

Unexplained male infertility: potential causes and management

Alaa Hamada^a, Sandro C. Esteves^b and Ashok Agarwal^a

^aCenter for Reproductive Medicine, Cleveland Clinic, Cleveland, USA and ^bANDROFERT – Andrology and Human Reproduction Clinic, Campinas, Brazil

Correspondence to Ashok Agarwal, PhD, HCLD, Lerner College of Medicine, Director, Center for Reproductive Medicine, Cleveland Clinic, Desk A19.1, 9500 Euclid Avenue, Cleveland, Ohio 44195, USA
Tel: 216-444-9485;
fax: 216-445-6049;
e-mail: agarwaa@ccf.org

Received 28 February 2011

Accepted 12 March 2011

Human Andrology 2011, 1:2–16

Objective

To highlight the concept of unexplained male infertility and discuss the potential causes and its proper management.

Design

Review of literature.

Results

Male infertility of unknown origin is a condition in which fertility impairment occurs spontaneously or due to an obscure or unknown cause. It includes two categories, unexplained male infertility and idiopathic male infertility. The dividing line between them is semen analysis, which is normal in the unexplained category and abnormal in idiopathic infertility. After ruling out female infertility factors, erectile problems and coital factors, modern andrology may help to analyze the unexplained male fertility problem on the basis of cellular and subcellular mechanisms. Furthermore, this analysis may lead to the selection of proper treatment options fitting the needs of patients with unexplained infertility.

Conclusion

Despite the advances and innovation of sophisticated laboratory tests in the field of andrology, further research is still needed to solve the dilemma of infertility.

Keywords:

male infertility, normospermia, sperm function, oxidative stress, autoimmune infertility, assisted reproductive techniques

Hum Androl 1:2–16
© 2011 Human Andrology
2090-6048

Introduction

Infertility is a common clinical problem affecting 13–15% of couples worldwide [1]. The prevalence varies throughout developed and underdeveloped countries, being higher in the latter in which limited resources for diagnosis and treatment exist [2]. A male factor is solely responsible for infertility in approximately 20% and contributory in another 30–40% of couples; as such, a male factor is implicated in more than 50% of couples attempting to conceive [3]. A reduction in the male fertility potential may be due to congenital or acquired conditions such as urogenital abnormalities, varicocele, infections of the genital tract, genetic abnormalities, endocrine disturbances, testicular failure, immunologic problems, cancer, systemic diseases, altered lifestyle, and exposure to gonadotoxic factors [4]. The cause of fertility impairment cannot be determined in several cases, despite the advances in diagnosis by the introduction of novel sophisticated tests.

Infertility of unknown origin includes unexplained male infertility and idiopathic male infertility; it is a condition in which fertility impairment occurs spontaneously or due to an obscure or unknown cause. Infertility of unknown origin accounts for 37–58% [5–7]. The category ‘unexplained male infertility’ (UMI) is reserved for infertile men with infertility of unknown origin with normal semen and in which female infertility factors have been

ruled out [8]. The reported prevalence of UMI ranges from 6 to 27% [5] and it strongly depends on how exhaustive is the evaluation of the patient. Men classified as having idiopathic male infertility have an unexplained reduction in semen quality with no history associated with fertility problems and have normal findings on physical examination and endocrine laboratory testing. Their routine semen analysis shows decreased number of spermatozoa (oligozoospermia), decreased motility (asthenozoospermia), or an increased proportion of abnormal forms (teratozoospermia). These abnormalities usually occur together and are described as the oligoastheno-teratozoospermia syndrome [6]. This category comprises approximately 31% of infertile men [6]. Table 1 clearly shows the frequency distribution of different causes of male infertility [8]. Furthermore, the frequency of male infertility of unknown origin is different between countries. In a group of 2383 subfertile male individuals attending one of the investigators’ (S.C.E.) tertiary center for male reproduction, 12.1% of the individuals were categorized as having infertility of unknown origin (Table 2).

In brief, the initial assessment of subfertile men includes history, physical examination, and at least two semen analyses after 12 months of unprotected intercourse. However, the initial male work-up may be carried out earlier, particularly in the cases of advanced female age (more than 35 years), the presence of known male

Table 1 Distribution of final diagnostic categories found in male infertility clinic [8]

Category	Frequency (%)
Immunological	–
Idiopathic	32.6
Varicocele	26.6
Obstruction	15.3
Normal female factor (unexplained male infertility)	10.7
Cryptorchidism	2.7
Ejaculatory failure	2.0
Endocrinologic	1.5
Drug/radiation	1.4
Genetic	1.2
Testicular failure	1.1
Sexual dysfunction	0.7
Pyospermia	0.5
Cancer	0.4
Systemic disease	0.3
Infection	0.2
Torsion	0.1
Ultrastructural	0.1
Total	100.0

Table 2 Distribution of diagnostic categories in a group of infertile men attending 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
Infertility of unknown origin	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

infertility risk factors (such as an undescended testicle), or if a man questions his fertility potential [3]. In roughly half of the patients, the initial assessment will identify the cause of infertility, whereas many other patients will need to go through several complementary tests to find its cause.

The goals of meticulous evaluation of subfertile men are (i) identification of the cause of subfertility, which is a prerequisite for the correct indication for appropriate surgical or medical treatment, (ii) identification of inadequate lifestyle or sexual behavior to allow counseling toward an improvement in the reproductive potential and overall health status, and (iii) identification of significant medical pathologies that threaten a man's overall health or life (endocrinopathies, testicular and prostate cancer, brain and spinal cord tumors), which can be identified in up to 6% of infertile men [9–12].

Potential etiologies of unexplained male infertility

Two vital questions come across the clinicians' mind when dealing with a subfertile male whose medical evaluation is unrevealing. First, how predictive are the semen analyses

results in anticipation of spontaneous conception? Second, what should be done next after performing a comprehensive work-up or when failing to identify the causes of infertility? Normal semen analysis results do not guarantee fecundity. A significant proportion of patients with normal semen on routine analysis remain childless over several months attempting to conceive [13]. In one study involving 430 couples, 45% of men with a sperm concentration of greater than 40 million sperm/ml were unable to impregnate their wives [14]. It had been demonstrated that the routine semen analysis was unable to detect sperm functional deficiencies in 40% of men presenting with subfertility [15]. Table 3 shows the frequency of semen analysis abnormalities in 8758 infertile patients attending the fertility clinic.

For men with unexplained infertility and normal semen analyses the following possibilities should be considered: (i) presence of a female factor, (ii) inappropriate coital habits, (iii) erectile dysfunction, (iv) the presence of antisperm antibodies (ASAs) (autoimmune infertility), and (v) sperm dysfunction [12]. To exclude the first three conditions, proper history taking as well as a thorough gynecological evaluation is needed, whereas modern andrology could be of help in managing the last two conditions.

Autoimmune infertility

Autoimmune infertility has long been postulated as one of the causes of subfertility [16]. It represents approximately 4.5% of male factor infertility [6]. Why does a man's body treat his sperm as foreign invaders? To explain this, three theories are hypothesized. The first states that sperm are not present at the time of embryological development during which the immune system establishes tolerance to self antigens [17]. The second claims that spermatozoa are haploid and have a different chromosomal make up from the somatic cells [16]. Meanwhile, the third theory, called 'immunosuppression theory', postulates that T suppressor lymphocytes, which inhibit immune responsiveness, are activated by small amounts of spermatozoal antigens continuously leaked from the genital tract [18]. As soon as spermatozoa, which are considered immunologically foreign cells, are formed during puberty, they must be completely isolated from the immune system. This isolation occurs within the testis, one of the immunologically privileged sites, by the blood–testis barrier. In other regions of the male genital tract, the epithelial lining, probably supplemented by a local immunosuppressive barrier, is responsible for this isolation [19]. Despite its immune-privileged status, the

Table 3 Distribution of abnormalities of semen parameters in 8758 patients [8]

Abnormality in semen parameters	Frequency (%)
Azoospermia	4
Predominance of a single abnormal parameter	29
Motility	18
Volume	2
Morphology	7
Density	2
Defects in two or more parameters	37
All parameters normal (unexplained male infertility)	30

4 Human Andrology

testis is clearly capable of mounting inflammatory responses, as proven by its effective cellular and humoral defense against infections. In pathological circumstances, the imbalance between the tolerogenic and the efferent limbs of the testicular immune response can lead to the development of ASAs, and in rare instances, to autoimmune epididymoorchitis [16,20–22]. Both humoral and cellular immunity have been implicated in the etiology of immune infertility.

Humoral immune infertility

Approximately 10% of all infertile men may have ASA (vs. 2% of fertile men) [23]. Unexplained male factor infertility may be related to ASAs. Moghissi *et al.* [24] confirmed that the incidence of sperm antibodies was significantly higher (42.5%) among patients with unexplained and persistent infertility. The pathogenesis of the formation of ASAs is still a matter of debate. Antisperm immune responses occur probably as a result of the disruption of the epithelial or blood–testis barrier [25,26], an immunosuppression defect [27], or as a result of an insult to the genital tract that would provide an excess of spermatozoal antigens that could override the mechanism of immunosuppression [28]. A major cause of ASA is vasectomy [29–31]. Other causes include vas obstruction [29], testicular trauma, torsion, malignancy, infection of the genital tract, semen deposition at nongenital tract sites (homosexuality), and perhaps, varicocele [31–33] and intolerance to heavy metals [34]. It is still unclear whether ASA is produced locally in the genital tract or transuded from serum. ASA can be found in serum, seminal plasma, and can be sperm bound. Among these, sperm-bound antibodies are the most clinically relevant. The antibody classes that appear to be clinically relevant include immunoglobulin G (IgG) and IgA. IgG antibody is derived from local production and from transudation from the bloodstream. IgA, in contrast, is thought to be purely locally derived [35]. The epididymis is postulated to be the production site in cases of obstruction because of the combined effect of increased intraluminal pressure and leak of spermatozoa antigens. In contrast, a woman can also produce ASAs in her cervical fluid. Such antibodies have been reported in 7–17% of infertile women, varying with the type of test performed and the population screened [36,37]. ASA impair sperm function by induction of apoptosis, or by inducing premature acrosome reaction (AR). ASA may also interfere with fertilization by inhibition of cervical mucus penetration, zona pellucida (ZP) binding or sperm–oocyte fusion. ASA may change some macromolecular and subcellular function by altering chaperon function, protein folding, and disulfide bonds [38]. The end result is that pregnancy rates may be reduced by ASA [39]. When dealing with patients with unexplained male infertility, one should keep in mind that the presence of elevated levels of ASA may occur even in the face of normal semen analysis. Sperm agglutination is the only well-established semen alteration related to the presence of ASA [40]. However, sperm agglutination, which is a time-dependent phenomenon, only rarely involves a large proportion of motile spermatozoa soon after liquefaction,

even when all ejaculated spermatozoa are antibody coated. Therefore, sperm agglutination, although extremely suggestive of sperm autoimmunization, does not represent an important mechanism of the antibody interference with fertility in most cases. Apart from sperm agglutination, there is little evidence that suggests a cause/effect relationship between ASA and the abnormality of semen parameters [39]. A negative impact of ASA on sperm motility/vitality, for instance, should involve a complement-mediated sperm injury, which is prevented by anticomplementary activity in human seminal plasma [41,42]. Nevertheless, adequate amount of complement is present in the cervical fluid, which can be activated through antibody antigen reaction and can exert a toxic effect on sperm.

The diagnosis of immunological infertility requires two conditions to be satisfied [43].

- (1) Fifty percent or more of the motile spermatozoa (progressive and nonprogressive) have attached beads. It should be noted, however, that particle binding restricted to the tail tip is not associated with impaired fertility and can be present in fertile men;
- (2) Sperm-bound antibodies interfere with sperm function; this is usually demonstrated by using functional tests such as the sperm–mucus penetration test, zona binding assays, and the AR [43].

Frequently, antibody-coated sperm may appear as a poor postcoital test (PCT). Complement, which is normally found in higher amounts in the cervical mucus than in the seminal plasma, is needed to immobilize spermatozoa. The antibody complement reaction may take at least 6 h to manifest. Physicians performing a PCT within 2 h after intercourse or using in-vitro mucus penetration assays may miss the immobilizing antibodies. Consequently, these patients may appear as having an absence of male factor infertility. It is therefore advisable to perform a PCT after at least 6 h after intercourse [44]. ASA can cause infertility without obvious problems with cervical mucus penetration. Such antibodies may interfere with the AR and may inhibit sperm penetration into the zona pellucida and fusion with the oocyte [45].

Currently, the most popular tests to identify sperm-bound ASA are both the direct immunobead test (IBT) and the direct mixed agglutination reaction [46]. In the direct IBT, beads coated with covalently bound rabbit antihuman immunoglobulins against IgG or IgA are mixed directly with washed spermatozoa. The binding of beads with antihuman IgG or IgA to motile spermatozoa indicates the presence of IgG or IgA antibodies on sperm surface [43]. IBT is more time consuming but it identifies the proportion of antibody-bound sperm in a given sample, the antibody class, and the location of antibodies on the sperm surface. In contrast, the direct mixed agglutination reaction test is an inexpensive, quick, and sensitive screening test in which sheep erythrocytes are used instead of immunobeads to detect and localize antibody-bound sperm [47,48].

Cellular immune infertility

There is evidence that cell-mediated immunity may play a role in immunological infertility. Histopathological studies demonstrated the presence of inflammatory cells infiltrating the contralateral testis in animal models and patients with unilateral testicular torsion because of release of germ cell inoculum in response to ischemia and necrosis of the torsed testis [49,50]. Despite this immunologic event, ASA are rarely seen in such cases. Other supporting evidence come from immunoreactivity studies using purified macrophages isolated from individuals with repaired unilateral and bilateral cryptorchidism and exposed to homologous sperm. It had been found that 50% of patients with unilateral and 80% with bilateral surgically repaired cryptorchidism had cell-mediated immunoreactivity [51]. Sperm granuloma at the vasectomy site is another evidence of cell-mediated immunity; it represents a dynamic structure and a site of spermatozoal phagocytosis. Intraluminal macrophages, also known as spermatophages, absorb degradation products rather than the whole sperm. T-type lymphocytes, in addition to ASA, may contribute to testicular damage after vasectomy [52]. Spermatozoa exposed to cytokines such as tumor necrosis factor and interferon γ show impairment in motility and inability to penetrate hamster eggs [53,54]. Despite all this, the full-blown spectrum of cell-mediated immunity is difficult to prove using laboratory tests and its role in unexplained infertility is still speculative [47]. More sophisticated investigation is needed to detect the impact of cellular immunity in men with unexplained infertility.

Deficient sperm function

Conventional semen parameters such as sperm count, motility, vitality, and morphology are inadequate to monitor sperm function and to be used as markers of fertility potential [55]. Conversely, sperm function tests may provide more clinically useful prognostic and/or diagnostic information. Such tests may be used to distinguish between fertile and infertile men and to aid in showing the cause of male subfertility and in suggesting therapeutics. Sperm function tests available in the andrology armamentarium include assays that investigate sperm DNA integrity, seminal reactive oxygen species, AR, hyperactivated motility, and ZP binding and penetration.

Sperm chromosomal complement and DNA integrity defects

Germ cells undergo meiotic divisions to form four haploid spermatids. During meiosis, shuffling of some genes occurs between homologous chromosomes giving rise to genetic diversity. During spermiogenesis, the haploid sperm chromatin undergoes significant changes in which most histones are replaced first by transition proteins, and then by positively charged protamines [56]. By this remodeling process, the sperm DNA condenses so tightly that it is resistant to mechanical stresses such as sonication [57] and even to boiling [57], which destroy the DNA in somatic cells. The condensation of sperm DNA protects it during its transit through the male and female reproductive tracts. Cytogenetic analysis and molecular biology genetic testing may identify subfertile

men misdiagnosed as having unexplained and idiopathic infertility. Abnormalities causing male infertility include:

- (1) Chromosomal complement changes in number (e.g. aneuploidy) or structure such as translocations or inversions.
- (2) Gene mutation and polymorphisms.
- (3) DNA integrity defects.

Chromosomal complement: the risk of sperm chromosomal aneuploidy is inversely related to sperm concentration and total progressive motility [58,59]. The overall frequency of chromosomally abnormal sperm in the general population is estimated to be 7%. The mean frequency of disomy for autosomes and sex chromosomes are 0.13 and 0.37%, respectively, whereas the rate for diploidy is 0.2%. For normospermic infertile men, correspondent figures are 0.11, 0.44, and 0.3–1%, respectively [60,61]. Increased sperm aneuploidy rates may impact male fertility and pregnancy viability. Their causes are unknown, but smoking, alcohol, chemotherapy, and aging may play a role. Interchromosomal variation in the rates of disomies has been observed with sex chromosomes and chromosomes 21 and 22; the higher rate of abnormalities related to such chromosomes may be due to their lower rate of meiotic recombination, which renders them more prone to nondisjunction [62].

Abnormal spermatozoa that retain excess cytoplasm show a greater extent of aneuploidy and diploidy than those without excess cytoplasm from the same ejaculate, whether selected by density gradient centrifugation [63], swim-up [64], or binding to hyaluronic acid [65]. In contrast, both morphologically normal and abnormal spermatozoa can be disomic or diploid [66], or contain damaged DNA [67]; as such, selecting normal-looking spermatozoa for assisted reproductive technique (ART) does not guarantee the absence of chromosomal abnormalities.

Chromosomal inversions, deletions, balanced or unbalanced translocations, and Y-chromosome microdeletions are often associated with abnormal semen parameters and higher rates of abortion, and in some cases, with a higher risk for the birth of a severely handicapped child [68]. In Y-chromosome infertility, the AZFc region is prone to many smaller subdeletions that are thought to be caused by intrachromosomal recombination [69]. These partial deletions produce a wide array of phenotypes, ranging from normospermia to azoospermia, because of factors that include the interaction of the environment and the genetic background [70].

Most chromosomal abnormalities may be detected by using one of the following methods: (i) Sperm karyotyping for detection of numerical chromosomal abnormalities; (ii) Fluorescence in situ hybridization analysis, which can be used to assess numerical and structural chromosomal changes by using specific probes; and (iii) quantitative polymerase chain reaction, which is a promising technique to detect and quantify damage to nuclear and mitochondrial DNA [71].

Specific gene defect (mutations and polymorphisms): the definition of nucleotide sequence for the human genome

has facilitated the identification of human fertility-related genes and this will open the way to identify these genes in the future. Nevertheless, DNA sequence analysis is rarely performed in the evaluation of male infertility [72]. In animal studies involving mice, up to 300 null mutations and 50 conditional targeted deletions have produced models of male infertility. Not only the DNA sequence has effect on male infertility but there is also a role for epigenetic events and modifiers of gene expression. With regard to unexplained male infertility, genes certainly play a role as they control meiosis events, spermiogenesis, remodeling, motility, capacitation, and fertilization. It is now possible to monitor the expression of thousands of genes simultaneously with DNA microarray analysis. Garrido *et al.* [73] used the microarray technology in the analysis of spermatozoa from fertile and infertile men with normal semen analysis. They found that hundreds of gene sequences (targets) were differentially expressed between groups. Preliminary results confirmed that there are few genes that are overexpressed, whereas all others are underexpressed in infertile men.

DNA integrity defects: sperm DNA integrity is increasingly being recognized as an important marker of fertilizing efficiency, and it is associated with better diagnostic and prognostic values than standard sperm parameters [74]. Saleh *et al.* [75] reported that an increase of spermatozoa with abnormal chromatin structure or DNA damage (expressed as DNA fragmentation index) negatively correlated with intracytoplasmic sperm injection (ICSI) and in-vitro fertilization (IVF) outcomes.

Populations of sperm with DNA damage are more often seen in subfertile/infertile men than in fertile ones [76–78]. Spermatozoa with damaged DNA may lead to paternal transmission of defective genetic material with adverse consequences to embryo development [79,80]. Approximately 8% of infertile men have abnormal DNA integrity despite normal semen parameters [81].

DNA damage is often assessed by the determination of chromatin compaction or DNA fragmentation. The former examines the accessibility of dyes (Toluidine blue, aniline blue, and chromomycin A3) to nucleoproteins or chromatin after challenging spermatozoa with physical insults; as such, it reflects how susceptible the DNA is, or has been, to noxious agents [82]. Toluidine and aniline blue stains bind to lightly packed chromatin and to lysine residues of histone that are not fully replaced by protamines, respectively. Chromomycin A3 binding is specific for protamine-deficient areas because of its affinity to guanine–cytosine (GC)-rich areas of DNA [83]. In contrast, DNA fragmentation is measured by detecting single-strand or double-strand DNA breaks. Transferase-mediated dTUP nick-end labeling, comet assay, acridine orange test, and sperm chromatin structure assay are methods clinically available to detect DNA fragmentation. Although they differ in costs and methods, most of the mentioned tests are clinically significant and correlate with sperm function and fertility [84].

Reactive oxygen species

It has been shown that 40–88% of nonselected infertile patients have high levels of seminal ROS [85]. Moreover, normospermic infertile men have higher ROS levels and reduced total antioxidant capacity levels than the normospermic fertile counterpart [86]. However, the true prevalence of oxidative stress problem among normospermic men remains to be determined. Mammalian spermatozoa are redox cells that are able to produce oxygen radicals and to export them to the extracellular medium [87–91].

The main source of oxygen radicals in spermatozoa appears to be the mitochondria, as the result of the monovalent reduction of molecular oxygen during oxidative phosphorylation [88]. ROS refer to a group of metabolites formed by reduction of oxygen, including free radicals such as superoxide anion ($O_2^{\bullet-}$), the hydroxyl radical (OH^{\bullet}) as well as powerful oxidants such as hydrogen peroxide (H_2O_2). It also includes reactants of carbon-centered radicals with molecular oxygen, including peroxy radicals (ROO^{\bullet}), alkoxy radicals (RO^{\bullet}), and organic hydroperoxides. ROS may also include other powerful oxidants such as peroxynitrite or hypochlorous acid as well as the highly biologically active free radical, nitric oxide ($\bullet NO$). ROS in semen originate from immature spermatozoa and seminal leukocytes [92]. These free radicals have considerable reactivity and the ability to react with and modify the structure of many different kinds of biomolecules, including proteins, lipids, and nucleic acids. The wide range of targets that can be attacked by ROS is a critical facet of their chemistry that contributes significantly to the pathological importance of these molecules [92]. ROS in low levels have a physiological role. They are required by sperm to attain their functional maturity and are essential for capacitation, hyperactivation, and AR [93,94]. ROS also exert their effect on sperm–oocyte interaction. Low levels of lipid peroxidation cause modifications of plasma membranes facilitating sperm adhesion to the oocyte [95]. However, the physiological ROS levels are still undetermined.

A natural antioxidant defense system offers protection against ROS. It consists of radical scavengers, chain-breaking antioxidants, and ROS-metabolizing enzymes in the vicinity of the spermatozoa during their sojourn in the male reproductive tract. Radical scavengers include small molecular mass such as vitamin C, uric acid, tryptophan, and taurine [96,97]. Chain-breaking antioxidants include membrane-associated antioxidants epitomized by α -tocopherol, a hydrophobic vitamin that is capable of terminating the peroxidation chain reaction [98]. Spermatozoa also harbor antioxidant enzymes such as superoxide dismutase and those of the glutathione cycle, but very little catalase. Imbalance between the oxidant load and natural antioxidant defense system has a pathological effect on sperm function by causing damage to sperm DNA in the nucleus and mitochondria as well as by inducing lipid peroxidation in the sperm plasma membrane [99]. DNA damage includes single-stranded or double-stranded DNA breaks, DNA base-pair oxidation,

chromatin cross-linking, chromosome microdeletions, and even various types of gene mutations such as deletions, point mutations, or polymorphisms that may result in decreased semen quality [100,101]. 8-Hydroxy-2-deoxyguanosine is considered a key biomarker of oxidative DNA damage [102]. ROS may induce lipid peroxidation and hence loss of sperm motility and may even initiate a chain reaction by activating caspases that ultimately lead to apoptosis [103].

The most often used methods for detecting ROS in an andrology setting are divided into two major categories, that is, direct methods such as chemiluminescence and flow cytometry, and indirect methods such as the colorimetric one. Chemiluminescence uses the probes lucigenin or luminol to detect ROS [104,105]. Luminol ($C_8H_7N_3O_2$) is a versatile chemical that shows chemiluminescence when mixed with an appropriate oxidizing agent. It can penetrate inside the cell and react with intracellular reactive oxygen species, in addition to extracellular ones. This probe has to undergo a one electron oxidation before it becomes sensitized to the presence of ROS, which can be accomplished by the addition of horseradish peroxidase to promote luminol oxidation in the extracellular space. In the absence of exogenous horseradish peroxidase, the assay is dependent on the presence of intracellular peroxidase to activate the probe [104–107]. The one electron oxidation of luminol leads to the creation of a radical species ($L\bullet$). The latter then interacts with ground-state oxygen to produce $O_2\bullet$ that induces the oxygenation of $L\bullet$ to create an unstable endoperoxide, which ultimately breaks down with the release of light. Lucigenin is a positively charged molecule and hence it cannot enter the cell and therefore could only measure the extracellular ROS only. Photons produced are converted to an electrical signal and measured using a luminometer [108], with ROS generation being measured as counted photons per minute. The normal range is less than 0.2×10^6 cpm per 20 million spermatozoa [109]. Intracellular ROS can be measured by flow cytometry using different fluorescent probes such as 2', 7'-dichlorofluorescein-diacetate, hydroethidine, that react with ROS to emit a red fluorescence [110]. The colorimetric technique is also widely used for indirectly quantifying ROS. It is based on the principle of spectrophotometry and measures lipid peroxide end products, mainly malondialdehyde, lipid hydroperoxides, and isoprostanes [111].

Fertilization defects

The sperm fertilizing potential is related to its ability of undergoing capacitation, which includes the acquisition of hyperactivated motility, and the acrosomal reaction to penetrate the ZP and its ability to fuse with oolema. Normospermic infertile men may have defective sperm that are unable to fertilize. This assumption is based on the observation of low success rates of IVF and intrauterine insemination (IUI) in certain cases of unexplained infertility. The major cause of fertilization failure in conventional IVF [112] is due to abnormalities of sperm–ZP binding and penetration. Although most sperm–ZP binding and penetration defects are due to

obvious sperm abnormalities such as asthenozoospermia and teratozoospermia, many patients have normal semen analysis and subtle sperm defects that affect sperm–ZP interaction. These defects cannot be shown by routine semen analysis but are apparent with sperm–ZP interaction tests [113].

Zona pellucida binding defects: sperm binding to the ZP is attributed to the presence of complementary binding sites or receptors on the surface of the gametes; typically, these receptors manifest a high degree of species specificity [114–116]. Human ZP (hZP) is composed of four major glycoproteins (hZP1, hZP2, hZP3, and hZP4) [117]. The ZP3 of human oocytes is believed to be the primary ZP receptor for capacitated acrosome-intact sperm binding [117,118]. In contrast, the exact nature of human sperm receptors for the ZP has not been established. Although a number of candidate sperm proteins have been found to be able to interact with either solubilized or intact ZP, it is not clear whether or not they are the primary receptors for sperm binding to the ZP [118–121]. Sperm binding to ZP3 induces a signal transduction cascade within the spermatozoon, involving multiple proteins and other factors, including protein kinases A and C pathways [122], that leads to the AR. Acrosome-reacted spermatozoa are believed to bind to ZP2, which facilitates the penetration to the zona matrix and progression into the perivitelline space [123]. Defective ZP-bound sperm are present in approximately 15 and 25% of subfertile men with a normal semen analysis and with an abnormal one, respectively [124–126]. Such individuals have a reduced chance of achieving successful fertilization when undergoing IVF [126]. Mackenna *et al.* [127] reported that two of 18 men with unexplained infertility showed lack of sperm binding to the zona despite having sperm morphology and hyperactivation status similar to fertile individuals. The presence of defective sperm–ZP binding in infertile men with normal semen may be due to defective signal transduction pathways upstream of protein kinases A and C. However, most defective sperm–ZP binding infertile men with normal semen and those with severe teratozoospermia are likely to have downstream disorders, structural defects, or absence of sperm receptors for binding the ZP.

Two tests of sperm binding to the human zona have been described: (i) the hemizona assay and (ii) the sperm–zona binding ratio test. In the former, a single zona is bisected and each zona half is incubated with control and patient sperm suspensions [128]. In the latter, a complete zona is incubated with equal numbers of motile spermatozoa from control and test populations, each labeled with a different fluorescent dye [129]. In each case, the number of spermatozoa from each population bound per whole or half zona is counted and the number of test sperm is expressed as a ratio of that of the control.

Capacitation defects: capacitation is a combination of concomitant processes; mainly, the sperm acquisition of a motility pattern known as hyperactivated motility, which enables efficient zona drilling and allows spermatozoa to reach the oolema, and preparation for the AR

[130]. Defects in capacitation may explain subfertility in some normospermic infertile men.

Defects in hyperactivation: hyperactivation is considered the first step of the complex capacitation process. It involves a typical swimming pattern of movement shown by most sperm retrieved from the oviductal ampulla at the time of fertilization [131]. Hyperactivated sperm show high-amplitude and asymmetrical flagellar bending movement. Hyperactivation is characterized by switching of sperm movement from progressive motility to more vigorous (nonprogressive) flagellar motion. The role of hyperactivation is to enhance the ability of sperm to detach from the oviduct wall, to move around in its labyrinthine lumen, to penetrate into the cumulus oophorus, and finally, to penetrate the ZP of the oocyte [132]. However, little is known about the mechanisms that lead the sperm to hyperactivation. It is speculated that specific signals appear within the oviduct shortly before ovulation. There is evidence that various components of the female reproductive tract serve as physiological stimuli of hyperactivation, such as hormones (e.g. progesterone), ions, and secretions in the oviduct luminal fluid [133]. When the oocyte enters the oviduct, it usually brings along cumulus oophorus and FF that have been shown to influence sperm motility. A number of physiological factors such as Ca^{2+} , c-AMP, bicarbonate, and metabolic substrates have been found as essential for the initiation or the maintenance of hyperactivated motility *in vitro* [134]. Recent studies have demonstrated that increased intracellular calcium entry through voltage gated calcium channels (Cation channel of sperm; CatSper1–4) in the principal piece of the sperm flagellum is the prime mechanism for hyperactivation [135–137]. This entry is induced by intracellular alkalinization because of extrusion of H^+ through voltage gated proton pumps, which are also located in the principal piece of the flagellum [135]. Increased intracellular pH and intracellular Ca^{2+} regulate not only the hyperactivation process but also the AR and the ability of the sperm to fertilize the egg [135]. Interestingly, molecular studies on CatSper ion channel show that it is a novel protein complex composed of six subunits. Of these, four are α subunits (CatSper1–4) with calcium-selective pore and two are transmembrane proteins with large extracellular domains, called CatSper β and CatSper γ , of unknown functions [138,139]. For humans, hyperactivation is not well defined as it is for other species [134], and only a small proportion of the sperm population may be hyperactivated at each time. The extent of hyperactivated motility in a population is positively correlated with the extent of zona binding, the AR, zona-free oocyte penetration, and fertilizing capacity *in vitro* [140]. Migration of spermatozoa toward the oocyte in the oviduct's ampulla is probably assisted by thermotaxis, which is the migration of spermatozoa toward a thermal gradient. The ampulla has a higher temperature than the isthmus of the fallopian tube and this may mediate long-range migration to the ampulla [141]. It has been shown that only capacitated spermatozoa respond to these influences [141]. The spermatozoa may also be directed to the egg within the cumulus by chemotaxis,

which is the migration of spermatozoa toward a higher concentration of chemoattractant [142].

Assessment of hyperactivation motility *in vitro* involves the use of computerized motion analysis in conjunction to kinematics module to distinguish different subpopulations of motile spermatozoa. Hyperactivated spermatozoa can be distinguished from nonhyperactivated ones by their high curvilinear velocity (VCL), low linearity (calculated as straight-line velocity/VCL), and large amplitude of the lateral head displacement. The clinical significance of such data is reflected by their correlation with IVF outcomes and spontaneous pregnancy rates [143]. Munire *et al.* [144] showed that there is a significant decrease in the percentage of hyperactivated sperm, sperm motility, progressive motility, and VCL from infertile men in comparison with sperm from fertile donors after overnight incubation with capacitating conditions, whereas linearity was increased in the former. Computerized assessment of follicular fluid (FF)-induced hyperactivation has been proved to be significantly lower in patients with unexplained infertility in comparison with normal fertile men [128]. The absence of hyperactivation after the addition of FF was observed in 39% of patients with unexplained infertility [127]; it is likely that spermatozoa from such patients have reduced ability to penetrate through the oocyte vestments and ZP as a result of this abnormal hyperactivation response to FF. In fact, Avenarius *et al.* [145] discovered that male patients with mutated CatSper1 gene are infertile with poor hyperactivation response despite their normal sperm count, morphology, and even their initial sperm motility. Furthermore, an animal study on mice concluded that mutation in each of CatSper (1–4) ion channel protein can lead to infertility despite normal semen parameters, normal testicular histology, size, and weight [146]. Interestingly, there are two known CatSper2 gene-related mutations in humans that cause male infertility, termed CatSper-related nonsyndromic male infertility and deafness–infertility syndrome [147]. However, both syndromes are associated with gross semen abnormalities. Further investigation is needed to show the genetic and molecular nature of fertilization in patients with defective hyperactivation response and unexplained infertility. Moreover, minor mutations in human CatSper (1–4) genes are yet to be deciphered in men with unexplained infertility.

Acrosome reaction: the AR is defined as the process of fusion of sperm plasma membrane with the outer acrosomal membrane leading to the release of exocytotic proteolytic enzymes (acrosine and hyaluronidase) in response to sperm–ZP binding. ZP3 is considered the natural stimulus for the AR, which leads to the proteolytic dissolution of the ZP. Human sperm initiate primary binding to the ZP with intact acrosome [148]. Glycoprotein (ZP3), present in the ZP, is involved in the induction of the AR [149]. Artificial stimuli used *in vitro* to challenge the AR are calcium ionophore A23187 and progesterone. There are two types of defective AR, which have clinical significance. The first is the AR prematurity, which is defined as a high level of spontaneous AR

(> 20% of spermatozoa showing spontaneous AR). The second is the AR insufficiency, which is defined as poor responsiveness to AR stimulants (when < 15% of spermatozoa responded to the ionophore A23187 challenge) [150]. Both conditions are associated with poor fertilization capacity on conventional IVF treatment. Under normal conditions, more than 15% AR in response to ionophore treatment is expected [151]. Various techniques are used for visualizing the human sperm acrosome, including lectins, monoclonal antibodies, or the triple stain [152–156]. Replicate (minimum two) slides must be scored for each determination, with at least 100 spermatozoa counted per slide. Some patients with unexplained infertility that have normal sperm–ZP binding have defective ZP-induced AR (ZPIAR), which will result in reduced sperm–ZP penetration and failure of fertilization. Patients with this condition usually have a long duration of unexplained infertility, normal semen analysis, and normal sperm–ZP binding, but show failure of ZP penetration by sperm and have zero or low rates of fertilization with standard IVF [157]. The diagnostic feature is that very low proportions of sperm undergo AR after binding to the ZP. However, these patients achieve high fertilization and pregnancy rates with ICSI [158]. Although the frequency of defective ZPIAR was high in subfertile men with idiopathic oligozoospermia (65%) and severe teratozoospermia (62%, strict normal sperm morphology $\leq 5\%$), defective ZPIAR was found in only 25% of normozoospermic subfertile men [125]. Although the exact mechanisms of defective AR are unknown, defective ZPIAR is more likely to be related to major structural defects of the sperm head, such as small or abnormal acrosomes, or associated abnormalities in the overlying plasma membrane in severe teratozoospermic subfertile men. In normozoospermic men, it has been shown that the seminal zinc concentration was significantly higher in men with defective ZPIAR. Addition of 0.5 mmol of zinc to the culture media had no effect on spermatozoa–ZP binding, but significantly reduced ZPIAR rates *in vitro*. Zinc has a role as a decapacitation factor by binding to the sperm plasma membrane; as such, high seminal zinc concentration may have an adverse effect on the ZPIAR [155]. Addition of zinc to the culture medium during capacitation of human spermatozoa inhibited spontaneous AR as well as the AR induced by the calcium ionophore [159]. It is possible that zinc binding to the sperm plasma membrane affects calcium influx through ion competition during capacitation. It is known that the seminal plasma contains decapacitation factors as its addition to the culture medium inhibits sperm capacitation and hyperactivated motility, as well as spermatozoa–ZP binding and penetration *in vitro* [160–163]. It is therefore apparent that defective ZPIAR may be caused by different mechanisms in men with or without severe sperm morphological defects. In men with normal sperm morphology, defective ZPIAR is most likely to be due to subtle biochemical or molecular defects in ZP receptors, signal transduction pathways, inefficient cholesterol or zinc removal from the plasma membrane during capacitation, actin polymerization, or acrosomal enzyme activation [116,164–169].

Furthermore, it is possible that other prostatic secretions (e.g. citric acid) might affect zinc levels or even sperm function. Zinc level determination in the seminal plasma is unlikely to be clinically useful for the prediction of defective ZPIAR; currently, only spermatozoa–ZP interaction tests using human oocytes have been shown to be accurate [159].

Defect of the fusogenic ability of the acrosome-reacted sperm with the oolema: the ability of the equatorial region of the acrosome-reacted human sperm to fuse with the vitelline membrane of the oocyte is tested by using the sperm penetration assay (SPA) also known as the zona-free hamster oocyte penetration test. Although this test does not assess sperm–ZP, it measures the spermatozoon's ability to undergo capacitation, AR, fusion and penetration through the oolema, and decondensation within the cytoplasm of an oocyte. The ZP is removed from a hamster oocyte, which is then incubated with human spermatozoa. In the original test, scoring is achieved by calculating the percentage of ova that are penetrated; normal sperm are able to penetrate 10–30% of hamster ova [68]. Recent refinement of this test is performed by incubating sperm in more potent capacitating media, which allow the majority of ova to be penetrated; scores are obtained by calculating the number of sperm that penetrate each ovum [68]. Aitken *et al.* [170] reported that 34.1% of patients with unexplained infertility had less than 10% oocyte penetration against 0% in a control group of fertile men. Various studies have evaluated the ability of the SPA to predict success or failure of IVF. Some investigators have shown no correlation with an abnormal test [171], whereas others have claimed 100% predictability [172]. Taking an average from different studies, a normal SPA may have 70% predictability of fertilization *in vitro* [44]. Nevertheless, semen samples that fail to fertilize hamster ova usually are unable to fertilize human ova [68]. Although the SPA is considered a research tool, it may be of clinical value for men with unexplained infertility with poor fertilization rate on IVF.

A practical approach for the assessment of men with unexplained infertility

PCT, if appropriately timed and performed, can be the initial test for couples with unexplained male infertility. Cervical mucus is normally hostile to sperm, except near the time of ovulation. The absence of sperm on a PCT in the presence of normal semen parameters suggests incorrect coital technique or failure to ejaculate into vagina, whereas the presence of normal sperm numbers but reduced motility or a shaking motion on a PCT is suggestive of the presence of ASAs [173]. The finding of a normal PCT raises the possibility of a functional sperm defect. Assessment of sperm function can be divided into two steps. The first step should be to check the competence of the sperm before the fertilization event by measuring the levels of ROS and DNA integrity defects. The second step should include the assessment of the fertilization potential of sperm, especially for those patients with a history of failure of conventional IVF. These tests include sperm–ZP binding assay,

capacitation, hyperactivation motility, inducibility of AR, and the ability of sperm to fuse with the vitelline membrane (zona-free hamster egg penetration test). Figure 1 shows the appropriate management plan for unexplained male infertility.

Treatment strategies

The treatment of men with unexplained infertility does not follow the typical rules for standard clinical practice decision making. Such a convention requires a specific scientific plan to identify and correct a known defect and the calculation of risks versus benefits. Owing to the facts that no recognizable reason for infertility is identified and few randomized clinical trials are available, no uniform protocol could be followed.

Counseling is an important part of management, particularly with regard to orientation about the physiology of ovulation and the need to time intercourse with the periovulatory period. A detailed medical history may help to disclose any hidden problems such as sexual dysfunction and inadequate coitus habits.

Expectant management

Expectant management is advised for young couples with a short duration of infertility. Pregnancy may occur spontaneously without any interventions in cases of unexplained infertility [174]. Hull *et al.* [175] found a cumulative pregnancy rate (PR) ranging from 50–80% over a 3-year period as a function of female age and 30–80% PR as a function of infertility duration. Cumulative PRs of 60% may be achieved within 2 years. However, infertility periods longer than 3 years are associated with very low PR of 1–3%, particularly if the female partner is aged 35 years or older [174]. For couples whose time to conceive is longer than 3 years, the cumulative PR decreases by 2% for each year of age after 25.7 years [176]. Owing to the costs of infertility treatments and given high proportion of couples with unexplained infertility who spontaneously conceive within a 2-year period, it is advisable to defer treatment of couples in this time period, unless the female partner is aged 35 years or older.

Interventional management

Interventions, which include medication and/or surgery or assisted conception, are justified in cases of unexplained infertility of long duration and/or advanced maternal and paternal age.

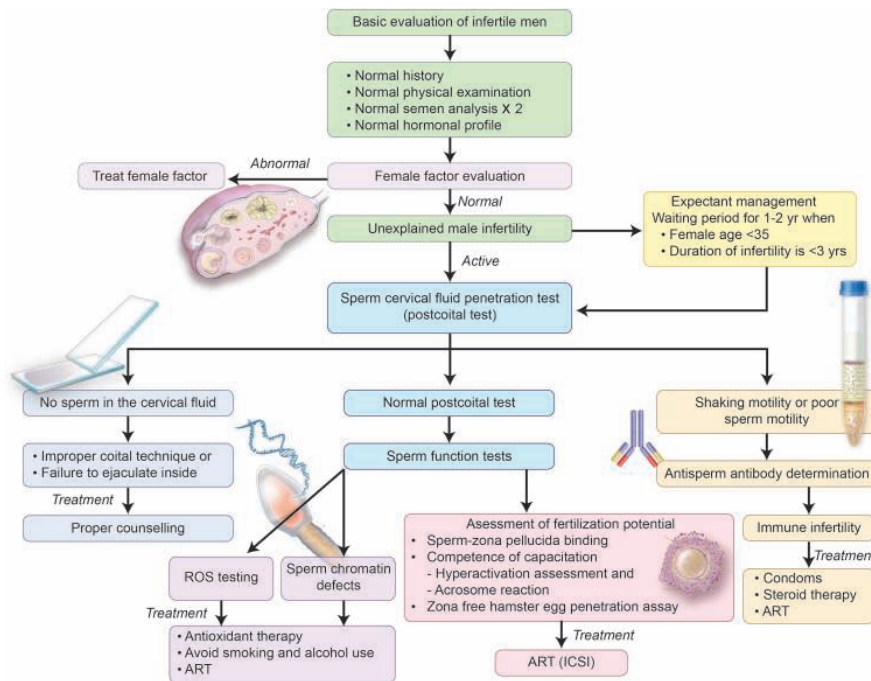
Immunological infertility

Treatment of immune infertility includes methods to either decrease ASA production or to remove sperm-bound ASA. ASA titers may be decreased by using condoms and systemic steroid. Condoms are of theoretical benefit because they may help to lessen the chances for frequent exposure of sperm to the female reproductive tract and hence decrease the sensitization and formation of ASA in the cervical mucus [177]. Immunosuppressive therapy had been tried in early years but it is seldom used nowadays, mainly because of the high incidence of side effects [178]. Moreover, efficacy of

steroids remains unclear as most studies lack appropriate placebo controls or have used different regimens and drugs. Despite these shortcomings, two prospective and randomized placebo-controlled studies were conducted and showed conflicting results. In the study of Hendry *et al.* [178], 40 mg of prednisolone was given for a 6-month period from cycle days 1–10 of the female partner, and then was tapered rapidly for the next 2 days. The PR of treated and untreated groups was 31 and 9%, respectively. In another study, the investigators reported similar PR after administration of methylprednisolone for three cycles, despite a significant decrease in sperm-associated IgG (but not IgA) in the steroid treatment group [179]. It has been shown that steroids may be only effective in removing sperm-bound ASA in the presence of low antibody titer [180]. Treatment with high-dose steroids for long time is associated with side effects that include mood changes, fluid retention, dyspepsia, gastrointestinal bleeding, aseptic necrosis of the hip joint, and significant decrease of bone mineral density in up to 60% of the patients [178,181,182].

Alternatively, methods to remove ASA already bound to sperm include sperm washing and IgA protease treatment. The effectiveness of these techniques in recovering antibodies-free spermatozoa is conflicting; most reports show limited success because of the difficulty of eluting the sperm cell surface by any washing method [183]. Esteves *et al.* [184] demonstrated that the population of antibody-free spermatozoa was increased by 29% after discontinuous colloidal gradient centrifugation. However, the investigators observed that sperm washing was ineffective to remove ASA in approximately 30% of the cases, and advise that the potential benefit of this strategy has to be tested individually. Microinjection of the compromised spermatozoa into the oocyte cytoplasm (ICSI) bypasses sperm–oocyte membrane interaction, and ICSI has been shown to increase fertilization when compared with conventional IVF in cases of male immunologic infertility. Nagy *et al.* [185] analyzed the outcome of ICSI in 37 men with a proportion of antisperm antibody-bound spermatozoa of 80% or higher. They concluded that fertilization, cleavage, and PRs after ICSI were not influenced by the percentage of ASA-bound spermatozoa, by the dominant type of antibodies present, or by the location of ASA on the spermatozoa. However, embryo quality was lower in the ASA-positive group. In another study, similar results were observed but a higher rate of first trimester pregnancy loss was observed in the ASA-positive group [186]. Clarke *et al.* [187] and Check *et al.* [188] studied 39 patients with a strong positivity on IBT (> 80%) and 93 patients with various degrees of autoantibodies, respectively. They found that fertilization and PRs were comparable between different levels of ASA on sperm. Esteves *et al.* [189] analyzed a large cohort of 351 patients and confirmed that fertilization, cleavage, and PRs after ICSI were not influenced by the ASA levels on sperm. These investigators observed neither the negative impact of ASA on embryo quality and cleavage rate nor an increase in pregnancy loss, as reported by other investigators. They also compared ICSI outcomes between patients with ASA

Figure 1



Work-up plan for unexplained male infertility. ROS, reactive oxygen species; ICSI intracytoplasmic sperm injection; and ART, assisted reproductive techniques.

positivity and a group of patients in which ICSI was indicated for other reasons. Fertilization, embryo development, pregnancy success, and miscarriage rates after ICSI in men showing varying levels of autoimmunity against spermatozoa were within the same range as our population of ICSI patients with severely abnormal seminal parameters. The investigators conclude by suggesting that ASA may become inactive within the ooplasm after microinjection, or that a segregation process may occur during the first cleavage divisions, similar to the inactivation and segregation processes that also occur with the acrosome and sperm tail after microinjection.

Excessive oxidative stress

Men with unexplained infertility may have higher oxidative stress than controls [86,190]. Lines of therapy include lifestyle habit modification, use of antioxidants, and ART. Patients are advised to quit smoking, eat antioxidant-rich food, and avoid pollutant environmental conditions. An antioxidant therapy has attracted attention in the recent years. Antioxidants are compounds and reactants that dispose, scavenge, and suppress the formation of ROS, or oppose their actions. Various antioxidants such as carnitine, vitamin C, vitamin E, coenzyme Q10, selenium, glutathione, *N*-acetyl cysteine, carotenoids, and trace metals are available. A recent Cochrane review on the use of antioxidants for male subfertility suggests that antioxidant supplementation may improve the outcomes of live birth and PR for subfertile couples undergoing ART cycles, but further head-to-head comparisons are necessary to identify the superiority of one antioxidant over another [191]. In

addition, therapeutic dosing, duration, and the toxic levels of ROS are still to be determined.

DNA damage

The management of unexplained male subfertility because of DNA damage often requires ART. The probability of fertilization *in vivo* and by IUI seems to be low when the proportion of sperm cells with DNA damage exceeds 30 and 12%, as detected respectively by sperm chromatin structure assay or transferase-mediated dTUP nick-end labeling [192,193]. Sperm DNA damage is negatively correlated with embryo quality and blastocyst formation in IVF cycles and with fertilization rates both in IVF and ICSI cycles [194]. However, successful pregnancies in IVF/ICSI cycles can be obtained using semen samples with a high proportion of DNA damage. Bungum *et al.* [195] demonstrated that significantly higher clinical PRs (52.9 vs. 22.2%) and delivery rates (47.1 vs. 22.2%) were obtained after ICSI as compared with IVF when semen samples with high levels of sperm DNA damage were used, as previously suggested.

The activation of embryonic genome expression occurs at the four-cell to eight-cell stage in human embryos. Therefore, the paternal genome may not be effective until this stage and it is speculated that an elevated level of sperm DNA strand breaks seems to be of importance in the later stages of embryonic development. Aitken and Krausz [196] proposed that sperm DNA damage is promutagenic and can give rise to mutations after fertilization, as the oocyte attempts to repair DNA damage before the initiation of the first cleavage.

Mutations occurring at this point will be fixed in the germline and may be responsible for the induction of infertility, childhood cancer in the offspring and for a higher risk of imprinting diseases. So far, however, follow-up studies of children born after ICSI compared with children born after conventional IVF have not been conclusive regarding the risks of congenital malformations, imprinting diseases, and health problems, in general. IVF, in general, is associated with multiple gestations and an increased risk of congenital abnormalities (including hypospadias) [197]. ICSI, in particular, carries an increased risk of endocrine abnormalities as well as epigenetic imprinting effects [197]. Although the absolute risk of any of these conditions remains low, current data are limited and study populations are heterogeneous [197–200]. It is therefore recommended that well-defined groups of couples undergoing ICSI with ejaculated sperm, ICSI with epididymal sperm, and ICSI with testicular sperm, and a control group of naturally conceived children are closely followed up.

Fertilization defects

ART is indicated for fertilization defects involving sperm capacitation, sperm–ZP interaction, or sperm–oocyte fusion. Couples should be advised that a significantly higher rate of successful pregnancy is achieved with IVF-ICSI compared with conventional IVF and IUI in such cases [124,158,201].

Donor insemination

Donor insemination is an alternative when all the above treatment options fail.

Conclusion

Clinical management of couples with unexplained infertility is usually limited to full gynecological evaluation of the female partner and to traditional clinical and laboratory assessment of the male factor infertility. As such, the work-up for men may be prematurely ended based on normal semen parameters and normal hormonal profile. This strategy has been historically supported by the high spontaneous conception rate for couples experiencing unexplained infertility, particularly when the duration of infertility is less than 3 years and the female partner is aged 35 years or less with no detectable functional abnormalities. However, proper scrutiny for uncommon male fertility problems should commence as soon as possible in couples with diminished chances of spontaneous pregnancy. Modern andrology has novel techniques and methods for the diagnosis of hidden sperm functional problems, which may tailor the subsequent application of various treatment options, including ART.

Future perspectives

The understanding of sperm physiology and fertilization is far from complete. However, molecular and genetic studies are on the pace to give a detailed and thorough perception of the entire process of human fertilization. Consequently, this perception may suggest, in the future, specific molecular therapy or even genetic target needed

to be precisely modified to improve male reproductive potential. Moreover, major advances in biomolecular techniques as well as in the sensitivity and accuracy of mass spectrometry are transforming our understanding of sperm physiology. The ‘omics’ era is under way, which refers to the study of genes (genomics), transcripts (transcriptomics), proteins (proteomics), and the various metabolites (metabolomics). Diagnostic genomics may help us to identify genotypes associated with specific sperm defects, as already reported in animal models [202]. A comprehensive proteomic analysis of normal and defective spermatozoa may provide insights into the structure–function relationships [203,204]. It has been suggested that sperm DNA damage is promutagenic and can give rise to mutations after fertilization, as the oocyte attempts to repair DNA damage before the initiation of the first cleavage. Mutations occurring at this point will be fixed in the germline and may be responsible for the induction of infertility, childhood cancer in the offspring, and for a higher risk of imprinting diseases [196]. Sperm metabolomics may elucidate which metabolic defects are associated to oxidative stress and sperm damage. This novel information may be useful both to identify the causes or consequences of oxidative stress in the male germline and to tailor individualized therapeutic intervention, such as an optimized regimen of antioxidants. Moreover in the context of unexplained infertility, glycomic analyses may be useful to reveal the causes of defective sperm–zona interaction [205]. It is then likely that future laboratory semen evaluation will move from the simple assessment of conventional semen profile into the assessment of sperm biochemistry, which may aid in the understanding of the underlying physiopathology of male infertility and in suggesting options for treatment and prevention.

References

- 1 WHO. *Report of the meeting on the prevention of infertility at the primary health care level*. Geneva: World Health Organization; 1984.
- 2 Cates W, Farley TM, Rowe PJ. Worldwide patterns of infertility: is Africa different? *Lancet* 1985; 2:596–598.
- 3 Jarow JP, Sharlip ID, Belker AM, Lipshultz LI, Sigman M, Thomas AJ, et al. Best practice policies for male infertility. *J Urol* 2002; 167:2138–2144.
- 4 Nieschlag E. Classification of andrological disorders. In: Nieschlag E, Behre HM, editors. *Andrology: male reproductive health and dysfunction*. 2nd ed. Berlin: Springer; 2000.
- 5 Moghissi KS, Wallach EE. Unexplained infertility. *Fertil Steril* 1983; 39:5–21.
- 6 Dohle GR, Diemer T, Giwercman A, Jungwirth A, Kopa Z, Krausz C. Guidelines of male infertility. In: *European Association of Urology*. April 2010. Retrieved from: <http://www.uroweb.org>.
- 7 WHO. *Manual for the standardised investigation and diagnosis of the infertile couple*. Cambridge, UK: Cambridge University Press; 2000.
- 8 Sigman M, Lipshultz L, Howard S. Chapter, 10. Office evaluation of the subfertile male. In: Lipshultz LI, Howards SS, Niederberge CS, editors. *Infertility in the male*. 4th ed. Cambridge, UK: Cambridge University Press; 2009. pp. 153–176.
- 9 Walsh TJ, Croughan MS, Schembri M, Chan JM, Turek PJ. Increased risk of testicular germ cell cancer among infertile men. *Arch Intern Med* 2009; 169:351–356.
- 10 Peng X, Zeng X, Peng S, Deng D, Zhang J. The association risk of male subfertility and testicular cancer. A systematic review. *PLoS One* 2009; 4:e5591.
- 11 Kolettis PN, Sabanegh ES. Significant medical pathology discovered during a male infertility evaluation. *J Urol* 2001; 166:178–180.
- 12 Honig SC, Lipshultz LI, Jarow J. Significant medical pathology uncovered by a comprehensive male infertility evaluation. *Fertil Steril* 1994; 62:1028–1034.
- 13 Aitken RJ, Best FS, Warner P, Templeton A. A prospective study of the relationship between semen quality and fertility in cases of unexplained infertility. *J Androl* 1984; 5:297–303.

- 14 Bonde JP, Ernst E, Jensen TK, Hjøllund NH, Kolstad H, Henriksen TB, *et al.* Relation between semen quality and fertility: a population-based study of 430 first-pregnancy planners. *Lancet* 1998; 352:1172–1177.
- 15 Van der Steeg JW, Steures P, Eijkemans MJ, Habbema JDF, Hompes PGA, Kremer JAM, *et al.* Role of semen analysis in subfertile couples. *Fertil Steril* 2011. [Epub ahead of print]
- 16 Mahmoud A, Comhaire F. Immunological causes. In: Comhaire FH, Hargreave TB, editors. *Andrology for the clinician*. Berlin: Schill W-B Springer; 2006. pp. 47–52.
- 17 Billingham RE, Brent L, Medawar PB. Actively acquired tolerance of foreign cells. *Nature* 1953; 172:603–606.
- 18 Tung KSK. Autoimmunity of the testis. In: Dhindsa DS, Schumacher GFB, editors. *Immunological aspects of infertility and fertility regulation*. North Holland, New York: Elsevier; 1980. pp. 33–91.
- 19 El-Demiry MI, Hargreave TB, Busuttill A, James K, Ritchie AW, Chisholm GD. Lymphocyte sub-populations in the male genital tract. *Br J Urol* 1985; 57:769–774.
- 20 McLachlan RI. Basis, diagnosis and treatment of immunological infertility in men. *J Reprod Immunol* 2002; 57:35–45.
- 21 Naz RK. Modalities for treatment of antisperm antibody mediated infertility: novel perspectives. *Am J Reprod Immunol* 2004; 51:390–397.
- 22 WHO. Towards more objectivity in diagnosis and management of male infertility. *Int J Androl* 1987; 7 (Suppl):1–53.
- 23 Guzick DS, Overstreet JW, Factor-Litvak P, Brazil CK, Nakajima ST, Coutifaris C, *et al.* Sperm morphology, motility, and concentration in fertile and infertile men. *N Engl J Med* 2001; 345:1388–1393.
- 24 Moghissi KS, Sacco AG, Borin K. Immunologic infertility. I. Cervical mucus antibodies and postcoital test. *Am J Obstet Gynecol* 1980; 136:941–950.
- 25 Mengel W, Zimmermann FA. Immunologic aspects of cryptorchidism. *Urol Clin North Am* 1982; 9:349–352.
- 26 Haas GG Jr. Antibody-mediated causes of male infertility. *Urol Clin North Am* 1987; 14:539–550.
- 27 Witkin SS. Mechanisms of active suppression of the immune response to spermatozoa. *Am J Reprod Immunol Microbiol* 1988; 17:61–64.
- 28 Haas GG. Male fertility and immunity. In: Lipshultz LI, Howards SS, editor. *Infertility in the male*. St Louis: Mosby-Year Book; 1991. pp. 277–296.
- 29 Hendry WF. Detection and treatment of antispermatozoal antibodies in men. *Reprod Fertil Dev* 1989; 1:205–220. discussion 20–22.
- 30 Meinertz H, Linnet L, Fogh-Andersen P, Hjort T. Antisperm antibodies and fertility after vasovasostomy: a follow-up study of 216 men. *Fertil Steril* 1990; 54:315–321.
- 31 Wen RQ, Li SQ, Wang CX, Wang QH, Li QK, Feng HM, *et al.* Analysis of spermatozoa from the proximal vas deferens of vasectomized men. *Int J Androl* 1994; 17:181–185.
- 32 Knudson G, Ross L, Stuhldreher D, Houlihan D, Bruns E, Prins G. Prevalence of sperm bound antibodies in infertile men with varicocele: the effect of varicocele ligation on antibody levels and semen response. *J Urol* 1994; 151:1260–1262.
- 33 Mahmoud AM, Tuytens CL, Comhaire FH. Clinical and biological aspects of male immune infertility: a case-controlled study of 86 cases. *Andrologia* 1996; 28:191–196.
- 34 Podzimek S, Prochazkova J, Pribylova L, Bartova J, Ulcova-Galova Z, Mrklas L, *et al.* Effect of heavy metals on immune reactions in patients with infertility. *Cas Lek Cesk* 2003; 142:285–288.
- 35 Turek PJ. Male infertility. In: Tanagho EA, McAninch JW, editor. *Smith's general urology*. 17th ed. United States: McGraw-Hill; 2008. pp. 684–716.
- 36 Jones WR, editor. The use of antibodies developed by infertile women to identify relevant antigens. Karolinska symposia on research methods in reproductive endocrinology immunological approach to fertility control. Stockholm: Karolinska Institute; 1974.
- 37 Beer AE, Neaves WB. Antigenic status of semen from the viewpoints of the female and male. *Fertil Steril* 1978; 29:3–22.
- 38 Zsolt Kopa MaBe. Inflammatory parameters of the ejaculate. In: Björndahl L, Giwercman A, Tournaye H, Weidner W, editor. *Clinical andrology EAU/ESAU course guidelines*. UK: Informa Healthcare; 2010. pp. 301–308.
- 39 Ayalotiotis B, Bronson R, Rosenfeld D, Cooper G. Conception rates in couples where autoimmunity to sperm is detected. *Fertil Steril* 1985; 43:739–742.
- 40 Francavilla F, Santucci R, Barbonetti A, Francavilla S. Naturally-occurring antisperm antibodies in men: interference with fertility and clinical implications. An update. *Front Biosci* 2007; 12:2890–2911.
- 41 D'Cruz OJ, Haas GG Jr. Lack of complement activation in the seminal plasma of men with antisperm antibodies associated in vivo on their sperm. *Am J Reprod Immunol* 1990; 24:51–57.
- 42 Petersen BH, Lammel CJ, Stites DP, Brooks GF. Human seminal plasma inhibition of complement. *J Lab Clin Med* 1980; 96:582–591.
- 43 World Health Organization. *WHO laboratory manual for the examination and processing of human semen*. 5th ed. Geneva: World Health Organization; 2010.
- 44 Check JH, Nowroozi K, Lee M, Adelson H, Katsoff D. Evaluation and treatment of a male factor component to unexplained infertility. *Arch Androl* 1990; 25:199–211.
- 45 Salonen I, Kallajoki M. Monoclonal antibody against human sperm acrosome inhibits sperm penetration of zona-free hamster eggs. *Int J Androl* 1987; 10:731–739.
- 46 Bronson RA. Antisperm antibodies: a critical evaluation and clinical guidelines. *J Reprod Immunol* 1999; 45:159–183.
- 47 Walsh TJ, Turek PJ. Immunological infertility. In: Lipshultz LI, Howards SS, Craig S, editors. *Infertility in the male*. 4th ed. UK: Cambridge University Press; 2009. pp. 277–294.
- 48 Rajah SV, Parslow JM, Howell RJ, Hendry WF. Comparison of mixed antiglobulin reaction and direct immunobead test for detection of sperm-bound antibodies in subfertile males. *Fertil Steril* 1992; 57:1300–1303.
- 49 Rodriguez MG, Rival C, Theas MS, Lustig L. Immunohistopathology of the contralateral testis of rats undergoing experimental torsion of the spermatic cord. *Asian J Androl* 2006; 8:576–583.
- 50 Anderson MJ, Dunn JK, Lipshultz LI, Coburn M. Semen quality and endocrine parameters after acute testicular torsion. *J Urol* 1992; 147:1545–1550.
- 51 Singer R, Dickerman Z, Sagiv M, Laron Z, Livni E. Endocrinological parameters and cell-mediated immunity postoperation for cryptorchidism. *Arch Androl* 1988; 20:153–157.
- 52 McDonald SW. Cellular responses to vasectomy. *Int Rev Cytol* 2000; 199:295–339.
- 53 Perdichizzi A, Nicoletti F, La Vignera S, Barone N, D'Agata R, Vicari E, *et al.* Effects of tumour necrosis factor-alpha on human sperm motility and apoptosis. *J Clin Immunol* 2007; 27:152–162.
- 54 Hill JA, Cohen J, Anderson DJ. The effects of lymphokines and monokines on human sperm fertilizing ability in the zona-free hamster egg penetration test. *Am J Obstet Gynecol* 1989; 160 (5 Pt 1):1154–1159.
- 55 Lewis SE. Is sperm evaluation useful in predicting human fertility? *Reproduction* 2007; 134:31–40.
- 56 Meistrich ML, Mohapatra B, Shirley CR, Zhao M. Roles of transition nuclear proteins in spermiogenesis. *Chromosoma* 2003; 111:483–488.
- 57 Tateno H, Kimura Y, Yanagimachi R. Sonication per se is not as deleterious to sperm chromosomes as previously inferred. *Biol Reprod* 2000; 63:341–346.
- 58 Vegetti W, Van Assche E, Frias A, Verheyen G, Bianchi MM, Bonduelle M, *et al.* Correlation between semen parameters and sperm aneuploidy rates investigated by fluorescence in-situ hybridization in infertile men. *Hum Reprod* 2000; 15:351–365.
- 59 Collodel G, Capitani S, Baccetti B, Pammolli A, Moretti E. Sperm aneuploidies and low progressive motility. *Hum Reprod* 2007; 22:1893–1898.
- 60 Egozcue S, Blanco J, Vendrell JM, Garcia F, Veiga A, Aran B, *et al.* Human male infertility: chromosome anomalies, meiotic disorders, abnormal spermatozoa and recurrent abortion. *Hum Reprod Update* 2000; 6:93–105.
- 61 Rives N, Saint Clair A, Mazurier S, Sibert L, Simeon N, Joly G, *et al.* Relationship between clinical phenotype, semen parameters and aneuploidy frequency in sperm nuclei of 50 infertile males. *Hum Genet* 1999; 105:266–272.
- 62 Thomas NS, Hassold TJ. Aberrant recombination and the origin of Klinefelter syndrome. *Hum Reprod Update* 2003; 9:309–317.
- 63 Kovanci E, Kovacs T, Moretti E, Vigue L, Bray-Ward P, Ward DC, *et al.* FISH assessment of aneuploidy frequencies in mature and immature human spermatozoa classified by the absence or presence of cytoplasmic retention. *Hum Reprod* 2001; 16:1209–1217.
- 64 Jakab A, Kovacs T, Zavaczki Z, Borsos A, Bray-Ward P, Ward D, *et al.* Efficacy of the swim-up method in eliminating sperm with diminished maturity and aneuploidy. *Hum Reprod* 2003; 18:1481–1488.
- 65 Jakab A, Sakkas D, Delpiano E, Cayli S, Kovanci E, Ward D, *et al.* Intracytoplasmic sperm injection: a novel selection method for sperm with normal frequency of chromosomal aneuploidies. *Fertil Steril* 2005; 84:1665–1673.
- 66 Celik-Ozenci C, Catalanotti J, Jakab A, Aksu C, Ward D, Bray-Ward P, *et al.* Human sperm maintain their shape following decondensation and denaturation for fluorescent in situ hybridization: shape analysis and objective morphometry. *Biol Reprod* 2003; 69:1347–1355.
- 67 Avendano C, Franchi A, Taylor S, Morshedi M, Bocca S, Oehninger S. Fragmentation of DNA in morphologically normal human spermatozoa. *Fertil Steril* 2009; 91:1077–1084.
- 68 Zini A, Sigman M. Evaluation of sperm function. In: Lipshultz LI, Howards SS, Craig S, editors. *Infertility in the male*. UK: Cambridge University Press; 2009. pp. 177–198.
- 69 Ferlin A, Raicu F, Gatta V, Zuccarello D, Palka G, Foresta C. Male infertility: role of genetic background. *Reprod Biomed Online* 2007; 14:734–745.
- 70 O'Flynn O'Brien KL, Varghese AC, Agarwal A. The genetic causes of male factor infertility: a review. *Fertil Steril* 2010; 93:1–12.
- 71 Wieacker P, Simoni M. Cytogenetic and molecular genetic investigations. In: Nieschlag E, Behre HM, Nieschlag S, editors. *Andrology male reproductive health and dysfunction*. Berlin Heidelberg: Springer-Verlag; 2010. pp. 119–126.
- 72 Dolores J. Look towards the future advances in andrology expected to revolutionize the diagnosis and treatment of infertile men. In: Lipshultz LI, Howards SS, Craig S, editor. *Infertility in the male*. UK: Cambridge University Press; 2009. pp. 642–653.
- 73 Garrido N, Martinez-Conejero JA, Jauregui J, Horcajadas JA, Simon C, Remohi J, *et al.* Microarray analysis in sperm from fertile and infertile men

- without basic sperm analysis abnormalities reveals a significantly different transcriptome. *Fertil Steril* 2009; 91 (4 Suppl):1307–1310.
- 74 Agarwal A, Allamaneni SS. The effect of sperm DNA damage on assisted reproduction outcomes. A review. *Minerva Ginecol* 2004; 56:235–245.
 - 75 Saleh RA, Agarwal A, Nada EA, El-Tonsy MH, Sharma RK, Meyer A, et al. Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. *Fertil Steril* 2003; 79 (Suppl 3):1597–1605.
 - 76 Evenson DP, Darzynkiewicz Z, Melamed MR. Relation of mammalian sperm chromatin heterogeneity to fertility. *Science* 1980; 210:1131–1133.
 - 77 Henkel R, Hajimohammad M, Staf T, Hoogendijk C, Mehnert C, Menkveld R, et al. Influence of deoxyribonucleic acid damage on fertilization and pregnancy. *Fertil Steril* 2004; 81:965–972.
 - 78 Tesarik J, Greco E, Mendoza C. Late, but not early, paternal effect on human embryo development is related to sperm DNA fragmentation. *Hum Reprod* 2004; 19:611–615.
 - 79 Aitken RJ, Baker MA, Sawyer D. Oxidative stress in the male germ line and its role in the aetiology of male infertility and genetic disease. *Reprod Biomed Online* 2003; 7:65–70.
 - 80 Bakos HW, Thompson JG, Feil D, Lane M. Sperm DNA damage is associated with assisted reproductive technology pregnancy. *Int J Androl* 2008; 31:518–526.
 - 81 Practice Committee of the American Society for Reproductive Medicine. The clinical utility of sperm DNA integrity testing. *Fertil Steril* 2006; 86 (5 Suppl 1):S35–S37.
 - 82 Yeung C, Cooper T. *Sperm quality and function tests. Andrology, male reproductive health and dysfunction*. Berlin Heidelberg: Springer-Verlag; 2010. pp. 139–154.
 - 83 Manicardi GC, Tombacco A, Bizzaro D, Bianchi U, Bianchi PG, Sakkas D. DNA strand breaks in ejaculated human spermatozoa: comparison of susceptibility to the nick translation and terminal transferase assays. *Histochem J* 1998; 30:33–39.
 - 84 Henkel R, Maass G, Hajimohammad M, Menkveld R, Staf T, Villegas J, et al. Urogenital inflammation: changes of leucocytes and ROS. *Andrologia* 2003; 35:309–313.
 - 85 Lewis SE, Boyle PM, McKinney KA, Young IS, Thompson W. Total antioxidant capacity of seminal plasma is different in fertile and infertile men. *Fertil Steril* 1995; 64:868–870.
 - 86 Pasqualotto FF, Sharma RK, Kobayashi H, Nelson DR, Thomas AJ Jr, Agarwal A. Oxidative stress in normospermic men undergoing infertility evaluation. *J Androl* 2001; 22:316–322.
 - 87 Alvarez JG, Storey BT. Spontaneous lipid peroxidation in rabbit and mouse epididymal spermatozoa: dependence of rate on temperature and oxygen concentration. *Biol Reprod* 1985; 32:342–351.
 - 88 Holland MK, Alvarez JG, Storey BT. Production of superoxide and activity of superoxide dismutase in rabbit epididymal spermatozoa. *Biol Reprod* 1982; 27:1109–1118.
 - 89 Alvarez JG, Storey BT. Lipid peroxidation and the reactions of superoxide and hydrogen peroxide in mouse spermatozoa. *Biol Reprod* 1984; 30:833–841.
 - 90 Alvarez JG, Touchstone JC, Blasco L, Storey BT. Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa. Superoxide dismutase as major enzyme protectant against oxygen toxicity. *J Androl* 1987; 8:338–348.
 - 91 Aitken RJ, Clarkson JS. Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J Reprod Fertil* 1987; 81:459–469.
 - 92 Aitken RJ, Bennetts L. The sperm cell production, maturation, fertilization, regeneration. In: De Jonge C, Barratt C, editors. *Reactive oxygen species: friend or foe*. UK: Cambridge University Press; 2006. pp. 170–193.
 - 93 Griveau JF, Le Lannou D. Reactive oxygen species and human spermatozoa: physiology and pathology. *Int J Androl* 1997; 20:61–69.
 - 94 Agarwal A, Nallella KP, Allamaneni SS, Said TM. Role of antioxidants in treatment of male infertility: an overview of the literature. *Reprod Biomed Online* 2004; 8:616–627.
 - 95 Kodama H, Kuribayashi Y, Gagnon C. Effect of sperm lipid peroxidation on fertilization. *J Androl* 1996; 17:151–157.
 - 96 Rhemrev JP, Van Overveld FW, Haenen GR, Teerlink T, Bast A, Vermeiden JP. Quantification of the nonenzymatic fast and slow TRAP in a postaddition assay in human seminal plasma and the antioxidant contributions of various seminal compounds. *J Androl* 2000; 21:913–920.
 - 97 Van Overveld FW, Haenen GR, Rhemrev J, Vermeiden JP, Bast A. Tyrosine as important contributor to the antioxidant capacity of seminal plasma. *Chem Biol Interact* 2000; 127:151–161.
 - 98 Aitken RJ, Clarkson JS, Fishel S. Generation of reactive oxygen species, lipid peroxidation, and human sperm function. *Biol Reprod* 1989; 41:183–197.
 - 99 Aitken RJ, Gordon E, Harkiss D, Twigg JP, Milne P, Jennings Z, et al. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol Reprod* 1998; 59:1037–1046.
 - 100 Spiropoulos J, Turnbull DM, Chinnery PF. Can mitochondrial DNA mutations cause sperm dysfunction? *Mol Hum Reprod* 2002; 8:719–721.
 - 101 Sharma RK, Said T, Agarwal A. Sperm DNA damage and its clinical relevance in assessing reproductive outcome. *Asian J Androl* 2004; 6:139–148.
 - 102 Helbock HJ, Beckman KB, Shigenaga MK, Walter PB, Woodall AA, Yeo HC, et al. DNA oxidation matters: the HPLC-electrochemical detection assay of 8-oxo-deoxyguanosine and 8-oxo-guanine. *Proc Natl Acad Sci USA* 1998; 95:288–293.
 - 103 Said TM, Paasch U, Glander HJ, Agarwal A. Role of caspases in male infertility. *Hum Reprod Update* 2004; 10:39–51.
 - 104 Aitken RJ, Buckingham DW, West KM. Reactive oxygen species and human spermatozoa: analysis of the cellular mechanisms involved in luminol- and lucigenin-dependent chemiluminescence. *J Cell Physiol* 1992; 151:466–477.
 - 105 Aitken RJ, Baker MA, O'Bryan M. Shedding light on chemiluminescence: the application of chemiluminescence in diagnostic andrology. *J Androl* 2004; 25:455–465.
 - 106 Faulkner K FI. Luminol and lucigenin as detectors for O₂•. *Free Radic Biol Med* 1993; 15:447–451.
 - 107 Fridovich I. Fundamental aspects of reactive oxygen species, or what's the matter with oxygen? *Ann N Y Acad Sci* 1999; 893:13–18.
 - 108 Fraga CG, Motchnik PA, Shigenaga MK, Helbock HJ, Jacob RA, Ames BN. Ascorbic acid protects against endogenous oxidative DNA damage in human sperm. *Proc Natl Acad Sci USA* 1991; 88:11003–11006.
 - 109 Agarwal A, Cocuzza M, Abdelrazik H, Sharma RK. Oxidative stress measurement in patients with male or female factor infertility. *Handbook Chemiluminescent Methods Oxidative Stress Assess*. Transworld Research Network. 2008; 195–218.
 - 110 Marchetti C, Obert G, Deffoesez A, Formstecher P, Marchetti P. Study of mitochondrial membrane potential, reactive oxygen species, DNA fragmentation and cell viability by flow cytometry in human sperm. *Hum Reprod* 2002; 17:1257–1265.
 - 111 Agarwal A, Makker K, Sharma R. Clinical Relevance of Oxidative Stress in Male Factor Infertility: An Update. *Am J Reprod Immunol* 2008; 59: 2–11
 - 112 Liu DY, Baker HW. Defective sperm-zona pellucida interaction: a major cause of failure of fertilization in clinical in-vitro fertilization. *Hum Reprod* 2000; 15:702–708.
 - 113 Liu DY, Baker HW. Disordered zona pellucida-induced acrosome reaction and failure of in vitro fertilization in patients with unexplained infertility. *Fertil Steril* 2003; 79:74–80.
 - 114 Ahuja KK. Carbohydrate determinants involved in mammalian fertilization. *Am J Anat* 1985; 174:207–223.
 - 115 Oehninger S. Molecular basis of human sperm-zona pellucida interaction. *Cells Tissues Organs* 2001; 168:58–64.
 - 116 Yanagimachi R. *Mammalian fertilization*. In: Knobil E, Neill JD, editor. *The physiology of reproduction*. 2nd ed. New York: Raven Press; 1994. pp. 189–317.
 - 117 Lefievre L, Conner SJ, Salpekar A, Olufowobi O, Ashton P, Pavlovic B, et al. Four zona pellucida glycoproteins are expressed in the human. *Hum Reprod* 2004; 19:1580–1586.
 - 118 Bleil JD, Wassarman PM. Identification of a ZP3-binding protein on acrosome-intact mouse sperm by photoaffinity crosslinking. *Proc Natl Acad Sci U S A* 1990; 87:5563–5567.
 - 119 Wassarman PM. Mammalian fertilization: molecular aspects of gamete adhesion, exocytosis, and fusion. *Cell* 1999; 96:175–183.
 - 120 Lasserre A, Gonzalez-Echeverria F, Moules C, Tezon JG, Miranda PV, Vazquez-Levin MH. Identification of human sperm proteins involved in the interaction with homologous zona pellucida. *Fertil Steril* 2003; 79 (Suppl 3): 1606–1615.
 - 121 Van Gestel RA, Brewis IA, Ashton PR, Brouwers JF, Gadella BM. Multiple proteins present in purified porcine sperm apical plasma membranes interact with the zona pellucida of the oocyte. *Mol Hum Reprod* 2007; 13:445–454.
 - 122 Thaler CD, Cardullo RA. The initial molecular interaction between mouse sperm and the zona pellucida is a complex binding event. *J Biol Chem* 1996; 271:23289–23297.
 - 123 ESHRE (European Society of Human Reproduction and Embryology) Andrology Special Interest Group. Consensus workshop on advanced diagnostic andrology techniques. *Hum Reprod* 1996; 11:1463–1479.
 - 124 Liu DY, Garrett C, Baker HW. Clinical application of sperm-oocyte interaction tests in in vitro fertilization-embryo transfer and intracytoplasmic sperm injection programs. *Fertil Steril* 2004; 82:1251–1263.
 - 125 Liu de Y, Liu ML, Garrett C, Baker HW. Comparison of the frequency of defective sperm-zona pellucida (ZP) binding and the ZP-induced acrosome reaction between subfertile men with normal and abnormal semen. *Hum Reprod* 2007; 22:1878–1884.
 - 126 Liu DY, Clarke GN, Lopata A, Johnston WI, Baker HW. A sperm-zona pellucida binding test and in vitro fertilization. *Fertil Steril* 1989; 52:281–287.
 - 127 Mackenna A, Barratt CL, Kessopoulou E, Cooke I. The contribution of a hidden male factor to unexplained infertility. *Fertil Steril* 1993; 59:405–411.
 - 128 Burkman LJ, Coddington CC, Franken DR, Krugen TF, Rosenwaks Z, Hogen GD. The hemizona assay (HZA): development of a diagnostic test for the binding of human spermatozoa to the human hemizona pellucida to predict fertilization potential. *Fertil Steril* 1988; 49:688–697.

- 129 Liu DY, Lopata A, Johnston WI, Baker HW. A human sperm-zona pellucida binding test using oocytes that failed to fertilize in vitro. *Fertil Steril* 1988; 50:782-788.
- 130 Esteves SC, Sharma RK, Thomas AJ Jr, Agarwal A. Effect of in vitro incubation on spontaneous acrosome reaction in fresh and cryopreserved human spermatozoa. *Int J Fertil Womens Med* 1998; 43:235-242.
- 131 Esteves SC, Sharma RK, Thomas AJ Jr, Agarwal A. Effect of swim-up sperm washing and subsequent capacitation on acrosome status and functional membrane integrity of normal sperm. *Int J Fertil Womens Med* 2000; 45:335-341.
- 132 Suarez SS, Ho HC. Hyperactivated motility in sperm. *Reprod Domest Anim* 2003; 38:119-124.
- 133 Nichol R, Hunter RH, Gardner DK, Leese HJ, Cooke GM. Concentrations of energy substrates in oviductal fluid and blood plasma of pigs during the periovalulatory period. *J Reprod Fertil* 1992; 96:699-707.
- 134 Mortimer ST, Swan MA. Variable kinematics of capacitating human spermatozoa. *Hum Reprod* 1995; 10:3178-3182.
- 135 Lishko PV, Kirichok Y. The role of Hv1 and CatSper channels in sperm activation. *J Physiol* 2010; 588 (Pt 23):4667-4672.
- 136 Suarez SS. Control of hyperactivation in sperm. *Hum Reprod Update* 2008; 14:647-657.
- 137 Carlson AE, Burnett LA, Del Camino D, Quill TA, Hille B, Chong JA, *et al.* Pharmacological targeting of native CatSper channels reveals a required role in maintenance of sperm hyperactivation. *PLoS One* 2009; 4:e6844.
- 138 Liu J, Xia J, Cho KH, Clapham DE, Ren D. CatSperbeta, a novel transmembrane protein in the CatSper channel complex. *J Biol Chem* 2007; 282:18945-18952.
- 139 Wang H, Liu J, Cho KH, Ren D. A novel, single, transmembrane protein CATSPERG is associated with CATSPER1 channel protein. *Biol Reprod* 2009; 81:539-544.
- 140 Cooper T, Yeung C. Physiology of sperm maturation and fertilization. In: Nieschlag E, Behre HM, Nieschlag S, editor. *Andrology: male reproductive health and dysfunction*. 3rd ed. Berlin Heidelberg: Springer-Verlag; 2010. pp. 61-86.
- 141 Bahat A, Eisenbach M. Sperm thermotaxis. *Mol Cell Endocrinol* 2006; 252:115-119.
- 142 Eisenbach M, Giojalas LC. Sperm guidance in mammals-an unpaved road to the egg. *Nat Rev Mol Cell Biol* 2006; 7:276-285.
- 143 Garrett C, Liu DY, Clarke GN, Rushford DD, Baker HW. Automated semen analysis: 'zona pellucida preferred' sperm morphometry and straight-line velocity are related to pregnancy rate in subfertile couples. *Hum Reprod* 2003; 18:1643-1649.
- 144 Munire M, Shimizu Y, Sakata Y, Minaguchi R, Aso T. Impaired hyperactivation of human sperm in patients with infertility. *J Med Dent Sci* 2004; 51:99-104.
- 145 Avenarius MR, Hildebrand MS, Zhang Y, Meyer NC, Smith LL, Kahziri K, *et al.* Human male infertility caused by mutations in the CATSPER1 channel protein. *Am J Hum Genet* 2009; 84:505-510.
- 146 Qi H, Moran MM, Navarro B, Chong JA, Krapivinsky G, Krapivinsky L, *et al.* All four CatSper ion channel proteins are required for male fertility and sperm cell hyperactivated motility. *Proc Natl Acad Sci USA* 2007; 104:1219-1223.
- 147 Hildebrand MS, Avenarius MR, Smith RJH. *CATSPER-related male infertility*. Seattle (WA): GeneReviews-NCBI Bookshelf; 1993.
- 148 Liu DY, Baker HW. Inducing the human acrosome reaction with a calcium ionophore A23187 decreases sperm-zona pellucida binding with oocytes that failed to fertilize in vitro. *J Reprod Fertil* 1990; 89:127-134.
- 149 Cross NL, Morales P, Overstreet JW, Hanson FW. Induction of acrosome reactions by the human zona pellucida. *Biol Reprod* 1988; 38:235-244.
- 150 Sigman M, Baazeem A, Zini A. Semen analysis and sperm function assays: what do they mean? *Semin Reprod Med* 2009; 27:115-123.
- 151 Esteves SC, Sharma RK, Thomas AJ Jr, Agarwal A. Cryopreservation of human spermatozoa with pentoxifylline improves the post-thaw agonist-induced acrosome reaction rate. *Hum Reprod* 1998; 13:3384-3389.
- 152 Esteves SC, Sharma RK, Thomas AJ Jr, Agarwal A. Evaluation of acrosomal status and sperm viability in fresh and cryopreserved specimens by the use of fluorescent peanut agglutinin lectin in conjunction with hypo-osmotic swelling test. *Int Braz J Urol* 2007; 33:364-374. discussion 75-76.
- 153 Mortimer D, Curtis EF, Camenzind AR. Combined use of fluorescent peanut agglutinin lectin and Hoechst 33258 to monitor the acrosomal status and vitality of human spermatozoa. *Hum Reprod* 1990; 5:99-103.
- 154 Cross NL, Morales P, Overstreet JW, Hanson FW. Two simple methods for detecting acrosome reacted human sperm. *Gamete Res* 1986; 15:213-226.
- 155 Talbot P, Chacon R. A new procedure for rapidly scoring acrosome reactions of human sperm. *Gamete Res* 1980; 3:211-216.
- 156 Mortimer D. *Practical laboratory andrology*. New York: Oxford University Press; 1994.
- 157 Liu DY, Baker HW. Disordered acrosome reaction of spermatozoa bound to the zona pellucida: a newly discovered sperm defect causing infertility with reduced sperm-zona pellucida penetration and reduced fertilization in vitro. *Hum Reprod* 1994; 9:1694-1700.
- 158 Liu DY, Bourne H, Baker HW. High fertilization and pregnancy rates after intracytoplasmic sperm injection in patients with disordered zona pellucida-induced acrosome reaction. *Fertil Steril* 1997; 67:955-958.
- 159 Liu DY, Sie BS, Liu ML, Agresta F, Baker HW. Relationship between seminal plasma zinc concentration and spermatozoa-zona pellucida binding and the ZP-induced acrosome reaction in subfertile men. *Asian J Androl* 2009; 11:499-507.
- 160 Kanwar KC, Yanagimachi R, Lopata A. Effects of human seminal plasma on fertilizing capacity of human spermatozoa. *Fertil Steril* 1979; 31:321-327.
- 161 Cross NL. Multiple effects of seminal plasma on the acrosome reaction of human sperm. *Mol Reprod Dev* 1993; 35:316-323.
- 162 Levay PF, Fourie FR, Meintjes J. The effect of seminal plasma on human sperm-zona pellucida binding. *Hum Reprod* 1995; 10:2590-2594.
- 163 Mortimer ST, Swan MA, Mortimer D. Effect of seminal plasma on capacitation and hyperactivation in human spermatozoa. *Hum Reprod* 1998; 13:2139-2146.
- 164 Lee MA, Kopf GS, Storey BT. Effects of phorbol esters and a diacylglycerol on the mouse sperm acrosome reaction induced by the zona pellucida. *Biol Reprod* 1987; 36:617-627.
- 165 Tollner TL, Overstreet JW, VandeVoort CA. Effect of protein kinase C stimulators on zona pellucida binding and the acrosome reaction of macaque sperm. *Biol Reprod* 1995; 52:1418-1425.
- 166 Cross NL. Human seminal plasma prevents sperm from becoming acrosomally responsive to the agonist, progesterone: cholesterol is the major inhibitor. *Biol Reprod* 1996; 54:138-145.
- 167 Liu DY, Baker HW. Protein kinase C plays an important role in the human zona pellucida-induced acrosome reaction. *Mol Hum Reprod* 1997; 3:1037-1043.
- 168 Liu DY, Martic M, Clarke GN, Grkovic I, Garrett C, Dunlop ME, *et al.* An actin monoclonal antibody inhibits the zona pellucida-induced acrosome reaction and hyperactivated motility of human sperm. *Mol Hum Reprod* 2002; 8:37-47.
- 169 Cohen G, Rubinstein S, Gur Y, Breitbart H. Crosstalk between protein kinase A and C regulates phospholipase D and F-actin formation during sperm capacitation. *Dev Biol* 2004; 267:230-241.
- 170 Aitken RJ, Best FS, Richardson DW, Djahanbakhch O, Mortimer D, Templeton AA, *et al.* An analysis of sperm function in cases of unexplained infertility: conventional criteria, movement characteristics, and fertilizing capacity. *Fertil Steril* 1982; 38:212-221.
- 171 Wolf DP, Sokoloski JE, Quigley MM. Correlation of human in vitro fertilization with the hamster egg bioassay. *Fertil Steril* 1983; 40:53-59.
- 172 Chan SY, Fox EJ, Chan MM, Tsoi WL, Wang C, Tang LC, *et al.* The relationship between the human sperm hypoosmotic swelling test, routine semen analysis, and the human sperm zona-free hamster ovum penetration assay. *Fertil Steril* 1985; 44:668-672.
- 173 Jarow JP. Diagnostic approach to the infertile male patient. *Endocrinol Metab Clin North Am* 2007; 36:297-311.
- 174 Brandes M, Hamilton CJ, van der Steen JO, de Bruin JP, Bots RS, Nelen WL, *et al.* Unexplained infertility: overall ongoing pregnancy rate and mode of conception. *Hum Reprod* 2011; 26:360-368.
- 175 Hull MG, Glazener CM, Kelly NJ, Conway DI, Foster PA, Hinton RA, *et al.* Population study of causes, treatment, and outcome of infertility. *Br Med J (Clin Res Ed)* 1985; 291:1693-1697.
- 176 Collins JA, Rowe TC. Age of the female partner is a prognostic factor in prolonged unexplained infertility: a multicenter study. *Fertil Steril* 1989; 52:15-20.
- 177 Li TS. Sperm immunology, infertility, and fertility control. *Obstet Gynecol* 1974; 44:607-623.
- 178 Hendry WF, Hughes L, Scammell G, Pryor JP, Hargreave TB. Comparison of prednisolone and placebo in subfertile men with antibodies to spermatozoa. *Lancet* 1990; 335:85-88.
- 179 Haas GG Jr, Manganiello P. A double-blind, placebo-controlled study of the use of methylprednisolone in infertile men with sperm-associated immunoglobulins. *Fertil Steril* 1987; 47:295-301.
- 180 Ras anen M, Lahteenmaki A, Agrawal YP, Saarikoski S, Hovatta O. A placebo-controlled flow cytometric study of the effect of low-dose prednisolone treatment on sperm-bound antibody levels. *Int J Androl* 1996; 19:150-154.
- 181 Sharma KK, Barratt CL, Pearson MJ, Cooke ID. Oral steroid therapy for subfertile males with antisperm antibodies in the semen: prediction of the responders. *Hum Reprod* 1995; 10:103-109.
- 182 Pearce G, Tabensky DA, Delmas PD, Baker HW, Seeman E. Corticosteroid-induced bone loss in men. *J Clin Endocrinol Metab* 1998; 83:801-806.
- 183 De Almeida M, Gazagne I, Jeulin C, Herry M, Belaisch-Allart J, Frydman R, *et al.* In-vitro processing of sperm with autoantibodies and in-vitro fertilization results. *Hum Reprod* 1989; 4:49-53.
- 184 Esteves SC, Schneider DT, Verza S Jr. Titulos de anticorpos antiespermatozoides antes e após o processamento seminal pela técnica do gradiente descontinuo coloidal (abstract). *Int Braz J Urol* 2005; 30:87.
- 185 Nagy ZP, Verheyen G, Liu J, Joris H, Janssenswillen C, Wisanto A, *et al.* Results of 55 intracytoplasmic sperm injection cycles in the treatment of male-immunological infertility. *Hum Reprod* 1995; 10:1775-1780.
- 186 Lahteenmaki A, Reima I, Hovatta O. Treatment of severe male immunological infertility by intracytoplasmic sperm injection. *Hum Reprod* 1995; 10:2824-2828.
- 187 Clarke GN, Bourne H, Baker HW. Intracytoplasmic sperm injection for treating infertility associated with sperm autoimmunity. *Fertil Steril* 1997; 68:112-117.

- 188** Check ML, Check JH, Katsoff D, Summers-Chase D. ICSI as an effective therapy for male factor with antisperm antibodies. *Arch Androl* 2000; 45:125–130.
- 189** Esteves SC, Schneider DT, Verza S Jr. Influence of antisperm antibodies in the semen on intracytoplasmic sperm injection outcome. *Int Braz J Urol* 2007; 33:795–802.
- 190** Huszar G, Vigue L. Correlation between the rate of lipid peroxidation and cellular maturity as measured by creatine kinase activity in human spermatozoa. *J Androl* 1994; 15:71–77.
- 191** Showell MG, Brown J, Yazdani A, Stankiewicz MT, Hart RJ. Antioxidants for male subfertility. *Cochrane Database Syst Rev* 2011; 1:CD007411.
- 192** Bungum M, Humaidan P, Spano M, Jepson K, Bungum L, Giwercman A. The predictive value of sperm chromatin structure assay (SCSA) parameters for the outcome of intrauterine insemination, IVF and ICSI. *Hum Reprod* 2004; 19:1401–1408.
- 193** Duran EH, Morshedi M, Taylor S, Oehninger S. Sperm DNA quality predicts intrauterine insemination outcome: a prospective cohort study. *Hum Reprod* 2002; 17:3122–3128.
- 194** Seli E, Gardner DK, Schoolcraft WB, Moffatt O, Sakkas D. Extent of nuclear DNA damage in ejaculated spermatozoa impacts on blastocyst development after in vitro fertilization. *Fertil Steril* 2004; 82:378–383.
- 195** Larson-Cook KL, Brannian JD, Hansen KA, Kasperson KM, Aamold ET, Evenson DP. Relationship between the outcomes of assisted reproductive techniques and sperm DNA fragmentation as measured by the sperm chromatin structure assay. *Fertil Steril* 2003; 80:895–902.
- 196** Aitken RJ, Krausz C. Oxidative stress, DNA damage and the Y chromosome. *Reproduction* 2001; 122:497–506.
- 197** Alukal JP, Lamb DJ. Intracytoplasmic sperm injection (ICSI)—what are the risks? *Urol Clin North Am* 2008; 35:277–288. ix-x.
- 198** Knoester M, Helmerhorst FM, Vandenbroucke JP, van der Westerlaken LA, Walther FJ, Veen S. Cognitive development of singletons born after intracytoplasmic sperm injection compared with in vitro fertilization and natural conception. *Fertil Steril* 2008; 90:289–296.
- 199** Belva F, Henriët S, Liebaers I, Van Steirteghem A, Celestin-Westreich S, Bonduelle M. Medical outcome of 8-year-old singleton ICSI children (born \geq 32 weeks' gestation) and a spontaneously conceived comparison group. *Hum Reprod* 2007; 22:506–515.
- 200** Woldringh GH, Besselink DE, Tillema AH, Hendriks JC, Kremer JA. Karyotyping, congenital anomalies and follow-up of children after intracytoplasmic sperm injection with non-ejaculated sperm: a systematic review. *Hum Reprod Update* 2010; 16:12–19.
- 201** Liu DY, Baker HW. Evaluation and assessment of semen for IVF/ICSI. *Asian J Androl* 2002; 4:281–285.
- 202** O'Bryan MK, de Kretser D. Mouse models for genes involved in impaired spermatogenesis. *Int J Androl* 2006; 29:76–89. discussion 105-108.
- 203** Aitken RJ, Baker MA. The role of proteomics in understanding sperm cell biology. *Int J Androl* 2008; 31:295–302.
- 204** Martínez-Heredia J, de Mateo S, Vidal-Taboada JM, Balleca JL, Oliva R. Identification of proteomic differences in asthenozoospermic sperm samples. *Hum Reprod* 2008; 23:783–791.
- 205** Liu DY, Baker HW. High frequency of defective sperm-zona pellucida interaction in oligozoospermic infertile men. *Hum Reprod* 2004; 19: 228–233.