Semen analysis and sperm function tests: How much to test?

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ABSTRACT

Semen analysis as an integral part of infertility investigations is taken as a surrogate measure for male fecundity in clinical andrology, male fertility, and pregnancy risk assessments. Clearly, laboratory seminology is still very much in its infancy. In as much as the creation of a conventional semen profile will always represent the foundations of male fertility evaluation, the 5th edition of the World Health Organization (WHO) manual is a definitive statement on how such assessments should be carried out and how the quality should be controlled. A major advance in this new edition of the WHO manual, resolving the most salient critique of previous editions, is the development of the first well-defined reference ranges for semen analysis based on the analysis of over 1900 recent fathers. The methodology used in the assessment of the usual variables in semen analysis is described, as are many of the less common, but very valuable, sperm function tests. Sperm function testing is used to determine if the sperm have the biologic capacity to perform the tasks necessary to reach and fertilize ova and ultimately result in live births. A variety of tests are available to evaluate different aspects of these functions. To accurately use these functional assays, the clinician must understand what the tests measure, what the indications are for the assays, and how to interpret the results to direct further testing or patient management.

Key words: Acrosome reaction, male infertility, miscarriage, reactive oxygen species, semen, semen analysis, sperm function tests, sperm DNA integrity, sperm penetration assay

INTRODUCTION

Semen analysis is an imperfect tool but remains the cornerstone of the investigation of male infertility.[1] It must be performed to a consistently high standard in order to evaluate descriptive parameters of the ejaculate.[2,3] To this day, controversy persists as to what constitutes the "*normal*" spermatozoa in semen, as normal and "*pathologic*" forms coexist in semen.[4]

Routine semen analysis provides useful information concerning sperm production, sperm motility and viability, patency of the male genital tract, secretions of the accessory organs, as well as ejaculation and emission. Although this assay reveals useful information for the initial evaluation of the infertile male, it is not a test of fertility.[5] It provides no insights into the functional potential of the spermatozoon to fertilize an ovum or to undergo the subsequent maturation processes required to achieve fertilization. It is important to understand that while the results may correlate with "*fertility*," the assay is not a direct measure of fertility.[6-8]

Seminal analysis needs to be complemented with sperm functional assay, which indirectly measures the ability of one spermatozoon to deliver the correct complement of chromosomes to an ovum. To do this, spermatozoa must be produced in sufficient numbers, exhibit normal motility and shape, pass through the cervical mucus, uterus, and ampullae of the oviducts, after undergoing capacitation, acrosome reaction (AR), zona pellucida binding, and nuclear decondensation. Defects in any of these complex events can result in male infertility and it is essential to understand these tests and their rationale.[9,10]

SEMEN–MACROSCOPIC EXAMINATION

As semen samples can show substantial variation, a minimum of 2 properly collected and transported samples, ideally

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collected over 2 spermatogenic cycles, should be examined at 37°C. This may unnecessarily prolong the investigation for patients and is recommended only if there is a recent insult to spermatogenesis.

Color

Pathologically, seminal discoloration may be due to fresh blood, drugs (pyridium), jaundice, or contamination of semen with urine (eg, bladder neck dysfunction). Physiologic yellowish tinge in samples with prolonged abstinence is due to carotene pigment, and sperm oxidation causes odor.

SEMEN VOLUME, PH, AND VISCOSITY

Volume

The normal volume of ejaculate after 2-7 days of sexual abstinence is about 2-6 mL.

Aspermia: No sperm seen in ejaculate after orgasm.

Hypospermia: <0.5 mL of semen.

Improper collection, hypogonadism, retrograde ejaculation, obstruction of lower urinary tract may yield low volume.

Hyperspermia: >6 mL of semen ejaculated (prolonged abstinence or excessive secretion from the accessory sex glands).

pH

Normal semen pH is in the range of 7.2-8.2 and it tends to increase with time after ejaculation. Changes are usually due to inflammation of the prostate or seminal vesicles.

Semen viscosity

Viscosity measures the seminal fluid's resistance to flow. High viscosity may interfere with determination of sperm motility, concentration, and antibody coating of spermatozoa. Normally, semen coagulates upon ejaculation and usually liquefies within 15-20 min. Semen that remains a coagulum is termed nonliquefied, whereas that which pours in thick strands instead of drops is termed hyperviscous. The clinical significance of abnormalities in liquefaction remains controversial.[11] Exact liquefaction time is of no diagnostic importance unless >2 h elapse without any change. Failure to liquefy is usually a sign that there is inadequate secretion by the prostate of the proteolytic enzymes fibrinolysin, fibrinogenase, and aminopeptidase.[12] On the other hand, absence of coagulation may indicate ejaculatory duct obstruction or congenital absence of seminal vesicles. Importantly, liquefaction should be differentiated from viscosity, as abnormalities in viscosity can be the result of abnormal prostate function and/or the use of an unsuitable type of plastic container.

MICROSCOPIC EXAMINATION

Sperm concentration

A phase contrast microscope using volumetric dilution and hemocytometry is recommended for all examinations of unstained preparations of fresh/washed semen and is reported as millions of sperm per mL. Samples in which no sperm are identified should be centrifuged and the pellet examined for the presence of sperm. Pregnancy rates by intercourse and intrauterine insemination decline as sperm density decreases.[13,14]

Azoospermia refers to the absence of sperm in the seminal plasma.

Oligozoospermia (also often called oligospermia) refers to seminal plasma concentration less than 20 million per milliliter.[7]

Motility

The efficient passage of spermatozoa through cervical mucus is dependent on rapid progressive motility,[15,16] that is, spermatozoa with a forward progression of at least 25 μm/s. Reduced sperm motility can be a symptom of disorders related to male accessory sex gland secretion and the sequential emptying of these glands.

Rapid and slow progressive motility is calculated by the speed at which sperm moves with flagellar movement in a given volume as a percentage (range 0%-100%) by counting 200 sperms.
(a) Rapid progressive motility (ie, >25 μm/s at 37°C and >20 μm/s at 20°C; Note: 25 μm is approximately equal to 5 head lengths or half a tail length).
(b) Slow or sluggish progressive motility
(c) Nonprogressive motility (<5 μm/s)
(d) Immotility

A normal semen analysis must contain at least 50% grade A and B, progressively motile spermatozoa. If greater than 50% sperms are immotile then the sperms should be checked for viability. Persistent poor motility is a good predictor of failure in fertilization, an outcome that is actually more important when making decisions regarding a couple's treatment options.[17]

Morphology

The clinical implications of poor morphology scores remain highly controversial. The initial studies using rigid criteria reported that patients undergoing *in vitro* fertilization (IVF) who had greater than 14% normal forms had better fertilization rates.[18] Later studies reported that most impairment in fertilization rates occurred with morphology scores of less than 4%.[19]

The staining of a seminal smear (Papanicolaou Giemsa,

Shorr, and Diff-Quik) allows the quantitative evaluation of normal and abnormal sperm morphological forms in an ejaculate. Smears can be scored for morphology using the World Health Organization (WHO) classification, or by Kruger's strict criteria classification.[19] WHO method classifies abnormally shaped sperm into specific categories based on specific head, tail, and midpiece abnormalities, which is based on the appearance of sperm recovered from postcoital cervical mucus or from the surface of zona pellucida (>30% normal forms). In contrast, Kruger's strict criteria classifies sperm as normal only if the sperm shape falls within strictly defined parameters of shape and all borderline forms are considered abnormal (>14% normal forms).

(a) *Head defects*: Large, small, tapered, pyriform, round, amorphous, vacuolated (>20% of the head area occupied by unstained vacuolar areas) heads with small acrosomal area (<40% of head area), double heads, any combination of these.

(b) *Neck and midpiece defects*: Bent neck; asymmetrical insertion of midpiece into head; thick, irregular midpiece; abnormally thin midpiece; any combination of these.

(c) *Tail defects*: Short, multiple, hairpin, broken, bent, kinked, coiled tails, or any combination of these.

(d) *Cytoplasmic droplets*: Greater than one-third of the area of a normal sperm head.

Morphology should be used along with other parameters, and not as an isolated parameter, when determining clinical implications. It is important to realize that, in general, pregnancy is possible with low morphology scores and that both motility and morphology have also demonstrated prognostic value, as do combinations of parameters.[20,21]

LEUKOCYTOSPERMIA—FIGURE

Infection of the male reproductive tract can directly or indirectly cause infertility.[22] Pyospermia is a laboratory finding categorized as the abnormal presence of leukocytes in human ejaculate and may indicate genital tract inflammation.[23]

To differentiate round cells from polymorphonuclear (PMN) leukocytes, which are primary sources of reactive oxygen species (ROS) generation, peroxidase staining is used. Neutrophils, polynuclear leukocytes, macrophages are peroxidase-positive granulocytes (PMN should be 1×10^6/mL), whereas degranulated PMNs, lymphocytes, and "*immature*" germ cells are peroxidase negative.[24-26]

SPERM AGGLUTINATION—ANTISPERM ANTIBODY

Immunologic protection to sperm antigens are provided by the tight junctions of sertoli cells forming the blood-testis barrier. The spermatozoon evokes an immune response when exposed to the systemic immune defense system in conditions in which this barrier gets disrupted, leading to the formation of antisperm antibodies (ASA). Certain ASAs have a cytotoxic effect on the spermatozoa and can cause cell death and immobilization of sperm cells. Other effects of ASAs include creating agglutinated clumps of moving sperm in the semen sample, hampering passage of sperm through the cervical mucus, and zonal binding and passage.[27]

Two current methods of detecting antibodies bound to the surface of motile sperm are the mixed agglutination reaction assay (MAR test; only for IgGs) and the immunobead-binding assay (for IgA, IgG, and IgMs).[28] A positive finding of >50% of motile sperm with attached beads is considered to be clinically significant, but with the advent of assisted reproduction technology (ART), ASA testing has lost its relevance.

BIOCHEMICAL ASSESSMENT OF SEMINAL PLASMA, PROSTATE, EPIDIDYMIS, AND SEMINAL VESICLES

Biochemical assessment is carried out to assess the impairment of epididymal, vesicular, and prostatic function and it may be clinically relevant in patients with hyperviscous semen and to understand genital fluid interactions during the semen coagulation-liquefaction process. Decreased levels of zinc, citric acid, and glucosidase may indicate either seminal vesicle, prostate dysfunction, or prostatic duct obstruction.[29-31]

SPERM FUNCTIONAL TESTS

Clinicians are still searching for semen parameter thresholds in the so-called *normal fertile populations* to be able to define fertility, subfertility, and infertility more accurately.[32,33] Notwithstanding such lack of uniform criteria, if sperm abnormalities are observed in the "*basic*" semen analysis or if the couple is diagnosed as "*unexplained*" infertility, the workup should proceed to the analysis of sperm functional tests (second-tier level). The diagnosis of subfertility or infertility, based on the first-tier (initial "*basic*" evaluation) and the "*expanded*" screening or second-tier level (functional), will direct management toward a variety of therapeutic options.[34-37] To accurately use the functional assays, the clinician must understand what the test measures, what the indications are for the assay, and how to interpret the results to direct further testing or patient management. It is at this time that sperm function/biochemical tests may be of highest value to direct the couple to ART. Assisted reproduction can be indicated as a result of (1) failure of urologic/medical treatment, (2) the diagnosis of "*unexplained*" infertility in the couple, (3) the presence of "*basic*" sperm abnormalities of moderate-high degree, or (4) abnormalities of sperm function as diagnosed by predictive bioassays of the "*expanded*" screening.[38]

SPERM CERVICAL MUCUS INTERACTION

The postcoital test (PCT) evaluates the sperm-cervical mucus interaction and the presence of more than 10-20 sperm per 400 high-power fields, the majority of which demonstrate progressive motility, is usually considered normal. The finding of immobilized sperm with a side-to-side shaking motion suggests the presence of antisperm antibodies either on the sperm or in the cervical mucus.[39] An abnormal PCT result suggests, but does not prove, cervical factor infertility. As timing of PCT is problematic, *in vitro* penetration tests, probably using mucus substitutes, such as methyl cellulose or hyaluronic acid, provide an alternative that has shown promise but require more extensive validation.

1. Computer-assisted semen analysis
2. Sperm viability testing
3. Tests of sperm capacitation
4. Tests of hemizona and zona pellucida binding
5. Sperm penetration assay or sperm capacitation index or zona-free hamster oocyte penetration assay
6. Tests of sperm DNA damage
7. Assessment of ROS
8. Sperm proteomics

COMPUTER ASSISTED SEMEN ANALYSIS

Manual semen analysis lacks the ability to measure the kinematics of sperm motion. CASA is potentially useful because of its capacity to analyze sperm motion (sperm head and flagellar kinetics), some of which have been shown to be related to IVF outcome.[40]

Some of the important kinematic parameters are as follows:

i. *Curvilinear velocity:* Curvilinear velocity (VCL) is the measure of the rate of travel of the centroid of the sperm head over a given time period.
ii. *Average path velocity:* Average path velocity(VAP) is the velocity along the average path of the spermatozoon.
iii. *Straight-line velocity:* Straight-line velocity (VSL) is the linear or progressive velocity of the cell.
iv. *Linearity:* Linearity of forward progression (LIN) is the ratio of VSL to VCL and is expressed as percentage.
v. *Amplitude of lateral head displacement:* Amplitude of lateral head displacement (ALH) of the sperm head is calculated from the amplitude of its lateral deviation about the cell's axis of progression or average path.

Motility

Although CASA is very accurate for determining the details of sperm kinetics, manual assessment of semen is much more accurate in discerning among debris, crystals, and immotile, dead sperm heads. Therefore, manually assessed sperm concentrations and number of immotile spermatozoa are much more reliable than corresponding data obtained by CASA, provided individual is adequately trained with appropriate internal and external quality control measures.[41,42]

Viability assays

Sperm viability testing is used to determine if nonmotile sperm are alive or dead and are indicated when sperm motility is less than 5%-10%. They are useful in primary ciliary dyskinesia where ultrastructural defects in sperm flagella result in absent or extremely low motility but with high viability. Also used to select sperm for intracytoplasmic sperm injection (ICSI), in surgically retrieved testicular tissue, sperms are alive but generally nonmotile, because of lack of epididymal transit.[43,44] Viability testing is done by dye exclusion assays or hypoosmotic sperm swelling (HOS test).

Dye exclusion assays rely on the ability of live sperm to resist absorption of certain dyes, whereas these dyes penetrate and stain nonviable sperm. Trypan blue and Eosin Y stains, which do not stain live sperm, are commonly employed. However, as the technique requires air drying after staining, sperms are killed and not practically useful.[45]

In HOS test when live cells are placed in hypoosmotic media, water enters the cytoplasm causing the cell to swell, particularly the tail, which is calculated as a percentage. This assay does not damage or kill the sperm and is very useful for identifying viable, nonmotile sperm for ICSI. HOS has a limited ability to predict male fertility, but an HOS result <50% is associated with increased miscarriage rates.

RESULTS

Normal values (fertile): >60% spermatozoa with swollen tails

Abnormal values (infertile): <50% spermatozoa with swollen tails.

TESTS OF SPERM CAPACITATION

Capacitation is a series of biochemical and structural changes that spermatozoa go through to undergo the AR and be able to fertilize. The process takes place in the female genital tract but can be induced *in vitro* by incubating spermatozoa with capacitation-inducing media. It is thought to have a role in preventing the release of lytic enzymes until spermatozoa reach the oocyte.[46] One of the signs of capacitation is the display of hyperactivation by spermatozoa. At the present time, the clinical value of sperm capacitation testing remains to be determined.

TESTS OF HEMIZONA AND ZONA PELLUCIDA BINDING

The interaction between spermatozoa and the zona pellucida

is a critical event leading to fertilization and reflects multiple sperm functions (ie, completion of capacitation as manifested by the ability to bind to the zona pellucida and to undergo ligand-induced AR).[47-49]

The 2 most common sperm-zona pellucida binding tests currently utilized are the hemizona assay (or HZA)[50] and a competitive intact-zona binding assay.[51] The HZA, which uses nonfertilized oocytes is useful in couples who have failed to fertilize during regular IVF, to determine the cause of the failure. Because the binding is species specific,[52,53] human zona must be used, thus limiting the utility of these assays.

The induced-AR assays appear to be equally predictive of fertilization outcome and are simpler in their methodologies. The use of a calcium ionophore to induce AR is at the present time the most widely used methodology.[54,55]

SPERM PENETRATION ASSAY OR SPERM CAPACITATION INDEX OR ZONA-FREE HAMSTER OOCYTE PENETRATION ASSAY

The concept of the sperm penetration assay was introduced by Yanagamachi.[56] It yields information regarding the fertilizing capacity of human spermatozoa by testing capacitation, AR, sperm/oolemma fusion, sperm incorporation into the ooplasm, and the decondensation of the sperm chromatin during the process. However, penetration of the zona pellucida and normal embryonic development are not tested. The spermatozoa penetration assay (SPA) utilizes the golden hamster egg, which is unusual in that removal of its zona pellucida results in loss of all species specificity to egg penetration. Thus, a positive SPA does not guarantee fertilization of intact human eggs nor their embryonic development, whereas a negative SPA has not been found to correlate with poor fertilization in human IVF.[57]

The acrosin assay an indirect measure of sperm penetrating capability measures acrosin, which may be responsible for penetration of the zona pellucida and also triggering the AR.[58] Measurement of acrosin is thought to correlate with sperm binding to and penetration of the zona pellucida.[59,60]

TESTS OF SPERM DNA DAMAGE

Mammalian fertilization involves the direct interaction of the sperm and the oocyte, fusion of the cell membranes, and union of male and female gamete genomes. Although a small percentage of spermatozoa from fertile men also possess detectable levels of DNA damage, which is repaired by oocyte cytoplasm, there is evidence to show that the spermatozoa of infertile men possess substantially more DNA damage and that this damage may adversely affect

reproductive outcomes.[61,62] There appears to be a threshold of sperm DNA damage, which can be repaired by oocyte cytoplasm (ie, abnormal chromatin packaging, protamine deficiency) beyond which embryo development and pregnancy are impaired.[63,64]

DNA damage—Direct tests
Comet assay
a. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling (TUNEL) assay
b. DNA oxidation measurement

DNA damage—Indirect tests
a. Sperm chromatin structure assay (SCSA)
b. Sperm chromatin dispersion assay.
c. Sperm fluorescence *in situ* hybridization analysis (FISH)

Overall, the data suggest that there is no significant relationship between sperm DNA damage and fertilization rate or pregnancy outcomes at IVF or IVF/ICSI.[65-69] However, there is evidence to suggest that sperm DNA damage is associated with poor pregnancy outcome after standard IVF.[70,71]

Sperm FISH analysis may be useful in the following: (a) infertile men with sex chromosome numerical anomalies, prior to ICSI; (b) infertile men with structural chromosome anomalies, prior to ICSI; (c) infertile men with severe oligozoospermia, prior to ICSI; and (d) couples with a history of recurrent miscarriages and trisomic pregnancies.

ASSESSMENT OF REACTIVE OXYGEN SPECIES

ROS also referred to as free radicals, are formed as a byproduct of oxygen metabolism. Contaminating leukocytes are the predominant source of ROS in these suspensions.[72,73] They can be eradicated by enzymes (eg, catalase or glutathione peroxidase) or by nonenzymatic antioxidants, such as albumin, glutathione, and hypotaurine, as well as by vitamins C and E. Small amounts of ROS may be necessary for the initiation of critical sperm functions, including capacitation and the AR. On the other hand, a high ROS level produces a state known as oxidative stress that can lead to biochemical or physiologic abnormalities with subsequent cellular dysfunction or cell death. Significant levels of ROS can be detected in the semen of 25% of infertile men, whereas fertile men do not have a detectable level of semen ROS.[73-75]

Sperm ROS can also be measured by using cellular probes coupled with flow cytometry by detection of chemiluminescence.[76] Briefly, this is done by incubating fresh semen or sperm suspensions with a redox-sensitive, light-emitting probe (eg, luminol) and by measuring the light emission over time with a light meter (luminometer).

The clinical value of semen ROS determination in predicting IVF outcome remains unproved but identifying oxidative stress as an underlying cause of sperm dysfunction has the advantage that it suggests possible therapies. Administration of antioxidants has been attempted in several trials with mixed results. But at this point there are no established semen ROS cutoff values that can be used to predict reproductive outcomes.[77,78]

SPERM PROTEOMICS

Sperm proteomics, an experimental technique, used extensively in several branches of medicine, may identify some of the molecular targets implicated in sperm dysfunction.[79] Sperm proteomics allows comparison of protein structure of normal and defective spermatozoa.[80]

CONCLUSION

Even to this day conventional semen analysis to diagnose male infertility is "*a numbers game*," resulting in a preoccupation with threshold counts for sperm number, motility, and morphology with emphasis on classification of patients into descriptive groups, such as oligozoospermic, asthenozoospermic, and others. However, we should never confuse such descriptive categories with our ultimate goal, which is a diagnosis. The issues in male infertility will not get resolved till the research finds solutions at molecular level. The meaningful analyses of structure function relationship will only be possible when we as clinicians have all the relevant information to formulate a correct strategy for treatment of a infertility.

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