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## Assessing Sperm Function

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Every male infertility work-up should start with the basics: a good history, physical examination, and at least two semen analyses. Throughout the past 50 years or so that it has been in existence, the semen analysis largely has remained unchanged. This basic test is inexpensive, non-invasive, and remains the cornerstone of the infertility evaluation. As advances are made, however, other tests are introduced—not to supplant or replace this test—but rather to delve further into the specific causes of male infertility. Just like any other aspect in the dynamic field of medicine, this role of the semen analysis has been challenged, its validity questioned, and its techniques scrutinized.

This article reviews basic semen tests and new fertility tests that are providing great insights to the rapidly developing understanding of male infertility. Finally, promising new tests under development are mentioned with their potential clinical applications.

### The basic test: the semen analysis

#### *Collection and timing*

Suboptimal sperm collection remains a frequent cause of error in the semen analysis. It should be emphasized to patients that there should be 2 to 7 days of sexual abstinence before

collection. Two separate samples should be analyzed. These samples should be not less than 7 days apart [1,2]. The duration of the abstinence should be constant if possible, because each additional day can add as much as 25% in sperm concentration [3]. Lubricants should be avoided, as they may interfere with motility results. Coitus interruptus often leads to inaccurate results, as the first part of the ejaculate that contains most of the sperm may be lost. A clean, sterile container should be used as a receptacle. A complete list of the guideline is provided in the World Health Organization (WHO) laboratory manual for examination of human semen and sperm–cervical mucus interaction [4].

#### *Semen collection*

Semen specimens can be produced in various ways. At times patients will require assistance.

*Masturbation in a clinical setting.* This is the recommended procedure where the collection is done in a private room in the same facility where the semen will be analyzed. The glans and the penis should be cleaned with a wet paper towel (soap should be avoided). Lubricant use is discouraged but if needed should not be applied to the glans. The container should be provided by the laboratory to avoid contamination or spermicidal effects. The main advantage of this collection method is its simplicity, noninvasiveness, and inexpensiveness. Optimal specimens, however, may be difficult to procure for some men who are uncomfortable providing a sperm specimen in this environment [1].

*Masturbation with assistance.* Some men may not be able to achieve adequate erection and

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ejaculation. Assistance can be provided for by oral medications such as PDE5 inhibitors given 30 to 60 minutes before collection. Cavernal and subcutaneous injections are less popular but possible options to administer to patients who have erectile dysfunction. Seminal pouches that do not contain any spermicides also can be used and allow the patient to engage in sexual activity should he be incapable or uncomfortable producing specimens by masturbation.

*Vacuum erection devices.* These can be used to obtain erection by creating a vacuum around the penis generating a pressure differential that fills the corpora with blood. A constrictive band is placed at the base, however, to maintain erection, and this can inhibit the flow of semen with ejaculation.

*Vibratory stimulation and electroejaculation.* Mechanical/vibratory stimulation may be used for patients who have suffered spinal cord injury (if the spinal cord lesion is T8 and above) [5]. Rectal probe electrostimulation (RPE) may induce ejaculation by stimulation of the efferent fibers of the hypogastric plexus. Precautions for autonomic dysreflexia should be performed while doing these procedures, as some patients with high spinal cord lesions can have life-threatening hypertension [1].

#### *Technical aspects of the semen analysis*

It should be emphasized that nonspecialized laboratories often will have inadequate equipment and inexperienced personnel to perform the semen analysis. Semen analysis is one of the few manually performed examinations remaining in medical laboratories, and ideally it should be performed in an experienced laboratory [6]. There is no reliable and cost-effective automation. Experienced laboratories often will use the Neubauer chamber (Zeiss, Jena, Germany) as recommended by WHO for sperm counting. This requires careful dilution and frequent cleaning. Incorrect use can increase chamber depth, producing erroneous results [6]. Counting chambers, such as the Makler (Sefi Medical Instruments, Haifa, Israel), that do not require dilution are also subject to the same variation [7]. Disposable counting chambers (Cell Vu (Millennium Sciences, New York), Microcell (Conception Technologies, San Diego, California)) are fairly inexpensive and offer less exposure of the clinician to bodily fluids by eliminating the cleaning process. The availability of an appropriate centrifuge also can be crucial. Semen

samples without spermatozoa in an initial assessment should be centrifuged at 2000 g for 10 minutes and reexamined for the presence of sperm. If no sperm is visible at this point, further centrifugation and microscopic examination at 3000 g for 15 minutes are advised. There should be repeated centrifugation and sperm counting performed before azoospermia can be reported in a single semen analysis [8]. The assessment of motility and morphology is an acquired skill for the medical technologist requiring both didactic lectures and practical experience. Quality control testing is a critical component of an accurate semen analysis and often is underemphasized in nonspecialized laboratories [6,9]. It is therefore crucial that patients are referred to a laboratory that can provide reliable results. This may eliminate the need for repeated tests and in the end allow the clinician to make an accurate and cost-effective diagnosis [9]. Clinical laboratories engaged in diagnostic work in the reproductive field in the United States are accredited by agencies such as the College of American Pathologist (CAP). They follow rigorous procedures and protocols and provide superior test results over a laboratory without any external inspection of its records and protocols.

#### *Standard procedures*

The semen sample should be examined within 1 hour of production and receipt in the laboratory. Some of the semen parameters can be affected by a delay in assessment. Motility decreases significantly after 2 hours and progressively diminishes afterwards while reactive oxygen species (ROS) level increases. Ideally, semen is placed in a 37°C gently shaking incubator for 30 minutes to allow liquefaction and mixing. The semen analysis characteristics can be classified into three groups: macroscopic, microscopic and physiologic.

#### *Macroscopic*

Table 1 lists the five macroscopic measurements in a standard sperm analysis. These parameters have remained fairly constant, with the normal values remaining relatively unchanged since the inception of the semen analysis. Some variation in macroscopic parameters (ie, liquefaction) is relatively common and has little clinical significance, although it also can be found in accessory gland dysfunction [4,10]. The specifics of how the tests are conducted (for all variables) are found in the WHO manual [2].

Table 1  
Macroscopic parameters of the semen analysis and clinical significances of some of the abnormal values

Parameters	Normal values	Abnormalities	Clinical significance
pH	7.8	Acidic: <6.5–7	w/ low volume and noncoagulation; congenital absence of the vas Ejaculatory duct obstruction Partial retrograde ejaculation
Coagulation/liquefaction	Coagulates and liquefies within 20 mins at room 37°	No coagulation	Congenital absence of the seminal vesicles
Color	Whitish-gray; pearl-white	Prolonged liquefaction Yellowish color Reddish brown	Poor prostatic secretions Jaundice, carotenemia, drugs Haemospermia secondary to urethral bleeding or inflammation of the seminal vesicles, but other GU tumors will need work-up to be excluded
Viscosity	4 mm threading	>6 mm No threading	Of importance when associated with low motility
Volume	2–4 mL	0 (aspermia) <2 mL (hypospermia)	Retrograde ejaculation Incomplete collection Partial retrograde ejaculation Short duration of sexual abstinence
		>6 mL	Prolonged sexual abstinence

### Microscopic

Microscopic examination of the semen in essence assesses spermatogenesis. This part of the semen analysis is subject to technical error, and even reliable laboratories can display variable results. The normal values are also subject to some patient variation, with variability from one ejaculate to the next. The following are the parameters of the microscopic analysis.

*Sperm agglutination.* 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 1000 × magnification. Sperm agglutination, sperm presence, and subjective motility can be assessed under this method. When sperm adheres to nonsperm elements (non-specific agglutination), it may be indicative of accessory gland infection. Sperm-to-sperm agglutination (site-specific agglutination) can be secondary to antisperm antibodies; however, it should be remembered that a small degree of agglutination is normal [4]. When agglutination is observed, semen cultures and antibody assessment should be preformed.

*Sperm count and concentration.* Assessment of sperm concentration (number of sperm per

milliliter) and sperm count (number of sperm/ejaculate) is conducted after liquefaction. Dilution of the semen is required if a Neubauer counting chamber is used, while Makler and other disposable sperm counting chambers (Micro cell and Cell-Vu) do not require dilution [11]. These disposable counting chambers are accurate and fairly inexpensive and minimize the clinician's exposure to body fluids as cleaning process is not required. The Neubauer chamber remains the gold standard of sperm-counting chambers [8], but it is not without its flaws. The normal sperm concentration is reported at greater than 20 million sperm/mL. Oligospermia (less than 20 million sperm/mL) may be indicative of incomplete collection or a short abstinence period. When collection problems are eliminated, further evaluation as outlined elsewhere in this issue should be undertaken. Azospermia (absence of sperm) may be the result of abnormal spermatogenesis, ejaculatory dysfunction, or obstruction. Polyspermia (abnormally elevated sperm concentration) rarely is reported but may be caused by long sexual abstinence and often is associated with sperm of poor quality.

When oligospermia is reported, the levels of motility and morphology become especially important, as total motile sperm counts guide

decisions on appropriate therapies, including the use of assisted reproductive techniques (ART). In cases of azoospermia and severe oligospermia, hormonal evaluation (follicle-stimulating hormone [FSH] and testosterone) should be requested. Karyotyping and Y microdeletion may provide valuable information regarding the etiology of the patient's abnormal semen parameters and important information if in vitro fertilization (IVF) is being entertained as a treatment option (see article on genetic causes of male infertility). Foci of microdeletions in the Y chromosome are associated with impaired spermatogenesis, and depending on their location, may predict poor sperm retrieval even with testicular biopsy [12]. Karyotyping may detect autosomal or x-linked genetic aberrations causing infertility. Knowledge of the chromosome status is important, as male offspring conceived with intracytoplasmic sperm insemination (ICSI) or even natural conception most likely will inherit the same microdeletion [12,13].

*Sperm morphology.* Table 2 compares the two widely used criteria in morphology (WHO and Tygerberg Strict criteria). Among the semen parameters, this is the most subjective and the most difficult to standardize [6,8]. Accurate assessment of morphology is critical in the evaluation of the infertile male. This also can be a significant predictor of pregnancy. When correctable causes of male infertility are not identified, couples with

teratozoospermia (less than 15% normal morphology) by WHO method may be directed to proceed with IVF with ICSI versus intrauterine insemination (IUI) [8,14]. Studies on sperm selection for ICSI reports of lower pregnancy outcomes and higher abortion rates [15,16] when morphologically abnormal sperm were used. There is no evidence, however, that abnormal morphology is associated with an increase spontaneous miscarriage rate in natural conception. Abnormal morphology, however, is associated with decreased fertilization or pregnancy rates [14,15].

*Nonsperm cells.* Immature germ cells, epithelial cell, and leukocytes are some of the nonsperm elements noted on seminal microscopic examination [17,18]. Epithelial cells are indicative of poor collection when present in high numbers. Leukocytes are the most significant nonsperm cellular elements in the semen and are a frequent finding in patients who have unexplained infertility [18]. In the initial microscopic analysis, the immature spermatozoa may be confused with leukocytes. To confirm findings, additional tests may be requested when there are greater than 5 round cells/hpf. Immunocytochemistry is the procedure of choice, but given its expense, most laboratories do not have this test. The Endtz test is a reliable alternative, as it allows accurate identification of leukocytes that contain enzymes that will react with the peroxide and be visualized with the

Table 2  
Sperm morphology classification

	World Health Organization 3rd	Strict World Health Organization 4th (Kruger)
Normal reference range	> 30%	> 14%
Head		
Shape	Oval	Oval, smooth borders
Acrosome	40% to 70% of head surface	40% to 70% head surface
Size	4–5.5 $\mu\text{m}$ length 2.5–3.5 $\mu\text{m}$ width Length/width = 1.5–1.75	3–5 $\mu\text{m}$ length 2–3 $\mu\text{m}$ width
Vacuoles	<20% head area	Up to 4
Midpiece		
Shape	Straight regular outlined Axially arched	Slender, straight, regular outline Axially arched
Size	<1/3 of head area	<1 $\mu\text{m}$ wide Length 1.5 $\times$ head
Cytoplasmic droplet	<1/3 of head area	<1/2 of head area
Tail		
Appearance	Slender, uncoiled	Uniform, uncoiled
Width		Thinner than midpiece
Length	>45 $\mu\text{m}$	10 $\times$ head

orthotoluidine dye [19]. Initially considered solely as a marker for genital tract infection, contemporary research has shown that leukocytes can be present with no other signs of infection or immune response [20] and have intimate links to reactive oxygen species (ROS) [19,21–23]. The WHO has defined leukocytospermia as levels above  $1 \times 10^6$  white blood cells (WBC)/mL. Studies have shown, however, that ROS levels are elevated even at WBC counts of less than  $.20 \times 10^6$ , suggesting that much lower levels of leukocytes are pathologic [22,24]. In a 12-month follow-up, men who had a negative Endtz test (zero) had a 23.7% chance of initiating pregnancy, while levels less than  $1 \times 10^6$  lowered the chances to 15.5% [25]. In IUI, high seminal leukocyte levels (greater than  $2 \times 10^6$ /mL) result in lower pregnancy rates. Leukocytospermia determination still has to be requested separately in many andrology laboratories. Its significance and the ease of determination that is reproducible in most laboratories should place this test among the standard testing that accompanies a basic semen analysis. When leukocytospermia is identified, semen cultures should be performed.

Red blood cells (RBC) are often present in semen. Although small amounts are usually a normal finding, they can be indicative of infection, inflammation, ductal obstruction, or rarely vascular abnormalities.

### *Physiologic variables*

#### *Sperm motility*

Sperm motility is a reflection of the normal development of the axoneme and the maturation the sperm undergoes within the epididymis. This is a parameter that is subject to significant potential for technical mistakes in the laboratory. The most common method employed by laboratories is to simply estimate the motility of sperm on several fields. This is a subjective method and prone to inaccuracy. Some argue that in vitro measurement of motility is not reflective of the true motility within the female reproductive tract [26,27]. The progressive motility grading system recommended by the WHO is ideal for technicians trained in andrology but can be daunting for those who do not perform the tests regularly [6]. Some authors recommend that temperature should be reported and the time from submission to examination, as slight temperature increases and delay of examination can decrease the number of motile sperm counted dramatically [8]. Asthenospermia (sperm

motility less than the WHO cutoff levels) also can be artifactual when spermicides, lubricants, or rubber condoms are used. Jouannet and colleagues and Nallella and colleagues compared semen parameters and found that sperm motility and concentration combined provided accurate prediction of fertility [28,29] with a high sensitivity (.74) and specificity (.90). Total motile sperm count is thought by many to be the best independent predictor [26,30] of pregnancy outcome with IUI (less than  $1 \times 10^6$  motile sperm with pregnancy rate of 2% and  $5\text{--}8 \times 10^6$  motile sperm with pregnancy rate of 19%) [30]. High cumulative pregnancy rates (motility greater than 30% with pregnancy rate of 74%) also were reported in females undergoing ovarian stimulation with clomiphene citrate and human menopausal gonadotropin in conjunction with IUI [31]. Postwash total motile sperm count with a cut-off rate of 40% also was found to be a better predictor of IUI success [27].

#### *Viability*

When the motility is reported as less than 5% to 10%, viability testing is recommended, as profoundly low motility can be from dead sperm or necrospemia [6,8]. The most common viability assessment involves staining with Eosin Y (Sigma-Aldrich, St. Louis, Missouri) followed by the blue-black counter stain of Nigrosin (Sigma-Aldrich, St. Louis, Missouri). The viable sperm with its intact cell membrane will not take up the dye and remain unstained. This test will differentiate necrospemia from immotile sperm secondary to structural flagellar defects such as in Kartagener's syndrome and primary cilia dyskinesia.

Hypo-osmotic swelling test (HOST) is an alternative method to assess sperm viability. It is based on the principle that viable sperm have intact cell membranes. Exposure of the sperm to hypo-osmotic fluid will cause water to flow into the viable cells seen as swelling of the cytoplasmic space and curling of the sperm tail. Nonviable sperm with nonfunctional cell membranes will not exhibit this effect, as they cannot maintain an osmotic gradient. One application of this test is to aid selection of viable sperm for use in IVF or ICSI, especially when there are no motile sperm seen in the testicular sperm or cryopreserved specimens. As this test is reproducible and relatively inexpensive, it also is recommended by some to be done routinely [32]. Nevertheless, it has not been employed widely in the routine management of infertile males.

### *Computer-assisted sperm analysis*

Computer-assisted sperm analysis (CASA) has two advantages: high precision and quantitative assessment of sperm kinematics. It is a semiautomated technique that provides data on sperm density, motility, straight-line and curvilinear velocity, linearity, and average path velocity, amplitude of lateral head displacement, flagellar beat frequency, and hyperactivation. Sperm concentration, sample preparation and frame rate can affect accuracy of the CASA [33]. Stains used also have affected the accuracy of determining morphology. Although this technology had theoretic advantages, it does not realize these advantages in clinical practice. This test requires expensive equipment and still requires the active participation of a technician. At present, these machines are found more commonly in andrology laboratories, and not in general pathology laboratories, where most of the initial semen analysis is analyzed [6,34]. The most important role of CASA at this time seems to be in standardizing aids in quality control and quality assurance in andrology laboratories, as the emerging use of IVF and ICSI diminished the role of motility assessment in sperm selection in assisted reproduction [34]. Future applications are being explored in reproductive toxicology [35].

### *Sperm home testing kits*

A decade or so ago, a few male fertility home kits were introduced to the market. Some kits even have microscope sets, and others are not true home kits but merely transport systems. These tests were developed to decrease the patient's embarrassment of having a semen analysis in a clinic or laboratory. The first notable home tests (Fertilmarq and Babystart, Lake Consumer Products Inc, Wisconsin) were based on sperm staining, and the color intensity will test positive when the sperm count is equal or above 20 million/mL. The kits have two separate tests per pack and claim 97% accuracy. The latest test to be introduced on the market is a combined male and female test kit (Fertell, Genosis Inc, Surrey, UK). The female kit tests for FSH on day 3 of the menstrual cycle from the early morning urine sample as an ovarian reserve test. The male kit is comprised of a container with a lid and is based on the principle of using hyaluronic acid as a cervical mucus substitute that will allow sperm to swim up. These sperm will be coded red in an antigen-antibody reaction. The test will show positive (indicated by a second line in the result box) when

the motile sperm has a concentration of greater than 10 million/mL [36]. This test claims 95% accuracy [37]. The disadvantage of this test is that it deals only with motility parameter, which is only one of the aspects of the male infertility spectrum.

Proponents of these home tests feel that these home kits may increase awareness of couples that the male factor needs to be considered in infertility evaluations. Its detractors are skeptical that it will achieve that purpose. Proper treatment is often as multifaceted as the causes of infertility, and these home tests may result in wrong focus and unnecessary delay. Males who test positive may be lulled into thinking that they are normal and may not seek urologic assessment. Only time will tell if these tests truly can promote male factor infertility awareness.

### *Limitations of the basic sperm analysis*

The true litmus test for male fertility remains the ability to cause pregnancy in vivo. Although the semen analysis is used as a surrogate measure of a man's fertility potential, it is not a direct measure of this. Clinical research has shown that a normal semen analysis may not reflect defects in sperm function (idiopathic infertility), and men with poor sperm parameters still may cause spontaneous pregnancy. Only fifty percent of infertile men have recognizable causes detectable by the basic semen analysis [8]. One out of every seven couples are subfertile [38] when based on WHO standards. The presence of several criteria further reinforces the emerging opinion that the current standards (WHO and Tygerberg) do not reflect the true fertility potential of subjects. The current normal values fail to satisfy clinical and statistical standards [8,28] and pose the risk of misclassifying a subject's true fertility status. In fact, using the WHO cut-off of  $20 \times 10^6$ , 20% of 18-year olds would be classified as subfertile [39]. Studies on sperm donors with known fertility status reveal a significant overlap in the sperm characteristics between fertile and subfertile men [28,40].

Guzick and colleagues [40] in 2001 conducted a study of 1461 fertile and infertile men with no female infertility factor and found different cut-off levels in:

- Sperm concentration (less than  $13.5 \times 10^6$  in subfertile and  $48 \times 10^6$  in fertile men)
- Percent motility (less than 32% subfertile and greater than 63% fertile men)
- Normal morphology (less than 9% subfertile and greater than 12% fertile men)

Nallella and colleagues [28] in 2006 did a similar study (n=572) and used the WHO and Tygerberg criteria on these subjects with known fertility. They noted that there is low sensitivity (0.48) in detecting subfertile subjects using WHO reference values for sperm concentration and low sensitivity (0.83) using Tygerberg criteria for % normal morphology. Among the variables, motility had the least overlap range and gave the best prediction of the subject's fertility potential. This is in contrast with the earlier study by Guzick and colleagues, where morphology was reported to provide the highest discriminating power in detecting subfertility among all the semen variables. Clearly, each variable alone is neither a powerful sole discriminator nor predictor of fertility status, and they must be considered in the context of other parameters and the clinical setting.

As the need for new reference values emerges, it is inevitable that the definition of normal semen parameters continues to be revisited. Nallella and colleagues [28], in analyzing receiver operating characteristic curves (ROC) for concentration, motility, and morphology, suggested the following values based on the equal sensitivity and specificity of each:

- Concentration greater than  $31.2 \times 10^6/\text{mL}$
- Motility greater than 57.8%
- Normal morphology greater than 33% (WHO) and greater than 11% (Tygerberg strict criteria)

These values are also close to earlier studies by Zinaman and colleagues [14] who noted a decrease in fertility rate when the concentration fell to less than  $30 \times 10^6$  and normal morphology (Tygerberg strict criteria) less than  $4 \times 10^6$ . There remains a need for further studies in larger populations and different demographics before a consensus can be reached on the necessity of resetting current values to increase the predictiveness and utility of the semen analysis.

#### *Additional tests (nonroutine)*

##### *Sperm–mucus interaction*

Also known as the postcoital test (PCT), this test can assess cervical environment as a cause of infertility. Accurate timing is crucial, as it has to be conducted when the cervical mucus is thin and clear just before ovulation. In this test, cervical mucus is examined 2 to 8 hours after normal intercourse. Progressively motile sperm greater than 10–20/hpf is designated as normal. Practical

guidelines of American Society of Reproductive Medicine (ASRM) (2004) recommend PCT in the setting of hyperviscous semen, unexplained infertility, or low volume semen with normal sperm count. Medical history and semen analysis can predict PCT results in half of the infertile couples [41]. Poor-quality semen most likely will have poor PCT, and it is not recommended routinely for males who have abnormal semen analyses. Couples who will show defective sperm mucus interaction may be advised to proceed with IUI, as additional tests are unlikely to impact the management [39]. This test has fallen out of favor in general infertility practice [10]. It may be useful in patients who are unable to produce an ejaculate or are unwilling because of religious proscriptions.

##### *Antisperm antibodies*

The tight Sertoli-cell junction provides the testis with a barrier that prevents the immune system from coming in contact with the postmeiotic germ cells. In certain conditions such as testicular torsion, vasectomy and testicular trauma, this unique barrier can be violated resulting in an immune response to sperm, displayed as antisperm antibodies (ASA). These antibodies affect fertility by blocking spermatozoal penetration of the cervical mucus or by preventing sperm binding and penetration of the zona pellucida. Approximately 10% of infertile men will present with ASA (versus 2% of fertile men) [40]. Excessive sperm agglutination or an abnormal PCT can suggest the presence of ASA. Often, sperm parameters are normal [42], thus leading some to suggest that this be tested routinely in all men undergoing infertility work-ups [8,32]. Techniques for this assay are described in the WHO manual [4]. Indirect testing detects the biological activity of circulating ASA, and false positives can come from nonimmunologic factors [43]. Direct ASA detects sperm-bound immunoglobulins. IgG-MAR (mixed antiglobulin reaction) and SpermMAR (Conception Technologies, San Diego, California) tests are more economical and readily available and thus may be recommended for screening. Immunobead Test (IBT), which measures IgG, IgA, and IgM, is employed widely and may be additionally recommended when either of the previous tests gives a positive result to determine if IgA are bound to the sperm surface. Fewer than 10% (IgG MAR) or 20% (IBT) of spermatozoa with adherent

particles have acceptable normal values by WHO (1992) standards. A weakly positive IgG MAR/IBT in men who have low motile sperm rules out immunologic factors, and no further testing is needed [43].

Clinical implications of ASA on male infertility are varied. ASA are present in 34% to 74% of vasectomized men and persist in 38% to 60% after vasectomy reversal [43,44]. Most clinicians do not test for ASA routinely in this setting, because they are of uncertain significance and usually do not affect the decision to do a vasectomy reversal. After orchidopexy for cryptorchidism, there are conflicting reports regarding ASA levels [45]. In genito-urinary infections, ASA is thought to be a consequence of the inflammatory process rather than cross-reactivity to the microorganism [43].

Management options include corticosteroid therapy in cyclic doses to increase antibody free spermatozoa and the selection of ICSI over IUI and IVF. Corticosteroid treatments are not always successful, and the adverse reactions associated with their usage should be considered [45]. There are reports of successful pregnancies with IUI, with 64.3% pregnancy rates after four IUI cycles [46] in superovulated females with partners who are IgG-MAR/IBT positive. The decision to proceed with IUI versus ICSI in immunologic infertility can be aided by a zona pellucida (ZP) test. If the sperm exhibit inability for ZP binding, ICSI is the ART procedure of choice. Presently, flow cytometry techniques are being developed to quantify ASA in individual spermatozoa [47]. These techniques also are being explored to identify sperm surface antigens for possible immun contraceptive development.

#### *Acrosome reaction*

After capacitation, the sperm fuses with the ova's plasma membrane and releases acrosomal enzymes that will allow sperm penetration and fertilization. Transmission electron microscopy, although the procedure of choice to detect acrosome reaction defects, is labor-intensive and expensive. Other techniques such as fluorescence microscopy and beads coated with antiacrosomal antibodies have been developed, but these tests are not readily available. This test may be recommended in cases of profound abnormalities of head morphology or in the setting of unexplained fertility in patients with poor IVF pregnancy rates.

#### *Sperm penetration assays and sperm zona binding tests*

The sperm penetration assay (SPA) uses zona-free hamster oocytes to measure fertilization capability. The zona pellucida is stripped, to allow cross species fertilization. Normally, 10% to 30% of ova are penetrated [4]. The ZP test uses oocytes that failed to fertilize in IVF clinics. Oligozoospermic and severely teratospermic men have a higher number of defective sperm ZP interactions, which may account for their low fertility potential in both spontaneous and IVF pregnancies [48]. Sperm capacitation index (SCI) is a variant of the SPA test, assessing the mean number of penetrations per ovum. It has been suggested that ICSI should be offered to couples with a SCI less than 5 instead of doing standard IVF procedures [46]. Meta-analysis of sperm function assays by Oehninger and colleagues [49] showed a high predictive power of sperm-zona pellucida-binding assays over SPA for fertilization and IVF outcome. The need for human oocyte supply, however, remains a limitation to the use of this test. SPA, although with low predictive power, is correlated positively with spontaneous pregnancy outcomes [50].

#### *Biochemical tests*

Acrosin is a serine protease-like enzyme that exhibits a lectin-like carbohydrate binding activity to the zona pellucida glycoproteins. Low acrosin activity has been associated with low sperm density, motility, poor normal morphology [51], HOST (hyper osmotic test) [51,52], and increased ROS [52] in subfertile men. Assays for this include a gelatinolysis technique and a spectrophotometric assay. Its activity has been correlated inversely with low fertility rates in IVF and has been suggested as a predictor of IVF success, independent of sperm morphology [52].

Citric acid, zinc, alpha glutamyl transferase, and acid phosphates are biochemical substances associated with the prostate. These have antioxidative properties that neutralize ROS in seminal plasma. Zinc is necessary for chromatin stability and decondensation, and a possible role in head-tail detachment in fertilization. It is measured by colorimetric methods with a reference value of 13  $\mu\text{mol}$  per ejaculate [4]. Reports on the effects of zinc in sperm function and semen parameters are conflicting. Mankad and colleagues [53] reported positive correlations between seminal zinc levels, alpha glucosidase, and sperm count; however there are other reports that showed no significant

changes in count and motility [54–56]. Zinc levels in seminal plasma are decreased significantly in asthenozoospermic and oligoasthenozoospermic men, but spermatozoal zinc levels are increased [57]. Low zinc–calcium ratio is associated with better motility [54] than high ratio. Dietary supplementation of zinc, however, did not improve semen variables [58].

The seminal vesicles contribute the bulk of seminal fluid that serves as the transport medium and nutrition in the form of fructose. There is a positive correlation between sperm motility and seminal fructose levels [56]. Low or absent fructose is seen in ductal obstruction and congenital conditions like congenital absence of vas deferens (CBAVD). Semen fructose testing may be requested when hypofunctioning seminal vesicles are suspected, although morphometric analysis of seminal vesicles using transrectal ultrasound has gained popularity.

L-carnitine is secreted by the epididymis and is concentrated in the seminal plasma to 10 times serum levels. It has a role in sperm maturation. Low L-carnitine levels are found in oligoasthenozoospermic men [59,60]. The levels of carnitine possibly can serve as indicators to the level of obstruction in the ductal system. Extremely low concentrations of L-carnitine are found in azoospermic men who have postepididymal obstructions, while normal levels are found in azoospermic men who have intratesticular obstructions [59]. Administration of L-carnitine supplements did not improve sperm density, but contrasting results have been reported for sperm motility [60]. L-carnitine determinations remain far from becoming mainstream tests in male infertility until significant well-designed studies are conducted. Alpha glucosidase, tested by fluorimetric methods, has been used to distinguish nonobstructive from obstructive azoospermia. It is used as a specific marker for epididymal function and believed to play a role in sperm maturation in the epididymis. A cut-off value of 12 mIU/mL distinguishes ductal obstruction from primary testicular failure [61]. The usefulness of this test was questioned by Krause and Bohring (1999), but Comhaire and colleagues [61], in their review, showed a strong association between alpha glucosidase and semen parameters. The cut off level ( $12\text{U l}^{-1}$ ) had 95% specificity in identifying obstructive azoospermia. This suggests that the test can predict IUI response (higher pregnancy rate greater than 78 U per ejaculate) as high levels indicate better zona-binding capacity [61]. The presence of

commercial test kits using colorimetric methods promises to make testing accessible and affordable.

### *Other tests*

#### *Reactive oxygen species*

Small amounts of ROS are normal and in fact necessary for the hyperactivation and capacitation of spermatozoa. In large amounts, it causes spermatozoal damage by lipid peroxidation of the plasma membrane, germ cell apoptosis, and DNA strand breakage [62]. Leukocytes or WBCs are the main source of ROS. Abnormal spermatozoa are a minor source of ROS, and they are caused by retention of cytoplasmic droplets during defective spermiogenesis. Smoking, alcohol abuse, and exposure to radiation and toxic chemicals have been associated with increased seminal ROS [60,61]. The oxidants are in hydroxyl ( $\text{OH}^\cdot$ ), superoxide ( $\text{O}_2^\cdot$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), and hypochlorite forms, as well as nitrogen-derived forms of nitric ( $\text{NO}^\cdot$ ) and nitrous oxide ( $\text{N}_2\text{O}$ ). Increased ROS levels have negative correlation with sperm concentration, motility, morphology, and overall normal semen parameters [63–66]. In addition, patients who have unexplained infertility may have increased levels [62,64]. There is an inverse relationship between ROS and in vivo fertilization [67]. Meta-analysis on ROS levels and IVF revealed an inverse relationship between the two [68].

Measurement of ROS is done by several methods, the most common of which is chemiluminescence, which measures total seminal ROS (from WBC, abnormal spermatozoa, and seminal fluid). Leukocytospermia is associated with increased ROS levels and can serve as indirect measurement of ROS [23].

Normal or reference values are not established at this point. A study by Shekarriz and colleagues [64] showed  $0\text{--}5.5 \times 10^4$  cpm at a sperm concentration of  $20 \times 10^6$  in normal fertile donors. The use of seminal WBC levels as basis of ROS levels, although proven and well-accepted, has yet to establish definite cut-off points. Even low WBC levels (below the WHO cut-off) are associated with ROS [22]. The seminal fluid contains antioxidants (zinc, glutathione) that neutralize the detrimental effects of ROS. Oxidative stress (OS) describes a condition in which there is greater ROS than the total antioxidant capacity (TAC). 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 (greater than 30)

can help in the prediction of pregnancy outcomes [25]. As the standardization of testing and the availability of these tests remain limited, it will be some time before this test will become a mainstream investigation in the evaluation of male infertility.

#### *DNA fragmentation*

DNA fragmentation initially was described in 1993 and has since been researched as a test to aid fertility predictions in subfertile males. The spermatozoal chromatin is a tightly packed structure because of the disulfide cross linkages between protamines that allow compaction of the nuclear head and protect the DNA fragments from stress and breakage. DNA damage is multifactorial and theories on its etiology include protamine deficiency and mutations that may affect DNA packaging or compaction during spermiogenesis [62,69,70]. Factors associated with increased sperm DNA damage are tobacco use, chemotherapy, testicular carcinoma, and other systemic cancers. DNA damage is correlated positively with poor semen parameters (low sperm concentration and low sperm motility), leukocytospermia, and high ROS levels [62,69,71,72]. Approximately 8% of subfertile men who have normal semen parameters will have high abnormal DNA [67].

Direct methods for DNA damage assay include single cell electrophoresis (COMET) and terminal deoxynucleotidyl transferase mediated 2-deoxyuridine 5-triphosphate (TUNEL). Indirect methods are sperm chromatin structure assay (SCSA), which measures sperm chromatin integrity, and DNA intercalating dyes (acridine orange) that differentiate single and double stranded DNA. The sperm DNA denaturation test and the sperm chromatin dispersion test are other tests reported in literature [73,74].

A cut off rate of greater than 30% was associated with a significant decrease in in vivo fertilization rates [71,75]. A DNA fragmentation index (DFI) of greater than 30% has a sensitivity of 15% and a specificity of 96% [76]. Meta-analyses by Evenson and Wixon [77] and Li and colleagues [78] showed that couples are twice as likely to become pregnant with regular IVF methods if the DFI is less than 30%. In IUI, DFI also has been found to be a useful pregnancy predictor, and an odds-ratio of DFI greater than 30% correlates with lower rates of clinical pregnancy, biochemical pregnancy, and delivery [79].

Based on the previous studies, testing for DNA fragmentation defects can help couples decide on what fertility modality and possible lifestyle

modifications they can employ that may increase their chances of conception. ICSI is advised when DFI is above cut-off levels. There is a higher rate of DNA damage in ejaculated or epididymal sperm than intratesticular spermatozoa, hence use of intratesticular spermatozoa from high DFI men is recommended for ICSI [80,81]. One study has demonstrated a higher miscarriage rate after ICSI in men who have high DFI [82]. Contrasting reports, however, have failed to show significant correlation between DNA damage and idiopathic infertility [83,84]. In addition, significant intraindividual variation exists (using SCSA) making conclusions problematic [70]. Treatment employed to counteract or decrease DNA fragmentation defects are likewise varied. The ASRM (2006) best summarizes the current viewpoint on DNA integrity testing, concluding that there are not enough data to make DNA testing routine in infertility testing and that treatments have yet to prove their clinical value. Still, its applications to research can provide greater insights to infertility and andrology. If tests on this become standardized, inexpensive, accessible, and reliable in their application, the possibility of their use in clinical practice will be highly likely.

#### *Electron microscopy*

Ultrastructural details of the sperm only can be seen under the electron microscope (EM). Patients who have low sperm motility (less than 5% to 10%) with high viability (HOST or Eosin-Nigrosin staining) and density may be appropriate candidates for EM assessment. These spermatozoa may test positive for viability even with the ultrastructural defects [10]. Mitochondrial and microtubular defects that are not visible under the usual Papanicolaou smear will be evident. Subfertile men will have more serrated and blurred circular sulcus, less intact acrosome membrane, a bigger proportion of the spermatic head, and more droplets attached to acrosome membrane [85].

#### **What is in the future of sperm analysis? (emerging technologies)**

##### *Microarray*

This method analyzes the transcriptome of cells and tissues. Comparison of the transcriptomes at the different stages of spermatogenesis may provide clues to molecular mechanisms to genetic infertility (ie, Yq microdeletions) and

potential biochemical markers for infertility. The primary application at this time in the field of andrology is in uncovering the still unknown genes, pathways, and mechanisms in sperm production. Creation of mRNA profiles possibly can distinguish spermatogenic infertility from other causes. The use of ICSI bypasses the natural selection of preventing transmission of defective genes, and clues in men who have genetic infertility may be provided for by these profiles [86].

### Proteomics

Body fluids with complex proteins are ideal candidates for studies as they have the potential to contain biomarkers. The advent of electrospray ionization (ESI) and matrix-associated laser desorption/ionization (MALDI) has pushed this field into sequencing peptides and proteins of body tissues at different biological states,

including seminal fluid [87]. Seminal fluid has been found to have 923 proteins [88], and at least 20 proteins have altered expressions in infertile men [89]. This noninvasive technique not only provides the potential to detect causes of infertility, but may play a role in the development of male contraception [90]. It has been shown that there are possible biomarkers for normal cellular function in in vivo embryonic development [91]. Insight to signaling complexes in physiologic process is a good start in understanding the biologic functions of these proteins, and the future of developing biomarkers does not seem to be far from becoming a reality [36].

### Metabolic profiling (metabolomics)

This technology brings together microassay and proteomic technologies. Metabolites are formed or released by cell processes. These

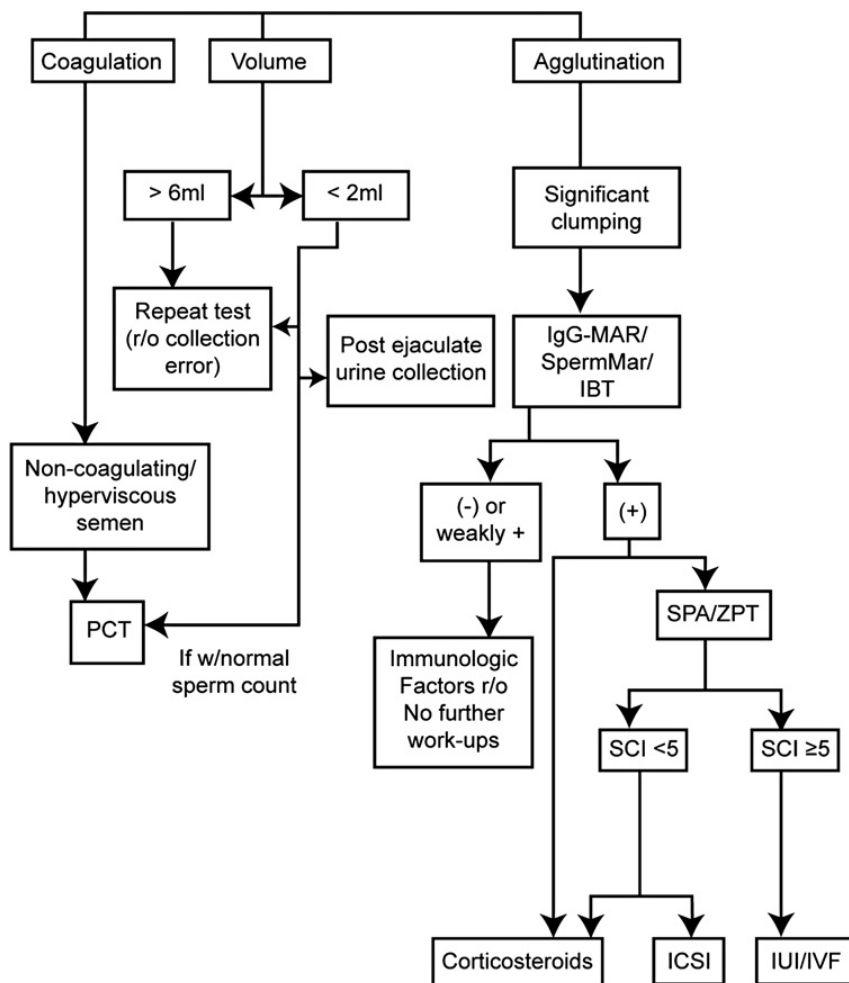


Fig. 1. Semen analysis. *Abbreviations:* IBT, immunobead test; ICSI, intracytoplasmic sperm injection; IgGMAR, IgG mixed antiglobulin reaction; IUI, intrauterine insemination; IVF, in vitro fertilization; PCT, post coital test; SPA, sperm penetration assay; SCI, sperm capacitation index; ZPT, zona pellucida testing.

biochemical intracellular substances can provide both qualitative and quantitative data for a glimpse of the network processes in vivo [92]. In male infertility, the production of oxidative stress byproducts and naturally occurring antioxidants can serve as biomarkers to potentially differentiate fertile from subfertile men with idiopathic infertility [93]. The same principle can be applied to assisted reproductive techniques, as oxidative stress can affect pregnancy outcomes markedly. The noninvasiveness of this test is its main advantage should it be developed and proven helpful for use in the clinical setting.

#### *Atomic force microscopy*

In 1986, a high-resolution type of scanning microscope with resolutions in the fractions of a nanometer was invented. Its main advantage over EM is the three-dimensional images it can provide, and the simplicity of the sample preparation (air drying). It also allows observation in an air or liquid milieu and thus the potential of observing biomolecules in vivo. The disadvantage is that the image quality is limited by the radius of curvature of the probe tip, and an incorrect tip can result in image artifacts. Spermatozoa have been reported as good subjects because of their small size and rigidity [87]. Studies in sperm plasma membrane during maturation and capacitation have identified new areas with phosphorylated proteins, and large aggregates of lipid did not cross postacrosome and equatorial segments [94]. Although this method for andrology testing requires expensive equipment at the present time, its use in research will provide information on the intricate processes and structures in sperm and uncover some of the unknown causes of male infertility.

#### **Summary**

In the primary care level, a proper diagnosis on male infertility can be made with comprehensive and properly performed semen analyses in conjunction with a thorough history and physical examination. Fertility, however, is not dependent on a single test but is often a combined sum of different factors.

Fig. 1 presents algorithms in sperm assessment. It should be remembered that the values set for each are not absolute and that there is still much to learn about sperm, its biochemical processes,

and its interaction with environment and physical stresses.

The advent of new tests should be geared toward better understanding of the intricacies in this haploid cell. The emergence of home kits for sperm assessment can be seen not as deterrent to seeking adequate advice but rather an information tool to promote awareness of this problem.

One must continue to carefully assess the male factor component and to continue to attempt to cure infertility and use assisted reproductive technologies judiciously. Although a carefully performed semen analysis remains the initial choice in the evaluation of male infertility, exciting new developments in semen testing promise continued advances in the targeted diagnosis and management.

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