Introduction

While the semen analysis (SA) is the cornerstone of the assessment of male factor infertility, it remains imperfect in predicting fecundity.\textsuperscript{1,2} There are a subset of patients in which there is a deficiency in one of the tasks necessary for spermatozoa to reach and fertilize an ovum, which is not detected by the standard SA, estimated at 40–50 percent of men presenting for subfertility.\textsuperscript{3} To try and determine the precise functional impairment, specialized semen tests evaluate specific aspects of spermatozoa function. This chapter reviews selected specialized semen tests, outlining the principals of each, as well as clinical data to support or refute their clinical utility. For the purposes of this chapter, more commonly utilized assays will be listed first, closing with emerging tests.

Semen Analysis

The traditional belief that there are a discrete number of spermatozoa with certain characteristics that are necessary to achieve fertilization has been debunked in the last decade with the realization that 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,\textsuperscript{1,3} however, there are still a significant proportion of patients with normal SAs exhibiting unexplained infertility.\textsuperscript{2} As one classic example, Guzick et al. reviewed the semen parameters of 765 subfertile men, and found there was significant overlap between fertile and infertile men with respect to sperm concentration, motility and morphology,\textsuperscript{2} a finding which has been corroborated by others.\textsuperscript{1,4} These studies illustrate that the SA cannot independently predict male fertility, as it does not evaluate spermatozoa functional competence. For this reason, specialized semen tests have been developed in an attempt to evaluate individual aspects of spermatozoal function.

Computer-assisted Sperm Assessment

Computer-assisted sperm assessment (CASA), the use of computer analysis of videomicrography to assess sperm kinetic parameters, was developed in an attempt to more precisely analyze sperm head and flagellar kinematics. The microscopic field is digitized and kinematic values are determined for each spermatozoon, which are then computer analyzed.\textsuperscript{5} Classic SA parameters are generated, as well as sperm trajectory characteristics and straight-line velocity, which cannot be determined by standard microscopic evaluation. While these characteristics have been positively correlated with IVF fertilization rates, CASA cannot reliably predict spontaneous fertilization outcomes.\textsuperscript{6}

Viability Assays

When a semen sample has a motility of < 30 percent, viability testing is indicated to distinguish between necro spermia and an ultrastructural defect. Low motility and high viability suggests living sperm with an ultrastructural defect, such as primary ciliary dyskinesia or Kartagener’s syndrome, which may be further evaluated with electron microscopy. Immotile sperm may also be seen after testicular extraction, when they have not acquired motility in the epididymis. In this situation, viability testing may be useful in selecting viable sperm, to be used for assisted reproductive techniques (ART).\textsuperscript{7}

Living sperm have an intact cytoplasmic membrane, which is the basis for viability assays such as evaluation of hypo-osmotic swelling or dye exclusion. Hypo-osmotic testing (Fig. 8.1A) evaluates spermatozoa response to hypo-osmotic fluid, which enters the cytoplasm of living cells to reach osmolar equilibrium, causing viable sperm to visibly swell, best visualized in the tail. Dye exclusion (Fig. 8.1B) tests sperms’ ability to resist the absorption of certain dyes, including Eosin, Nigrosin, or Trypan blue. These tests are considered normal if ≥ 60 percent of sperm are viable.\textsuperscript{8} During dye exclusion sperm are air dried (killed), and thus cannot be used for ART. In contrast, the hypo-osmotic swelling assay does not lyse cells, allowing for the selection of sperm for ART.\textsuperscript{9}

Leukocytospermia Testing

Seminal leukocytes are a frequent finding in patients both with and without subfertility. Quantification in the standard SA may be inaccurate since under light microscopy it is difficult to differentiate leukocytes from immature germ cells. The Endtz test stains for peroxidase within polymorphonuclear leukocytes, allowing for this distinction.

Seminal leukocytes are powerful generators of reactive oxygen species (ROS), and their full role in fecundity is still being
elucidated. Leukocytospermia, defined as $>1 \times 10^6$ WBC/mL, is negatively associated with multiple parameters of spermatozoa function. However, levels as low as $20.2 \times 10^6$ WBC/mL have been associated with elevated ROS, suggesting that lower levels of leukocytes are pathologic. Leukocytospermia has been correlated with sperm tail defects, acrosomal damage, teratospermia, and impaired motility. Functionally, men with leukocytospermia have a lower chance of spontaneous pregnancy compared with fertile counterparts, and antibiotic treatment for men with leukocytospermia and genital infections has been shown to reduce seminal leukocyte and ROS levels, leading to an improvement in sperm motility and natural conception rates.

### Antisperm Antibodies

Sperm autoantibodies are present in 10 percent of infertile men, compared with 2 percent of fertile men. Sperm agglutination, impaired motility, an atypical postcoital test, or abnormalities of cervical mucus interaction, may prompt ASA testing. Routine semen parameters are often normal, leading some authors to recommend ASA testing in all men undergoing infertility work-up.

Only antibodies that bind to sperm membrane antigens are of functional significance, and thus only tests which examine sperm antibody presence are clinically useful. The immunobead test consists of incubating spermatozoa with microbeads coated with IgG class-specific secondary antibodies, and the observing microscopically for agglutination (Fig. 8.2). Antibodies are considered significant when $> 50$ percent of spermatozoa are coated, when sperm are unable to penetrate the preovulatory human cervical mucus, or demonstrate impaired fertilizing capacity. ASA agglutinate, immobilize, and opsonize sperm, which may interfere with sperm differentiation, migration, capacitation, zona penetration, or sperm-oocyte membrane interactions. Because of this, the presence of ASA may lead to impaired spontaneous and IVF pregnancy rates and higher miscarriage rates. Steroids may be given to lower ASA titers prior to IUI, but are unnecessary if ICSI is used. Isotype specificity and spermatozoa localization of ASA is possible, however, there are currently no tests to determine the quantity of antibody molecules bound. Research is ongoing to determine the stimuli for and effects of specific ASA on individual sperm proteins, which may lead to targeted therapies.

### Seminal Fluid Testing

The prostate, seminal vesicles, and epididymis each produce unique seminal components, which may then serve as surrogates for the glands that produce them:
- **Prostate**: Citric acid, zinc, calcium, magnesium, gamma glutamyl-transferase, PSA, acid phosphatase
- **Seminal vesicles**: Fructose, semenogelin, prostaglandin, seminal plasma motility inhibitor
- **Epididymis**: Free L-carnitine, glycerophosphocoline, $\alpha$-glucosidase

Of these, zinc and citric acid are commonly assayed for the prostate, fructose and semenogelin for the seminal vesicles, and $\alpha$-glucosidase for the epididymis. Levels of these compounds may highlight the pathogenesis of some semen samples. For example, $\alpha$-glucosidase and L-carnitine can be used to distinguish ductal obstruction from primary testicular failure, and they may also serve as indicators of IVF success. In reality, biomarkers are best interpreted with respect to each other. One example of this involves semenogelin, a coagulum released by the seminal vesicles, and PSA, from the prostate. PSA rapidly cleaves semenogelin, leading to semen liquefaction and sperm...
motility. An abnormality in either of these components would lead to hyperviscous semen, preventing sperm motility and fertilization.\(^{18}\)

**Acrosomal Integrity and Function**

Spermatozoa lacking an acrosome, either never having one or due to spontaneous release, will not bind to or penetrate the zona pellucida. Acrosomal integrity can be assessed by staining with fluorescent lectins that selectively bind to either the outer membrane or acrosomal contents (Figs 8.2A to C).\(^4\) If the acrosome is intact, the timing of enzymatic release can also be assessed. A proportion of sperm from any sample will exhibit ‘acrosomal prematurity’, or spontaneous enzymatic release. Normally, this comprises < 4 percent of the sample,\(^4\) however, men with repeated IVF failure have been shown to have > 20 percent of spermatozoa with acrosomal prematurity.\(^{19}\) Because acrosomal release can be due to sperm death, these tests are often used in conjunction with viability testing.

To test the acrosomal release of its contents, enzymatic release is induced, either by ionophore A23187, progesterone, or human zona pellucida, and the proportion of reacting spermatozoa is measured. This value, the stimulated acrosomal reaction (SAR) score, ranges from 20–98 percent in fertile men and lower values have been correlated with impaired IVF rates, although predictive values vary.\(^{20}\) As such, acrosomal testing is primarily used after repeated IVF failure, and has limited utility in the initial assessment of subfertility.

**Post-Coital Test**

Also known as the cervical mucus reaction, the post-coital test evaluates sperm motility within the cervical environment and its ability to access the uterus. It is indicated in the settings of hyperviscous semen or unexplained infertility. Anatomic abnormalities, including hypospadias, or inappropriate sexual technique may result in the absence of sperm in the cervical mucus.\(^9\)

The test is conducted when the cervical mucus is thinnest, just prior to ovulation; the number and motility of sperm in the cervical mucus is assessed 2–8 hours after intercourse. Greater than 10–20 motile sperm per high-powered field is considered normal\(^9\) and has then been positively correlated with spontaneous and *in vitro* pregnancy rates.\(^{21}\) However, while interesting in theory, a thorough history and SA can predict the results of the post-coital test in half of infertile couples,\(^{22}\) and thus its true clinical utility is limited.

**HEMIZONA ASSAY**

Binding of spermatozoa to the species-specific zona pellucida triggers the acrosome reaction, wherein the enzymatic contents of the acrosome are released.\(^9\) This reaction is evaluated using the hemizona assay and sperm-zona binding ratio. The former (Fig. 8.3) utilizes human oocytes from which the zona pellucida is isolated and split. One half is incubated with fertile donor sperm and the other with patient sperm. The ratio of fertile to donor binding is measured, with < 30 percent considered abnormal.\(^9\) For the sperm-zona binding ratio, different fluorochromes are used to label fertile donor sperm and patient sperm. Sperm are then incubated with zona-intact oocytes, and the ratio of bound to unbound spermatozoa are quantified.\(^9\)

Defects in sperm-zona binding and penetration are among the most common causes of IVF failure,\(^{23}\) IUI failure,\(^{24}\) and impaired spontaneous pregnancy in infertile men.\(^{24}\) These tests are primarily used to elucidate the origin of IVF failure, rather than as part of the initial infertility evaluation, and men found to have abnormal binding should be counseled to consider ICSI.\(^{24}\)

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**Figures 8.2A to C:** ASA testing using the immunobead test: Sperm are mixed with beads that have been coated with IgG class-specific secondary antibodies: (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.
Chapter 8: Testing Beyond the Semen Analysis: The Evolving Role of New Tests

Sperm Penetration Assay

The sperm penetration assay (SPA) tests a sperm’s ability to undergo capacitation, acrosomal release, fusion and penetration with the oocyte vitelline membrane, and decondensation within the oocyte. The zona pellucida is stripped from a hamster oocyte, which is then incubated with human spermatozoa. The percentage of ova penetrated or average number of sperm penetrations per ovum is used to score the assay, which in limited studies has been shown to correlate with spontaneous pregnancy outcomes and IVF fertilization rates. However, the SPA is hampered by a wide range of sensitivities and specificities, and therefore is not commonly used.

Tests of Spermatozoal Reactive Oxygen Species (ROS)

Human spermatozoa are exquisitely sensitive to damage by ROS. While small amounts of ROS are necessary for the acrosome reaction and capacitation, high levels can overwhelm the limited spermatozoal antioxidant defenses Elevated levels of ROS are detected in the semen of 25–40 percent of infertile men. This excess has been associated with seminal leukocytes, smoking, varicocele, alcohol, infection, and radiation exposure. Functionally, elevated levels of ROS have been correlated with decreased motility, impaired DNA integrity, impaired spontaneous pregnancy rates, and impaired IVF potential.

The chemiluminescent assay is used to directly measure ROS levels within spermatozoa. Luminol or lucigenin probes bind to ROS, including that in leukocytes, seminal fluid, and spermatozoa, are then assessed using a luminometer. The intensity of the signal produced is negatively associated with sperm function, and reflects the fertilizing potential of human spermatozoa in vivo and in vitro. Seminal leukocyte levels are also assessed to determine their contribution to the total ROS.

The ROS levels have been negatively associated with impaired spontaneous, and IVF pregnancy rates. Men with elevated levels of ROS should be considered for antioxidant therapy with vitamins A, C, or E as these agents have been shown to improve semen quality, pregnancy and implantation rates after ICSI. As the full ramifications of oxidative stress on male fertility are still being elucidated, these tests will likely play an expanding role.

Tests of DNA Damage

Excessive ROS levels induce germ cell DNA damage, manifested as DNA fragmentation. While early embryos can repair some spermatozoal DNA damage, there appears to be an upper limit beyond which pregnancy loss occurs. The spermatozoa of infertile men has shown to possess more DNA damage than fertile counterparts, leading to the suspicion that ROS induced DNA damage may play a role in infertile males. Over 30 assays to assess oxidative stress have been described, which can be largely grouped into direct, indirect, and implied. Oxidative damage can also be measured indirectly using the nitroblue tetrazolium assay, in which superoxide radicals within sperm react and become visible under light microscopy. A cutoff value of 30 percent for DNA fragmentation index (DFI) has been
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Figures 8.4A and B: (A) Comet assay: Electrophoresis is applied to fluorochrome-stained spermatozoal DNA, causing the DNA fragments to form a characteristic comet shaped streak, the pattern of which is indicative of DNA fragmentation levels. (B) TUNEL assay: DNA breaks are tagged with F-dUTP, which binds to exposed 3'-hydroxyl ends, and is then quantified using flow cytometry. The first frame shows 7.4% damage (negative) and the second shows 47.4% damage (positive).

Approximately 8 percent of subfertile men with normal semen parameters will have high levels of abnormal DNA. Corroborating this, subfertile men have been shown to have abnormal DNA denaturation and fragmentation rates of 25 and 28 percent, respectively, as compared with 10 and 13 percent in fertile men. Oxidative damage in the male germ line has been associated with leukocytospermia, oligoasthenozoospermia, and negatively correlated with sperm concentration, motility, and morphology. With respect to pregnancy, ROS have been linked to impaired preimplantation development, spontaneous abortion, and an increased incidence of disease in the offspring. Men with a high percentage of spermatozoa with DNA damage have a reduced potential for natural fertility, and poorer outcomes after IUI, IVF, and ICSI.

At this time, DNA testing is most helpful in cases of unexplained infertility, recurrent pregnancy loss, prognostication of ART outcome, and assessment of genetic integrity in post-chemotherapy patients or those of advanced age. Extensive DNA fragmentation may also suggest the need for testicular sperm extraction, as testicular sperm often have lower levels of DNA fragmentation than ejaculated sperm. Assays of DNA damage may also be useful in identifying sperm with an optimal quality to be used for ICSI. As DNA testing becomes more standardized, less expensive, more accessible, and more reliable, its role in clinical practice will likely continue to expand.

Microarray Technology

Spermatozoa RNA provides a historical record of spermatogenesis, based on constructs obtained using transcriptional profiling, and these are being investigated as markers of fertility. This technology may be used to investigate the response of cells to conditions that alter mRNA expression, allowing insight into the mechanisms and effects of specific diseases. Microarrays from fertile and infertile men may also allow for the identification of genes which are important for successful fertilization and pregnancy, or biomarkers for infertility. Similarly, comparing transcriptomes at different stages of spermatogenesis, may allow for the identification of genetic aberrations which may provide some insight for couples with recurrent spontaneous abortions. Finally, this technology may have implications for ART, as spermatozoa used for ICSI bypass the bodies natural selection process, and may therefore transmit flawed genes.
Proteomics

Gel electrophoresis is being investigated to identify individual proteins allows from semen samples from fertile men to be compared with infertile men. Currently, seminal fluid has been found to contain 923 proteins, at least 101 of which have an altered expression pattern in infertile men. Examples include protamine 2 precursors, which have been shown to accumulate in some infertile patients, as well as the structural protein, actin, which is altered in asthenozoospermic men. These proteins may prove to be diagnostic markers in understanding some of the pathogenic mechanisms involved in male infertility.

Metabolomics

Metabolites are breakdown products from intracellular metabolic processes which may provide insight into their biochemical precursors. In the evaluation of male infertility, this area may serve as an indirect measure any number of physiologic processes, including oxidative stress by comparing either ROS byproducts or antioxidant levels in fertile and infertile men. The noninvasive nature of this test makes it attractive, and other markers will emerge in the coming years.

High Magnification Microscopy

High magnification microscopy allows for the analysis of sperm morphology at great magnification, up to X8000. As mentioned earlier, the relationship between sperm morphology and pregnancy is not universally predictive. Ultrafine microscopy may be able to detect subtle ultrastructural malformations which are not currently identifiable, which may impact fertilization. Currently this technology is being used to select sperm with the highest morphologic integrity for intracytoplasmic morphologically selected sperm injection (IMSI). In general, the morphologic features most important include the degree of chromatin condensation, which precludes a liability to DNA fragmentation, and nuclear vacuole presence. Studies in which spermatozoa were selected based on high resolution microscopy have demonstrated higher pregnancy rates as compared with conventional ICSI, and the use of this technology in andrology will likely continue to expand.

CONCLUSION

A carefully performed SA remains the cornerstone of the evaluation of male factor infertility. However, there remains a subset of men in which the standard SA is unable to detect an innate functional defect. In these men specialized semen testing may provide insight into the impairment which is preventing fertilization. This may in turn lead to targeted management of a specific defect. Emerging technologies will likely to incorporate an increasing amount of genetic testing, as well as non-invasive testing using biomarkers. These tests will likely hold promise in the continued advancement toward the diagnosis and management of the multitude of causes for male infertility. Finally, with the increasing use of ICSI, which bypasses many of the sperm requirements for egg fertilization, more commonly used tests will be those that select for the optimal spermatozoa lead to a successful pregnancy with IVF or ICSI.

REFERENCES

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