

Interpretation of Basic Semen Analysis and Advanced Semen Testing

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Introduction

A consensus exists that the basic semen analysis is the most important tool in male fertility investigation. In the last two decades, researchers and clinicians alike have relied on the World Health Organization (WHO) criteria for the interpretation of basic semen analysis. Nevertheless, the criteria for what constitutes a normal semen analysis remain controversial. Although a single test such as the routine semen analysis can deliver several sperm attributes, male fertility cannot be determined based solely on its results. In this

chapter, we present an overview of the interpretation of results derived from the most standardized tests used for the evaluation of the fertility potential of a seminal ejaculate.

Basic Semen Analysis

Routine semen analysis continues to be the main pillar in male fertility investigation. In order to establish consistency in laboratory procedures, the WHO first published a manual for the examination of human semen and semen-cervical mucus interaction in 1980. The manual also identified standards to exclude influences such as the health of patient over the previous spermatogenic cycle, length of sexual abstinence, time, and temperature. The manual has been regularly updated (1980, 1987, 1992, 1999) (Lewis 2007). The addition of normal reference values in the WHO manuals has been of significant help in establishing some consistency of what constitutes a normal value

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(Table 1). Nevertheless, it is critical to note that the WHO manuals report reference values identified in fertile population rather than the minimum requirements for male fertility. Most recently, the data have been modified based on the assessment of 4,500 men in 14 different countries. In men, whose partners were able to conceive within 12 months ($n=428-1,941$), the lower reference limits were: semen volume=1.5/mL; total sperm number=39 million per ejaculate; sperm concentration=15 million/mL; vitality=58% live; progressive motility=32%; total (progressive+non-progressive) motility=40%; morphologically normal forms=4.0% (Cooper et al. 2010).

The heterogeneity of human semen further diminishes the clinical significance of the WHO reference values. Data indicate that there are subtle variations in semen parameters between men in different geographic areas and even between samples from the same individual (Alvarez et al. 2003; Jorgensen et al. 2001). The limited power of semen analysis in predicting fertility has been reported from the 1980s to the present (Glazener et al. 1987; Swan 2006). Analysis of the seminal fluid must include the evaluation of both macroscopic and microscopic parameters. Analysis should be performed on multiple ejaculates before characterizing a man as normal or infertile due to the large within-subject variation in sperm parameters (Keel 2006).

Macroscopic Parameters

The macroscopic properties of a semen sample include volume, appearance, color, coagulation/liquefaction,

TABLE 1. Normal values for semen parameters according to the WHO (1999).

Parameter	Reference value
Volume	≥2.0 mL
pH	7.2–8.0
Concentration	≥20 × 10 ⁶ /mL
Total spermatozoa per ejaculate	≥40 × 10 ⁶
Motility	≥50% motile (grade a + b), or ≥25% grade a
Morphology	Data suggest that <15% is associated with decreased fertilization following assisted reproductive techniques
Viability	≥75% live spermatozoa
Leukocytes	<1.0 × 10 ⁶ /mL
Antisperm antibodies	<50% bound sperm using immunobead or MAR tests
Zinc	≥2.4 μM per ejaculate
Citric acid	≥52 μM per ejaculate
Fructose	≥13 μM per ejaculate

and viscosity. The volume (normal >2 mL) of the ejaculate is an accurate indicator of various abnormalities. Absence of any semen volume after orgasm, termed aspermia, occurs in patients with diabetic neuropathy, following the intake of sympatholytic drugs and following surgical procedures that damage the sympathetic nervous plexus or resection of the prostate. In some of these cases, there may be retrograde flow of the semen into the bladder, and the examination of the postejaculatory urine should be conducted. Hypospermia (semen volume <0.5 mL), could be due to the loss of a portion of the ejaculate during collection, short abstinence period or incomplete orgasm. Hypospermia with pH less than 7.4 may indicate partial/complete ejaculatory duct obstruction or absent seminal vesicles. If hypospermia is associated with pH more than 7.8, it could indicate accessory gland impairment as in the case of hypogonadism, inflammation, or narcotics intake.

Regarding the appearance, it was thought that a translucent sample denotes the absence of sperm cells; however, other nonsperm cellular components may render the sample opaque. Therefore, the appearance of an ejaculate, whether translucent or opaque, does not seem to have any clinical value. Semen color is also considered insignificant in assessing sperm fertilization potential. However, it may be a good sign of associated clinical conditions, such as excessive erythrocytes, i.e., hematospermia (red color) or jaundice (yellow color).

A normal semen sample coagulates immediately after ejaculation and then liquefies within 15–30 min. Failure of coagulation denotes the lack of secretions from the seminal vesicles, which may be due to either obstruction or the absence of seminal vesicles. Prolonged liquefaction indicates poor prostatic secretion as in the case of inflammation. Viscosity is another parameter that is considered abnormal if the length of a thread exceeds 60 mm. If these cases are associated with low sperm motility, the sperm transportation will be compromised.

Microscopic Parameters

Microscopic attributes of the seminal fluid include sperm concentration, motility, viability, morphology as well as nonsperm cellular components in the form of leukocyte concentration and immature germ cells. Among the parameters reported in a routine semen analysis, it is not yet known which one would be the most associated with fertility. While multiple reports

point to sperm morphology as the parameter with the most discriminatory power, others indicate that sperm concentration and/or motility are the most valuable (Lewis 2007).

Sperm Concentration

The cutoff point of 20 million spermatozoa/mL has been repeatedly suggested as the lower normal value for sperm concentration in an ejaculate (World Health Organization 1999). In a study that evaluated two semen specimens from each of the male partners in 765 infertile couples and 696 fertile couples, subfertile men had sperm concentrations of less than 13.5×10^6 /mL (Guzick et al. 2001). On the other hand, another study that evaluated 166 male factor infertility patients and 56 proven fertile donors has suggested the concentration of 31.2×10^6 /mL as a prognostic factor for fertility status (Nallella et al. 2006). Therefore, the literature describes significant overlap in threshold sperm concentration between fertile and infertile men.

The observation of a low sperm concentration, oligozoospermia, is indicated when sperm concentration falls below $5\text{--}10 \times 10^6$ /mL depending on the cutoff point used. It may be due to the loss of a portion of the ejaculate, partial obstruction of the genital tract, drugs or genetic abnormalities. Other factors include medications such as nitrofurantoin and excessive heat exposure. On the other hand, azoospermia, complete absence of spermatozoa, may be due to the obstruction of the sperm transport, hypogonadism, and iatrogenic causes, such as chemotherapy or idiopathic factors, that are most probably genetic in origin. If azoospermia is detected, the semen analysis must be repeated to confirm that no iatrogenic cause, such as loss of the sample, was the reason of the finding. Documented azoospermia is one of the conditions, where chemical analysis of the seminal plasma may be of importance. Fructose, normally present in seminal plasma, originates mainly from the seminal vesicles. Absence of fructose in azoospermic patient may be indicative of ductal obstruction (Jarow et al. 1989).

Sperm Motility

The presence of progressively motile sperm in the ejaculate is critical to ensure adequate sperm transport and fertilization. Sperm motility is considered as compromised if the percentage of forward progressive sperm falls below 50% within 60 min of sample

collection (World Health Organization 1999). The presence of low sperm motility, asthenozoospermia, could occur as a result of prolonged time to processing of collected samples. Sample containers may be toxic to the sperm, and sample exposure to extreme temperature or sunlight may result in decreased sperm motility. Long periods of abstinence also proved to be a cause of poor sperm motility. Other causes of asthenozoospermia include sperm axonemal deformities, excessive leukocytes, and unknown idiopathic factors. Asthenozoospermia is also most commonly seen with antisperm antibodies. The observation of sperm clumping combined with low sperm motility is a further indication of the presence of antisperm antibodies.

Asthenozoospermia warrants the investigation of sperm viability to identify the presence of necrozoospermia (nonviable spermatozoa). Immotile sperm may still be viable and could be used in assisted reproductive techniques (ART). Sperm viability could be assessed using supravital stains such as Eosin-Y. The percentage of sperm with intact membranes that exclude the stain should be equal to or exceed 75% (World Health Organization 1999).

Sperm Morphology

Different methods for staining and the evaluation of sperm morphology have been described (Ombelet et al. 1995). One method for assessing morphology is based on the sperm meeting strict criteria (Kruger et al. 1987). Both the WHO suggested criteria and the strict method constitute the two most commonly used criteria for the evaluation of normal sperm morphology. Data highlight a reasonable predictive power of sperm morphology in centers using the same or different criteria, however, the cutoff values for normality are different (Ombelet et al. 1997).

Debate is still ongoing regarding the evaluation criteria that should be used and the one that offers the most predictive power for in vivo and in vitro fertility. Previously, the WHO manuals recommended 30% normal forms as the cutoff point for normality (World Health Organization 1992). On the other hand, authors advocating the use of the strict criteria suggest >4% as a cutoff point for correlation with positive IVF outcomes. A systematic review has evaluated the data produced around the 5% normal sperm morphology threshold using the strict criteria. Results showed that the overall fertilization rates were 59.3% for the $\leq 4\%$ group, 77.6% for the $>4\%$ group, and the overall

pregnancy rates were 15.2% and 26.0%, respectively (Coetzee et al. 1998). It is important to note that other studies have found the strict criteria to be of less value in predicting IVF outcomes. A study conducted on 132 couples undergoing IVF found that the comparison of traditional morphology and strict criteria with regard to IVF outcome favored traditional morphology in several areas. In particular, low scores were more predictive of poor IVF outcome (Morgentaler et al. 1995).

Nonsperm Cellular Components

Immature germ cells in the ejaculate are usually associated with below normal sperm counts. Special attention should be given to the concentration of leukocytes in the seminal ejaculate. Leukocytes are normally present in the seminal fluid; however, a concentration of $>1 \times 10^6/\text{mL}$ is considered abnormal (World Health Organization 1999). A positive correlation was observed between leukocyte count and the total count of microorganisms in semen sample. An optimal sensitivity/specificity ratio appears at 0.2×10^6 WBC/mL semen (Punab et al. 2003). The excessive presence of leukocytes may be detrimental to spermatozoa due to their excessive production of reactive oxygen species (ROS) and cytotoxic cytokines. The presence of erythrocytes is indicative of reproductive tract pathology, while the presence of microorganisms is an indication of genital tract infection.

Sperm Motion Kinetics

Several systems referred to as computer-aided semen analyzers (CASA) have been developed using digital image analysis for the automated analysis of seminal ejaculate (Mortimer 2000). Although this technology was initially welcomed as a major contribution to the semen diagnostics, its application has been challenged by a wide margin of error in setup procedures and object detection (Davis and Katz 1993). Thus, to date, manual semen analysis performed by a well-trained technician remains the most reliable method for the assessment of sperm concentration, percentage motility and morphology (Consensus Workshop on Advanced Diagnostic Andrology Techniques 1996).

Relying on serial digital images, the CASA system can plot the movement of a sperm head creating a trajectory and reconstructing the movement track. This function appears to be the only area, where CASA can be of benefit since the resulting sperm motion

kinetics are impossible to assess using routine microscopy. Sperm kinetics include measuring the distance between each head point for a given sperm during the acquisition period (curvilinear velocity, VCL, $\mu\text{m/s}$), the distance between first and last head points divided by the acquisition time (straight line velocity, VSL, $\mu\text{m/s}$), and the measure of sperm head oscillation (amplitude of lateral head displacement, ALH, μm). Linearity (LIN, %) measures the departure from linear progression and is calculated as $\text{VSL}/\text{VCL} \times 100$, while the average path velocity (VAP, $\mu\text{m/s}$) is a smoothed path constructed by averaging several positions on the sperm track (Kay and Robertson 1998).

Several studies have shown that the quantitative assessment of sperm kinetics is of clinical value in identifying men with unexplained infertility and predicting in vivo and in vitro fertility (Consensus Workshop on Advanced Diagnostic Andrology Techniques 1996; Peedicayil et al. 1997; Shibahara et al. 2004). Despite the documented clinical value of assessment of sperm kinetics, there is an agreement that individual motion characteristics are of little value (Consensus Workshop on Advanced Diagnostic Andrology Techniques 1996). Moreover, it was agreed that studies that have only reported differences in sperm kinetics between populations do not provide any useful information (Guidelines on the Application of CASA Technology in the Analysis of Spermatozoa 1998). A relevant analysis of the sperm motion should focus on the identification of normal values for a movement pattern. As an example, spermatozoa capable of penetrating preovulatory cervical mucus had $\text{VAP} \pm 25.0 \mu\text{m}$ and $\text{ALH} \pm 4.5 \mu\text{m}$ (Mortimer et al. 1986; Aitken et al. 1985).

Another specific movement pattern is hyperactivation, which is acquired during the capacitation process and enables spermatozoa of mechanical thrust to penetrate the zona pellucida (Ho and Suarez 2001). Several values have been suggested to define hyperactivated sperm (VSL: 0.1 to $>91.5 \mu\text{m/s}$, LIN: <60 to 345%, ALH: 0.5 to $>9.9 \mu\text{m}$) (Kay and Robertson 1998). Clearly, the difference in CASA instruments used, their setup as well as the counting chambers between studies have made it impossible to reach an agreement. To date, there is no consensus regarding the proportion of hyperactivated sperm that should be present in the ejaculates of fertile men. Although a correlation was established between hyperactivation and successful fertilization in vitro (Sukcharoen et al. 1995), this parameter cannot be considered of clinical significance since there are no universal criteria to define hyperactivation.

Investigations for Antisperm Antibodies

The presence of antisperm antibodies (ASA) has been documented to impede human fertility via several mechanisms (Naz and Menge 1994). Nevertheless, the testing for ASA is controversial due to variations between different testing methodologies and the interpretation of the results in the context of male infertility. Most of the methods previously described for the detection of ASA are now obsolete due to the relatively high interassay variability and their limited clinical benefits. Only two methods are now accepted to test for the presence of ASA, the mixed antiglobulin reaction (MAR) test and the immunobead test (IBT) (World Health Organization 1999).

The currently recognized cutoff point for a positive ASA test stands at 50% of spermatozoa showing the binding in the MAR test or the IBT (World Health Organization 1999). In general, there is a limited role for ASA testing in the diagnosis of male infertility and any subsequent treatments that might be contemplated accordingly. Elevated ASA levels were seen in 18% of men presenting with unexplained infertility in one study (Fichorova and Boulanov 1996). Therefore, a valid indication for ASA testing appears to be cases with unexplained infertility, where a reason for delay in fertility could be attributed to the presence of ASA. Other indications could include severe asthenozoospermia.

Sperm Function Tests

The diagnosis of male infertility is mostly based on the descriptive evaluation of human semen, including the number of spermatozoa that are present in the ejaculate, their motility, and their morphology. However, it is not so much the absolute number of spermatozoa that determines fertility, but their functional competence (Aitken 2006). 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. These tasks include penetrating the cervical mucus, reaching the ova, undergoing capacitation and the acrosome reaction, zona pellucida penetration, and ooplasm incorporation. Defects in any of these steps may result in infertility (Sigman and Zini 2009).

In the era of IVF and ICSI, sperm function testing appears to have lost some of its significance. However,

many couples are looking for less invasive and inexpensive solutions, therefore establishing an exact diagnosis in these cases would be extremely important to identify success rates for spontaneous pregnancy or intrauterine insemination (Muller 2000). Several sperm function tests have been developed, including tests for cervical mucus penetration, capacitation, zona recognition, the acrosome reaction, and sperm–oocyte fusion. However, few were adopted in routine clinical practice and no single sperm function has been proven to be a reliable predictor of male fertility. Three functions have been widely investigated as diagnostics for male fertility: sperm–mucus penetration, acrosome reaction, and zona penetration capabilities (Agarwal et al. 2008a).

Sperm–cervical mucus penetration tests (SMTP) measure the ability of spermatozoa in the semen to swim through cervical mucus or substitute. Each test, whether in vitro or in vivo SMTP, has a different reference range which adds to the dilemma of interpreting the results. According to the WHO criteria, the presence of >50 motile spermatozoa/high power field in cervical mucus collected during the postcoital test indicates a normal clinical condition. Another important clinical use of the sperm–mucus penetration test is in the diagnosis of sperm autoimmunity (Kremer and Jager 1992).

Acrosome reaction (AR), the release of proteolytic enzymes allowing egg penetration, should occur at the time of sperm–zona binding. Two types of anomalies could be seen in AR tests. AR insufficiency occurs when the difference in AR between calcium ionophore treated and untreated sperm is <15%, while AR prematurity is described when >20% of spermatozoa show spontaneous AR (Tesarik and Mendoza 1995). Both deficiencies could be bypassed by performing ICSI.

The zona-free hamster oocyte sperm penetration assay (SPA) examines the ability of human spermatozoa to capacitate, undergo the acrosome reaction, fuse with the vitelline membrane of the oocyte and initiate nuclear decondensation (Johnson et al. 1995). Normal cutoff values have been reported to be 4–20% (Muller 2000). Applications of the SPA include the prediction of the likelihood of spontaneous pregnancy in vivo and the likelihood of successful fertilization during IVF. While correlation with IVF results has been documented, results of SPA may not be considered meaningful as the test was reported to have 20–30% false positive rates and 0–100% false negative rates (Consensus Workshop on Advanced Diagnostic Andrology Techniques 1996).

Evaluation of Oxidative Stress

Oxidative stress has been widely implicated in the pathogenesis of male infertility (Sharma and Agarwal 1996; Agarwal et al. 2008b). Normally, equilibrium exists between ROS production and antioxidant scavenging activities in the male reproductive tract. However, the production of excessive amounts of ROS produced by leukocytes and immature spermatozoa may overwhelm the antioxidant defense mechanisms and cause oxidative stress. Although the number of leukocytes in the ejaculate correlates with higher levels of ROS, the leukocyte number alone cannot be used to assess the level of ROS because leukocyte levels below the accepted WHO reference values were also shown to be associated with ROS (Sharma et al. 2001).

The OS status of an individual can be identified by measuring the ROS levels and total antioxidant capacity (TAC). ROS levels are usually measured by chemiluminescence method (Kobayashi et al. 2001), while total antioxidant capacity is measured by enhanced chemiluminescence assay or colorimetric assay (Said et al. 2003). Recently, normal reference values for seminal ROS in fertile population have been developed. In semen samples without leukocytes, the normal cutoff for ROS was 0.55×10^4 counted photons per minute with 76.4% area under the curve (AUC) in the neat samples and 10.0×10^4 counted photons per minute with 77% AUC in the washed samples. In semen samples with leukocytes ($<1 \times 10^6$), the cutoff for ROS in neat samples was 1.25 with 72.7% AUC and 51.5 with 81% AUC in the washed samples (Athayde et al. 2007). The reference values for TAC is 1,420 μM trolox equivalent/mL. Infertile men with seminal plasma TAC below the above value may have depleted TAC and are therefore more vulnerable to OS-induced damage.

A ROS-TAC score has been proposed as a parameter derived both from levels of ROS produced and the antioxidant levels. The composite score is calculated using the principal component analysis, which provides linear combinations or weighted sums that account for the most variability among correlated variables (Sharma et al. 1999). Therefore, the ROS-TAC score minimizes the variability present in the individual parameters of oxidative stress. ROS-TAC score was used to identify patients with idiopathic infertility as patients who had lower scores compared to controls (32.8 ± 14.2 vs. 50.0 ± 10.0 , $p < 0.001$) (Pasqualotto et al. 2008a). A similar approach was used to characterize infertile patients with varicocele

providing a mechanism for infertility in these cases (Pasqualotto et al. 2008b). Most importantly, ROS-TAC scores were correlated with pregnancy outcomes. The expected pregnancy rates for a patient with ROS-TAC score of 30 would be 13.9, 21.0, and 31.6% for 12, 24, and 36 months, respectively; whereas a score of 50 would have expected pregnancy rates of 35.1, 48.9, and 54.3% over the same intervals (Sharma et al. 1999).

Assessment of DNA Integrity

Assessment of the sperm DNA integrity is one of the most investigated sperm parameters during the last decade. Yet, there is little consensus regarding testing methodologies for sperm DNA integrity and cutoff points that are clinically relevant with in vivo and in vitro fecundity. Different methods may be used to evaluate the status of the sperm DNA for the presence of abnormalities or simply immaturity. Most of these assays have many advantages as well as limitations. The choice of which assay to perform depends on many factors, such as the expense, the available laboratory facilities, and the presence of experienced technicians. Among the different methods that could be used, only the flow cytometric-based sperm chromatin structure assay (SCSA) and terminal deoxynucleotidyl transferase-mediated fluorescein-deoxyuridine triphosphate-nick end labeling assay (TUNEL) assay have been well standardized and have made their way in routine clinical practice.

Testing for sperm DNA integrity is to be considered for the screening of infertile men as well as for the cases of unexplained infertility (Host et al. 1999; Saleh et al. 2002). Testing has also been recommended as a prognostic factor of ART outcomes (Evenson and Wixon 2006; Evenson and Wixon 2008). The cutoff values for abnormal samples vary between 27 and 40% of sperm showing fragmented DNA using the SCSA and between 20 and 36% using the TUNEL assay (Sergeie et al. 2005; Committee 2008). However, a recent systematic review and meta-analysis reported that the commonly used tests, such as SCSA and TUNEL, may not be predictive of pregnancy outcome after IVF/ICSI (Collins et al. 2008). The study suggests testing of DNA integrity may be appropriate in the subgroups of infertile patients, such as couples with history of repeated pregnancy loss and those with idiopathic infertility (Carrell et al. 2003; Saleh et al. 2003).

Summary

Routine semen analysis is almost always performed as a part of the investigation of the infertile couple and its interpretation plays a major role in charting the treatment plan(s). Human semen is very different from other body fluids, mainly because of its heterogeneity, which has negative effects on the quality of the semen analysis. Thus, there is a legitimate concern about the value of semen analysis in any clinical situation, although it remains an essential tool for the identification of infertility and the diagnosis of its severity. Technical variations in semen testing methodologies add to the challenges that face clinicians while attempting to deduct clinically significant information from these various assays. The results of routine semen analysis and other investigations currently available should not be overinterpreted and should be considered in conjunction with the history and clinical examination of the infertile couple.

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