miscarriages rates in TS vs. ES* (9.4 vs. 29.9; $p < 0.01$)	[45, 97]

Table 58.4 (continued)

Selection method for ICSI	Design of study	Study population (size)	Mechanism/criteria of sperm selection	SDF relative reduction (%) (in comparison with different methods as control)	Clinical outcomes (%) (significant)	References
SCE	Experimental laboratory study Prospective cohort study	Healthy donors ($n = 31$) Infertile patients ($n = 128$)	The positive cell fraction of the anode in the electrophoresis allows to select spermatozoa negatively charged (NCS) by their nature of glycosylate residues of membrane which offers them to negative charge, with mature chromatin competent to suffer capacitation, reach the oocyte, and fertilize it	*SCE vs. NE* (5 vs. 13; $p < 0.05$) *NCS vs. nonselected sperm* (3.9 vs. 17.3; $p < 0.001$)	NS NCS is associated with IVF *fertilization rate* ($r = 0.47$; $p < 0.01$) and blastocyst *development* ($r = 0.31$; $p < 0.01$) and inversely associated with *embryo arrest* ($r = -0.25$; $p < 0.05$). The *implantation rate was* higher in patient groups with >15% NCS (53.13 vs. 8.33; $p < 0.01$) and couples which achieved *clinical pregnancy* had higher NCS than couples who did not achieve (53 vs. 33.16; $p < 0.01$)	[101, 102]
MFSS	Prospective randomized study Experimental laboratory study Prospective randomized controlled study	Infertile men ($n = 98$) Healthy donors ($n = 37$) Men with unexplained infertility ($n = 122$): MFSS ($n = 61$), SU ($n = 61$)	Microfluidic devices mimic the natural conditions of female reproductive system and are able to select competent spermatozoa to fertilize to the oocyte, separating physically themselves (based in their motility, morphology) of debris and immotile incapacitated spermatozoa	*MFSS vs. DGC vs. NE* (0 vs. 15 vs. >30; $p < 0.05$) *MFSS vs. SU* (0.8 vs. 10.1; $p < 0.05$) *MFSS vs. SU* (ns)	NS *Fertilization rates* (63.3 vs. 57.4; $p > 0.05$), *clinical pregnancy rates* (48.3 vs. 44.8; $p > 0.05$), and *live birth rates* (38.3 vs. 36.2; $p > 0.05$) improve but not significantly. The number of *available grade 1 embryos after ICSI* is higher (1.5 vs. 0.8; $p < 0.05$)	[105–107]

PICSI physiological intracytoplasmic sperm injection; *IMSI* intracytoplasmic morphologically selected sperm injection; *MACS* magnetic cell sorting; *NE* neat ejaculate; *SU* swim up; *DGC* density gradient centrifugation; *HA* hyaluronic acid; *PVP* polyvinylpyrrolidone; 200× 200× magnification; *TESE* testicular sperm extraction; *TS* testicular sperm; *ES* ejaculated sperm; *MFSS* microfluidic sperm sorter; *NS* Not studied. All studies represent the reduction of SDF with the use of testicular sperm, except (∗) which did not study the SDF value of the patients after biopsy procedure; *n* size of study cohort; *N* normozoospermics; *OA* oligoasthenozoospermics; *OAT* oligoasthenoteratozoospermics; *SCE* sperm cell electrophoresis; *NCS* negatively charged sperm

[77]; others have not found an advantage in DGC-MACS application [79–81] (Table 58.4). This later study, which includes 237 infertile patients down ICSI cycles with donor oocytes, showed similar outcomes after ICSI, in terms of fertilization rates, good-quality embryos, and clinical pregnancy and live birth rate, between SU-MACS and SU groups [81].

These differences in ICSI outcomes could be explained due to this later study performing DGC after MACS; in fact, one recent study confirms that MACS-DGC protocol led to a significantly higher percentage of spermatozoa with progressive motility and normal morphology than DGC-MACS protocol [82]. Furthermore, the highest reduction (about 60% regarding to the NE) of SDF regarding other methods and NE has been achieved when MACS was applied alone [74].

In this later study, Gonzalez-Martinez et al. (2018) have shown a significant reduction in MACS fraction compared with NE (7.1% vs. 41.4%; $p < 0.001$), especially in patients with very high SDF (>30%) in general. Although the authors concluded that this reduction was not homogeneous, due to the presence of outliers without a reduction of SDF in the negative MACS fraction. Therefore, the possible explanation of these results could be the presence of fragmented spermatozoa in the negative MACS fraction, which are produced by other DNA degradation processes, commented previously, and not necessarily associated with apoptosis [74]. Furthermore, the presence of spermatozoa free of damaged DNA in MACS+ fraction reinforces this hypothesis. For this reason, just some patients whose have a justified type of male infertility related with apoptosis, as teratozoospermic and immotile population, could be benefited from MACS selection of spermatozoa due to the reduction of apoptosis fraction [78, 83].

Additionally, different studies have suggested that the use of sperm attached to hyaluronic acid (HA) by means of a sperm selection process known as *physiological ICSI with hyaluronic acid binding assay (PICSI)* improves embryo quality and development after ICSI, although all these stud-

ies were relatively small [84, 85]. In this assay, HA in a culture dish mimics artificially the nature of oocyte extracellular matrix, contributing to the binding of competent, mature sperm for fertilization by the presence of hyaluronidase enzymes on their plasma membrane [85].

In general, an improvement in embryo quality, implantation, and clinical pregnancy rates has been shown with PICSI technique compared with conventional morphology sperm selection in ICSI [85, 86] despite these studies not related to both PICSI outcomes with SDF assays at the same time [84–87]. In this way, a previous study has shown a significant reduction of SDF about 60% regarding the NE, and after using this sperm fraction in ICSI cycles, it has shown an improvement in ICSI outcomes, in terms of embryo quality and development (35.8% vs. 24.1%; $p < 0.05$) and (95% vs. 84%; p = 0.001) [88] (Table 58.4). However, in this later study, the patients under ICSI treatment had low levels of SDF (<20%).

Furthermore, the fertilization rates and abortion rates did not improve with this sperm selection method in PICSI cycles [85, 88]. In fact, the true cause in embryo development and pregnancy rate improvement has not been related exactly with a reduction of SDF value. In this way, PICSI has been considered a sensitive method to evaluate morphological integrity, high progressive motility, and nuclear maturation but is not a reliable test to predict SDF, MMP risks, intracellular ROS, and then healthy spermatozoa selection [87], being teratozoospermic patients benefiting most with PICSI [85]. Thus, evidence does not support routine use of hyaluronic acid binding assays; all ICSI cycles and the identification of patients that might benefit from this technique need further study [84].

A similar situation occurs with the use of *intracytoplasmic morphologically selected sperm injection (IMSI)*. While some studies have shown the improvement of ICSI outcomes with IMSI [89], others have not demonstrated a significant improvement of ICSI clinical outcomes compared with conventional ICSI, including very recent researches such as retrospective studies and meta-analysis [90, 91] on top of the lack of assays to check the SDF reduction in all these works.

With regard to the effect of IMSI in the reduction of SDF, there is a direct relationship between morphological parameters of spermatozoa and their DNA integrity, finding the SDF values higher in teratozoospermic patients [92]. In fact, in a randomized prospective study, the improvement of ICSI outcomes in cases of severe male factor, such as teratozoospermia, has been demonstrated [89], justifying the indication of IMSI in these cases. However, the IMSI technique alone is not enough for the selection of spermatozoa with intact nuclei [92], despite the apparent reduction of about 78% SDF (sperm selection by IMSI vs. 200x magnification; 4.1% vs. 18.7%; $p < 0.001$) in normozoospermic patients [93] (Table 58.4). In this way, it has been demonstrated the presence of SDF in sperm whose morphology was apparently normal and it has been suggested a negative impact on embryo quality and the pregnancy rates in ICSI cycles in case of being selected and injected [94].

Thus, a prospective study in cohort of 33 normozoospermic men showed that one of the best predictors of DNA integrity could be the combination of a sperm head birefringent pattern with *normal motile sperm organelle morphology examination* (*MSOME)* and without nuclear vacuoles (as a result of the 97.2% of sperm with intact DNA leading this pattern) [95]. The combination of both methods, using a single polarized light microscope based on the real-time evaluation of sperm under a magnification up to >6000x, allows to select spermatozoa for ICSI with double refraction or birefringence. The presence of birefringence is due to the decomposition of a ray of light into two rays when it passes through an anisotropic nature of sperm cells due to nucleoprotein filaments arranged in rods and oriented longitudinally into the sperm head, indicating a normal nuclear organization [95]. However, neither the fertilization capacity of these men was not tested nor the improvement of ICSI outcomes due to this reduction of SDF.

The use of *testicular sperm extraction/testicular sperm aspiration (TESE/TESA)* for ICSI, even on males with the presence of sperm within their ejaculate as a treatment strategy for high SDF cases, showed promising results [36, 96, 97]. Despite the doubts regarding the use of testicular sperm that currently exists, due to the potential harm produced by the intervention, sperm retrieval in non-azoospermic men is highly successful and requires minimal tissue excision [98]. In fact, this sperm selection strategy would be the most appropriate in men with high SDF values in the ejaculate, since it has been shown that elevated levels of ROS can cause SDF during sperm transport through the seminiferous tubules and epididymis, thus causing posttesticular harm [99].

This, together with a very recent meta-analysis of Esteves et al. (2017), including 143 patients which provided paired SDF rates for testicular and ejaculated sperm, has reported a relative reduction in SDF ranging from 67% to 80% [6, 45, 96] being remarkably greater than other techniques described above (Table 58.4). Indeed, the beneficial effect of using testicular sperm over other sperm selection techniques has been demonstrated. In a retrospective analysis of Bradley et al. (2016), 1924 patients with high SDF (≥29%) under ICSI treatment were evaluated. A higher live birth rate of 49.8% was reported with the use of testicular sperm, which was significantly higher than that of PICSI (38.3%) and IMSI (28.7%) ($p < 0.05$). In addition, high SDF patients who had an intervention had significantly improved blastocyst transfer outcomes, similar to those of low SDF patients; single embryo transfer live birth rate for high SDF intervention patients was higher (43.8% vs. 24.9%; $p < 0.05$) as compared with high SDF and similar to low SDF patients (43.8% vs.

40.6%; $p > 0.05$) [96]. Also, some other clinical outcomes using testicular sperm have improved compared with the use of ejaculated sperm in ICSI, getting higher results in clinical pregnancy (50% vs. 29.4%; $p < 0.001$) while lower in terms of miscarriage rate (9.4% vs. 29.1%; $p < 0.001$) [45]. A very recent prospective study of Pabuccu et al. (2017), with 71 normozoospermic men with high SDF (>30%) and at least two previous ART failures, has reported significant better outcomes in clinical and ongoing pregnancy rates using testicular sperm compared to ejaculate sperm group (41.9% vs. 20% and 38.7% vs. 15%; $p < 0.05$), despite not demonstrating the clear reduction in SDF with this strategy, due to the lack of its analysis before and after the intervention in the same sample of the patient with their ICSI outcomes at the same time [97] (Table 58.4).

Thus, sperm DFI should be a part of male partner's evaluation following unsuccessful ART attempts because of the clear evidence in its negative impact over the ICSI outcomes. In addition, when high DFI is detected (>30%), ICSI using testicular spermatozoa obtained by TESE/TESA seems an effective option, among other less invasive treatments for alleviating DNA damage, particularly for those with repeated ART failures in terms of clinical, ongoing pregnancies, and miscarriages even though conventional sperm parameters are within normal range [36, 97]. Hence, the use of testicular spermatozoa is the unique strategy which has been demonstrated in several studies up to now with a significant reduction of SDF degree and an improvement of ICSI outcomes, at the same time, comparing other less invasive treatments in case of high SDF [36, 97]. Nonetheless, a call for continuous monitoring is required concerning the health of generated offspring and the potential complications of this sperm extraction [36].

Apart of the ability to bind to HA, the absence of PS externalization of an apoptotic stage, and the apparently normal morphology, the spermatozoa express other markers whose presence indicate a good capacity to fertilize the oocyte and are currently chosen to perform a sperm selection. Ainsworth et al. (2011) studied that sperm surface glycoproteins are the main responsible of sperm cell's electronegative charge by studies of mass spectrometry [100]. For this reason, many authors, including his team, have attempted the design of a sperm selection method based on the electric charge of spermatozoa through electrophoresis assays [101–103]. It has been described that the CD52, a highly sialated glycosylphosphatidylinositol-anchored protein, is introduced in the sperm surface during epididymal maturation and is the main precursor of the negative charge generated in this process [104]. Thus, the *sperm cell electrophoresis (SCE)* allows to select the negatively charged sperm (NCS), which is mature and competent to migrate to the anode in an electrophoresis assay, facing the positively charged sperm (PCS), which is composed to immotile and immature sperm and debris [102]. In addition, Ainsworth et al. (2005) in an experimental study of 31 healthy donors evaluated this NCS, and it seems to display a significant reduction of SDF versus other semen processing methods such as DGC and repeated centrifugation and, in relation to the NE ($p < 0.05$), suggests a new selection method to choose the intact DNA sperm for ICSI [102] (Table 58.4). Ainsworth et al. (2007) also demonstrated a significant reduction in NCS of SDF after cryopreserving-thawing procedures of semen samples (15% vs. 30%; $p < 0.01$) and in spermatozoa of testicular biopsy (12% vs. 30%; $p < 0.05$), but with a limited sample size [103]. Some years later, Simon et al. (2015) performed this assay in a prospective study of 128 infertile patients, in a microelectrophoresis instrument. These authors demonstrated again the significant reduction of fragmentation in NCS versus nonselected sperm (3.9% vs. 17.3%; $p < 0.001$), neutrally charged (3.9% vs. 12.1%; $p < 0.001$), PCS populations (3.9% vs. 27.8%; $p < 0.001$), and progressively motile and morphologically normal ICSI-selected sperm (3.9% vs. 9.8%; $p < 0.001$) [101]. Furthermore, this sperm fraction has been related to an improvement of ICSI outcomes (Table 58.4). The percentage of NCS was positively associated with blastocyst development ($r = 0.308$; $p < 0.01$) and inversely associated with embryo arrest ($r = -0.253$; $p < 0.05$). In addition, implantation rate was higher in the patient group containing >15% NCS (53.13% vs. 8.33%; $p < 0.01$) compared with the patient group containing <15% NCS. Finally, couples achieving clinical pregnancy had a higher NCS (53.20% vs. 33.16%; $p < 0.01$) and lower %PCS (42.47% vs. 64.23%; $p < 0.01$) than couples who did not achieve clinical pregnancy. In addition, this method is extremely versatile and easy to use and neither requires complex instruments nor additional qualified technicians, being the most important. In conclusion, despite the results of selection of free DNA damage by NCS, this principle has yet to be confirmed in the context of ART and management of male infertility [101]. So, future researches about this are needed to confirm the ICSI outcomes.

Other promising strategy is by the use of *microfluidic sperm sorter (MFSS),* which consists in a device to isolate motile and morphologically normal sperm, based on fluid dynamics, from a sample without centrifugation, thereby potentially avoiding oxidative stress and DNA damage in contrast to SU and DGC procedures [105]. This innovative technique is based on laminar flows within microfluidic channels, mimicking the natural routes that select healthy spermatozoa in female reproductive system to sperm selection depending on their own ability to swim across the semen stream into the medium stream, considering it as the best spermatozoa with potential capacity to reach and fertilize the oocyte [105, 106]. Furthermore, it is an easy, less time-consuming procedure with high clinical applicability and repeatability instead of the centrifugation stages involved in

the conventional techniques [106]. Quinn et al. (2018) have recently reported a significant reduction of SDF in MFSS compared to DGC and NE sperm (0% vs. 15% vs. >30%; $p < 0.05$, respectively) of 70 infertile men [107], and Shirota et al. (2016) found a significant reduction compared to SU (0.8% vs. 10.1%; $p < 0.05$) in 37 healthy volunteers but without proven fertility [105]. The reduction of SDF over conventional sperm procedures in both studies was nearby 100% [105, 107]. Although none of both studies have included ICSI outcomes yet, it seems that it is a promising strategy of non-invasive sperm selection for men with high SDF values. In a very recent prospective, randomized controlled trial of Yetkinel et al. (2018), the effects of sperm selection by microfluidic in ICSI outcomes compared to SU procedures (control group) in 122 men with unexplained infertility have been analyzed. This study has not showed that MFSS technique improves significantly the ICSI outcomes in terms of fertilization rates (63.3% vs. 57.4%; $p > 0.05$), clinical pregnancy rates (48.3% vs. 44.8%; $p > 0.05$), and live birth rates (38.3% vs. 36.2%; $p > 0.05$) versus conventional SU procedures. However, these rates were better in males whose seminal samples were processed with MFSS and showed a significant higher number of available grade 1 embryos after ICSI treatment compared to control whose samples were processed with SU (1.5% vs. 0.8%; $p < 0.05$). However, this study has neither evaluated the SDF value of the patients (Table 58.4) nor its possible impact in the ICSI outcomes [106].

Sperm selection techniques including PICSI, IMSI, and MACS have brought about conflicting results [6]. Likewise, the effect of sperm preparation with DGC and SU on ART outcomes remains inconclusive [6]. Some studies have found a reduction in SDF rates with regard to the NE [79, 80, 105, 107], thus demonstrating higher reduction of SDF in combination of conventional procedures with MACS [79, 80]. However, there is no definitive clinical evidence that any of these methods can avoid the potentially harmful effects of abnormal sperm on ART outcomes. Sperm selection technologies face limitations because none of the current techniques can completely prevent the selection of sperm with DNA damage [35].

Furthermore, it suggested to verify the ICSI outcomes of the new promising strategies such as MFSS, SCE, and the use of IMSI along with the birefringence, which appear to have presented a reduction of DFI near 100%, a percentage that has not yet been reached by any technique of sperm selection.

58.6 Conclusions

In summary, the sperm DNA damage origin seems to be a multifactorial process, in which there are several internal and external factors implicated, being oxidative stress one of the main factors in most cases. This difficulty to identify a sole true origin of the strand breaks makes difficult not only its diagnosis but also selection of the sperm with intact DNA.

For this reason, there is a lack of agreement and consensus about how to introduce the sperm DNA fragmentation assay in a routine sperm analysis in clinics today and how to clinically deal with their results, since no one-fits-all solution seems to appear valid. Currently, there are different patients such as men with unexplained infertility, recurrent pregnancy loss, repeated ART failures, clinical varicocele, or suffering possible harming effects of a variety of lifestyle factors, who could be potentially benefited from the information provided by sperm DNA fragmentation assays, probably in a different manner and amount, since they could be identified as possible cases of infertility caused by high SDF values.

Thus, it suggested the customized application of SDF cut-off for each type of risk patients, due to other influential unknown factors in ART outcomes and the difference with respect to the sensitivity of the methods.

With regard to the impact of SDF in fertility, there are evidences about this negative effect in natural conception and intrauterine insemination outcomes. Despite the controversy in this negative effect with IVF outcomes, overall it seems that ICSI treatment improves the clinical outcomes versus IVF, in couples with high sperm DNA fragmentation values, and has been noted in men. As a result, it justifies the need to choose the best strategy of sperm selection to rule out the possibility to microinject a spermatozoa with damaged DNA in ICSI.

Currently, there are different strategies to reduce the SDF value in the ejaculate, but they have not demonstrated that a significant reduction improves the ICSI outcomes up to now conclusively and robustly. The use of testicular sperm has showed the best outcomes for ICSI via a significant evidence of SDF reduction in patients with previous failures and high values of SDF despite of not ensuring its complete reduction.

Further research will undoubtedly clarify some of these knowledge gaps and will contribute to the improvement of the reproductive results in infertile couples where the male factor had a significant contribution to the reproductive failure.

58.7 Review Criteria

A comprehensive research of studies examining the relationship between sperm DNA fragmentation and assisted reproduction technique outcomes was performed using search engines such as Pubmed, Google Scholar, and Sciencedirect. The search period comprised 3 months (from November 2018 until January 2019). A first revision was performed to compile the most current articles about this topic, based on

the keyword "sperm DNA damage." The articles were classified according to their content and the type of study. Review studies were specially selected to answer the first two questions in this review. While a data extraction, based on prospective studies and meta-analysis, was included to answer the last questions, using the additional following keywords: "sperm DNA damage AND ART outcomes" and "sperm DNA damage AND sperm selection," focusing the data analysis mainly in ICSI cycles.

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Testicular Sperm in Non-azoospermic Infertile Men with Oxidatively Induced High Sperm DNA Damage

59

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Key Points

- Sperm DNA integrity is essential for healthy human embryo development and successful pregnancy outcome. In addition to the risk of infertility and impaired reproductive outcomes, there is an increased risk of diseases in offspring when natural or artificial inseminations are carried out with specimens containing high frequencies of sperm with fragmented DNA.
- Sperm DNA fragmentation (SDF) testing has been used to obtain information about sperm DNA quality, particularly for the evaluation of a possible male factor contributing to infertility. Knowledge of the SDF status can be used to strengthen patient counseling and allow clinicians to provide a more realistic prognosis of every treatment strategy the couple wishes to pursue.
- A clinical practice guideline issued by the Society for Translational Medicine has provided recommendations for SDF testing. In the assisted reproductive technology (ART) settings, SDF testing is recommended after failed IUI, IVF, or ICSI cycles provided no other apparent reason exists to explain that failure. In such cases, the use of testicular sperm rather than ejaculated sperm may be beneficial in men with oligozoospermia, high SDF, and recurrent IVF failure (grade B–C recommendation).
- Apoptosis triggered by testicular conditions and oxidative stress (OS) during sperm transit through the male reproductive tract are the primary causes of sperm chromatin susceptibility to damage. The source of OS can range from a specific clinical condition such as a varicocele and a subclinical genital infection to environmental exposure to toxicants, advanced paternal age, obesity, and smoking.
- Correction of underlying factors can decrease SDF and potentially enable natural conception or increase the likelihood of pregnancy by ART using ejaculated sperm.
- When SDF remains high after treatment of the underlying factors or no apparent condition is identified to allow potential treatment, the use of testicular sperm (Testi-ICSI) in preference over ejaculated sperm for ICSI might overcome the oxidatively induced SDF.
- Sperm DNA fragmentation is markedly lower in testicular sperm than in the ejaculated sperm. Testi-ICSI may bypass posttesticular chromatin damage caused by OS during sperm transit through the epididymis.
- Data from observational studies suggest that pregnancy outcomes by Testi-ICSI in men with high SDF in the semen are significantly better than that of ejaculated sperm. Additionally, miscarriage rates are lower with the former.
- Testi-ICSI results in higher live birth rates than the current laboratory methods used to select specimens with lower SDF levels.
- Further research in the form of well-designed prospective randomized trials is needed to confirm the clinical utility of Testi-ICSI as a means to overcome the oxidatively induced SDF. Furthermore, more investigation is required to determine the influence of this approach on offspring health.

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59.1 Introduction

The laboratory evaluation of male infertility still relies on conventional semen parameters according to the criteria established by the World Health Organization (WHO) [1]. However, the normalcy values as per the WHO cannot differentiate fertile from infertile men accurately. Indeed, 15–40% of men with difficulties to father a child have semen parameters within normal ranges and no obvious abnormalities on physical examination [1–7].

Nowadays, irrespective of the cause or severity of male factor infertility, a substantial number of couples use assisted reproductive technology (ART), in particular intracytoplasmic sperm injections (ICSI), as a means to alleviate infertility [8]. However, live birth rate (LBR) with ICSI does not exceed 30% per cycle overall, and semen abnormalities – as conventionally assessed – are not predictive of ART outcomes [9].

Modern sperm functional testing with clinical value for pregnancy prediction would be advantageous for couples embarking on ART. Currently, the focus is on molecular mechanisms implicated in the etiology of male infertility [6, 10, 11]. Along these lines, the assessment of sperm chromatin at the molecular level has gained increased interest over recent years. In clinical practice, the use of sperm DNA fragmentation (SDF) tests has become common among ART practitioners for two main reasons [2, 3, 11–13]. First, infertile men have higher SDF in semen than fertile counterparts [2, 11, 14, 15]. Second, the evidence of an adverse effect of SDF on natural fertility as well as ART outcomes has become less equivocal with the accumulation of published data [16–20].

Varicocele [21], systemic diseases, male accessory gland infections, advanced paternal age [22], obesity, lifestyle and environmental factors, radiation, and heat exposure [5] are some of the conditions associated with abnormal levels of SDF [4, 5]. Most of these stressors share a common trait, that is, excessive oxidative stress (OS), which is regarded as a major factor in the pathophysiology of SDF.

Several strategies have been proposed to alleviate SDF, including treatment of the underlying conditions, changes in lifestyle and dietary patterns, use of antioxidants, and ART [23], which can be applied alone or combined. The first step is to identify the affected individuals by measuring the proportion of spermatozoa with DNA fragmentation in the neat ejaculate using probes or dyes with the aid of fluorescence microscopy, optical microscopy, or flow cytometry [24]. Despite the need for further standardization and establishment of consensus cutoff values, SDF testing can provide a common pathway to measure the oxidatively induced SDF [2, 25, 26].

As for ART, although ejaculated sperm are regarded as having better fertilization potential than testicular sperm as they have completed their transit through the male reproductive tract, recent studies have shown that higher pregnancy outcome might be achieved with the use of testicular sperm than ejaculated sperm for ICSI in non-azoospermic infertile men with high SDF [15, 18, 21, 27–34]. In this chapter, we discuss the biological plausibility of using testicular sperm (Testi-ICSI) in preference of ejaculated sperm for ICSI in men with high SDF and summarize the current literature concerning the clinical utility of Testi-ICSI in this specific clinical scenario.

59.2 The Oxidatively Induced Sperm DNA Fragmentation

Sperm chromatin damage mainly comprises single- and double-stranded DNA breaks in the sperm nuclei [4]. The critical mechanisms explaining SDF include abortive apoptosis, protamination defects, and OS [4, 5]. Adequate protamination stabilizes and protects the nuclear DNA from damage during epididymal passage and post-ejaculation [27, 35]. In normal conditions, there is a balance between reactive oxygen species (ROS) production and antioxidant defenses in the male reproductive tract [4, 11]. OS occurs when ROS production overwhelms the antioxidant defense. Excessive ROS attack both sperm membranes and nuclear and mitochondrial DNA, mostly during sperm transit through the male genital tract [4, 6, 10, 11, 26, 35, 36]. ROS can also inflict oxidative damage to DNA base pair in sperm, which might result in gene mutations or polymorphisms [5].

A 2018 systematic review and meta-analysis polling data from 28 observational and interventional studies showed that infertile men have higher SDF rates than fertile men (weighted mean difference [WMD] −1.67%, 95% CI −2.12%, −1.21%; $I^2 = 97\%$; $p < 0.00001$) [3]. Given the high statistical heterogeneity probably due to various SDF tests utilized, the authors analyzed the data separately according to each SDF test, namely, sperm chromatin dispersion test (SCD), sperm chromatin structure assay (SCSA), and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Their results confirmed that the difference remained statistically significant after subgrouping patients according to the SDF method. Additionally, a subgroup analysis comparing men with proven fertility to men with unexplained infertility revealed that SDF rates were higher in the latter ($p = 0.003$). In the study mentioned above, the SDF threshold of 20% best discriminated fertile from infertile men when all datasets and SDF assays were grouped (area under the curve [AUC] 0.844, $p < 0.001$). With this cutoff, the sensitivity and specificity of SDF was 79% and 86%, respectively [3].

Animal and clinical studies have confirmed the relationship between SDF and OS. In a recent report, Majzoub and colleagues showed that the oxidation-reduction potential – a marker of OS – was five times higher in infertile patients than in fertile controls (5.44 ± 0.34 mV/10^6 sperm/mL vs. 1.18 ± 0.94 mV/10^6 sperm/mL; $p < 0.001$) [13]. Likewise,

SDF (by the SCD assay) was markedly higher among infertile men (27.6% ± 1.0%) than controls (15.7% ± 0.9%; $p < 0.001$).

59.3 Clinical Impact of Sperm DNA Fragmentation in ART

Many studies have investigated the influence of SDF on embryonic and pregnancy outcomes among infertile couples undergoing ART [13, 16, 17, 37–40]. Overall, high levels of SDF negatively affect fertility by reducing fertilization rates, embryo development, implantation, and pregnancy [2, 3, 5, 9, 10, 21, 26, 32, 41–43].

Meta-analyses of ART studies concur overall that sperm DNA damage reduces pregnancy success. A 2014 study indicated that pregnancy likelihood was lower in couples whose male partners had high SDF rates in the ejaculated semen (combined relative risk [RR] = 0.81; 95% CI 0.70–0.95; $p = 0.008$) [32]. In this report, there was a significant increase in the risk of miscarriage among couples whose male partner had high sperm DNA damage irrespective of the insemination method, IVF or ICSI (OR 2.68; 95% CI 1.40–5.14; $p = 0.003$). In a 2017 report polling data from 56 studies and over 8, 000 treatment cycles, SDF adversely affected clinical pregnancy following IVF (OR 1.65, 95% CI 1.34–2.04; $p < 0.0001$) and ICSI (OR 1.31, 95% CI 1.08–1.59; $p = 0.0068$) [5]. Additionally, the risk of miscarriage increased among couples undergoing ART with high (versus low) SDF rates (RR 2.16; 95% CI 1.54–3.03; $p < 0.0001$).

Given these recent observations, the potential value of SDF testing before ART and other clinical scenarios has been acknowledged by the Society for Translational Medicine in its 2017 clinical practice guidelines for SDF testing in male infertility (Table 59.1) [9].

Table 59.1 Society for Translational Medicine: clinical practice guidelines for sperm DNA fragmentation testing based on clinical scenarios

1. Clinical varicocele
SDF testing is recommended in patients with grade 2/3 varicocele with normal conventional semen parameters (grade C recommendation)
SDF testing is recommended in patients with grade 1 varicocele with borderline/abnormal conventional semen parameter results (grade C recommendation)
2. Unexplained infertility/IUI failure/recurrent pregnancy loss (RPL)
SDF testing should be offered to infertile couples with RPL or prior to initiating IUI (grade C recommendation)
Early IVF or ICSI may be an alternative to infertile couple with RPL or failed IUI (grade C recommendation)
3. IVF and/or ICSI failure
SDF testing is indicated in patients with recurrent failure of assisted reproduction (grade C recommendation)
The use of testicular sperm rather than ejaculated sperm may be beneficial in men with oligozoospermia, high SDF and recurrent IVF failure (grade B–C recommendation)
4. Borderline abnormal (or normal) semen parameters with risk factors
SDF testing should be offered to patients who have a modifiable lifestyle risk factor of male infertility (grade C recommendation)

SDF sperm DNA fragmentation, *RPL* recurrent pregnancy loss, *IVF* in vitro fertilization, *ICSI* intracytoplasmic sperm injection, *IUI* intrauterine insemination

Grades of recommendations according to quality of evidence: Grade A, based on clinical studies of good quality and consistency with at least one randomized trial; Grade B, based on well-designed studies (prospective, cohort) but without good randomized clinical trials; Grade C, based on poorer quality studies (retrospective, case series, expert opinion). Modified from Oxford Centre for Evidence-Based Medicine (http://www.cebm.net/oxford-centre-evidence-based-medicine-levels-evidence-march-2009/)

Based on data from Ref. [9]

59.4 Biological Plausibility of Testicular Sperm from Non-azoospermic Men for ICSI

59.4.1 SDF Rates in Ejaculated, Epididymal, and Testicular Sperm

Early human studies comparing paired epididymal and testicular specimens from men with obstructive azoospermia (OA) revealed that SDF was lower in testicular sperm than in epididymal sperm. In a group of 20 men with OA using the comet assay, the percentage of testicular sperm with intact DNA was significantly higher (83.0 ± 1.2%) than that of proximal epididymis (75.4 ± 2.3%; $p < 0.05$) [44]. The authors also compared the proportion of sperm with intact DNA between testicular specimens of 39 men with OA (84.0% ± 0.9) and ejaculated specimens from a control group of 10 fertile men about to undergo vasectomy (86.8% ± 1.8) and found that results were not statistically different [44]. In another study involving 25 men with OA, the authors used a modified long polymerase chain reaction to study mitochondrial DNA (mtDNA) and a modified alkaline comet assay to measure nuclear DNA (nDNA) fragmentation in testicular and epididymal sperm [45, 46]. They found that mtDNA and nDNA of testicular sperm have fewer mutations and fragmentation than epididymal sperm.

More recently, a 2017 study comparing SDF rates between testicular and epididymal sperm using the TUNEL assay provided confirmatory evidence in favor of testicular sperm (versus epididymal sperm) in patients with OA [47]. The authors evaluated 21 patients with OA due to a congenital bilateral absence of the vas deferens, postinfection, and idiopathic obstruction. For each patient, the authors obtained specimens from the testis, caput epididymis, and corpus/cauda epididymis. Overall, SDF rates were lower in the testis (6.71% ± 0.75) than in caput epididymis (14.9% ± 1.9; $p = 0.0007$) and corpus/cauda (32.6% ± 3.1; $p < 0.0001$). There was no apparent difference in SDF rates according to the etiology of obstruction. In their series, all deliveries were

Table 59.2 Studies assessing DNA fragmentation in paired ejaculated and testicular specimens on same subjects

Study	Population	No. patients	SDF assay	DFI cutoff (%)	Median SDF rate in testicular sperm compared to ejaculated sperm	No. patients (%) with SDF values lower in testicular sperm than in ejaculated sperm
Greco et al. [30]	Non-smokers; mean sperm count: 26.8 M/ml; sperm motility: 36.7%; sperm morphology: 20.9%	18	TUNEL	15	4.8% vs. 23.6% ($p < 0.001$)	17 (94.5%)
Moskovtsev et al. [34]	High DFI despite AOX	12	TUNEL	30	13.3% vs. 39.7% ($p < 0.001$)	11 (91.7%)
Esteves et al. [18]	Idiopathic oligozoospermia (5–15 M/mL); high DFI despite AOX	81	SCD	30	40.7% vs. 8.3% ($p < 0.001$)	81 (100.0%)
Mehta et al. [31]	Idiopathic oligozoospermia (<5 M/ml), failed IVF	24	TUNEL	7	5.0% vs. 24.0% ($p = 0.0013$)	ND

DFI DNA fragmentation index, *SDF* Sperm DNA fragmentation, *AOX* Oral antioxidant therapy, *TUNEL* terminal deoxynucleotidyl transferase dUTP nick end labeling, *SCD* sperm chromatin dispersion test, *ND* not described

achieved with specimens with low SDF rates; the authors noted a tendency to better delivery rates by ICSI with testicular sperm rather than epididymal sperm (35.7% vs. 12.1%; $p = 0.06$).

Studies examining paired ejaculated and testicular specimens from non-azoospermic men also showed that SDF is lower in the testis than in the ejaculate (Table 59.2). In 2005, Greco et al. were the first to suggest that SDF might be avoided by using testicular sperm instead of ejaculated sperm for ICSI [30]. The authors also showed that SDF rates were significantly lower in testicular sperm than in ejaculated counterparts. Moskovtsev et al. [34] confirmed these findings in a group of infertile men with high SDF in the semen despite the use of oral antioxidants for 3 months. In their study, SDF in testicular sperm were threefold lower than in ejaculated sperm (13.3 ± 7.3% vs. 39.7 ± 14.8%, $p < 0.001$). Later in 2015, Esteves et al. evaluated a group of infertile men with idiopathic oligozoospermia ($5–15 \times 10^6$ sperm per ml) presenting with persistent high SDF (>30% DNA fragmentation index [DFI] by SCD) despite the use of oral antioxidant therapy for 3 months [20]. The DFI was fivefold higher in the semen (40.7% ± 9.9%) than in the testis (8.3% ± 5.3%; $p < 0.001$). Lastly, in a population of severe oligozoospermic men and using the TUNEL assay, Mehta et al. [31] found lower DFI in testicular sperm (5%, 95% CI 3–7%) than in ejaculated sperm (24%, 95% CI 14–34%; $p < 0.001$).

A 2017 systematic review and meta-analysis examined SDF rates in paired ejaculated and testicular specimens of non-azoospermic infertile men with high SDF in neat semen [15]. The pooled estimates indicated that the mean difference in SDF rates between testicular and ejaculated sperm was −24.6% (95% CI −32.53 to −16.64; $I^2 = 92\%$; $p < 0.001$). Given the high statistical heterogeneity, the authors analyzed the data separately by the SDF method. With TUNEL, the pooled mean difference remained statistically different in favor of a reduced SDF in testicular sperm ($p < 0.001$). In the single study using the SCD test, the mean difference was −32.4% (95% CI −34.85% to −29.95%; $p < 0.001$) favoring testicular sperm [15].

59.4.2 Aneuploidy Rates in Ejaculated, Epididymal, and Testicular Sperm

Suganuma et al., in a mice study, showed that motile sperm from the cauda epididymis had a higher frequency of structural chromosome aberrations than testicular sperm or caput epididymis sperm. In their study, the percentage of structural chromosomal aberrations in embryos resulting from fertilization by sperm taken from the cauda epididymis was significantly higher than that with the use of testicular sperm (24–33% vs. 10–13%, $p < 0.05$) [35].

By contrast, Moskovtsev et al., studying a small group of 12 infertile men, showed higher aneuploidy rates in testicular sperm than in ejaculated counterparts (12.4 ± 3.7% vs. 5.7 ± 1.2%; $p < 0.05$), despite of the fact that SDF by TUNEL was about threefold lower in the former (13.3% ± 7.3 vs. 39.7% ± 14.8) [34]. Contrary findings were reported recently in a 2018 study by Cheung et al. who used whole exome sequencing molecular karyotype to evaluate sperm aneuploidy rates in ejaculated and testicular sperm [48]. The authors studied fertile donors and infertile patients (both men with nonobstructive azoospermia and non-azoospermic men with high SDF). Next-generation sequencing data revealed that aneuploidy rates in testicular specimens were as low as those of ejaculated samples from fertile donors (1.9% vs. 1.2%), whereas aneuploidy rates were 11.1% in ejaculated specimens of infertile patients ($p < 0.0001$). Additionally, paired assessments in ejaculated and testicular specimens of non-azoospermic men with high SDF showed that both SDF rates and aneuploidy rates were significantly lower in testicular sperm (8% and 1.2%) than in ejaculated sperm (20% and 8.4%).

The literature is deficient in studies evaluating embryo aneuploidy rates according to the type of sperm used for ICSI. However, some evidence does exist suggesting that aneuploidy rates are similar irrespective of the type of sperm [49]. In one report screening 572 embryos for aneuploidy in chromosomes 13, 18, 21, X, and Y by FISH analysis, the proportion of euploidy embryos was not statistically different among epididymal (41% ± 31%), testicular (48% ± 38%), and

ejaculated (48% ± 31%) groups [48]. In this study, however, the mean female age, which plays a remarkable role in embryo genetic status, was higher among couples using ejaculated sperm, and thus might have biased the overall results in favor of epididymis and testicular groups.

Currently, there is a lack of reports individually evaluating embryo genetic status and health of offspring resulting from Testi-ICSI in couples of non-azoospermic partners with high SDF. While the data from studies assessing the health of infants born from ICSI using epididymal and testicular sperm from azoospermic fathers is overall reassuring [25, 50–52], further research is warranted to confirm whether these observations will hold for non-azoospermic men.

59.5 ICSI Outcomes Using Testicular Sperm from Non-azoospermic Men with High SDF

In 2005, Greco et al. were the first to use testicular sperm in a group of 18 couples with repeated ICSI failure after use of ejaculated sperm [30]. The male partners were non-azoospermic with high DFI in ejaculated sperm. The authors noted that clinical pregnancy rates by ICSI were significantly higher with testicular sperm than with ejaculated sperm (44.4% vs. 5.6%, $p < 0.05$).

Confirmatory findings were published later in 2010 by Sakkas and Alvarez [53]. The authors retrospectively evaluated 72 patients with high SDF (>20% by TUNEL), 42 of which had sperm injections with ejaculated sperm, whereas 30 had ICSI with testicular sperm harvested by TESA. The CPRs and implantation rates in the testicular sperm group (40.0% and 28.1%, respectively) were significantly higher than in the ejaculated sperm group (13.8% and 6.5%, respectively; $p = 0.03$ for pregnancy and $p = 0.002$ for implantation).

Subsequently, in 2015, Esteves et al. conducted a prospective comparative study which enrolled 172 infertile men with idiopathic oligozoospermia and persistent high SDF (>30% by SCD) after oral antioxidants [31]. In their study, miscarriage rates were lower and live birth rates (LBR) were higher in couples who underwent ICSI with testicular sperm than in those in whom sperm injections were carried out with ejaculated sperm. The adjusted relative risks (RR) for miscarriage and live birth between the testicular sperm group and the ejaculated sperm group were 0.29 (95% CI 0.10–0.82; $p = 0.019$) and 1.76 (95% CI: 1.15–2.70; $p = 0.008$), respectively. Additionally, the authors reported that the number needed to treat by testicular in preference over ejaculated sperm to obtain an additional live birth per fresh transfer embryo cycle was 4.9 (95% CI 2.8–16.8).

In the same year, Mehta et al. reported a case series of 24 couples with previous failed ICSI cycles with ejaculated sperm whose male partners had severe oligozoospermia and high SDF (>7% by TUNEL) [31]. Sperm injections performed with testicular sperm resulted in a CPR of 50%, and all pregnancies ended in deliveries of healthy babies.

In 2016, Bradley et al. queried their database to examine ICSI outcomes in cycles involving high seminal SDF [23]. They compared the results of sperm injections using ejaculated sperm selected with some laboratory method to reduce sperm with DNA fragmentation (220 cycles), ICSI using ejaculated sperm without any specific sperm selection method (80 cycles), and ICSI using testicular sperm (Testi-ICSI; 148 cycles). The laboratory methods used to select sperm with better chromatin integrity for ICSI were intracytoplasmic morphologically selected sperm injection (IMSI) and hyaluronic acid sperm selection ICSI (PICSI). In their study, higher LBRs ($p < 0.05$) were obtained with Testi-ICSI (49.8%) than with IMSI (28.7%) or PICSI (38.3%). The lowest LBRs (24.2%) occurred when no intervention was carried out to deselect sperm with DNA fragmentation ($p = 0.020$).

A 2017 study by Pabuccu et al. assessed ICSI outcomes of 71 couples with repeated ICSI failure [15]. The male partners were normozoospermic and presented high SDF (>30% by TUNEL). In this report, CPR (41.9% vs. 20.0%; $p = 0.04$) and ongoing pregnancy rates per started cycle (38.7% vs. 15.0%; $p = 0.02$) were higher when sperm injections were performed with testicular sperm rather than ejaculated sperm.

The studies [18, 23, 28, 30] mentioned above were compiled in a 2017 meta-analysis, in which a total of 507 ICSI cycles and 3840 injected oocytes were analyzed [15]. The pooled estimates showed that the OR for CPR and LBR between the testicular sperm group and the ejaculated sperm group were 3.6 (95% CI 1.94–6.69; $I^2 = 0$; $p < 0.0001$) and 2.6 (95% CI 1.54–4.35, $I^2 = 0\%$; $p = 0.0003$). Additionally, the OR for miscarriage between the testicular and ejaculated groups was 0.40 (95% CI 0.10–1.65, $I^2 = 34\%$; $p = 0.005$) [15].

Recently, in 2018, two studies on the matter concerned were added to the literature. In a prospective cohort study, Arafa et al. assessed ICSI outcomes using testicular sperm in 36 couples with a history of ICSI failure with use of ejaculated sperm [29]. The male partners were either normozoospermic or oligozoospermic, but all of them had high seminal SDF (>30% by SCD). In their study, CPR was significantly higher in the testicular sperm group than in the ejaculated sperm group (38.9% vs. 13.8%). Additionally, the authors reported 17 live births in the testicular sperm group and only 3 live births in the ejaculate sperm group ($p < 0.0001$). In another report involving 102 couples with no history of ICSI failure, Zhang et al. compared ICSI outcomes by testicular sperm versus ejaculated sperm [54]. All male partners had high SDF (DFI ≥30% by SCSA). The CPR (36% vs. 14.6%; $p = 0.017$) and delivery rate (36% vs. 9.8%; $p = 0.001$) after embryo transfers on day 3 were significantly higher in the testicular sperm group than in the ejaculated sperm group.

Given the apparent superiority of testicular sperm over ejaculated sperm for ICSI in men with high seminal SDF, a

critical appraisal of the studies mentioned above is warranted. First, the studies of Greco et al., Mehta et al., and Arafa et al. used testicular sperm after failed ICSI with ejaculated sperm; thus, the authors compared two interventions in the same patients after a given intervention has already been applied. Ideally, studies aiming at using patients as their controls should apply a crossover design. For that, patients should receive the sequence of treatments over a successive period, crossing over to an alternative treatment as part of the sequence. At the start of the study, every patient is assigned to a sequence (e.g., ejaculated sperm followed by testicular sperm and testicular sperm followed by ejaculated sperm), with successive treatments typically separated by a washout period.

The studies of Bradley et al. and Pabuccu et al. provide some evidence in favor of testicular sperm, but the authors have used a retrospective design (evidence level 2c), which has its inherent limitations. Lastly, a careful examination of the study of Zhang et al. does not allow determining whether the study is a prospective or retrospective cohort. To our knowledge, the best study currently available is the work by Esteves et al., which provide confirmatory evidence concerning the safe utilization of testicular sperm in couples undergoing ICSI whose male partners have high SDF (evidence level 2b).

The characteristics of the papers discussed above are summarized in Table 59.3. Well-designed randomized controlled trials are warranted to confirm these

Table 59.3 Characteristics and main outcome measures of studies reporting ICSI outcomes with testicular versus ejaculated sperm in non-azoospermic men with high sperm DNA fragmentation in the semen

Author and Year	Design (evidence level)	Subjects and cohort size (N)	SDF testing method	SDF cutoff values	SDF results (%)	Sperm retrieval method	Fertilization rate (%)	Clinical pregnancy rate (%)	Live birth rate (%)
Greco et al. [30]	Case series; intervention applied in consecutive patients (4)	Predominantly normozoospermic infertile men (18) Couples with history of ICSI failure	TUNEL	15%	23.6 ± 5.1% (E) and 4.8 ± 3.6% (T)[a]	TESE and TESA	74.9[b]	44.4 (T)[c]	NR
Sakkas and Alvarez [52]	Case series (4)	Infertile couples (72)	TUNEL	20%	NR	TESA	NR	13.8 (E) vs. 40.0 (T)	NR
Esteves et al. [18]	Prospective cohort (2b)	Oligozoospermic infertile men (172) Couples with no history of ICSI failure	SCD	30%	40.9% ± 10.2% (E) and 8.3% ± 5.3% (T)[a]	TESE and TESA	69.4 (E) vs. 56.1 (T)	40.2 (E) vs. 51.9 (T)	26.4 (E) vs. 46.7 (T)
Mehta et al. [31]	Case series (4)	Severe oligozoospermic infertile men (24) Couples with history of ICSI failure	TUNEL	7%	24.0 [95% CI 14%–34%] (E) and 5.0 [95% CI 3.0%–7.0%] (T)[a]	Micro-TESE	54.0[b]	50.0	50.0
Bradley et al. [23]	Retrospective cohort (2c)	Predominantly oligozoospermic men infertile men (228)[d]	SCIT	29%	NR	TESE and TESA	66.0 (E) vs. 57.0 (T)	27.5 (E) vs. 49.5 (T)	24.2 (E) vs. 49.8 (T)
Pabuccu et al. [28]	Retrospective cohort (2c)	Normozoospermic infertile men (71) Couples with history of ICSI failure	TUNEL	30%	41.7 ± 8.2 (E)	TESA	74.1 ± 20.7 (T) and 71.1 ± 26.9 (E)	41.9 (T) and 20.0 (E)	38.7 (T) vs. 15.0 (E)
Arafa et al. [29]	Prospective cohort; intervention applied in consecutive patients (2c)	Oligozoospermic and normozoospermic infertile men (36) Couples with history of ICSI failure	SCD	30%	56.3 ± 15.3 (E)	TESA	46.4 (T) and 47.8 (E)	38.9 (T) and 13.8 (E)	38.9 (T) vs. 8.0 (E)
Zhang et al. [53]	Prospective cohort (2b)[e]	Oligozoospermic and normozoospermic infertile men (102) Couples with no history of ICSI failure	SCSA	30%	NR	TESA	70.4 (T) vs. 75.0 (E)	36.0 (T) vs. 14.6 (E)	36.0 (T) vs. 9.8 (E)

Levels of evidence: 2b, Individual cohort study or low quality randomized controlled trials (e.g. <80% follow-up); 2c, "Outcomes" Research; ecological studies; 4, Case-series (and poor quality cohort and case-control studies). Modified from Oxford Centre for Evidence-Based Medicine (http://www.cebm.net/oxford-centre-evidence-based-medicine-levels-evidence-march-2009/)

[a]SDF results from paired ejaculated and testicular specimens

[b]2PN fertilization rate with use of testicular sperm; data from previous cycles with use of ejaculated sperm not provided

[c]The authors reported only one pregnancy with ejaculated sperm which miscarried

[d]Number of ICSI cycles; SDF: sperm DNA fragmentation; TESE: Testicular sperm extraction, TESA: testicular sperm aspiration; micro-TESE: microdissection testicular sperm extraction; NR: not reported; SCD: sperm chromatin dispersion test; SCIT: sperm chromatin integrity test, a variation of sperm chromatin structure assay (SCSA); TUNEL: terminal deoxyribonucleotide transferase-mediated dUTP nick end labeling assay; E: ejaculated sperm; T: testicular sperm

[e]Inferred from the study reported data; authors not contacted for providing clarification

observations. In such trials, at least 770 patients (385 per group) will be required to provide an 80% chance of detecting, as significant at the 5% level, an increase in LBRs of 10% (e.g., from 30% in the control group to 40% in the experimental group) [33].

59.6 Sperm Retrieval Methods in Non-azoospermic Men

Percutaneous and open sperm retrieval methods are highly effective for harvesting sperm from non-azoospermic men with high SDF in the semen [33, 55]. These procedures are commonly performed on an outpatient basis in association with oocyte retrieval and immediate sperm injection. Since men with high SDF in the semen have complete spermatogenesis, unlike men with nonobstructive azoospermia (NOA), sperm retrieval can be carried out using percutaneous or open methods with minimal tissue excision without the aid of microsurgery. Additionally, the testes of such men are usually adequate in size. Therefore, major adverse effects occasionally seen after SR in men with NOA, such as reduction of testosterone production and potential testis atrophy, are unlikely to occur [33, 44, 56, 57]. However, given the potential for complications, including pain, swelling, infection, and hematoma, SR should be performed by reproductive urologists who are familiar with testicular anatomy. The overall reported complication rate after SR in non-azoospermic men with high SDF in the semen has been 5% or less [18, 23, 28–30].

59.7 Offspring Health

ICSI has become the most widely used insemination method to overcome severe male factor infertility. Nevertheless, its use has been associated with possible increased risk of congenital malformations, epigenetic disorders, chromosomal abnormalities, subfertility, cancer, delayed psychological and neurological development, and impaired cardiometabolic profile compared with naturally conceived children, probably due to the influence of parental subfertility [8].

The integrity of the sperm genome and epigenome is vital for the birth of healthy infants [58]. As the spermatozoon loses most cytosolic antioxidants during spermiogenesis, the male gamete is highly vulnerable to oxidatively induced DNA damage. Low levels of critical DNA repair enzymes might explain the persistence of DNA damage in ejaculated sperm from infertile men [10, 59]. The fertilization of oocytes by such sperm through ICSI might result in an increased risk of fertilization failure, embryo arrest, miscarriage, congenital malformations, as well as perinatal and postnatal morbidity [21, 60].

Aneuploidy rates have been discussed earlier in this chapter; some reports suggest that both testicular sperm and embryos derived from Testi-ICSI have higher euploidy rates than ejaculated and epididymal sperm and their derived embryos cohorts [48, 49]. Additionally, mitochondrial DNA mutations (mtDNA) have been associated with defective sperm function and debilitating diseases, including neuromuscular and neurodegenerative conditions, such as Alzheimer's and Parkinson's disease [45]. As previously mentioned, the average number of mtDNA deletions was shown to be lower in testicular sperm than in epididymal sperm (1.5 vs. 3.6, $p < 0.01$). Furthermore, the mean size of mtDNA deletions seems to be smaller in testicular sperm than in epididymal sperm (1.7 kb vs. 4.27, $p < 0.01$) [45]. Along the same lines, nuclear DNA fragmentation (nDNA) was shown to be significantly lower in testicular sperm than in epididymal sperm (16% vs. 26%, $p < 0.01$), with a strong relationship between nDNA fragmentation and the number and mean size of mtDNA deletions detected in both epididymal and testicular sperm.

Therefore, the use of sperm for ICSI with better chromatin integrity and lower aneuploidy rates, such as testicular sperm from non-azoospermic men with high SDF, might help explain the improved reproductive outcome with testicular sperm as seen in various studies. Moreover, the use of such gametes for ICSI might result in a positive effect in the offspring health. However, no study has yet investigated on the health of infants born from ICSI using testicular sperm from non-azoospermic men with high SDF. Since the evidence favoring Testi-ICSI is still limited, continuous monitoring and more extensive investigation concerning the offspring health are necessary until the safety of this strategy is fully confirmed.

59.8 Proposed Algorithm for SDF Testing and Use of Testicular Sperm for ICSI in Non-azoospermic Men

Current evidence suggests that male partners of couples with unexplained infertility and recurrent pregnancy loss should be tested for SDF. Additionally, testing is recommended after failed intrauterine insemination, conventional IVF, or ICSI cycle provided no other apparent reason exists to explain that failure (Table 59.1) [9, 21, 61].

In the presence of high SDF, reduction of SDF rates might be attempted by treating the underlying condition associated with oxidatively induced sperm chromatin damage. Such conditions include varicocele and subclinical genital infections, obesity, smoking, and environmental exposure to toxicants [24]. If SDF is still high despite treatment, or when no apparent underlying condition is identified, the use of testicular sperm for ICSI seems to be a valid strategy to bypass

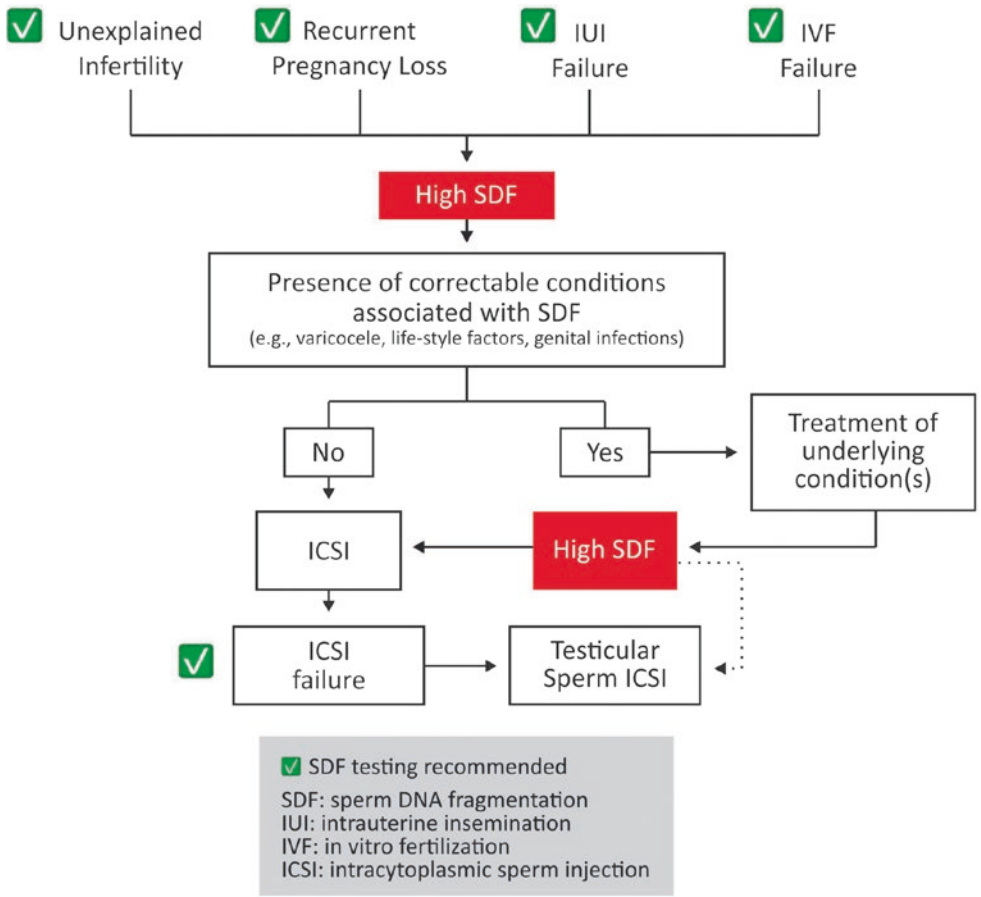

Fig. 59.1 Proposed algorithm for sperm DNA fragmentation testing and use of testicular sperm for ICSI. Solid arrows indicate the preferential decision tree, whereas the dotted arrow indicates an optional approach (Reprinted with permission, ANDROFERT© 2018. All Rights Reserved)

posttesticular chromatin damage caused by OS during sperm transit through the epididymis (Fig. 59.1). The benefits and risks of this option in light of the existing evidence should be discussed with the affected patients.

59.9 Other Strategies to Reduce Sperm DNA Fragmentation

Shortening ejaculatory abstinence time, recurrent ejaculations, and personal sperm bank are some of the strategies aimed at reducing SDF in the semen [62, 63]. Additionally, advanced laboratory sperm selection techniques, including electrophoresis and zeta potential, magnetic cell sorting (MACS), intracytoplasmic morphologically selected sperm injection (IMSI), and physiological ICSI with the hyaluronic acid binding assay (PICSI), have been utilized with the same goal [62]. The effectiveness of these methods to decrease the proportion of sperm with damaged chromatin is variable, but none of them is able to eliminate DNA-damaged sperm for use with ICSI completely [57, 64].

A 2016 study by Bradley et al. evaluated 2175 ICSI cycles, of which 20.6% were carried out in patients with high SDF in semen [23]. In their study, interventions to reduce SDF in samples used for ICSI included testicular sperm retrieval, IMSI, and PICSI. Then, they compared ICSI outcomes using ejaculated sperm with and without intervention to Testi-ICSI. Live birth rates were significantly higher ($p < 0.05$) with Testi-ICSI (49.8%) than with IMSI (28.7%) and PICSI (38.3%). The lowest LBR (24.2%) was achieved when no intervention was performed to deselect sperm with DNA fragmentation ($p = 0.020$). To the best of our knowledge, the paper by Bradley et al. is the only study published to date to compare the use of testicular sperm with other laboratory methods to reduce SDF in the semen. Despite providing insightful information, additional data will have to be collected prospectively to evaluate which intervention – alone or combined – would result in the highest increment in pregnancy success among patients with high SDF.

59.10 Conclusions

The existing evidence suggests that Testi-ICSI in non-azoospermic men may be beneficial for those with confirmed high SDF in the ejaculate, resulting in better pregnancy outcomes than with the use of ejaculated sperm. Given these

promising results, the work-up of infertile couples embarking on ART should include SDF testing. ICSI with testicular in preference over ejaculated sperm should be considered, in particular to those couples suffering from ICSI failure. Prospective randomized trials are needed to confirm the clinical utility and also the cost-effectiveness of testicular sperm in preference over ejaculated sperm for ICSI in non-azoospermic men with high SDF in the semen. Moreover, the ploidy status of embryos resulting from the use of testicular sperm from these men should be investigated. Lastly, the short- and long-term offspring health resulting from Testi-ICSI to overcome oxidatively induced SDF also warrants further research.

59.11 Review Criteria

An extensive search of studies examining the role of testicular sperm retrieved from non-azoospermic men with high sperm DNA fragmentation in the semen for use with intracytoplasmic sperm injection was performed using PubMed and MEDLINE. The start date for the search was January 2005, and the end date was November 2018. The overall strategy for study identification and data extraction was based on the following keywords: "sperm DNA fragmentation," "sperm DNA damage," "male infertility," "sperm retrieval," "testicular sperm," "non-azoospermic men," "reproductive techniques," "assisted," "ICSI," "in vitro fertilization," "sperm injections," "intracytoplasmic," and "IVF," with the filters "humans" and "English language." Citations dated outside the search dates were only included if provided with the conceptual content. Papers evaluating the use of testicular sperm for ICSI from non-azoospermic men with either cryptozoospermia or untested for SDF were not considered.

Conflict of Interest The authors declare no conflict of interest.

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Development of Artificial Gametes

60

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Abbreviations

AA	Activin A
ACR	Acrosin
ACT	Activator of cAMP-responsive element modulator in testis
ADMSCs	Adipose tissue-derived mesenchymal stem cells
AKT	Protein kinase B
AMH	Anti-Mullerian hormone
AMHR2	Anti-Mullerian hormone receptor 2
AP2γ	Activating protein 2γ
APSCs	Adult pluripotent stem cells
ART	Artificial reproductive technology
bFGF	Basic fibroblast growth factor
BLIMP1	B lymphocyte-induced maturation protein-1
BM	Bone marrow
BMMSC	Bone marrow mesenchymal stem cells
BMP15	Bone morphogenetic protein 15
BMP4	Bone morphogenetic protein 4
BMP7	Bone morphogenetic protein 7
BMP8b	Bone morphogenetic protein 8b
BOLL or BOULE	Boule homolog from the deleted in azoospermia-like gene family
BVSCH18	ESC line harboring Blimp1-mVenus (BV) and Stella-ECFP (SC)
CD117	Cluster of differentiation 117
CD44	Cluster of differentiation 44
CD45	Cluster of differentiation 45
CD49f	Cluster of differentiation 49f (Integrin alpha 6)
CD61	Cluster of differentiation 61 (Integrin beta 3)
CHIR99021	Aminopyrimidine derivative inhibitor of GSK3
C-KIT	Tyrosine-protein kinase Kit
cMYC	MYC proto-oncogene, bHLH transcription factor
COC	Cumulus-oocyte-complex
CXCR4	C-X-C chemokine receptor type 4
CYP19A1	Cytochrome P450 family 19 subfamily A member 1
DAZ	Deleted in azoospermia
DAZL	Deleted in azoospermia-like
DDX4	DEAD-box helicase 4
DMC1	DNA meiotic recombinase 1
DNA	Deoxyribonucleic acid
DND1	Dead end protein homolog 1
DNMT3B	DNA (cytosine-5-)-methyltransferase 3 beta
EB	Embryonic bodies
ECFP	Enhanced cyan fluorescent protein
EGF	Epidermal growth factor
EpiLC	Epiblast-like cells
ESC	Embryonic stem cells
FBS	Fetal bovine serum
FE-J1	Acrosomal protein FE-J1
FGF2	Fibroblast growth factor 2
FGF5	Fibroblast growth factor 5
FIGα	Factor in the germline alpha
FLCs	Follicle-like cells
FOXL2	Forkhead box L2
FRAGILIS	Interferon-induced transmembrane protein 1 human homologous
FSH	Follicle-stimulating hormone
FSHR	Follicle-stimulating hormone receptor
GASZ	Germ cell-specific ankyrin
GATA4	GATA binding protein 4
GATA6	GATA binding protein 6

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GCs	Granulosa cells
GDF9	Growth differentiation factor 9
GDNF	Glial cell-derived neurotrophic factor
GFP	Green fluorescent protein
GFRA1	GDNF family receptor alpha 1
GLCs	Granulosa-like cells
GSCLCs	Spermatogonia-like cells
GSK3	Glycogen synthase kinase 3
H19	Imprinted maternally expressed transcript H19
hAFSCs	Human amniotic fluid stem cells
HAP	Haprin
hBMSCs	Human bone marrow mesenchymal stem cells
hESCs	Human embryonic stem cells
hFGS	human fetal gonadal stromal cells
hiPSCs	Human induced-pluripotent stem cells
hMSCs	Human mesenchymal stem cells
HSP90α	Heat shock protein 90 alpha
hUCSCs	Human umbilical cord stem cells
ICM	Inner cellular mass
ICSI	Intracytoplasmatic sperm injection
ID1	Inhibitor of DNA binding 1
ID2	Inhibitor of DNA binding 2
ID4	Inhibitor of DNA binding 4
iPSC	Induced pluripotent stem cells
ITGA6	Integrin alpha 6
KLF4	Kruppel-like factor 4
LIF	Leukemia inhibitory factor
LIN	Lineage depletion
LIN28	Lineage depletion 28
MAD3	Mitotic arrest deficient protein 3
MEF	Mouse embryonic fibroblasts
MESA	Microsurgical epididymal sperm aspiration
mESC	Mouse embryonic stem cells
MII	Metaphase II
miPSCs	Mouse induced pluripotent stem cells
MSCs	Mesenchymal stem cells
MVH	Mouse Vasa homologue
NANOG	Homeobox transcription factor Nanog
NANOS	Nanos CCHC-type zinc finger
NANOS1	Nanos CCHC-type zinc finger 1
NANOS3	Nanos CCHC-type zinc finger 3
NEUROD1	Neuronal differentiation 1
NOA	Non obstructive azoospermia
OA	Oleic acid
OCT4	Octamer-binding transcription factor 4
ODF2	Outer dense fiber of sperm tails 2
OLCs	Oocyte-like cells
PAX6	Paired box 6
PGCLCs	Primordial germ cell-like cells
PGCs	Primordial germ cells
PGP 9.5	Protein gene product 9.5
PI3K	Phosphoinositide 3-kinases
PIWIL2	Piwi-like RNA-mediated gene silencing 2
PIWIL4	Piwi-like RNA-mediated gene silencing 4
PLZF	Promyelocytic leukaemia zinc finger
POI	Primary oocyte insufficiency
PRDM1	Positive-regulatory domain I binding factor
PRM1	Protamine 1
PTEN	Phosphatase and tensin homolog
R115866	Cytochrome P450 26A1 inhibitor
RA	Retinoic acid
RAD51	DNA repair protein RAD51 homolog 1
RBM	RNA-binding motif
REC8	Meiotic recombination protein REC8 homolog
RET	Proto-oncogene tyrosine-protein kinase receptor Ret
REX	Reduced expression-1 (zinc finger protein 42)
RNF17	Ring finger protein 17
SCA1	Stem cells antigen-1
SCF	Stem cell factor
SCP1	Synaptonemal complex protein 1
SCP3	Synaptonemal complex protein 3
SDSCs	Skin-derived stem cells
SMAD1/5	Mothers against decapentaplegic homolog 1 and 5
SMC1β	Meiosis-specific cohesin subunit SMC1 beta
SNRPN	Small nuclear ribonucleoprotein polypeptide N
SNTs	Somatic nuclear transfer cells
SOX17	Sex determining region Y-box 17
SOX2	Sex determining region Y-box 2
SPO11	Meiotic recombination protein SPO11
SSCs	Spermatogonial stem cells
SSEA1	Stage-specific embryonic antigen 1
STAG3	Cohesin subunit SA-3
STELLA	Developmental pluripotency-associated protein 3 (DPPA3)
STRA8	Stimulated by retinoic acid 8
TCFAP2C	Transcription factor AP-2 gamma
TDRD5	Tudor domain containing 5
TEKT1	Tektin 1
TESE	Testicular sperm extraction
TEX14	Testis-expressed sequence 14
TEX18	Testis-expressed sequence 18
TFAP2C	Transcription factor AP-2 gamma
TGF-β1	Transforming growth factor beta 1
TH2	Tyrosine hydroxylase 2
TP1	Transition protein 1
TP2	Transition protein 2
TSPY	Testis-specific Y-encoded protein 1
TTFs	Tail tip fibroblasts

VASA	DDX4
VSELs	Very small embryonic-like stem cells
W/W^{v}	Mice lacking the receptor tyrosine kinase c-Kit
WNT3	Wnt family member 3
ZP1	Zona pellucida glycoprotein 1
ZP2	Zona pellucida glycoprotein 2
ZP3	Zona pellucida glycoprotein 3

Key Points

- Artificial gametes are commonly derived from pluripotent stem cells, including embryonic stem cells, induced-pluripotent stem cells, adult pluripotent stem cells and "very small" embryonic-like stem cells.
- Embryonic stem cells, until the eight cells-morula stage, are considered totipotent. At the blastocyst stage, the inner cellular mass is divided into epiblast and hypoblast. The epiblast cells contain pluripotent cells that can give rise to differentiated cells from the three germ layers. Moreover, human embryonic stem cells are equivalent to mouse epiblast cells.
- Unipotent cells can be reprogrammed to assume a pluripotent state by introducing genes important for maintaining the essential properties of embryonic stem cells and/or by somatic nuclear transfer to a fertilized egg, and/or under specific culture conditions and/or environment.
- Most studies, in animal-models and humans, are focused on trans-differentiation of stem cells into primordial germ-like cells. Furthermore, only some studies in animal models have successfully achieved the differentiation of the primordial germ-like cells into mature, functional gametes. Concerning human artificial gametes, the progression of the primordial germ cells through meiosis in vitro is still a big challenge to be faced.
- The generation of offspring using human artificial gametes involves important ethical issues, making the clinical application of these amazing technologies still some distance away from medical practice.

60.1 General Introduction

Fertilization of the oocyte by the sperm is the first step in the development of a mammalian embryo, therefore perpetuating the genetic information across generations [1]. This event is one of the most challenging of the biological system because many processes occur before the production of functional gametes. Male and female gametes are formed during gametogenesis comprising different steps, such as specification of primordial germ cells (PGCs) within the posterior epiblast, migration of PGCs, colonization of the genital ridges by PGCs, differentiation into oogonia or spermatogonia, mitotic divisions, meiosis and final functional maturation of germ cells [2–4]. After gamete maturation, the oocyte awaits sperm fertilization to initiate a cascade of events, including the degradation of maternal transcripts, formation of male and female pronuclei, production of zygote transcripts and epigenetic parental genome modifications [3, 5, 6]. Lastly, the animal–vegetal poles orientate the first divisions, giving rise to a blastocyst to be implanted in a prepared uterus.

Reproductive success (fertility) depends on the accurate production of gametes, effective gamete union as well as correct embryo development. A number of events can go wrong within these steps, causing infertility. By definition, infertility is the failure to establish pregnancy after 1 year of unprotected and regular sexual intercourse [7, 8]. It is estimated that 8–15% of reproductive-aged couples worldwide present some problem in gametes maturation, and they are recognized as infertile [7, 9, 10]. In some regions, such as South and Central Asia, the Middle East and North Africa, Central and Eastern Europe, the infertility rates can reach ~30% [7, 11]. While premature ovarian insufficiency, polycystic ovary syndrome, endometriosis, uterine fibroids and endometrial polyps play a significant role in female infertility, male infertility can be caused by several testicular and post-testicular deficiencies [12].

Since the birth of Louise Brown in 1978, the refinements in laboratory technologies have allowed increasing numbers of infertile couples to have families [13]. In the past few decades, technological innovations have led to the introduction of several reproductive tools that provide effective treatment for infertility [13]. Among them we could identify intracytoplasmatic sperm injection (ICSI), microsurgical epididymal sperm aspiration (MESA), testicular sperm extraction (TESE), gamete cryopreservation, testis and ovarian tissue grafting and germ cell transplantation [13, 14]. More recently, exciting results regarding the in vitro production of gametes are attracting researchers worldwide and providing hope to individuals without mature gametes. Potential medical applications include the development of this technology to assist men presenting incomplete spermatogenesis and, in women, to help, for instance, those with primary oocyte insufficiency (POI) [8, 15].

Although spermatogenesis in mammals is a very complex process involving numerous factors and interactions between somatic and germ cells [16], several scientists are trying to produce sperm in vitro using spermatogonial stem cells (SSCs) in association with specific culture systems [8]. The mechanism of SCCs in vitro differentiation to produce sperm

is of great value for patients with non-obstructive azoospermia [17]. Kanatsu-Shinohara and collaborators (2003) [17] cultivated SSCs in culture media containing several factors, such as GDNF, FGF2, EGF, LIF and FBS, but sperm formation was not achieved in the culture system. Deng and colleagues (2016) [18] were able to produced sheep sperm in vitro supplementing the media with melatonin. Although the viability of offspring was not evaluated, the haploid sperm-like cells activated eggs and a high number of blastocysts were formed. Other successful experiments were developed in cattle [19] and mice [20]. However, until now, it has not been possible to obtain human gametes using this system [21]. In general, in vitro culture of SSCs has limitations because of their low count per testis and difficulties in their pure isolation [8].

As noted for spermatogenesis, mammalian oogenesis is also a very complex process involving a large number of intra- and extra-ovarian factors [22]. Since the majority of mammalian oocytes are not fully utilized, it has been proposed that in vitro oogenesis could increase the female germ cell pool, increasing the possibilities of assisted reproductive technologies [23]. Unfortunately, as demonstrated and reviewed by Lonergan and Fair (2016) [24], the success rate for in vitro oogenesis is small and oocyte maturation is not always achieved. Usually, female germ cells are unable to progress beyond the first stage of meiotic prophase I outside the gonadal microenvironment [23]. Furthermore, it is worthy of mention that the majority of immature oocytes fail to develop into the blastocyst stage.

The term artificial gametes is used to describe mature gametes (sperm or eggs) originated by the manipulation of their progenitors or by directed differentiation of pluripotent somatic cells into the germ-cell lineage [21]. As is well-established in the literature, stem cells can be considered totipotent, pluripotent or multipotent (Fig. 60.1), whereas embryonic stem cells (ESCs), until 8 cells-morula stage, are considered totipotent, i.e. they are capable of generating an entire organism [25]. At blastocyst stage, the inner cellular mass (ICM) is divided into epiblast and hypoblast. While the epiblast cells present pluripotent cells that can give rise to differentiated cells from the three germ layers [8, 23, 26, 27], the hypoblast cells are responsible for yolk sac formation. Multipotent stem cells can develop into more than one cell type, but are more limited than pluripotent cells because they can differentiate into all cell types within one particular lineage [25, 28]. By introducing genes important for maintaining the essential properties of ESCs (e.g. *Oct4*, *Sox2*, *Klf4*, *cMyc*, *Nanog,* etc.), unipotent differentiated cells can be reprogrammed to assume a pluripotent state (Fig. 60.1, green line), giving rise to the induced pluripotent stem cells (iPSCs). Similarly, somatic nuclear transfer cells (SNTs) can be generated through nuclear reprogramming of unipotent

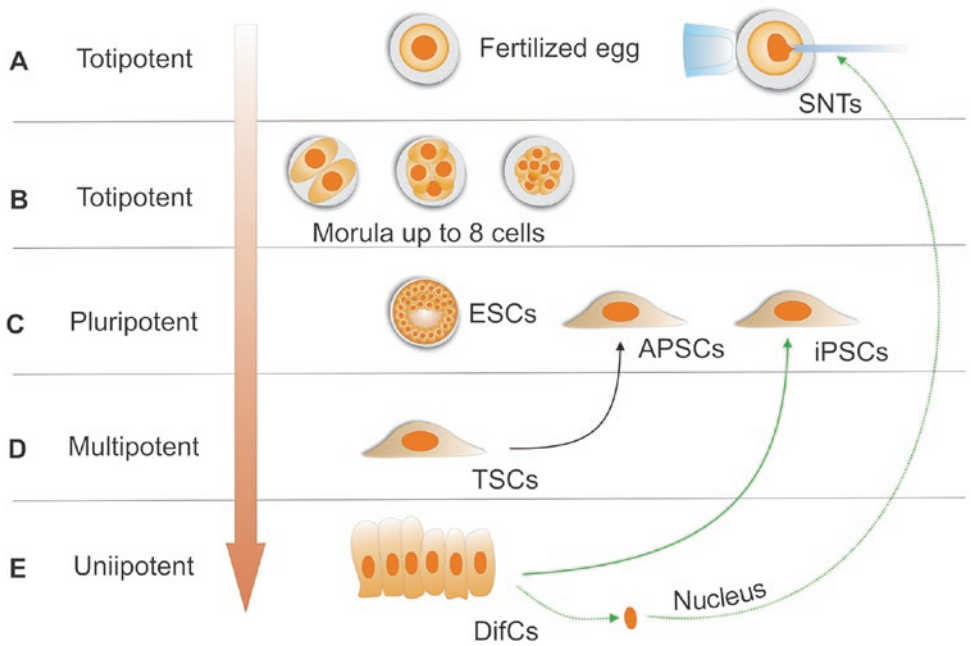

Fig. 60.1 Differentiation steps of stem cells. From newly fertilized eggs (A) up to the 8 cells-morula (B), the stem cells are considered totipotent. Embryonic stem cells (B, ESCs) are considered pluripotent due to their capacity to differentiate into cells from the three germ layers. Since they show limited capacity of differentiation, tissue stem cells (D, TSCs) are multipotent. Differentiated cells (DifCs) are considered unipotent because they are highly specialized for certain functions. TSCs can produce adult pluripotent cells (APSCs) when exposed to specific culture conditions and/or environments and DifCs can be reprogrammed to assume a pluripotent state through genetic induction (green arrow) or through nuclear transference (dotted green arrow)

cells that become pluripotent stem cell (Fig. 60.1, green dashed line). In addition, under specific culture conditions and/or environments, several experiments have demonstrated that multipotent cells can adopt distinct states of pluripotency (Fig. 60.1, black line).

The most common stem cells used to produce artificial gametes are ESCs, iPSCs, adult pluripotent stem cells (APSCs) and very small embryonic-like stem cells (VSELs) and most studies are focused on stem cell trans-differentiation into PGC-like cells (PGCLCs) (Fig. 60.2). The possibility of pluripotent cells adopting an epiblast-like developmental program in vitro, generating cells (epiblast like cells – EpiLCs) competent to undergo specialization into PGCLCs, is being discussed [27]. Remarkably, it has been established that human ESCs are equivalent to mouse epiblast cells. Another crucial point is the differentiation of PGCLCs into gametes (Fig. 60.2). This differentiation event is achieved in vitro or in vivo when suitable factors/niche are provided [2, 29]. To determine the progress of artificial gamete production, the cells are evaluated using a combination of morphological, histochemical and molecular markers. Figure 60.3 lists the most common molecular markers associated with cell differentiation progress. Generally, these markers are expressed in a sequential fashion (not random), allowing a better definition of the cell type. Herein, the main findings of several studies are reviewed, detailing the most advanced cell type observed as well as their molecular marker expression.

Artificial gamete biotechnology has already been successfully applied in male and female reproduction [8, 23]. It is believed that this technique will allow a full genetic parenthood to homosexual couples and to women of postmenopausal age, as well as other benefits [21]. This chapter provides an overview of the progress made in the generation of biologically relevant artificial gametes in male and female individuals and will provide insight into all technological developments in the assisted reproductive field. Although this topic has attracted special attention from scientists, the news media and the general population, it is important to mention that the topic is relatively new and under considerable debate. Therefore, extensive experimentation is being developed and the clinical application of newly developed technologies will require careful validation.

60.2 Development of Artificial Gametes in Animal Models

In order to generate artificial gametes in vitro, research efforts by several groups around the world have focused on using animal models to investigate the potential use of ESCs, iPSCs, EpiLC and pluripotent somatic stem cells. The development of male and female animal germ cells in culture has contributed to advancements in numerous areas, including infertility treatment, nuclear transfer, genetic manipulation of germ cells and germline epigenetics programming. Additionally, these studies are helping to better understand gene functions involved in the segregation of germ and somatic cell lineages, as well as their interaction and differentiation during gametogenesis.

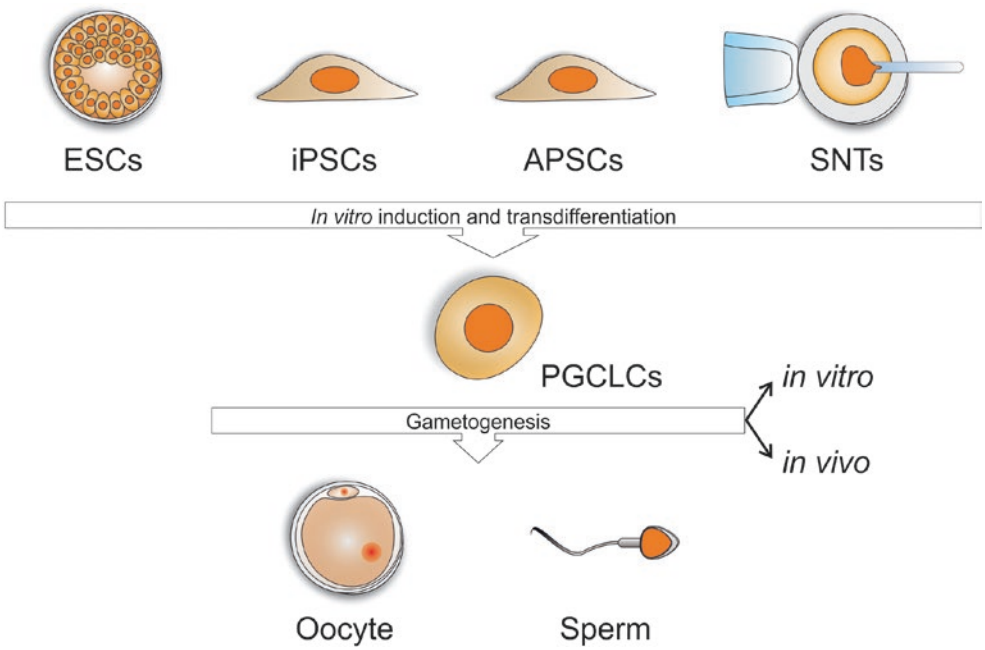

Fig. 60.2 The main objectives involving artificial gametes production. The majority of studies focus on the events related to PGC-like cells specification (PGCLCs). Research describes the early differentiation events of embryonic stem cells (ESCs), induced pluripotent cells (iPSCs), adult pluripotent stem cells (APSCs) and somatic nuclear transfer cells (SNTs) into PGCLCs. Another important challenge is the differentiation of PGCLCs into gametes (gametogenesis) through in vivo and in vitro assays

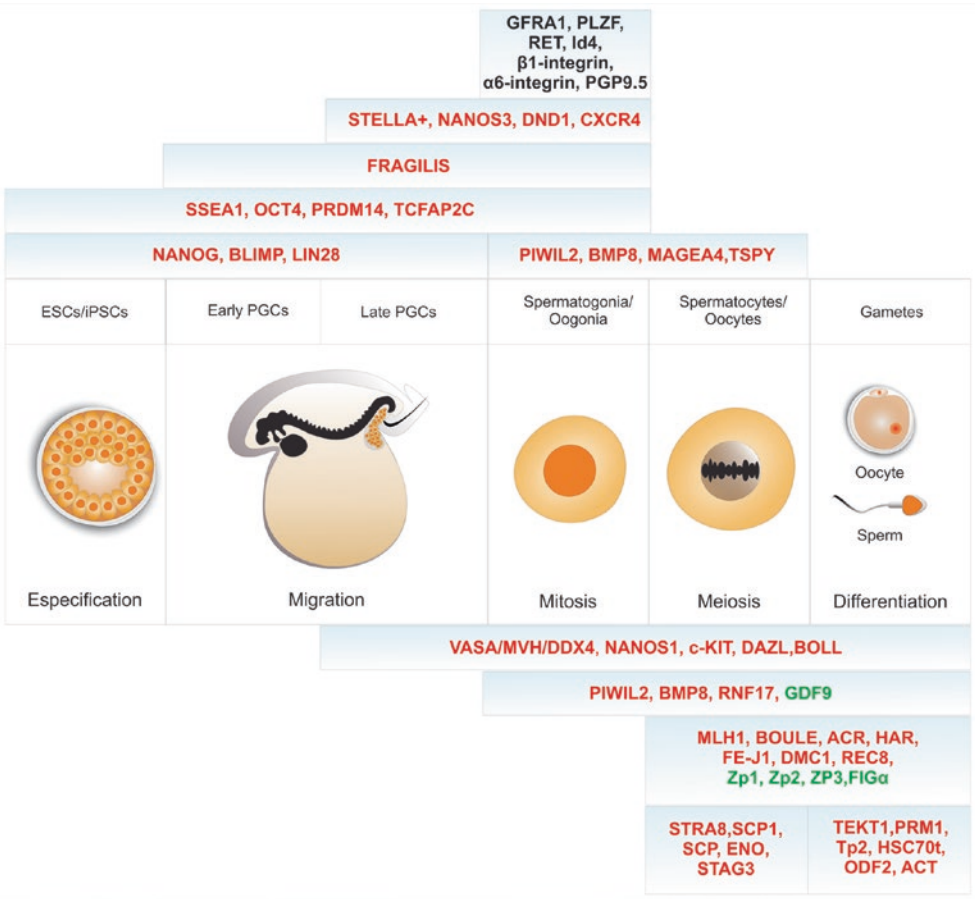

Fig. 60.3 Main cellular molecular markers used to recognize the steps of artificial gametes production. Although precise identification of germ cells is quite difficult using only their morphology in vitro, several cellular markers can be used to determine their differentiation progress. The molecular markers in red are considered the most general to male and female differentiation pathways. Specific male molecular markers are depicted in black, while specific female molecular markers are shown in green

60.2.1 Artificial Male Germ Cells and Gametes Derived from Embryonic Stem Cells

In mice, germ cell competence is induced in proximal epiblast cells around embryonic day 6.5 by specific signals emanating from the extraembrionic ectoderm, including bone morphogenic proteins (BMPs) to form PGCs [30, 31]. Therefore, the first fundamental step for artificial gamete generation involves differentiation of pluripotent cells into functional PGCs (Fig. 60.2). Likewise, cell generation in vitro, equivalent to epiblast cells, represents an essential step for germ cell induction. Several research groups have shown that these unique early germ cells can be developed in vitro in animal models [32–34] from different sources of pluripotent stem cells (Fig. 60.1).

In 2003, Toyooka and collaborators [35] were pioneers in the generation of male PGCs from mESCs in culture and the formation of sperm in vivo from these ES-derived germ cells. mESC differentiation into male germ cells depended on the formation of embryonic bodies (EB), a three-dimensional structure, in which ESCs gain the ability to differentiate into a wide range of cell types. ESCs carrying reporter genes under the control of MVH, specifically expressed in differentiating germ cells, were co-cultured with BMP4-producing cells to form EB and later exhibited a limited number of MVH$^+$ cells. Following aggregation with dissociated embryonic testicular cells and transplantation under a host testis capsule, ES-derived PGC differentiated into spermatogenic cells, including elongated spermatids expressing HSC70t. SSEA1$^+$ and Oct4$^+$ PGCs isolated from mESCs-derived EB showed an absence of epigenetic imprints of the *Igf2r* and *H19* genes, suggesting phenotypic and biological proprieties comparable to PGCs developing in vivo. In retinoic acid (RA) treated culture, these cells underwent in vitro differentiation (*Acr*$^+$, *Hap*$^+$ and *FE-J1*$^+$), culminating in the formation of haploid round spermatid-like cells. When these cells were injected into oocytes, the diploid chromosome was restored allowing the development of blastocysts [36]. Nevertheless, significant unanswered questions remained about the functionality of ESC-derived germ cells. The ectopic expression of Dazl (a germ cell-specific gene that plays critical roles in germ cell differentiation) also promotes the differentiation of mESCs into male haploid cells in vitro. ICSI revealed that the *Dazl*-induced sperm were able to fertilize eggs, although the full-term development of embryos could not be achieved [37]. Other authors showed the in vitro production of male gametes of mESCs

using RA induction into EB without any genetic manipulation or preselection. Formed gamete-like cells expressed germinal-specific genes (*Vasa*, *Stella*, *Dazl*, *Piwil2*, *Tex14*, *Bmp8b* and *Rnf17*) and showed positive labelling for meiotic and post-meiotic male germinal-specific markers (*Scp1*, *Scp3*, *Stra8* and *Hap*). Notably, this study stated that XY mESCs could also produce presumptive female gametes [38].

Wolfgang Engel's group was the first to report the birth of live offspring using haploid male gametes derived in vitro from mESCs. A strategy for the establishment of spermatogonial stem cell (SSCs) lines (*Oct4*$^+$*, Fragilis*$^+$*, Stella*$^+$*, Vasa*$^+$ and *Rnf17*$^+$) from ESCs was developed to evaluate the functionality of artificial male gametes. SSCs were able to undergo meiosis in vitro (*Scp3*$^+$*, Acr*$^+$ and *Dmc1*$^+$) and produce a haploid cell population (*Tp2*$^+$ and *Prm1*$^+$) after RA induction. The intracytoplasmic injection of in vitro-generated haploid cells into oocytes resulted in the birth of pups that died prematurely [39]. Because the differentiation of the ES-derived gamete might affect crucial reprogramming events in gametogenesis, it has been suggested that deaths occur due to the production of malformed gametes that could not exhibit the accurate methylation imprints required for normal development.

To investigate the events that occur before the development of germ-like cells from pluripotent stem cells, research efforts were later focused on establishing a defined in vitro system that could recreate the PGC specification pathway. mESCs induced into pre-gastrulating epiblast-like cells (EpiLCs) that expressed *Wnt3*, *Fgf5* and *Dnmt3b* were able to generate primordial germ-like cells (PGCLCs) in vitro, (*Blimp1*$^+$, *Prdm14*$^+$, *Tcfap2c*$^+$, *Nanos*$^+$, *Stella*$^+$, *Tdrd5*$^+$ and *Dnd1*$^+$ cells), recapitulating the phenotypic and epigenetic profile of PGC formation from epiblasts cells in vivo [2, 40]. PGCLCs resumed spermatogenesis after being transplanted into the seminiferous tubules of infertile mice (W/W^v). They formed sperm capable of fertilizing oocytes through ICSI and generated healthy offspring with normal imprinting patterns. F1 individuals reached adult age and exhibited normal fertility [2]. When aggregated with testicular somatic cells, mEPCs-derived PGCLCs can form spermatogonia-like cells in vitro (GSCLCs) expressing *Id4*, *Ddx4*, *Ret, Gfra1,Plzf*, *Dazl*, *Itga6*, *Piwil4*, *Piwil2* and *Kit* [41].

GSCLCs, but not PGCLCs, were able to colonize adult testes after transplantation, resuming spermatogenesis and contributing to fertile offspring. Analyses of global DNA methylation profiles demonstrated that GSCLCs exhibited partially aberrant epigenetic properties, which could limit their spermatogenic potential [41]. Zhou et al. successfully provided proof for all key hallmarks of complete meiosis progression of mESCs-derived PGCLCs in vitro, and demonstrated the functionality of haploid spermatid-like cells obtained. Through exposure to RA, BMPs and activin A (AA) and co-culture with neonatal testicular somatic cells, PGCLCs differentiated and began to express meiotic markers (*Stra8*, *Dmc1* and *Scp3*). These cells exhibited normal chromosomal synapsis, DNA double stranded breaks and homologous recombination repair, which was monitored by SPO11 and RAD51 expression. Hormonal stimulation with follicle-stimulating hormone (FSH), testosterone and bovine pituitary extract induced the formation of haploid spermatid-like cells in vitro expressing *Tp1*, *Prm1*, *Acr* and *Hap*. Global transcription profile clustering analyses revealed similarities between in vitro spermatid-like cells and in vivo spermatid. Bisulfite sequencing also confirmed differential methylation at the *H19* and *Snrpn* loci equivalent of in vivo round spermatids. Finally, intracytoplasmic injection of the resulting spermatid-like cells into oocytes were able to produce embryos that underwent normal embryonic development, resulting in fertile offspring, which gave birth to the next generation [27].

60.2.2 Artificial Male Germ Cells and Gametes Derived from Induced Pluripotent Stem Cells

The generation of iPSCs via the expression of specific transcription factors has been demonstrated in animal models and has provided new potentials in regenerative medicine and reproductive science based on their ability to differentiate into a wide-range of cell types in vitro. Several studies have demonstrated that the germ cell-specific pathway can also be reconstituted by iPSCs in mice.

Different iPSC lines derived from mouse embryonic fibroblasts (MEF) [42–44] were induced into EpiLCs and supported the formation of PGCLC after BMPs treatment. Although these cells could not develop beyond this precursor stage in vitro, isolated SSEA1$^+$ and Integrin-β3$^+$ PGCLCs, when transplanted into the seminiferous tubules of W/W^v mice, differentiated into mature sperm, whereas unsorted cells led to the formation of teratomas in the recipient testes. Applying ICSI, followed by embryo transfer, iPSC-derived sperm contributed to the generation of offspring [2]. Remarkably, some of the offspring died prematurely, indicating that although miPSCs exhibit induction properties they may form PGCLCs with improper function. The differentiation potential of miPSC from neural progenitor cells to generate male germ cells in vivo was also explored using testis allograft [45, 46] and germ cell transplantation into sterile mice testes [47, 48]. Nevertheless, these studies did not demonstrate the functionality of the iPSC-derived male gametes. The molecular mechanisms underlying male germ cell development were recently explored in vitro and suggested that the development of

male germ cells from iPSCs is promoted by BMP4 via the Smad1/5 pathway with activation of Id1, Id2 and Gata4 genes [49]. Interestingly, it has been demonstrated that fibroblasts from sterile trisomic XXY and XYY mice can lose the extra sex chromosome during reprogramming to induce pluripotent stem cells [50]. Thus, XY iPSCs derived from sex chromosome trisomy fibroblasts could be differentiated through the epiblast-like state to create PGCLCs in vitro. Blimp1$^+$ PGCLCs when isolated and transplanted into germ-cell deficient W/W^v testes resulted in complete spermatogenesis. Particularly, sperm from SCT-derived XY iPSCs gave rise to chromosomally normal, healthy and fertile offspring. Loss of extra chromosomes also applies to autosome ones, developing euploid iPSCs from cells of the Down syndrome model [50]. Therefore, these findings have relevance to overcoming infertility and other trisomic phenotypes, such as the Klinefelter syndrome.

The ability of iPSC to transdifferentiate without any in vivo stage remains unclear and is still little investigated. EB formation and RA and/or testosterone induction seems to be able to promote differentiation of miPSC originated from MEF into male germ cells. DNA content and transcript analysis indicated that these approaches resulted in derived haploid male germ cells (*Odf2*+, *Act*+ and *Prm1*+) in vitro [51]. Nevertheless, the functionality of spermatids-like cells obtained with this system has not been assessed.

Despite its successes in mice, the induction of PGCLCs from the iPSCs of large animals is still under-explored. In 2016, a defined culture system for the induction of PGCLCs from porcine iPSCs was stabilized. Cell morphology, germ cell marker gene expression and epigenetic properties were used to identify the pPGCLCs. SSC-like cells induced from the pPGCLCs could initiate meiosis in vitro and, after xenotransplantation into germ cell-ablated testis, iPSCs-derived SSC-like cells survived and colonized the recipient seminiferous tubules [52]. Bovine iPSCs have also been induced into bPGCLCs through EB formation in the presence of RA and BMP4. However, low rates of germ cell differentiation were achieved in vitro based on the detection of bovine *Vasa/DDX4* [53]. Recently, a protocol for differentiating male rhesus macaque PGCLCs from riPSCs was reported [54]. This study showed that rPGCLCs generated in vitro corresponded to *SOX17*$^+$, *TFAP2C*$^+$ and *PRDM1*$^+$ newly specified germ cells that have not yet been initiated in the global epigenetic reprogramming. To functionally validate the differentiation of rPGCLCs, xenogeneic (monkey to mouse) and allogeneic (monkey to monkey) transplantations into germ cell-ablated testes were performed. Post-transplantation analyses showed that rPGCLCs gave rise to *VASA*$^+$ and *MAGEA4*$^+$ cells, corresponding to early spermatogonia. Meanwhile, both mouse and monkey adult testicular niche could not support the spermatogonial differentiation (ENO2-) [54].

60.2.3 Artificial Male Germ Cells and Gametes Derived from Adult Pluripotent Stem Cells

Considering the tumorigenic potential of ESCs and iPSC, limited sources and ethical concerns involving their application in regenerative medicine, stem cells with the same pluripotent capacity became a desirable alternative for differentiation of various cell populations. APSCs are already widely studied worldwide and their therapeutic signalling, particularly with respect to their trophic properties, have been intensively studied in animal models (Fig. 60.1) [55–57]. During the past decade, studies have shown that some adult stem cells not only innately display germ cell characteristics but also, in the presence of certain stimulators, they can be induced in vitro to transdifferentiate into germ cells (Fig. 60.2). Interestingly, several extra-gonadal somatic organs have been reported as a source of male germ cells, such as bone marrow mesenchymal stem cells, skin-derived stem cells and adipose tissue-derived stem cells, which has opened new possibilities for use of these cells in reproductive medicine.

Adult bone marrow stem cells (BMSCs) are well characterized cells that can be selected autologously and, therefore, have long been used therapeutically. RA treatment is able to induce isolated BM mesenchymal stem cells (MSCs) to transdifferentiate into male germ cells in vitro [39]. BMMSC-derived germ cells express molecular markers of PGCs (*Oct4, Mvh, Stella, Fragilis* and *Rnf17*) as well as molecular markers of SSCs and spermatogonia (*Dazl, Piwil2, Rbm*, *Hsp90alpha, Tex18, c-Kit*, *Stra8*, *beta1* and *alpha6 integrins*), demonstrating the differentiation arrest of these cells at the premeiotic stages. In vivo, BMMSC-derived germ cells exhibit the ability to proliferate, migrate to the basement membrane and to colonize recipient seminiferous tubule after transplantation. Although few pre-meiotic spermatocytes could be generated, no further differentiation was observed after transplantation, indicating a possible failure in the meiotic programming of mouse BMMSC-derived germ cells [39]. Besides RA, based on the expression of *Mvh,* the most effective concentration of BMP4 for inducing PGC features in mouse BMMSCs was also established [58]. It has been demonstrated that BMSCs can contribute to testicular germinal epithelium regeneration in vivo. BM cells isolated from mice, when transplanted in the seminiferous tubules and interstitial space of recipient testis, survived and differentiated into both male germ cells (spermatogonia and spermatocytes) and somatic cell types (Sertoli and Leydig cells) [59]. Similarly, rat BMMSCs can potentially contribute to the reconstruction of testis germinal epithelium after transplantation. Researchers claim that not only did rBMMSCs differentiate into SSCs but they also further formed spermatocytes, spermatids and spermatozoa.

Nevertheless, the conclusions were strictly based on morphological analyses of the testicular cells and did not investigate gene expression patterns or molecular features of the produced cells [60].

More recently, the pluripotent potential of SSEA1$^+$ cells isolated from mouse BM mononuclear cells was supported through their differentiation towards cells of three germ layers. In addition, after induction with RA, SSEA1$^+$ cells exhibited PGC and SSCs-specific markers (*Mvh*, *Fragilis*, *Dppa3*, *Stra8*, *Dazl*, *Piwil2*, *β1* and *α6-integrins*), as well as positivity for Scp3, a meiotic marker [61]. However, RA or BMP4 were insufficient to induce PGCLCs to develop further into late male germ cells in vitro [61].

The potential of TGF-β1, BMP4 and BMP8b to promote transdifferentiation of ram BMSC into germ cells in vitro has also been reported [62]. TGF-β1 treatment efficiently induced cell colonies to express the protein gene product 9.5 (PGP 9.5), a marker of ram spermatogonia. Similarly, sheep BMMSCs treated with the RA in vitro also showed the ability to transdifferentiate into male germ cells that expressed *VASA,* β1-integrin and PGP 9.5 [62]. To assess their functionality, ram BMMSC-derived germ cells were transplanted into the ram testes [63]. A few PGP9.5$^+$ germ-like cells in the testes formed colonies after homing nearby the basement membrane of recipient tubules. Nevertheless, none of the homed BMMSC-derived germ cells underwent additional differentiation, and there was no labelled sperm in the epididymis from all transplanted testes [63]. Overexpression of *STRA8*, *BOULE* and *DAZL* genes was also able to promote the in vitro transdifferentiation of goat BMMSCs towards putative male germ cells. Though cell DNA content and fertilizing capacity of haploid cells were not further checked, the authors stated that all stages of the germ cell development process from gPGCLCs to post-meiotic stage had occurred in culture [64].

Although rather incipient, the in vitro germline potential of stem cells derived from skin cells has also been reported in the literature. Mouse skin-derived stem cells (SDSCs) could be induced to differentiate into EB and formed germ cells after being co-cultured with fibroblasts. MVH$^+$ and SSEA1$^+$ cells were found to be SDSCs-derived PGCLCs that responded to RA treatment increasing in vitro proliferation and upregulating the expressions of cyclin D1 and cyclin-dependent kinase 2 [65]. AA could also induce mouse SDSCs to differentiate into PGCLCs in both EB-like structure and the co-culture stage, and these cells exhibited similar dynamic changes in epigenetic modulation to that of developing PGCs in vivo. MAX dimerization protein 3 (MAX3) was suggested to be the key downstream signal of AA involved in promoting PGCLC generation from mouse SDSCs. During the later stage of co-culture, some meiotic genes expression (*Stra8*, *Dmc1*, *Scp3* and *Scp1*) could be identified [66].

Similar to SDSCs, adipose-derived mesenchymal stem cells (ADMSCs) also have one important advantage over other tissue-derived MSCs in that they are easily accessible. In an in vivo study, the capability of rat ADMSCs for restoring spermatogenesis in experimentally infertile male after transplantation was tested. The authors claimed that ADMSCs not only supported endogenous spermatogonia for the spermatogenesis recovery but could also transdifferentiated into male germ cells [67]. Remarkably, the overexpression of integrin-β3 (CD61) in canine ADMSCs promoted their differentiation into PGCLCs by activating the TGF-β signal pathway and, accordingly, upregulated the expression of PRDM1, PRDM14, AP2γ, CD49f, SOX2 and Nanog [68].

Recently, a novel population of pluripotent stem cells, termed very small embryonic-like stem cells (VSELs), from adult gonads has been proposed as an attractive alternative for obtaining gametes in vitro [34, 69]. Studies show that VSELs are very small in size (3–5 μm) and exhibit a surface phenotype LIN$^-$, CD45$^-$, SCA1$^+$, SSEA1$^+$ and OCT4$^+$, differing from SSCs. In the adult testis, as well as in other somatic tissues, VSELs are very few in number and are described as quiescent cells that survive chemotherapy [70–73].

60.2.4 Artificial Female Germ Cells and Gametes Derived from Embryonic Stem Cells

Compared to male, fewer studies were developed for female germ cell differentiation from pluripotent stem cells in animal models. In 2003, Hübner et al. [74] were the first to propose that mouse pluripotent stem cells could generate gametes and reported the successful derivation of oocyte-like cells (OLCs) from both female and male mESCs in vitro. Oct4-GFP mouse ESCs had been used to establish the conditions required for the spontaneous PGC formation recognizable by a GFP$^-$, *c-kit*$^-$ and *Vasa*$^+$ population, corresponding to postmigratory germ cells. Although the functionality was not evidenced, isolated cell colonies formed follicle-like structures in vitro and *Gdf9*$^+$, *Zp2*$^+$, *Zp3*$^+$ and *Figα*$^+$ oocytes underwent meiosis (*SCP3*$^+$ and *SCP1*$^+$ cells), and later parthenogenetically developed into blastocysts [74]. Using a similar approach, it has been demonstrated that primary and primordial follicles formed in vitro from mESCs expressed SCP3 but in an abnormal manner not associated with chromosomes. Expression of others essential meiotic proteins, such as SCP1, SCP2, STAG3, REC8 and SMC1β, and regular meiotic chromosomal organization were not detected either, suggesting that the OLCs formed from ESCs fail to progress through meiosis [75]. Genetic modification using EGFP reporter construct under the control of *Gdf9* gene promoter revealed that a specific population of cultured XX

mESCs contain the transcriptional machinery to drive expression of oocyte-specific genes spontaneously, and that these cells resemble oocytes, i.e. exhibit zona pellucida with Zp3 expression, typical metaphase plate with condensed chromosomes and a polar body-like structure. Some ESCs-derived OLCs are also capable of undergoing parthenogenesis and form blastocysts [76].

Some studies have focused on *in vitro* oocyte induction using specific media and factors. Oct4-GFP intensity combined with SSEA1 expression could be used to allow the isolation of PGCCLs that exhibit the ability to initiate meiosis under defined culture conditions (BMP4, Kit-ligand, SCF, RA, R115866 and βFGF); however, oocyte maturation eventually fails in vitro. When transplanted under the renal capsule of recipient mice, these cells formed primordial and primary-like follicles [77]. mESCs cultivated in conditioned medium collected from testicular cell cultures prepared from newborn males derived follicle-enclosed oocytes. *Fig-α* and *ZP3* expression was detected but without visible zona pellucida [78]. Induction of OCLS from mESCs by co-culture with ovarian granulosa cells was also reported [79]. RA treatment is also shown to induce mESCs to differentiate towards female germ cells, while oleanolic acid (OA) induced the differentiation towards male and female early germ cells in vitro, suggesting the OA could be used alone as a germ cell inducer [80].

In 2012, Hayashi et al. [81] successfully managed to develop ESCs-derived PGCLCs as a precursor step for fully functional oocyte derivation. Similar to those studied in the male, transgenic *Stella*- ECFP XX ESCs were induced into EpiLCs and further into PGCLCs [81]. These cells, when aggregated with female gonadal somatic cells to form reconstituted ovaries in vitro, underwent imprint erasure, X-reactivation and exhibit meiotic potential, corroborated by the expression and localization pattern of SCP3. Transplantation of reconstituted ovaries under ovarian bursa or kidney capsules of recipient mice allowed the PGCLCs development into fully grown oocytes with several layers of granulosa and theca cells, although some instability in cumulus complex formation were observed. Upon in vitro maturation and fertilization (IVF), PGCLC- derived OLC contributed to healthy offspring. However, the achievement of PGCLC-derived pups was less efficient when compared to those obtained from in vivo PGC or wild type oocyte. About half of the zygotes/oocytes derived from PGCLCs failed to extrude the second polar body, resulting in triploid phenotype [1, 81]. To date, the underlying mechanism of this failure has not been thoroughly investigated, but, in a promising way, the full potency of ESC-derived OLCs was fulfilled.

In this way, the reconstitution of the entire cycle of the female germ cells from ESCs in vitro was later reported [82]. First, the line of transgenic ESCs (BVSCH18) were aggregated with female gonadal somatic cells and submitted to PGCLCs differentiation in vitro. *Foxl2* expression and analysis of the meiotic chromosome revealed the progression of meiotic prophase I and indicated the primary oocytes induction from ESCs. Under FSH treatment, primary oocytes in secondary follicle-like structures grew to germinal vesicle oocytes and extruded the first polar body. Although global transcription dynamics during oogenesis in vitro revealed misregulation of genes, ESCs-derived MII oocytes were capable of developing into BVSCH18 fertile offspring. Nevertheless, embryogenesis was frequently retarded at many stages, such as the cleavage stage and early and late gestation. Developmental arrest could be partly attributable to aneuploidy and aberrant gene expression [82]. Clearly, in vivo developed PGCs and in vitro derived PGCLCs are not identical as discussed previously [1, 83]. Indeed, converting pluripotent stem cells into PGCLCs involves extensive epigenetic changes, which might be difficult to achieve in vitro [84].

60.2.5 Artificial Female Germ Cells and Gametes Derived from Induced Pluripotent Stem Cells

With the emergence of iPSC technology that allowed the reprogramming of differentiated somatic cells into a pluripotent state (Fig. 60.1), some studies also focused on creating female PGCLCs from iPSCs and on the induction of their development in gamete-like cells without in vivo steps. The development of MVH^+ round-shaped cells resembling immature oocytes from miPSCs derived from adult hepatocyte was first reported [85]. miPSCs were further induced to differentiate into OLCs that expressed *Mvh*, *Dazl*, *Stra8*, *Scp3* and *Zp3* by culturing adherent EBs in RA and porcine follicular fluid differentiation medium [48]. iPSCs could correspondingly be induced into fully functional oocytes as described above for mESCs. Transgenic Pou5f1(Oct4)-EGFP miPSCs derived from female MEF were induced in vitro by BMP4 and integrin-β3+ and SSEA1+ PGCLCs were purified to generate reconstituted ovaries. After transplantation under ovarian bursa or kidney capsules, iPSC-derived PGCLCs contributed successfully to oogenesis forming OLCs that reached the fully-grown germinal vesicle stage. OLCs in vitro maturation and IVF, followed by embryo transference, resulted in offspring that expressed *EGFP* and *Pou5f1* transgenes and grew into fertile adults [81]. With the purpose of differentiating functional endocrine tissue and assessing for evidence of preferential epigenetic memory, ovarian granulosa cells (GCs) were used to generate iPSCs lines [86]. Comparative analysis of EBs generated from different cell lines revealed that GC-derived iPSC synthesize tenfold more estradiol than iPSC differentiated from fibroblasts or mESCs under identical culture conditions.

Moreover, efficiency of early germ cell generation from GC-derived iPSC subpopulations was markedly improved when compared to the other cell lines, as evidenced by the higher incidence of OCLs and germ cell antigen expression. These data support the hypothesis for epigenetic-mediated mechanisms of homotypic differentiation of iPSCs, which may prove useful when applied to specific targeted tissue derivation for use in therapies [86]. To verify reproducibility of the culture system developed by Hikabe and colleagues [82] for mESCs, two iPSC lines from MEF and two iPSC lines from adult tail tip fibroblasts (TTFs) were also induced to germ cell differentiation and oogenesis in vitro. Fully mature oocytes were generated from MEF-derived iPSC lines and TTF-derived iPSC lines. Upon fertilization, followed by transfer to surrogate mothers, iPSC-derived oocytes gave rise to fertile pups [82].

Studies involving the derivation of OLCs from iPSCs of non-rodent models are still emerging. The potential of goat iPSCs, derived from fibroblasts, to generate germ cell-like cells and OLC was already investigated. giPSCs could differentiate into PGCLCs in the presence of RA and BMP4 and some GDF9$^+$ oocyte-like structures that resembled goat cumulus-oocyte-complex (COCs) were identified. Moreover, zona pellucida specific genes *ZP3* and *ZP2* were detected in goat OLCs [87]. Remarkably, treatment of bovine skin-derived fibroblasts with 5-azacytidine (a DNA methyltransferase inhibitor) could induce the expression of the pluripotency factors SOX2, NANOG, OCT4 and REX. The maintenance of these cells in differentiation medium supplemented with BMP2, BMP4 or follicular fluid resulted in morphological changes and promoted the expression of markers for germ cells (VASA, DAZL and cKIT), meiosis (SCP3) and oocytes (ZPA and GDF9) [88].

60.2.6 Artificial Female Cells and Gametes Derived from Adult Pluripotent Stem Cells

As previously noted, adult stem cells exhibit a broad differentiation potential. Some studies identified BM as a potential source of germ cells that could sustain oocyte production in adulthood. The transplantation of BMSCs into a sterilized recipient female contributed to the formation of primordial follicles with immature oocytes. Although BMSC-derived cells exhibited many intrinsic features of oocytes, such as morphology and normal expression of germ cell and oocyte molecular markers, their functionality was not determined [89]. Similar findings confirmed the presence of donor BM-derived oocytes in chemotherapy-treated recipient ovaries after transplantation; however, all offspring were derived from the recipient germline, suggesting that BMSC besides differentiating into female germ cells can play a supportive role for endogenous germ cells and restoration of fertility [90]. Indeed, further studies demonstrate fertility rescue and ovarian follicle growth promotion after BMSCs infusion in mice [91–93].

In porcine species, follicular fluid successfully simulated differentiation of oocyte during culture of stem cells obtained from SDSCs [94]. SDSCs differentiation into cell aggregates resembling COCs was verified by the clear expression of *Oct4*, *GDF9*, *Dazl* and *Vasa* transcripts. On further differentiation, these cells formed follicle-like aggregates that secreted estradiol, progesterone and extruded large OLCs expressing zona pellucida and the meiosis markers. Some OLCs spontaneously developed into parthenogenetic blastocyst-like structures, whose cells expressed *Oct4* [94]. Porcine SDSCs-derived PGCLCs when epigenetically investigated showed imprint erasure and had the same methylation pattern in *H19* DMRI as porcine oocytes [95]. Mice *Oct4*-GFP SDSCs were also capable of differentiating into early OLCs (ZP3$^+$) after aggregation with ovarian cells, and transplantation under the kidney capsule of ovariectomized mice. GFP$^+$ oocytes were identified within a subpopulation of follicles in reconstituted transplanted ovarian structures [96].

Adult pancreatic stem cells are able to differentiate spontaneously in vitro into various somatic cell types. Likewise, Danner and colleagues demonstrated that rat PSCs could differentiate into female germ cells in culture. A clonal pancreatic stem cell line spontaneously aggregated in vitro, and released large single cells into the culture medium. Analysis of these cells revealed oocyte-like morphology and expression of meiosis-specific markers SCP3 and DMC1 [97].

The potential of porcine muscle derived stem cells (pMDSCs) to differentiate into female germ cells in vitro after exposure to Reversine was also investigated. After confirmation of their pluripotency, and treatment with bovine follicular fluid, reversine-treated pMDSCs differentiated into large round germ-like cells. Immunocytochemistry analysis showed that, similar to porcine oocytes, in vitro derived cells were positive for Vasa, Dazl, Stra8, Scp3, Zp2 and Zp3 proteins [98]. In general, the use of APSCs to generate gametes in vitro has introduced a novel platform for research on germ cell development and the scientific findings on animal models are encouraging enough to merit further investigation; however, much more needs to be done before bring this into the clinics.

60.3 Development of Artificial Gametes in Humans

Over the past 20 years, significant efforts have been made towards the development of an artificial biological system with the ability to differentiate pluripotent stem cells into human artificial gametes [99]. To this end, several studies

have been described regarding the use of pluripotent stem cells of several origins, including human embryonic stem cells (hESCs), human induced-pluripotent stem cells (hiPSCs) and adult pluripotent stem cells (Fig. 60.1). Advances in this important field have been observed, mainly with respect to the differentiation of stem cells into PGCLCs in vitro.

60.3.1 Artificial Male Germ Cells and Gametes Derived from Embryonic Stem Cells

hESCs have been shown to differentiate into several lineages in vitro, including neural, pancreatic, muscular, endothelial and hematopoietic cells [100–105]. The first study regarding the production of germ cells from hESCs compared the transcriptional profiles between human ICM (inner cell mass) and three pluripotent human ES cells lines. ICM cells expressed *NANOS1*, *STELLAR* and *OCT4*, whereas hESCs expressed these genes along with *DAZL*, pointing out that undifferentiated hESCs lines expressed markers of pre-meiotic germ cells. Moreover, it was shown that hESCs were able to spontaneously differentiate into EBs, due to a shift in expression from RNA and protein markers of immature germ cells to those of mature germ cells, including *VASA*, *BOLL*, *SCP1*, *SCP3*, *GDF9* and *TEKT1* [106]. Despite the expression of early meiotic markers, such as SCP1 and SCP3, no evidence of complete meiosis were observed. In order to increase the percentage of PGCLCs differentiated from hESCs and progression through meiosis, several approaches have been developed, including the addition of growth factors and cytokines to the culture medium [107–109], methodologies of enrichment of the PGCLCs [110, 111], as well as co-culture with Sertoli cells [111, 112] and over-expression of genes involved in the hESCs differentiation to PGCLCs [108, 113, 114].

In this context, it has been shown that the addition of recombinant human BMP4 enhanced the expression of the germ cell-specific markers (*VASA* and *SCP3*) during differentiation of hESCs to EBs. BMP7 and BMP8b also showed additive effects on germ cell induction in association with BMP4. Moreover, the addition of BMPs also increased the percentage of cells that stained positively for VASA, pointing to the influence of these growth factors in the improvement of the differentiation of PGCLCs from hESCs. Despite the presence of a germ cell-specific marker that is expressed initially in meiosis (*SCP3)*, no evidence of haploid cell production was observed [107]. Studies also using BMPs supplementation in the culture system and inducing overexpression of genes *DAZL, DAZ* and *BOULE* (also called *BOLL*) have been performed demonstrating that human *DAZL* plays a role in primordial germ cell formation, whereas *DAZ* and *BOULE* promote later stages of meiosis and development of haploid gametes that resemble round spermatids in morphological and molecular characteristics [113]. In XY cells, overexpression of these three genes resulted in the highest percentage of cells with SCP3 staining. Over expression of *DAZ* alone gave rise to more than 20% of cells with punctate SCP3, whereas *DAZL* or *BOULE* was sufficient to induce elongated synaptonemal complex formation. Moreover, RNAm expression of the mature sperm markers *TEKT1* and *ACR* was highly elevated in cells that overexpressed all three family members and, as 2% of cells have shown 1 N content, these findings suggest a potential formation of haploid gametes [113].

Addition of RA to the culture medium also promoted the differentiation of human hESCs cells to PGCLCs by activating the expression of *STELLA*, a marker of germline differentiation that follows *VASA* expression [108]. Moreover, the over-expression of *STELLA* does not interfere with maintenance of the stem cell state of hESCs, but following RA induction it leads to up-regulation of germline and endodermal-associated genes, whereas neural markers *PAX6* and *NEUROD1* are down-regulated [108]. The production of haploid cells from hESCs was also achieved using a three-step media and calcium alginate-based 3D-culture system with addition of BMP4 and RA. After PGCLCs differentiation, cells were transferred to a 3D-co-culture system with testicular somatic cells where 3% of male haploid germ cells were obtained after 2 weeks [112]. Other studies have used hESCs with Sertoli cell co-culture. However, the production of haploid cells was not observed [111]. Also, using hESCs culture with somatic testicular cells and mouse embryonic fibroblasts feeder layers, it has been demonstrated that the addition of PDGF to PGCLCs culture increased the number of pluripotent colonies of these cells through the activation of the PI3K/AKT pathway that consequently caused upregulation of the *OCT4* and suppression of *PTEN* genes [115]. A previous report showed the maintenance of ESCs pluripotency through the regulation of *SOX2* [116]. Furthermore, *PRDM1* suppresses transcription of *SOX2,* favouring germline differentiation instead of the neural lineage, suggesting a new pathway to modulate the divergence of neural or germline fate of hESCs differentiation [117]. Moreover, knockdown of *SOX17* induced repression of the pluripotency genes *NANOG*, as well as of PGCLCs-genes *BLIMP1*, *NANOS3*, *TFAP2C*, *STELLA* and *KIT*, suggesting that *SOX17* might also be important for regulating the established germline gene expression network [29].

Exogenous supplementation of AA also up-regulated the genes *STELLA/DPPA3* and cKIT receptor in PGCLCs derived from hESCs. Furthermore, AA in association with BMP4 strongly increased PGCLCs' differentiation potential of hESCs based on the high expression of *DAZL* and *VASA/DDX4* at mRNA and protein level. PGCLCs also exhibited expression of *SCP3*. However, the absence of SCP3 at the protein level clearly suggests that additional maturation steps are needed to efficiently push the derived PGCs towards meiosis. Furthermore, an up-regulation of endoderm-specific genes *GATA4* and *GATA6* was also observed [109]. In a pre-

vious investigation also using AA and BMP4 supplementation, *GASZ,* a gene reported to participate in meiosis of postnatal spermatocytes, led to the upregulation of PGCLCs formation from both human and murine ESCs [114]. Differentiation of PGCLCs from hESCs was also observed by using CHIR99021, an inhibitor of glycogen synthase kinase 3 (GSK-3), and RA supplementation. The results showed that DAZL protein can be detected in CHIR99021 co-culture with RA but not expressed in single CHIR99021 or RA treated cultures. Moreover, PGCLCs exhibited several germ cell markers (*DDX4/VASA*, *BLIMP-1*, *NANOS*, *TFAP2C*, *c-Kit)* and *CXCR4*, which is mainly expressed on the migrating germ cells [118]. Furthermore, based on SCP3 staining and FISH analysis, the results confirmed that meiosis was initiated and putative haploid cells were formed [119]. Despite several studies reporting the differentiation of hESCs into PGCLCs in vitro using distinct protocols, the progression of these cells through meiosis and formation of male haploid germ cells is still facing significant challenges. Thus, future studies should focus on the elucidation of specific molecular mechanisms of differentiation of hESCs to PGCLCs, considering that the distinct pluripotent states in mouse and human embryonic stem cells might also result in differences in the mechanism of germline specification.

60.3.2 Artificial Male Germ Cells and Gametes Derived from Induced Pluripotent Stem Cells

The production of male germ cells from the differentiation of human induced pluripotent stem cells (hiPSCs) still faces many obstacles to achieve the progress already obtained in mice [120]. This occurs because the hiPSCs exhibit a primed pluripotency with less potential for a germ cell fate [121–123].

Based on protocols developed to produce mouse iPSCs [2], several approaches have been applied to hiPSCs in order to obtain male germ cells, including spontaneous differentiation [124], overexpression of germ cell regulators [125–127] and addition of cytokines and growth factors [29, 128]. Moreover, co-culture of hiPSCs with gonadal cells in vitro and the use of xeno-transplantation of the germ cells in vivo had also been reported in some studies [124]. In the first attempts to produce human PGCLCs derived from hiPSCs, adult and embryonic fibroblasts were reprogrammed to pluripotency through the introduction of the genes *OCT4, SOX2, KLF4 and MYC*, via the pMIG vector. Besides that, it was demonstrated that *OCT4* and *SOX2* were essential to the production of PGCLCs, whereas *MYC* and *KLF4* enhanced the efficiency of colony formation of these cells [125, 129, 130]. However, only a small portion of human fibroblasts that had been transduced with the four retroviruses acquired iPS cell identity, pointing to the low efficiency of this technique [125]. Moreover, non-retroviral methods to introduce genes, such as adenoviruses, cell permeable recombinant proteins, or tools of gene editing should be improved in future studies [125]. In this context, Park et al. (2009) [124] reported the first successful assay to create PGCLCs from hiPSCs by co-culturing them with human fetal gonadal stromal cells (hFGS). Furthermore, the efficiency to produce PGCLCs from the reprogramming of human skin fibroblasts were similar to human embryonic stem cells (hESCs), indicating that the hFGS cells produce important iPGC survival and/or self-renewal factors. However, the erasure of the genetic imprint did not initiate efficiently in PGCLCs originated from hiPSCs [124].

Concerning pluripotent stem cells survival and maintenance of self-renewal, in apparent contrast to mouse ESCs [131], BMPs have been shown to induce hESC differentiation [132]. The association of BMP with overexpression of members of the DAZ gene family, induced hiPSCs to form meiotic cells with synaptonemal complexes and post-meiotic haploid cells with acrosin staining, similar to human spermatids [126]. Furthermore, other studies showed that the overexpression of *VASA*, *DAZL*, *MVH*, *DAZL*, *GFRa1*, *NANOS3* and DMRT1, enhanced hiPSCs differentiation into PGCLCs and progression through meiosis [127, 133]. However, using standardized mouse spermatogonial stem cell culture conditions [33, 83, 134], some studies have shown that post-meiotic haploid cells could also be obtained from hiPSCs without overexpression of germ line-specific factors. Using these protocols, PGCLCs were able to differentiate into haploid cells expressing proteins that are specifically found in spermatids and/or spermatozoa, such as acrosin, transition protein 1 (TP1) and protamine [134].

Although some studies have reported the differentiation of PGCLCs from hiPSCs in vitro, the complete development of PGCLCs into human sperm in vitro has not been achieved. Considering that the gonadal environment in vivo is important for successful meiosis [14], another alternative method for male germ cell differentiation is xeno-transplantation of hiPSCs into immunodeficient mice with endogenous spermatogenesis chemically depleted. In order to evaluate their potential to differentiate into germline cells, hiPSCs were transplanted directly into the seminiferous tubules of immunodeficient mice. The transplanted hiPSCs migrated to the basement membrane of the seminiferous tubules, but differentiated cells only expressed PGCLCs and pre-meiotic germ cell markers [135]. Also using xenotransplantation, hiPSCs derived from azoospermic and fertile men were transplanted to murine seminiferous tubules. Human iPSCs with azoospermia factor deletions produced significantly fewer PGCLCs in vivo with defects in gene expression, pointing to the influence of the donor genetic background [136]. Moreover, supporting the influence of the donor genetic background, a recent in vitro experiment showed that NOA-iPSC lines from patients with Sertoli cell-only syndrome had reduced formation of haploid cells compared to cell lines from men with obstructive azoospermia [135].

In order to investigate germ cell fate regulatory pathways, several studies were developed to evaluate the influence of cytokines and growth factors released by early extraembryonic tissues [29, 128, 137]. However, despite the initial elucidation of some molecular mechanisms, most of the differentiated cells from hiPSCs remained at the early stages, such as PGCLCs.

In conclusion, although a few studies showed differentiation of PGCLCs into haploid cells in vitro, human male germ cells were not derived from hiPSCs. Therefore, the improvement of cell culture conditions supporting the efficient production of PGCLCs from hiPSCs [138] and the maintenance of human PGCLCs differentiation should be addressed in future investigations.

60.3.3 Artificial Male Germ Cells and Gametes Derived from Adult and Fetal Pluripotent Stem Cells

Male germ cells can also be originated from human MSCs (hMSCs) isolated from different tissues sources, such as human adult and fetal bone marrow (hBMSCs) and human amniotic fluid stem cells (hAFSCs) [139–142]. Initially, it was demonstrated that adult and fetal hBMSCs were able to transdifferentiate into male PGCLCs by induction with RA, promoting the enhancement of the early germ cell markers expression in hBMSCs and inducing further differentiation [139, 141]. Adult hBMSCs showed the expression of early germ cell markers *OCT4*, *FRAGILIS*, *STELLA* and *VASA* and male germ cell specific markers *DAZL*, *TSPY*, *PIWIL2* and *STRA8* [139]. Meanwhile, fetal hBMSCs exhibit germ cell markers *OCT4*, *STELLA*, *NANOG* and *VASA* and male germ-cell-specific markers, such as *DAZL*, *TH2*, c-kit, β1-integrin, *ACR*, *PRM1*, *FSHR*, *STRA8* and *SCP3* [141]. *SCP3* is a known specific marker of meiosis in male and female germ cells [143]. However, although these studies suggested the possibility that PGCLCs derived from fetal hBMSCs were undergoing meiosis, there was no evidence of meiosis completion.

Human amniotic fluid is also considered as a source for the isolation of human hAFSCs [140]. Moreover, after 5 days of hAFSCs culture, embryoid bodies (EBs) were obtained, showing the presence of *OCT4* and *SOX2*, as well as the expression of genes involved in early stages of germ cell development [142].

60.3.4 Artificial Female Germ Cells and Gametes Derived from Embryonic Stem Cells

The production of OLCs [144] and granulosa-like cells (GLCs) [145, 146] from hESCs in vitro have already been described in a few studies. Thus, using a monolayer culture system supplemented with RA, BMP4 and NMT, structures resembling primordial follicle (follicle-like cells, FLCs) derived from hESCs were observed. Moreover, the differentiation of these putative follicles also correlated with expression of mRNA for the oogenesis markers *GDF9* and *ZP1*. However, it was not possible to obtain immunolocalization of the zona pellucida matrix [147]. Furthermore, successful differentiation of hESCs into functional ovarian GLCs was achieved using multistep approaches comprising in vitro treatments with some growth factors, such as AA and BMP4. Gene expression analyses showed the progress of hESCs into primitive streak-mesendoderm, intermediate plate mesoderm and finally to becoming functional GLCs that expressed the granulosa cell-specific markers *FOXL2*, P450 19A1 (*CYP19A1*), *AMH*, *AMHR2* and *FSHR*. Moreover, suggesting biological functionality [145], these GLCs were also capable of producing AMH and aromatizing testosterone to estradiol. Thus, these functional GLCs could be useful to improve in vitro conditions for the development of human female gametes from hESCs. In this context, granulosa cell co-treatment enriched the number of Oct4-EGFP expressing hESCs. These manually collected H9 Oct4-EGFP^{+} cells expressed significantly higher levels of *VASA* and *GDF9*. Moreover, H9 Oct4-EGFP^{+} cells developed into ovarian FLCs, but with low efficiency. Although a significant number of PGCLCs could be derived from hESCs, this study showed that the expression of *SCP3* was still low. However, indicating the potential use of the association of RA and granulosa cell co-culture in hESCs differentiation into haploid germ cells [146], RA co-treatment significantly enhanced *VASA*, *GDF9* and *SCP3* expression in the differentiated cells after 28 days.

Confirming that *DAZL* has an important role in human female fertility [148], the first report of FLCs resembling primary follicle derived from hESCs showed that a combination of *DAZL* and *BOULE* can be utilized to induce hESCs to enter meiosis in vitro. Moreover, the combination of recombinant GDF9 and BMP15 in the culture system promoted the induction of folliculogenesis in vitro. In addition, FLCs contained OLCs in the middle and GLCs at the outer layer, suggesting that the mixed population of cells in the differentiated culture had the ability to self-organize into an ordered biological entity [144].

60.3.5 Artificial Female Germ Cells and Gametes Derived from Induced Pluripotent Stem Cells

The first attempt to produce female germ cells from hiPSCs emerged from the need to create an efficient alternative method to treat the POI, which is the main disease causing female infertility [149, 150]. Through the overexpression of the genes *OCT4*, *SOX2*, *NANOG* and *LIN28* using lentiviral

vectors, fibroblast cells were able to differentiate into POI-iPSCs. However, gene expression profiling indicated that the PGCLCs differentiated from these cells were pre-meiotic [149]. Meanwhile, without using viral transfection, POF-iPSCs cultured in an in vitro system supplemented with TGFβ-1, BMP4 and RA, showed PGCLCs expressing *STELLA*, *VASA* and *SCP3*, which are markers of meiosis initiation [150]. In a recent report, hiPSCs differentiated progressively into oogonia-like cells during a four-month in vitro culture in a xenogeneic reconstituted ovary with mouse embryonic ovarian somatic cells. Although these cells showed epigenetic reprogramming and acquired an immediate precursory state for meiotic recombination, haploid cells were not observed [151].

60.3.6 Artificial Female Cells and Gametes Derived from Adult Pluripotent Stem Cells

Because hAFSCs have the capacity to differentiate into the three germ layers [152, 153], several studies have investigated the generation of OLCs from the hAFSCs [154–156]. It was demonstrated that hAFSCs have extensive self-renewal ability and remain highly viable in long-term cultures [154]. Moreover, when hAFSCs were induced by a medium supplemented with porcine follicular fluid, OLCs with a diameter of 50–60 μm and zona pellucida-like morphology were observed after 15 days of differentiation [154]. Furthermore, these OLCs showed expression of the gene *BMP15* that is involved in oocyte maturation and fertilization [157]. OLCs derived from hAFSCs also had different expression profiles of *ZP1*, *ZP2* and *ZP3* mRNA along the differentiation path [154]. In order to improve the purity and assay reproducibility, $CD117^{+}$/$CD44^{+}$ hAFSCs showing fibroblastoid morphology and intrinsically expressed stem and germ cell markers were selected and differentiated using a two-step method. During the induction period, formation of cell aggregates and synthesis of steroid hormones were detected, and OLCs ranging from 50 to 120 μm presenting zona pellucida were observed. Moreover, some OLCs developed spontaneously into multicellular structures similar to pre-implantation embryos and approximately 2% of the hAFSCs differentiated to meiotic germ cells that expressed folliculogenesis- and oogenesis-associated markers [156]. However, despite the high-efficiency of differentiation of hAFSCs into OLCs, the physiological function of induced OLCs still needs to be investigated.

MSCs can also be obtained from the human umbilical cord (hUCSCs) and are able to differentiate into several cell types, including germ cells [155, 158]. The results showed that hUCSCs differentiated into OLCs expressing the germ cell markers *OCT4*, *VASA*, *DAZL*, *ZP2*, *ZP3* and *STRA8* [155]. Moreover, it has been demonstrated that OLCs could be originated from male and female hUCSCs [155].

60.4 Biological Progress and Ethical Aspects of Clinical Application of Human Artificial Gametes

Currently, the treatment of infertility has mainly been done by assisted reproductive technology (ART). Although substantial progress has been made in this area, the potential of ART seems to have some limitations since it is unable to help the infertile couples lacking functional gametes [120]. In this context, the development of stem cell technology arises as a promising alternative treatment, since it makes possible the ability to derive artificial gametes from human pluripotent stem cells. Furthermore, artificial gametes technology can also provide a genetically related child to couples who lack functional gametes production [120, 159]. In addition to helping infertile heterosexual couples, artificial gametes could also potentially assist post-menopausal women and same-sex couples to conceive a child genetically related to them. However, as clinical application of these new technologies may have social consequences, a proactive consideration of the possible impacts should be considered [160].

Concerning the distinct stem cell sources, hESCs can differentiate into male and female germ-like cells in vitro. However, their utilization has a strong social impact due to ethical issues concerning the use of human embryos [161, 162]. Thus, since adult somatic cells are able to be reprogrammed in vitro and acquire pluripotency genes expression and differentiation into human gametes [129, 163], the use of hiESCs represents an alternative approach. Moreover, because their use appears to involve fewer ethical issues, hiPSCs have been considered an alternative source of stem cells for reproductive medicine application. However, it has been known for some time that somatic cells from patients, lacking functional gametes due to genetic disorders, retain some epigenetic marks, which leads to a less efficient hiPSCs differentiation to germ cells [133, 164]. Therefore, although there have been major advances in protocols to induce the differentiation of human stem cells into gametes in vitro, the progression of primordial germ cells into haploid cells still faces significant challenges. In addition, the generation of offspring using human artificial gametes involves important ethical issues, making the clinical application of these amazing technologies still a considerable distance away from the medical practice.

60.5 Concluding Remarks

Owing to a multitude of factors affecting reproduction and particularly gametogenesis, human fertility is decreasing drastically worldwide, mainly in industrialized countries, representing a big concern for those who plan to have children genetically related to themselves. Fortunately, in the past decade considerable hope has arrived for individuals

without mature gametes, through significant advances in innovative technologies that allow for the production of artificial gametes in vitro. However, even in animal models where these studies are more advanced, the techniques available using different cell sources and techniques still show limited success, mainly for females. Even so, besides many potential implications related to regenerative medicine, studies involving the development of animal artificial gametes have contributed to the advancement of several areas, including the treatment of infertility, genetic manipulation of stem cell nuclear transfer and germline epigenetics programming. Nevertheless, in order to bring these approaches to clinical medicine and to overcome the ethical issues, further investigations are necessary to meet the challenges of improving the protocols required for producing the artificial gamete.

60.6 Review Criteria

An extensive research concerning studies pointing to the production of artificial gametes from different sources of stem cells was performed, using in particular search tools, such as Science Direct, Google Scholar and PubMed. The period of research activities started at November 2018 and finished at March 2019. Considering studies carried out with both animal models and human, the strategy for the study recognition and data extraction was based on the following keywords: "artificial gametes"; "artificial sperm production"; "artificial oocyte production"; "primordial germ cells"; "embryonic stem cells"; "induced pluripotent stem cells"; and "adult pluripotent stem cells". Book-chapter citations provide conceptual content only. Articles published in languages other than English were also considered, whereas data published only in conferences, meeting proceedings or books were not included.

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ICSI and Male Infertility: Consequences to Offspring

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Key Points

- Intracytoplasmic sperm injection (ICSI) has revolutionized the management of many conditions associated with male infertility. Nevertheless, ICSI utilization raises concerns about the health and well-being of the children born after the procedures.
- Parental sperm defects, rather than the method of assisted reproductive technology (ART), could be the responsible factors for increases in the incidence of these conditions. Concerns also exist regarding the invasive nature of the ICSI procedure.
- The quality of the studies evaluating congenital malformations in children born through ART techniques is moderate or low, mainly because the studies are heterogeneous and present selection bias and problems related to the design of the studies. However, the risk of congenital malformation seems to be increased when evaluating systematic review and meta-analysis studies evaluating singleton pregnancies and limited to good quality studies. Further studies should take into account the couple's reproductive history and infertility factors to identify whether the risk of congenital malformations is associated with ART or the infertility factor.
- Differences in the methylation pattern of naturally conceived and ART children were found. However, if there are differences in the frequency of imprinting and epigenetic diseases between children conceived naturally and through ART designed, the effects are small in magnitude. The detection of these differences should be evaluated in larger studies with well-defined populations.
- The risk of chromosomal abnormalities is greater in children born to ART than naturally conceived children, especially when evaluating sex chromosome-related aneuploidies. ICSI appears to be at increased risk of chromosomal abnormalities than conventional IVF.
- Apparently, the semen quality of young adults born through ART is lower than naturally conceived counterparts.
- The risk of developing cancer in children conceived through ART is still unknown. Studies evaluating the main outcome of increased risk of cancer have shown similar risks to children conceived naturally; however, subgroup analyses have shown increased risks in some cancer subtypes.
- The evidence related to the risks of neuropsychomotor development impairment in children or adolescents born through ART is equivocal. While data is overall reassuring when the studies are controlled for multiple births, there seem to be differences in subgroup analyses.
- Few studies have evaluated the cardiometabolic outcomes of children born through ART. Limited evidence points to increased risks of high blood pressure and increased fasting blood glucose.

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61.1 Introduction

Intracytoplasmic sperm injection (ICSI) has revolutionized the management of many conditions associated with male infertility. Nevertheless, ICSI utilization raises concerns about the health and well-being of the children born after the procedures. These concerns relate to the fact that ICSI bypasses natural selection mechanisms that would otherwise eliminate unfit products. While the primary goal of ICSI treatments is to produce healthy infants, concerns include possible increased risks of congenital malformations, epigenetic disorders, chromosomal abnormalities, infertility, cancer, delayed psychological and neurodevelopment, as well as impaired cardiometabolic profiles compared with naturally conceived children [1].

Parental sperm defects, rather than the method of assisted reproductive technology (ART), could be the responsible factors for increases in the incidence of these conditions [2]. Specifically, it has been speculated that fertilization of oocytes by sperm from subfertile men, either naturally or through in vitro fertilization (IVF) and ICSI, might pose increased risks to the embryo, thus ultimately leading to implantation failure, arrest of embryo development, congenital malformations, miscarriages, pediatric cancers, and other perinatal morbidities [3, 4].

Concerns also exist regarding the invasive nature of the ICSI procedure. The nuclear decondensation of spermatozoa might be disrupted by sperm injection, and this could lead to aneuploidy in the embryo [5]. The microinjection pipette itself might indirectly cause abnormal segregation patterns of chromosomes. This phenomenon would occur if the pipette accidentally disrupted the oocyte meiotic spindle during oocyte injection [6]. Furthermore, handling oocytes outside the incubator for prolonged periods, as in ICSI, can alter (however slightly) the temperature and pH, possibly increasing the rates of stress-induced aneuploidy [7]. In this section, we summarize the most relevant studies evaluating this putative phenomenon.

61.2 Risks and Sequelae of ICSI to the Health of Offspring

61.2.1 Congenital Abnormalities

The main goal of ART treatment is to produce healthy infants. A major concern of reproductive endocrinology specialists is that children conceived through ART are at increased risk for congenital malformations. Scientific evidence from systematic reviews and meta-analysis demonstrates an increased risk of congenital malformations in children born through ART when compared with those conceived via natural pregnancies [8–10]. Nevertheless, a meta-analysis analyzing congenital malformation rates in twin pregnancies as the primary outcome found no differences between infants conceived through ART treatments and those conceived naturally [11]. Furthermore, meta-analyses comparing rates of congenital malformations among ART procedures (ICSI vs. IVF) did not show increased risks of general congenital malformations [12, 13].

It is worth mentioning that these meta-analyses included prospective and retrospective cohort studies with conflicting results, some with small cohorts and high-bias risks [8–14]. Furthermore, the characterization of malformations was often heterogeneous, futher increasing the risk of bias.

A Danish national birth cohort study published by Zhu et al. demonstrated that the increase in congenital malformations rates of children conceived through ART treatments might be related to the diagnosis of infertility or its determinants [15]. According to the authors, children conceived naturally by couples diagnosed with subfertility presented a 20% increased risk of congenital malformations when compared to children conceived by fertile couples. The authors also found an increased prevalence of congenital malformations with a longer time to pregnancy. Rimm et al. revisited the meta-analysis published in 2004, considering the time to pregnancy. The result of this new meta-analysis demonstrated that the risk difference of congenital malformations between infants conceived through ART treatments and those conceived naturally was not significant [13].

Taken together, the prospective and/or retrospective cohort studies included in the meta-analyses show significant heterogeneity, with inappropriate control groups composed of "normal" populations that might overestimate the risk of congenital malformations solely attributable to subfertility. The association between infertility and congenital malformations should be further investigated in new studies (Table 61.1).

61.2.2 Epigenetic Disorders

The behavior of a person's genes depends not only on their DNA sequences. Epigenetic factors are pieces of information present in the genome over and above the DNA genetic sequence that have a critical role in genomic function. Epigenetic changes can activate or inhibit genes, determining which proteins will be transcribed [16]. Epigenetics acts in a variety of normal cellular processes. Genes may be silenced by epigenetic control, possibly contributing to the differential gene expression. Three systems interact to silence genes in cells, namely, DNA methylation, histone modifications, and RNA silencing [17].

Table 61.1 Summary of effects of ART/ICSI treatment on health outcomes for the offspring

Outcomes	Summary effect	Explanation
Congenital abnormalities	↑	The risk of congenital malformation seems to be increased when evaluating systematic review and meta-analysis studies comprising singletons and limited to high-quality studies. One study showed higher risk of overall genitourinary congenital malformation among ICSI infants than IVF infants [14]
Epigenetic disorders	↑	Differences in the methylation pattern of naturally conceived and ART children were found. However, it is still unknown how such differences translate into imprinting and epigenetic diseases between children conceived naturally and through ART. One study showed higher mean methylation indices in maternal peripheral blood and umbilical cord of IVF infants compared to ICSI infants [31], but no risk difference between ICSI and IVF groups were reported by other groups
Chromosome abnormalities	↑	The risk of chromosomal abnormalities is increased in children born to ART than naturally conceived children, in particular, when evaluating sex chromosome-related aneuploidies. ICSI children appears to be at increased risk of chromosomal abnormalities than conventional IVF. Oligozoospermia was related to higher risks of disomy for sex chromosome [52] and aneuploidy in trophectoderm cells of blastocysts [7]. No risk difference was seen between ICSI and IVF groups with normal sperm
Infertility	↑, in male ↔, in female	Alterations in semen quality of young men born through ICSI were found. In young women born through ICSI, data are still too limited to draw any conclusions about possible negative effects
Cancer	↔	It remains to be determined if children born through ART have an increased risk of developing cancer. However, limited evidence suggests that some types of cancer are more common in children born through ART. In subgroup analysis by ART method, no significant differences between ICSI and IVF were reported
Psychological and neurodevelopment	↔	Concerning the variables related to the neuropsychomotor development of children and adolescents born through ART, the results are conflicting. However, several risks, such as autistic disorders and mental retardation, decrease after adjusting for multiple births or were only observed in subgroup analyses (ICSI for male factor infertility) [70]
Metabolic and cardiovascular	↑	Limited data suggest a potential increase in blood pressure and higher fasting glucose levels in ART children. No risk difference between ICSI and IVF groups were reported

↑ increased, ↔ no effect, *ART* assisted reproductive technology, *ICSI* intracytoplasmic sperm injection, *IVF* in vitro fertilization

Epigenetic changes seem to be necessary for normal and healthy embryo development; however, they also may be implicated in the pathogenesis of some diseases. Disruption of any of the three systems might activate or inhibit genes. These disorders have been associated with cancer and chromosomal syndromes involving disabilities and mental retardation.

Epigenetic activity is associated with critical stages of development that occur during gametogenesis and early embryonic development [18]. A fundamental phenomenon is imprinting (Fig. 61.1), when genes are epigenetically regulated and expressed according to the maternal/paternal origin [19]. For most autosomal genes, expression occurs in both alleles simultaneously. If the allele inherited by one of the parents undergoes genomic imprinting and is therefore silenced, only the allele inherited from the other parent is expressed. If the remaining allele has a significant deletion or mutation, the individual may present with an epigenetic disease, which, although rare in the general population [20], may occur more frequently in subfertile populations [21].

Recent studies suggest that ART treatments might cause epigenetic abnormalities that affect proper genomic imprinting [22]. Ovarian stimulation and oocyte retrieval, sperm manipulation, and embryo culture conditions might be associated with abnormal methylation processes and imprinting disorders [23]. Most studies evaluating the effects of ART treatments on epigenetic changes are inconclusive; therefore, it is not possible to establish a causal relationship. Several reasons explain these results, including the differences among studied individuals, tissue samples, and laboratory methodologies. In the specific case of male infertility, an association between oligozoospermia/azoospermia and epigenetic abnormalities has been described [22, 24–28].

What is clear is that, if there are changes in imprinting epigenetic status between naturally and ART-conceived children, the effects are small in magnitude [29–44]. Nonetheless, detecting imprinting defects is likely to be demanding and will require more controlled studies with standardized methodologies, in larger and better-defined populations. It is also vital to select populations of children of couples with subfertility in both ART and non-ART (exposed and unexposed) groups in order to explore the influence of fertility problems per se as opposed to those of ART on epigenetic changes in the offspring (Table 61.1).

61.2.3 Chromosome Abnormalities

Chromosomal segregation errors contribute to numerical chromosomal abnormalities in mature germ cells that can be transmitted to the embryo. Aneuploidies and chromosomal structural aberrations are the primary factors involved in

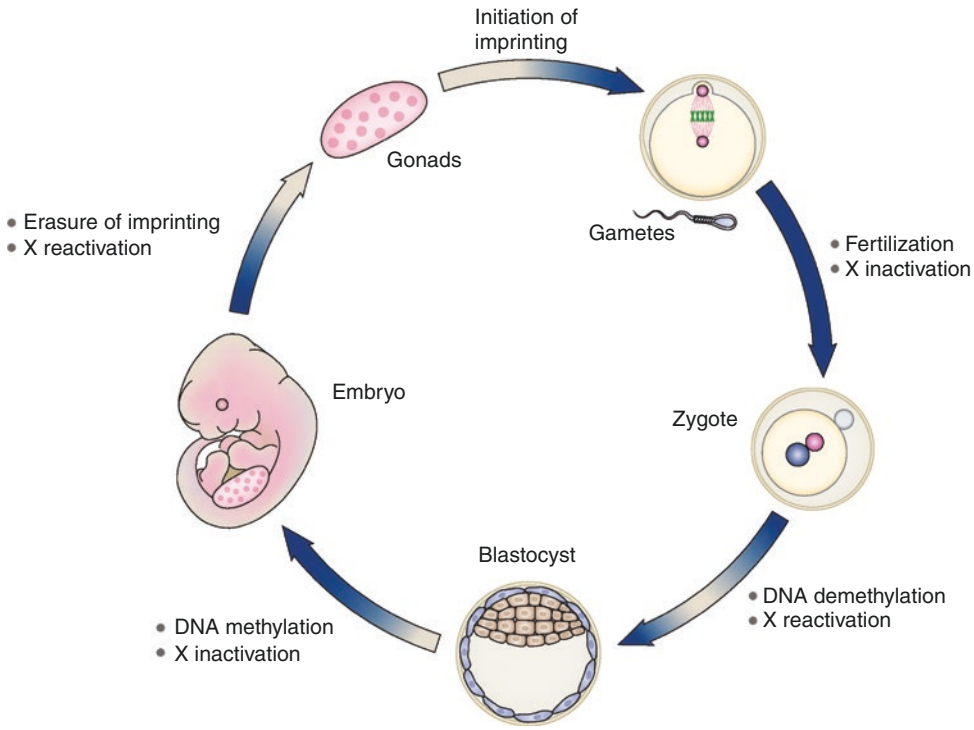

Fig. 61.1 The epigenetic reprogramming cycle. The two major waves of epigenetic reprogramming occur during gametogenesis and after fertilization. During gametogenesis, the majority of parental epigenetic marks are erased and reestablished at the time of oogenesis and spermatogenesis. A second epigenetic reprogramming wave occurs soon after fertilization with a fast, active paternal demethylation and a slower, passive maternal demethylation. At the blastocyst stage, novel methylation patterns are established in the inner cell mass, while the trophectoderm stays relatively unmethylated. Fluctuation in DNA methylation levels is represented by the differences in the arrows, with the blue color indicating high methylation levels. (Reprinted from Esteves et al. [82]. With permission from Springer Nature)

fertilization failures, implantation failures, spontaneous abortions, stillbirths, congenital malformations, and mental and behavioral dysfunctions [45, 46].

It remains unclear whether pregnancies conceived through ART are associated with a higher incidence of chromosomal abnormalities when compared to naturally conceived pregnancies. However, the invasive nature of the procedure has been implicated as a possible contributory factor. Fertilization by ICSI could (a) compromise nuclear decondensation of spermatozoa, possibly leading to embryo aneuploidy; (b) affect the meiotic spindle of the oocytes by the use of the micromanipulator needle during sperm injection, possibly leading to abnormal chromosomal segregation patterns; and (c) affect embryo development owing to the handling of oocytes outside the incubator for longer periods when compared to standard IVF procedures; sperm handling outside the incubator might be associated with suboptimal temperature and pH, possibly increasing the rates of stress-induced aneuploidy. All these interventions associated with ICSI might impair chromosomal segregation of the oocyte and subsequently the embryo.

Data related to infertility conditions treated through ICSI suggest that the technique circumvents natural selection mechanisms and could increase the rates of aneuploidy in the first trimester of gestation [47, 48]. Although ICSI has become the method of choice for the treatment of male infertility, there is concern that infertile men may have a higher frequency of chromosomal abnormalities in their spermatozoa.

Consistent with this concern, some studies showed a higher proportion of aneuploidy in sperm cells in men diagnosed with male factor infertility than in healthy controls [49–51]. Therefore, the risk of abnormal chromosomal offspring might increase in patients using ICSI for treatment, as this technique could perpetuate aneuploidy through the injection of disomic sperm [52].

Recent evidence has shown an increased risk of sexual chromosome aneuploidy in couples that underwent male-assisted infertility treatments. This risk had no relation to the ICSI procedure itself, but rather to the quality of the spermatozoa used for sperm injections [7] (Table 61.1).

61.2.4 Infertility

Some studies have evaluated various parameters related to the reproductive potential of children born through ART [53–57].

However, information related to the reproductive potential of these children in adult reproductive stage remains scarce.

A longitudinal cohort study evaluated serum hormone levels of 3-month-old boys to compare the testicular function of boys born through ART with that of naturally conceived boys [53]. Since men with male factor infertility have impaired Leydig cell function and lower serum testosterone, the researchers decided to assess the hormone levels of ICSI offspring with 3 months of age as predictive markers of adult reproductive function. They found a subtle impairment of Leydig cell function in boys conceived by ICSI, possibly inherited from their parents. Although the finding was of uncertain meaning, the authors suggested the need to investigate further in men born through ART [53].

A prospective cohort study evaluated the semen analysis, hormone serum levels, and physical examination of 18-year-old men who were conceived through unspecified infertility treatments [54]. When compared to the control group, the male offspring born as a result of infertility treatments showed significantly altered semen parameters and lower testicular volumes. Hormone levels were also altered, although this difference was not statistically significant [54]. Published data from the long-term follow-up of a cohort of men aged 18–22 years conceived through ART demonstrated the reproductive function of these men through semen analysis and hormone serum levels [55]. Semen analysis parameters showed lower sperm concentrations and lower sperm motility in men born through ART when compared to those of the control group consisting of naturally conceived men [55]. Hormonal analysis of these men was reassuring, with mean and median levels of FSH, LH, inhibin B, and testosterone similar to those of the control group. Despite these findings, it is worth noting that men conceived through ART were more likely to have inhibin B levels below the 10th percentile and FSH levels above the 90th percentile [56].

A long-term assessment of women born through ART also brought reassuring data. Women born through ART aged 18–22 years had serum levels of anti-Müllerian hormone and follicle-stimulating hormone similar to those of the control group consisting of naturally conceived women. Similar results were observed for antral follicle counts [57] (Table 61.1).

61.2.5 Cancer

Childhood cancer is a significant cause of death in children in developing countries. The etiology of childhood cancer remains poorly understood; however, it has been hypothesized that some cancers initiate in the early stages of fetal development [58]. Consequently, events that precede and occur around the time of conception can play an important role and can justify a specific assessment.

Several isolated reports of cancer in children born through infertility treatments have been published; however, few large-scale epidemiological studies are available, and the results are inconsistent. The first systematic review and meta-analysis assessing the risk of cancer in children born after ART did not demonstrate an overall increase in cancer risk [59]. Nevertheless, a recent systematic review and meta-analysis showed an overall increased risk of cancer in children born after ART (RR 1.33; 95% CI 1.08–1.63) [60]. Statistically, significant associations were established between the risks of specific types of cancer, including leukemia, neuroblastoma, and retinoblastoma [60–64]. Recently, large longitudinal cohort studies not included in the systematic review and meta-analysis have shown no increase in the overall risk of cancer [61–63]. In the subgroup analysis, however, increased risks of hepatoblastoma and rhabdomyosarcoma [61], nervous system tumors and malignant epithelial neoplasms [62], and leukemia and Hodgkin's lymphoma [63, 64] were shown, although the absolute risks were small.

Generally, significant differences in subanalyses should be interpreted with caution when no significant differences are found in the primary analysis. Nevertheless, particular cancers have varying etiologies, and therefore, it might be conceivable that the risk of cancer in children born after ART is increased for some specific types of cancer, while there is no increase in the overall risk. Therefore, it is unclear whether children born through ART present increased risks of developing cancer (Table 61.1).

61.2.6 Psychological and Neurodevelopment

Studies have shown a divergence of data regarding the association between neurodevelopmental disabilities in children and ART. The results of studies of long-term neurodevelopmental disabilities, including cognitive, motor, and language development, remain sources of controversy [65–73]. Longitudinal cohort studies are crucial for the education of couples planning to undergo treatment with ART.

The most extensive systematic review analyzing the long-term neurodevelopment of children conceived through ART concluded that additional data were needed to determine the actual impact of ART on these outcomes [65]. Several factors may explain the complexity in defining the possible neurodevelopment deficits in children conceived through ART. First, ART is associated with higher twin pregnancy rates, increasing the risks for preterm birth, low birth weight, and small for gestational age. These are risk factors for neurodevelopmental deficits [65]. Even when the evaluation only includes singletons, children conceived through ART had higher rates of preterm birth and fetal growth restriction, with an important bias factor for the evaluation of

neurodevelopmental deficits [65]. Another critical factor to consider is the impact of subfertility, regardless of ART, on neurodevelopment differences [65]. Lastly, the use of varying controls and analytical approaches for controlling confounding and mediating variables has to be considered by researchers planning to study the effect of infertility and ART on childhood outcomes [67].

The long-term effects on neurodevelopment in children born through testicular sperm extraction (TESE) and ICSI have been poorly studied; however, current evidence is reassuring regarding the overall development and health of these children. Some evidence suggests an increased relative risk for autistic disorders and mental retardation in children born through TESE and ICSI when compared to those conceived via IVF without ICSI [70]. Nonetheless, the prevalence of these disorders was reported to be small, as were the absolute increase in the risk compared with conventional IVF. These associations need to be evaluated in future studies.

It is worth mentioning that studies evaluating adolescent psychological adjustment have shown reassuring data, indicating that children born through ART had positive parent-adolescent relationships and were well-adjusted [71]. The mental health of young teens, including behavior, relationships with colleagues, and social and emotional functioning, was also evaluated. In contrast to the observations above, a study based on national data assessing academic performance showed that adolescents conceived through ART had significantly lower mean scores when compared to spontaneously conceived adolescents [72]. Nevertheless, the differences were small and were most likely not clinically significant (Table 61.1).

61.2.7 Metabolic and Cardiovascular

The potential health risks associated with ART are of great importance to public health because millions of babies worldwide are born as a result of these procedures. All procedures inherent to ART, including ovarian stimulation, gamete manipulation, and embryonic culture, occur in a critical window for the establishment of genomic methylation patterns. These aspects could increase the risk of cardiometabolic diseases in advanced stages of life.

Several studies have evaluated the long-term cardiovascular and metabolic outcomes in children born through ART [74–79]. Despite a large number of reports, the findings remain preliminary and sometimes inconsistent. Moreover, studies assessing the individual effects of ART by fertilization method have been few. Furthermore, the features associated with subfertility could also play important roles [80]. Women presenting with subfertility tend to be older, more obese, and often more nulliparous than fertile women, possibly explaining why children born with ART have a worse cardiometabolic profile when compared to those of naturally conceived children. Within this context, embryos of obese and overweight women express compromised developmental and metabolic profiles [75]. Oocytes of obese or overweight women are smaller than those of women with normal body mass indexes. These oocytes have lower chances of reaching the blastocyst stage, and those who reach this stage have accelerated development, with fewer cells in the trophectoderm [81]. These embryos also show significant metabolic changes, with decreased glucose consumption, altered amino acid metabolism profiles, and increased triglyceride levels, all of which could have long-term implications for the offspring [75] (Table 61.1).

61.3 Conclusions

Current evidence suggests that children conceived through ICSI, compared to naturally conceived children, have an increased risk of chromosomal abnormalities, particularly those affecting the sex chromosomes. The data suggest that these effects are parental-related rather than method-related. Conflicting reports, however, suggest that the reproductive potential of boys born from ICSI treatment may be impaired. At present, it is unclear whether the risk of cancer is increased overall among ICSI children; however, limited evidence demonstrates that certain cancer types are more common in ICSI children than in their naturally conceived counterparts. The association between a higher risk of congenital disabilities and epigenetic defects among ICSI children remains equivocal, as it does for the reproductive potential of girls born through ICSI. By contrast, psychological and neurodevelopmental levels of ICSI children and young adults are well-preserved, despite a few reports suggesting that children born with the use of testicular sperm extracted from men with nonobstructive azoospermia have a slightly higher risk of autistic disorders and mental retardation than do children born through ART using ejaculated sperm.

61.4 Review Criteria

We performed an extensive search of PubMed and MEDLINE for studies examining the relationship between intracytoplasmic sperm injection and male infertility and children and health. The start and end dates for these searches were January 2006 and February 2017, respectively. We based the overall strategy for study identification and data extraction on the following keywords: (“intracytoplasmic sperm injection [MeSH]” OR ICSI) AND (offspring OR child∗) AND (psychologic∗ OR neurodevelopment) OR (infertil∗) OR (“epigenetic∗ disorders” OR “epigenetic∗ disease” OR “imprint∗ disorders” OR “imprint∗

methylation") OR ("congenital abnormalities"[Mesh] OR "congenital malformation*" OR "birth defect*") OR ("metabolic* profile" OR "cardiometabolic* profile" OR metabolic) OR (cancer OR oncol*), with the filters "humans" and "English language." A total of 860 relevant articles were identified. We excluded data that were produced only for conferences or proceedings of academic meetings, as well as websites and monographs. Citations dated outside the search dates, website citations, and book-chapter citations provided conceptual content only.

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Part VI

Guidelines and Best Practices in Male Infertility and Antioxidants

Best Practice Guidelines for Male Infertility Diagnosis and Management

62

Edward D. Kim and Oliver Benton IV

Key Points

- Infertility is defined as the inability to achieve a natural pregnancy within 1 year in sexually active couple.
- The American Urological Association (AUA), the American Society for Reproductive Medicine (ASRM), and the European Association of Urology (EAU) are three predominant organizations that regularly develop and update guidelines for the diagnosis and management of the infertile male.
- Comprehensive medical history and physical examination with two semen analyses are the essential components of the initial evaluation for the infertile male.
- The AUA and ASRM recommend andrological evaluation if the patient has abnormal findings on initial assessment or one of two semen analyses. The EAU differs in its recommendation, requiring two abnormal semen analyses prior to proceeding with andrological evaluation.
- Differences between accepted WHO semen analysis reference values create potential for discrepancies between patients selected for andrological evaluation. The EAU and ASRM reference the latest criteria published in 2010, while the AUA still cites the 1999 version.

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62.1 Introduction

Classically, infertility is defined the inability to conceive a natural pregnancy within 1 year in a sexually active couple [1]. The American Society for Reproductive Medicine describes infertility as the result of any disease process (an interruption, cessation, or systemic disorder) of the male or female genital tracts that prevents natural conception over a 1-year period or, in females, the inability to maintain a pregnancy to delivery [2]. Recent estimates predict between 8 and 15% of couples are unable to conceive with regular, unprotected intercourse at 12 months [2]. While recent cross-sectional studies within limited populations suggested male infertility rates are around one in ten or 10.1% (CI 9.2–11.1), a recent collaboration by the WHO suggests that numerous confounding factors, variation in geographical fertility rates, and lack of uniformly accepted criteria for infertility make global estimates extremely difficult [3, 4].

Male factor infertility can be due to a number of congenital or acquired urogenital irregularities. Systemic diseases, environmental/lifestyle (e.g., obesity, gonatotoxins, smoking, etc.) erectile dysfunction, genetic abnormalities, variations in scrotal temperature (i.e., varicocele), urogenital tract infections, urogenital trauma, and improper coital habits can all result in some degree of male infertility [5]. Nearly half of all cases fail to determine an identifiable cause for male infertility. In large part, this is due to limited understanding of the intricacies that underlie natural conception and the limited capability of current diagnostic testing to identify abnormalities [6]. The AUA estimates that, despite best management efforts, nearly 5% of couples will remain unable to conceive due to some combination of male or female factor infertility [7]. There are emerging interests into developing new treatments for unexplained male factor infertility. These efforts are largely centered upon stem cell biology and gene therapy, but have yet to transition into guideline-based practice and are typically used empirically after conventional management has failed [8].

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Recent recognition for the need and utility of clinical guidelines to aid practitioners in the assessment of the infertile male has been spurred by increased understanding of the medical complexities that underlie infertility. Standardized diagnosis and treatments have been outlined in these guidelines in order to help improve efficiency. Well-known organizations from around the world have developed guidelines through multidisciplinary collaborations in order to achieve this goal [2, 7, 9, 10]. Of these sources, urologists and practitioners specializing in reproductive medicine commonly utilize three predominant guidelines for the evaluation and treatment of male infertility: (i) American Urological Association (AUA) best practice statements for the evaluation of the infertile male [7], (ii) the ASRM Practice Committee Report on the diagnostic evaluation of the infertile male [2], and (iii) the European Association of Urology (EAU) guidelines on male infertility [9].

While several concurrent collaborations from different organizations have developed expert opinion panels and best practice statements, the previously cited institutions present the most comprehensive and up-to-date guidelines. These organizations utilize multidisciplinary teams using clinical evidence to develop recommendations. These recommendations meet the criteria for "Clinical Practice Guidelines" created by the Institute of Medicine (IOM). The IOM defines clinical practice guidelines as "statements that include recommendations, intended to optimize patient care, that are informed by a systematic review of evidence and an assessment of the benefits and harms of alternative care options" [11]. Guidelines are not intended to be used as a legal agent. They should be employed as a set of principles that provide a template for standardization of care and help to improve diagnostic efficiency while preserving physician autonomy. A combination of physician judgement and guideline-based management is likely most representative of the current standard of care [12].

62.2 AUA Best Practice Statement: Optimal Evaluation of the Infertile Male

The AUA Board of Directors initially created the Male Infertility Best Practice Policy Committee in 1999. This subsequently became a collaborative initiative between the AUA and the ASRM in 2001 with a goal of developing a series of best practice statements in regards to management of male factor infertility. The initial goal of the committee was "to develop recommendations, based on expert opinion, for optimal clinical practices in the diagnosis and treatment of male infertility." In the most recent update entitled "The optimal evaluation of the infertile male: Best practice statement," the AUA Practice Guidelines Committee selected a ten-person panel composed of nine urologists and one research andrologist [7]. The members of the panel were not reimbursed for their contributions and provided disclosures regarding conflicts of interest to the AUA before participating.

In 2015, the AUA released the American Urological Association Clinical Practice Guidelines Development Standard Operating Procedure [13]. This document details the methodology for the formulation of AUA best practice statements and guidelines across all non-oncologic subdisciplines within urology. This is outlined on the AUA website and an unabridged version is available for free download. Initially, topics for guidelines are nominated by either Practice Guidelines Committee members or by AUA members online. Depending on the topic in question, a panel is formed with special attention paid to the particular expertise of the candidate members. As previously stated, these potential panel members cannot have a conflict of interest with the guideline under consideration. The panel then develops the scope of study by setting parameters for exclusion/inclusion criteria and creating research questions to be investigated. An initial literature review is performed and the results of which are subjected to data extraction, analysis, and synthesis prior to the development of an evidence report. At this point, a final literature review is performed and the guidelines are written for peer review [13]. This methodology, adopted in 2015, has yet to be implemented into the development of AUA infertility guidelines as the most recent update was released in 2011.

In the 2011 update of the AUA Best Practice Statement: Optimal Evaluation of the Infertile Male, the panel suggests that initial infertility workup should be performed if natural pregnancy has not occurred by 1 year of regular unprotected vaginal intercourse. Consideration for earlier workup is recommended if the male and/or his female partner have known infertility risk factors. The best practice statement provided in the manuscript recommends the initial evaluation for male infertility includes both a thorough reproductive history with a urogenital physical exam and two properly obtained semen samples. Additional tests should be considered if (i) abnormalities are identified during the initial evaluation, (ii) the etiology of infertility cannot otherwise be identified, and (iii) problems with infertility continue despite appropriate treatment of the female partner. Table 62.1 details the breadth and methodology used in the creation of the AUA guidelines.

62.3 ASRM Guidelines

The ASRM recommendations and best practice statements have undergone multiple revisions since its inception in 2006. Initially presented in conjunction with the AUA as detailed above, the Practice Committee of the ASRM has released updated guidelines and best practice statements in 2012 and again republished in 2015 in Fertility and Sterility [2].

Table 62.1 AUA, ASRM, and EAU: Scope and methods for development of guidelines for the evaluation of the infertile male

	AUA	ASRM	EAU
Guideline title	Optimal evaluation of the infertile male: AUA best practice statement	Diagnostic evaluation of the infertile male: a committee opinion	Guidelines on male infertility
Goal	To offer recommendations for the optimal diagnostic evaluation of the male partner of an infertile couple	To provide clinicians with principles and strategies for the evaluation of couples with male infertility problems	To assist urologists and healthcare professionals from related specialties in the treatment of male infertility
Intended users	Physicians	Physicians	Physicians
Collection and evidence selection	Medline literature review from 1999 through October 2007 was supplemented by hand searches of published literature	Not stated	Literature search using EMBASE, Medline, Cochrane review databases limited to RCT, and meta-analyses spanning at least 3 years. Other high-level evidence and other organizations' high-quality guidelines
Analysis of the evidence	Review of published meta-analyses and systematic reviews	Not stated	Preferred reporting items for systematic reviews and meta-analyses (PRISMA)*
Assessment of quality and strength of evidence	Not stated	Not stated	Modified Oxford Centre for Evidence-Based Medicine – Levels of Evidence approach [aa]
Methods used to formulate the recommendations	Expert consensus	Expert consensus	Expert consensus
Description of methods used to formulate the recommendations	The Practice Guidelines Committee (PCG) chair appoints panel chair based on expertise and leadership ability. The panel chair nominates up to seven additional members. The number of panel members from each institution is limited to two The objective the panel is to develop evidence-based recommendations either by analytical means or group consensus that help guide best practices on management of male infertility	A panel of 16 members drafted the first edition offering consensus and evidence-based recommendations in the context of clinical practice. Ethical and fiscal considerations are included when applicable	A collective panel of actively practicing academic urologists, endocrinologist, and gynecologists with special interest and experience in infertility and andrology. Literature reviewed and quality of evidence assessed using PRISMA* methodology. Grading recommendations are performed via the modified Oxford Centre for Evidence-Based Medicine – Levels of Evidence [a]
Methods for determining strength of evidence	Not stated	Not stated	Modified grading of recommendations assessment, development and evaluation (GRADE)**
Guideline validation technique	External and internal peer review	Proposed documents are circulated among members of the society for review before final approval and publication	External and internal peer review
Guideline algorithm(s)	Not provided	Not provided	Not provided
Implementation strategy	Abridged and unabridged versions of the optimal evaluation of the infertile male are available for viewing or download from the AUA at: https://www.auanet.org/education/guidelines/male-infertility-d.cfm Guidelines and best practice statements from the SSMR are available for viewing or download at: http://www.ssmr.org/professionals/male-infertility-guidelines.aspx	The practice committee of the ASRM published a report in Fertility and Sterility in 2015 and have it on the ASRM website for viewing: http://www.asrm.org/Guidelines/	Annual reprint of the long version of EAU guidelines. Text available on CD with hyperlinks for references (http://www.uroweb.org/guidelines/online-guidelines) Additional condensed pocket versions of the EAU guidelines reprinted each year available for free to all EAU members. Abridged versions published in European urology as original papers. Guidelines, in all mediums of content, are published and presented in 25 different languages

(continued)

Table 62.1 (continued)

	AUA	ASRM	EAU
Cost analysis provided	No	No	Yes
Publication history	Initial release in 2001 in collaboration with the practice committee of the ASRM. Revised in 2010 with confirmation of validity in 2011	Since its first edition in collaboration with the AUA in 2001, the practice committee has provided updates in 2006 and 2012. The report was most recently published in Fertility and Sterility in 2015	First published in 2001, followed by full-text updates in 2004, 2007, 2010, 2014, and 2015
Where guidelines can be found	Available for viewing or download at AUAnet.org: https://www.auanet.org/common/pdf/education/clinical-guidance/Male-Infertility-d.pdf	Available for viewing and download by ASRM members or view-only for nonmembers (http://www.asrm.org/Guidelines/)	Available for online viewing or download at EAU society website: http://www.uroweb.org/guidelines/online-guidelines/
Date released	2001 April (revised 2010; reviewed and validity confirmed 2011)	2012 June	2010 April (revised 2013 March)

RCT randomized controlled trials

[a]Preferred Reporting Items for Systemic Reviews and Meta-Analyses (PRISMA) is an evidenced-based minimum criteria for evaluating and performing systematic reviews and meta-analyses. This particular method is aimed at the reporting of RCTs, but can be utilized for reporting systematic reviews of other evidence [14]

[b]The Modified Grading of Recommendations Assessment, Development, and Evaluation (GRADE) approach is a method adopted by the EAU to assess the quality of evidence included in guidelines. This helps to provide a structured approach to defining a grading system [15]

[c]Levels of Evidence

1a: Evidence obtained from meta-analysis of randomized trials

1b: Evidence obtained from at least one randomized trial

2b: Evidence obtained from one well-designed controlled study without randomization

2b: Evidence obtained from at least one other type of well-designed quasi-experimental study

3: Evidence obtained from well-designed nonexperimental studies, such as comparative studies, correlation studies, and case reports

4: Evidence obtained from expert committee reports or opinions or clinical experience of respected authorities

[d]Rating scheme for the grade of recommendations

Grade A: Based on clinical studies of good quality and consistency addressing the specific recommendations and including at least one randomized trial

Grade B: Based on well-conducted clinical studies, but without randomized clinical trials

Grade C: Made despite the absence of directly applicable clinical studies of good quality

This committee was composed of 125 physicians and basic science researchers from the fields of urology, reproductive andrology, gynecology, family medicine and primary care, andrology, and reproductive medicine. The 2012 revision entitled "Diagnostic evaluation of the infertile male: a committee opinion" has garnered the approval of the Board of Directors of the AUA and the ASRM. The stated goal of the Practice Committee's report is "to provide clinicians with principles and strategies for the evaluation of couples with male infertility problems" [2]. This document suggests that it stands to serve as an adjunct to clinical care stating, "although this document reflects appropriate management of a problem encountered in the practice of reproductive medicine, it is not intended to be the only approved standard of practice or to dictate an exclusive course of treatment. Other plans of management may be appropriate, taking into account the needs of the individual patient, available resources, and institutional or clinical practice limitations" [2]. An itemized summary of the breadth and methodology used to develop the ASRM guidelines can be found in Table 62.1. A comparison of AUA and ASRM guidelines and major recommendations can be found in Table 62.2.

Table 62.2 AUA (2011) and ASRM (2012) guidelines

	AUA	ASRM
Goals for evaluation	Initial screening for male infertility should be performed if pregnancy has not occurred within 1 year of regular and unprotected intercourse. Evaluations before the 1-year threshold may be considered in certain circumstances (i.e., history of bilateral cryptorchidism or advanced female age). A full evaluation should be performed by a urologist or other reproductive specialist when initial screening an abnormal semen analysis or medical history. A full evaluation may also be considered in cases of persistent infertility despite diagnosis and treatment of female factor	Evaluation for infertility is indicated for couples who fail to achieve a successful pregnancy after 12 months or more of regular unprotected intercourse. Earlier evaluation and treatment may be considered, based on medical history and physical findings, and is warranted after 6 months or more in couples with females greater 35 years of age. Men having concerns about their future fertility also merit evaluation. At a minimum, the initial screening evaluation should include reproductive history and analysis of at least one semen sample
Components of a full evaluation	A full evaluation of the infertile male should start with a comprehensive medical and reproductive history and physical examination performed by a urologist or reproductive specialist. This should be accompanied by at least two semen analyses. These samples should be produced at least one month apart. Ideally, an "abnormal" sample should have at least two abnormal semen parameters prior to proceeding with a full evaluation. Additional components of the full evaluation (detailed below) should be employed at the discretion of the urologist or reproductive specialist to help elucidate the etiology of infertility	When an initial evaluation elicits an abnormal history or abnormal parameters on semen analysis, a more detailed evaluation should be considered. This should be performed by a urologist or other male reproductive specialist. The full evaluation should include the medical history, physical exam, and semen analysis obtained in the initial screening in addition to a variety of diagnostic tests and procedures (detailed below) to be utilized at the discretion of the healthcare professional
Endocrine evaluation	Endocrine evaluations should include at least a morning serum testosterone and FSH. This evaluation is encouraged for abnormal semen analysis (especially when sperm concentration is <ten million/ml), when sexual function is impaired, or other clinical findings suggest underlying endocrinopathy (e.g., hyperprolactinemia)	Endocrine evaluation should be considered in men having (1) abnormal semen parameters, especially with sperm concentrations below 10 million/mL, (2) impaired sexual function, or (3) clinical findings that suggest an endocrinopathy. At minimum, it should include a measurement of serum testosterone and FSH concentrations. When T level is low (<300 ng/mL), a second early morning total T level with serum free testosterone (T), LH, and prolactin should be obtained. Inhibin B has been shown to correlate better with sperm parameters. However, due to cost of measuring inhibin B, FSH should be utilized first
Post-ejaculatory urinalysis	Post-ejaculatory urinalysis should be considered when absent or low volume (<1 ml). This test should *not* be performed in those patients with diagnosed CBAVD or clinical signs of hypogonadism	Post-ejaculatory urinalysis is indicated in men having an ejaculate volume less than 1 mL, except in those diagnosed with hypogonadism or CBAVD
Transrectal ultrasonography	Transrectal ultrasonography should be considered in azoospermic patients with palpable bilateral vasa and low ejaculate volumes. Seminal vesicles measuring greater than 2.0 cm in anteroposterior diameter should raise concern for ejaculatory duct obstruction	TRUS is indicated in low-volume, acidic azoospermia or in samples without fructose. Seminal vesicles measuring greater than 1.5 cm in anteroposterior diameter should raise concern for complete or partial ejaculatory duct obstruction
Scrotal ultrasonography	Scrotal ultrasonography should be employed when clinical examination of scrotal structures is difficult or when a testicular mass is suspected	Scrotal ultrasonography can be considered when careful physical examination is unable to identify structures or pathology

(continued)

Table 62.2 (continued)

	AUA	ASRM
Strict sperm morphology	Sperm morphology using strict criteria has not been shown to reliably predict fertility. It should not be utilized as the sole diagnostic test to guide therapeutic decisions	No specific recommendation presented
DNA integrity	Insufficient evidence in literature to support the routine application of DNA integrity testing in the full evaluation of the infertile male. Furthermore, no proven therapies have been developed to treat abnormal tests	Sperm DNA damage is more common in infertile men and may contribute to infertility. However, data regarding reproductive outcomes and DNA integrity is too limited to routinely recommend testing of the male partner
Reactive oxygen species (ROS)	ROS have not been shown to be predictive of fertility. Insufficient evidence exists to support the routine use of ROS testing in infertility evaluations. Furthermore, presently no proven medical or surgical interventions exist to treat ROS in semen samples	No specific recommendation provided
Specialized tests	*Quantitation of leukocytes* Patients with true pyospermia (greater than one million leukocytes per ml) should be evaluated for genital tract infection	*Quantification of leukocytes* Men with true pyospermia (> one million WBCs/mL) should be evaluated for genital tract infection or inflammation
	Antisperm antibody assay Should be considered in cases of isolated asthenospermia with otherwise normal semen parameters	*Antisperm antibody assay* Routine testing not indicated. Should not be performed when ICSI is planned
	Sperm viability test May be utilized in cases with viable, nonmotile sperm in consideration for ICSI	*Sperm viability test* Can be utilized to assess whether nonmotile sperm would be viable for ICSI
	Sperm-cervical mucus interaction Subject to variable interpretation and often negated by the use of assisted reproductive technology	*Sperm penetration assay* May be beneficial for evaluating ICSI candidates, but often superseded due the routine use of ICSI in IVF
	Zona-free hamster oocyte test Sperm penetration assay (SPA) should be reserved for patients in whom abnormal tests will direct therapeutic decisions. Subject to variable interpretation	*Sperm chromosome aneuploidy* Sperm with severely abnormal morphology, men with karyotypic abnormalities, or nonobstructive azoospermia may benefit from sperm aneuploidy testing. However, testing is cost-limiting and identifying sperm to be used in ICSI is difficult. It is not routinely recommended
	Computer-aided sperm analysis (CASA) Useful for assessing motility and motion parameters. Not routinely used	
	⁎not required for diagnosis of male infertility. May aid in selecting therapy in specific circumstances	
Genetic screening and testing	Congenital bilateral absence of vasa deferentia should warrant cystic fibrosis transmembrane conductance regulator (CFTR) mutation testing. If positive, female partners should be offered CFTR testing prior to assisted reproductive efforts to harvest sperm. Conversely, unilateral absence of vasa deferentia should be followed up with renal imaging. CFTR evaluation should, at minimum, test common point mutations associated with cystic fibrosis and the 5 T allele Gene sequencing may be considered in couples where the wife is a carrier and the husband with CBAVD tests negative for the routine CFTR panel Karyotyping and genetic counseling should be offered to all patients with nonobstructive azoospermia and severe oligospermia (<five million/ml) Insufficient data available to recommend a minimum number of sequence tagged sites to test for patients undergoing Y chromosome microdeletion analysis. Patients with large deletions involving azoospermia factor (AZF) region a or b often have a poor prognosis. However, this result cannot reliably exclude the presence of viable sperm	Men with nonobstructive azoospermia or severe oligozoospermia (<five million/mL) should be evaluated for genetic abnormalities Testing for chromosome 7 CFTR gene mutations should be considered in cases of CBAVD. Patients with unilateral absence of vasa deferentia should be offered renal imaging and it is not recommended that they be tested for CFTR mutations Karyotype testing for chromosomal abnormalities should be employed in men with nonobstructive azoospermia or severe oligozoospermia when getting evaluated for ICSI Y chromosome microdeletions, also known as azoospermia factor (AZF) regions, can have proximal, central, or distal regional mutations. Distal mutations (AZFc) have the only potential for fecundity using IVF. AZF should therefore be tested in men with nonobstructive azoospermia or severe oligozoospermia before performing ICSI

CLIA Clinical Laboratory Improvement Amendments, *CBAVD* congenital bilateral agenesis of the vas deferens, *TRUS* transrectal ultrasound, *ICSI* intracytoplasmic sperm injection

62.4 European Association of Urology Guidelines

The European Association of Urology (EAU) guidelines office (given this title in 2004 after its conception in 1996) was challenged with the task of developing European clinical urological guidelines [16]. This panel, consisting predominantly of urologists, gynecologists, and reproductive endocrinologists, created the "EAU Male Infertility Guidelines." Since its initial release in 2001, these guidelines have undergone regular updates with the most recent edition published as a full-text update in 2015 [9]. While many non-urologic medical practitioners commonly utilize these guidelines. The EAU has made it their focus to create a resource for urologists. The respective members of the panel (all of which were members of the EAU) were required to submit nondisclosure statements and inform the EAU of any potential conflicts of interest prior to participating in the development of guidelines. Panel members were considered on the basis of their scientific and clinical merits and their willingness to commit considerable amounts of time to produce well-founded and thorough guidelines. Each member's commitment is for a 4-year term which may be renewed for one additional term. The panel is led by an EAU guidelines office appointment chairman. In an interest to keep the focus of these guidelines within the field of urology, the chairman appointed is always a board-certified and full-time urologist. Once the panel has formulated a preliminary guideline, new edition, or best practice statement, a minimum of 3–4 reviewers are asked to provide an assessment and formal review of the document submitted. These reviewers may or may not be associated with the EAU and receive no monetary compensation [9]. As of the last update in 2015, the EAU significantly reduced the volume of text in non-oncology guidelines and standardized formatting for ease of use [9].

Development of evidence-based recommendations has long been an emphasis of the committee. This is due to the fact that the EAU clinical guidelines are predominantly intended to enhance the practitioner's clinical decision-making. In accordance with this goal, the development of incremental levels of evidence and the associated grades for each recommendation helps quantify each recommendation based on the quality of underlying evidence. This helps to preserve physician autonomy and allows clinicians to gauge how strictly they adhere to each individual recommendation [9, 16]. Table 62.1 provides a summary of the scope and methods used by the committee to formulate the EAU guidelines.

In creating new guidelines or new editions of current guidelines, the panel gathers and appraises evidence from current literature. In the 2015 update, a total of 409 unique records were initially collected from an extensive literature review and screened for validity and relevance. Of these, nine publications were selected for inclusion into the formulation of new recommendations [16]. This information gets formulated into a series of statements. The statements are summarized as recommendations and presented along with their associated levels of evidence. The strength of each recommendation is graded (grade of recommendation = GR) depending upon the quality of underlying evidence (level of evidence = LE) (Appendix 1). The GR does not always follow a linear relationship with LE. This is due, in large part, to the variability of study design, limitations in methodology, and/or disparity in available data on a given recommendation. The inverse is also true. Statements without high-level evidence may receive high-grade recommendations if dictated by overwhelming clinical experience and/or general consensus. These instances are typically documented in the text as "upgraded based on panel consensus" [9]. A comprehensive evaluation of each recommendation is performed after a grade is assigned to ensure that each statement, while supported by underlying scientific evidence or group consensus, is equitable with value, preference, and costs. As of the 2018 update, the EUA reported using a modified GRADE methodology, a structured approach in assessing the evidence used in formulating recommendations [15, 17]. This essentially aims to eliminate the ambiguity of a grade A, B, or C recommendation and recategorizes the statements as either "strong" or "weak" recommendations [16]. Additionally, meta-analyses are only utilized as part of a systematic review if multiple randomized control trials address the same question and the outcomes are reported in a similar manner. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidance is followed in these instances [18].

The clinical practice guidelines supplied by the EAU address 13 different topics within male infertility. These include epidemiology and etiology, disorders of ejaculation, testicular dysfunction, varicocele, obstructive azoospermia, genetic disorders, germ cell malignancy with testicular microcalcification, and semen cryopreservation. Table 62.3 provides selected recommendations from the EAU that are aimed at helping the clinician evaluate and manage male factor infertility. Many national urological associations have filed formal replies to incorporate EAU guidelines into their respective guidelines. Over 50 national societies from around the world have submitted endorsements of EAU guidelines [16].

Table 62.3 EAU (2018) guideline recommendations on the evaluation of the infertile male

Area	Recommendation	Grade of Recommendation
Epidemiology and etiology	Both partners should be evaluated simultaneously to characterize infertility	Strong
	Men who are diagnosed with infertility or with abnormal semen parameters should be examined	Strong
Diagnostic evaluation of the infertile male	Female partner fertility status should be included in the evaluation and treatment of subfertile males as this may affect fertility outcomes	Strong
	Semen analyses should be performed in accordance with guidelines from *WHO Laboratory Manual for Examination and Processing of Human Semen (fifth ed)*	Strong
	Full andrological assessment should be reserved for patients with at least *two* abnormal semen analyses	Strong
	Adherence to the 2000 WHO manual for the standard evaluation, diagnosis, and management of the subfertile male	Weak
Primary testicular deficiency	Even with a negative genetic panel, men who are undergoing sperm retrieval should be given appropriate genetic counseling	Strong
	Testicular biopsies (TESE or micro-TESE) should be performed in men with nonobstructive azoospermia. This can aid in determining degree of spermatogenesis, cryopreserve sperm, and diagnose germ cell neoplasia in situ	Strong
Genetic disorders and male infertility	Karyotype analysis should be considered in all men with impaired spermatogenesis (spermatozoa <10 million/mL). This should be performed for diagnostic purposes	Strong
	Genetic counselling should be provided for all couples when genetic abnormalities are elicited on clinical or genetic evaluation and in patients who may be a carrier for an inheritable disease process	Strong
	Patients with Klinefelter's syndrome should be provided with long-term endocrine follow-up and appropriate medical treatments, when appropriate	Strong
	Microdeletion testing in men with obstructive azoospermia (OA) should not be performed as spermatogenesis is often unaffected	Strong
	Patients with Yq microdeletions wishing to attempt intracytoplasmic sperm injection (ICSI) should be informed that microdeletions will be passed to male offspring, but not female	Strong
	Patients with structural abnormalities of the vasa deferentia should be tested along with their partner for cystic fibrosis transmembrane conductance regulator (CTFR) gene mutations	Strong
Obstructive azoospermia (OA)	Microsurgical vasovasostomy or tubovasectomy should be performed for OA secondary to epididymal or vasal obstruction	Strong
	Sperm retrieval techniques (i.e., microsurgical epididymal sperm aspiration, testicular sperm extraction, and percutaneous epididymal sperm aspiration) should be performed only when cryopreservation facilities are available	Strong
Varicocele	Adolescents with a varicocele and physical findings revealing ipsilateral testicular volume loss or other signs of testicular dysfunction should be treated	Weak
	Subclinical varicoceles and infertile men with normal semen analysis should not be treated	Strong
	Men with clinical varicoceles, findings of oligospermia on semen analysis, and otherwise unexplained infertility should be treated	Weak
Hypogonadism	Symptomatic patients with primary or secondary hypogonadism who are not considering fertility should be offered testosterone replacement therapy	Strong
	Men diagnosed with hypogonadotropic hypogonadism should be offered effective drug therapy (human chorionic gonadotropin, human menopausal gonadotropins, recombinant follicle-stimulating hormone, highly purified FSH).	Strong
	Testosterone replacement therapy should not be used to treat infertility	Strong
Cryptorchidism	Hormonal therapy should not be used to treat cryptorchidism in adults	Strong
	Simultaneous testicular biopsy should be performed for detection of intratubular germ cell neoplasia in situ in adult patients undergoing correction for undescended testes	Weak
Idiopathic male infertility	Patients with hypogonadotropic hypogonadism should be offered medical treatment	Strong
	The use of gonadotropins, antioxidants, and anti-estrogens lacks sufficient evidence to provide sound recommendations	Strong
Male contraception	Use cauterization and fascial interposition during vasectomy have been proven to be most effective techniques in preventing recanalization postprocedure	Strong
	Patients pursuing vasectomy should be informed about the surgical technique, risk of failure, possible irreversibility, the necessity for contraception after the procedure until clearance, and the risk of potential complications	Strong
	Microsurgical epididymal sperm aspiration, percutaneous epididymal sperm aspiration, or testicular sperm extraction utilized in conjunction with intracytoplasmic sperm injection can be used as a second-line option for men who decline vasectomy reversal and those who failed vasectomy reversal surgery in order to achieve pregnancy	Weak
Male accessory gland infections	Provide instruction for patients with epididymitis secondary to proven or suspected *N. gonorrhoeae* or *C. trachomatis* infections. Refer sexual partners for evaluation and treatment	Strong

Table 62.3 (continued)

Area	Recommendation	Grade of Recommendation
Germ cell malignancy and testicular microcalcification	Men with evidence of testicular microcalcification (TM) should be encouraged to perform self-examination for early detection of testicular germ cell tumor (TGCT)	Weak
	Testicular biopsy, follow-up scrotal ultrasound, biochemical tumor markers, or abdominal/pelvic CT imaging should not be used in men with isolated TM without associated risk factors (i.e., infertility, cryptorchidism, testicular cancer, and atrophic testis)	Strong
	Testicular biopsy should be considered in men with testicular microcalcification (TM) who belong to one of the following high-risk groups: Bilateral ™, atrophic testes (less than 12 cc), history of undescended testes or TGCT	Strong
	Concerning findings on physical examination or sonographic evaluation in patients with TM or associated lesions should be followed with surgical exploration consisting of testicular biopsy and possible orchiectomy	Strong
	Men with TGCT should be followed for increased risk of hypogonadism and/or sexual dysfunction	Strong
Disorders of ejaculation	Specific treatments for ejaculatory disorders should be offered before performing sperm collection and assisted reproduction technique (ART). Short-acting SSRIs such as dapoxetine with or without topical anesthetics for premature ejaculation	Strong
Semen cryopreservation	Cryopreservation should be offered to men who are scheduled to undergo chemotherapy, radiation, or surgery that may interfere with spermatogenesis or cause ejaculatory dysfunction	Strong
	Sperm cryopreservation should be offered if testicular biopsies are performed for fertility indications	Strong
	If cryopreservation is not available locally, inform patients about the possibility of visiting or transferring to a cryopreservation unit before therapy starts	Strong
	Take precautions to prevent transmission of viral, sexually transmitted or any other infection by cryostored materials from donor to recipient and to prevent contamination of stored samples. These precautions include testing of the patient and the use of rapid testing and quarantine of samples until test results are known. Do not store samples from men who are positive for hepatitis virus or HIV in the same container as samples from men who have been tested and are free from infection	Strong

62.5 An Assessment of the Guidelines for the Evaluation of the Infertile Male

Given the AUA's and ASRM's history of collaboration, it is not surprising that many of the guidelines and best practice statements overlap. In fact, the first editions from each organization produced in 2001 were developed by the AUA's Male Infertility Best Practice Policy Committee in concordance with the Practice Committee of the ASRM [19]. These documents were subsequently reviewed and updated with AUA revisions in 2010/2011 and ASRM revisions in 2006/2012. These documents do differ in varying capacities from the ones provided by the EAU [9].

While many similarities exist between the AUA/ASRM and EAU guidelines, there are some notable discordances. For instance, the AUA/ASRM guidelines recommend a minimum initial evaluation of the infertile male including a medical/surgical history and semen analysis [2, 7]. The EAU guidelines opt not to specify a minimum initial workup. It makes mention that history and physical exam are "standard assessments" in all patients and that a semen analysis should be included [9]. AUA and ASRM documents suggest that a full evaluation must be done by a urologist or other reproductive specialists when an initial evaluation reveals an abnormal semen analysis or the clinical history/findings are suggestive of endocrinopathy. On the contrary, the EAU guidelines state a complete andrological evaluation should only be performed if a minimum of two semen analyses are abnormal per WHO criteria [20]. This implies that normal semen analyses exclude dysfunctional sperm as the etiology for infertility, while many patients with unexplained infertility have normal semen characteristics. Unexplained infertility occurs when female factors of infertility have been excluded and the male has no identifiable cause on history, physical examination, and semen analysis [6]. The reported prevalence of unexplained infertility is highly variable (between 6 and 30%) and dependent on diagnostic criteria and population demographics [5, 6, 21–23].

Despite the aforementioned discrepancies between guidelines, all three committees clearly place an emphasis on the diagnostic importance of the traditional semen analysis. In all three guidelines, an abnormal semen analysis (two in the EAU guidelines) is required before a full andrological evaluation can be performed. The latest guidelines from the EAU and ASRM consider the updated 2010 WHO [20] semen analysis criteria, while the AUA guidelines still adhere to the version published in 1999 [24]. This discrepancy can have major clinical implications as the lower reference ranges for normal semen parameters in the updated 2010 version may exclude many patients from further evaluation. Up to 15% of men with at least one abnormal parameter in the 1999 WHO criteria were reclassified within normal limits in the 2010 WHO criteria in comparison study [24, 25]. Another study

with similar methodology found that upwards of 19% of men were reclassified as "normal" after having at least one abnormal semen analysis on the 1999 WHO criteria [26]. While many men who were originally eligible for a full evaluation may be excluded with the adoption of new criteria, an argument can be made that the new reference values provide a more accurate representation of natural variance. This may provide a more cost-effective parameter to eliminate unnecessary evaluations and will certainly be a topic for further research going forward.

Regardless of reference values and guideline specifics, it is clear that all three associations place a significant emphasis on the diagnostic value of the conventional semen analysis. This calls into question the validity of the test as a marker for male infertility [27]. Semen parameters aimed to delineate between fertile and infertile males are not always well defined and only ~40% of infertile men fall within the accepted reference ranges [28–30]. While inherent natural variability among semen samples does exist, confounding factors like diagnostic errors, the functionality of accessory sex organs, and ejaculatory abstinence do exist and should not be ignored [31–35]. Recent evidence has suggested that variability can exist both within individuals and particular laboratories performing the semen analysis. One study comparing intra-facility variation in semen analysis suggested that the highest variability in measurements were seen with morphology (coefficient variability above 80%) and count (coefficient variability greater than 60%) [36]. Another component of this study suggested that standardizing training for evaluating specific semen parameters only showed subsequent improvement with morphology. Another study assessing intraindividual variability using healthy participants over a 10-week interval showed the highest variation among sperm concentration (26.8%), then morphology (19.6%), and progressive motility (15.2%) [32]. The lowest variability was seen among assessments for vitality (10.3%).

The utility of parameters formed from population means and analysis of semen characteristics is largely linked to the individual variability within each characteristic. Reference values for those semen characteristics with significant variability may offer limited clinical value [37, 38]. Analysis of semen from donors for artificial insemination showed regression towards the mean when selecting those samples with abnormal characteristics in the first test. This result was amplified when repeated in a second test [37]. Assessing multiple samples from each individual helps account for variability within each characteristic and, ultimately, increases the accuracy of the parameter [38]. While this has a limited effect in preventing regression towards the mean, the averages from multiple samples help reduce its magnitude.

Therefore, it stands to question the legitimacy of a single "normal" semen analysis, as suggested by guidelines from both the AUA and the ASRM. A recent retrospective review using 2010 WHO criteria analyzed 5132 semen samples from 2566 patients who had provided at least two semen samples and found that 51.2% of second analyses confirmed the first [39]. When initial samples were found to be "normal," roughly 27% of second samples were found to be pathological. Conversely, when an initial sample was found to be abnormal, 23% of the second samples were found to be normal. Even with a "normal" semen analysis, many men remain infertile for reasons not explained by conventional semen characteristics and parameters. Intrinsic sperm dysfunction seen in DNA damage or immature chromatin has been described in roughly 30% of males with "unexplained infertility." These men's sperm dysfunction can only be explained by functional sperm evaluations (oxidative stress, DNA/chromatin integrity, and antisperm antibody assays) [40–42]. While the use of semen analysis does have certain limitations, the AUA and ASRM guidelines do suggest that further workup for male factor should be considered in cases when unexplained infertility persists and female factors have been ruled out or treated.

While addressing the application and parameters of the semen analysis, all three guidelines emphasize the importance of obtaining a properly performed analysis. Institutional quality control standards from the WHO [20] or the Clinical Laboratory Improvement Amendments (CLIA) [43] have been adopted by all three guidelines. However, existing data from surveys of laboratory practice indicate that semen analyses are still poorly standardized. The need for global standardization among laboratories has been well documented [44–48]. A clinician should have reasonable confidence in the accuracy and reproducibility of the semen analysis given its clinical value in evaluating the infertile male.

Beyond varying interpretations of the conventional semen analysis, discrepancies between AUA/ASRM and EAU guidelines persist in regard to what defines a "full evaluation." AUA/ASRM guidelines provide detailed descriptions of the components of the evaluation including when further procedures or invasive tests should be utilized. These include diagnostics like post-ejaculatory urinalysis, transrectal/scrotal ultrasound, sperm function tests, genetic testing, and endocrine evaluations (Table 62.2). Conversely, EAU guidelines refer to WHO manual for the standardized investigation, diagnosis, and management of the infertile couple (Box 62.1 and Table 62.4). This manual, first developed in 1993 and revised in 2000, aimed to provide detailed guides for medical history, physical examination techniques, and laboratory tests [1]. While it was reliable and accurate at the time, many argue that this manual is in need of revision to reflect significant advancements in technology and understanding over the last 18 years.

Table 62.4 Lower reference limits (fifth centiles and their 95% CIs) for semen characteristics

Parameter	Lower reference limit (range)
Semen volume (mL)	1.5 (1.4–1.7)
Total sperm number (106/ejaculate)	39 (33–46)
Sperm concentration (106/mL)	15 (12–16)
Total motility (PR + NP)	40 (38–42)
Progressive motility (PR, %)	32 (31–34)
Vitality (live spermatozoa, %)	58 (55–63)
Sperm morphology (normal forms, %)	4 (3.0–4.0)
Other consensus threshold values	
pH	>7.2
Peroxidase-positive leukocytes (106/mL)	<1.0
Optional investigations	
MAR test (motile spermatozoa with bound particles, %)	<50
Immunobead test (motile spermatozoa with bound beads, %)	<50
Seminal zinc (μmol/ejaculate)	≥2.4
Seminal fructose (μmol/ejaculate)	≥13
Seminal neutral glucosidase (mU/ejaculate)	≤20

Box 62.1. WHO recommendation: semen analysis

Standard evaluation in all men should include a medical history and physical exam in addition to scrotal ultrasonography and semen analysis. Andrological evaluation is should be performed when semen analysis demonstrates abnormalities when compared to reference values (Table 62.4). Standardization of laboratory reference values helps guide important treatment decisions. The WHO has provided the WHO laboratory manual for the examination and processing of human semen (fifth edn.). It is the consensus that modern spermatology must abide by these reference values (per EAU recommendations)

While many of the recommendations from both the AUA/ASRM and EAU are evidence-based, some of the guidelines are still supported by nonrandomized clinical trials, retrospective studies, and expert opinion (Table 62.3). The aforementioned GRADE methodology adopted by the EAU has attempted to address this by delineating between those guidelines with and without quality supporting data. The assigned "strong" or "weak" GR intends to simplify the grading system, yet it requires inherently subjective evaluation of the recommendation using a template of principles [15, 16]. Conversely, the AUA Practice Guidelines Committee found insufficient evidence to develop a formal evidence-based guideline, stating that the majority of recommendations are derived from nonrandomized trials, expert opinion, or some combination of the two [7]. This certainly leaves the opportunity for further research and improvement going forward.

62.6 Conclusion

The goal of guidelines is to provide urologists and other reproductive specialists with a reference to help improve quality and efficiency of care while protecting the patient from potentially harmful or unnecessary interventions. Of the many sources available, the most commonly referenced and up-to-date guidelines are the AUA best practice statement for the evaluation of the infertile male, the ASRM Practice Committee Report on the diagnostic evaluation of the infertile male, and the EAU guidelines on male infertility.

While these guidelines are intended to help guide the practitioner in clinical practice, variable methodology used to develop the recommendations can alter both the strength and quality of the statements provided. Of the three associations detailed in this chapter, only the EAU has committed to developing evidence-based grades for recommendations given. However, the evidence cited is often based on nonrandomized clinical trials, expert opinion, and retrospective studies. This certainly offers opportunity for further research into various areas within male infertility and for the development of higher-quality recommendations.

Despite the aforementioned differences, the AUA, ASRM, and EAU guidelines recommend similar initial evaluations for male infertility. This starts with a thorough medical/surgical history and a properly executed semen analysis. If initial screening yields abnormal medical history or semen analysis (two abnormal semen analyses in EAU guidelines), a full evaluation may be considered. Ultimately, these guidelines act as a reference. A physician's clinical judgment should always be incorporated into the implementation of these guidelines in order to provide optimal care on a case-by-case basis.

62.7 Review Criteria

A systematic search of the most current and updated guidelines on the diagnosis and management of male infertility was performed for the American Urological Association (AUA), the American Society for Reproductive Medicine (ASRM), the European Association of Urology (EAU), and the World Health Organization (WHO) [semen analysis parameters] as provided on their respective web addresses. Extensive searches of the most recent relevant studies using search engines such as PubMed, Google Scholar, CINAHL Complete, and Cochrane Library were performed between September 2018 and December 2018 with the following

keywords: "male infertility," "infertility rate," "semen analysis," "semen analysis parameters," "infertility diagnosis," and "infertility guidelines." Articles published in languages other than English were not considered. Data published for presentations, conferences, meetings, books, or websites were not included. Book chapters and specific websites were cited to help provide contextual content for discussion.

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Best Practice Guidelines for Sperm DNA Fragmentation Testing

63

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Key Points

- Sperm DNA is a vital component of human conception, so high levels of sperm DNA fragmentation may affect various markers of conception including embryo quality and blastocyst development.
- Several methods have been utilized to measure sperm DNA damage, the most common of which include the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, the sperm chromatin dispersion (SCD) test, the single cell gel electrophoresis (SCGE), and the sperm chromatin structure assay (SCSA).
- Sperm DNA fragmentation test is found to be most valuable during the evaluation of patient's recurrent spontaneous abortion, unexplained infertility, varicocele, and assisted reproductive therapy (ART) failure and patients with lifestyle risk factors.
- Conservative measures, antioxidant therapy, varicocelectomy, sperm selection strategies, and the use of testicular sperm for intracytoplasmic sperm injection are examples of treatment modalities that can be used for patients with high sperm DNA fragmentation.

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63.1 Introduction

Infertility is recognized by the World Health Organization (WHO) as a global public health issue [1]. It is defined as failure of a couple to conceive after 12 months of regular unprotected intercourse in women <35 years of age and after 6 months of regular unprotected intercourse in women ≥35 years old [2]. Male infertility is an important issue in the field of human reproduction. It is responsible for approximately half of all infertility cases [2]. The prevalence of male factor-associated infertility is increasing with almost 50% of all cases of fertility treatments being linked to this factor [2].

The conventional semen analysis test measures basic sperm parameters such as count, motility, and morphology. Although these measures are considered to be the cornerstone of male fertility evaluation, they may fail to provide a complete understanding of fertility potential [3]. In fact, nearly 15% of infertile men may have normal sperm parameters suggesting that other factors may be contributing to their infertility [4]. The sperm DNA integrity has been most commonly accused in this regard as it is essential for normal fertilization, implantation, pregnancy, and fetal development [5]. As a consequence, tests of sperm DNA fragmentation (SDF) have been utilized in clinical practice to unravel the significance of male factors to couple's infertility and to aid treatment decisions in a number of situations [6].

Sperm DNA is a vital component of human conception, so high levels of SDF may affect various markers of conception including embryo quality and blastocyst development, by which it makes pregnancy more difficult to be achieved [6, 7].

Several factors can alter the sperm DNA, such as errors in chromatin packaging and remodeling, a seminal oxidative stress state, and other apoptotic events taking place in the epididymis [8]. Recently, oxidative stress was recognized as an important factor that could affect sperm DNA [9]. It results from an imbalance between ROS and the protective antioxidant molecules and elicits detrimental effects such as sperm lipid peroxidation and SDF [10]. Up to 80% of patients

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with infertility were found to have elevated ROS levels suggesting that oxidative stress may in fact be an intermediary state between various disease conditions and altered reproduction [10].

SDF testing has been acknowledged as a valuable apparatus for the evaluation of infertile men. Its utility for male fertility evaluation has been indicated in recurrent spontaneous abortion, unexplained infertility, varicocele, assisted reproductive therapy (ART) failure, and patients with lifestyle risk factors [11]. This chapter aims to review the evidence surrounding the utility of SDF testing in clinical practice.

63.2 SDF Testing Methods

Several methods have been utilized to measure sperm DNA damage whether directly through utilizing probes and dyes or indirectly through measuring the DNA vulnerability to denaturation [12]. Each method uses a different procedure and results in a different measurement interpretation. The most commonly utilized SDF testing methods (Table 63.1) include:

63.2.1 The TUNEL Assay

The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay detects strand breaks in sperm DNA that are induced by oxidative stress (ROS) and abortive apoptosis [13].

During apoptosis, a group of enzymes called caspases induce cell death by activating other enzymes called nucleases [14]. These nucleases induce the fragmentation of nuclear DNA leaving free 3′ ends of the fragmented DNA. The TUNEL assay uses an enzyme, end terminal deoxynucleotidyl transferase (TdT), which catalyzes the binding of labeled nucleotides to the 3-hydroxyl terminal of fragmented damaged DNA. Flow cytometry or fluorescence microscopy can be used to quantify the signal generated from this enzymatic reaction [15].

63.2.2 The Comet Assay

The comet assay, also known as the single cell gel electrophoresis (SCGE), is a sensitive and rapid technique that quantifies and analyzes the DNA damage in cells [16]. In this method, sperm cells are embedded in agarose gel on a microscope slide. These cells are lysed by detergent allowing the DNA to set free. Then, electrophoresis in a neutral or alkaline medium is carried and fluorescence is applied. The damaged DNA migrates away from the nucleus and from the undamaged DNA, creating a shape of a comet (hence the name of the assay) [17]. The comets are formed as the negatively charged ends of the broken DNA migrate freely and at a faster rate than undamaged DNA toward the anode [17]. The head of the comet represents the undamaged DNA fragments, whereas the tail of the comet represents the damaged DNA fragments. There is a proportional relationship between the number of DNA in the tail and the amount of damage in the DNA.

Table 63.1 Comparison between the different SDF testing methods

Test	Principle	Result	Thresholds	Advantage	Disadvantage
TUNEL	Quantifies the enzymatic incorporation of dUTP into DNA breaks	Fluorescent DNA represents fragmented DNA	36.5% [78] 36% [79] 35% [80] 15% [81]	Direct measure. Can be performed on fresh or frozen samples. Detects both single- and double-strand breaks. Commercial assays available.	Standardization among laboratories is still required. Time consuming. Does not evaluate immature spermatozoa.
Comet	Electrophoretic assessment of fragments of DNA	Size of tail represent amount of fragmented DNA	56% [82] 52% [17]	Direct measure. Detects multiple types of DNA damage. Can be performed in patients with severe oligozoospermia as only 5000 sperm are required.	Only performed on fresh samples. Inter-observer variability. Time consuming. Experienced observer required.
SCD	Assesses dispersion of fragmented DNA after denaturation	Sperm with fragmented DNA do not produce Halos	35% [83] 30% [37]	Simple test. Commercial kit available.	Indirect measure. Inter-observer variability. Time consuming.
SCSA	Assess susceptibility of DNA to denaturation	Normal DNA ➔ green Fragmented DNA ➔ orange-red	30% [40] 27% [84]	Standardized protocol available. Rapid test. Large number can be tested. Fresh or frozen samples.	Indirect measure. No commercial assay available. Expensive instruments. Experienced technicians required.

63.2.3 The Sperm Chromatin Dispersion Test

The SCD test, also known as Halosperm assay, determines DNA fragmentation indirectly [18]. This is done by quantifying the amount of nuclear dispersion (halo) after sperm lysis and acid denaturation to remove nuclear proteins. After denaturation, a lysing solution is added which will lead to nuclear dispersion. The size of this dispersion is measured under fluorescence or optical microscope. Since only normal DNA will disperse and fragmented DNA will not lead to the formation of halos, the amount of dispersion is inversely proportional to the level of DNA damage. This method does not require the use of complex laboratory equipment, and it is a simple and fast assay that is able to detect SDF [19]. Many kits are available in the market to assist physicians in testing chromatin dispersion rapidly (e.g., Halotech) [20]. The interpretation of SCD testing is presented in Fig. 63.1.

63.2.4 The Sperm Chromatin Structure Assay

The sperm chromatin structure assay (SCSA) is another indirect test of SDF that has been validated as being a powerful assay in determining DNA integrity [21]. The percentage of sperm with fragmented DNA can be measured in about 5 minutes, which is much faster than any other assay [22].

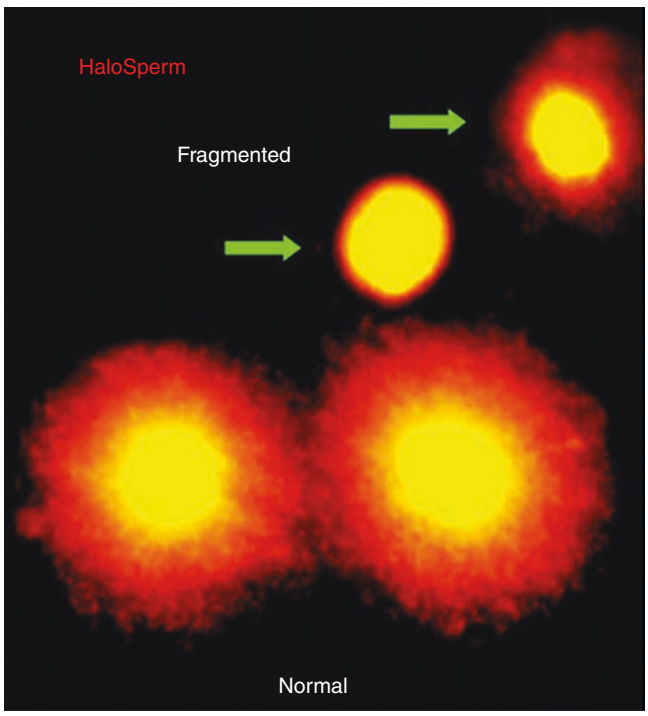

Fig. 63.1 Interpretation of the Halosperm test. (Reprinted from Vandekerckhove et al. [20]. With permission from Creative Commons License 4.0: https://creativecommons.org/licenses/by/4.0/)

After denaturation using an acidic solution, staining is done by the addition of a fluorescent dye (acridine orange). Then using a flow cytometer, 5000 to 10,000 sperm cells can be analyzed. Using a specific SCSA software, a scatter plot is created showing the ratio between red and green sperm. The percentage of red sperm represents the DNA fragmentation index (DFI) which is the sperm with fragmented DNA. As for the red sperm, they are called high DNA stainable (HDS) sperm and represent immature spermatozoa [23]. A DFI greater than 30% is a poor predictive of fertility [22].

SDF testing can be considered a more reliable test of fertility potential in comparison to conventional semen analysis as it is less liable to biologic variability [24]. Furthermore, several SDF testing methods have been standardized and validated for clinical practice [13, 25]. More importantly, since the outcome measured may differ according the testing method utilized (such as chromatin decondensation or DNA strand breaks), studies have established significant correlations between different SDF testing methods [26, 27]. Finally, several thresholds have been identified for each SDF testing method (Table 63.1). While this may complicate result interpretation, it is likely to be influenced by the clinical scenario at hand, and andrology laboratories are advised to establish their own cutoff levels after validating their results.

63.3 Indication of SDF Testing

SDF tests are increasingly being used in clinical practice for the evaluation of infertility in men. A recent survey of 65 professionals from 18 countries around the world reported that SDF testing is commonly ordered by 81.6% of responders who most commonly utilized the TUNEL and SCSA methods for SDF assessment [28]. This survey was part of a special issue on "Sperm DNA Fragmentation" (http://tau.amegroups.com/issue/view/612) which included a guideline for the utility of SDF testing in clinical practice. This guideline [11], endorsed by the Society for Translational Medicine, has identified the following clinical indications for SDF testing (Table 63.2).

63.3.1 Men with Clinical Varicoceles

A testicular varicocele is an abnormal expansion of the pampiniform plexus responsible for testicular venous drainage. This condition is detected in around 40% of men with infertility, and its mainstay treatment is surgery [29]. However, patient selection for surgery has been a subject of considerable debate principally because a remarkable number of men with clinically palpable varicocele are able to conceive naturally. Efforts made to find adjunct laboratory tests that can aid

Table 63.2 Clinical practice guidelines on sperm DNA fragmentation

Indications for SDF	Level of recommendation
Clinical varicocele	Grade C
Grade 2/3 and normal semen parameters	
Grade 1 and borderline abnormal semen parameters	
Recurrent pregnancy loss	
Unexplained infertility	
Recurrent IUI/IVF failure	
Recurrent abortions following ICSI	
Patients with lifestyle risk factors	

Based on data from Agarwal et al. [11]

in selecting patients who would benefit from surgery have recognized the clinical value of SDF testing.

DNA damage from varicoceles can occur due to many factors mostly related to testicular hyperthermia and intratesticular blood stasis, resulting in hypoxia and oxidative damage [30, 31]. A significant direct relationship has been observed between varicocele and SDF both in fertile and infertile men [32]. A cross-sectional study of 2399 men attending infertility clinics identified significantly worse SDF levels in varicocele patients (n = 391) compared with infertile men without varicocele (n = 2399) [33]. Such an observation coincided with significantly worse conventional semen parameters among the varicocele group as well. Within the varicocele group, the authors also identified significant positive associations between SDF and early apoptosis and abnormal mitochondrial membrane potential [33].

On the other hand, the evidence retrieved from studies showing a significant reduction in SDF levels after varicocelectomy has justified the utility of this test in patients with clinical varicocele [32, 34, 35]. One study reported significant improvement in SDF (evaluated with SCSA) 3 months following surgery (35.2% preoperatively vs. 30.2% postoperatively; $P = 0.019$) in 49 infertile varicocele patients [36]. Ni et al. compared infertile varicocele patients who underwent varicocelectomy to normal fertile controls and detected significant improvement in conventional semen parameters and SDF following surgery (preoperative: 28.4%; postoperative: 22.4%; $P = 0.018$). Patients who conceived after varicocelectomy did not have a significantly different SDF level than fertile controls; however, their postoperative SDF values were lower than the preoperative results and the SDF levels of nonpregnant patients [37].

While the impact of SDF on the fertility of men with lower grades of varicocele has not been thoroughly investigated, few studies have reported equally elevated SDF levels in different varicocele grades [37, 38]. More importantly, an improvement in SDF was observed in lower grades of disease together with a benefit in fertility potential following surgery, a finding that would justify the utility of SDF testing in all grades of disease [37, 38].

These results suggest that varicocele is strongly associated with SDF and that varicocele repair is useful in improving the DNA integrity in patients. SDF testing served as a tool to evaluate the prognosis and the outcome of varicocelectomy.

63.3.2 Unexplained Infertility, Recurrent Pregnancy Loss, or Intrauterine Insemination Failures

Unexplained infertility is defined when all the evaluation tests for fertility are normal, but the man is still infertile. This occurs in around 10–30% of infertile couples [39].

It is important to know that infertile men with normal semen parameters may still have high SDF levels [40]. Thus, SDF testing may be indicated in men with unexplained infertility.

In a recent study including 25 couples with unexplained infertility, 43% and 29% of patients had SDF levels above 20% and 30% (evaluated by the SCD test), respectively [20]. Similarly, Saleh et al. compared men with unexplained infertility to normal fertile controls reporting significantly higher SDF levels, using SCSA, in the former (23%) than in the latter (15%) [41].

As for IUI failures, a study revealed that a higher likelihood of pregnancy (7.0- to 8.7-fold) is expected in infertile couples when the male partners' sperm have lower levels of DNA damage [42]. Duran et al. evaluated semen samples from 154 men undergoing IUI. The authors observed significantly higher SDF levels, using TUNEL, among failed cycles in comparison to successful cycles and identified a SDF cutoff value of 12% above which no pregnancy could be achieved with IUI [43]. Using the SCSA method of SDF testing, Bungum et al. identified a cutoff value of 30% above which couples would have significantly lower biochemical pregnancy, clinical pregnancy, and delivery rates following IUI [40].

Studies have also linked SDF with recurrent spontaneous abortion (RSA), defined by two or more spontaneous miscarriages prior to 20 weeks' gestation. Couples with RSA were found to have 1.2-fold higher levels of SDF (measured by SCSA) than normal controls (28.1 ± 4.9 vs. 21.7 ± 4.7, respectively; $P < 0.05$) [44]. Another study comparing 30 couples with RSA with 30 control couples echoed similar results [45].

63.3.3 In Vitro Fertilization (IVF)/ Intracytoplasmic Sperm Injection (ICSI) Failure

Evidence suggests that the SDF level can significantly impact the outcomes of ART such as in IVF and ICSI. SDF can alter fertilization, embryo quality, clinical pregnancy, and live

birth and miscarriage rates [5]. Data from systemic reviews and meta-analyses indicated that high SDF levels were associated with decreased pregnancy rates with IVF and increased miscarriage rates after both IVF and ICSI [46–48]. The reported odds ratio for lower pregnancy rates following IVF in patients with high SDF was 1.27–1.57 [48, 49], while that for pregnancy loss following IVF and ICSI was 2.48 [49].

Technical differences between IVF and ICSI could explain the variable effect on pregnancy rate following the two procedures. With IVF, sperm are subjected to prolonged culture which may aggravate the SDF levels and hence exacerbate its detrimental effects [50]. Furthermore, the oocyte quality was also found to play an important role on ART outcome with worse results reported in females with reduced ovarian reserve [51].

63.3.4 Patients with Risk Factors

SDF testing is indicated in infertile men exposed to several risk factors that are known to induce DNA fragmentation. Factors such as smoking, obesity, occupational exposure (mainly lead and cadmium), organochlorine pollutants or pesticides (polychlorinated biphenyls and metabolites of dichlorodiphenyltrichloroethane), and bisphenol A, a compound used in plastic containers in food and drink industries, have been particularly identified [6, 52–55]. Evaluating such risk factors during the interview is important before SDF testing which would help in integrating lifestyle modifications and following their response in subsequent visits.

The previously published survey has explored the utility of SDF testing among healthcare practitioners in the abovementioned clinical scenarios and revealed that this test was most commonly utilized during the workup of couples with recurrent IVF failure (87.2%), followed by recurrent pregnancy loss whether naturally or following ICSI (79.5% for both) [28]. The remaining responses are presented in Fig. 63.2.

63.4 Treatment

63.4.1 Conservative and Counseling Methods

Several conservative and/or counselling measures can be utilized in the setting of high SDF values. Since a number of modifiable exposures have been identified in this regard. Patients presenting with high SDF levels should be screened for the occurrence of such risk factors. These include physical factors such as radiation and heat exposures, cigarette smoking and airborne pollutants, chemical agents such as anticancer drugs and sexually transmitted infections, and biological factors such as elevated body mass index and diabetes. As such, lifestyle modifications should be applied, and genitourinary infections, if present, should be treated with appropriate antibiotics.

Several studies have shown that male genital infection and inflammation can increase SDF [56]. Also, inflammation can lead to the production of ROS which are known to cause DNA modification and damage [57].

Finally, patients can be advised to perform frequent ejaculation especially during the time of ovulation as this might aid in decreasing DNA damage by reducing the sperm transit time through the epididymis, thus decreasing exposure to oxidative stress [58, 28]. Agarwal et al. have confirmed the effect of short abstinence with significantly lower SDF observed in patients with 1 day of abstinence compared to those with longer abstinence times [59].

63.4.2 Antioxidant Treatment

Oxidative stress has been recognized as a leading cause of SDF [9]. Therefore, measures taken to reverse this imbalance in the redox state should confer protective effects on sperm DNA integrity and fertility potential overall. Antioxidants are compounds that can be consumed through diet or as oral supplements. They are commonly prescribed for the treatment of male infertility as several reviews have demonstrated their ability to improve semen parameters, oxidative stress, clinical pregnancy, and live birth rates [60–63].

In general, fewer studies have explored the effect of antioxidant supplementation on SDF levels. We previously conducted a review which identified vitamins C and E, selenium, zinc, folic acid, and L-carnitine to have a significant beneficial effect on SDF levels [60].

63.4.3 Surgical Treatment (Varicocele Ligation)

As discussed earlier, varicocele is the most commonly treatable cause of male infertility, prevalent in about 25.4% to 81% of infertile men [64]. In addition to the beneficial effects of varicocele treatment on semen parameters and pregnancy rates, studies have also shown a favorable effect on SDF levels among infertile men [32, 65].

A meta-analysis of six studies including 177 patients evaluated the effect of varicocelectomy on SDF. The authors confirmed that patients with varicocele tend to have higher SDF levels than controls, with a mean difference of 9.84% (95% CI 9.19 to 10.49; $P < 0.00001$), and that varicocelectomy would reduce these levels, with a mean difference of −3.37% (95% CI −4.09 to −2.65; $P < 0.00001$) [65]. The authors finally suggested varicocelectomy as a sound treatment option to reduce the level of SDF.

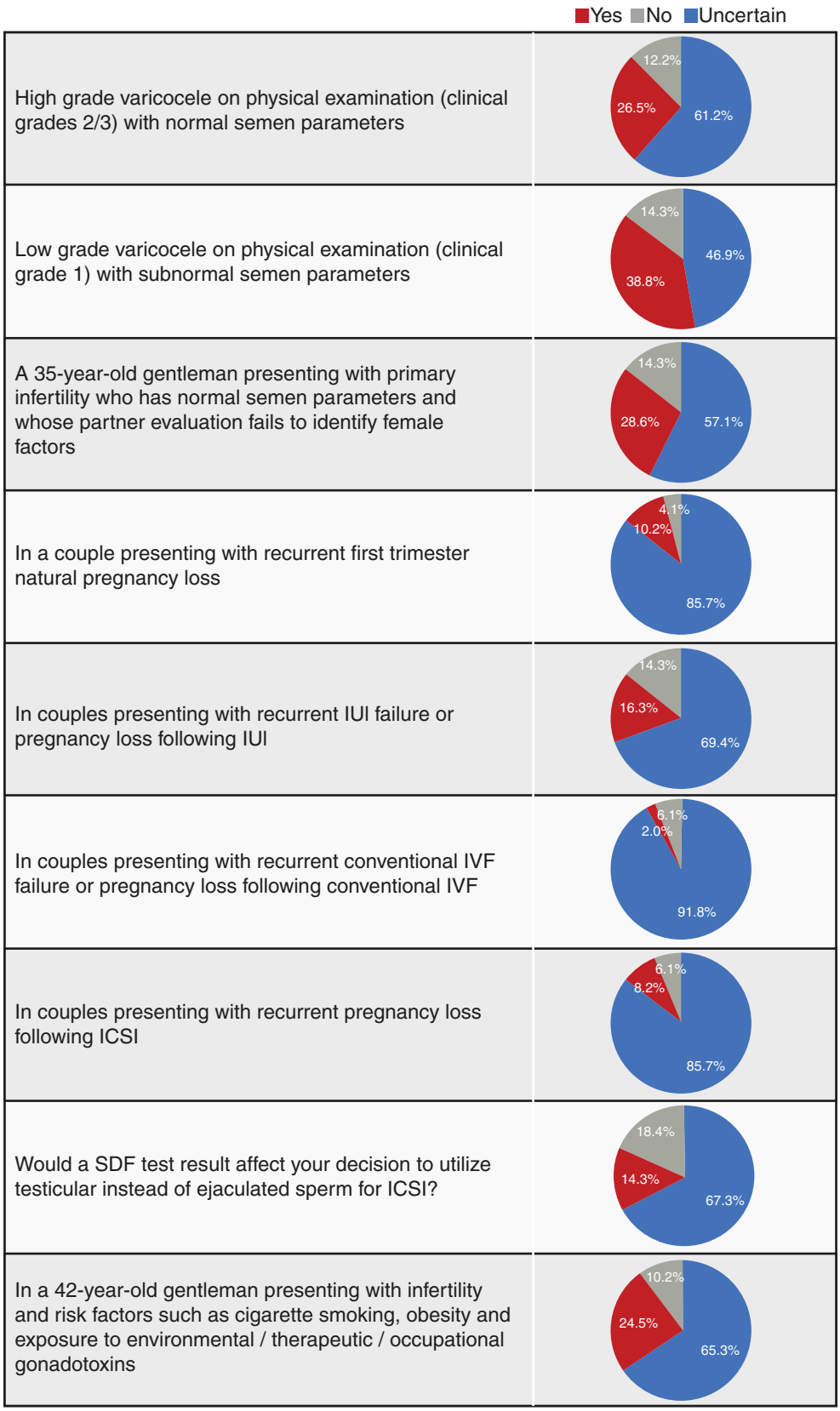

Fig. 63.2 The utility of SDF in various clinical scenarios. *SDF* sperm DNA fragmentation, *IUI* intrauterine insemination, *ICSI* intracytoplasmic sperm injection, *IVF* in vitro fertilization. (Reprinted from Majzoub et al. [28]. With permission from AME Publishing Company)

Another large systematic review done in 2011 by Zini and Dohle also supports the evidence that varicocele is associated with high SDF which may occur secondary to varicocele-mediated oxidative stress. The study also concluded that a beneficial effect from varicocele ligation on sperm DNA damage can be expected [32].

Thus, the efficacy of varicocele repair in alleviating oxidative stress, increasing seminal antioxidants, and decreasing SDF has been demonstrated. The SDF value is still not enlisted among the accepted indications for varicocele treatment which include abnormal semen parameters in a couple with normal or treatable female factors [64]. It is worth witnessing whether the SDF test result would be included in any future society recommendations for varicocele treatment.

63.4.4 In the Setting of ART

The SDF level poses important prognostic information for the couple undergoing ART. We have previously formulated an ART algorithm taking into consideration the SDF value (Fig. 63.3) [7]. Patients with recurrent IUI or IVF failure who are found to have high SDF levels should undergo ICSI. When recurrent pregnancy loss following ICSI is faced, then efforts should be made to select sperm with lower levels of SDF for the subsequent ICSI trial. These efforts include in vitro sperm selection techniques or the use of testicular sperm [11].

Spermatozoa retrieved from the testicles of men with high levels of SDF in the ejaculated sperm tend to have better DNA quality and integrity [66]. The rationale behind this fact is that retrieval of sperm from the testes will overcome the posttesticular SDF caused by oxidative stress during transit through the epididymis. Hence, fertilization by sperm with better DNA integrity and quality will be optimized which would result in better reproductive outcome.

To evaluate the effect of using testicular sperm versus ejaculated sperm, a prospective study evaluated 147 couples undergoing ICSI and investigated the effectiveness of ICSI using testicular vs. ejaculated sperm [66]. Results have shown that the SDF level was higher in ejaculated than testicular sperm (40.7% vs. 8.3%). Moreover, a higher clinical pregnancy (51.9% vs. 40.2%) and live birth rate (46.7% vs. 26.4%) and lower miscarriage rate (10% vs. 34.3%) were noted when testicular sperm were used instead of ejaculated sperm [66].

Thus, this study concluded that ICSI outcomes were significantly better in the group of men who used testicular sperm for ICSI compared with ejaculated sperm and that SDF was significantly lower in testicular sperm [66].

Another study evaluated ART outcomes using testicular sperm in oligospermic men who previously had a failed ART using ejaculated sperm with high SDF (measured by TUNEL) [67]. Authors confirmed that testicular sperm had lower SDF values than ejaculated sperm and that the use of testicular sperm for ICSI resulted in a 50% pregnancy and live birth rate in couples with previously failed cycles using ejaculated sperm.

Arafa et al. compared the clinical outcome of a consecutive testicular sperm ICSI cycle performed on 36 men with high SDF to the clinical outcome of a prior ejaculated sperm ICSI cycle [68]. Higher pregnancy rates (38.9% vs. 13.5%, $P < 0.001$) and live birth rates (47.2% vs. 8.3%, $P < 0.001$) were achieved with the testicular sperm in comparison to the ejaculated sperm ICSI cycle [68].

In a SWOT review analysis, authors summarized the studies that have been published on the use of testicular sperm for ICSI among men with high SDF [69]. The SWOT analysis presents the strengths, weaknesses, opportunities, and threats of the topic (Fig. 63.4). The authors have concluded that (1) the use of testicular sperm for ICSI in patients with posttesticular SDF is supported by the literature; (2) this treatment modality can be offered only when other less invasive treatment methods have failed; and (3) monitoring is required to determine potential risks to the health of the generated offspring and the complications of sperm retrieval.

Sperm selection techniques are not uncommonly performed during the course of ICSI cycles [70]. These techniques are aimed at selecting structurally intact and mature sperm with high DNA integrity for fertilization. They mainly depend on sperm surface charge, apoptosis, birefringence, morphology under ultra-high magnification, or ability to bind to hyaluronic acid [71].

Many laboratory techniques to reduce SDF have been investigated in the setting of ICSI. For instance, shortening the ejaculatory abstinence, repeated ejaculation, and density centrifugation to isolate intact mature sperm have shown to provide a reduction in the proportion of damaged

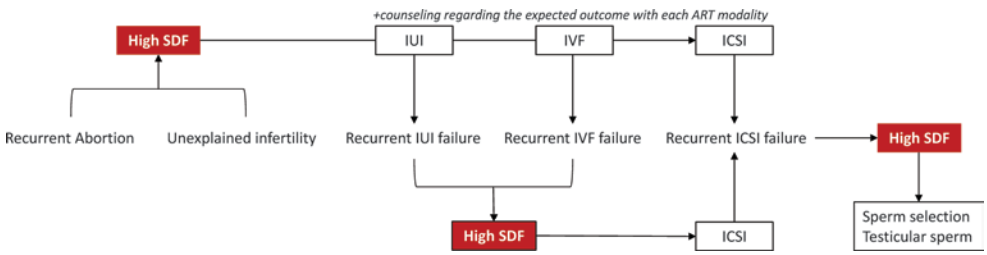

Fig. 63.3 Algorithm for utility of sperm DNA fragmentation in assisted reproductive therapy

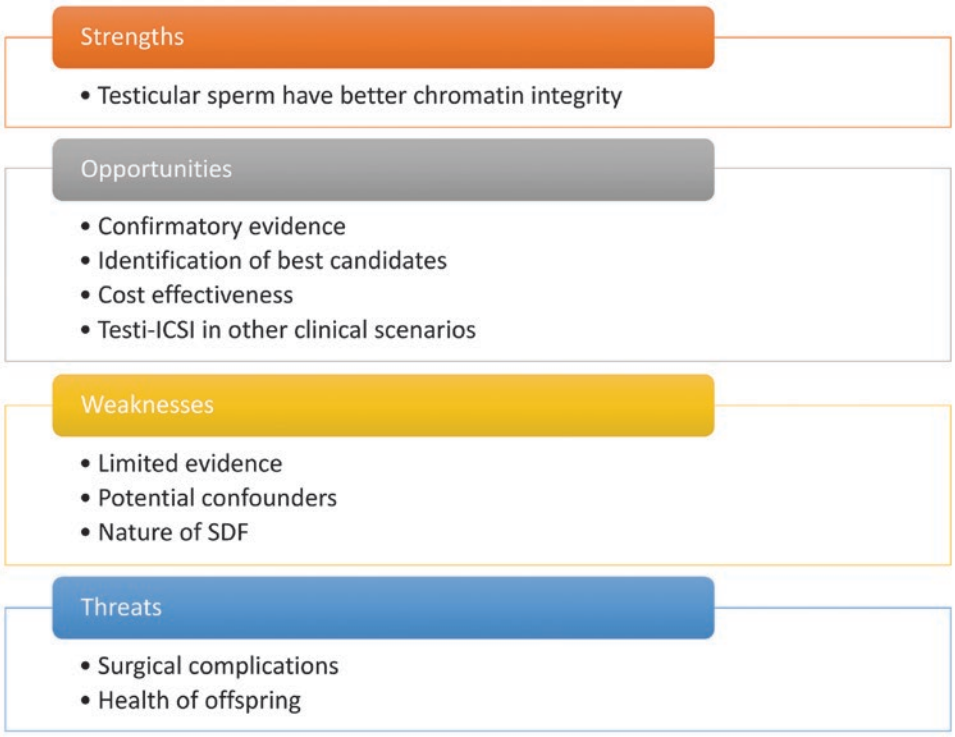

Fig. 63.4 SWOT analysis of the use of testicular sperm in ICSI. (Based on data from Ref. [69])

DNA from 47% to 22% [56, 69]. Likewise, swim-up technique, which selects sperm with better motility, yielded a reduction of about 35% in the proportion of sperm with damaged DNA [72, 73]. Another study evaluated the ability of swim-up technique and density gradient centrifugation to remove single- and double-strand DNA damage. The results showed that both are equally efficient in eliminating spermatozoa containing double-strand DNA damage and sperm with highly damaged (degraded) DNA and that density gradient centrifugation is more efficient than swim-up technique in selecting spermatozoa that are free from single-strand DNA damage [74]. Other methods such as the morphologically (IMSI) or physiologically (PICSI) selected sperm for ICSI and magnetic cell sorting have selected sperm with lower SDF for use in ICSI with variable efficiency [75, 76]. Bradely et al. [77] compared ICSI outcomes between PICSI, IMSI, and testicular sperm. The authors have established that a better outcome is achieved with all PICSI and testicular sperm. However, the live birth rate was highest when testicular sperm are used, followed by PICSI.

63.5 Conclusion

SDF is an important test of fertility potential. Recent guidelines were published recommending its utility in patients with unexplained infertility, recurrent spontaneous abortion, clinical varicocele, ART failure, and lifestyle risk factors. The available evidence indicates that the SDF test result can influence treatment decisions which results in better reproductive outcomes. The SDF results can help the clinician to recommend/monitor lifestyle modifications, offer varicocelectomy, prescribe antioxidant supplementation, select the appropriate ART method, and perform sperm selection or use testicular sperm prior to ICSI.

63.6 Review Criteria

An extensive search of studies exploring sperm DNA fragmentation was performed using search engines such as ScienceDirect, OVID, Google Scholar, PubMed, and MEDLINE. The overall strategy for study identification and data extraction was based on the following keywords: "sperm DNA fragmentation," "sperm function tests," "treatment," "diagnosis," "guidelines," "semen parameters," and "assisted reproduction." Articles published in languages other than English were also considered. Data that were solely published in conference or meeting proceedings, websites, or books were not included.

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64 Antioxidants in the Medical and Surgical Management of Male Infertility

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Key Points

- Sperm DNA and membrane are highly susceptible to reactive oxygen species (ROS), which are able to cause DNA fragmentation and membrane lipoperoxidation
- Treatment with antioxidants represents a valid therapeutic option for male infertility, especially after the sperm ROS-induced damage has been ascertained
- Several compounds with antioxidant properties have been developed
- Founded on an evidence-based medicine criterion, vitamin C and E, carnitines and coezyme Q10 may be proposed as first-line treatment, while gluthatione, zinc, lycopene and myoinositol as second-line

64.1 Introduction

Oxidative stress (OS), resulting from an imbalance between the production of radical oxygen species (ROS) and antioxidant scavenging activities [1], causes male and female infertility. The role of OS in the pathophysiology of human sperm function has been extensively explored. Indeed, spermatozoa are extremely sensitive to ROS because of their high content of polyunsaturated fatty acids (PUFA) and their limited ability to repair deoxyribonucleic acid (DNA) damage [2, 3]. High seminal ROS levels have been shown to damage sperm motility, morphology and DNA integrity [4]. Furthermore, meta-analytic data provide evidence for a negative impact of seminal ROS levels on the outcome of assisted reproductive techniques [5]. Therefore, the administration of antioxidants has been proposed to improve sperm quality and, consequently, fertility.

Treatments varied over the years, involving the use of many different compounds. The most used are zinc, folic acid, N-acetylcysteine, coenzyme Q10 (CoQ10), vitamins E and C, myoinositol, selenium, carnitines and pentoxifylline in various dosages and combinations [6]. Most of them act by decreasing ROS concentration and, in turn, by improving sperm motility. However, some compounds exert a prokinetic effect by also acting through different mechanisms. For example, myoinositol acts by sensitizing post-receptorial mechanisms [7], whereas carnitines might improve mitochondrial fatty acid transport [8].

The administration of antioxidants to infertile patients represents a great challenge for the andrologist. Indeed, the first step before antioxidant treatment might be prescribed is to consider the clinical history and laboratory and instrumental data to understand if a given patient is suitable for a scavenging treatment or whether a different therapeutic strategy should be undertaken. A suitable strategy would be to eradicate all causes that increase ROS overproduction and/or decrease seminal plasma scavenging action. On this account, the Italian Society of Andrology and Sexual Medicine (SIAMS) suggested antioxidant prescription after a correct and complete diagnostic work-up [9]. The efficacy of antioxidant administration could clearly be evaluated by using markers that can reliably measure OS before treatment and/or of markers that evaluate the damage caused by OS on sperm membrane and DNA. In the past few years, several strategies have been developed to assess semen ROS levels or ROS-induced sperm damage. In greater detail, seminal 8-hydroxy-2-deoxyguanosine (8-OHdG) and malondialdehyde (MDA) levels represent markers of sperm DNA and lipid peroxidation, respectively [6]. In addition, the mitochondrial membrane potential (MMP) as well as the sperm DNA fragmentation (SDF), indirectly provide information on ROS-induced damage in spermatozoa [10]. The use of antioxidants has been suggested to be appropriate in

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the case of documented increased SDF levels [9]. Despite a general agreement, a clear SDF cut-off value has not been reached to date; values above 20% have been suggested to be abnormal [9].

Many studies carried out in animal models suggest a possible successful use of antioxidants in humans [11–16]. The evidence coming from the meta-analysis of randomized controlled studies is in favour of antioxidant efficacy in male infertility [17, 18]. Accordingly, data derived from 34 RCTs, including a total of 2900 couples, found a fivefold live birth rate (LBR) increase in patients who were prescribed antioxidants (vitamin E, vitamin C, vitamin B, L-acetylcarnitine, N-acyl-cysteine, docosahexaenoic acid, selenium or their combinations) compared to controls (OR = 4.85; $p = 0.0008$) [18]. Another meta-analysis published by the same group of authors on 48 RCTs (including 4200 infertile patients) largely confirmed these findings (OR 4.21, $p < 0.0001$) [17]. Antioxidant administration has also been associated with an increased clinical pregnancy rate (PR) compared to placebo or no treatment (OR = 3.43, $p < 0.0001$) [18].

However, scientific literature on this topic is greatly heterogeneous [19]. Indeed, despite that many studies describe positive results following administration of antioxidants on sperm parameters, there is no well-defined scavenging approach during OS, and often the experimental design is not double-blind and/or placebo-controlled. In addition, non-homogeneous cohorts of patients have been enrolled in some studies. Finally, a large number of compounds have been used, but for many of them, scanty evidence has been published.

We used an evidence-based medicine (EBM) method to better understand the role of the various antioxidants for the treatment of infertile men (Table 64.1). To accomplish this, the National Clinical Guidelines for Type 2 Diabetes, formulated by the Royal College of General practitioners, Effective Clinical Practice Unit, ScHAAR University of Sheffield (http://www.shef.ac.uk/guidelines) were used as they seem more suitable for pharmacological trials. These guidelines classify evidence and recommendations as indicated in Table 64.2.

Table 64.1 List of the antioxidants reviewed in this chapter

Antioxidants
Ascorbic acid (vitamin C)
α-tocopherol (vitamin E)
Ascorbic acid (vitamin C) plus α-tocopherol (vitamin E)
α-tocopherol (vitamin E) plus selenium
Glutathione
L-Carnitine plus L-acetyl-carnitine
Coenzyme Q10
Myoinositol plus folic acid
Myoinositol, vitamin E, L-carnitine, L-arginine, folic acid and selenium
Lycopene
Picnogenol
N-acetyl-cysteine
Vitamin A and vitamin E
Pentoxifylline
Zinc
Zinc plus folic acid
Pycnogenol and L-arginine
Selenium
Shao-Fu-Zhu-Yu-Tang
Astaxanthin
Lepidium meyenii
α-linolenic acid and lignans
Vitamin C and E, lycopene, selenium, folic acid, garlic oil plus zinc
Morindae officinalis extract

Table 64.2 Evidence and recommendations of the National Clinical Guidelines for Type 2 Diabetes (The Royal College of General practitioners, Effective Clinical Practice Unit, ScHAAR University of Sheffield) used in this chapter

Evidence classification	
Ia	Evidence from meta-analysis of randomized controlled trials
Ib	Evidence from at least one randomized controlled trial
IIa	Evidence from at least one controlled study without randomization
IIb	Evidence from at least one other type of quasi-experimental study
III	Evidence from non-experimental descriptive studies, such as comparative studies, correlation studies and case-control studies
IV	Evidence from expert committee reports or opinions and/or clinical experience of respected authorities

Recommendation grading	
A	Directly based on category I evidence
B	Directly based on category II evidence, or extrapolated recommendation from category I evidence
C	Directly based on category III evidence, or extrapolated recommendation from category I or II evidence
D	Directly based on category IV evidence, or extrapolated recommendation from category I, II or III evidence

64.2 Ascorbic Acid (Vitamin C)

Ascorbic acid concentrations are 10-times higher in the seminal plasma than in serum [20]. It is an effective scavenger when peroxyl radicals are in an aqueous phase [21], but vitamin C does not have the same powerful scavenging action within membrane lipids [22]. The amount of vitamin C in the seminal plasma has been reported to decrease significantly when ROS concentrations increase [23]. Similarly, the concentrations of seminal ascorbic acid are significantly depleted in leucocytospermic samples. When this occurs, a significantly higher SDF has been found compared with semen samples with normal or high levels of ascorbic acid [24]. Interestingly, vitamin C plays an antioxidant action at low concentrations, but it can start auto-oxidation processes at higher concentrations [25]. Moreover, in humans, the vitamin C plasma saturation occurs at a daily dose of 1 g. Higher

amounts could promote the development of kidney stones, because of the enhanced excretion of oxalate [26].

The administration of vitamin C (1 g/day) increases ascorbic acid level by 2.2-fold [27]. In addition, it has been reported that seminal plasma vitamin C concentrations positively correlate with the number of normal spermatozoa, in a controlled clinical trial [28] (IIa). In earlier studies, vitamin C (1 g/day) supplementation has been proposed to ameliorate the sperm quality in infertile patients [29] (III), [30] (Ib). Sperm parameters also increase with a higher vitamin C intake, as shown by the higher sperm concentration and total progressive motile sperm count (TPMS) [31] (IIb) as well as sperm motility and morphology [32] (III). In a placebo-controlled study, vitamin C given to heavy smokers at a dose of 200 or 1000 mg/day for 4 months improved sperm parameters. The group that took the dose of 1000 mg/day had a higher increase [30] (Ib). In addition, vitamin C safeguards human spermatozoa from endogenous oxidative DNA damage [33] (IIb). Recently, a randomized, double-blind, placebo-controlled clinical trial evaluated the effectiveness of ascorbic acid, orally administered at the dose of 250 mg twice a day for 3 months after varicocelectomy. The group receiving this treatment showed a significantly higher sperm motility and morphology than the one receiving placebo. Bio-functional sperm parameters were not evaluated [34] (IIa).

64.3 α-Tocopherol (Vitamin E)

In a single-blind study, eight patients treated with 100 mg of vitamin E for 4 months did not show any sperm parameter improvement [35] (IIa), while the administration of vitamin E, at a dose of 100 mg 3-times/day, produced a slight increase in seminal plasma vitamin E concentration. In a study performed on 15 subjects, the number of spermatozoa, the percentage of sperm with forward motility, the half-life of the percentage displaying forward motility and the rate of swollen spermatozoa in hypo-osmotic medium did not show any significant enhancement during vitamin E administration. The authors explained the lack of effects on these parameters with the small increase of vitamin E achieved in the seminal plasma. They hypothesized that higher doses of vitamin E may be more effective [36] (Ib).

Many trials have been conducted to ameliorate sperm parameters of infertile men by vitamin E administration. In a double-blind randomized, placebo-controlled, cross-over trial, 30 healthy men with elevated semen ROS concentrations and healthy female partners, were given vitamin E (600 mg/day) or placebo for 3 months. Vitamin E significantly increased blood serum α-tocopherol concentrations and sperm function, evaluated by the zona binding assay [37] (Ia). A single-blind study took into account eight patients receiving 300 mg/day of vitamin E, divided in three daily doses of 100 mg each, for 4 months. Patients receiving α-tocopherol did not show any improvement [35] (IIa). A placebo-controlled, double-blind study showed that the elevated sperm MDA in asthenozoospermic and oligo-asthenozoospermic men decreased significantly after vitamin E administration, which also improved sperm motility in asthenozoospermic patients. In addition, 11 out of 52 wives (21%) of the treated group got pregnant during the 6-months treatment; nine of them had normal term deliveries, while two spontaneously aborted in the first trimester. No pregnancy was reported in the placebo group [38] (IIa). Moreover, elevated MDA concentrations significantly dropped to normal levels, and the fertilization rate per cycle increased significantly following administration of 200 mg/day of vitamin E for 3 months, in a prospective study conducted in 15 fertile normozoospermic men. The elevated MDA concentrations significantly decreased to normal levels and the fertilization rate per cycle increased significantly after 1 month of treatment [39] (IIa). Furthermore, an elevated consumption of daily nutrients with scavenging potential (food and nutraceutical complements, such as zinc, folate, vitamin C, vitamin E and β-carotene) proposed to 97 healthy, non-smoking men, showed that the vitamin E intake correlated with the highest progressive motility and TPMS [31] (IIb). Finally, a randomized-controlled study found no benefit on conventional sperm parameters from 12-month-long vitamin E administration at the dose of 300 mg twice a day on 22 patients compared to 23 controls after varicocelectomy [40].

64.4 Ascorbic Acid (Vitamin C) and α-Tocopherol (Vitamin E)

Vitamin C and vitamin E may be administered together to decrease the peroxidative injury on spermatozoa, taking advantage of their hydrophilicity and lipophilicity, respectively. In addition, if these compounds act directly on spermatozoa to prevent ROS-induced damage, the improvement could be rapid, given that the two vitamins reach spermatozoa both within the epididymis and following ejaculation.

A double-blind, placebo-controlled, randomized trial has been performed in asthenozoospermic or moderate oligo-asthenozoospermic men. Vitamin C (1 g) and vitamin E (800 mg) were prescribed simultaneously for 2 months, but no improvement of semen parameters was reported [41] (Ib). These unsatisfactory findings match with the findings of other studies [38, 42] but diverge from other published data [39, 43]. It is also possible that the duration of the treatment was too short to produce an effect, particularly if the action occurs within the testis.

Sixty-four patients with idiopathic infertility and an increased (≥15%) proportion of spermatozoa with DNA

fragmentation were randomly divided into two groups: one was given vitamin C (1 g) and vitamin E (1 g) daily and the other one placebo. After 2 months of treatment, the proportion of DNA-fragmented spermatozoa decreased significantly in the antioxidant-treated group, while no variation was detected in the placebo group [44] (Ib). An additional trial was performed on 38 patients with a raised (≥15%) percentage of DNA-fragmented spermatozoa in the ejaculate. They were prescribed vitamin C (1 g) and vitamin E (1 g) daily for 2 months following one ICSI cycle failure. In 29 of them (76%), the scavenging therapy led to a decline in the proportion of DNA-fragmented spermatozoa and a successful ICSI attempt with higher clinical pregnancy (48.2% versus 6.9%) and implantation (19.6% versus 2.2%) rates [45] (IIb).

64.5 α-Tocopherol (Vitamin E) and Selenium

Few studies have been performed using the association of vitamin E and selenium [46–48]. A trial was conducted in nine oligoasthenoteratozoospermic patients who were prescribed vitamin E (400 mg) plus selenium (100 μg) daily for 1 month. Thereafter, selenium supplementation was increased to 200 μg/day for the next 4 months. This kind of association produced a significant improvement of sperm motility, morphology and vitality [46] (Ib). Another study, using the same association, was performed in 28 men who were given vitamin E (400 mg) and selenium (225 μg) daily for 3 months, resulting in a significant reduction in MDA concentrations and an enhancement of sperm kinetic parameters compared to controls [47] (Ib). Furthermore, the daily combined administration of vitamin E (400 units) and selenium (200 μg) for at least 100 days significantly improved sperm motility and spontaneous PR among 690 infertile patients who were prescribed the treatment [48].

64.6 Glutathione

Glutathione (GSH) is one of the most commonly used drugs due to its antitoxic and scavenging action in different diseases. Although it cannot cross cell membranes, its concentration increases in biological fluids following a systemic intake. GSH is able to reach the seminal plasma and to act at this level. Here, it safeguards spermatozoa from ROS attack; hence, GSH may play a beneficial function in several andrological diseases, particularly during male genital tract inflammation [49].

GSH (600 mg/day i.m.) was prescribed to 11 patients with dyspermia associated with different andrological diseases in a 2-month pilot trial. Sperm kinetics improved, particularly in men with male accessory gland infections (MAGI) and in men with varicocele [50] (III), two circumstances wherein ROS or other noxious substances may play a pathogenic role. Following these encouraging findings, the same investigators conducted a placebo-controlled, double blind, cross-over study on infertile men experiencing unilateral varicocele and amicrobial MAGI. The patients were allocated to treatment with GSH, 600 mg i.m. on alternate days, or placebo ampoules. Men who received GSH showed higher sperm number, motility, kinetic parameters and percentage of normal forms. These effects on sperm motility and morphology lasted for some time after the treatment was discontinued. The authors hypothesized that these findings may relate to a post-spermatocyte action of GSH, since the length of the treatment did not cover the full length of a complete spermatogenesis [49] (Ib). This kind of sperm modification can be partially corrected by GSH administration when cell membrane injury is not too critical [51] (IIa).

The above reported data indicate that, at least to some extent, the beneficial effect of GSH is suitable for the biochemical changes in membrane organization and its following defensive action on the lipid components of the cell membrane. The decline of lipoperoxide levels in seminal plasma leads to considering that GSH minimizes the consequence of lipoperoxidation generated by vascular or inflammatory diseases.

64.7 Carnitines

Carnitines are involved in many metabolic pathways in several cellular organelles. These compounds play a primary function in sperm maturation within the male genital organs and a relevant role in the metabolism of spermatozoa by furnishing immediately accessible energy to be utilized by spermatozoa. This positively correlates with sperm motility and concentration [52]. An increase of sperm progressive motility occurs simultaneously to L-carnitine augmentation and storage in the epididymal lumen [53].

Several different kinds of studies (controlled, uncontrolled, human, animal) have been carried out to evaluate the potential application of carnitines as scavenging molecules [54]. In 1992, a study was conducted on the male partners of 20 couples affected by idiopathic oligoasthenozoospermia (concentration < 20 × 10^6 spermatozoa/ml, progressive motility <50%) who were given 4 g/day of L-acetyl-carnitine for 2 months. No significant effect on sperm concentration, total motility and morphology resulted, whereas a significant improvement of progressive motility (21.7 ± 3.2% vs. 38.2 ± 4.7) was appreciated [55] (IIb). Afterwards, a multicentre open study was performed on 100 men with idiopathic asthenozoospermia. L-carnitine was administered orally at the dose of 1 g 3-times/day for 4 months with a significant

improvement of several sperm kinetic parameters [56] (IIb). Another study came to similar results, giving an oral solution of L-carnitine (1 g) 3-times a day for 3 months, to 47 patients with idiopathic asthenozosperma [57] (IIb). A review article proposed carnitines treatment as an alternative method in the broader medical treatment of patients with infertility due to OS [58].

Clinical evidence suggests that infertile patients with prostato-vesiculo-epididymitis (PVE) benefit from carnitines administration, since antimicrobial and/or nonsteroidal anti-inflammatory drugs, although effective to eliminate microbial infection, have a poor scavenging action [59] (Ib). Another study conducted on 98 patients with PVE and leukocytospermia showed that carnitine scavenging treatment was totally successful once these patients were pretreated with nonsteroidal anti-inflammatory compounds [60] (Ib).

In a placebo-controlled, double-blind, crossover trial, L-carnitine was able to enhance sperm parameters, even if it was unsuccessful in reducing LPO concentrations. These findings suggested an incomplete action of L-carnitine to counteract the ROS attack [61] (Ib). The same group proposed a double-blind, randomized, placebo-controlled trial. They gave a combined treatment with L-carnitine (2 g/day) and L-acetyl-carnitine (1 g/day) or placebo to 60 infertile males with oligoasthenoteratozoospermia. All sperm parameters improved, but the most important enhancement was found in both progressive and total sperm motility, particularly in patients with the highest degree of asthenozoospermia [62] (Ib). Another placebo-controlled study, also conducted in patients with oligoasthenoteratozoospermia, showed that the same treatment improved sperm concentration, motility and morphology, particularly when cinnoxicam (1 suppository every 4 days) was added [63] (Ib). Furthermore, 60 patients with asthenozoospermia were enrolled in a double-blind clinical trial with L-carnitine (3 g/day), L-acetyl-carnitine (3 g/day), a combination of L-carnitine (2 g/day) plus L-acetyl-carnitine (1 g/day) or placebo, for 6 months. Total and forward motility, including kinetic parameters analysed by computer-assisted sperm analysis, improved in men receiving L-acetyl-carnitine either alone or in association with L-carnitine. The total oxyradical scavenging capacity of the semen towards hydroxyl and peroxyl radicals also improved and correlated with the enhancement of sperm kinetics. Patients with lower motility and total oxyradical scavenging capacity of the seminal fluid had more chances of responding to the treatment [64] (Ib). In another trial, L-carnitine (2 g/day) and L-acetyl-carnitine (1 g/day) were given orally tid for 3 months to 90 men with oligoasthenozoospermia. In the treatment group, 10 female partners (11.6%) achieved pregnancy, whereas only two pregnancies (3.7%) were recorded in the control group. Moreover, their percentage of forward and total motile spermatozoa increased significantly [65] (Ib). In the trial lead by De Rosa and colleagues, 66 patients with <50% motility receiving L-carnitine (1 g/day) and L-acetyl-carnitine (500 mg tid) for 6 months, had a significant increase in sperm total motility, viability, membrane integrity, linearity of sperm movement, both after 3 and 6 months of treatment, and the ability to penetrate the cervical mucus increased after 6 months [66] (IIb). Twenty-one patients with infertility and with sperm motility ranging from 10% to 50% were given carnitines (2 g of L-carnitine and 1 g of L-acetyl-carnitine per day) orally for 6 months, but conversely from the other studies, no significant effects on sperm motility resulted [67] (Ib). In a further trial, L-carnitine (2 g/day) and L-acetyl-carnitine (1 g/day) were administered for 3 months in men with PVE and increased ROS production. Carnitines showed to be a successful treatment once seminal leukocytes were within the normal range [68] (IIb).

In the light of the many studies exploring the effects of carnitines on sperm parameters, a systematic review has recently been published. The meta-analysis that compared L-carnitine and/or L-acetyl-carnitine treatment to placebo reported significant improvement in total and forward sperm motility, atypical sperm cells and PR. No significant difference has been found in sperm concentration [69] (Ia).

64.8 Coenzyme Q10

CoQ10 is a lipid-soluble constituent of the respiratory chain. Ubiquinol is the reduced form and the active one. It behaves as a powerful scavenger in some biological components, for instance lipoproteins and membranes.

The concentrations of reduced and oxidized forms of CoQ10 (ubiquinol/ubiquinone) and of hydroperoxide have been measured in the seminal plasma and seminal fluid of 32 infertile patients. A positive correlation between ubiquinol concentration and sperm count has been observed, whereas a negative correlation was reported between sperm count and ubiquinol concentration or hydroperoxide levels. An important correlation between sperm concentration, motility and seminal fluid ubiquinol-10 content has been found, whereas in total fluid, an inverse correlation between ubiquinol/ubiquinone ratio and the severity of teratozoospermia has been reported. These findings indicate that ubiquinol-10 impedes hydroperoxide occurrence in seminal fluid and in seminal plasma [70].

CoQ10 has been given orally at the dose of 60 mg/day to 17 patients with low fertilization rate after ICSI performed for male infertility for an average of 103 days previous to the subsequent ICSI procedure. The results showed a significant enhancement of the fertilization rate [71] (IIb).

In the human seminal fluid, CoQ10 has been found at relevant concentrations, and it shows a direct association with sperm concentrations and kinetics. Differently, in patients

with varicocele, despite a higher proportion of CoQ10 in the seminal plasma, the correlation with sperm motility was not observed [72]. Elevated CoQ10 levels have been found in spermatozoa of oligozoospermic and asthenozoospermic patients without varicocele. This correlation was not found in patients with varicocele, who additionally showed slightly lower intracellular absolute concentrations of CoQ10. Higher intracellular levels could be linked to a spermatozoa protective system. In patients with varicocele, this kind of system could be inadequate, leading to an excessive susceptivity to OS [72].

A double-blind, randomized trial has been carried out in 60 infertile patients with idiopathic asthenozoospermia. Patients underwent a double-blind CoQ10 (200 mg/day) administration or placebo for 6 months. After treatment, CoQ10 and ubiquinol rose appreciably in the seminal plasma as well as in spermatozoa. Interestingly, spermatozoa improved their motility. Patients with poorer sperm motility and lower concentrations of CoQ10 had a statistically significant higher chance to better respond to its administration [73] (Ib).

More recently, a randomized placebo-controlled trial confirmed the efficacy of CoQ10 orally administered at the dose of 300 mg in 212 infertile patients with idiopathic oligoasthenoteratozoospermia on sperm concentration, motility and morphology compared to placebo [74] (Ib). Higher doses of CoQ10 (300 mg twice daily for 12 months) have also been reported to raise conventional sperm parameters and to have a beneficial effect on PR as well [75] (IIb). Accordingly, CoQ10 supplementation has been shown to increase seminal levels of catalase and SOD activity [76]. In contrast with these findings, a randomized, double-blind, placebo-controlled study on 47 infertile patients did not find any difference in conventional sperm parameters after a 12 week-long daily administration of CoQ10 (200 mg), though lower levels of plasma MDA and higher plasma antioxidant capacity were found compared to placebo group [77] (Ib). Finally, meta-analytic data support the benefit of CoQ10 administration on sperm concentration and motility [78] (Ia).

64.9 Myoinositol

Myoinositol is a precursor of phosphatidylinositol polyphosphates, which are involved in the intracellular signal transduction [7]. In vitro sperm incubation with myoinositol has been shown to improve MMP and sperm motility, as well as the number of spermatozoa recovered by swim-up [79–81]. Oral supplementation at the dose of 2000 mg (plus folic acid 200 μg)/daily for 3 months resulted in the enhancement of both sperm concentration and motility in patients with idiopathic infertility compared to the placebo-controlled group (Ib) [82]. An increase in sperm count, but not of motility, was achieved after the 2-month-long myoinositol supplementation at the dose of 4000 mg (plus 400 μg of folic acid)/daily (IIa) [83].

Sperm concentration, motility and morphology improved greatly when myoinositol was administered in combination with vitamin E, L-carnitine, L-arginine, folic acid and selenium (Andrositol®) (IIb) [84]. Accordingly, Andrositol® administration ameliorated sperm motility in a cohort of asthenozoospermic patients (IIb) [85].

64.10 Lycopene

Lycopene is an element of the human redox defensive system against oxidative stress. Oral lycopene administration might play a role in the treatment of patients with idiopathic infertility. Following the administration of 2 g of lycopene twice a day for 3 months, a significant increase occurs in sperm number and motility, but the sperm concentration increase occurred only in men with a sperm concentration >5 million/ml [86] (IIb). At the daily dosage of 8 mg, lycopene also seems effective in increasing the PR (III) [87]. Finally, a randomized clinical trial on 44 infertile patients with oligoasthenozoospermia ameliorated sperm motility and decreased sperm leukocyte concentration after the 12-week-long administration of lycopene at the daily dosage of 30 mg compared to both the control group (who did not receive any treatment) and the antioxidant one (taking vitamin C 600 mg, vitamin E 200 mg and glutathione 300 mg, daily) (Ib) [88].

64.11 Other Compounds

64.11.1 N-Acetyl-Cysteine (NAC) or Vitamin A Plus Vitamin E and Essential Fatty Acids

An open, prospective study, conducted in 27 infertile patients who were given a combined oral antioxidants treatment with N-acetyl-cysteine (NAC) or vitamin A plus vitamin E and essential fatty acids, showed an increase of sperm concentration in oligozoospermic patients. Moreover, this treatment significantly decreased ROS and 8-OHdG production, and in the mean time, it increased the percentage of acrosome-reacted spermatozoa, the quantity of PUFA in phospholipids and sperm membrane [89] (IIb). Very recently, 120 idiopathic infertile patients were randomly given NAC alone (600 mg/d orally) or placebo for 3 months. NAC increased semen volume, sperm motility and semen viscosity [90] (Ib).

64.11.2 Pentoxifylline

Spermatozoa from 15 patients with asthenozoosperma and high ROS levels were treated in vitro with pentoxifylline to evaluate the effects of this compound on ROS generation and sperm movement. Pentoxifylline was able to

reduce the production of ROS by spermatozoa, and it slowed down the in vitro decline of the curvilinear velocity and the beat cross frequency for 6 hours. These same 15 patients and 18 asthenozoospermic patients, whose spermatozoa did not generate ROS at steady state, were then prescribed pentoxifylline at two distinct doses (300 and 1200 mg daily) to validate its in vivo outcome on ROS generation, sperm kinetics and sperm fertilizing competence. Pentoxifylline administration had no effect on spermatozoon-induced ROS formation, and it did not show any effect on sperm motility and fertilizing capacity. Nevertheless, it increased motility and beat cross frequency at the dose of 1200 mg daily (IIb) [91]. At the dose of 400 mg twice a day for 24 weeks, pentoxifylline improved sperm concentration, motility and morphology compared to placebo (Ib) [92].

64.11.3 Selenium

Selenium supplementation was given alone to 33 subfertile patients for 3 months, but it did not produce any improvement of sperm count, motility and morphology [93] (IIa). Subsequently, a trial was performed on 69 asthenozoospermic patients who received either placebo, selenium alone or selenium plus vitamin A, vitamin C and vitamin E daily for 3 months. Treatment did not show any improvement of sperm concentration, while sperm motility increased in both selenium-treated groups. This study showed that oral selenium administration is effective, especially in patients with low selenium [94] (Ib). A clinical trial investigated the usefulness of selenium (200 μg) and/or NAC (600 mg) in 468 infertile men with idiopathic oligoasthenoteratozoospermia for 6 months. This treatment was shown to be effective on all sperm parameters measured and showed a clear correlation between seminal plasma selenium concentrations, NAC and semen characteristics [95] (Ib).

64.11.4 Zinc

Zinc therapy has been shown valuable in decreasing OS, sperm apoptosis and SDF in asthenozoospermic men. Zinc associated with vitamin E or with vitamin E plus vitamin C did not result in any further significant effect [96].

On the other hand, oral zinc supplementation (220 mg twice a day for 4 months) in 14 infertile patients was effective in increasing sperm count, motility and morphology in a not-controlled study (IIb) [97]. Similarly, the 3-month-long administration at the dose of 250 mg twice a day improved sperm count and progressive motility in 100 patients with asthenozoospermia compared to placebo (Ib) [98]. These findings have been recently confirmed (IIa) [99].

Finally, a randomized, double-blinded, placebo-controlled study administering zinc (220 mg daily) plus folic acid (5 mg daily) for 16 weeks found no efficacy on sperm quality (Ib) [100]. Contrasting data were reported on post-varicocelectomized patients (Ib) [101].

Evidence on *Shao-Fu-Zhu-Yu-Tang, Picnogenol, Lepidium meyenii, Morindae officinalis* extract and combinations of antioxidants, such as *Menevit®, Proxeed® and Prelox®,* is summarized in Table 64.3 [108–111].

64.12 Conclusion

Many studies have been performed using different antioxidant compounds with the aim to improve semen parameters. Unfortunately, the end-points taken into account by these studies are often different and this does not help in understanding the efficacy of a given antioxidant. Moreover, it should be kept in mind that any andrological disease, independently of the OS, may be reversible or not according to the degree of the damage at the time of the therapeutic intervention. A prolonged exposure to OS can also cause extensive damage that, over time, can compromise the efficiency of the male accessory glands on sperm function. This represents an additional bias for many trials that have not considered the duration of the disease. All these reasons make scavenging therapy a great challenge for the andrologist.

Bearing this in mind, we attempted a primary distinction dividing the antioxidants in compounds that play positive effects and compounds that play negative effects, as reported in Table 64.3. Using an EBM method, we proposed that some compounds may be considered as first line treatment, because of the extensive investigation and the higher EBM evidence. These include: vitamin C, vitamin E, carnitines and CoQ10. The efficacy of other antioxidants is not yet supported by a sufficient number of studies. These include picnogenol, pentoxyfilline, etc., which need additional controlled trials. Other scavenging molecules, such as glutathione, zinc, lycopene and myoinositol, can be proposed as second line treatment because of the well-designed, though few, studies performed on them. Nevertheless, studies that can clarify the dark points previously analyzed are also welcome for these compounds.

64.13 Review Criteria

A systematic search was performed through the Pubmed, MEDLINE, Cochrane, Academic One Files, Google Scholar and Scopus databases, from each database inception to December 15, 2018, using Medical Subjects Headings (MeSH) indexes and keywords searches.

Table 64.3 Summary of the evidence and grading of the recommendations of the effects of each antioxidant used alone or in combination on sperm quality and function, according to the National Clinical Guidelines for Type 2 Diabetes (The Royal College of General practitioners, Effective Clinical Practice Unit, ScHAAR University of Sheffield)

Compound	Classification of evidence		Grading of recommendations
	Positive effects	No or negative effects	
Vitamin C			
Dawson et al. (1987) [29]	III		B
Fraga et al. (1991) [33]	IIb		C
Dawson et al. (1992) [30]	Ib		A
Thiele et al. (1995) [28]	IIa		B
Eskenazi et al. (2005) [31]	IIb		B
Akmal et al. (2006) [32]	III		C
Cyrus et al. (2015) [34]	Ib		A
Vitamin E			
Giovenco et al. (1987) [35]		IIa	B
Moilanen et al. (1993) [36]		Ib	A
Kessopoulou et al. (1995) [37]	Ia		A
Suleiman et al. (1996) [38]	IIa		B
Geva et al. (1996) [39]	IIa		B
Eskenazi et al. (2005) [31]	IIb		B
Ener et al. (2016) [40]		Ib	A
Vitamin C plus vitamin E			
Rolf et al. (1999) [41]		Ib	A
Greco et al. (2005) [44]	Ib		A
Greco et al. (2005) [45]	IIb		B
Vitamin E plus Selenium			
Vézina et al. (1996) [46]	Ib		A
Keskes-Ammar et al. (2003) [47]	Ib		A
Moslemi and Tavanbakhsh (2011) [48]	III		C
N-acetyl-cysteine plus vitamin E			
Comhaire et al. (2000) [89]	IIb		B
Selenium plus N-acetyl-cysteine			
Safarinejad and Safarinejad (2009) [95]	Ib		A
N-acetyl-cysteine			
Ciftci et al. (2009) [90]	Ib		
Glutathione			
Lenzi et al. (1992) [50]	III		C
Lenzi et al. (1993) [49]	Ib		A
Lenzi et al. (1994) [51]	IIa		B
Carnitines			
Moncada et al. (1992) [55]	IIb		B
Costa et al. (1994) [56]	IIb		B
Vitali et al. (1995) [57]	IIb		B
Vicari et al. (2001) [59]	Ib		A
Vicari and Calogero (2001) [68]	IIb		A
Vicari et al. (2002) [60]	Ib		A
Lenzi et al. (2003) [61]	Ib		A
Lenzi et al. (2004) [62]	Ib		A
Cavallini et al. (2004) [63]	Ib		A
Balercia et al. (2005) [64]	Ib		A
Li et al. (2005) [65]	Ib		B
De Rosa et al. (2005) [66]	IIb		A
Sigman et al. (2006) [67]		Ib	A
Zhou et al. (2007) [69]	Ia		B
Coenzyme Q10			
Lewin and Lavon (1997) [71]	IIb		B
Balercia et al. (2009) [73]	Ib		A
Safarinejad (2009) [74]	Ib		A
Safarinejad (2012) [75]	IIb		B
Nadjarzadeh et al. (2011) [77]		Ib	A

Table 64.3 (continued)

Compound	Classification of evidence		Grading of recommendations
	Positive effects	No or negative effects	
Lafuente et al. (2013) [78]	Ia		A
Myoinositol plus folic acid			
Calogero et al. (2015) [82]	Ib		A
Gulino et al. (2016) [83]	IIa		B
Andrositol®			
Montanino Oliva et al. (2016) [84]	IIb		B
Dinkova et al. (2017) [85]	IIb		B
Lycopene			
Mohanty et al. (2001) [87]	III		C
Gupta and Kumar (2002) [86]	IIb		B
Yamamoto et al. (2017) [88]	Ib		A
Picnogenol			
Roseff (2002) [102]	IIb		B
Pentoxyfilline			
Okada (1997) [91]	IIb		B
Safarinejad (2011) [92]	Ib		A
Selenium			
Iwanier and Zachara (1995) [93]		IIa	B
Scott (1998) [94]	Ib		A
Zinc			
Tikkiwal et al. (1987) [97]	IIb		B
Omu et al. (1998) [98]	Ib		A
Hadwan et al. (2015) [99]	IIa		B
Zinc plus folic acid			
Azizollahi et al. (2013) [101]		Ib	A
Raigani et al. (2014) [100]	Ib		A
Shao-Fu-Zhu-Yu-Tang			
Yang et al. (2003) [103]	IIb		B
Astacarox®			
Comhaire et al. (2005) [104]	Ib		A
Proxeed®			
Comhaire et al. (2005) [104]	Ib		A
Lepidium meyenii			
Gonzales et al. (2001) [105]	IIb		B
Lee et al. (2016) [108]	Ib		A
Linseed oil			
Comhaire and Mahmoud (2003) [106]	IV		None
Menevit®			
Tremellen et al. (2007) [107]	Ib		A
Prelox®			
Nikolova et al. (2007) [109]	Ib		A
Stanislavov et al. (2009) [110]	Ib		A
Stanislavov and Rohdewald (2014) [111]	Ib		A

The search strategy used combined MeSH terms and keywords and was based on the following keywords: "male infertility", "antioxidants", "oligozoospermia", "sperm", "semen", "spermatozoa", "oxidative stress", "sperm DNA fragmentation", "pregnancy rate", "ascorbic acid", "vitamin C", "α-tocopehrol", "vitamin E", "selenium", "glutathione", "carnitines", "coenzyme Q10", "myoinositol", "lycopene", "N-acetyl-cysteine", "folic acid", "picnogenol", "pentoxyfilline", "zinc". Additional manual searches were made using the reference lists of relevant studies. No language restriction was used for any literature search.

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Part VII

Special Topics

Fertility Preservation for Boys and Adolescents

65

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Key Points

- Fertility preservation (FP) is very challenging in adolescents and young boys.
- Testicular shielding and testicular transposition are simple methods for FP but with limited value.
- Cryopreservation is the gold standard method for FP, but sperm may not be retrieved in prepubertal boys.
- Experimental stem cell cryopreservation for future use to initiate spermatogenesis is the new hope for FP in adolescents and young boys.

65.1 Introduction

Cancer in children is rare. One percent of new cases of cancer diagnosed is under the age of 20 [1]. According to the National Cancer Institute 2017 guidelines, 15,270 children and adolescents between the ages of 0 and 19 will be diagnosed with cancer [2]. According to the International Agency for Research on Cancer (IARC), in the recent years there is a 13% increase in the reported worldwide incidence of childhood cancer (from 165,000 new cases per year to 215,000 cases for children 14 years and younger). They also stated that there are many unreported cases globally due to a lack of childhood cancer registries in many countries [3] (Table 65.1).

With the advances in cancer detection and treatment (including chemotherapy, radiotherapy, and surgical interventions), the overall 5-year survival rate in childhood, adolescent, and young adults' cancer significantly increased up to 80% in developed countries in 2017 compared to only 50% in 1975. Even though the survival rate is cancer-specific, the majority of patients can reach adulthood. The most two common cancers to occur in this age group are acute lymphoblastic leukemia and non-Hodgkin lymphoma. The 5-year survival rate has increased between year 1975 and 2017 from 10% to 88% and <50% to 89%, respectively. In contrast, in some other cancers like diffuse intrinsic pontine glioma, Wilms tumor, and sarcoma, the survival rate remains very poor with higher incidence of metastasis [4, 5].

Table 65.1 (1) Shows the most common types of cancer diagnosed in children ages 0–14 years in the USA arranged by incidence (2)

Age	0–14	15–19
Common types of Cancer	Leukemia Brain and other central nervous system tumors Lymphomas Soft tissue sarcomas (Mainly rhabdomyosarcoma) Neuroblastoma Kidney tumors	Lymphomas Brain and other central nervous system tumors Leukemia Gonadal (testicular and ovarian) germ cell tumors Thyroid cancer Melanoma

65.2 Physiology of Reproduction in Children and Adolescents

65.2.1 Hypothalamic-Pituitary-Gonadal Axis

The hypothalamic-pituitary-gonadal axis activation occurs at the age of puberty with subsequent stimulation of spermatogenesis. However, there are two activation phases for the axis in the prepubertal life, intrauterine and postnatal. During the second half of gestation, there is activation of the axis with production of high levels of testosterone. This activation phase is important for the development of genitalia and second phase of testicular descent from inguinal canal to the scrotum. The second activation phase occurs at age of 3 months and ends by 6 months where testosterone levels reach almost pubertal levels associated with slight increase in testicular size. The significance of this phase is still obscure. At puberty, under the effect of pulsatile release of gonadotropin-releasing hormone, there is increase in the

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release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH stimulates steroidogenesis in Leydig cells resulting in the production of testosterone, while FSH stimulates Sertoli cells and spermatogenic cells initiating and maintaining spermatogenesis [6].

65.2.2 Spermatogenesis

During early fetal life, the spermatogonial cells migrate from primordial germ cells to the testis where they are imbedded in the center of the seminiferous tubules and become associated with the Sertoli cells. They undergo proliferation and migration to the periphery of the tubules toward the basement membrane where they differentiate into the spermatogonia types A and B. The prepubertal testis consists of seminiferous tubules lined with Sertoli cells and spermatogonial stem cells imbedded in peritubular stroma containing Leydig cells. The spermatogonia then remain quiescent till puberty where, under the control of the hormonal surge, they start mitosis to form spermatocytes and then a series of meiotic divisions to become spermatids and finally mature sperm. This proliferation is associated with increase in testicular volume as the seminiferous tubules become distended with spermatogenic cells. It has been found that sperm could be produced even before the ability of young boys to ejaculate them as sperm were detected in urine samples of prepubertal boys [7, 8].

65.3 Effect of Cancer on Reproductive Function in Adolescents and Young Boys

Both the cancer itself and its treatment can affect fertility in many different ways:

65.3.1 Effect of Cancer on Fertility

Testicular cancer harbors the most direct relation to infertility even before initiating any treatment. Decreased semen parameters in patients with testicular cancer have been reported in several studies. The postulated mechanisms are disruption of spermatogenic cells in seminiferous tubules by the tumor, antisperm antibody production due to damage in the blood testis barrier, associated defective spermatogenesis (testicular dysgenesis syndrome), or production of reproductive hormones by the tumor that disrupts the normal hypothalamic-pituitary-gonadal axis. Notably, testicular cancer and infertility may be due to congenital defect in testicular maturation as in cases of cryptorchidism [9, 10].

Apart from testicular origin, any other cancers can affect spermatogenesis and male fertility. Pelvic and retroperitoneal tumors affecting the venous drainage of the testis may lead to secondary varicocele which is usually of high grade in these cases. Varicocele is a well-known cause for male infertility affecting sperm count, motility, and morphology as well as seminal oxidative stress and sperm DNA fragmentation [11].

Other mechanisms that may explain the deleterious effect of cancers on fertility include hypermetabolic state due to increased proliferation of cancer cells leading to production of gonadotoxic substances. Also, fever that may be associated with Hodgkin's disease may affect spermatogenesis. Some tumors lead to vitamin deficiency with secondary effect on spermatogenesis. Lastly, the stress associated with the diagnosis and management of cancer may lead to hormonal disturbances [12].

65.3.2 Effect of Chemotherapy on Fertility

Chemotherapy affects rapidly dividing cells including cancer cells and spermatogenic cells. However, the effect of chemotherapy on testicular function is dependent on type of chemotherapy used as well as the dose of the therapeutic agent. Another important factor is the initial state of spermatogenesis. In patients with defective spermatogenesis, more aggressive and prolonged effect on semen parameters is expected. Lastly, the type of spermatogenic cells affected by the gonadotoxin plays a very important role in the prognosis of fertility restoration following cancer treatment with the worst effect occurring if spematogonia or stem cells are affected. The problem with prepubertal boys and adolescents is that the spermatogenesis may not have been initiated, and therefore, spermatogonia are more liable to be affected with more chance of total loss of the testis' ability to produce sperm [13–15].

Alkylating agents and platinum analogues are having the worst prognosis with expected long-term azoospermia especially if taken during or before puberty. Bleomycin, antimetabolite agents, and plant derivatives usually cause temporary azoospermia. However, if they are added to other chemotherapeutic agents, they may lead to permanent azoospermia [11, 16]. Also, prolonged and repeated courses of chemotherapy usually exhibit bad prognosis for fertility. There is also proven dose-dependent affection of Leydig cells for chemotherapy leading to worsening of reproductive function with prolonged treatment. High cumulative doses of chemotherapy cause a significant and persistent impairment of Leydig cell function [17].

65.3.3 Effect of Radiotherapy on Fertility

The effect of radiotherapy on spermatogenesis is dependent on the dose and duration of irradiation as well as area irradiated and method of delivery of irradiation. It takes around 2–3 years

following irradiation for regain of spermatogenesis. The adverse effect on spermatogenesis starts from a dose as low as 0.1–1.2Gy with the severe damage at dose 4–6Gy according to different studies. There is no exact cut-off value for the dose causing irreversible sterilization. As expected, the longer the duration of therapy, the worst the prognosis regarding fertility with longer time for spermatogenesis recovery and more risk for irreversible azoospermia [11, 18].

The testes might be irradiated either directly as in a number of malignancies, namely, pelvic malignancies, or indirectly affected by the scattered irradiation for other parts of the body. The risk of irreversible damage of spermatogenesis after irradiation increases when the testis is a target organ. The risk of damage increases when combining chemotherapy to radiotherapy. Similarly, whole body irradiation bears more risk for spermatogenesis than targeted therapy [11].

The reproductive hormones can also be affected by irradiation. The Leydig cells are more resistant to irradiation, but with doses of 15Gy, Leydig cell damage starts and is irreversible at doses of 24–30Gy. Hypothalamic and pituitary irradiation can lead to transient or permanent depletion of gonadotropins with secondary hypogonadotropic hypogonadism. This axis in children and pubertal boys is usually affected with lower irradiation doses than adults (24–30 vs. 45Gy, respectively) [19].

65.3.4 Effect of Cancer Surgery on Fertility

Radical unilateral orchidectomy for testicular tumors can lead to decrease in semen parameters in 50% of cases that could progress to even azoospermia. This effect can be permanent or transient [20]. Another type of surgery that affects fertility is retroperitoneal surgeries as retroperitoneal lymph node dissection that may lead to an ejaculation due to injury sympathetic ganglia responsible for emission and ejaculation [20, 21].

65.4 Fertility Preservation Options

Fertility preservation in this group of patients can be challenging specially for young children and adolescent before the onset of spermatogenesis. Simple measures such as testicular shielding and sperm cryopreservation are very effective. Other more complex procedures such as testicular transposition can be offered prior to pelvic radiotherapy. Other experimental measures are available for the subset of patients before the onset of spermatogenesis.

65.4.1 Testicular Shielding

This is a very simple and old method that may help decrease the hazardous effect of radiotherapy on spermatogenesis. A shield made of lead is placed around the testis to protect it from irradiation. Many studies have assessed the effectiveness of such modality and found it to help decrease the radiotherapy dose by 3–10-folds depending on the area irradiated. There are different types of shields, the "clam shield" which is a clam shell-shaped device applied to the testis (Fig. 65.1). Some studies debated that due to cremasteric reflex the periphery of the testis is not properly shielded and recommended adding a lower pelvic block using a lead 10 cm bar. However, other studies have stated that there is no extra benefit from such modification. We believe that any modification should be used as long as it does not harm the patient and, on the contrary, may lead to extra protection of spermatogenesis even with small percentage [22, 23].

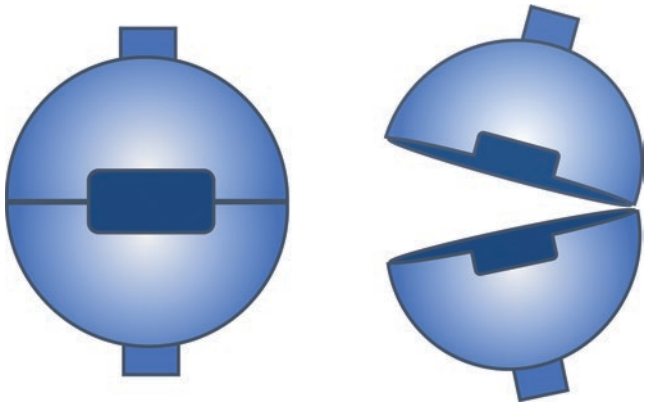

Fig. 65.1 Testicular shield

65.4.2 Sperm Cryopreservation

Sperm cryopreservation is the gold standard reliable method for fertility preservation. It is well studied and reported that post-thaw sperm count is markedly reduced especially with prolonged cryopreservation period as in the case of adolescents who will not seek fertility except after many years and thus may risk the chances of restoring fertility. However, this is not the main challenge faced in sperm banking for this age group. Sperm source for cryopreservation could be the ejaculated semen or testicular sperm.

65.4.2.1 Ejaculate

Obtaining a semen sample for cryopreservation is the most straightforward scenario for fertility preservation. Spermatogenesis starts before puberty, and although sperm production by ejaculation could not be done before puberty, sperm could be found in the urine samples of prepubertal boy [24]. Boys who are at ≥ Tanner stage III of development are theoretically, mentally, and physically mature enough to produce a semen sample by masturbation. However, there are a number of other challenges in this age group of patients (young boys and adolescents) that limit successful collection

of sperm sample. Spermatogenesis may not have been started in prepubertal boys, and therefore, this method will not be beneficial. The young age of the patients may limit their ability to ejaculate especially in the presence of cultural and religious barriers that prohibit the act of masturbation and psychological stress from the cancer itself. The tumor itself may hinder the ability of ejaculation if the tumor is affecting the conscious level of the patient as in brain tumors or if it is disrupting the neurological pathway of ejaculation as seen in spinal cord lesions [25].

In case of failure to produce a semen sample, electroejaculation can be used where a rectal probe is used to deliver electrical pulses to the pelvic and prostatic area to stimulate semen emission [26]. This procedure is done under general anesthesia which may not be convenient for the patients. However, as these patients usually undergo other surgical procedures for diagnosis or treatment of the primary cancer, electroejaculation can be done during the same setting. There is limited research on the effectiveness of electroejaculation in adolescents, but the available data states 50% success [27].

The quality of semen and its suitableness for cryopreservation is another challenge since there is no clear data on semen quality in pubertal and adolescents. The few studies on adolescent boys showed that apart from lower ejaculate volume, semen parameters were comparable to adults. Therefore, multiple semen samples may be needed for proper cryopreservation for these patients [13, 28]. There are other factors that may also affect the semen quality of these patients. Some tumors, e.g., Hodgkin's lymphoma, may be associated with poor semen quality as well as other effects of cancer on spermatogenesis as discussed previously. Successful sperm cryopreservation in adolescents was reported to be 66–93% according to different studies [29, 30].

65.4.2.2 Testicular Sperm Extraction

In adolescents and peripubertal boys who failed to produce a semen sample or where semen samples were not fit for cryopreservation, surgical sperm retrieval could be tried. This could be done by testicular sperm aspiration or more invasively microsurgical testicular sperm extraction. Potential challenges for this approach are the need for anesthesia, the possibility of delaying definitive treatment for the primary disease due to testicular sperm retrieval procedure, or in cases with testicular tumors where testicular procedures may lead to spread of the disease and are therefore contraindicated [31]. However, in case of testicular tumors, testicular sperm extraction (onco-TESE) could be done during the same setting for orchidectomy. Although there is no study on this issue in adolescents, few studies reported successful sperm retrieval by onco-TESE in adult patients. The main drawback of such procedure is the disruption of the histopathology specimen that may hinder proper classification of the cancer [32].

65.4.3 Testicular Transposition

Testicular transposition is a new fertility preservation method that was assessed in prepubertal boys receiving pelvic irradiation for different cancers. It is based on a simple idea of moving the testis away from the radiotherapy beam. This is done surgically where the spermatic cord is mobilized till external inguinal ring. The gubernaculum is then dissected and cut to render the testis freely mobile. Great care should be taken so as not to injure the testicular or more importantly the vasal vessels. The testis can be then wrapped in silicon material or not and then fixed laterally to the thigh or upward beneath anterior abdominal wall. It is kept in this new position until finishing the radiotherapy and is then brought back and fixed to the scrotum. There are few reports on semen outcome following the procedure, but the initial data is very promising [33, 34]. Although the idea may be appealing to some fertility preservation specialists, personally we think that there is limited benefit from such a procedure. The testis will still be near the radiation and may be affected by the scattered irradiation. Secondly, this procedure involves complex surgical intervention in a young boy without proven efficacy as well as delaying the definite treatment.

65.4.4 Experimental Methods

The main challenge for fertility preservation is faced with young boys where spermatogenesis has not yet started and has no sperm production. A number of experimental methods have been proposed for such cases:

65.4.4.1 Cryopreservation of Testicular Tissue and Spermatogonial Stem Cells (SSCs)

In prepubertal boys where spermatogenesis is still dormant, it has been suggested to cryopreserve testicular tissue for future use to initiate spermatogenesis. Prepubertal testis contains SSC. After puberty, SSCs attain the ability to differentiate into two spermatogonial A cells, A-dark which is responsible for renewing the SSC population and A-pale which differentiates into spermatocytes and finally sperm. The main theory in this procedure is based on the postulation that these stem cells can be cryopreserved and then in the future with proper stimulation can differentiate and start spermatogenesis [35, 36].

Testicular tissue is harvested by routine testicular sperm extraction techniques. Then, it can be cryopreserved as whole tissue or cell suspension [37]. Cell suspensions are performed using either enzymatic digestion or mechanical mincing of tis-

sue followed by cryopreservation in a media containing cryoprotectants, e.g., ethylene glycerol, DMSO, and propanediol [38]. This procedure is now tried in some oncology centers but only on experimental basis and after IRB approval. Cryopreservation of whole testicular tissue is very challenging due to the presence of different tissues each having its own freezing point. Therefore, there is increase in the possibility of formation of intracellular ice crystals in some cells during the procedure jeopardizing the success of cryopreservation [37]. Cryopreservation of cell suspension and SSC reduces this risk, but however, it is very difficult to identify SSC in the tissue and multiple cell markers are needed to be identified [38].

Cryopreservation of testicular tissue in cancer patients carries many risks. There is difficulty in obtaining and preparing the sample, and there is the lack of evidence that this tissue could be used in the future. There is also the risk of genetic instability of SSC during cryopreservation. Although some studies have shown significant genetic stability with cryopreservation, thorough multicenter research must be carried out to study this side effect.

65.4.4.2 Spermatogonial Stem Cell Maturation

The main challenge in the testicular tissue or SSC transplantation is in vitro growth of these cells. Normally, SSC relies on a number of signals from surrounding environment "niche" in order to grow and differentiate. This niche is very difficult to simulate; however, animal studies succeeded in in vitro culture of SSC in mice and larger animals like monkeys and goats [39]. The original culture media used contains Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin [40]. Supplementation of the media has been done with addition of GDNF, FGF2, lipid mixtures, and coculture with cells such as testicular stroma [41].

Using this in vitro culture resulted in differentiation of mice SSC and formation of mature sperm that was used for insemination resulting in fertile offspring [42]. This has been reproduced in goats with formation of blastocysts and with rats with production of spermatids [43].

65.4.4.3 Spermatogonial Stem Cell Transplantation

Transplantation of SSC into the testis to populate the testis with spermatogenic cells and initiate spermatogenesis in sterile testis is the future hope for fertility preservation in young boys, adolescents, and even adult males. After transplantation, SSCs migrate to the basement membrane of the seminiferous tubules and initiate spermatogenesis [39, 44]. This method was first reported in 1994 where donor SSCs were able to proliferate in the recipient testis and spermatogenesis was initiated resulting in mature sperm [45]. This procedure can be done through two mechanisms, autologous transplantation and xenografting.

Autotransplantation or autologous transplantation of SSC means reintroducing of cultured SSC into the testis of the same individual or species [46]. This was initiated in mice [47] and then was repeated in primates. One study on five cynomolgus monkeys is reported where SSCs were successfully retrieved and cryopreserved prior to sterilization by irradiation. Autologous SSC transplantation resulted in increase of testicular size in four monkeys with restoration of spermatogenesis in two of them although no mature sperm could be found [48]. In another study on rhesus monkey, autologous SSC transplantation in adult and prepubertal monkey after reaching puberty resulted in recovery of spermatogenesis in 9/12 adult and 3/5 prepubertal monkeys [49]. Up till now, there are no human studies in this regard except for one from Manchester in 2000 who unfortunately did not report their results till now [50].

Xenografting of SSC where SSCs of one species are transplanted to the testis of another species is another method of SSC transplantation [39]. This has been tried in smaller animals like rats and rabbits where SSCs were successfully transplanted in the rete testis of nude mice. This was successfully replicated in larger animals like baboons, pigs, and goat. Spermatogenesis was initiated and spermatocytes were isolated but not mature sperm [51, 52]. In humans, a similar experiment was done where human SSCs were injected into mice testis. The SSCs were maintained for 6 months but in reduced numbers and no functionality indicating that further development of spermatogenesis is dependent on human testicular microenvironment [53].

When addressing the problem of fertility preservation in prepubertal boys, another problem may arise. The efficiency of this technique requires abundant number of SSC which may not be present in younger age group of patients. Therefore, in vitro expansion of SSC may be needed to enhance their number adding more difficulties to the technique [40]. This has been successfully demonstrated in studies using rodent SSCs where SSCs retained the capacity to colonize the basal membrane of seminiferous tubules after 2 years from transplantation [54–56]. Human SSCs have been isolated from testis biopsies in adults and adolescents and were then subjected to primary culture to increase their number for the sake of SSCT using different culture systems [57, 58].

Different methods have been tried for SSC transplantation. In mice, intratubular microinjection of SSC at multiple was successful [59]. Other methods were later tried including injection in the efferent ducts or rete testis with promising results [60, 61]. In primates with larger testicular size, intratesticular injection of SSC in rete testis was tried. Ultrasound-guided rete testis injection of SSC was only successful with a recent modification of rete testis drip injection of SSC [62, 63].

SSC transplantation carries some serious risks. The possibility of reseeding of malignant cells in the body can be postulated especially in cases of testicular tumors or lymphomas. Also, genetic instability from the culture and transplantation cannot be ruled out [64]. In animal studies, the mice offsprings resulting from insemination from SSC transplanted mice were found to have slight growth retardation. Also, sperm produced were found to have lower quality [64]. In spite all this, SSC transplantation carries a huge hope for fertility preservation in young boys and adolescent cancer patients.

65.4.4.4 Transplantation of Immature Testicular Tissue (ITT)

Transplantation of whole testicular tissue instead of isolated SSC is also a proposed method for fertility preservation. It has the advantage of maintaining the testicular structure and microenvironment preserving the SSC niche needed for spermatogenesis.

Multiple animal studies have succeeded in initiating spermatogenesis after autologous or xenografting of ITT. Scientists have reported successful spermatogenesis up to sperm production when ITT of pig, rabbit, and lamb were xenografted under the skin of immunodeficient mice [51, 65–69]. Healthy offspring produced after IVF use of sperm were generated after xenografting of ITT in mouse and rabbit [66].

In humans, spermatogenesis could not be completed after xenografting of human ITT from prepubertal boys. There was only preservation of Sertoli cells and few SSC. This is most probably due to lack of appropriate microenvironment needed for spermatogenesis. However, this remains a big step for future modification of the technique to make it suitable for use in fertility preservation [70–74].

65.4.4.5 In Vitro Gamete Maturation (IVM)

In vitro maturation of cryopreserved testicular tissue aiming to grow mature sperm is another promising method for fertility preservation in prepubertal boys. It also helps to overcome the risk of cancer cell contamination faced with SSC transplantation. This can be achieved through two methods, organotypic culture (whole tissue culture) or culture of isolated spermatogenic cells [75]. This method was first described in rats in 1997 with success in IVM of cryopreserved testicular tissue and production of sperm [76]. However, in humans the results are still limited to survival of SSC and production of spermatocytes with no further progression of spermatogenesis.

Organotypic culture has the advantage of maintaining the testicular microenvironment needed for spermatogenesis [42]. IVM of testicular tissue in humans have been reported to induce maturation of Sertoli cells and Leydig cells with production of testosterone and reduction of anti-Mullerian hormone. SSC showed considerable proliferation and survived for more than 4 months, but the number decreased significantly over the course of culture. Some studies reported the presence of spermatocytes, and only one study reported the progression to round spermatids [77, 78]. With prepubertal boys, another issue arises which is the immature blood testis barrier which is a prerequisite for complete spermatogenesis [79]. Further studies and improvements in culture techniques are needed to make this method suitable as fertility preservation option; nevertheless, it remains as a good hope for future use.

In vitro culture of isolated spermatogenic cells is the other method for IVM of SSC where SSC is isolated from the testicular tissue of prepubertal boys and is then cultured in alginate, collagen, and agar matrix mimicking the testicular microenvironment [80]. This method was not successful in initiating full spermatogenesis in mice. In humans, few studies are found regarding IVM with different results. One study reported the presence of round spermatid-like cell that was used for round spermatid injection of oocyte and resulted in viable embryo that unfortunately failed to implant [81]. Another study reported the presence of round and elongated spermatids but were not used to fertilize the oocytes [82].

65.4.4.6 Artificial Gametes

Another hope for restoration of fertility in the prepubertal boys and adolescents with cancer is artificial gametes which is generation of gametes (sperm) through manipulation of its progenitor cells or somatic cells [83]. There are three different methods to generate artificial gametes. First is through transfer of nuclear material from differentiated somatic cell of the affected individual to embryonic stem cells (ESCs) of a donor resulting in the formation of a stem cell containing the individual's genetic material. Second is through transfer of nuclear material from differentiated somatic cell of the affected individual to induced pluripotent stem cells (iPSCs). Cells produced by these two methods can then be cultured in vitro or transplanted into the testis to produce sperm. Third is through direct differentiation of differentiated somatic cells to gametes without passing through stem cell or germ cell development [84].

The first two methods have been used successfully in mice with the production of sperm that was able to fertilize and produce viable offspring. In cattle, sperm could also be produced using artificial gamete technology. In human, few studies reported generation of artificial sperm but with no studies done on their fertilizing ability [83].

65.5 Challenges in Cryopreservation in Children and Adolescents

While fertility preservation is a straightforward and well-established process in adult oncology, it still remains undermined in pediatric setting with a lot of challenges and

limitations. There are a lot of points that have to be put in mind before discussing such issue.

65.5.1 Patient/Family Related

The patient and his parents are usually under severe psychological stress from the disease itself and its management. Their primary concern is usually survival of the patient, and sometimes fertility is not thought of given that the patient is a child and not in the child-bearing period. Discussing fertility preservation with them may increase their anxiety, and the perception of such discussion may vary from one person to another. Studies on young cancer male patients have shown that the child's concern for fertility is higher than the parents (44% vs. 21%) [85]. This concern or awareness of the patients increases with patients' age although parents' response is almost consistent caring more for their child's well-being and survival than for fertility [86].

In adult males, cryopreservation of ejaculated or testicular sperm is the option proposed for all oncology patients. However, in young boys and adolescents, producing a semen sample by masturbation is not always feasible. Spermatogenesis may have not still initiated in these boys. Furthermore, even if spermatogenesis is functional, some prepubertal boys cannot masturbate, and asking the boys to masturbate may sometimes seem inappropriate according to certain parents based on social and religious beliefs [87]. The patient himself may feel shy to share with his family members or treating physician that he masturbates. Therefore, proper approach toward this subject should be cautiously done. Again, discussing electroejaculation or surgical sperm retrieval with the need for anesthesia adding another procedure to what is already needed for cancer treatment adds more barriers to the discussion of fertility preservation [31, 88].

The patients' well-being is another issue where sometimes the medical or physical condition of the cancer patient is so compromised hindering his ability to produce a semen sample. Also, the necessity for starting the definitive treatment of cancer may be delayed by the fertility preservation procedure. Therefore, proper orchestration of the treatment plan is needed where fertility preservation procedures should be done simultaneously with the diagnostic and therapeutic intervention, e.g., surgical sperm retrieval can be done while the patient is receiving general anesthesia for diagnostic biopsy or lumbar puncture [89].

The cost for cryopreservation and surgical sperm retrieval is very high especially in Western countries. This will be even more for young boys based on the fact that the cryopreservation will be of long term. Also, the reuse of cryopreserved sperm in IVF later in life is costly. This financial burden may affect the decision-making for fertility preservation especially when there is no medical insurance coverage [90].

65.5.2 Physician Related

Physicians play a very important role in highlighting the importance and options for fertility preservation for cancer patients. However, there are a lot of challenges related to the physicians while dealing with such issue. The lack of knowledge is one of the important factors. Many physicians do not know where to refer the patient for fertility preservation. Also, there is lack of knowledge regarding different fertility preservation techniques especially in this patient age group of young boys [91]. A US study in 2009 found that 25% of oncologists did not know where or how to refer a patient for fertility preservation [92].

Communication barrier is another difficulty that the physicians face hindering their ability to discuss fertility preservation. Physicians indicated that they feel uncomfortable discussing fertility preservation with cancer patients especially in young boys that may be also more complicated by the presence of parents or interpreter [93, 94]. Another barriers include well-being of the patients or aggressiveness of the disease with the need to start cancer definitive treatment as soon as possible which may make the physicians feel more uncomfortable or less eager to trigger this conversation. Physician's attitude toward discussing fertility preservation varies from one country to another; where it is an increasingly usual practice in the USA and UK, physicians from developing countries lack the knowledge regarding fertility preservation [95, 96].

65.5.3 System Related

Even with the proper physicians' knowledge and patients' awareness regarding fertility preservation, the availability of fertility preservation center may be a big barrier. While in developed countries there are known sperm cryopreservation centers and usually there are preset protocols for patient referral to these centers, this is not usually available in developing countries especially rural areas. Patients may have to travel from their cities to urban cities for sperm cryopreservation adding to the difficulties of the process. Even in developed countries, the lack of sample collection and receiving areas in the pediatric hospitals may act as a barrier for proper fertility preservation procedures. New modalities and experimental methods for fertility preservation are limited to some developed countries where advanced fertility preservation studies are available making it very challenging to undergo fertility preservation for young boys [95, 97].

65.6 Ethical Considerations

With absence of clear guidelines for fertility preservation, a lot of ethical concerns emerge that requires proper handling. Most international bodies agree on discussion of the effect of cancer treatment on fertility in adolescent boys, but there are no clear rules for discussion of fertility preservation. In the UK, National Institute for Health and Care Excellence recommended that there is no lower age limit for sperm cryopreservation, but it did not discuss the use of experimental fertility preservation procedures [98]. In the USA, Ethics Committee of American Society for Reproductive Medicine has clearly stated that all fertility preservation procedures with no proven efficacy should be only used under ethically approved experimental protocols and not as regular practice [99].

The patient being a minor and not being able to make his own decision is another ethical problem. The parents or the guardians have the upper hand in taking the decision for fertility preservation. However, the patients' approval and signature is usually needed for the commencement of such procedures which should be thoroughly explained and discussed with him [89].

Another ethical concern is providing false hope for fertility in patients who undergo fertility preservation especially using experimental procedures with no proven efficacy. This is more evident in prepubertal boys where future fertility is still dependent on future discoveries [100]. Also, the risk of fetal malformations and congenital anomalies exists with the use of experimental procedures. All these risks must be clearly explained to the patient and his parents before any attempt for fertility preservation [89].

Weighing the risk-benefit for fertility preservation and effect of delaying definitive cancer treatment is a major ethical concern. With most of fertility preservation techniques for young boys being experimental, delaying of therapy and effect of this delay on child's survival is a very sensitive and important issue. This is why a multidisciplinary team including oncologist, fertility preservation specialist, and psychiatrist should be created to assess each case individually and provide their recommendations regarding fertility preservation based on case-to-case study [101].

65.7 Conclusion

The need for fertility preservation in children is increasing exponentially due to the recent improvement of the survival rate after childhood malignancies. Researchers have been experimenting new methods for fertility preservation for this age group including testicular tissue cryopreservation, spermatogonial stem cell manipulation and transplantation, in vitro gamete maturation, and artificial gametes. Although the science behind these approaches is logical, the results are still questionable making it difficult to shift these methods from the experimental to the applicable side. More effort is needed to validate such methods before use for human subjects.

65.8 Review Criteria

Extensive search of the literature was done using Pudmed, Medline, Google Scholar, and Science Direct from 1970 to 2018. The search terms included "fertility preservation," "cryopreservation," "adolescent," "childhood cancer," "spermatogonial stem cell," "spermatogenesis," "stem cell," "in vitro," "xenograft," "autologous transplantation," "allograft," "pluripotent," "pluripotency," and "embryonic stem cell." Paper titles were screened for relevance, and abstracts were read for pertinent papers. Papers written in English were only selected. Book chapters and websites were also included.

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Novel Home-Based Devices for Male Infertility Screening

66

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Key Points

- Approximately 30% of infertile couples fail to proceed with a male fertility evaluation prior to proceeding with ART.
- Many men struggle with providing a semen sample in a laboratory setting and believe it to be stressful and difficult.
- Home-based semen testing typically only provides users with sperm concentration, but it may facilitate a formal evaluation and potentially avoid unnecessary diagnostic and medical treatment for the female partners.
- Conventional laboratory semen analysis evaluates pH, volume, concentration, motility, morphology, viability, and markers of oxidative stress. Continued comparison between laboratory and smartphone-based screening should be performed.
- The YO Home Sperm Test provides accurate and precise results of motile sperm count when compared to computer-assisted semen analysis.

66.1 Introduction

Infertility is the inability of a couple to conceive after 12 months of unprotected sexual intercourse. Of the total infertility cases, 50% are attributable to male factor alone or combined with female factor [1, 2]. Both male and female partners should seek evaluation and treatment to optimize their chances of achieving a pregnancy. However, men are more hesitant to seek medical evaluation when compared to their female counterpart [3]. In a survey of men aged 25–44 years, only 9.4% underwent a fertility assessment compared to 13% of age-matched women [4]. Moreover, roughly 30% of infertile couples entirely forgo a male fertility evaluation prior to proceeding with assisted reproductive techniques, which has its own limitations [5]. The semen analysis has been the primary screening test to evaluate male fertility potential, and recent evidence suggests that infertile men may be at higher risk for adverse health later in life. Further research is necessary to elucidate the nature and etiology of the association between male infertility and its possible long-term impact on health. However, increasing interest and development of commercially available, affordable at-home semen analysis screening tests is aimed to screen male fertility parameters at home and serving as guide to further screening at specialized andrology laboratory [6].

Semen analysis provides useful information to clinicians and patients, but natural conception is an intricate process, and conventional semen analysis may not truly predict the fertility outcome [7–9]. Among standard sperm parameters, total motile sperm count (TMSC) is more predictive of fecundity when compared to concentration, motility, and morphology [10, 11]. Low motility is also inversely associated with the sperm DNA damage [12]. Manual microscopic semen evaluation and computer-assisted semen analysis (CASA) are both acceptable methods to perform conventional semen analyses. Both technologies have limitations such as human error, relatively expensive equipment, and inadequate accessibility to patients [13, 14]. Formal testing also requires trained andrology lab personnel and dedicated

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equipment, both of which are not readily available in many parts of the world. Also, many men struggle with providing a semen sample in a laboratory setting and cite it to be stressful and difficult [15].

In order to overcome these limitations and prevent unnecessary interventions on the female side in couples with male factor infertility, home-based semen screening tests were devised. At-home semen analysis offers a convenient, rapid, and cost-effective solution to this issue. This approach facilitates identification of men with subfertility who may otherwise be hesitant to seek medical evaluation, prompting a more formal assessment [3–5]. Men interested in assessing their fertility potential or those who live in areas with limited andrology services may also find at-home tests useful. Furthermore, these devices may be of interest and helpful in easy screening post vasectomy and after radiation/chemotherapy in cancer patients. This chapter reviews the evolving landscape of home and smartphone-based semen analysis. We discuss the limitations and future directions of these devices.

66.2 Conventional Semen Analysis

Since 1980, the WHO has endeavored to standardize how laboratories analyze and report semen parameters globally. The WHO manual is currently in its fifth edition which was published in 2010 [1]. The reference values for semen parameters have evolved over time and are not without controversy. For results that fall outside of the reference values, the American Urological Association recommends a clinical and/or laboratory evaluation of the male [16]. Semen analyses provide valuable information for clinicians and patients but are only a surrogate for male fertility and do not guarantee fecundity. It is unclear why some men with "normal" semen analyses may be unable to conceive and those with "abnormal" semen analyses may remain fertile [17].

The Clinical Laboratory Improvement Amendments (CLIA) has specific guidelines for laboratories to ensure quality and accurate semen analysis results [18]. Despite these guidelines, there is still a wide variability in techniques used, and inter-observer/laboratory variations and standardization are difficult. Semen testing can be performed either manually or via automated testing systems. Automated testing systems refer to computer-assisted semen analysis (CASA) or sperm analyzers such as SQA-Vision and Integrated Visual Optical System (IVOS) [19]. Although manual semen analysis results are subjective, they are considered accurate when performed by trained medical andrology technicians [1, 19]. Automated systems are also not inoculated from challenges such as costly equipment, inadequate sample preparation, optics, or poorer performance with low sperm concentrations [20]. Macroscopic and microscopic semen parameters are analyzed to determine quantitative aspects of the semen. Once the semen liquefies, typically within 20–60 minutes, a macroscopic assessment of the semen sample is performed which includes volume, pH, color, and viscosity. Subsequently, microscopic examination determines the concentration, motility, morphology, and vitality of the sperm [11].

It is important for clinicians to understand that semen parameters can vary with time in different samples taken from the same man [21, 22]. This issue can be related to many factors, such as differences in the duration of abstinence and physiological variation [23]. As a result, analysis of at least two separate semen samples is usually advised [1]. In an effort to avoid multiple visits to the laboratory as well as reducing health-care related expenditures, home-based screening tests have been developed.

66.3 Home-Based Semen Tests (Fig. 66.1)

There are several home-based semen tests that have been approved for use by the US Food and Drug Administration (FDA). They allow men to perform and interpret the test in the comfort of their own home. This can be valuable for patients who are reluctant to seek medical evaluation or lack access to andrology services. Currently available at-home semen analysis tests include SpermCheck Fertility (Princeton BioMeditech), SwimCount Sperm Quality Test (MotilityCount ApS), Micra First Step (Micra), and the Trak Male Fertility Testing System (Sandstone Diagnostics) [24–27]. Many of these products only provide users with sperm concentration, which is only one aspect of the semen analysis used to assess fertility potential. However, a simple assessment may help determine when a more formal evaluation should be obtained and potentially avoid unnecessary diagnostic and medical treatment for the female partners.

66.3.1 SpermCheck Fertility

This device is FDA-approved and commercially available with price $39.99. This product is an immunodiagnostic test that works similar to a pregnancy test. It uses a sperm concentration greater than 20 million/ml (M/ml) as its threshold for a normal result which is higher than the current WHO standard cutoff of 15 M/ml. However, it carries some limitations as it does not calculate a numerical sperm count nor provide information regarding motility or morphology. It utilizes monoclonal antibodies to detect a surface antigen,

Fig. 66.1 Home-based semen tests: (**a**) SpermCheck Fertility kit with SpermCheck device, semen transfer device, semen collection cup, and SpermCheck solution bottle. (**b**) SwimCount Sperm Quality Test with plastic cup, syringe, and test device. (**c**) Micra First Step kit with slides and microscope. (**d**) Trak Male Fertility Testing System with engine and props. (**a**: Courtesy of SpermCheck Fertility (Princeton BioMeditech), **b**: Courtesy of MotilityCount ApS, **c**: Courtesy of Micra First Step (Micra), **d**: Courtesy of Trak Male Fertility Testing System (Sandstone Diagnostics))

SP-10, located on the head of spermatozoa. SP-10 concentration has been shown to correlate with sperm concentration [24]. The SpermCheck Fertility kit consists of SpermCheck device, semen transfer device, semen collection cup, and SpermCheck solution [25].

The semen is mixed with the SpermCheck solution thereby releasing the SP-10 protein from the sperm. The mixture is transferred to the sample wells where SP-10 binds to a colloidal gold protein, forming gold-SP-10. When the newly formed gold-SP-10 complex traverses the test membrane, a red line will appear in the results window if the sperm concentration is greater than 20 M/ml. If the sperm concentration is less than 20 M/ml, there will be no red line. The test results are available in approximately 10 minutes. The manufacturers report that the test was accurate in detecting normozoospermia, oligozoospermia, or severe oligozoospermia in 96% of patients [24, 25].

66.3.2 SwimCount™ Sperm Quality Test

This product is an easy to use home test device that reports progressively motile sperm cells (PMSCs) per mL [28]. The European CE-marked version of the SwimCount™ Sperm Quality Test kit contains a plastic cup, syringe, instructions for use, and test device. Once the patient has provided a sample, they must wait 30 minutes to allow for the semen to liquefy. The syringe is used to draw 0.5 mL of the sample (avoiding bubbles, which will affect the volume). The sample is transferred onto the device where there are three distinct chambers. Only PMSCs are capable of moving from the first chamber (sample chamber) into the second chamber (separation chamber). The PMSCs are stained with a dye in the second chamber, which produces the blue color in the third chamber (detection and result window). The more PMSCs in the semen sample, the darker the color in the detection and results window. After approximately 30 minutes and pulling the slider back, the user must compare the shade of color in the results window to the reference colors on the device. The result is characterized as <5 million progressive motile sperm/mL (light color which means "low" sperm quality), 5–20 million progressive motile sperm/mL (medium color which means a "normal-mid" sperm quality), and >20 million progressive motile sperm/mL (darkest color which means a "normal-high" sperm quality). An accuracy of 95% was determined after comparison with traditional semen analysis [28]. The sensitivity and specificity of the test is 88.1% and 93.3%, respectively [28]. It is currently pending FDA approval and is available in Europe for €49.99 [14] and countries outside of Europe including Brazil and New Zealand.

66.3.3 Micra First Step

This product is a home microscopic kit that assesses semen volume, concentration, and motility [14]. The kit consists of a plastic microscope, pipette, and slides. The user transfers their ejaculated sample onto the slide using the pipette. The microscope lens contains an "analysis grid" that assists the user in calculating the sperm concentration and motility in specified visual fields. The process is similar to a manual semen analysis performed in a laboratory. However, the equipment is of lower quality, and the user is unlikely to be as highly trained as laboratory technicians for analyzing cells under a microscope. This allows for more user error and poorer accuracy when interpreting the results. The device is FDA-approved and is available for approximately $85 [14].

66.3.4 Trak System

This product was developed based on principles of centrifugal microfluidics and provides sperm concentration only [29]. The Trak System includes an instrument (the Trak engine), disposable cartridges (the Trak props), and a mobile app to record and monitor results. The user collects their ejaculate and transfers it to the liquefaction cup. A pipette is used to place 0.25 ml of the liquefied sample onto the disposable test prop which is loaded onto the Trak engine. Once the lid is closed, the engine will centrifuge the sample for approximately 6 minutes. The spermatozoa will form a pellet in the channel at the bottom of the test prop. The height of the pellet corresponds with either optimal (>55 M/ml), moderate (15–55 M/ml), or low (<15 M/ml) sperm concentrations for conception. The accuracy of typical users was determined to be 93.3%, 82.4%, and 95.5% in the low, moderate, and optimal categories, respectively [25]. The authors also demonstrated that device had a positive linear relationship with CASA (r ¼ 0.99) [25]. The product is FDA-approved and retails for $124.99 [29].

66.4 Smartphone-Based Semen Testing Devices

It is estimated that there were 2.1 billion smartphone users in 2016 and that number is expected to grow to 2.5 billion in 2019. At the time of publication of this book, over 35% of the global population use a smartphone [30]. With much of this growth stemming from developing countries with limited resources and poor accessibility to health-care services, smartphones are emerging as a powerful tool in the search of point-of-care diagnostic testing [31]. Advances in smartphone technologies have allowed for rapid processing and

transmission of data through user-friendly interfaces called "apps." Additionally, smartphones provide secure memory storage, high-resolution cameras, and built-in sensors that can be used for the detection and assessment of a variety of human biosignals [31]. Smartphones can communicate this data between the user and a centralized laboratory for professional guidance [32, 33]. Several groups have developed devices compatible with smartphones that have the potential to provide affordable and convenient home-based semen testing [34–36].

In 2016, Kobori and colleagues [33] constructed an economical single-ball lens that attaches to a smartphone in order to assess semen concentration and motility. The device consists of a polyethylene sheet and 0.8 mm in diameter single-ball lens, which provides 555 times magnification. It costs roughly $7 to produce. A small fraction of the ejaculate is placed onto the polyethylene sheet which attaches to the single-ball lens microscope device by magnetic force. The smartphone is then connected to a personal computer where the user can manually assess sperm concentration and motility from a 3-second movie clip of the sperm. The sensitivity and specificity of the device to measure oligozoospermia ($<15 \times 10^6$ spermatozoa/mL) when compared to CASA was dependent on the type of smartphone used and varied between 75.5% to 90.9% for sensitivity and 87.8% to 90.9% for specificity [34]. This device is not currently FDA-approved, and the need for a personal computer to interpret the results coupled with the potential for user error is a limitation of this device.

Another point-of-care smartphone semen testing system was designed utilizing microfluids and a wireless weight scale system [35]. This device consists of an optical attachment for the smartphone and disposable microfluidic slides. The developers analyzed 350 semen specimens which were compared to CASA testing and determined the device had an accuracy of 97.71% [35]. The smartphone app associated with this product analyzes the stored video clip to calculate sperm concentration and motility. The mean reporting time is less than 5 seconds and can be reviewed by the user or clinician. Another significant advantage is that the cost to produce this device is less than $5. However, limitations of this device include misidentifications of nonsperm objects of a similar size to a sperm head and a saturation point for sperm concentration >100 million/ml [34, 36]. It is currently in the prototyping phase and is not FDA-approved.

66.4.1 Yo Home Sperm Test (Fig. 66.2)

The YO Home Sperm Test (Medical Electronic Systems) was approved by the FDA in 2016 and is the first commercially available smartphone-based semen testing device [36]. The YO measures motile sperm concentration (MSC) utilizing the smartphone's camera and flash to record a video of the sperm. The YO kit contains the YO device, collection cup, pipettes, liquefaction powder, and YO slides to complete two YO sperm tests.

Instructions (Video 66.1):

1. Collect semen specimen in the collection cup via masturbation (no lubricants). Pour one vial of the red powder into your sample and let it "rest" for 10 minutes.
2. Place the YO clip on your smartphone (2019 version of the YO device will replace clip with an external testing module which plugs into phone).
3. Use the pipette to transfer a drop of your sample onto the red dot on the YO slide.
4. Insert the YO slide into the YO clip and press "start testing."
5. Results are typically ready within 3 minutes.

The YO device utilizes the smartphone camera to capture the light fluctuations caused by movement of sperm. The device determines the sperm concentration and motility to ultimately calculate MSC. The YO test results will report whether MSC is "low" or "moderate/normal," using six million/mL motile sperm as its threshold [37]. Agarwal and colleagues [38] performed a double-blind trial comparing the YO Home Sperm Test and an automated laboratory analyzer (SQA-Vision). They analyzed 144 aliquots of semen samples from 24 healthy donors and demonstrated that the YO device provided good correlation when compared with SQA-Vision. The Pearson and concordance correlation coefficient was above 0.92. The YO exhibited an accuracy of 97.8%. The YO device yielded a precision of 16%. The manufacturers cite an accuracy and precision of 97% and 20%, respectively [38].

The device is currently available for $59.95 [36]. Like the aforementioned home tests, point-of-care semen tests can provide the concentration of motile sperm, but do not evaluate all of the parameters commonly used to evaluate male fertility.

66.5 Limitations

Identifying men with subfertility who may otherwise be hesitant to seek medical evaluation and preventing unnecessary interventions for the female partner will certainly improve patient care, but point-of-care screening also has several limitations. The smartphone-based sperm testing devices provide only basic semen parameters such as sperm count or MSC, whereas a laboratory semen analysis evaluates significant sperm parameters such as pH, volume, concentration, motility, morphology, viability, and markers of oxidative stress. Continued comparison between laboratory and smartphone-based screening should be performed to ensure

Fig. 66.2 2019 The YO® Home Sperm Test kit. (Courtesy of Medical Electronic Systems)

accuracy and reproducibility with varying clinical conditions. In some cases, user error or potentially lack of quality control may lead to false-negative results, which could delay the actual diagnosis or treatment process. A thorough history and physical examination is a cornerstone of the male fertility assessment, and a home-based screening test cannot replace an office visit with a physician.

66.6 Conclusion

Novel smartphone-based semen screening devices are a step forward in the right direction and may overcome some of the limitations of laboratory testing including convenience, cost, access to lab, and hesitation to ejaculate in the lab. There is continuous need to devise an ideal low-cost, easy-to-use, reliable screening test which could provide substantial information on more standard sperm parameters. Current and future advancements in smartphone and point-of-care technologies will enable more men to assess semen parameters at home which will ideally facilitate identification of men with subfertility who may otherwise be hesitant to seek medical evaluation or indicate when a more formal assessment should be obtained. A reliable and more accurate home-based male infertility screening test will potentially reduce the undue female investigations and financial and psychological pressure on the couple.

66.7 Review Criteria

We extensively searched Google Scholar, PubMed, Medline, ClinicalKey, and ScienceDirect for articles focusing on semen analyses, male infertility, and home semen testing. We began our literature search September 2018 and completed it by January 2019. The following keywords were utilized in our search: "semen analysis," "microfluidics," "SpermCheck Fertility," "SwimCount Sperm Quality Test," "Micra," "Trak Male Fertility Testing System," and "YO Home Sperm Test." We reviewed only English language articles. Images were obtained with written consent.

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Ethical Dimensions of Male Infertility

67

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Key Points

- Ethics relates to moral ideas and ideals that we value as individuals and as a society. This is an important consideration in assisted reproductive technologies (ART).
- The four core principles of medical ethics or bioethics, initially described by Beauchamp and Childress, are autonomy, non-maleficence, beneficence, and justice.
- The main issues in male infertility today include oncofertility, posthumous reproduction and sperm cryopreservation, advanced paternal age, transgender fertility, and economic disparities.
- Health providers should be aware of the current ethical issues and framework in order to adequately work through any dilemma encountered in male infertility.

67.1 Introduction

Ethics relates to moral ideas and ideals that we value as individuals and as a society. Couples and individuals seeking care for infertility have grown significantly due to the availability of assisted reproductive technologies (ART) and other resources. With the rapid advancement and utilization of such techniques have come newer ethical dilemmas and policies as well. Additionally, there has been a surge in health providers focused on reproductive issues, surpassing the realm of only MDs and DOs. It is, therefore, crucial that all health providers are well versed in ethical issues and standards pertaining to male infertility. As a field, we must be prudent to keep pace with the ethical problems that follow our technological advancements in fertility.

Although there are endless potential scenarios, this chapter will focus on what is thought to be the primary issues faced in male fertility today. The four core principles of medical ethics or bioethics, initially described by Beauchamp and Childress, are autonomy, non-maleficence, beneficence, and justice [1]. This will serve as a framework for thinking about the main ethical issues providers may face with male patients seeking care for male infertility. The main issues in male infertility are the following: oncofertility, posthumous reproduction and sperm cryopreservation, advanced paternal age, transgender fertility, and economic disparities (Table 67.1).

Table 67.1 Core principles and definitions of bioethics, according to Beauchamp and Childress

Bioethical principle	Definition
Autonomy	Respecting and supporting autonomous decisions and thoughts, free of coercion, which allows the patient to make a fully informed decision
Justice	The fair distribution of risks, benefits, and costs of treatment across populations and incorporating existing laws
Beneficence	Providing care with the intent of maximizing benefits against costs and risks and reducing or preventing harm and balancing benefits against risks and costs
Non-maleficence	Providing care with the intent to avoid causing harm

Based on data from Ref. [1]

67.2 Oncofertility

In the USA, the lifetime probability of being diagnosed with invasive cancer as a male is about 40% [2]. It is well known that underlying oncological processes can negatively impact fertility in men, with damage to sperm possibly occurring before initiation of treatment [3, 4]. The respective treatments,

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including radiation, chemotherapy, and surgery, all have significant deleterious effects on male fertility. These include direct damage to the male sexual organs ultimately affecting spermatogenesis and erectile function, hormonal changes via disruptions in the hypothalamic-pituitary-gonadal (HPG) axis, cytotoxicity, and psychological distress and depression affecting sexual performance and attempts at conception [3, 4]. Cancers of the genitourinary tract, in particular, may affect fertility at various steps in the reproductive process, from the HPG axis to spermatogenesis and erections. Of note, testicular cancer is the most common form of urologic cancer in men ages 15–45. Therefore, sperm is quite vulnerable to the various treatment modalities, requiring preservation prior to cancer treatment.

Oncofertility is easier in men due to the ease of sperm banking. Also, it seems that future fertility is often desired by young men with cancer. In a survey study by Schover et al., approximately 50% of men with newly diagnosed cancer ages 14–40 wanted to father children in the future. Therefore, urologists specializing in fertility are essential in the multidisciplinary care of male cancer patients [5].

There are various ethical issues pertaining to male oncofertility. It is estimated that 30–50% of people may not receive adequate information about fertility risks and preservation practices before initiating cancer treatment. This represents a significant knowledge gap in our patients. The lack of information for sperm cryopreservation could be attributed to several factors. Patients may have limited access to providers who have a sufficient understanding of the risks, benefits, and options for fertility preservation in the setting of cancer. There could also be limitations of providers and facilities offering sperm preservation based on insurance coverage, which may influence how this topic is presented, if at all. Appropriate and comprehensive counseling from providers about the potentially irreversible effects of cancer treatments should be an essential aspect of medical care. The inability to provide complete information and access violates the ethical principle of justice as this sets up an imbalance in risk and cost. Furthermore, incomplete information makes it difficult for patients to have autonomy if they are not fully informed. Another issue at hand is that of autonomy in young male patients who may not appreciate the downstream effects of electing against sperm preservation in the setting of cancer. In an attempt to adhere to the principle of beneficence, providers may be forcing their ideas or coercing patients into sperm preservation, which are violations of autonomy.

67.3 Posthumous Reproduction/Sperm Retrieval (PHR/PSR)

Posthumous reproduction (PHR) has been an ethical issue for decades. This occurs when a child is born after the death of one or more of its parents. This can result either from one of two avenues: sperm extraction directly from a dying or deceased male for use at a future date or utilizing cryopreserved sperm of a dying or deceased male for insemination. It is a particularly relevant issue in high-risk populations, including men with terminal illnesses and those in the armed forces. Comparable to the generally accepted practice of posthumous organ donation, PHR is unique in that it creates life rather than prolonging life (Table 67.2).

Table 67.2 American Society for Reproductive Medicine (ASRM) guidelines on posthumous reproduction

Posthumous gamete (sperm or oocyte) procurement and reproduction are ethically justifiable if written documentation from the deceased authorizing the procedure is available
Programs are not obligated to participate in such activities, but in any case should develop written policies regarding the specific circumstances in which they or will not participate in such activities
In the absence of written documentation from the decedent, programs open to considering requests for posthumous gamete procurement or reproduction should only do so when such requests are initiated by the surviving spouse or life partner
It is very important to allow adequate time for grieving and counseling before the posthumous use of gametes or embryos for reproduction

Based on data from Ref. [6]

Multiple ethical issues arise in the setting of PHR as well. Primarily, PHR is a threat to the autonomy of the deceased male. Did the male consent to a sperm retrieval procedure? If he did, did he do so with the intent of PHR and posthumous parenting? This can be quite challenging especially in male minors who have banked sperm without time to spell out or think about their ultimate intention. Did he want his partner raising his child if he was dead? Did he want his testicles surgically opened if he died? However, if these are the intentions of the decedent and it was expressed, not acting in accord with his wishes could also be a violation of autonomy.

On the other hand, the desire for PHR may be the immediate grief reactions from a spouse or family members who want to preserve and protect the decedent's legacy. Other ethical principles that must be taken into account involve the concept of property and rights around the stored semen of a deceased individual. Does the stored semen legally belong to the spouse or the family if intentions have not been clearly spelled out by the decedent? Additionally, the long-term psychological impact on the child who is the product of PHR can be detrimental. Guidelines, such as those summarized by the American Society for Reproductive Medicine (ASRM), can guide urologists about proceeding with care in these instances.

There have been a few landmark court cases addressing PHR and may provide some key examples of the ethical dilemmas in this area. In the case of Hecht vs. the Superior Court of Los Angeles County (California) in 1993, the children of a deceased man fought his partner over 15 vials of stored semen she wanted to use for PHR [7]. The appeals

court sided with the spouse. In the case of Mrs. Blood or Regina vs. Human Fertilization and Embryology Authority (UK) in 1997, the sperm of Mrs. Blood's comatose husband was surgically retrieved and frozen upon her request [8]. When the husband later died, she used his sperm to conceive a child [8]. However, the court decided that cryopreservation of the sperm and its later use were not allowed under the current laws.

67.4 Advanced Paternal Age

Paternal age has been increasing for the last two decades. Similar to the phenomenon seen with the rising rates of advanced maternal age, these trends are multifactorial. The most significant underlying factor is the impact of career on family planning, which leads to delayed childbirth. Additionally, the advancements and availability of ART have made it possible for older men to make parenthood a reality if family goals change in the future.

From a biological standpoint, there are no physical risks to the potential father when it comes to childbearing. This is in stark contrast to advanced maternal age, where the obstetrical risks and burdens to the mother are significant and increase over time. For this reason, consideration of paternal age was never really a consideration given the focus placed on women in these situations. For example, the ASRM Ethics Committee discourages, and even advises denying, embryo transfer to women over age 50 [9]. There is no statement in the guidelines denying embryo transfer for male partners over age 50 [9]. Currently, there are no age limits on paternal age for ART.

Reduced fertility in men occurs in the late 30s and early 40s [10, 11]. Multiple studies have identified the decreased birth rates and increased risk of intrauterine losses with older male parents, possibly due to increased aneuploidy rates and structural chromosomal abnormalities [10, 11]. For example, a large prospective cohort study in California of over 5000 pregnant women found that the spontaneous abortion rate increased with advanced paternal age and showed an association with first trimester losses [12]. Additionally, some studies have noted an association with advanced paternal age and prematurity rates [11]. Advanced paternal age has also been associated with multiple autosomal dominant diseases, including achondroplasia, multiple endocrine neoplasia type 2B, and craniosynostotic diseases [10]. There have also been some data suggesting an increased risk of more complex diseases, such as schizophrenia and autism [10, 13, 14].

Procreative liberty describes an individual's right against interference by state or other parties with reproductive decisions [15]. However, this personal right comes at a cost to the future child and society. Procreative liberty in the context of advanced paternal age has become problematic for multiple reasons, many of which have also been identified as issues in advanced maternal age. One major issue of honoring someone's autonomy and procreative liberty is exposing a future child to some of the aforementioned medical risks at birth and/or during their lifetime. People that choose to have children during early adult years face issues with genetic abnormalities and disabilities in their offspring. These are known and generally acceptable risks for anyone having children. However, engaging in these common obstetrical risks is very different from the risks taken on by older parents. There is substantial evidence indicating the increased risk of abnormalities with advanced age, although advanced maternal age is typically considered to be more detrimental. Are we, as providers, truly practicing non-maleficence and justice toward the offspring of older parents, when we are also well aware of the increased risks? Is respecting someone's procreative liberty and autonomy more important than minimizing the exposure of certain disabilities and abnormalities in their future children? The tough question is whether or not we should accept the risks and subsequently facilitate fertility in our older male patients.

Another notable ethical issue involves the life of a child with an older parent. The psychological distress of losing a parent is significant at any age. It is more detrimental, however, for an adolescent to lose a parent, compared to that of an older, middle-aged person. The long-term effects of such a loss could have a significant impact on the course of that child's life. Furthermore, some of the responsibilities adults must handle for aging parents may be quite difficult for a younger person. Children born to older parents may also be living with certain conditions and may harbor some resentment, especially if this condition limits their ability to contribute to society.

Societal limitations on childbirth pose issues of justice and fairness to older parents. Currently, there are ample state and local programs that assist preteens in managing the risks of earlier births. Although their theoretical medical risks are not comparable to those with advanced age, one could argue that society should rather conform to the changing times of older parenthood. This could entail initiating programs for older parents that also help mitigate the unique risks their offspring face with childbirth.

The growing phenomenon of advanced paternal age will have significant policy implications in the future as we continue to realize the full spectrum of long-term effects in this population of children.

67.5 Transgender Fertility (Male to Female)

Transgender medicine brings a new wave of ethical issues to fertility conversations. Transgender describes someone whose gender identity differs from their birth-assigned gender [16]. The focus of this section will be on transgender

women, i.e., assigned male at birth. The phase of life that people transition to transgender from their birth-assigned gender can affect reproductive choices. According to the American Society for Reproductive Medicine, providers should offer fertility preservation options to individuals before they decide to transition via surgical and hormonal treatments [16]. If providers are not capable of adequately providing information and resources regarding fertility preservation, they are obligated to refer patients to the appropriate providers [16].

A major issue present in transgender fertility pertains to providing complete and accurate information, including sperm cryopreservation. Access to information on fertility-preserving technologies in male to female patients is challenging and leads to uninformed decision-making. Many patients are unaware that hormone treatments for male to female transitions are less easily reversible than female to male transitions. The effects of exogenous hormonal treatments and gonadectomy (orchiectomy in the case of transgender women) have well-established impacts on fertility [16]. It is the ethical duty of health providers to ensure that transgender individuals are aware of these effects.

The main ethical issue in transgender medicine involves a violation of autonomy. Many people, including health providers, have biases against transgender individuals and their procreative liberties. Some of these biases include questioning the psychological stability of transgender individuals and their suitability to parent. Multiple studies have shown no association between gender dysphoria, depression, and psychosocial problems in the children of transgender individuals [16, 17]. Social stigmas and overt discrimination from providers have led to incomplete information and counseling for transgender individuals, which inhibits their ability to make fully informed decisions on reproduction.

Furthermore, given the unknown medical risks in the offspring of people who have received hormonal treatments, trying to minimize harm for the future potential child is also challenging for the provider. Insurance coverage for transgender fertility options also remains unclear and not standardized. The ethical challenges and relevant policies continue to evolve as more transgender individuals actively seek fertility options.

67.6 Economic Disparities

It is well known that assisted reproductive technologies are expensive ventures. The average cost of a particular procedure varies based on procedure type and insurance coverage. In a retrospective insurance claims study by Lemos et al., the average maternal all-cause costs (e.g., stimulation, retrieval, egg transfer and cryopreservation, sperm transfer and cryopreservation) were estimated to be about $16,000 for a successful singleton birth [18]. In a worldwide economic and policy study, Chambers et al. estimated that the cost of a standard in vitro fertilization cycle was about $12,500 [19]. Repeat cycles are not infrequent given the success rates of embryo transfers and other reproductive methods [20]. In a multicenter study by Boyle et al., percutaneous and testicular sperm aspiration costs an estimated $725, on average, exclusive of cryopreservation costs [21]. These expenses above are generally cost-prohibitive and somewhat restricted to people in higher socioeconomic brackets. Insurance coverage and assistance does vary by place of employment and state, which does help to level the playing field a bit. Given the high costs of these procedures, the primary ethical principle of justice is at play. Should the cost of assistive reproductive technologies be standardized to ensure a fair distribution of this benefit to all Americans? Unfortunately, current practice and law do not regulate these treatment costs. This is a broader ethical issue, surpassing the health provider, but must be taken into account when counseling patients about their fertility options.

67.7 Conclusions

There are multiple ethical issues at play within male infertility, which providers commonly face. This chapter highlighted some of the current leading issues faced by urologists and other health providers. The bioethical principles and arguments presented in this chapter can serve as a framework for some of the complex dilemmas encountered in male reproductive medicine. It is important for providers to be aware of these ethical principles and constantly apply them in daily practice.

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Harmful Effects of Antioxidant Therapy

68

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Key Points

- Male infertility is a major issue of concern as it affects more people during their lifetime as a common disease.
- Oxidative stress is a major cause of male infertility.
- Oxidative stress can be treated with antioxidants.
- A fine balance between oxidation and reduction must be maintained in order for spermatozoa to function properly.
- Antioxidants can be harmful to the body as they can induce oxidative stress if they are taken in in excessive amounts.
- The latter is called the "antioxidant paradox."

68.1 Introduction

Oxidative stress has an integral role in the pathophysiology of most human diseases, aging, and even reproduction. With a rapidly aging population, increased attention and study have been directed toward the use of antioxidant therapy. The appeal is that antioxidants are considered "natural" and therefore "healthy" substances and are associated with a healthy diet. This general assumption by people is reinforced by aggressive marketing campaigns in a multibillion dollar market. The hypothesis has been that decreasing oxidative stress may prevent diseases such as cancer or coronary heart disease [1, 2]. Considering that these supplements can be purchased over the counter without any prescription and even food is enriched with these supplements, it is critical that they are nontoxic and show no side effects. While initial studies of antioxidant supplementation suggested a beneficial role in disease prevention, more recent clinical trials and a meta-analysis have questioned the benefit of these therapies. Several studies have suggested that excess supplementation may in fact be harmful [3–6]. Such concerns have been confirmed in later analyses [7]. Other studies indicated positive effects of antioxidant treatments to improve male fertility [8] and also showed that too high levels of antioxidants can be teratogenic to embryos [9]. Therefore, recent attention has also focused on the use of antioxidants for the treatment of male infertility. This chapter focuses on the potentially harmful effects of antioxidant therapy.

68.2 Reactive Oxygen Species

Reactive oxygen species (ROS) are highly reactive derivatives of oxygen exhibiting half-life times in the nano- (10^{-9} s) to millisecond (10^{-3} s) range [10]. These molecules can either be of radical (molecules with one or more unpaired electrons in their outer orbit causing it to be electronically unstable such as superoxide [$\cdot O_2^-$] or the hydroxyl radical [$\cdot OH$]) or non-radical such a hydrogen peroxide (H_2O_2) in nature. As a consequence of this electronic instability of the radicals, they react instantly at the site of their generation. In contrast, non-radical ROS have paired electrons in the outer orbit with antiparallel spin [11] and are therefore more stable and rather persistent with few of them even showing half-life times up to 7 s (alkoxyl radicals, RO$\cdot$). If molecules are uncharged such as H_2O_2, they can freely travel over longer distances and even penetrate plasma membranes, which charged molecules cannot do.

In biological systems, ROS are physiological products of the cellular metabolism. In the course of the cellular energy production in the mitochondria, oxygen is reduced by taking up four electrons to highly reactive radicals as intermediates, eventually leading to the production of water. Mitochondria

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possess at least nine sites where the superoxide radical can be produced and dismutated under the influence of superoxide dismutase into H_2O_2. In addition, the production of these ROS is coupled by the so-called Fenton and Haber-Weiss reactions, where under the influence of Fe^{2+}/Fe^{3+}, the production of hydroxyl radicals is promoted. Considering that the mitochondrial electron transfer chain can be disrupted leading to subsequent leakage of electrons, this process is resulting in the fact that between 1% and 5% of the inhaled oxygen is converted into radicals [12, 13]. Normally, the generation of these cytotoxic by-products of cellular metabolism are eliminated by scavengers such as manganese superoxide dismutase, copper/zinc superoxide dismutase, glutathione peroxidase and catalase. Yet, this elimination process is not complete and cells have to deal with this situation. If the amount of ROS is at levels that can be tolerated by the cells, ROS are essential role players in normal physiological functions of cells including cell signalling and gene transcription as well as modulating male reproductive functions [14, 15]. Physiological levels of ROS are also required for normal sperm function in order to trigger essential processes such as capacitation and acrosome reaction leading to successful fertilization [16, 17]. On the other hand, in cases of illness; poor lifestyle including alcohol consumption, smoking, and vaping; exposure to electromagnetic waves from cell phones or radiation; environmental pollution; and physical stress, the body is exposed to excessive ROS levels [18], which can negatively affect male reproductive functions [19, 20]. The latter conditions can result in oxidative stress, a condition which can be and is often treated with antioxidants to scavenge detrimental levels of ROS.

68.3 Oxidative Stress

Under normal, physiological conditions, cells are functioning under rather reduced conditions. Yet, a finely balanced equilibrium has to be maintained especially for spermatozoa to fertilize oocytes [21]. If this balance is disrupted due to an excessive bodily production of ROS, excessive exposure to exogenous oxidants, or an undersupply of antioxidants and therefore a lack of antioxidant capacity, the condition is called oxidative stress (OS). OS is a concept in redox biology, which was coined by Sies in 1985 [22], and describes the imbalance between oxidants and reductants (antioxidants) in favor of the oxidants.

Considering that spermatozoa have an own general lack of antioxidative protection because the male germ cell has only very little cytoplasm and an extraordinary high content of polyunsaturated fatty acids in their plasma membrane, these cells are highly susceptible to oxidative stress. Oxidants will attack the double bonds of the membrane lipids, thereby oxidizing them in a process called lipid peroxidation. This process consists of three steps, initiation, propagation, and termination [23]. In the initiation process, a radical reacts with the conjugated double bonds in the lipids forming lipid radicals where the free electron coming from the radical is delocalized in so-called resonance structures, which are electronically more stable. In the propagation of the process, these lipid radicals react with elementary oxygen forming lipid peroxyl radicals, which in turn react with neighboring lipid molecules forming a lipid hydroperoxyl radical and thereby passing the free electron on to another lipid molecule, which is turned into a radical. This process of propagating the radical properties to the next molecule is called a radical chain reaction. Finally, if the number of lipid radicals in the plasma membrane increases, two lipid radicals can react with one another to stop the radical chain reaction. In this whole process, lipid hydroperoxyl radicals also react leading to end products such as malondialdehyde, 2-alkenales, and 4-hydroxy-2-alkenales, compounds which are highly mutagenic and genotoxic and are also forming DNA adducts [24, 25]. By way of inducing lipid peroxidation, oxidative stress is decreasing the membrane fluidity of plasma and organelle membranes, thus damaging its functions and eventually impairing sperm fertilizing capability [26, 27]. Through these molecular mechanisms, OS is negatively affecting male fertility and has generally been accepted as major contributing factor to male infertility [28–31].

68.4 Antioxidants and Male Infertility

Per definition, antioxidants are compounds that inhibit oxidation, and as such, these compounds are electron donors, whereas oxidants are electron acceptors. In order for the body to keep the bodily redox potential in equilibrium to maintain homeostasis, oxidants and antioxidants have to be kept in balance. To achieve this, the body has to take up antioxidants with the nutrition, or if that is not enough due to exposure to elevated levels of oxidants or a poor diet, antioxidant supplementation should be taken. Therefore, one needs to distinguish between dietary antioxidants and antioxidant supplements that are often heavily advertised and freely available over the counter.

In order to alleviate the effects of OS, clinicians have started to treat patients presenting for infertility for various reasons with antioxidants to scavenge excessive levels of ROS [32–34]. In this regard various groups showed that supplementary intake of antioxidants such as L-carnitine, vitamin C, vitamin E, or coenzyme Q10 has significant positive effects on semen quality including sperm DNA fragmentation, chromatin packaging, sperm concentration, and motility [8, 33–36]. A Cochrane study by Showell and co-workers [37] indicates that antioxidant supplementation in infertile men may improve pregnancy and live birth rates. In a recent

meta-analysis, analyzing 1024 articles from 65 studies [38], Huang and co-workers confirmed that seminal oxidative stress resulting from low antioxidant concentrations is associated with male infertility. It appears that particularly patients suffering from idiopathic male infertility and varicocele benefit from an antioxidant treatment [8, 39–41]. Although it seems logical and results are straightforward that oxidative stress needs to be treated with antioxidants, other authors, on the other hand, report no or even negative effects on specific sperm parameters of such treatments [42–44].

68.5 Antioxidants: A Double-Edged Sword

68.5.1 The "Antioxidant Paradox"

Since it is well known that antioxidants scavenge ROS and thereby decrease oxidative stress, clinicians prescribe antioxidants for that purpose, and patients are buying these products in health shops or elsewhere over the counter, and such antioxidant supplementation makes sense because living an unhealthy lifestyle is setting the body into oxidative stress conditions. Obesity, for instance, is causing a systemic inflammatory response with high levels of ROS [45], and exposure to toxicants such as smoking, alcohol, radiation, or environmental pollutants does the same [46, 47]. Since there is no regulation and these products are massively advertised as being "healthy," it is possible that patients might unintentionally take very high doses of such antioxidants [48].

Conversely, it has been shown that taking antioxidants does not only have beneficial effects but also harmful effects. For example, high dosages of vitamin E have been shown to increase the overall death rate in patients [49]. In addition, vitamin A supplementation appears to trigger cancer in smokers rather than preventing or reducing it [50], and too high bodily vitamin C levels have the same effect on inducing oxidative stress and DNA damage than too low levels [51, 52]. These contradictory effects of antioxidants are referred to as the 'antioxidant paradox' of antioxidants [55] and maintaining the finely balanced equilibrium between oxidation and reduction is essential [53]. This "antioxidant paradox" has serious consequences for bodily health and male reproduction as not everything being assumed good for the body is indeed good, at least it depends on an appropriate concentration of both oxidants and antioxidants.

68.5.2 Reductive Stress

Maintaining the redox equilibrium is not only crucial for the body but also for spermatozoa as essential sperm functions such as capacitation and acrosome reaction are triggered by small quantities of ROS, and high levels of antioxidants will quench these effects, thus rendering sperm unable to fertilize oocytes [54–56]. The cases of too high levels of ROS are well described, and oxidative stress has been recognized as a major cause of male infertility [57]. On the other hand, however, unphysiologically high concentrations of antioxidants have also significant detrimental effects on cells, and male fertility seems to be no exception. A shift of the bodily redox balance toward the reductive side is called "reductive stress" [58], a condition which has been regarded to be as dangerous as oxidative stress [59].

In other medical disciplines like cardiology or neurology, the concept of reductive stress has already been accepted to explain pathologies and diseases such as cancer, cardiomyopathy, heart failure, brain microvasculature, blood-brain barrier dysfunction, or Alzheimer's disease [60–65]. In the reproduction arena, the necessity for a tight control of the cellular redox system has been shown for mammalian embryos [66] with high antioxidant levels being able to cause teratogenic development [9]. In turn, glycolytic energy production in early embryos seems to be beneficial for cellular compaction and blastulation as these early embryos are naturally facing a rather hypoxic environment in utero [67]. The authors hypothesize that with this metabolic shift even changes in redox-sensitive transcription factors take place and gene expression may be modified. This may affect major processes in embryonic development such as the fertilization process itself, genome activation, and/or cellular differentiation. In general, review of the available literature highlights that the redox biology of reproduction is only poorly understood. Although much has been published on oxidative stress in respect to male infertility, its counterpart, reductive stress, is widely unknown and not recognized, despite indications that exceedingly high levels of antioxidants or the wrong composition of antioxidant mixtures may have detrimental effects [68]. In this respect, Menezo and co-workers [69] investigated daily treatment of patients with a formulation consisting of vitamins C and E, β-carotene, zinc, and selenium and found that sperm DNA fragmentation improved. On the other hand, however, sperm nuclear decondensation increased possibly due to vitamin C reducing the disulphide bonds in the protamines [70] and thereby destabilizing the chromatin leading to failed fertilization.

68.6 Clinical Aspects of Antioxidant Treatment

68.6.1 Benefits of Antioxidant Treatment

Currently, no studies are available indicating the physiological range of serum and seminal redox levels including the physiological amounts of antioxidants. Clinicians and scientists know neither what the normal levels of oxi-

dative stress and antioxidants are nor how wide the physiological range of these parameters is. Consequently, the composition of antioxidant formulations and their dosage and efficacy in clinical trials are rather empiric with no clear and scientifically validated guidelines for the treatment of patients. This might be one reason for the inconsistency in the results of such studies, particularly in older and smaller studies. Nevertheless, more recent and larger studies showed encouraging positive results. Treatments with various formulations containing L-carnitine [8, 71], coenzyme Q10 [72, 73], and co-factors for antioxidant enzymes such as zinc, selenium [8, 74–76], or vitamin C [8, 77] succeeded in improving sperm count and motility. The duration of the treatment varied between 2 and 14 months. However, the evidence of such treatments in respect to pregnancy and live birth rates is still limited [78].

68.6.2 Risks of Antioxidant Treatment

As indicated above, antioxidants can have positive and negative effects on the human body; thus maintaining the equilibrium between oxidation and reduction is crucial for normal, healthy bodily functions. However, the problem is that we do not know where this equilibrium is and how wide the bodily tolerance is. To make matter worse, it has also been shown that stress, illness, exposure to toxic substances, or unhealthy diet can shift the bodily redox levels in either direction [79–84], all ultimately depending on the concentration of oxidants and antioxidants available in the body or taken up, either naturally via food or with supplementations, thus posing a distinct risk to human health including male fertility [68]. Therefore, excessive and uncontrolled intake of antioxidants may result in harmful effects making their effects a double-edged sword (Fig. 68.1).

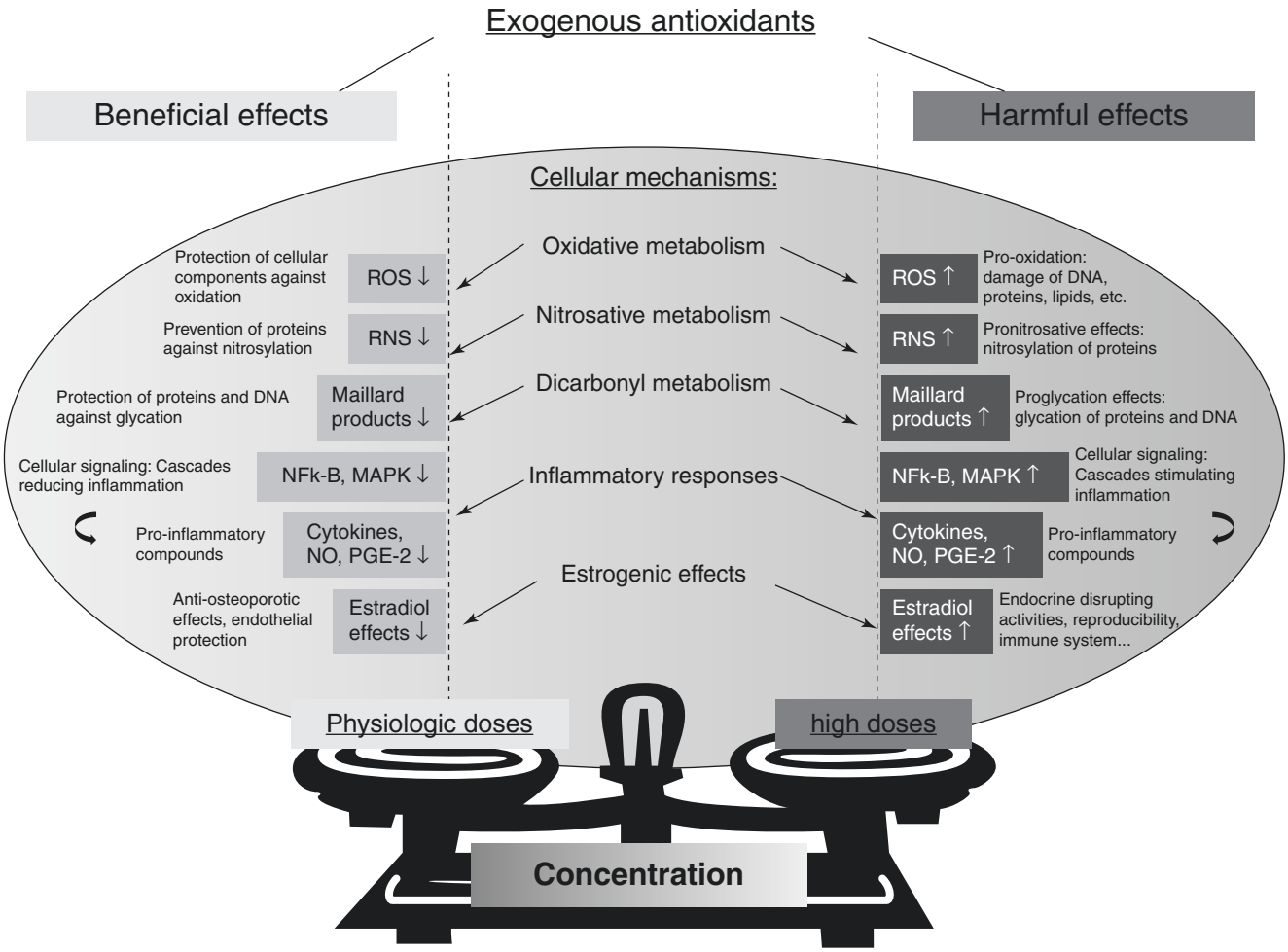

Fig. 68.1 Double-edged effects of exogenous antioxidants on cellular responses including oxidative, nitrosative, and dicarbonyl metabolisms and other pathways such as inflammatory processes depending potentially on their concentrations: physiologic doses lead to beneficial effects, whereas high doses may result in harmful effects. (Reprinted from Bouayed and Bohn [68]. With permission from Creative Commons License 3.0: https://creativecommons.org/licenses/by/3.0/)

Table 68.1 Dietary antioxidants

Foods	Reducing acid present
Cocoa bean and chocolate, spinach, turnip, rhubarb	Oxalic acid
Whole grains, maize, legumes	Phytic acid
Tea, beans, cabbage	Tannins

68.6.3 Risks of Dietary Antioxidants

Certain vegetables have high contents of oxalic acid, phytic acid, and tannins. These relatively strong reducing acids may have anti-nutrient effects by binding to dietary minerals in the gastrointestinal tract and diminishing their absorption [85, 86]. Calcium and iron deficiencies are not uncommon in developing countries where less meat is eaten, and there is high consumption of phytic acid from beans and unleavened whole grain bread [87]. In modern, industrialized nations where balanced diets are more common, the adverse effects of excessive dietary antioxidant intake are minimal. Table 68.1 lists foods containing oxalic acid, phytic acid, and tannins.

68.6.3.1 Oxalic Acid

Oxalic acid impairs calcium absorption by forming an insoluble salt of calcium oxalate. Cases of calcium deficiency have been associated with a high content of oxalates in foods [88]. A high level of oxalate intake constitutes a health risk for infants and metabolically disposed adults. Spinach is among the vegetables richest in oxalate. Sweet potatoes and peanut greens are also high in oxalic acid [89].

68.6.3.2 Phytic Acid

Phytic acid is a strong inhibitor of iron absorption in both infants and adults [90]. Iron and zinc deficiencies are widespread in infants and young children in developing countries where vegetable protein sources are often mixed with cereals. This iron deficiency in infants can lead to reduced psychomotor and mental development. Complementary foods increase the protein content and improve the protein quality of cereal-based foods. Cereals and common legumes, such as soybean, mung bean, black bean, lentils, and chickpeas, are high in phytic acid. Decreasing phytic acid by 90% (approximately 100 mg/100 g dried product) would be expected to increase iron absorption about twofold. Complete enzymatic degradation of phytic acid with cooking methods such as blanching has been recommended for at-risk populations [85, 89].

68.6.3.3 Tannins

Tannins, which include condensed tannins (proanthocyanidins) and derived tannins, belong to the flavonoid family [91]. Tannins are found in a wide variety of foods that are apples, berries, chocolate, red wines, and nuts. Derived tannins are formed during food handling and processing and are found primarily in black and oolong teas, red wine, and coffee. Flavonoids and tannins are quite sensitive to oxidative enzymes and cooking conditions.

Condensed tannins inhibit herbivore digestion by binding to consumed plant proteins and making them more difficult for animals to digest and by interfering with protein absorption and digestive enzymes. Tannins have traditionally been considered anti-nutritional, but it is now known that a large intake of tannins may cause bowel irritation, kidney irritation, liver damage, irritation of the stomach, and gastrointestinal pain in sensitive individuals [92].

68.6.3.4 Others

Nonpolar antioxidants such as eugenol, a major component of oil of cloves, have toxicity limits that can be exceeded with the misuse of undiluted essential oils. Toxicity associated with high doses of water-soluble antioxidants such as ascorbic acid is less of a concern as these compounds can be excreted rapidly in urine.

68.6.4 Risks of Antioxidant Supplements

It is well established that certain amounts of antioxidants, vitamins, and minerals are required in the diet. However, the benefit, dosing requirements, and risk profile of most antioxidant supplements are largely unknown. When used for disease prevention, the doses are several fold greater than the recommended daily allowance (RDA). The hypothesis that antioxidant supplements can prevent diseases has been proven false by researchers. In spite of this information, many companies manufacture and sell dietary supplements with antioxidants in a variety of different formulations. Common supplements include vitamins A, C, and E, selenium, resveratrol (found in grape seeds and knotweed roots), and herbal extracts like green tea and jiaogulan.

The potential for harmful effects of antioxidant therapy has been suggested, such as in the β-Carotene and Retinol Efficacy Trial (CARET) which was a randomized, double-blind, placebo-controlled chemoprevention trial in 18,314 men and women at high risk of developing lung cancer [93]. The study was initiated due to the observation of other studies that found people who have high serum β-carotene concentrations had lower rates of lung cancer [93]. The hypothesis of the CARET study was that these antioxidants would decrease the risk of lung cancer in an already high-risk population. Subjects were treated for up to 6 years. This study demonstrated that smokers who ingested a combination of 30 mg β-carotene and 25,000 IU retinyl palmitate (vitamin A) taken daily had 28% more lung cancer and 17% more deaths than placebo subjects. The CARET intervention was stopped 21 months early because of clear evidence of no benefit and substantial evidence of possible harm.

Other studies have found similar findings of adverse events. Table 68.2 lists observed side effects of supplemental antioxidants. The α-Tocopherol (Vitamin E) β-Carotene Cancer Prevention (ATBC) Study Group reported on a randomized, double-blind, placebo-controlled primary prevention trial [94]. The objective was to determine whether daily supplementation with vitamin E, β-carotene, or both would reduce the incidence of lung cancer and other cancers. A total of 29,133 male smokers 50–69 years of age from southwestern Finland were randomly assigned to one of four regimens: α-tocopherol (50 mg/day) alone, β-carotene (20 mg/day) alone, both α-tocopherol and β-carotene, or placebo. These patients were followed for 5–8 years. There was no reduction in the incidence of lung cancer among male smokers after 5–8 years of dietary supplementation with vitamin E. Those men given β-carotene had an 18% increase in the incidence of lung cancer compared to placebo. There was also an increased number of deaths due to ischemic heart disease and lung cancer in the β-carotene group compared to placebo. The vitamin E group had an increased incidence of death due to hemorrhagic stroke and an increased incidence of other cancers compared to placebo. While these data suggest that there may be harmful effects of these supplements, the authors state that further studies would need to be performed in order to validate these results [94].

Table 68.2 Observed side effects with supplemental antioxidants

Antioxidant metabolite	Recommended daily allowance (RDA)	Reported side effects
Glutathione	250 mg/day or 600 mg IM QOD for male infertility	Acute: gastrointestinal disturbances
Carotenes	15–30 mg/day	Acute: skin color changes
		Chronic: possible increased risk of death and certain cancers
α-Tocopherol (vitamin E)	22.4 IU/day	Acute: headache, fatigue, muscle weakness, creatinuria
		Chronic: impaired bone mineralization, increased bleeding, cardiovascular disease; increased overall mortality
Ascorbic acid (vitamin C)	75–90 mg/day	Acute: diarrhea
		Chronic: hyperoxaluria, urinary stone formation, iron overload
Ubiquinol (coenzyme Q10)	60–90 mg/day	Acute: gastrointestinal disturbances, heartburn, abdominal discomfort
		Chronic: hemorrhagic toxicity
Selenium	55 μg/day	Acute: fatigue, gastrointestinal disturbances, skin rashes, irritability
		Chronic: concern for diabetes, loss of hair and nails, neuropathy
Melatonin	10 mg/day (bedtime)	Acute: diarrhea, rash, dizziness, headache, heartburn, nausea
		Chronic: sleep disturbance
Zinc	8–11 mg/day	Acute: gastrointestinal disturbance, anosmia (intranasal)
		Chronic: concern for increased risk prostate cancer, copper deficiency, suppression of immune system, anemia

Observation of these adverse effects was not limited to smokers. Bjelakovic's meta-analysis from 2007 included 68 randomized trials with 232,606 participants. This publication showed that treatment with β-carotene, vitamin A, and vitamin E may increase all-cause mortality and the potential roles of vitamin C and selenium on mortality may need further study [3].

These results were later confirmed by the same authors with an additional publication using the Cochrane Collaboration methodology [3]. In this systematic review, several key findings were noted: (1) β-carotene, vitamin A, and vitamin E given singly or combined with other antioxidant supplements appeared to significantly increase mortality, (2) there was no evidence that vitamin C increases longevity, (3) selenium tended to reduce mortality, and (4) trials with inadequate bias control overestimated intervention effects [95–98]. It should be noted that only all-cause mortality, not the cause of the increased mortality, was assessed. It is likely that increased cancer and cardiovascular mortality are the main reasons for the increased all-cause mortality [99, 100].

Several other publications have disagreed with the Bjelakovic meta-analysis [49, 95, 99–101] and reported no effect on all-cause mortality. The Supplementation en Vitamines et Mineraux Antioxydants (SU.VI.MAX) study by Hercberg et al. was a randomized, double-blind, placebo-controlled primary prevention trial. A total of 13,017 participants took a single daily capsule of a combination of 120 mg of ascorbic acid, 30 mg of vitamin E, 6 mg of β-carotene, 100 μg of selenium, and 20 mg of zinc or a placebo. After a mean of 7.5 years, there were no major differences found between the groups in total cancer incidence, ischemic cardiovascular disease incidence, or all-cause mortality [101].

Miller et al. performed a meta-analysis on the dose–response relationship between vitamin E supplementation and total mortality by evaluating randomized, controlled trials. Vitamin E doses ranged from 16.5 to 2000 IU/day, and there were 135,967 who took vitamin E alone or in combination with other vitamins and minerals. While the results showed that there very well may be an increased risk of all-cause mortality with high doses of vitamin E (greater than or equal to 400 IU/day), lower doses did not reveal this same concern [49].

Although Bjelakovic et al. found no compelling evidence that antioxidant supplements have a significant beneficial effect on primary or secondary prevention of colorectal adenoma formation, in their meta-analysis of eight randomized clinical trials comparing antioxidant supplements with placebo or no intervention, they found no statistically significant effects of supplementation with β-carotene; vitamins A, C, and E; and selenium alone or in combination. Antioxidant supplements seemed to increase the development of colorectal adenoma in three low-bias risk trials (1.2, 0.99–1.4) and significantly decrease its development in five high-bias risk trials (0.59, 0.47–0.74). There was also no significant difference between the intervention groups regarding adverse events including mortality (0.82, 0.47–1.4) [95].

The mechanism of the possible negative impact of antioxidant supplements is speculative. First, it is known that oxidative stresses are a part of the pathogenesis of different chronic diseases; however, could the oxidative stress be the cause of the chronic disease or the chronic disease causing the oxidative stress [102]? Second, some essential defense mechanisms, such as phagocytosis, detoxification, and apoptosis, depend on free radicals. If impaired, a negative impact on homeostasis may ensue [103–105]. Third, unlike prescription drugs, antioxidant supplements are not put through the same thorough toxicity studies in order to be sold to consumers [106]. A better understanding of the mechanisms and actions of antioxidants toward specific disease processes is needed [107]. Finally, if antioxidants reduce the redox stress in cancer cells, then they may decrease the effectiveness of chemotherapy and radiation therapy. However, other researchers argue that the antioxidants would reduce the unintentional side effects of the cancer treatment and increase survival times [108, 109].

68.7 Conclusion

Although there is the general perception by patients as well as by clinicians that antioxidants are healthy because they are heavily marketed as such and have pronounced "anti-aging" effects, it is widely overseen that the body functions are at an equilibrium between oxidation and reduction. Yet, certain crucial physiological functions including gene activation, gene regulation, and sperm functions like capacitation, and even the fertilization process itself, essentially need a small amount of reactive oxygen species to trigger these events. An overdose of antioxidant would therefore prevent these functions from happening. Therefore, a paradigm shift needs in patients and clinicians to take place in order to understand that these so-called healthy compounds can also exert harmful effects which may not only lead to infertility but also to more serious diseases such as cancer, cardiomyopathy, and others. Hence, care must be taken when taking antioxidants.

68.8 Review Criteria

An extensive search of studies examining the impact of antioxidants on male fertility was performed using search engines such as Google Scholar and PubMed. The searches were not limited for time. Yet, the most recent records were preferred. The overall strategy for study identification and data extraction was based on the following keywords: "antioxidant," "oxidative stress," "male infertility," "vitamin C," "vitamin E," "therapy," and "treatment" as well as the names of most read authors. Articles published in languages other than English were also considered. Data that were solely published in conference or meeting proceedings, websites, or books were not included. Websites and book-chapter citations provide conceptual content only.

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Male Infertility from the Developing Nation Perspective

69

Kasonde Bowa, John S. Kachimba, Victor Mapulanga, Mumba Chalwe, and Elaikim Manda

Key Points

- Infertility is highest in the developing countries of Africa, Asia, and Eastern Europe.
- The main cause of infertility is secondary infertility in developing countries.
- The male infertility prevalence in developing country ranges from 4% to 12%.
- There has been a decline in sperm count in Africa of over 75% over the last 35 years.
- The most commonly used male infertility treatments are medical therapies such as antioxidants and antiestrogens.
- The assisted reproductive technologies are few and inaccessible to most infertile couples in developing countries.

69.1 Introduction

Infertility is defined by the World Health Organization as the inability of a sexually active couple to conceive after regular intercourse at least twice a week for a period of 12 months [1]. Infertility is an important problem worldwide that is of particular importance in societies of the developing world [1, 2]. There are 48.5 million infertile couples globally and 30 million men who are infertile. In sub-Saharan Africa, the prevalence of infertility, using the most recent demographic surveys done in this region, ranges from 22% to 35%. The male factor infertility rates using the "Sharlip factor" is a range from 4.4% to 11.5% [3–5]. The problem would appear to be most pronounced in parts of sub-Saharan Africa, in countries that lie in the "infertility belt" [5]. This is a region spanning from Cameroon in the west to Tanzania in the east and includes the following countries: Cameroon, Central African Republic, Gabon, Democratic Republic of the Congo, Togo, Sudan, Kenya, and Tanzania. In these countries, as many as one-third of couples are unable to conceive [4]. The estimated rate of male factor infertility is 20% to 30%. This factor can be used to compute male factor infertility rate (Sharlip factor), since infertility rates use the female partner as the surrogate. Current studies show that parts of West, Southern, and East Africa have very high infertility rates of between 26.4% and 36.5%. This includes in particular Mauritania, Ghana, Senegal, Gabon, Namibia, South Africa, Sudan, and Ethiopia [6–8].

In the rest of the developing countries, the highest infertility rates are found in Eastern Europe, West Asia, Central Asia, and parts of South America. The infertility rates in these regions range from 32.8% to 38.2%. The male factor infertility ranges from 6.56% to 11.5%. In particular the countries of Mexico, Brazil, Ecuador, Turkey, Pakistan, Thailand, and Myanmar have the highest infertility rates [6–8].

In general, the highest prevalence of male infertility occurs in developing countries. The highest rates are reported in Central and Eastern Europe (8%–12%) and Australia (8% to 9%) and in North America (4.5% to 6%) and Europe (7.5%). The rates reported for sub-Saharan Africa are some of the highest at 4.4% to 11.5%. Due to paucity of data in this region, it is expected that the actual rate could be even higher. The rates are influenced by the availability of fertility treatments which increases willingness of men to self-report in contrast to cultural reticence seen in developing countries [9, 10].

In addition to this, there has been an evident decline in sperm counts of up to 75% in Africa over the last 50 years. A similar decline has been seen in up to 57% in the rest of the world. In Europe the fall in sperm counts at 32.5% has been

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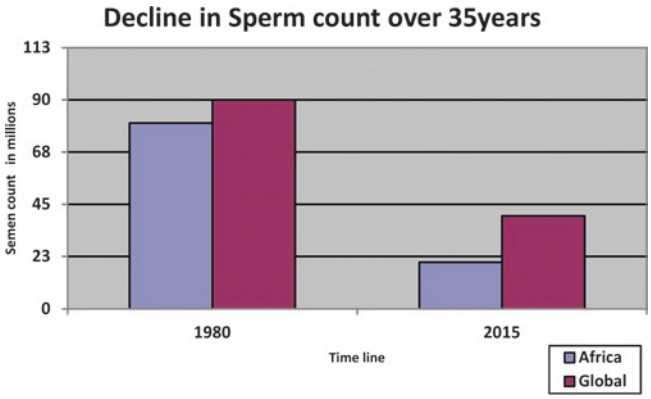

Fig. 69.1 The global vs African decline in sperm counts over the last 35 years

relatively lower. Some possible explanation for this difference includes poor health-seeking behavior in African men, high levels of STIs, HIV infection, environmental pollution, high alcohol intake, and an increase in smoking [11–13] (Fig. 69.1).

A need exists for more studies to evaluate the prevalence, risk factors, and prevention of this disease process in many parts of Africa, and the WHO has been at the forefront of this need for more research into infertility in Africa and developing countries of the Asian subcontinent. Though many infertile unions worldwide suffer from primary infertility, the predominance of secondary infertility in sub-Saharan Africa has been widely accepted [11–13]. This is also noted in developing countries of the Asian continent and South America. Furthermore, with the high prevalence of the human immunodeficiency virus infection in this part of Africa, the prevalence rates of tuberculosis, sexually transmitted infections, and septic complications associated with deliveries will rise and fuel this epidemic of secondary infertility. There remains a paucity of information on male infertility in sub-Saharan Africa. Though women bear the brunt of infertility socially, research on the continent has shown that a significant proportion of infertility is a result of male factor infertility. Male factor causes are generally present in 20% to 30% of cases of infertility [7]. Ikechebelu showed that a large proportion of couples in southeastern Nigeria presenting with infertility had male factor causation alone (42.4%), as compared to 25.8% resulting from female factors alone. It is accepted that male factor causes are present in 20% to 30% of cases of infertile couples, while in approximately 30–40% of couples, both male and female factors are responsible for infertility. Hence, in half of the couples presenting to a health facility, there is a male factor at play as a cause of infertility [14].

Assisted reproductive technologies (ART) are the main method of treatment for infertility. These have a success rate ranging from 5% in Japan to 30% in the United States. These technologies are expensive and require huge investments, and as a result, they are not widely available in developing countries. The mainstay of treatments, in developing countries, is medical therapies and antioxidants. These have a lower success rate, though they are widely used in developing countries. The WHO has proposed the use of low-technology ART services in developing countries, which may be easier to set up. However the burden of disease from infectious diseases makes it difficult for ministries of health to provide sufficient resource for fertility treatments [14].

69.2 Prevalence of Male Infertility in Developing Countries

The prevalence of male infertility has not been comprehensively studied in most of Africa. Most views on male infertility are extrapolated from studies on infertility in women. The first comprehensive epidemiologic study of infertility in the sub-Saharan Africa was conducted by Larsen. This study showed the prevalence of infertility in countries of this region to be 12.5–16% and countries of the so-called infertility belt having significantly higher prevalences [15–17].

However more recent studies have shown that though there has been a profound decline in sperm counts in Africa over the last 50 years, the estimate of the prevalence of male factor infertility is highest in developing countries of sub-Saharan Africa, Eastern Europe, and Central Asia (Table 69.1).

The prevalence of infertility in men increases with age due to decreased sperm function, an increase in genetic abnormalities, and various secondary causes such as sexually transmitted infections. Most infertile men resort to polygamy as a solution to their infertility with all the attendant risks of multiple partner relationships. The key determinants of fertility are economic, social, cultural, and medical. The medical determinants of fertility in Africa are HIV infection, sexually transmitted infections, tuberculosis, and, in areas of Africa with high prevalence, hemoglobinopathies (such as sickle cell disease and thalassemia). Unlike developed countries where there is a high number of urology specialists as recommended by WHO

Table 69.1 The prevalence of male infertility in selected regions of the world. Regional male infertility prevalence

Region	Estimated prevalence	Source
North America	9.4%	Agrawal et al. (2015)
Eastern Europe and Central Asia	8%–12%	
Sub-Saharan Africa	4.4%–11.5%	Stat compiler DHS
Europe	9%	

(1:100,000 population), who are able to manage and prevent male infertility, this is not the case in Africa. Aside from South Africa and Egypt, there are low numbers of urologists across Africa. The early detection of undescended testis, testicular torsion, and varicocele means that these treatable conditions will not result in male infertility. The management of established infertility through medical or surgical means is also limited by this manpower constraint. Furthermore, there are only a few centers in Egypt and South Africa that provide various methods of assisted reproductive treatments. The total fertility rates are only a proxy of male fertility rates. A more direct determinant of male fertility rates are sperm counts. Over the last 40 years, there has been a decline in sperm counts worldwide [15, 16].

69.3 Etiology of Male Infertility in Africa

In sub-Saharan Africa, the principal causes of male infertility are acquired and result in secondary infertility. This results from sexually transmitted diseases and iatrogenic causes due to poor surgical care in many areas. There is a small percentage of men who experience infertility as a result of anatomic, genetic, hormonal, and immunologic problems. These form a small percentage of infertile men that is reflected in virtually all populations and estimated to form approximately 5% of the infertile male population. In sub-Saharan Africa, sexually transmitted diseases are the main cause of male infertility [17, 18].

69.4 Reproductive Tract Infections

69.4.1 *Neisseria Gonorrhoeae*

Gonorrhea is a worldwide infection that has seen resurgence in prevalence in the last few decades. Approximately 22% of men develop gonorrhea after a single episode of vaginal intercourse with an infected female [19]. The infection starts as a cervicitis in females that is largely asymptomatic and facilitates its spread. In the male, gonorrhea begins as a urethritis that is symptomatic. Dunn et al. in a study of rural men in India found a high level of reproductive health problems. More than 10% of these men had urethral discharge that they did not seek medical treatment and had continued to have sexual relationships without the use of condoms. This is a scenario that is common in many settings in sub-Saharan Africa. Untreated urethritis ascends and involves the prostate, seminal vesicles, and epididymis. Chronic seminal vesiculitis and chronic epididymitis are associated with abnormal sperm parameters and later complicated into an obstructive azoospermia [20].

69.4.2 Chlamydia Trachomatis

Chlamydia trachomatis causes nongonococcal urethritis in sexually active men and frequently complicates into epididymitis. This is an indolent infection that goes unrecognized for long periods and is a common finding in infertile men [21].

69.4.3 Human Immunodeficiency Virus Infection

HIV infection is a global epidemic infecting millions of people worldwide. The majority of infections occur in the third world. Sub-Saharan Africa's deaths due to HIV/AIDS were estimated to account for 76% of the 2.1 million deaths occurring worldwide as a result of this pandemic [22]. HIV/AIDS is the leading cause of death in sub-Saharan Africa. The Joint United Nations Programme on HIV/AIDS (UNAIDS) estimates that in 2007, there were 2.5 million new infections with HIV/AIDS, 68% of these occurred in sub-Saharan Africa [22]. Men infected with HIV are at risk of infertility secondary to altered spermatogenesis. Though the exact mechanism remains unclear, it is postulated to occur by a direct effect of the infection and an increased susceptibility to sexually transmitted infections and their occlusive sequelae.

69.4.4 Genital Tuberculosis

Tuberculosis continues to be a worldwide problem, and in Africa, HIV infection has led to a dramatic increase in the numbers of patients. Alongside the high HIV prevalence, there is a high incidence of TB. The WHO reports that over 1.9 billion people are currently infected with TB [23]. Most of these are in the low- to middle-income countries of Asia and Africa. It has been reported that there has been an annual increase in TB of 10% in developing countries as a result of HIV [23]. There is a high coinfection with TB in patients with HIV infection, and this occurs mainly when the CD4 count falls below 200. Extrapulmonary tuberculosis accounts for approximately 15% of cases, of which 14% are patients presenting with genitourinary tuberculosis [23]. In approximately a third of patients with genitourinary TB, the infection affects the genital tract only. Genitourinary TB affects more men than women (ratio 2:1). The highest incidence is in the age group 30–50 years. The prostate, epididymis, and seminal vesicles are the commonly affected sites. The most common site is the epididymis. Sexual transmission from female to male has been described in 3.9% of cases [23]. The diagnosis is difficult, and a high index of suspicion is required to diagnose this condition early and commence therapy before obstructive complications

occur. Uncharacteristic urologic complaints, sterile pyuria, persistent cystitis, infertility without overt genital lesions, or sperm anomalies on evaluation should be investigated for the exclusion of genitourinary TB. TB of the genital tract can occur as an isolated disease leading to obstruction of the seminal ducts and vasa leading to infertility.

69.4.5 Genitourinary Bilharziasis

Bilharziasis is a communicable disease of major public health importance in the developing world that unfortunately remains neglected. It is estimated that over 200 million people are infected with this parasite and > 80% live in Africa [18]. Bilharziasis of the genitourinary tract is due to infection with *Schistosoma haematobium*. Bilharzial infection leading to infertility affects the prostate, seminal vesicles, spermatic cord, and epididymis. The testis has remarkable immunity against bilharzial infection. Disease in these structures frequently affects the reproductive age group from 20 to 40 years with chronic vague symptoms that may include perineal discomfort, low back pain, dysuria, and hemospermia. As the infestation enters its chronic stage, the process of fibrosis predominates. The process of fibrosis in the seminal vesicles is marked to a degree that is unsurpassed by any other organ, including the bladder [24]. The extensive fibrosis in these structures leads to obstructive azoospermia if not diagnosed and treated early [25].

69.4.6 Lepromatous Leprosy

Leprosy occurs predominantly in Asia, Africa, and South America. Leprosy is a chronic mycobacterial disease of man caused by the *Mycobacterium leprae* that affects the peripheral nerves mainly and secondarily involves the skin and other organs including the testes. The testes are the only internal organ outside the body, and *M. leprae* has a preference for the lower temperatures in these organs [26]. Atrophy of the testes is common in patients with lepromatous leprosy with resultant infertility and impotence [26, 27].

69.4.7 Sickle Cell Disease

In areas of Africa where malaria is endemic, there is a high prevalence of sickle cell disease (SCD). Malaria is a parasitic infection which is the leading cause of mortality and morbidity in children under 5 years of age. The sickle cell trait reduces the severity of malaria and incidence of malaria in carriers. Therefore, the trait has persisted at a high prevalence of sickle cell disease and other hemoglobinopathies because of this protective effect. The prevalence of SCD/thalassemias is 15–20% in the populations of Central, Western, and North Africa [28]. In SCD patients, 10–15% of patients will have recurrent priapism that may result in erectile dysfunction and affect fertility. Men with SCD and those with thalassemias have been shown to have reduced sperm counts compared to matched controls. This infertility is the result of an acquired hypogonadotropic hypogonadism at the level of the pituitary. In addition, it is worsened by recurrent infections and infarctions in the testes resulting in further reduction in testicular function [28].

69.4.8 Mycotoxin and Environmental Toxins

The levels of male infertility are higher in developing countries than developed countries; this is often sorely explained by the high prevalence of STI, TB, and HIV. However some workers have shown that this difference could be related to the economic activities. Many developing countries have a large rural population, with economic activity based on agricultural products. This exposes men to pesticides and mycotoxins which have been shown to interfere with reproductive function [29]. These chemical substances have been shown to damage reproductive cells which results in a decline in sperm count. Previous studies supporting this show that the sperm decline among men in close contact with mycotoxins such as aflatoxins B1 and ochratoxins A. These are present in local preserved foods in african communities [29, 30].

69.4.9 Smoking and Alcohol Intake on Male Infertility

The prevalence of smoking and alcohol intake in many developing countries is on the rise. Smoking and alcohol surveys have shown an increase among teenage boys. Many papers have shown some effect of alcohol on sperm counts and sperm production. However a number of papers have shown that alcohol and smoking may not be among the most significant factors which affect male fertility. It has been shown that the predominant factors are STIs and other testicular disease [29, 30].

69.4.10 Iatrogenic Causes of Male Infertility in Africa

While reviews of iatrogenic causes of infertility in Western Europe reveal that this contributes approximately 5% of cases of infertility in both men and women, in Africa, this rate is higher [31]. While most evidence highlights this cause in women, it is also a cause of infertility in men. Kuku and Osegbe showed a pattern of male infertility due to vascular injuries sustained during hernia repairs in Nigeria [31].

69.4.11 Management of Male Infertility in Africa

Frequently in the African culture, infertility is blamed on the woman and results in divorce. The male seeks fertility services reluctantly and often after a number of failed marriages. The male client will typically have attempted several local remedies before coming to seek medical advice. The longer the period of infertility that lapses, the lower the chances of conceiving. If the duration of infertility is over 4 years of unprotected sexual intercourse, the conception rate per month is only 1.5% . Thus, it is very important to start investigating and treating infertility at the earliest opportunity.

Evaluating and treating infertile couples is a costly procedure, and in Africa, a cost-effective approach is adopted. The couple is approached and treated as a whole, and investigation is undertaken in both to provide the best chance for a successful outcome. A successful outcome is the delivery of a healthy child. Secondary infertility is much more common in this region, and certain basics are followed to minimize expense on time and investigations. A detailed history from both partners along with a simple semen analysis is usually sufficient to make a clinical diagnosis. A history that looks at previous STIs, hernia surgery, infection with any endemic infections like TB, bilharziasis, and HIV which lead to infertility should reduce the need for more complex and invariably expensive investigations. This can be followed by hormonal profile where indicated. Postejaculatory urine and ultrasonography are additional methods of evaluation depending on the etiology. In many centers in Africa, the clinician will rely on the history and a semen analysis. The parameters for semen analysis are assessed using WHO guidelines. Two samples given at separate occasions, approximately 6–8 weeks apart, are required to make a diagnosis.

The treatment is divided into medical, surgical, and ARTs. In many cases where there is a demonstrable obstruction to a reproductive structure or a varicocele, surgery is the only treatment option available. Varicocelectomy has shown success in treating infertility in the absence of other causes. Up to two-thirds of patients experience some improvement in sperm quality after varicocelectomy [32, 33]. Surgical repair of an obstructed vas has provided a much greater challenge in sub-Saharan Africa where the expertise and technology is lacking. Medical therapy consists of several empirical treatments with varying success rates. Common drugs in use include clomiphene which has antiestrogen properties and works by lowering estrogen while raising the levels of follicular-stimulating hormone (FSH) which in turn increases spermatogenesis. Tamoxifen is used on similar grounds. Another drug available for the medical treatment of the infertile male is pentoxifylline that works by improving circulation and has been shown to improve sperm motility. Several antioxidant vitamins such as vitamins A, E, and C are used as they have been shown to improve sperm motility and quality.

The next logical measure in the management of infertility in Africa will be the introduction of ART [32, 33]. Though the indications will be no different from developing countries, the demand will be higher due to the prevalence of tubal obstructive causes and male factor infertility. Suboptimal semen parameters that are found in significant numbers of infertile African men can potentially be effectively treated by intracytoplasmic sperm injection (ICSI). The need therefore for efficient ART services in the African region cannot be overemphasized. Needless to say, the establishment of ART services in Africa is faced with enormous obstacles because these are expensive services to establish and maintain. It has been conclusively shown in Nigeria and Ghana that ART service delivery in a public hospital system is practicable and effective as a backup for infertility cases that are intractable to conventional treatment as in almost 60% of the infertile population. Despite all the challenges of specialized manpower, equipment, and infrastructure, ART services have been set up in South Africa, Nigeria, Ghana, and Egypt and are being accessed by the affluent portions of the population. Their impact on the treatment of infertility in this region is minimal as the majority of patients come from economically disadvantaged backgrounds. As countries in the region look to ARTs in the management of infertility, intrauterine insemination may be a logical first step as it is a procedure that is not so expensive.

69.4.12 Prevention of Male Infertility in Africa

There are numerous reasons that justify mounting a preventive campaign as a major part of managing male infertility in developing countries. Other than the fact that reproductive tract infections are a major cause of male infertility and are preventable, other reasons include:

- The difficulties in diagnosis, the complex medical and surgical treatments, and their poor long-term outcomes make it better to prevent these conditions from complicating into infertility. Further, these costly diagnostic modalities and treatments are concentrated in the few tertiary centers available and not accessible to many couples.
- The lack of specialist expertise to manage infertile couples and the virtual absence of research data on infertility in developing countries.
- The failure of most health authorities to prioritize infertility care and integrate it into the reproductive health care services.

Prevention strategies should involve a number of components that include:

- Ensuring all health-care personnel are continually updated on STI management to ensure prompt diagnosis and effective treatment is instituted.
- A countrywide education campaign targeting young people on the nature and consequences of STIs, their control, and treatment. This campaign should stress the importance of abstinence and condom use to prevent infection with STIs.
- Extensive education campaigns to ensure all persons have knowledge of and have access to treatment for STIs [34].

69.4.13 Male Infertility (the Case of Zambia)

Male infertility in sub-Saharan Africa, as illustrated by southern African state of Zambia, presents a very difficult clinical condition to manage. The expectations of the numerous patients that attend health facilities for treatment are unfortunately at odds with the goals of many health authorities that place emphasis on prevention of infertility rather than treatment. Further, the traditional view of reproductive health as predominantly a "women's health issue" permeates the decision-makers in government that results in minimal funding for male reproductive health. Zambia has a population of 14 million with an HIV prevalence of 13% and a per capita income of 1600 USD. Fertility rates are traditionally calculated using the number of births per woman in a given population. The Zambia Demographic and Health Survey (ZDHS) is a survey of health status of the population in Zambia which is done every 5 years. The survey includes an assessment of fertility rates in the population. The average fertility rate is 5.3. This is the average number of births per woman. There has been a general decline in the fertility rates in Zambia as in other African countries for the last 20 years. In the ZDHS of 2014, there was a decline in fertility rates from 7.2 in 1980 to 5.3 in 2014 [35]. The general female fertility rate in Zambia is 184 per 1000 (18.4%). The infertility rate is 24.1% based on the ZDHS of 2014. Using "Sharlip factor," the male infertility rates in Zambia can be calculated to range from 4.82% to 7.23%. This is just slightly lower than the average for the sub-Saharan African region of 4.4% to 11.5% [35]. The general fertility rates have declined in all provinces with the lowest rates being in the Copperbelt and Lusaka provinces. These two provinces also have the highest HIV prevalence rates (Fig. 69.2).

The infertility rates in Zambia are similar to those of the African countries in the region and subregion as illustrated by Table 69.2.

It is a known fact that HIV infection has some effect on reproduction. In female, this includes abortions and pathological disease in the reproductive system [36]. In male, HIV causes erectile dysfunction, testicular atrophy, and various sperm dysfunctions [37]. HIV infection is also associated with testicular tumors and tuberculosis of the testis. All of these adversely affect male fertility.

Despite numerous limitations in ancillary investigations and laboratory facilities, many hospitals in the region, through collaborative research agreements, are now able to evaluate the hormonal profile for patients and obtain histological diagnosis in azoospermatic men undergoing testicular biopsy. At the Lusaka University Teaching Hospital in Zambia, a fivefold increase in testicular biopsies for infertility was recorded over a 10-year period [36, 37]. Azoospermia was the only indication for testicular biopsy. In a review of the relationship between HIV and male infertility done during the same period, it was noted that among men presenting to the urology clinic, there was a high HIV prevalence of 26% compared to the general population of 13% [38]. There was a high prevalence both of TB and STIs in this group of patients as well. TB is the most common opportunistic infection seen at the UTH.

Medical treatment is largely empirical, and with the challenges in follow-up and poor long-term patient compliance, treatment efficacy has not been determined. The main treatment modality is the use of antiestrogens. It takes advantage of their effect of increasing pituitary output of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) and hence stimulating testosterone production and spermatogenesis. The antiestrogens used are clomiphene citrate and tamoxifen citrate. The other nonsurgical treatment commonly employed is the use of antioxidants. These supplements, especially vitamins E and C, are utilized for their known effects of improving sperm function and protecting sperm DNA from oxidative stress, respectively.

Surgical treatments offered are very limited in scope. Varicocelectomy is performed in patients presenting with infertility, an abnormal semen analysis, and a clinically demonstrable varicocele. Surgical treatment options are not available to the majority of infertile men in the region as these require specialist expertise and equipment that is lacking in sub-Saharan Africa. The microscopic techniques required in unblocking obstructed seminal tubes or vasa require a huge investment in manpower training and equipment. For most countries in the region, it is an investment deemed not prudent to make in the present economic climate. The other option for treating infertile couples in this region, especially with improvement in HIV outcomes that are now evident with improved HAART, is the use of assisted reproductive technologies (ARTs) [39]. These

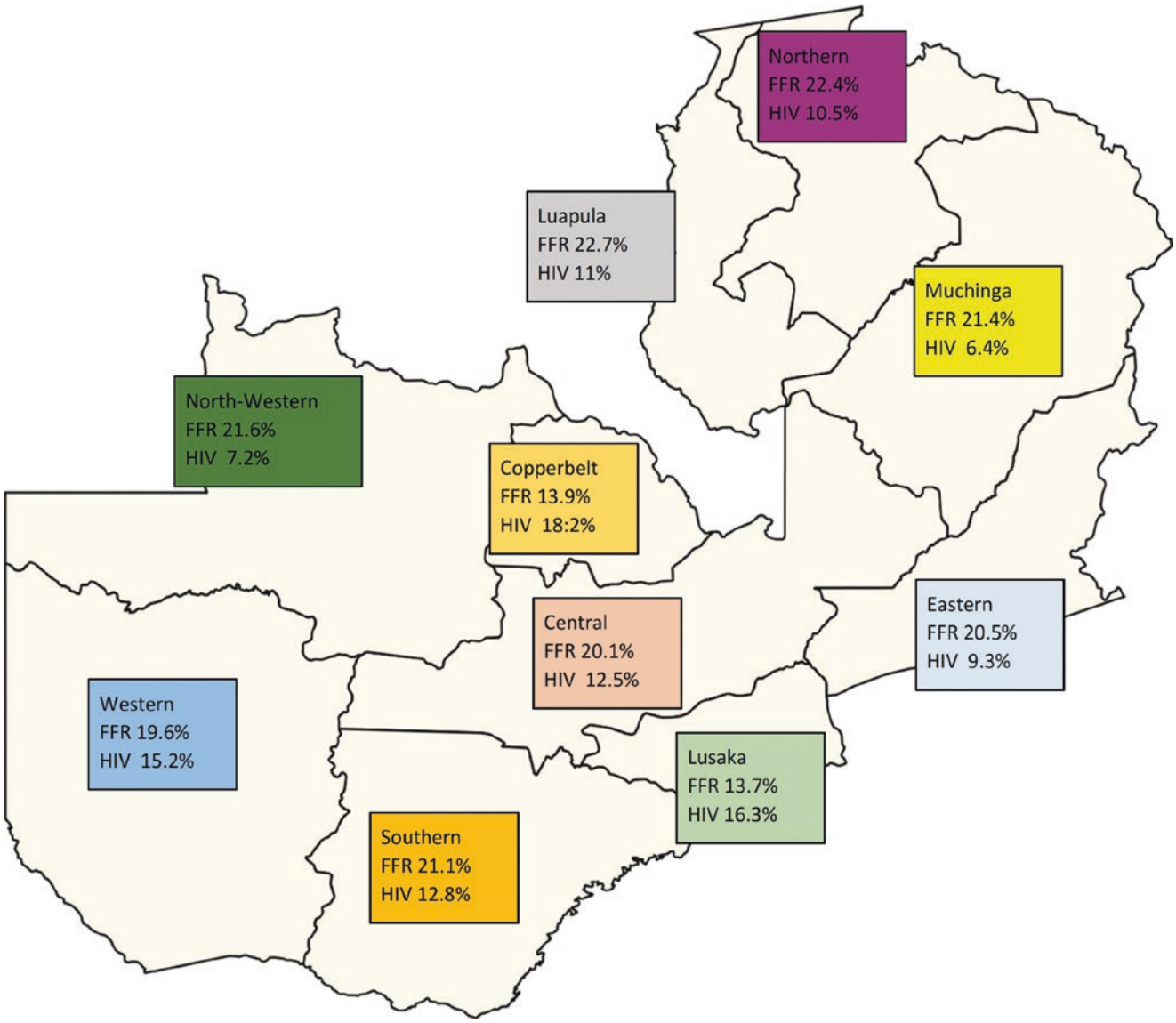

Fig. 69.2 The nine provinces of Zambia and the HIV prevalence and fertility rates by province (data from 35) (abbreviation FFR, female fertility rates)

Table 69.2 The range of male infertility in Zambia is similar to that found in the sub-Saharan Africa subregion

Country	Female fertility rate	Infertility rates	Male fertility rates
Zambia	184 per 1000	24.1%	4.82%–7.23%
Kenya	137	25.7%	5.14%–7.71%
Tanzania	170	24.5%	4.9%–7.53%
Ghana	146	28.7%	5.74%–8.61%
Nigeria	179	23.8%	4.76%–7.14%
Uganda	189	24.2%	4.84%–7.26%

ARTs are presently beyond the economies of countries in this region. Since 2017 ART services has become available in the private sector in the capital city, Lusaka. These services are available only to a limited number of middle- and upper-middle-class couples.

69.5 Conclusion

The determination for male-specific infertility is difficult because most data uses female infertility rates. However using the Sharlip factor, male infertility in a population can be inferred. The highest infertility rates in the world are found in the developing countries of Africa, Asia, and Eastern Europe. These rates range from 4 to 12%. Among the developing countries, the infertility belt in sub-Saharan African has some of the highest male infertility rates. There has been a decline in male sperm count globally over the last 30 years. The greatest decline has been noted in Africa. The main cause of male infertility in developing countries and Africa in particular has been sexually transmitted infections (STIs). Primary infertility is rare representing under 5% of the

causes of male infertility. Other causes include reproductive tract infection, environmental toxins, and alcohol abuse.

The large majority of male infertility is managed using conventional medicines. The ART services are generally uncommon in these low-resourced countries. Zambia provides a case study of the challenges of managing male infertility in the context of HIV infection, in a low-resourced setting. It is expected that in the next 5 years, there will be an increasing drive to develop ART services alongside STI prevention strategies in addressing male infertility.

69.6 Review Criteria

This chapter on male infertility from the developing nations perspective was reviewed using PubMed, Google Scholar, and Africa Journals OnLine (AJOL) search engines. This was supplemented by a literature search of the World Health Organization website. The search was done between the months of September and December 2018 and covered any literature on male infertility in Asia, Africa, Eastern Europe, and South America. The key search words were male infertility, developing countries, Africa, Eastern Europe, and South America. These terms were used in combination with the main search term male infertility. The data obtained was further substratified as related to prevalence, etiology, management, and Zambia. Full-length articles and abstract were included in the review. Only articles written in English were included in the review.

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Diabetes and Male Infertility

70

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Key Points

- Diabetes mellitus (DM) is a multifactorial metabolic disease characterized by chronically elevated blood glucose or hyperglycemia, due to disorders of insulin secretion.
- Some studies differentiate effects from Type 1 and Type 2 diabetes mellitus. However, most of the effects on the male reproductive system typically pertain to both forms of diabetes mellitus.
- The primary mechanisms through which diabetes mellitus exerts its effects on fertility include oxidative stress, the hypothalamic-pituitary-gonadal axis, and neuropathic changes.
- Direct impacts on fertility are through changes in sperm parameters, oxidative damage, and problems with steroidogenesis.
- Diabetes mellitus has a significant effect on the male reproductive system and fertility by affecting erectile function, ejaculatory function, libido, and sexual desire.
- Physicians should be aware of the reproductive and sexual problems associated with diabetes and discuss proper glucose control with their patients.

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70.1 Introduction

Diabetes mellitus (DM) is a multifactorial metabolic disease characterized by chronically elevated blood glucose or hyperglycemia, due to disorders of insulin secretion and action, leading to improper metabolism of carbohydrates, fat, and proteins [1]. DM is one of the most common medical conditions, affecting about 420 million people globally (8.5% of the world population) [1]. According to the World Health Organization, the number of people with DM has almost quadrupled since 1980. In the United States, about 1.5 million people are diagnosed every year, and it effects almost 10% of the population [2]. DM is classified into two major categories: Type 1 and Type 2 [2, 3].

Type 1 DM, previously known as juvenile diabetes or insulin-dependent diabetes (IDDM), comprises approximately 4% of the diabetic population, with an estimated 1.25 million people in the United States [2, 3]. It is an autoimmune disease characterized by T cell-mediated destruction of insulin-secreting islet β cell [2]. Several genetic factors are linked to IDDM, rather than other environmental factors typically described with Type 2 DM [2].

Type 2 DM, also referred to as adult-onset or non-insulin-dependent DM (NIDDM), is the most common subtype of DM, representing well over 90% of diabetics [2, 3]. It is a result of defective insulin production and secretion, with impaired response, known as insulin resistance. Obesity and being overweight are the strongest risk factors for Type 2 DM [1, 2, 4–6]. However, other factors such as family history, history of gestational diabetes in women, and ethnicity also play key roles in the development of Type 2 DM [1].

Prediabetes comprises several other conditions which result in hyperglycemia, but are not high enough to meet the diagnostic criteria for DM [3, 7]. Diagnostic criteria for DM and prediabetic states include at least two recorded values with one or more of the following blood tests: hemoglobin A1c, fasting plasma glucose, random plasma glucose, and oral glucose tolerance [3]. As the number of prediabetics

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rises, this condition becomes increasingly clinically significant because prediabetic conditions are at increased risk of developing Type 2 DM [3].

Up to 90% of diabetic males have sexual dysfunctions, including decreased libido and infertility [8]. Compromised fertility and sexual dysfunction are not uncommon in men with DM. DM has effects on the following areas: spermatogenesis, degenerative changes in the testes, steroidogenesis, sperm maturation, erections, ejaculation, sperm parameters, and glucose metabolism in Sertoli cells (blood-testis barrier) [9–11]. DM affects the reproductive system through a number of mechanisms: endocrinopathies, neuropathies, and increased oxidative stress.

70.2 General Mechanisms

70.2.1 Oxidative Stress

DM contributes significantly to male infertility through a variety of direct and indirect mechanisms [12]. Oxidative stress has been a well-established major factor linked to several degenerative conditions, such as aging and systemic conditions like DM [8, 13]. Oxidative stress is induced by the disruption in the equilibrium between reactive oxygen species (ROS) and antioxidant scavenging species, which ultimately causes strand breaks in DNA. This leads to activation of a wide range of cellular effects and pathways. The key metabolic pathways which contribute to cellular damage from DM and hyperglycemia include increased polyol, hexosamine, protein kinase C, and advanced glycation end products [8, 14, 15]. Several studies have implicated oxidative stress in the various complications seen in DM, such as infertility. For example, Zhao et al. used a mouse model to demonstrate the role of oxidative stress in diabetes-induced testicular apoptosis and infertility [14]. Sperm, specifically, are susceptible to damage from oxidative stress due to high levels of polyunsaturated fatty acids, lack of cytoplasm and thus poor antioxidant protection, and lack of DNA repair mechanisms [16–18]. ROS are present at physiological levels for normal sperm function, but excess ROS leads to sperm DNA fragmentation and impairments in morphology [16, 17, 19]. However, separate pathways through the sperm plasma membrane's NADPH oxidase system and mitochondrial NAD-dependent oxidoreductase, respectively, contribute to the imbalance of ROS that can cause infertility [16, 19, 20]. Antioxidants have been shown in animal and human studies to reduce ROS, protect against DNA fragmentation, and improve semen parameters [16, 21]. Multiple studies have been done with ascorbic acid (vitamin C) and α-tocopherol (vitamin E) showing improvements in semen parameters [21]. Other antioxidants studied in the setting of male infertility include glutathione, coenzyme Q10, lycopene, and pycnogenol [16]. Therefore, in the setting of ROS resulting from hyperglycemic states, antioxidants may be indirectly beneficial for men with infertility.

70.2.2 Hypothalamic-Pituitary-Gonadal Axis

The hypothalamic-pituitary-gonadal (HPG) axis is the center for various hormones integral to male fertility. Luteinizing hormone (LH) released from the pituitary gland acts on Leydig cells to produce testosterone. Follicle-stimulating hormone (FSH) acts on Sertoli cells, which control spermatogenesis and promote testicular maturation. Therefore, any issues along the HPG axis can cause semen impairment. It is reported that diabetic patients have blunted levels of FSH and LH, as well as other abnormalities with other hormones along this axis. There is conflicting evidence relating HPG axis dysfunction to infertility and/or erectile dysfunction. Pitteloud et al. described an inverse relationship where increased insulin resistance reduced Leydig cell population and testosterone metabolites [22]. Garcia-Diez et al. showed that men with Type 1 DM exhibited lower seminal fluid volume [23]. The concentration, motility, vitality, and proportion of normally shaped spermatozoa were also lower [23]. In contrast, Faerman et al. found no abnormalities in the number of Leydig cells or testicular morphology in infertile diabetics [24].

70.2.3 Neuropathy

Diabetic neuropathies affect up to 50% of diabetics, with peripheral neuropathies being the most common form of neuropathy [25, 26]. Neuropathies in DM contribute to erectile dysfunction and secondarily infertility. An intact cavernous nerve is required for natural physiologic penile erections. Additionally neuronal nitric oxide synthase (nNOS) is required for the initiation and maintenance of penile erections. Yin et al. used a mouse major pelvic ganglion tissue, which showed that nNOS-positive nerve fibers and neurite outgrowth were significantly lower in high-glucose tissue compared to tissue with normal glucose exposure [27].

70.2.4 Infectious

There are also multiple infectious etiologies, which occur in higher rates in patients with DM. Infections affecting the reproductive system can contribute to infertility, even with appropriate treatment in some cases. Table 70.1 summarizes some infections found in diabetic patients, which may affect fertility, adapted from a table by Gandhi et al. [12].

Table 70.1 Genitourinary complications of diabetes mellitus

Genitourinary site	Infections
Spermatic cord	Abscess, xanthogranulomatous funiculitis, calcification of the vas deferens
Epididymis	
Testicle/ epididymis	Epididymo-orchitis, emphysematous epididymo-orchitis, xanthogranulomatous orchitis, xanthogranulomatous epididymitis, epididymo-testicular malakoplakia, gangrene
Penis	Gangrene, balanitis
Scrotum	Abscess, intertrigo

Based on data from Ref. [61]

70.3 Steroidogenesis

DM can affect fertility through its effects on all aspects of the male reproductive system – pre-testicular, testicular, and post-testicular [11]. The pre-testicular component of the axis includes the HPG axis and its endocrine controls. The hypothalamus releases gonadotropin-releasing hormone (GnRH) to stimulate the anterior pituitary gland. The anterior pituitary gland then releases various stimulatory hormones, including FSH and LH. FSH initiates spermatogenesis in the testicles via the Sertoli cells, the backbone of the highly regulated blood-testis barrier. LH stimulates Leydig cells in the testicle to release testosterone. There is a relationship between insulin, glucose regulation, and the HPG axis [28]. Insulin activates the HPG axis and helps to modulate the HPG axis during times of stress [29, 30]. Insulin is produced directly by both sperm and testes, with receptors also found at the hypothalamus and pituitary gland, therefore playing an integral role in spermatogenesis [31]. Insulin has many roles in the body, including acting as a surrogate marker for leptin. Leptin is a hormone that regulates energy and associated with an animal's ability to meet the demands of reproduction [26, 31]. There has been a correlation with uncontrolled DM and decreased leptin levels, which could contribute to infertility [31].

Many mechanisms within the HPG axis have been described to explain the infertility and subfertility seen in diabetic men. One such mechanism stems from diabetic neuropathies, which may blunt receptor activity at the hypothalamus, anterior pituitary gland, and testicles [11]. Glucose dysregulation alters the glycoprotein component of the membrane-bound LH receptor and affects the hormonal interaction [11]. In a group of diabetic men with fertility issues, Baccetti et al. described significantly reduced responses to GnRH and diminished semen quality [32]. Hypogonadotropic hypogonadism has been associated with Type 2 DM and has been described in several studies. In a study by Tripathy et al., 50 men with Type 2 DM and low free testosterone were found to most commonly have the aberrance of low FSH and low LH [33]. Lopez-Alvarenga et al. studied a group of 20 men with Type 1 DM who were found to have suppressed levels of LH after endogenous and exogenous stimulation with GnRH [34]. Similarly, Maneesh et al. looked at men with Type 1 DM and also found them to have significantly low levels of testosterone, LH, and FSH, compared to healthy male controls [35]. All of the aforementioned studies included generally young men, with average age in the 20s and 30s. Dhindsa et al. studied a slightly older group of men (mean age 55) with Type 2 DM, also found to have abnormally low levels of testosterone, LH, and FSH [36].

Excess estradiol in men with DM has also been implicated as a cause of hypogonadism. Estradiol acts in a negative feedback loop that can lower testosterone levels by affecting GnRH, FSH, and LH. Men with DM have been found to have elevated estradiol levels from increased aromatase activity in fat cells. The increase in estradiol subsequently causes decreases in testosterone levels.

Conversely, several studies have shown no relationship between diabetes or testicular mphrology. Faerman et al. examined testicular biopies via light micrscopy and did not find abnormalities in the number of Leydig cells or testicular morphology in impotent diabetic men [24].

Additionally, the treatment of insulin in some animal models have not resulted in recovery of normal sperm parameters or improved fertility [31, 37].

70.4 Spermatogenesis and Sperm Parameters

In general, semen analyses from men with DM have been found to have decreased motility, density, abnormal morphology, and increased semen abnormalities. The main mechanisms which contribute to damages in spermatogenesis and sperm parameters in DM include endocrine dysfunction via an altered HPA, neuropathy, and oxidative stress [8]. These environmental changes in sperm production and maturation can lead to the increased rates of infertility and subfertility seen in diabetic men [8, 9, 38]. Additionally, changes in the blood-testis barrier (BTB) can have significant effects on spermatogenesis (Table 70.2).

The BTB, or Sertoli cell barrier, divides the seminiferous epithelium into basal and apical compartments, separating different stages of germ cell development [10]. Sertoli cells, which are found in the testicles, are an integral component of spermatogenesis [10]. The BTB is highly reliant on glucose metabolism and hormonal control. Therefore, glucose dysregulation from DM affect the BTB function, with major negative impacts on spermatogenesis, testicular architecture, and fertility [10].

In the setting of DM, spermatozoa may have defective mitochondria from oxidative stress, causing declines in motil-

Table 70.2 Effects of DM on sperm parameters

Summarized effects of diabetes on sperm parameters
Impaired spermatogenesis (hormonal and environmental)
Reduced sperm count
Reduced semen volume
Impaired motility
Altered sperm quality
Disruption in blood-testis barrier
Oxidative damage
Epigenetic dysregulation
Altered seminal plasma components

Based on data from Ref. [43]

ity and ultimately fertility [8, 39]. Furthermore, DNA damage in the setting of oxidative stress leads to germ cell apoptosis and decreased sperm counts [39]. In a comparative study by Agbaje et al., the semen of diabetic men were found to have increased levels of nuclear and mitochondrial DNA damage, compared to healthy controls [40]. However, in this study, with the exception of sperm volume, semen parameters were comparable between men with DM and the healthy controls [40]. Amiri et al. found that men with DM had markedly increased levels of nitrate/nitrite and 8-hydroxydeoxyguanosine (8-OHdG), chemicals associated with ROS and DNA damage [41]. Malondialdehyde, another marker of oxidative stress, was also found by La Vignera et al. to be increased in infertile men with Type 2 DM, negatively impacting sperm density, motility, and sperm count [42].

Epigenetic modifications, which define the phenotype of cells, are essential in the germline for normal embryonic development [43]. Damage from DM to male reproductive structures and spermatogenesis at this critical stage in development, stemming from maternal hyperglycemia, can have permanent effects on fertility in subsequent germlines and generations [43]. Therefore, maternal DM can potentially have downstream effects on fertility in their offspring [43].

Numerous animal and human clinical studies have looked at semen parameters and outcomes following insulin treatment. Kim et al. showed significantly lower rates of sperm concentration, motility, and fertilization in Type 1 diabetic mice, with significant improvements in these parameters following insulin treatment [44]. Similarly, Scarano et al. also studied the sperm of streptozotocin-diabetic rats, which showed decreases in epididymal sperm and testicular sperm counts [45]. Damage to sperm in the epididymis has deleterious effects for sperm maturation, storage, and transit. Bartak et al. was one of the first to describe changes in the sperm of diabetic men, including significantly worsened motility and morphology in these men [46]. Delfino et al. studied men with both Type 1 and Type 2 DM, showing alterations in sperm motility and morphology, with no significant differences in sperm concentration [38]. Agbaje et al. found a small but significant decrease in the semen volume of Type 1 diabetic men [40]. La Vignera et al. found that patients with DM and neuropathy exhibited atony of the seminal vesicles, with worsened ultrasound features in those with longer disease duration [47].

It is evident from the studies to date that sperm quality is altered in men with DM, but this varies on an individual basis according to disease duration of the disease and glycemic control. It is important to recognize that Type 1 and Type 2 DM may have different underlying mechanisms contributing to the changes seen in their sperm parameters. The sperm alterations in Type 1 DM can be autoimmune mediated. However, the changes seen in those with Type 2 DM can be multifactorial with the metabolic syndrome and changes in testosterone.

70.5 Sexual Dysfunctions

Sexual dysfunctions have been widely observed in diabetic men. Although studies focus on erectile dysfunction (ED), other abnormalities in sexual function involve ejaculatory dysfunction, decreased libido, and infertility. Up to 90% of diabetic men suffer from at least one of these sexual problems [8].

70.5.1 Erectile Dysfunction

Any process causing difficulties with erectile function can subsequently affect infertility [48]. ED is present in approximately 35–75% of diabetic men [49]. Endothelial dysfunction and neurological damage via the autonomic system play major roles in the pathophysiology of ED in diabetic men [26, 50]. In addition to these issues, endocrine changes and pharmacologic side effects may also lead to ED.

Smooth muscle relaxation is paramount to achieving an erection. In DM, endothelium-dependent smooth relaxation of blood vessels is impaired, affecting the microvasculature of the penis and leading to both arteriogenic and veno-occlusive ED types [51]. This damage is mediated through increased expression of endothelial adhesion molecules ICAM-1 and VCAM-1 and oxidative stress [26]. Hyperglycemia increases advanced glycation end products (as previously discussed in the "General Mechanisms" section) and is thought to also contribute to the vascular problems in the penis seen in DM. As a result, hyperglycemia is significantly higher with worsening erectile function [49, 52]. As an end organ, the penis is susceptible to both microvasculopathies and macrovasculopathies [26]. Macrovascular disease contributes to atherosclerosis and impaired penile circulation [53]. Microvascular disease contributes to disease morbidity, such as blindness and end-stage renal disease [54]. In a study by Vardi et al., the prevalence of ED was higher in the presence of microvascular disease in diabetic men [55].

An intact neurological system is required to achieve normal erections. The S2-S4 nerve roots, which yield the cavernosal nerves, are required for erections through a parasympathetic cascade. This parasympathetic response is also responsible for the sensation of the penis. Emission is controlled by a sympathetic response of the hypogastric nerves via the T10-L2 nerve roots. Lastly, ejaculations are controlled via the pudendal nerves in a somatic response from the S2-S4 nerve roots. Hyperglycemia and oxidative stress from DM cause direct nerve fiber damage and subsequent motor and sensory changes [26]. Reduced parasympathetic input is the leading mechanism in autonomic nervous system dysfunction causing ED in DM, as parasympathetic input is required for smooth muscle relaxation in the corpus cavernosum [26, 56]. Autonomic neuropathy in DM is associated with other sexual dysfunctions, bladder dysfunction, and retrograde ejaculation [26, 49]. Diabetic neuropathy also decreases nitric oxide synthesis, an essential component of the veno-occlusive cascades in erections.

Hormonal changes causing diabetic ED stem from abnormalities in the HPG axis. Hypogonadotropic hypogonadism, commonly associated with DM, also contributes to ED [33].

Finally, multiple drugs also cause ED in diabetic men. A common culprit is that of antihypertensive drugs, such as diuretics and beta blockers [49]. However, many psychotropic drugs, such as selective serotonin reuptake inhibitors and antipsychotics, can make erections more difficult and affect sexual function in diabetic men [49].

70.5.2 Ejaculatory Dysfunctions

Ejaculatory disorders are the most common sexual dysfunctions causing infertility in DM. Ejaculation is controlled through a somatic response via the pudendal nerve and rhythmic contractions of the ischiocavernosus and bulbospongiosus muscles. The central, peripheral, and autonomic nervous systems coordinate a complex set of integrated mechanisms as essential components of ejaculation. Ejaculatory dysfunction is a range of disorders including premature ejaculation, delayed ejaculation, retrograde ejaculation (RE), and anejaculation. Normal ejaculations are necessary for sperm to enter the vaginal vault and undergo capacitation and an acrosomal reaction prior to fertilization. RE and anejaculation have been strongly linked to DM and are significantly more problematic for fertility than other ejaculatory disorders.

Anejaculation due to DM occurs in approximately 10% in men [26, 57]. In diabetic men, anejaculation is due to autonomic dysfunction stunting peristalsis of the vassal and seminal vesicle lining, preventing ejaculation from reaching the urethra [26]. Autonomic dysfunction in diabetics is also a contributing factor to the pathophysiology of RE. It is caused by an abnormal mechanism involving the internal urethral sphincter. In the normal physiology of ejaculation, sympathetic fibers innervating the bladder cause the bladder neck to close and facilitate antegrade movement of ejaculate into the urethra. Autonomic nervous system damage from DM compromises the normal high-pressure bladder neck closure. The neuropathic changes result in a low-pressure closure of the bladder neck during ejaculation, causing partial or complete propulsion of ejaculate into the bladder. In an early study by Dinulovic et al., the incidence of ejaculatory disorders in younger, diabetic men was about 6% [58]. In a case control study by Fedder et al., diabetic men displayed a significantly increased rate of RE, whereas no cases of RE were noted in nondiabetic men [59]. Additionally, diabetic men with RE more frequently exhibited associated ED [59]. The true incidence of RE and other ejaculatory disorders in diabetic men is unknown. Due to the older age of men that develop Type 2 DM, for example, RE and fertility may be unreported. However, RE and other ejaculatory issues should be assessed by performing a post-ejaculate urinalysis in young diabetic men with infertility concerns [11, 26].

70.5.3 Libido

Decreased libido is also common in diabetic men and is largely attributed to changes in the HPG axis causing hypogonadotropic hypogonadism. The resultant decreased testosterone levels cause impaired libido, as well as other sexual dysfunction issues previously discussed. In a large cross-sectional observational study by Malavige et al., of about 250 men, the prevalence of reduced libido was about 25% in diabetic men [60]. In this same study, ED was strongly associated with libido and premature ejaculation. In the context of fertility, diminished libido can be problematic and, thus, must also be managed. Diabetic patients with low testosterone and libido may benefit from fertility preserving hormonal therapy to address this. Additionally, tighter glycemic control may also help to alleviate the effects of this diabetic complication.

70.6 Conclusions

DM is a multifactorial metabolic disease with multisystem effects and complications. Through hormonal changes, autonomic and peripheral neuropathies, and oxidative stress, diabetes negatively impacts the male reproductive system. Diabetes affects fertility both directly and indirectly, through alterations in steroidogenesis and spermatogenesis. Microvascular and macrovascular changes due to DM also cause sexual dysfunction, which inherently compromises fertility. Clinicians specializing in reproductive medicine

should be aware of the various mechanisms through which diabetes affects fertility. It is prudent that providers encourage strict management of DM in couples and individuals seeking reproductive options.

70.6.1 Review Criteria

A search of stuides examining the role of diabetes mellitus Type 1 and 2 in male infertility was performed using PubMed, MedlinePlus, Google Scholar and Scopus. The start date for the search was January 2000 and the end date was December 2018. The overall strategy for study identification and data extraction was based on the following keywords: "diabetes and infertility," "Type 2 diabetes sexual function," "Type 1 diabetes sexual function," "Type 2 diabetes infertility," "Type 1 diabetes infertility," "diabetes sexual dysfunction," "urological diabetes," "diabetes effects," "diabetes mechanisms," "diabetes hormonal changes," "diabetes erectile dysfunction," "diabetes libido," "diabetes ejaculatory function," "diabetes neuropathy," "diabetes libido," diabetes oxidative stress," and "diabetes-assisted reproduction." Articles included had to be in the English language. Citation dates outside the search dates were included if required for understanding and appropriate citation of original work. Papers that were editorial pieces, response to papers, or nonacademic articles were not considered.

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Empiric Medical Therapy for Idiopathic Male Infertility

71

Tejash Shah and David Shin

Key Points

- Idiopathic male infertility is the term used for infertile male patients when no identifiable male infertility factor is found but abnormalities are still seen on semen analysis.
- Hormonal medications used in the treatment of hypogonadotropic hypogonadism, such as gonadotropins, androgens, selective estrogen receptor modulators, and aromatase inhibitors, have been used as empiric medical therapy for idiopathic male infertility.
- Off-label use of selective estrogen receptor modulators and aromatase inhibitors has yielded promising results for the treatment of idiopathic male infertility, but large-scale, randomized controlled trials with pregnancy outcomes data are needed to make conclusive recommendations.
- Excessive reactive oxygen species leading to increased oxidative stress and subsequent damage to sperm DNA is believed to be another cause of idiopathic male infertility.
- Antioxidant therapy in men with idiopathic infertility has been shown to improve semen parameters in small, poorly designed studies. Well-designed large trials with pregnancy outcomes data are needed.

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71.1 Introduction

Male infertility is implicated in approximately 50% of all couples presenting for a fertility evaluation [1]. Often a genetic, endocrine, or anatomic cause for male infertility can be identified, and appropriately targeted treatments or assisted reproductive techniques can be provided. In 30–40% of men presenting with infertility, idiopathic male infertility is the diagnosis given when no identifiable male factor is found but abnormalities are still seen on semen analysis. Patients with idiopathic male infertility present with no previous history of illnesses known to affect fertility and have a normal physical exam and normal genetic, laboratory, and endocrine testing. The cause of idiopathic male infertility remains a mystery but is hypothesized to be due to several factors, including unknown genetic or epigenetic defects, reactive oxidant species, and environmental pollution [2]. Since the exact cause leading to idiopathic male infertility is unknown, treatment for such males can be challenging. A wide array of empiric treatments has been tried for this condition, ranging from hormonal therapy to vitamin supplements. A 2012 survey of the American Urological Association members showed that two-thirds of those surveyed used various empiric medical therapies for the treatment of idiopathic male infertility. However, there was no consensus on which patients would benefit from empiric therapy or what is the ideal empiric medical treatment [3]. In this chapter, we will review the available literature and common practice patterns regarding empiric medical treatment of idiopathic male infertility.

71.2 Hormonal Treatments

Hormonal treatment is widely used for empiric therapy of idiopathic male infertility since men with idiopathic male infertility often have hormonal abnormalities not detectable on standard hormone profiles. Some of the hormonal medications used include gonadotropins, androgens, selective

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estrogen receptor modulators, and aromatase inhibitors. The goal in using these medications is to modify the hypothalamic-pituitary-gonadal axis in order to lead to improved semen parameters and increased pregnancy rates.

71.2.1 Gonadotropins

Gonadotropins have shown well-documented benefits in the treatment of hypogonadotropic hypogonadism. Subsequently, the use of gonadotropins, such as gonadotropin-releasing hormone, follicle-stimulating hormone, and human chorionic gonadotropin has been extrapolated for the treatment of idiopathic male infertility, but their benefits have not been well established.

Gonadotropin-releasing hormone (GnRH) is a hormone released by the hypothalamus, which helps to regulate follicle-stimulating hormone (FSH) and luteinizing hormone (LH) release from the anterior pituitary gland (see Fig. 71.1). The rationale in using GnRH in idiopathic male infertility is that the increase in GnRH will lead to increased FSH and LH levels, which in turn will lead to increased testosterone levels and spermatogenesis. In one of the first studies in 1988, Badenoch et al. randomized 19 males to receive a GnRH agonist, buserelin, vs. saline for 12 weeks. At the end of the study period, there was no statistically significant difference in the sperm concentrations between the aforementioned groups [4]. Another study published in 1992 by Crotazz et al. looked at 28 patients randomized to GnRH vs. placebo. In this study, there was a slight improvement in semen parameters in the GnRH group, and five patients in the GnRH group achieved pregnancy compared to three patients in the placebo group; however, these results were not statistically significant [5]. A study published in 2000 examined the use of intranasal GnRH treatment with 100 micrograms of buserelin for 19 men over a 3-month period. Although these patients did not suffer any of the possible side effects associated with GnRH treatment such as superficial thrombophlebitis or injection site irritation, they too failed to show any significant improvement in semen parameters [6]. Overall, the evidence for the use of GnRH in idiopathic male infertility is poor and is not considered to be an effective treatment option for these men.

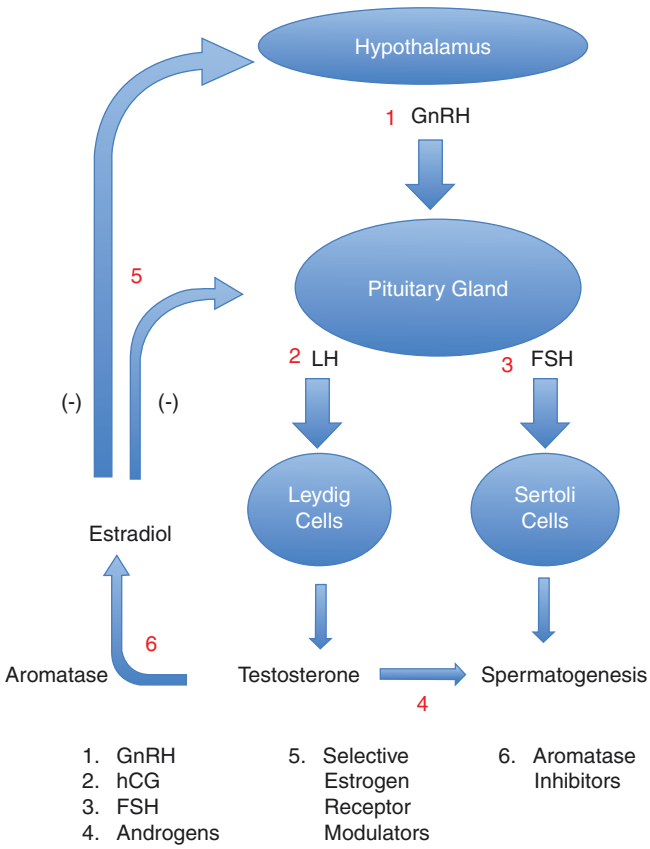

Fig. 71.1 Action of hormonal agents on hypothalamic-pituitary-gonadal axis. Represents the pathways along which hormonal agents used for treatment of idiopathic male infertility are believed to act

Human chorionic gonadotropin (hCG) is a hormone normally produced by syncytiotrophoblasts. The alpha subunit of hCG shares a similar structure to luteinizing hormone (LH), and therefore hCG has the equivalent biological activity of LH. hCG has been shown to significantly increase intratesticular testosterone concentrations and is routinely used as an effective treatment of hypogonadotropic hypogonadism in conjunction with other gonadotropins. In the case of idiopathic male infertility treatment, hCG has been shown to improve semen parameters in up to 69% of patients with pregnancy rates up to 36%; however, these results were found in older, uncontrolled studies [7]. In a randomized, placebo-controlled trial, 19 men were placed in the hCG treatment arm for 13 weeks. These patients received injections of 2500 IU hCG twice a week with 150 IU of human menopausal gonadotropin three times a week. At the conclusion of the study, there were no significant differences in the semen parameters of the hCG group compared to placebo group. The hCG group did have 2 pregnancies, while the placebo group of 20 patients had no pregnancies. Side effects of hCG treatment include acne, testis pain, gynecomastia, and arterial thromboembolism [8]. While hCG is an accepted therapy of hypogonadotropic hypogonadism, its utility in idiopathic male infertility has not been proven.

Follicle-stimulating hormone (FSH) is released by the anterior pituitary gland and stimulates Sertoli cells to help drive spermatogenesis (see Fig. 71.1). As with other gonadotropins, FSH has been used for the treatment of hypogonadotropic hypogonadism, and its success for these patients has led to its potential use as empiric therapy for idiopathic male infertility. In a 2006 study by Paradisi et al., 30 men received 300 IU of recombinant FSH (rFSH) for 4 months or greater. At the end of the treatment, there was a significant increase

in sperm count and slight increase in sperm motility but no change in other semen parameters. Pregnancy data was not available [9]. On the other hand, a 2012 randomized, prospective study compared 65 men treated with 150 IU of rFSH on alternative days for 90 days with 64 men who were treated with non-antioxidant vitamins, and no difference was seen in semen parameters [10]. A 2013 Cochrane review of the effects of gonadotropins on idiopathic male infertility analyzed six randomized studies, five with rFSH and one with hCG. When comparing gonadotropins to placebo groups, the live birth rate was 27% vs. 0% and spontaneous pregnancy rate was 16% vs. 7%, respectively [11]. While the results are encouraging, it is difficult to convincingly support the use of gonadotropins for idiopathic male infertility given the poor quality and small number and sample size of these studies.

71.2.2 Androgens

Endogenous androgens are critical for normal male development and spermatogenesis. However, exogenous testosterone has been shown to inhibit the release of pituitary FSH and LH resulting in a decrease in intratesticular testosterone and eventual cessation of spermatogenesis [12]. In one study of 59 men on exogenous testosterone therapy who presented to an infertility clinic, 88% were azoospermic [13]. Despite the known negative feedback inhibition of exogenous testosterone therapy on the hypothalamus-pituitary-gonadal axis, a 2012 American Urological Association survey showed that 25% of urologists surveyed routinely prescribed exogenous testosterone for the treatment of idiopathic male infertility [5]. This may be due to the misperception that exogenous testosterone treatment is a direct stimulation of spermatogenesis and rebound spermatogenesis (which occurs once the exogenous source of testosterone has been removed resulting in a transient increase in gonadotropins and subsequent spermatogenesis). One randomized, double-blind study from 1993 reported a marginally significant improvement in sperm morphology and pregnancy rate with exogenous testosterone therapy which seems to support this misconception [14]. However, several meta-analyses have subsequently demonstrated that androgens do not improve semen parameters or pregnancy rates for men with idiopathic infertility [15]. There are significant side effects associated with the use of exogenous androgens such as acne, weight gain, dyslipidemia, polycythemia, gynecomastia, deep venous thrombosis, and cardiac and hepatic dysfunction [16]. Overall, exogenous androgens are not recommended for the treatment of idiopathic male infertility as there is strong evidence that exogenous testosterone therapy decreases spermatogenesis or causes azoospermia.

71.2.3 Selective Estrogen Receptor Modulators

Clomiphene citrate and tamoxifen are selective estrogen receptor modulators (SERM) that have been some of the most studied empiric treatments for idiopathic male infertility. Clomiphene citrate and tamoxifen are antiestrogens that block the negative feedback cycle at the level of the hypothalamus and pituitary (see Fig. 71.1). Use of clomiphene citrate and tamoxifen has been shown to increase LH and FSH levels with subsequent increases in both testosterone levels and spermatogenesis. However, improvement in pregnancy rates following treatment with SERMs has been inconsistent [17].

For empiric therapy, clomiphene citrate is generally prescribed at a dose of 25–50 mg/day and tamoxifen at a dose of 10–30 mg/day for 3–6-month treatment periods [7]. A 2007 prospective study of 65 men demonstrated improvement in sperm concentration and motility after 3 months of treatment with 25 mg/day of clomiphene citrate [18]. In a 2010 prospective trial of 32 men, Moradi et al. also found statistically significant improvement in sperm concentration and motility after 3 months of 25 mg/day of clomiphene citrate treatment [19]. Turning our attention to studies using tamoxifen, a prospective, randomized, controlled trial by Cakan et al. using 20 mg/day of tamoxifen for 3 months demonstrated a significant improvement in sperm concentration and motility, but these parameters did not reach normal levels as defined by the World Health Organization [20]. In two recent prospective uncontrolled trails by Nada et al. [21] and Tang et al. [22], tamoxifen was administered for 6 months, and improvement in sperm concentration was seen but not in sperm motility.

While the data above is encouraging, none of the studies mentioned above reported pregnancy data [21, 22]. A Cochrane review of tamoxifen and clomiphene citrate for idiopathic male infertility was performed in 2000 which included ten randomized, controlled trials. The treated men demonstrated statistically significant improvement in hormonal parameters; however, no significant changes in pregnancy rates were observed. This review has since been withdrawn as it had not been updated for over 10 years and there was significant variations in treatment parameters and other factors [23]. In 2013, a more recent meta-analysis was performed which analyzed 11 randomized controlled trials, 9 of which had pregnancy data that used either tamoxifen or clomiphene citrate for idiopathic male infertility empiric treatment. This study found that SERM use led to statistically significant increase in sperm concentration and sperm motility. More importantly, the study demonstrated 2.4 times ($P = 0.004$) higher chance of pregnancy after treatment with SERMs compared with controls. Tamoxifen 20–30 mg/day and clomiphene citrate 50 mg/day were found to have 2.8

times and 5 times higher chances of pregnancy, respectively, both statistically significant findings. Clomiphene citrate at a dose of 25 mg/day was not significantly associated with increased pregnancy rate. No significant difference in adverse events was noted between treatment and control arms [24].

Some studies have looked at the use of SERMs to improve sperm counts to allow for intracytoplasmic sperm injection (ICSI) to be used in conjunction with in vitro fertilization. In one study of 42 patients treated with clomiphene citrate, the level of maturation arrest improved, from spermatocytes before treatment to spermatids after treatment [25]. However, the clinical significance of late versus early maturation arrest is questionable [26]. Interestingly, two poorly designed studies reported 100% ICSI sperm retrieval rates after SERM treatment, one study with clomiphene citrate and one with tamoxifen [25, 27]. While surgical management of idiopathic male infertility is outside the scope this chapter, it is important to understand that empiric medical therapy with SERMs may help patients with future surgical outcomes if medical therapy alone is not sufficient to attain pregnancy.

Overall, SERMs are usually well tolerated with minimal side effects such as hot flashes, gynecomastia, headaches, and nausea. Serious but rare adverse events that have been reported include seizures, palpitations, and pulmonary embolus [17]. Treatment with clomiphene citrate and tamoxifen has been the most studied therapy up to date with promising associated successful pregnancy outcomes.

71.2.4 Aromatase Inhibitors

Aromatase is a cytochrome P-450 enzyme that converts testosterone to estradiol and is found in the female reproductive tract, adipose tissue, testis, liver, and brain. Aromatase inhibitors are compounds that block the conversion of testosterone to estradiol [28] (see Fig. 71.1). Since estradiol is the major source of estrogen in men, aromatase inhibitors have been used to reduce estradiol levels while increasing endogenous testosterone levels. This increase of the testosterone to estradiol (T/E) ratio is hypothesized to help promote spermatogenesis in men with idiopathic male infertility [17]. Pavlovich et al. identified men with infertility as having a T/E ratio of 6.9, while men with normal spermatogenesis as having a mean T/E ratio of 14.5. Based on this observation, it has been proposed that a T/E ratio less than 10 is abnormal, and subsequent studies have focused on the use of aromatase inhibitors in men with abnormal T/E ratios and low serum testosterone levels [29].

Two types of aromatase inhibitors have been used as empiric medical therapy: steroidal irreversible inhibitors (testolactone) and nonsteroidal reversible inhibitors (anastrozole and letrozole). A 1989 randomized controlled study assigned 25 men to 2 g/d of testolactone for 8 months followed by crossover. In this study, estradiol and testosterone levels did not change significantly with treatment. No improvements in semen parameters were observed and no pregnancies occurred in either group [30]. Other studies using testolactone did not focus solely on idiopathic male infertility, as men with low testosterone levels were incorporated into the study. Testolactone is no longer clinically available in the United States.

While several studies have focused on the use of nonsteroidal aromatase inhibitors for treatment of male infertility, very few prospective studies have been conducted using nonsteroidal aromatase inhibitors strictly for idiopathic male infertility. Anastrozole is a more selective aromatase inhibitor compared to testolactone, while letrozole is a more potent aromatase inhibitor compared to anastrozole. One of the first papers to suggest the use of anastrozole was published in 2002 where Raman et al. compared 140 infertile patients with abnormal T/E ratio treated with either 100–200 mg testolactone daily or 1 mg anastrozole daily. Although the study population did not strictly include men with idiopathic infertility only, a significant increase in the hormonal profile and semen parameters was observed in both treatment groups. The authors concluded that anastrozole was just as effective as testolactone for treating infertile men with abnormal T/E ratio [28]. Saylam et al. conducted a prospective, uncontrolled trial of 27 men with low T/E ratio and idiopathic male infertility who were treated with 2.5 mg/d of letrozole for 6 months. They found significant improvement in serum testosterone levels, T/E ratio, and all semen parameters; however, semen parameters did not reach normal levels. Two of the ten men in the study achieved spontaneous pregnancies [31]. A 2012 nonrandomized study of men with low T/E ratio compared letrozole 2.5 mg/d for 6 months with anastrozole 1 mg/d for 6 months. Significant improvement in semen parameters was noted in both groups with numerically greater improvement observed in the anastrozole group [32]. Cavallini et al. conducted a randomized controlled trial comparing 22 patients on letrozole 2.5 mg/d for 6 months with 24 patients in a control group. Significant increase in sperm concentration and motility was noted after treatment, but no pregnancy data was available [33]. Finally, in a 2017 study of 86 subfertile men who were treated with 1 mg anastrozole daily, on a subgroup analysis of 21 oligospermic men with low T/E ratio, a significant improvement was seen in semen parameters and hormonal profile with anastrozole treatment. No improvement was seen in men with azoospermia, cryptospermia, and normospermia at presentation [34].

Overall, aromatase inhibitors have demonstrated improvement in semen and hormonal parameters in patients with low testosterone levels and abnormal T/E ratio while demonstrating promising pregnancy outcomes data. For treatment of idiopathic male infertility, the benefits of aromatase inhibitor

Table 71.1 Hormonal agents for idiopathic male infertility

Agent	Doses reported	Mechanism	Reported benefits	Level of evidence
GnRH	Variable: 100 micrograms)	Stimulate LH/FSH release	Improvement in semen parameters	Poor
hCG	Variable: 2500 IU	Stimulate Leydig cells	Improvement in semen parameters	Poor
FSH	Variable: 150 IU – 300 IU	Stimulate Sertoli cells	Improvement in semen parameters	Promising
Androgens	Variable	Stimulate spermatogenesis	Improvement in semen parameters	Poor
Clomiphene	25–50 mg/day	SERMs block estrogen negative feedback	Improvement in semen parameters and pregnancy data	Good
Tamoxifen	10–30 mg/day	SERMs block estrogen negative feedback	Improvement in semen parameters and pregnancy data	Good
Anastrozole	1 mg/day	Aromatase inhibitor	Improvement in semen parameters in men with low T/E ratio	Promising
Letrozole	2.5 g/day	Aromatase inhibitor	Improvement in semen parameters in men with low T/E ratio	Promising

Summary of hormonal agents used for idiopathic male infertility. Level of evidence: Good, based on reviews of well-done randomized controlled trials (RCT) or individually well-done RCT. Promising, well-designed individual studies but larger studies needed. Poor, poorer quality of studies, well-designed larger studies needed

use are encouraging and should be considered for men with abnormal T/E ratios less than 10. In general, aromatase inhibitors are well tolerated with minimal side effects, including headache, nausea, vomiting, hot flashes, change in libido, and transient change of liver function tests [32].

In summary, hormonal therapy has shown proven benefits in patients with hypogonadotropic hypogonadism. Off-label use for the treatment of idiopathic male infertility has yielded the most promising results with SERMs followed by aromatase inhibitors, but large-scale randomized controlled trials with pregnancy outcomes data are needed. Prior to the initiation of such therapies in patients, it is important to discuss current evidence supporting its use and to undergo a shared decision-making process to review benefits and risks of therapy (see Table 71.1).

71.3 Antioxidant Treatments

In addition to hormonal abnormalities as a cause of idiopathic male infertility, increased oxidative stress leading to sperm damage has been postulated to contribute to idiopathic male infertility as well. Reactive oxygen species (ROS) are oxygen-containing molecules which at normal physiologic levels are thought to benefit sperm function and sperm maturation and enhance overall cellular signaling pathways [35]. Normally, excessive ROS are deactivated or stabilized by the body's antioxidant system; however, when ROS production occurs unchecked, a state of oxidative stress develops [36]. Elevated ROS levels can lead to protein damage, lipid peroxidation, apoptosis, and DNA damage. Spermatozoa are susceptible to ROS damage as they lack antioxidant repair systems and their membranes are rich in polyunsaturated fatty acids, making them susceptible to lipid peroxidation [35]. It has been found that 20–40% of infertile men have significantly higher levels of ROS in their semen as compared to ROS semen levels of fertile men [37]. Furthermore, there are reports indicating that increased oxidative stress is correlated with decreasing semen parameters and decreasing fertilization and pregnancy rates [38, 39]. Therefore, by decreasing oxidative stress through antioxidant treatment, an improvement in semen parameters and fertility rates can be observed. A large abundance of oral antioxidants are available that claim to improve male fertility. This section will focus on presenting the data for the more frequently prescribed oral antioxidant supplements for idiopathic male therapy. One of the challenges in reviewing data of antioxidant supplements is the wide dosing ranges used and lack of strict inclusion criteria in studies for patients with only idiopathic male infertility.

Glutathione is a key part of the glutathione peroxidase/reductase enzyme, a naturally occurring antioxidant that confers protection against lipid peroxidation in the epididymis and testes, thus preserving sperm viability and motility [40]. One study by Rajimakers et al. and another by Ochsendorf et al. found significantly higher glutathione levels in seminal fluid of fertile men compared to subfertile men [41, 42]. A 1992 randomized, placebo-controlled, double-blind, crossover trial with 20 patients with varicoceles or genital tract inflammation demonstrated a significant increase in semen parameters after glutathione treatment. However, the study did not measure ROS or glutathione levels before and after therapy, pregnancy data was absent, and therapy did not focus on idiopathic male infertility. Glutathione is well tolerated with minimal side effects, but parenteral administration is preferred given poor enteric absorption [43]. While clinical studies encompassing all infertile males and in vitro studies have demonstrated

protective effects of glutathione on sperm characteristics and semen parameters, there is no high quality evidence supporting the use of glutathione in men with idiopathic male infertility.

Vitamin E (α-tocopherol) is a fat-soluble antioxidant molecule which protects polyunsaturated fatty acids against peroxidation [7]. Therond et al. identified that the percent of motile spermatozoa is significantly related to semen α-tocopherol levels [44]. Vitamin E is often used for empiric treatment of male infertility, but retrospective reviews have yielded conflicting results with encouraging data seen when vitamin E is given in combination with other antioxidants. However, a randomized controlled study from 1999 by Rolf et al. failed to show any benefit from combination therapy of vitamin E and vitamin C [45]. A more recent prospective study published in 2012 by Chen et al. also failed to show any improvement in semen parameters or pregnancy rates with vitamin E supplementation alone [46]. Overall, vitamin E is well tolerated with minimal side effects and low risk of toxicity [47]. In theory, vitamin E should be a beneficial empiric treatment of idiopathic male infertility; however, the current data does not provide any conclusive supporting evidence.

Vitamin C (ascorbic acid) is a water-soluble compound with the ability to neutralize hydroxyl, superoxide, and hydrogen peroxide radicals with subsequent protection against oxidative damage [48]. Vitamin C levels have demonstrated a positive correlation with sperm motility and percentage of normal sperm morphology [35]. A 2006 prospective, uncontrolled study of 13 men with idiopathic male infertility demonstrated a significant increase in sperm motility and sperm count [49]. Other studies supporting the use of vitamin C have been conducted with the use of multiple antioxidants, making it difficult to draw conclusions on the beneficial effect on male fertility due to vitamin C alone [35]. As noted in the vitamin E section, a randomized controlled study from 1999 by Rolf et al. failed to show any benefit in semen parameters in male infertility from combination therapy of vitamin E and vitamin C [45]. Similar to vitamin E, vitamin C is well tolerated with minimal side effects; however, current data is also lacking to conclusively support the use of vitamin C in empiric idiopathic male infertility treatment.

Carnitine is a water-soluble antioxidant, which is also involved in sperm metabolism and motility [35]. Although lower levels of carnitine have been observed in patients with male infertility [50], a 2006 prospective, randomized controlled study with 21 patients failed to demonstrate any significant improvement in semen parameters after carnitine treatment [51]. However, a 2010 prospective, uncontrolled study of 20 patients treated with 2 g/d of carnitine for 3 months demonstrated a significant increase in sperm count and motility [19]. Similarly, a 2012 prospective, uncontrolled study of 60 patients treated with carnitine 1 g/day demonstrated a significant increase in sperm motility and antioxidant capacity and reported a 23% pregnancy rate [8]. Overall, the data for the use of carnitine for idiopathic male infertility appears promising, but larger, prospective, double-blind studies are still needed.

Coenzyme-Q10 (CoQ-10) is an essential part of cellular respiration and energy production. Moreover, it is found in high concentration in sperm mitochondria, thought to be vital in sperm motility and possess antioxidant properties [52]. As seen with some of the other antioxidants discussed, a positive correlation has been seen between seminal CoQ-10 levels and antioxidant capacity and sperm motility [53]. A prospective, double-blind, placebo-controlled trial of 47 men treated with 200 mg/day for 12 weeks did not show any significant improvement in semen parameters but did report increased antioxidant capacity [54]. A prospective, uncontrolled study by Safarinejad et al. with 297 men treated with 600 mg/day for 1 year demonstrated significant improvement in semen parameters and a 34.1% pregnancy rate [55]. A 2013 meta-analysis of three randomized controlled trials of CoQ-10 supplementation demonstrated significant improvement in sperm motility and concentration but no increase in pregnancy rates [56]. Studies have supported benefits in empiric treatment of idiopathic male infertility with CoQ-10; however, no uniform dosing has been suggested and positive pregnancy outcomes data is lacking.

N-Acetylcysteine (NAC) is an amino acid with antioxidant properties, and in vitro studies with NAC treatment have demonstrated improved sperm motility and reduced ROS levels [57]. A 2009 randomized, placebo-controlled study of 120 patients with idiopathic infertility treated with 600 mg daily of NAC demonstrated significant improvement in sperm motility when compared to placebo [58]. Selenium is an essential trace element, and selenium deficiency has been associated with impaired sperm motility and morphologic sperm midpiece abnormalities [59]. Selenium as an empiric treatment has primarily been studied in combination with other antioxidant supplements. One study with three treatment arms, NAC 600 mg, selenium 200 micrograms, NAC with selenium (600 mg + 200 micrograms), and placebo demonstrated significant improvement in semen parameters in all treatment arms with additive improvement in the combination group [60]. While these results are encouraging for both NAC and selenium, no data is available regarding the degree of pregnancy rate improvement.

Zinc is an essential mineral with antioxidant properties and is involved in DNA/RNA metabolism. Zinc deficiency is associated with sperm structure abnormalities, including flagellar abnormalities [35]. A study from 1987 of 14 men with idiopathic male infertility treated with 220 mg of zinc daily demonstrated improvement in semen parameters and 3 of 14 patients' wives achieving pregnancy [61]. A 2008 study of 45 men randomized into 4 groups, zinc, zinc + vitamin E, zinc + vitamin E + vitamin C, and no therapy, demonstrated

significant improvement in semen parameters for the groups with zinc therapy alone or in combination with other vitamins. No pregnancy data was available [62].

Lycopene is one of the most potent antioxidants, and its levels are lower in the testes and seminal fluids of infertile males [63]. A 2002 prospective, uncontrolled study of 30 men treated with 2 mg twice daily for 3 months demonstrated a significant improvement in sperm concentration and motility. However, lycopene levels before and after therapy were not measured, and the improvement in semen parameters was noted only in men with baseline sperm concentration greater than 5 million/ml [64]. Some other poorly designed studies have shown benefits in semen parameters with lycopene treatment, but overall, the quality of current available data is poor.

Folic acid (vitamin B9) is important for nucleic acid synthesis and amino acid metabolism and has free radical scavenging properties. However, no significant evidence exists for the use of folic acid in the treatment of idiopathic male infertility [34].

Several other compounds with believed antioxidant properties have been used in the treatment of male infertility, including but not limited to magnesium, pentoxifylline, and arginine. While studies do exist in the literature that report improved semen parameters or seminal concentrations of the study antioxidants, the studies are either are of poor design, are not randomized controlled, used combination therapy of antioxidants, or are lacking pregnancy data. Therefore, it is difficult to support the use of these compounds for idiopathic male infertility treatment [65–67].

Another trend in the treatment of idiopathic male infertility is the use of combination antioxidant therapy to achieve synergistic benefits [68]. A 2013 study of 20 infertile men treated with 1500 mg of carnitine, 60 mg of vitamin C, 20 mg of CoQ-10, 10 mg of vitamin E, 10 mg of zinc, 200 μg of folic acid, 50 μg of selenium, and 1 μg of vitamin B12 for a duration of 3 months demonstrated significant improvement in semen parameters and significant improvement in DNA integrity. [69] Busetto et al. conducted a prospective uncontrolled study of 96 men treated with carnitine (145 mg), acetyl-carnitine (64 mg), fructose (250 mg), citric acid (50 mg), selenium (50 μg), CoQ-10 (20 mg), zinc (10 mg), ascorbic acid (90 mg), cyanocobalamin (15 μg), and folic acid (200 μg) once daily for a duration of 4 months. Sperm motility significantly increased, and 16/96 (16.7%) patients achieved pregnancy [70]. A 2014 Cochrane review of 48 randomized controlled trials concluded that only low-quality evidence exists to suggest antioxidant supplementation may improve pregnancy and live birth rates. Overall, adverse effect rates were low in antioxidant therapy [47].

In summary, studies have shown the benefits in the use of antioxidant therapy for idiopathic male infertility with minimal side effects. However, the poor quality of study design and size, variability in antioxidant dosing, and lack of consensus on individual versus combination antioxidant therapy make it difficult to provide clear recommendations on the use of antioxidants for empiric treatment of male infertility. Further large-scale, randomized controlled trials are needed prior to making any substantive recommendations (see Table 71.2).

Table 71.2 Antioxidant agents for idiopathic male infertility

Agent	Doses reported	Mechanism	Reported benefits	Level of evidence
Glutathione	Variable (IM or intranasal)	Scavenges lipid peroxides and hydrogen peroxide	Improvement in semen parameters	Poor
Vitamin E	Variable: 200–400 mg PO	Neutralizes hydroxyl radicals and superoxide anions	Improvement in semen parameters	Poor
Vitamin C	Variable: 500–1000 mg PO	Neutralizes hydroxyl, superoxide, and hydrogen peroxide radicals	Improvement in semen parameters and oxidative stress	Poor
Carnitine	Variable: 500–1000 mg PO	Antioxidant, sperm motility energy source	Improvement in semen parameters and pregnancy data	Promising
CoQ-10	Variable: 100–600 mg PO	Cellular respiration, energy production/sperm motility, antioxidant	Improvement in semen parameters	Promising
N-Acetylcysteine	Variable: 300–600 mg PO	Free radical scavenger	Improvement in semen parameters and oxidative stress	Promising
Lycopene	Variable: 2–8 mg	Potent free radical scavenger	Improvement in semen parameters	Poor
Zinc	Variable: 50–250 mcg	Sperm structure protective effect, antioxidant properties	Improvement in semen parameters	Poor
Selenium	Variable: 50–200 mcg	Important for sperm structure	Improvement in semen parameters and oxidative stress	Poor
Glutathione	Variable (IM or intranasal)	Scavenges lipid peroxides and hydrogen peroxide	Improvement in semen parameters	Poor

Summary of antioxidant agents used for idiopathic male infertility. Level of evidence: Good, based on reviews of well-done randomized controlled trials (RCT) or individually well-done RCT. Promising, well-designed individual studies but larger studies needed. Poor, poorer quality of studies, well-designed larger studies needed

71.4 Conclusion

Hormonal agents and antioxidants are widely used for the treatment of idiopathic male infertility. Some hormonal agents, such as SERMs and aromatase inhibitors, and some antioxidants, such as carnitine and CoQ-10, have shown promising results in the treatment of idiopathic male infertility. However, to truly assess the effectiveness of these medications and supplements, large prospective, randomized controlled trials are needed. If hormonal agents and antioxidants are to be used, prior to the initiation of treatment, a shared decision-making process must be undertaken with the patient to discuss the costs, risks, and benefits of empiric medical treatment as well as the risks and benefits of delaying more definitive treatment (i.e., surgical treatments) since the beneficial outcomes of empiric medical therapy may not be observed for at least 3 months due to the length of the sperm maturation process (typically 72–84 days). Currently, to our knowledge, no new medical treatments are being developed for treating idiopathic male infertility; however, if new treatments are to be developed, empiric medical therapy should ideally increase endogenous testosterone levels, improve semen parameters, decrease oxidative stress, and have minimal side effects for the male partners of couples trying to achieve pregnancy.

71.5 Review Criteria

An extensive search of studies and review papers examining the use of empiric medical treatments for idiopathic male infertility was performed using search engines such as Google Scholar and PubMed. The overall strategy for study identification and data extraction was based on the following keywords, “idiopathic male infertility,” “treatments,” “medical therapy,” “hormones,” “antioxidants,” “infertile men,” “infertility,” “semen parameters,” and “pregnancy rate,” and the names of specific hormones and antioxidants. Articles published in languages other than English were also considered. Data that were solely published in conference or meeting proceedings, websites, or books were not included.

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Testosterone Therapy in Male Infertility

72

Ranjith Ramasamy and John M. Masterson

Key Points

1. Normal spermatogenesis is dependent upon endogenous testosterone production and high concentrations of intratesticular testosterone.
2. Exogenous testosterone is a contraceptive which provides negative feedback to the HPG axis, reducing GnRH release.
3. Testosterone therapies that preserve spermatogenesis also preserve the HPG axis and take advantage of its physiology to promote endogenous testosterone production.
4. Intranasal gels are the only exogenous testosterone therapy to show the ability to preserve normal spermatogenesis and do so by preserving pulsatile GnRH release.
5. Elevated BMI is associated with secondary hypogonadism, and reductions in BMI can contribute to elevated serum testosterone and improvement in symptoms of hypogonadism.

72.1 Introduction

Testosterone levels typically begin to decrease over time in men starting in their late 30s [1, 2]; however, as many as 12.4% of men below the age of 39 suffer the effects of low testosterone and seek treatment [3]. This statistic suggests that a significant number of men seeking treatment for low testosterone are within their reproductive years, underscoring the importance of appropriate counseling for patients seeking testosterone therapy as it pertains to family planning. The challenge for testosterone replacement among men who desire fertility is that exogenous testosterone is a known contraceptive [4]. There are many currently available treatments for low testosterone: the risks, benefits, and impact on male fertility of these treatments are described below.

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72.2 HPG Axis and Association with Male Reproduction

Testosterone plays an essential role in several domains of male health: sexual function, bone health, fat metabolism, muscle mass and strength, and fertility [5]. Normal spermatogenesis is dependent upon endogenous testosterone production as well as an increased concentration of testosterone in the testes relative to serum, typically 50–100-fold greater [6, 7]. Testosterone production and modulation is regulated by signaling along the hypothalamic-pituitary-gonadal (HPG) axis. In the HPG axis, the hypothalamus initially releases gonadotropin-releasing hormone (GnRH) in a pulsatile fashion which stimulates the anterior pituitary gland to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH in turn bind to sites within the testes. LH stimulates Leydig cells to release testosterone and insulin-like growth factor 1 (IGF-1), which upregulates LH receptors within the testes [8]. FSH stimulates Sertoli cells to release inhibin B and androgen binding protein (ABP), which helps to concentrate testosterone within the seminiferous tubules. In addition to stimulating the production of ABP, FSH has been shown to have an independent role in promoting spermatogenesis, suggesting that factors beyond high levels of intratesticular testosterone play a role in normal spermatogenesis [7, 9].

The HPG axis functions as a self-regulating negative feedback loop wherein the end products of the axis downregulate the axis' overall activity. Testosterone and inhibin B produced by the Leydig and Sertoli cells, respectively, are sensed by the hypothalamus, which reduces release of GnRH

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in response [10]. GnRH release is also reduced in response to estradiol, which is generated by the conversion of testosterone to estradiol by the enzyme aromatase [10].

72.3 Effects of TRT on Male Fertility

Testosterone replacement therapy (TRT) is commonly prescribed for men with the constellation of symptoms known as hypogonadism (Table 72.1) and low testosterone [11]. Low testosterone is defined by the 2018 American Urological Association guideline statement on the Evaluation and Management of Testosterone Deficiency as repeated early morning serum testosterone levels below 300 ng/dL [11]. TRT is the simplest form of treatment for low testosterone as it is simply the introduction of exogenous testosterone in an effort elevate a patient's serum testosterone level and relieve their symptoms of hypogonadism. TRT is available in several forms: transdermal patches, intranasal gels, long and short-acting injections, and subcutaneous pellets. Each delivery method of testosterone comes with its unique advantages and disadvantages.

72.3.1 Transdermal Gels and Patches

Transdermal testosterone therapies are applied directly to and absorbed through the skin. Gels are typically applied to an area of the skin of the patient's choosing. Steady-state testosterone levels are typically achieved within 24–72 hours, returning to pretreatment baseline within 4 days of discontinuation [12]. Absorption rates of transdermal gels have been estimated to be 13%–20%, and use of an occlusive dressing has been shown to increase absorption by 2.5-fold [13, 14]. Dosing should be titrated to each individuals' hormone profile and symptomatology over time. Risks associated with transdermal gels are minimal and include application site reaction and transference of testosterone to others [15]. Users of topical testosterone gels should exercise caution not to inadvertently transfer their testosterone to those around them, especially women and children [11].

Taking advantage of the synergistic effects of occlusive dressings with transdermal gels, transdermal testosterone patches consist of a mixture of testosterone, penetration agents, and a gelatinous matrix separated from the skin by a microporous membrane [11]. Testosterone patches tend to increase testosterone to supratherapeutic levels several hours after application with a gradual decrease to therapeutic levels over the subsequent 12 hours [16]. The half-life following removal of a transdermal patch is estimated to be approximately 2 hours [17, 18]. An advantage of transdermal patch use and the nature of its testosterone level fluctuation is that nighttime application has been shown to closely mimic the circadian nature of endogenous testosterone, with peak levels in the morning and trough levels at night [19]. Dosing is one or two patches per day, depending on the patients hormonal and symptomatic response [20]. Risks associated with transdermal patches are mostly related to administration site irritation as the risk of transference is much lower than that of transdermal gels [11].

Table 72.1 Signs and symptoms associated with testosterone deficiency

Physical signs and symptoms
Reduced energy
Reduced endurance
Diminished work performance
Diminished physical performance
Loss of body hair
Reduced beard growth
Fatigue
Reduced lean muscle mass
Obesity
Cognitive signs and symptoms
Depressive symptoms
Cognitive dysfunction
Reduced motivation
Poor concentration
Poor memory
Irritability
Sexual signs and symptoms
Reduced sex drive
Reduced erectile function

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72.3.2 Long- and Short-Acting Injections

Injectable testosterone is available in both short-acting and long-acting formulations. Short-acting injectable testosterone is available in both intramuscular and subcutaneous injections. Intramuscular injections have been demonstrated to have a shorter time to peak testosterone levels as well as a higher peak testosterone than subcutaneous injections [21]. Intramuscular injections also have a higher seven-day mean testosterone value but a shorter overall half-life (10 days) [21]. There is currently no recognized optimal dosing strategy but short-acting injectable testosterone, but more frequent injections are thought to offer clinicians tighter control over peak and trough testosterone levels [11].

Long-acting testosterone, or testosterone undecanoate, is currently available in only an intramuscular formulation at single dosage under the brand names Nebido® and Aveed® [11]. Administration occurs every 10 weeks following two initial loading 2 weeks apart. The advantage of long-acting injectable testosterone as compared to short-acting is decreased variability between peak and trough levels [22, 23].

Disadvantages of both short- and long-acting injectable testosterone include injection site irritation and abnormally increased hemoglobin and hematocrit [24–26]. Disadvantage unique to short-acting testosterone is the testosterone trough that can occur when testosterone becomes subtherapeutic [11]. Long-acting testosterone is uniquely but rarely associated with intense coughing related to pulmonary microemboli caused by the oil-based preparation of the medication [27, 28]. For this reason long-acting testosterone must be administered in an office setting in which patients are monitored for 30 minutes after injection [11].

72.3.3 Subcutaneous Pellets

Subcutaneous pellets require the least frequent administration among currently available TRT delivery methods. The pellets are inserted into subcutaneous spaces using a sharp trocar to gain access and a stylet into which pellets containing 75 mg of testosterone are loaded. Multiple pellets are used to achieve therapeutic levels of testosterone for individual patients. Increasing the number of pellets will increase the amount of testosterone delivered, but it will not increase the half-life of testosterone or decrease the frequency of dosing [29]. Current dosing schedules are six to eight pellets every 3–6 months, with serum testosterone monitoring at 4 weeks after insertion for peak levels, 3 months after insertion, and every 2 weeks thereafter for therapeutic monitoring [29–31]. Disadvantages of subcutaneous pellet delivery of testosterone include pain and ecchymoses at the insertion site and even possible extrusion of the pellets themselves [32]. Other effects include thrombocytopenia seen also with injectable testosterone but at lower rates [29].

What is common among all of the aforementioned TRT methods is that they provide users with a steady-state, basal level of testosterone that slowly wanes over time. Introduction of a basal, non-pulsatile, level of exogenous testosterone signals to the hypothalamus to cease the production of GnRH. In the absence of GnRH production, the anterior pituitary will not release LH and FSH to stimulate testosterone production and ABP production in testes, halting normal spermatogenesis [33]. Exogenous testosterone, therefore, is effectively a contraceptive for males and will lead to azoospermia in the majority of users [33]. In fact, TRT is an increasingly common etiology of male infertility [34].

72.3.4 Intranasal Gels

A relatively new TRT delivery method that shows some promise on the issue of male fertility is via intranasal gel. Intranasal gels deliver exogenous testosterone as a nasal gel to be administered twice daily. Intranasal gels provide a short-lived boost in serum testosterone that, in theory, more closely mimics the pulsatile nature of endogenous testosterone, thereby preventing the negative feedback to the hypothalamus which leads to azoospermia [35]. In a recent phase II clinical trial, intranasal testosterone gel was found to restore normal testosterone levels in 90% of hypogonadal study subjects [36]. Preliminary results from an ongoing phase IV clinical trial have shown that intranasal testosterone gel can increase serum testosterone while maintaining normal semen parameters [35].

72.4 Treatment Options to Increase Intratesticular Testosterone

The challenge for clinicians in treating men with low testosterone who desire fertility is to avoid the pitfall of simply providing exogenous testosterone. Fortunately there are several medications that take advantage of HPG axis physiology and are able to bypass the negative feedback of exogenous testosterone that ultimately halts normal spermatogenesis.

72.4.1 Gonadotropins: GnRH and hCG

In a normally functioning HPG axis, GnRH is released from the hypothalamus and stimulates the anterior pituitary to release the gonadotropins LH and FSH. LH and FSH act upon Leydig and Sertoli cells, respectively, within the testes. Leydig cells release testosterone, and Sertoli cells release inhibin B and ABP, which increases intratesticular testosterone. As an alternative to exogenous testosterone administration, in the appropriate patients, we are able to simply supplement GnRH to stimulate production of LH and FSH and therefore endogenous testosterone [37, 38]. GnRH is administered via subcutaneous portable infusion pump, delivering GnRH in a pulsatile fashion that mimics the pulsatility of normal GnRH signals [39]. Due to the logistical challenges on constant dosing, GnRH administration is currently only indicated in experimental settings [39].

Human chorionic gonadotropin (hCG) shares structural similarities with LH and can thereby stimulate Leydig cells at the LH receptor within the testes. Still-functioning Leydig cells can then be stimulated to release endogenous testosterone. hCG is typically administered via subcutaneous injection one to three times weekly. The most notable adverse effect related to hCG therapy is gynecomastia, due to the supraphysiologic doses of hCG necessary to preserve fertility [40].

Gonadotropin therapy is appropriate for patients with hypogonadotropic hypogonadism and will be less effective in patients with primary hypogonadism. Functioning testicular tissue is required for the gonadotropins to have a substrate

on which to bind and exert their effects. hCG therapy has been demonstrated to preserve fertility in men with hypogonadotropic hypogonadism and requires administration for an average of 7–10 months until pregnancy is achieved [40, 41].

72.4.2 Clomiphene Citrate

The negative feedback demonstrated by the HPG axis is predominantly dependent on the presence of testosterone to downregulate the release of GnRH. Estrogen, however, also has the ability to produce negative feedback on the hypothalamus in a similar manner. Clomiphene citrate is classified as a selective estrogen receptor modulator (SERM). SERM mechanism of action is to either agonize or antagonize estrogen receptors. SERMs were initially developed for stimulation of ovulation during in vitro fertilization, but are used off-label for the treatment of hypogonadism in men [42, 43]. Clomiphene citrate is an estrogen receptor modulator at the level of the hypothalamus. It competitively inhibits estrogen from binding receptors in the hypothalamus, thereby preventing the negative feedback normally provided by estrogen. The downstream effect is to maintain GnRH release, gonadotropin release from the anterior pituitary, and downstream release of endogenous testosterone from the testes [44]. Clomiphene citrate essentially tricks the hypothalamus into sensing less circulating estrogen than is actually present, preventing negative feedback. Preservation of the HPG axis with clomiphene citrate allows for maintenance of intratesticular testosterone levels adequate for spermatogenesis while stimulating enough endogenous testosterone to treat symptoms of hypogonadism [45–47]. Clomiphene citrate is typically administered orally two times per day. The most common adverse effects are gynecomastia and fluid retention [48].

72.5 Aromatase Inhibitors

Similarly to SERMs, aromatase inhibitors limit HPG axis suppression by limiting the negative feedback of estrogens. Aromatase is most concentrated in the testis but exists in various tissues throughout the body including brain and adipose tissue. Aromatase converts testosterone to estradiol, which provides estrogenic feedback onto the hypothalamus, leading to decreased gonadotropin production [49]. Aromatase inhibitors decrease the peripheral conversion of testosterone to estradiol, thereby restoring the testosterone to estrogen ratio to within normal limits [50]. The subsequent increased level of testosterone effectively treats symptoms of hypogonadism, while the maintenance of an intact HPG axis protects normal spermatogenesis [51, 52]. Adverse effects of aromatase inhibitors are usually mild and include nausea, decreased bone mineral density, and decreased libido [48].

72.6 Lifestyle Modification

Several modifiable risk factors have been found to be associated with low testosterone and symptoms of hypogonadism. These risk factors include but are not limited to body mass index (BMI) greater than 30, status as a current smoker, and presence of one or more comorbid conditions (heart condition, high blood pressure, cancer ever, bronchitis, asthma, peptic ulcer, epilepsy, diabetes, liver disease, kidney disease, prostate disease, and stroke) [53, 54]. It stands to reason that avoidance or modification of these risk factors will increase endogenous testosterone levels [55]. There is data to suggest that weight loss can increase serum testosterone levels and vice versa, that testosterone supplementation can reduce BMI [56]. Most importantly, lifestyle modification as treatment for low testosterone preserves the HPG axis and thereby preserves normal spermatogenesis, leaving fertility unaffected.

72.7 Varicocele Repair

Varicocele is caused by a dilatation of the pampiniform plexus of spermatic veins and is a common reversible cause of male infertility. Varicocele is present in up to 20% of postpubertal males and is more likely to be found in men with abnormal semen parameters [57]. The mechanism by which varicocele causes low testosterone is somewhat controversial. Varicocele is thought to cause Leydig cell dysfunction, essentially creating testicular or primary hypogonadism [58]. Dysfunctional Leydig cells are unable to synthesize testosterone in response to LH which can lead to symptoms of hypogonadism and abnormal spermatogenesis. It should be noted, however, that not all men with varicoceles have low serum testosterone or abnormal semen parameters. Varicocele repair has been shown to improve semen parameters in men with varicocele and infertility; however the association between varicocele repair and improved testosterone levels is dubious [59, 60]. Theoretically varicocele repair should alleviate Leydig cell dysfunction in way that increases endogenous testosterone while maintaining an intact HPG axis; however consistent data is lacking.

72.8 Leydig Stem Cells

The use of Leydig stem cells has recently shown promise in animal models as a potential therapy for men with low testosterone who wish to preserve fertility. Several experiments

have demonstrated that transplanted Leydig stem cells into the testes have the ability to differentiate into testosterone-producing Leydig cells [61–64]. A recent pilot study by Arora et al. found that autografted Leydig stem cells implanted in the subcutaneous tissue of castrate mice were able to differentiate into mature Leydig cells, produce endogenous testosterone, and be regulated by the HPG axis [65]. These experiments are encouraging in that they offer another treatment modality that increases endogenous testosterone and thereby preserves spermatogenesis.

72.9 Conclusion

Low testosterone affects a significant number men who desire fertility. The challenge in treating the signs and symptoms of low testosterone in this population is that exogenous testosterone is a known contraceptive in men due to its negative feedback on the HPG axis. The only exogenous testosterone modality that has shown promise in preserving fertility is intranasal gel due to the pulsatile nature of testosterone delivery and thus preservation of the HPG axis. Other therapies that treat low testosterone like gonadotropins, clomiphene citrate, hCG, and aromatase inhibitors preserve the native physiology of the HPG axis and promote upregulation of endogenous intratesticular testosterone.

72.10 Review Criteria

An extensive search of studies investigating treatment of low testosterone in men desiring fertility was performed using search engines such as PubMed and Google Scholar. There were no publication date limitations placed upon this search. The overall strategy for study of identification and data extraction was based on the following keywords: "low testosterone," "hypogonadism," "fertility," "infertility," "spermatogenesis," "testosterone replacement therapy," as well as the names of specific testosterone therapies. Articles published in languages other than English were not considered. Data that were solely published in conference or meeting proceedings, websites, or books were not included. Websites and book-chapter citations provide conceptual content only.

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