

Laboratory Assessment of Male Infertility—A Guide for the Urologist

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Abstract

After receiving a thorough history-taking and physical exam, patients should have two to three properly collected semen analyses. Semen analysis is a demanding test requiring laboratory personnel who are well trained in all aspects of andrological examination. To ensure the highest level of accuracy in andrology testing, clinicians should refer their patients to diagnostic laboratories that are accredited by national organizations (such as the College of American Pathologists [CAP] in the US) and subscribe to external proficiency testing schemes in andrology as part of their quality management. Specialized andrology laboratory tests may be ordered as indicated to properly diagnose male factor infertility. These tests include fructose test in azoospermic samples, viability test for poor motility specimen, antisperm antibody test in cases of agglutination and poor motility, peroxidase test for white blood cells in cases of infection or inflammation, tests for seminal reactive oxygen species level, and total antioxidant capacity in seminal plasma; sperm DNA fragmentation assessment may be helpful in cases of idiopathic male factor.

Keywords

Semen analysis, male infertility, sperm morphology, DNA damage, oxidative stress, andrology

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The assessment of a male's fertility starts with a thorough history-taking and at least two separate sperm analyses. For the past 50 years, this inexpensive test has been the first ancillary procedure ordered when a man undergoes fertility assessment. Proper collection of semen must be emphasized to patients as it is often the cause of error in the semen analysis. The semen samples must be collected with a minimum interval of seven days while observing sexual abstinence of two to seven days per collection.^{1,2} The fertility physician must bear in mind that inter-patient variability exists and multiple collections may be required to obtain an accurate assessment. Other factors, such as well-equipped and well-trained laboratory personnel, play a hidden but significant role in the delivery of accurate results.³

Macroscopic Variables

Macroscopic variables are volume, pH, coagulation, liquefaction, color, and viscosity. The normal values—based on the World Health Organization (WHO) 1999 manual—are listed in *Table 1*.⁴ Variations in these parameters are relatively common and may be of little clinical significance; however, this may also be indicative of accessory gland malfunction. The specifics of the tests can be found in the WHO manual.² The most common condition that may give rise to abnormalities in the macroscopic variables is ductal obstruction and/or vas/epididymal abnormalities. The semen is often low in volume and non-coagulating with acidic pH. Volume variation is of value when it is consistently low and may indicate partial duct obstruction or

retrograde ejaculation. High-volume semen (>8ml) is often associated with poor-quality semen.²

Microscopic Variables

The microscopic parameters assess spermatogenesis and are said to be the most technically difficult part of the semen analysis. Inter-patient variation is present in even the most ideal conditions of collection and testing. The WHO laboratory manual is the most widely used resource for normal results, and is used for infertility and andrology work-up purposes.⁴ The microscopic examination starts with the creation of a wet smear (a drop of semen on a slide covered with a cover slip) visualized under x1,000 magnification. Sperm agglutination, sperm presence, and subjective motility can be assessed using this method. A small degree of agglutination is normal.⁴ Excessive agglutination is suggestive of the presence of antisperm antibodies, and subsequent work-ups for this should be requested. After liquefaction, assessment of sperm concentration (number of sperm/ml) and sperm count (number of sperm/ejaculation) is conducted. Oligozoospermia (<20 million sperm/ml) may be indicative of incomplete collection or a short abstinence period. It may also indicate partial duct obstruction and other medical conditions that affect sperm production such as varicocele, cryptorchidism, diabetes, primary testicular failure, and medications such as nitrofurantoin. Febrile illnesses may cause transient oligozoospermia (and, rarely, azoospermia). A repeat semen

Table 1: Macroscopic Parameters of the Semen Analysis and Clinical Significance of Some of the Abnormal Values

Parameters	Normal Values	Abnormalities	Clinical Significance
pH	7.8	Acidic: <6.5–7	Low volume and non-coagulation: congenital absence of the vas Ejaculatory duct obstruction Partial retrograde ejaculation
Coagulation/liquefaction	Coagulates and liquefies within 20 minutes at room temperature	No coagulation Prolonged liquefaction	Congenital absence of the seminal vesicles Poor prostatic secretions
Color	Whitish-gray; pearl-white	Yellowish Reddish-brown	Jaundice, carotenemia, drugs Hemospermia secondary to urethral bleeding or inflammation of the seminal vesicles, but other genito-urinary tumors will need work-up to be excluded
Viscosity	4mm threading	>6mm No threading	Of importance when associated with low motility
Volume	2–4ml	0 (aspermia) <2ml (hypospermia) >6ml	Retrograde ejaculation Incomplete collection Partial retrograde ejaculation Short duration of sexual abstinence Accessory gland overproduction Prolonged sexual abstinence

analysis should be requested after two to three months, bearing in mind that a complete spermatogenesis cycle lasts for 74 days. Azoospermia (absence of sperm) is observed when the genital tract is obstructed, as well as severe sperm testicular pathology such as Sertoli cell only syndrome or hypogonadism. Hormonal tests (follicle-stimulating hormone [FSH] and testosterone), karyotyping, and Y chromosome microdeletion testing are requested when severe oligozoospermia or azoospermia are seen. Deletions in the AFZa or AFZb (versus AFZc) regions of the Yq chromosome are indicative of markedly impaired spermatogenesis with poor sperm retrieval even with testicular biopsy, and these deletions may be passed on to the male offspring conceived with intracytoplasmic sperm insertion (ICSI).⁵

Sperm morphology can also be a significant predictor of pregnancy in subfertile couples when deciding which assisted reproduction technology (ART) procedure to employ. Couples with teratozoospermia (<15% normal morphology) by WHO standards, and with no other male factor infertility, may be recommended to proceed with ICSI versus intrauterine insemination (IUI).^{6,7} Leukocytes are the most significant non-sperm cellular elements in the semen, and are a frequent finding in patients with unexplained infertility.^{8,9} In the initial microscopic analysis, the immature spermatozoa may be confused with leukocytes. Additional tests such as immunocytochemistry may be requested to confirm findings when there are >5 round cells/high power field (hpf). A reliable alternative is the Endtz test, an orthotoluidine dye test that accurately identifies leukocyte-containing enzymes that will react with the peroxide and, consequently, allow visualization with the orthotoluidine dye.¹⁰ The WHO has defined leukocytospermia as levels >1×10⁶ white blood cells (WBCs)/ml. The condition has been associated with reactive oxygen species (ROS).¹¹ However, even lower levels of seminal WBC can generate significant levels of ROS.^{12,13} Red blood cells (RBCs) are often present in semen. While small amounts are usually a normal finding, they can be indicative of infection, inflammation, ductal obstruction, or, rarely, vascular abnormalities. With other clinical findings and risk factors, genito-urinary neoplasm may have to be ruled out as well.

Physiological Variables

Sperm motility is indicative of the maturation process the sperm undergoes in the epididymis. Asthenozoospermia (sperm motility less than the cut-off levels) can also be artifactual when spermicides, lubricants, or rubber condoms are used. This parameter, when combined with total sperm concentration, provided the most reliable predictor of IUI success in male factor infertility cases.^{14–16} Sperm viability testing is recommended when motility falls to <10%. Necrospermia should be differentiated from flagellar defects (such as seen in Kartagener's syndrome) with eosin–nigrosin or hypo-osmotic sperm swelling test (HOST).^{3,6} Both tests are based on the integrity of the cell membrane in live and viable sperm. The normal sperm will be unstained in eosin–nigrosin tests and will demonstrate swelling and curling of the tail in HOST. An additional application for HOST is as a tool in selecting ideal spermatozoa for ICSI in testicular or cryopreserved specimens that demonstrate little or no motility.¹⁷

Other Tests

Sperm–mucus interaction or a post-coital test (PCT) assesses the cervical environment. The 2004 practical guidelines of the American Society for Reproductive Medicine (ASRM) recommend PCT in the setting of hyperviscous semen, unexplained infertility, or low-volume semen with normal sperm count. Progressively motile sperm >10–20/hpf is designated as normal. Couples who show defective sperm mucus interaction are advised to proceed with IUI, as additional tests will have no impact on management.¹⁸ The sperm penetration assay (SPA) uses the zona-free hamster oocyte to measure fertilization capability. The stripping away of the zona allows cross-species fertilization. Normally, 10–30% of ova are penetrated.⁴ Sperm capacitation index (SCI) is a variant of the SPA, assessing the mean number of penetrations per ovum. It has been suggested that ICSI can be offered to couples with an SCI <5 instead of a standard *in vitro* fertilization (IVF) procedure.¹⁹

An immune response to the sperm may result in the production of antisperm antibodies (ASA). This occurs when the blood–testis barrier is

violated in conditions such as testicular trauma, torsion, and vasectomy. Sperm–cervical mucus interaction as well as zona pellucida binding is affected, thus affecting fertility potential.^{20,21} Approximately 10% of infertile men will present with ASA (versus 2% of fertile men).²⁰ Sperm agglutination on initial microscopic examination or an abnormal PCT is suggestive of ASA. Immunoglobulin G (IgG)–mixed antiglobulin reaction (MAR) or immunobead tests (IBTs) can confirm the presence of ASA. A weakly positive IgG–MAR/IBT with <50% motile sperm with ASA rules out immunological factors and no further test is needed.²¹ Computer-aided sperm analysis (CASA) is a semi-automated technique that provides data on sperm density, motility, straightline and curvilinear velocity, linearity, average path velocity, amplitude of lateral head displacement, flagellar beat frequency, and hyperactivation. Although theoretically an ideal method, this expensive test does not have a diagnostic advantage over adequately performed manual techniques.

Biochemical tests are available for citric acid, zinc, alpha glutamyl transferase, and acid phosphatase, which are antioxidants produced by the prostate gland that can neutralize ROS in the seminal plasma produced by immature spermatozoa and leukocytes.^{22–24} Dietary supplementation of these substances has not been proved to be of use when they are low in seminal plasma.²⁴ Fructose (a product of the seminal vesicles) is requested when absent or hypofunctioning seminal vesicles are suspected. However, transrectal ultrasound has gained great popularity in determining this and has supplanted fructose in seminal vesicle testing. L-carnitine has a role in sperm maturation and is secreted by the epididymis, and is concentrated in the seminal plasma to x10 serum levels. Extremely low concentrations of L-carnitine are found in azoospermic men with post-epididymal obstruction, while normal levels are found in azoospermic men with intratesticular obstruction.^{25,26} Alpha glucosidase is also a marker for epididymal function. A cut-off value of 12mIU/ml distinguishes ductal obstruction from primary testicular failure. It is said that this test can predict the IUI response as it is reflective of better zona pellucida binding capacity of the spermatozoa.²⁶ Acrosin is a serine protease enzyme that exhibits a lectin-like carbohydrate-binding activity to the zona pellucida glycoproteins. Assays for acrosin include the gelatinolysis technique and a spectrophotometric assay. Low acrosin activity has been associated with low sperm density and motility, poor normal morphology,²⁷ HOST,^{27,28} and increased ROS²⁸ in subfertile men. ROS are by-products of normal sperm processes and are necessary for hyperactivation and capacitation of spermatozoa. High levels of these radicals are present with increased abnormal sperm and leukocytes.²⁹ Increased ROS levels have been shown to have a negative correlation with sperm concentration, motility, and morphology.^{30–32} There is an inverse relationship between ROS and *in vivo* fertilization rates,³³ and a meta-analysis on ROS levels and IVF revealed an inverse relationship between the two.³⁴ Chemiluminescence, which measures total seminal ROS (from WBCs, abnormal spermatozoa, and seminal fluid), is the most common method of assay. Leukocytospermia is associated with increased ROS levels and can serve as an indirect measurement of ROS.³⁵ Oxidative stress (OS) indicates that there is greater ROS than the total antioxidant capacity (TAC). The measurement of oxidative stress (ROS-TAC score) has been proposed to be a more accurate determination of the total effectual ROS, and a higher score (>30) can help in the prediction of pregnancy outcomes.³⁶ Testing for this remains limited in most fertility centers due to the high cost of equipment and the need for a fresh semen specimen.

DNA damage is positively correlated with poor semen parameters (low sperm concentration and low sperm motility), leukocytospermia, and high ROS levels.^{37–40} Approximately 8% of subfertile men with normal semen parameters will have high abnormal DNA.³⁷ A DNA fragmentation index (DFI) of >30% has been associated with a significant decrease of *in vivo* fertilization rates.^{41,42} A couple of meta-analyses show that couples are twice as likely to become pregnant with regular IVF methods if the DFI is <30%.^{43,44} Direct DNA damage assays include single-cell electrophoresis (COMET) and terminal deoxynucleotidyl transferase-mediated 2-deoxyuridine 5-triphosphate (TUNEL). Indirect methods include the sperm chromatin structure assay (SCSA), which measures sperm chromatin integrity, DNA intercalating dyes (acridine orange), which differentiates single- and double-stranded DNA, and sperm DNA denaturation tests. However, the use of DNA damage testing is controversial.^{45,46} A recent meta-analysis found that the small but statistically significant association between sperm DNA integrity test results and pregnancy in IVF and ICSI cycles does not provide a clinical indication for routine use of these tests in male evaluation.⁴⁷ The current viewpoint on DNA integrity testing, summarized by the ASRM Best Practice Guidelines, is that there are insufficient data to make DNA testing routine in infertility testing and that treatments have yet to prove their clinical value. Electron microscopy has been employed by some to determine ultrastructural sperm details in cases of low sperm motility with high viability (eosin–nigrosin/HOST).⁴⁸ Microtubular and mitochondrial defects are some defects that may test positive for viability.⁴⁹

Flow cytometry (FC) is an analytical cytological technique that has been extensively used for decades. It allows cell-by-cell analysis of many optical or immunological features in the same sample at the same time.⁵⁰ Using FC, a variety of sperm features such as sperm count, viability, acrosomal integrity, mitochondrial function, and DNA damage can be measured.⁵⁰ It offers an unbiased, sensitive, and accurate analysis on a statistically relevant number of cells. The main drawback of the use of FC in routine andrology is that it requires expensive instrumentation and technical expertise.

Emerging Technologies

Microarray

This method analyzes the transcriptome of cells and tissues and allows comparison transcriptomes at different stages of spermatogenesis. Its primary application is in uncovering the still unknown genes, pathways, and mechanisms in sperm production. Creation of messenger RNA (mRNA) profiles can possibly distinguish spermatogenic infertility from other causes.⁵¹

Metabolomics

Metabolites are formed or released by cell processes that can provide both qualitative and quantitative data for a glimpse of the network processes *in vivo*.⁵² In male infertility, the production of OS by-products and naturally occurring antioxidants can serve as biomarkers to potentially differentiate fertile from subfertile men with idiopathic infertility.⁵³ The non-invasiveness of this test is its main advantage, should it be developed for use in the clinical setting.

Atomic Force Microscopy

The main advantage of atomic force microscopy (AFM) over electron microscopy is the 3D images it can provide and the simplicity of sample

preparation (air drying). Studies in sperm plasma membrane during maturation and capacitation have identified new areas with phosphorylated proteins and large aggregates of lipid that did not cross post-acrosome and equatorial segments.⁵⁴

Controversies Surrounding Semen Analysis

Clinical research has shown that a normal semen analysis may not reflect defects in sperm function (idiopathic infertility), and the partners of men with poor sperm parameters may still achieve spontaneous pregnancy.^{6,14} Fifty percent of infertile men have recognizable causes, while the other half will have intrinsic spermatogenic defects that may often be missed by the basic semen analysis.⁶ Studies on sperm donors with known fertility status reveal a significant overlap in the sperm characteristics between fertile and subfertile men.^{14,55,56} Studies noted that there is low sensitivity (0.48) in detecting subfertile subjects using the WHO reference values for sperm concentration and low sensitivity using WHO criteria for normal morphology.^{14,57} Among the variables, motility had the lowest overlap range and gave the best prediction of the subject's fertility potential.¹⁴ This is in contrast to an earlier study where morphology was reported to provide the highest discriminating power in detecting subfertility among all of the semen variables.⁵⁷ Clearly, each variable alone is neither a powerful sole discriminator nor a predictor of fertility status, and must therefore be considered in the context of the other variables. Ideally, the specimen should be collected within the premises of the laboratory in which it will be examined. Often, a small private room is provided to patients. However, most men are uncomfortable producing a specimen in this environment and suboptimal collection with incomplete collection often occurs. Home-test kits were developed and marketed to overcome this. The first test that came out in the market was based on sperm staining, and the color intensity will test 'positive' when the sperm count is ≥ 20 million/ml. The kits have two separate tests per pack and claim 97% accuracy. Several concerns were raised with the introduction of these tests. There is still a consensus that laboratory-performed semen analysis cannot be replaced by these home tests. Furthermore, there were concerns regarding its effects on the couple undergoing or desiring infertility work-ups. As these home kits test only one semen variable, a 'normal test' may falsely assure couples that male factor infertility is not involved. Proponents of these home tests feel that they may increase awareness in couples that the male

factor needs to be considered in infertility evaluations. Its detractors are sceptical that it will achieve that purpose. Proper treatment and the proper approach are often as multifaceted as the causes of infertility, and these home tests may result in a misapprehension and unnecessary delay. This may lull males who test positive into thinking that they are 'normal,' and therefore they may not seek urological assessment. Only time will tell whether these tests can truly promote male factor infertility awareness.

Conclusion

A proper diagnosis of male infertility can be made with comprehensive and properly performed semen analyses in conjunction with a thorough history and physical examination. While a carefully performed semen analysis remains the initial choice in the evaluation of male infertility, exciting new developments in semen testing promise further advances in targeted diagnosis and management. ■



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