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Review Article

New generation of diagnostic tests for infertility: Review of specialized semen tests

Mary K Samplaski, Ashok Agarwal, Rakesh Sharma and Edmund Sabanegh

Glickman Urological and Kidney Institute, Cleveland Clinic Foundation, Cleveland, Ohio, USA

Abstract: The initial evaluation of the subfertile male includes a through history and physical examination, semen analyses, and hormonal evaluation. However, a normal spermiogram does not necessarily correlate with fertility potential, because it does not assess sperm function. For this reason, specialized semen tests have been developed to test various aspects of spermatozoal function. This article reviews basic male reproductive physiology, as well as clinically utilized advanced seminal tests, including their methodology and implications for reproductive technology outcomes and fecundity.

Key words: infertility, male, semen analysis, specialized tests, sperm.

Introduction

Primary infertility affects approximately 15% of couples,[1] with male factor infertility accounting for 50% of cases.[2] Evaluation of the male partner includes a detailed history and physical examination, semen analyses, genetic and hormonal evaluation, and specialized semen tests as needed. The semen analysis has traditionally been the cornerstone of the evaluation of the infertile male and remains the initial text of choice. The results of the semen analysis are then considered in the context of the patient's global picture to develop a differential diagnosis, which may prompt further testing. Although most of these tests are interpreted by an infertility specialist, a basic understanding is important for all clinicians. The present article reviews the semen analysis and specialized sperm function tests, so that the clinician has a baseline familiarity with the causes of male factor infertility, how they can be diagnosed, and their meanings.

Normal reproductive physiology

Spermatogenesis, the transformation of germ cells into spermatozoa occurs in the seminiferous tubules of the testicles (Fig. 1) and takes 74 days. This process is under the control of follicle stimulating hormone (FSH), which stimulates Sertoli cells to maintain normal spermatogenesis. Spermatozoa are released into the lumen of the seminiferous tubules, where they are transported toward the caput of the epididymis. In the epididymis, sperm undergo maturation, where they acquire increased motility and enhanced capacity to fertilize. Sperm are then stored in the epididymis until ejaculation. The tail of the epididymis is the earliest location where sperm with motility and the ability to fertilize can be found.

Once in the female reproductive tract, spermatozoa penetrate the cervical mucus barrier. In uterus, they undergo capacitation, a priming process in which a higher level of responsiveness to signals emanating from the cumulus-oocyte complex is obtained.[3] This then triggers an increase in the spermatozoa motility, known as hyperactivation. When the sperm reaches the ovum, the acrosome releases enzymes that penetrate the zona pellucida. Fertilization then occurs, with subsequent zygote formation and development. Abnormalities in any of these steps can lead to infertility.

Semen analysis

The semen analysis is the cornerstone of the assessment of the male partner for infertility, and evaluates not only spermatozoa, but also seminal plasma and non-sperm cells. Human spermatozoa show marked heterogeneity,[4] and two semen samples should be evaluated, collected at least 7 days between specimens, and 3 months after any febrile illness. Samples are collected after a period of abstinence of greater than 48 h, but less than 7 days. Specimens should be analyzed within 1 h of collection, as semen liquefies at room temperature within 60 min.

The semen analysis is evaluated using standardized reference ranges. Most commonly the World Health Organization (WHO) values, which were published manuals in 1980, 1987, 1992, 1999 and 2009 providing standard reference values for semen characteristics (Table 1).[5]

Historically, the belief that there are a discrete number of spermatozoa with certain descriptive parameters that are

Correspondence: Edmund Sabanegh MD, Department of Urology, Glickman Urologic and Kidney Institute, Cleveland Clinic, Cleveland, OH 44195, USA. Email: sabanee@ccf.org

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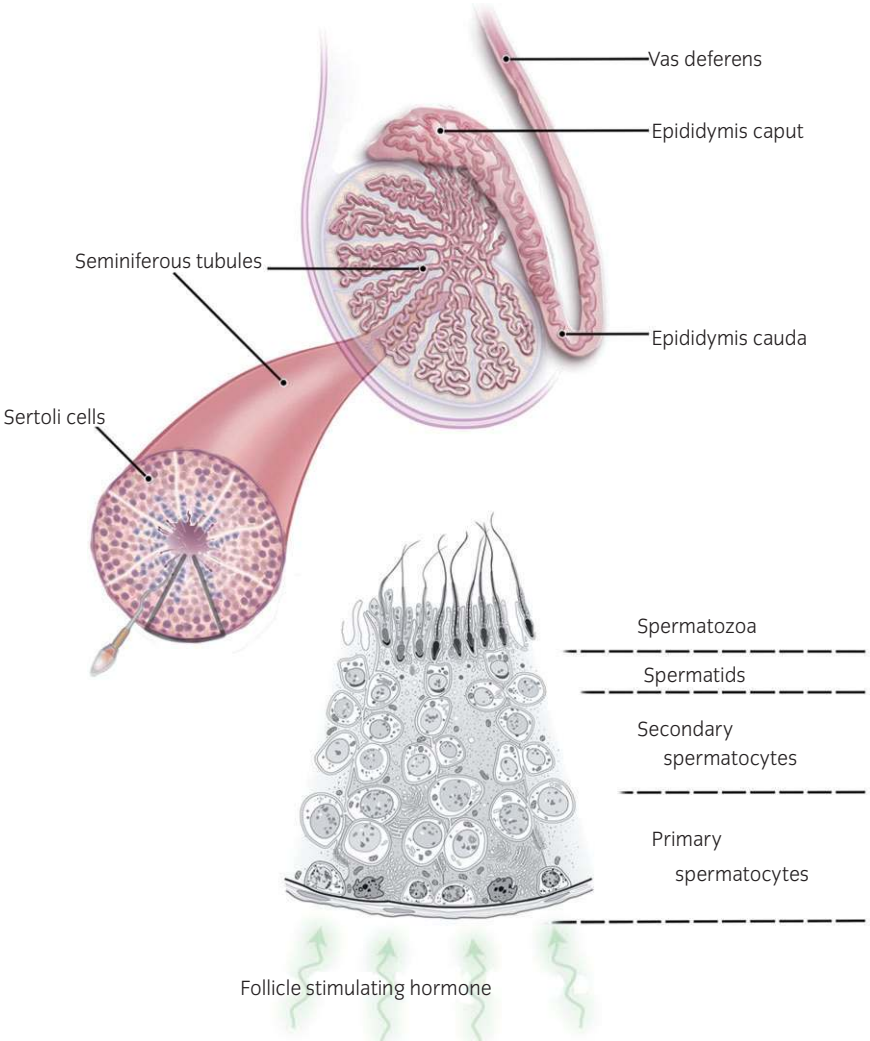

Fig. 1 Spermatogenesis: Within the seminiferous tubules, follicle stimulating hormone stimulates Sertoli cells. Spermatazoa are released into the lumen of the seminiferous tubules, where they are transported toward the caput of the epididymis. In the epididymis, sperm undergo maturation, where they acquire increased motility and enhanced capacity to fertilize. Sperm are then stored in the epididymis until ejaculation.

Table 1 2009 World Health Organization standard reference values for semen characteristics

Volume	1.5 mL or more
pH	7.2 or more
Sperm concentration	15 million or more spermatozoa/mL
Total sperm count	39 million or more spermatozoa/ejaculate
Progressive motility	32% or more
Total (progressive + non-progressive) motility	40% or more
Morphology	4.0% or more normal forms
Vitality	58% or more live

necessary for fertilization has driven the semen analysis. However, a normal spermiogram does not necessarily correlate with fertility potential, because it does not assess sperm function. Some factors, including sperm count and morphology, have been clearly found to relate to conception;[6] however, there are still a significant proportion of patients with normal semen analyses showing unexplained infertility.[7] In one study of 430 couples, 65% with a sperm concentration of greater than 40 million spermatozoa/mL achieved pregnancy, compared with 51% with a lower sperm concentration.[6] Similarly, in a study of male partners in 765 infertile couples, there was significant overlap between fertile and infertile men with respect to sperm concentration, motility and morphology.[8] These studies show that the semen analysis does not always correlate with fecundity. There are a subset of patients in whom the standard semen analysis has been unable to detect some functional deficiency necessary for fertilization, estimated at 40% of men presenting for subfertility.[9] In light of these findings, specialized semen tests have been developed to evaluate specific aspects of sperm function. As our understanding of male infertility has developed, the role for these tests has become better defined, and their role in the diagnosis and treatment of male infertility continues to evolve.

Antisperm antibodies

Antisperm antibodies (ASA) are present in 4–8% of infertile men. Their formation is induced by breaching of the blood–testis barrier, either during development, traumatic disruption, surgery, or infection. Sperm agglutination on a semen analysis, severely impaired sperm motion, an abnormal postcoital test, or abnormalities of cervical mucus interaction or penetration may prompt ASA testing. Unfortunately, there is a relatively poor correlation between ASA in blood and semen, relegating blood antisperm antibody testing to historical status.[10] Only antibodies that bind to sperm membrane antigens are of functional significance, and thus tests that directly examine sperm antibody presence are most useful.

The presence of ASA has been shown to negatively affect sperm function in several ways. Antibodies that agglutinate, immobilize, or opsonize sperm impair migration through the female reproductive tract and reduce the number of sperm at the fertilization site. Likewise, ASA may interfere with the normal process of sperm differentiation, capacitation, cumulus penetration, acrosome reaction, zona binding and penetration, or sperm-oocyte membrane interactions.[11,12] The end result is that pregnancy rates may be reduced by ASA.[13]

The diagnosis of ASA is made by the mixed antiglobulin reaction or immunobead test, with the immunobead test being used more commonly (Fig. 2). For this test, spermatozoa are mixed with beads that have been coated with IgG class-specific secondary antibodies. The sperm suspension is then observed microscopically for agglutination. Antibodies are considered clinically significant when greater than 50% of spermatozoa are coated, when spermatozoa are unable to penetrate the preovulatory human cervical mucus or show impaired fertilizing capacity. Isotype specificity and spermatozoa localization of ASA is possible; however, none of the currently available tests are able to detect the number of antibody molecules bound. This area is under investigation and might provide more information about the underlying cause of an individual patient's ASA and how their infertility could be best treated.[12]

Steroids may be given to lower titers before intrauterine insemination (IUI), but are unnecessary if intracytoplasmic sperm injection (ICSI) is used.[12] Using *in vitro* fertilization (IVF), patients with different ASA (IgG or IgA; in semen, cervical mucus, seminal plasma or female serum) have been shown to have lower pregnancy rates and higher miscarriage rates.[14] Current research is exploring the stimuli for and effects of specific ASA on individual sperm proteins. This might then provide a greater understanding or this disorder and possibilities for more targeted therapies.[12]

Viability assays

Non-motile sperm may either be dead (necrospermia) or immobile as a result of an ultrastructural defect. Viability testing is indicated when sperm motility is less than 5–10%. A high viability with a low motility is suggestive of a structural defect, such as primary ciliary dyskinesia, which may be further evaluated with electron microscopy. Surgically retrieved testicular sperm are oftentimes non-motile, because they do not pass through the epididymis. Viability testing may be used to determine which sperm are alive and suitable for ICSI,[15] using either dye exclusion or by evaluation of hypo-osmotic sperm swelling. Flow cytometry has also been applied to assess sperm membrane vitality, using dual staining for simultaneous assessment of the plasma and mitochondrial membrane integrity.[16]

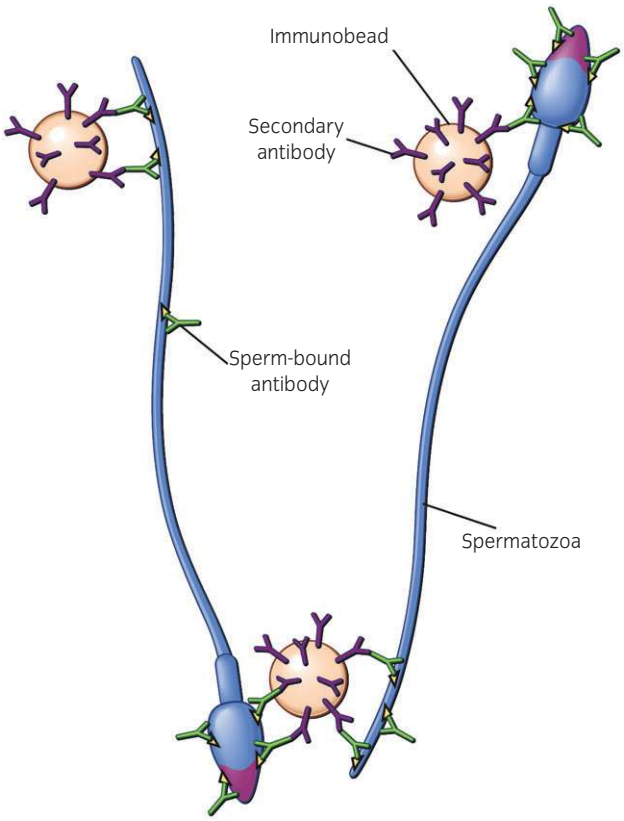

Fig. 2 Immunobead test. Spermatozoa are mixed with beads that have been coated with IgG class-specific secondary antibodies. Antibodies are considered clinically significant when greater than 50% of spermatozoa are coated, or when the spermatozoa are unable to penetrate the preovulatory human cervical mucus, or show impaired fertilizing capacity.

Dye exclusion involves mixing spermatozoa with a supravital dye, such as eosin or trypan blue (Fig. 3). Sperm with an intact cell membrane are able to exclude the stain and will not change color. Because this method kills the sperm, they cannot later be used for ICSI. The hypo-osmotic sperm swelling test involves placing sperm into a low osmotic media and assessing their ability to respond.[17] Water enters the cytoplasm of live cells to reach osmotic equilibrium, causing the spermatozoa tail to swell,[18] and the

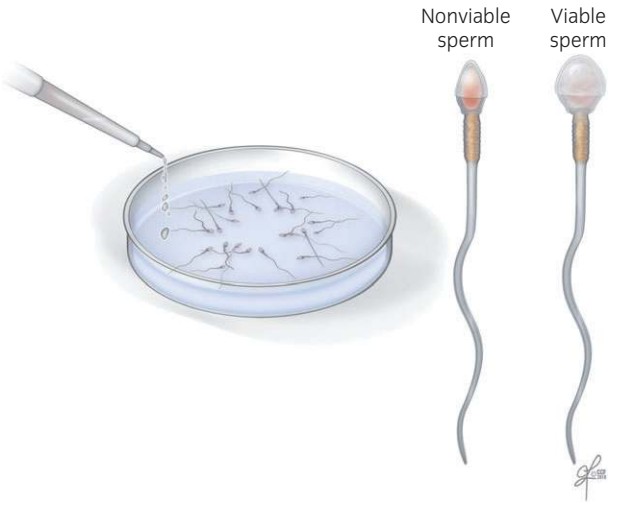

Fig. 3 Hypo-osmotic sperm swelling test to assess spermatozoa viability. Sperm with an intact cell membrane take up the hypo-osmotic fluid and swell.

test is considered normal if more than 60% of the sperm tails swell.[19] Because the hypo-osmotic media does not kill viable sperm, they can be used for ICSI.

Computer-Assisted Sperm Assessment

Computer-assisted sperm assessment (CASA) involves the use of computer analysis of videomicrography to assess a variety of sperm kinetic parameters, including motility, concentration, motion kinetics, and morphology.[20] Hyperactivation, characterized by high velocity, large amplitude, flagellar waves[3] creates a characteristic pattern that is quantified by CASA,[21] which has been found to correlate with IVF fertilization rates.[22] The straight line velocity is considered the most robust parameter for clinical analysis of sperm progression.[19] Although positively correlated with fertilization rates in IVF,[23] the assessment of sperm motion characteristics by CASA cannot reliably predict fertilization outcome,[11] and its role in fertility prediction is primarily investigational.

Acrosomal Integrity and function

Proteolytic enzymes in the acrosome digest through the zona pellucida, allowing for sperm–oolemma fusion (Fig. 4a). When this process is impaired, either by lack of an acrosome or acrosomal dysfunction, fertility can be marred. Illustrating this, round-headed sperm, indicative of acrosomal absence, will not bind to or penetrate through the zona pellucida.[24] Staining with different fluorescent lectins that bind to either the outer membrane or acrosomal contents can assess the acrosomal integrity (Fig. 4b).[3] Because acrosomal loss can be a result of normal sperm death, this test is often used in conjunction with a test of cell viability, such as with

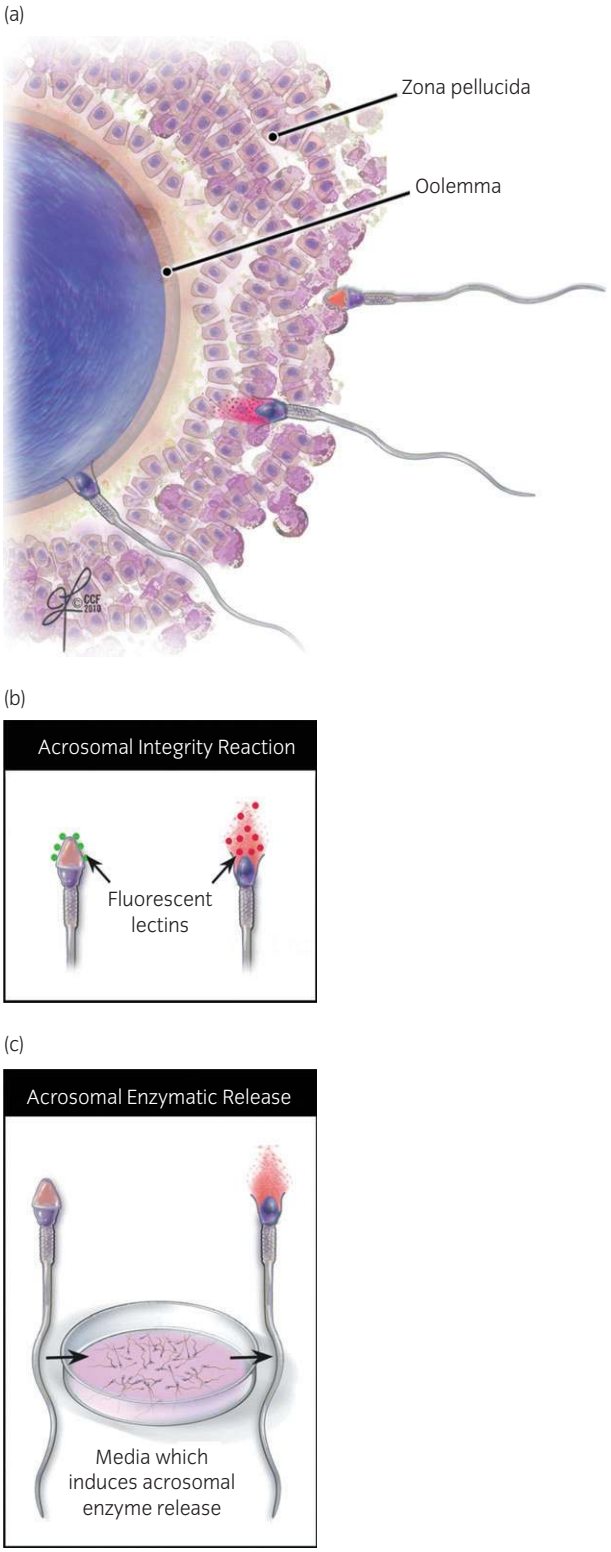

Fig. 4 (a) Normal physiology: Proteolytic enzymes in the acrosome digest through the zona pellucida, allowing for sperm-oolemma fusion. (b) Assessing acrosomal integrity: Different fluorescent lectins are applied to label either the outer membrane or acrosomal contents. (c) Assessing acrosomal enzymatic release: Enzymatic release is induced and the proportion of reacted spermatozoa are assessed.

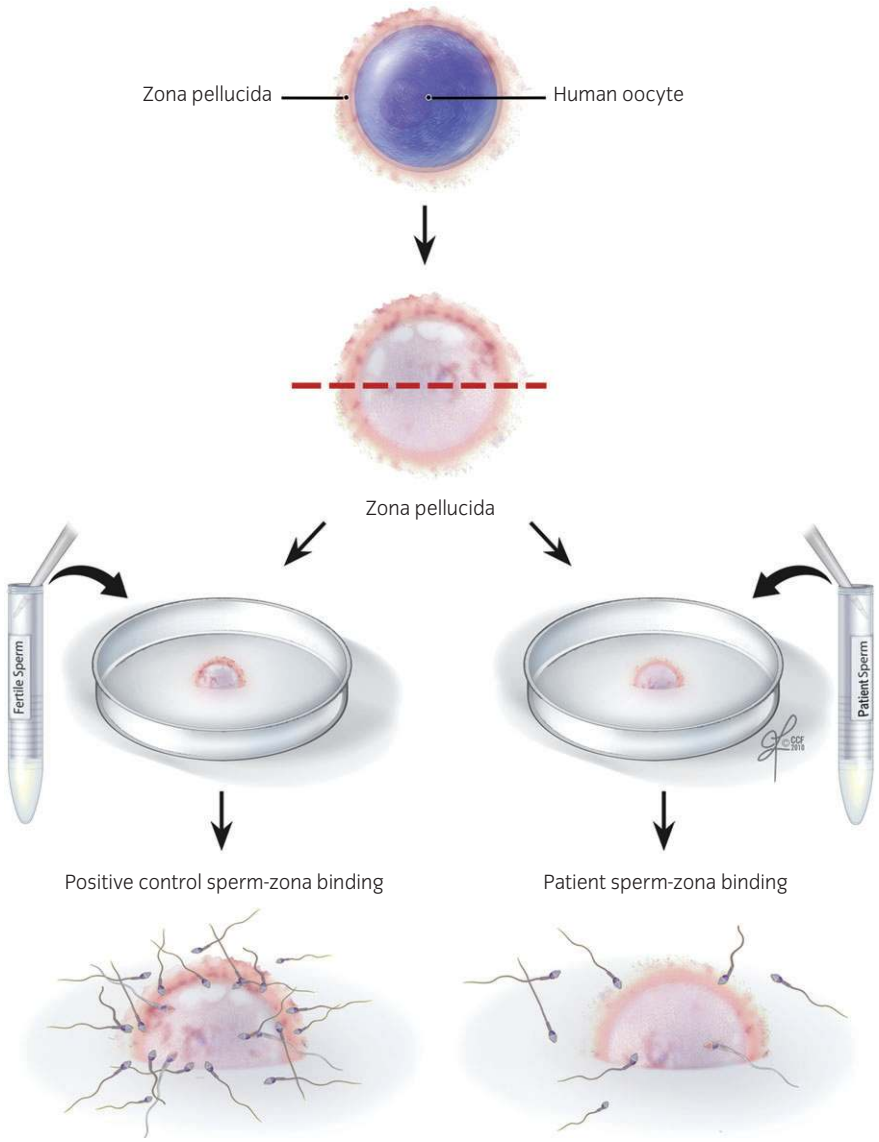

Fig. 5 Hemizona assay. The zona pellucida is isolated and divided in half. One half is incubated with fertile donor sperm (positive control) and the other half is incubated with patient sperm. The ratio of fertile to donor binding is measured.

the hypo-osmotic swelling test, to distinguish non-viable from reacted acrosomes. This test has been found to predict the fertilizing potential of human spermatozoa *in vitro*.[25]

To evaluate enzymatic release, the ionophore challenge is used. Acrosomal enzymatic release is induced and the proportion of reacted spermatozoa are assessed (Fig. 4c). Acrosomal stimulators include the ionophore A23187, progesterone, and the human zona pellucida.[25–27] Semen samples with 5–30% of reacted spermatozoa have a higher fertility potential, which then correlates with reproductive outcomes.[28] These tests are primarily used in men being evaluated after IVF failure, and have limited utility in the treatment of primary infertility.

Hemizona assay

Binding of the spermatozoa to the zona pellucida triggers the acrosome reaction.[11] When IVF is used, defective binding and penetration are among the most common causes of fertilization failure.[26,29] This binding is species specific, limiting the bioavailability of these assays.[28] The two available assays to evaluate binding include the hemizona assay and the sperm–zona binding ratio. The hemizona assay (Fig. 5) uses human oocytes from which the zona pellucida is isolated and divided in half. One half is incubated with fertile donor sperm (positive control) and the other half is incubated with patient sperm. The ratio of fertile to donor binding is measured, with less than 30% being considered abnormal.[30]

The sperm zona binding ratio uses patient sperm and fertile donor sperm labeled with different fluorochromes. Sperm are incubated with zona-intact oocytes, and the number of bound sperm is counted.[28] Poor zona binding has been found to correlate with poor fertilization rates using IVF,[31] and patients with an abnormal zona binding should be counselled consider ICSI. These tests are helpful primarily

in patients that have failed standard IVF, and have limited utility in the setting of primary infertility.

Sperm penetration assay

The sperm penetration assay, or zona-free hamster oocyte penetration assay, measures the spermatozoa's ability to undergo capacitation, acrosome reaction, fusion and penetration through the oolemma, and decondensation within the cytoplasm of an oocyte. The zona pellucida is removed from a hamster oocyte, which is then incubated with human spermatozoa. This allows the human sperm to fuse with the hamster ova. The test is scored by calculating the percentage of ova that are penetrated or the average number of sperm penetrations per ovum. This test is one of the most sensitive measures of sperm function available.[3] Prospectively, it has been shown to be positively associated with IVF fertilization rates,[32,33,34] and the achievement of pregnancy in infertile males with unexplained infertility.[35] Based on the results of this test, modifications can be made to the sperm preparation or IVF conditions, such as modification of supplementation of the culture media.[36]

Leukocytospermia testing

Quantification of seminal leukocytes is part of the standard semen analysis, but under light microscopy it may be difficult to differentiate leukocytes from immature germ cells. The Endtz test stains for peroxidase within polymorphonuclear granulocytes allowing for this distinction.[37] Leukocytospermia, defined as $>1 \times 10^6$ WBC/mL is negatively associated with multiple parameters of spermatozoa function.[38] Specifically, leukocytospermia has been correlated with sperm tail defects, acrosomal damage, high sperm deformity index, impaired sperm morphology and motility.[39,40] Seminal leukocytes are powerful generators of reactive oxygen species (ROS), and their full role in fecundity is being elucidated. However, treating men who have *Chlamydia* or *Ureaplasma* with antibiotics leads to a decrease in seminal leukocytes and ROS production, with a subsequent improvement in sperm motility and an improvement in natural conception, suggesting a correlation with infertility.[41]

Chemiluminescent signals

In recent years, it has been recognized that impaired sperm function is frequently correlated with oxidative stress created by excess reactive oxygen species (ROS).[42] Human spermatozoa are exquisitely sensitive to damage by ROS as a result of their limited antioxidant defenses.[43] Although low levels of ROS are necessary for sperm capacitation,[44] excessive levels have been correlated with sperm cell membrane lipid peroxidation, decreased sperm motility, impaired DNA integrity, as well as impaired spontaneous pregnancy rates and fertilizing potential *in vivo* and *in vitro*.[42,45] In turn, ROS-induced DNA damage may accelerate the process of germ cell apoptosis, leading to lowered sperm counts.[42] Elevated levels of ROS can be detected in the semen of 25–40% of infertile men.[42] The full implications of ROS on fecundity are still being elucidated.

To assess the oxidative stress to spermatozoa, the chemiluminescent assay has been developed. A signal is generated by the stressed spermatozoa in the presence of redox-sensitive probes, such as lucigenin and luminol. The intensity of signal produced is negatively associated with sperm function,[35,43,46,47] and reflects the fertilizing potential of human spermatozoa *in vivo* and *in vitro*.[35,47] The generation of ROS has been shown to be negatively associated with spontaneous pregnancy rates in infertile males,[35] and some studies have shown that elevated levels of ROS correlate with impaired IVF pregnancy rates.[48,49] Men with elevated levels of ROS should be considered for antioxidant therapy, such as Vitamins A, C or E, as these agents improve semen quality, pregnancy and implantation rates after ICSI.[50]

Tests of spermatozoa DNA damage

The effect of oxidative stress on spermatozoa can also be monitored by assessing levels of DNA damage, a surrogate for apoptosis.[45] There appears to be a threshold of sperm DNA damage beyond which embryo development and pregnancy are impaired,[51,52] and studies have shown that the spermatozoa of infertile men possess more DNA damage than their fertile counterparts.[52,53] Flow cytometry to evaluate the DNA of sperm can distinguish the mature haploid and the abnormal diploid mature spermatozoa, cellular fragments and immature germ cells.[54] DNA damage can be assessed directly, using the comet assay, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) assay; or DNA oxidation. It can also be measured indirectly using the sperm chromatin structure assay (SCSA) or sperm chromatin dispersion assay.

The SCSA measures the stability of spermatozoa chromatin. DNA is exposed to acridine orange media, which specifically binds to DNA.[52] The acridine orange fluoresces green when bound to double-stranded DNA and red when bound to single-stranded DNA, and the ratio of green to red fluorescence is determined. The sperm chromatin dispersion assay denatures spermatozoa DNA and evaluates for the pattern of fragments that result. Spermatozoa with fragmented DNA fail to produce the characteristic large halo of dispersed DNA loops that is observed in sperm with intact DNA.[55]

DNA damage in the male germ line has been associated with poor semen quality, impaired preimplantation development, increased abortion, and an increased incidence of disease in the offspring, including childhood cancer.[45] Men

with a high percentage of spermatozoa with DNA damage have a reduced potential for natural fertility,[52,56] and poorer outcomes after IUI.[57,58] However, the data seem to suggest that DNA damage does not impact the fertilization rate or pregnancy outcome after ICSI.[57,59,60]

Microarray technology

One potential method for evaluating male factor infertility is the use of spermatozoal RNA profiles.[61] Spermatozoa RNA provides a historical record of spermatogenesis, based on constructs obtained using transcriptional profiling.[62] In the future, this technology may be used to investigate the response of cells to changes in the environment or conditions that alter mRNA expression.[62] The expression of genes may be studied under different conditions, allowing insight into the mechanisms of diseases on fertility. Preliminary studies have shown an altered genomic expression pattern in the spermatozoa of infertile men.[62] This may become a technique useful in men who are exposed to known environmental or occupational risk factors, who could be tested and offered reproductive counseling. Similarly, this might provide some insight for couples with multiple, recurrent spontaneous abortions, in whom there might be some underlying spermatozoa dysfunction.

Conclusions

The conventional semen analysis is a critical first step in the evaluation of male fertility, but provides limited information about sperm function. In a subset of patients, specialized semen tests will be needed to elucidate the etiology of subfertility. These tests are useful for determining specific defects of human sperm physiology. Newer sperm function tests may eventually be useful clinically, but more information is needed to determine if these tests will truly predict fertility potential. Their addition to potential to the routine semen analysis could help in the diagnosis of what is currently classified as unexplained infertility.

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