INTRODUCTION

Infertility is defined as the inability of a couple to conceive after 12 months of regular, unprotected sexual intercourse. It is one of the major health issues affecting about 48.5 million couples around the world, with the male factor accounting for about 50% of the cases (Mascarenhas, Flaxman, Boerma, Vanderpoel, & Stevens, 2012). Reportedly, the global prevalence of male infertility ranges between 2.5% and 12%, with a higher prevalence in Central/Eastern Europe and Australia (Agarwal, Mulgund, Hamada, & Chyatte, 2015). A cross-sectional population survey revealed a strong association between increased incidence of male infertility with high socio-economic status and delayed parenthood (Datta et al., 2016). Furthermore, it is estimated that around 50% of men requiring treatment for infertility seek for help (Datta et al., 2016).

Several factors can cause male infertility, which include testicular and hypothalamic-pituitary diseases (such as cryptorchidism, orchitis, genital tract infections, varicocele, obstructions of the male genital tract and hypogonadism), genetic conditions (such as Kallmann or Klinefelter syndromes, globozoospermia and microdeletions of chromosome Y), cancer, systemic diseases, medical therapies or exposure to endocrine disruptors (Fainberg & Kashanian, 2019; Miyamoto et al., 2017). In addition, lifestyle-associated factors such as smoking, drinking, drug abuse, high energy-based diet, obesity and psychological stress negatively impact the male reproductive potential (Durairajanayagam, 2018). Evidence suggests that oxidative stress plays an important role in the establishment of male infertility, accounting as a causative factor in 30%–80% of infertile men (Agarwal et al., 2019).

Semen analysis is the most fundamental step in the evaluation of the male fertility potential. It reflects testicular function and reflects the production of spermatozoa in the testes, the patency of the duct system and the glandular secretory activity. Evaluation of seminal fructose, sperm vitality and leucocytes (Endtz test) are useful adjuncts to semen analysis that provide information on specific clinical conditions. Though several computer-assisted sperm analysis (CASA) systems have been developed, conventional methods for semen analysis are still widely accepted in clinical practice. This review summarises the conventional techniques used in routine semen analysis and their diagnostic value in clinical andrology.

Abstract

Infertility is a major health issue affecting over 48.5 million couples around the world, with the male factor accounting for about 50% of the cases. The conventional semen analysis recommended by the World Health Organization (WHO) is the cornerstone in the evaluation of male fertility status. It includes macroscopic and microscopic evaluation of the ejaculate, which reflects the production of spermatozoa in the testes, the patency of the duct system and the glandular secretory activity. Evaluation of seminal fructose, sperm vitality and leucocytes (Endtz test) are useful adjuncts to semen analysis that provide information on specific clinical conditions. Though several computer-assisted sperm analysis (CASA) systems have been developed, conventional methods for semen analysis are still widely accepted in clinical practice. This review summarises the conventional techniques used in routine semen analysis and their diagnostic value in clinical andrology.

KEYWORDS

Diagnostic value, male infertility, semen analysis, spermatozoa
identification of reversible medical conditions which can impact fertility (Jungwirth et al., 2018).

In this review, the World Health Organization (WHO, 2010) guidelines for the conventional methods used in routine semen analysis and adjunct tests (e.g., fructose test, sperm vitality tests and Endtz test) are discussed with reference to clinical interpretation of each variable. Furthermore, the diagnostic value and limitations of standard semen analysis as well as technological advances in the field are summarised.

2 | SEMEN

Semen is a heterogeneous fluid released at the time of ejaculation, which is composed of cellular and noncellular fractions. The cellular fraction includes mature spermatozoa, leukocytes, immature germ cells and epithelial cells (in rare pathological cases even erythrocytes), while the noncellular fraction is a water-based seminal plasma constituted by the secretions of epididymis, seminal vesicles, bulbourethral and prostate glands (Milardi, Grande, Vincenzoni, Castagnola, & Marana, 2013).

Testis and epididymis account for about 5% of the total seminal plasma volume (~0.15 ml). The epididymal fraction is rich in neutral α-glucosidase, the main marker of epididymal functionality (Qiu, Chu, Zhang, Luo, & Quan, 2018), and L-carnitine, a molecule involved in the mitochondrial β-oxidation pathway (Li, Li, & Huang, 2007). Other molecules include microRNAs, glyceryl phosphorylcholine and maltase (Hu, Wu, Guo, Li, & Xiong, 2014; Rosecrans, Jeyendran, Perez-Pelaez, & Kennedy, 1987).

Seminal vesicles contribute to 50%–65% of the total ejaculate (1.5–2.0 ml), while the prostatic secretions account for 20%–30% (0.6–0.9 ml) of the ejaculate. The secretion of seminal vesicles is rich in fructose (1.5–6.5 mg/ml), which is involved in sperm energy (0.6–0.9 ml) of the ejaculate. The secretion of seminal vesicles is (1.5–2.0 ml), while the prostatic secretions account for 20%–30% of the total ejaculate, producing a clear secretion rich in mucoproteins (Puppo & Puppo, 2016).

3 | SEMEN ANALYSIS

3.1 | WHO 2010 (5th edition)

The WHO laboratory manual has been the standard reference handbook for semen analysis since 1980. The latest, updated version (5th edition) of the WHO guidelines, published in 2010, provides recommendations to improve the standardisation of semen quality evaluation and the comparability of results obtained by different laboratories. The 2010 edition is composed of three parts: (a) the analysis of semen quality, including routine methods, more advanced optional tests and methods applied for clinics and research; (b) preparation of sperm, including ejaculated spermatozoa as well as those retrieved surgically, with a focus on sperm cryopreservation; and (c) a final section on quality control, to ensure a strict control of such analysis. In comparison with previous editions, the 5th edition focuses mainly on preparation of solutions, procedures, mathematical calculations and interpretations of results in order to explain how to carry out each procedure and reduce the intra- and inter-laboratories variability. The section on the evaluation of sperm count has been revised to consider a minimum of at least 200 spermatozoa for the assessment of correct sperm concentration. The evaluation of sperm motility was changed in the 5th edition to progressive, nonprogressive and immotile spermatozoa in order to reduce the bias linked to the subjectivity of the microscopic evaluation, while previous editions differentiated sperm motility in grades from a to d.

3.1.1 | Reference values

The 5th edition provides updated reference values (Table 1) obtained from semen parameters of 1953 proven fertile men in 8 different countries located on three continents (WHO, 2010). Due to the highly right-skewed distribution of semen parameters, the 5th percentile has been considered as the lower reference value for each variable. However, it must be considered that having a normal semen evaluation does not guarantee the fertility status. Decreased reference values have been reported in the 5th edition compared to the 4th for volume (0.5 ml lower), sperm concentration (5 million/ml lower), motility and morphology (10% lower). These changes in the reference values have had a great impact on the classification of male fertility, especially for those patients who were previously classified as having ‘abnormal’ semen quality but are being regarded as ‘normozoospermic’ according to
the current 5th edition reference values (Alshahrani et al., 2018; Murray et al., 2012).

### 3.1.2 | Strict criteria classification—sperm morphology

In agreement with the previous editions, the 5th edition of the WHO laboratory manual reinforces the sperm morphology ‘strict criteria’ defined in 1988 (Kruger et al., 1988; Menkveld, Stander, Kotze, Kruger, & van Zyl, 1990). It describes the spermatozoa that successfully penetrate the cervical mucus as morphologically ‘normal’ (Kruger et al., 1988). According to the ‘strict criteria’, a morphologically normal spermatozoon is defined as having a smooth oval-shaped head (length: 3–5 µm; width: 2–3 µm) with an acrosome, accounting for 40%–70% of the volume, an axially attached midpiece of 1.5 times the length of the head and a uniform 45 µm long tail, thinner than the intermediate midpiece (Menkveld, Holleboom, & Rhemrev, 2011). Several studies have shown that the normal spermatozoa defined as per the ‘strict criteria’ were able to bind selectively to the zona pellucida, therefore associating good morphology to better reproductive function (Liu & Baker, 1992; Menkveld, Franken, Kruger, Oehninger, & Hodgen, 1991).

The 5th edition has several tables showing abnormalities of sperm head, neck and tail (WHO, 2010). Head defects include abnormal shapes classified as tapered, pyriform, round, amorphous and vacuolated as well as double heads. Abnormal neck and midpiece can be categorised as asymmetrical insertion of the midpiece into the head, abnormally thin, thick, sharply bent or having excessive cytoplasmic residue. Finally, abnormal tail forms can be short, bent, coiled or double. Any borderline morphology should be considered as ‘abnormal’, in order to reduce the degree of operator subjectivity in the microscopic evaluation (Menkveld et al., 2011).

### 3.2 | Sample collection

#### 3.2.1 | Abstinence time and liquefaction

The WHO guidelines recommend sexual abstinence for 2–7 days before sample collection (WHO, 2010). Such range of time may be considered arbitrary as the influence of abstinence time on semen parameters is still debated. Evidence shows that a shorter period of abstinence is associated with an improvement of sperm motility, morphology and DNA integrity, while a longer abstinence time improves semen volume and sperm count (Hanson, Aston, Jenkins, Carrell, & Hotaling, 2018).

Semen evaluation must be carried out after liquefaction, which can occur within 15 min at room temperature after ejaculation, although it may need up to 60 min. Continuous gentle mixing or rotation of the sample (room temperature/incubator at 37°C) aids to obtain a homogeneous watery sample. If the liquefaction of semen does not occur within 60 min, then it is referred to as delayed liquefaction, for which enzymatic or mechanical digestion are considered (WHO, 2010).

#### 3.2.2 | Sample handling—the do’s and the don’ts

The sample handling is crucial for proper evaluation of macro- and microscopic features (Figure 1) of semen (WHO, 2010). Once the sample...
is collected in a sterile, nontoxic container, it should be kept at 37°C to favour liquefaction. If the sample is collected at home, it must be transported to the laboratory within one hour. However, the collection in a private room close to the laboratory is preferable in order to avoid fluctuations in temperature and record the exact time of collection. The collection during sexual intercourse is allowed only if the subject is unable to collect the sample by masturbation. However, commercial latex condoms should be avoided as they contain spermicidal substances (Jones, Kovacs, Harrison, Jennings, & Baker, 1986). The entire ejaculated fraction should be collected, as the loss of a part may result in a false evaluation of semen parameters. Semen evaluation should be conducted within an hour of liquefaction to avoid dehydration or thermal variations from affecting semen quality. Like any other biological fluid, semen should be considered potentially infectious due to the potential presence of agents such as HIV, hepatitis or herpes simplex virus. Therefore, sterile materials, safety guidelines and good laboratory practices must be strictly adhered to reduce biological risk.

3.3 | Macroscopic evaluation of semen—Assessment and clinical interpretation

3.3.1 | Semen age

Semen age is defined as the period between sample collection and initiation of semen analysis following liquefaction. It should not exceed 60 min in order to prevent variation of semen quality due to external factors (WHO, 2010).

3.3.2 | Appearance and colour

Normal liquefied semen should appear homogeneous with a grey-opalescent colour. In case of high sperm concentration, semen appears opaque, while it appears more transparent in case of low sperm concentration. The ejaculate may also appear in red-brown nuances due to the presence of erythrocytes (haematospermia), while a yellowish colour could be due to liver diseases or consumption of certain vitamins/drugs.

3.3.3 | Viscosity

Normally, semen coagulates upon ejaculation and liquefies within 15 min. Viscosity reflects the seminal fluid’s resistance to flow. It is assessed by aspirating the sample into a pipette and allowing it to drop by gravity. A normal semen sample forms small discrete drops, while formation of thread >2 cm is indicative of hyperviscosity. High viscosity interferes with the evaluation of sperm motility and concentration. It can be reduced by diluting the sample with a physiological medium or by the addition of proteolytic enzymes (Du Plessis, Gokul, & Agarwal, 2013). Hyperviscosity is indicative of accessory gland dysfunction resulting in inadequate secretion of prostate and seminal vesicles (Harchegani, Rahmani, Tahmasbpour, & Shahriary, 2019). Furthermore, infection and inflammation of accessory glands are also associated with semen hyperviscosity (Elia et al., 2009). Conversely, absence of coagulum may be due to obstruction in the ejaculatory duct or congenital absence of seminal vesicles (Andrade-Rocha, 2005).

3.3.4 | Volume

Semen volume reflects the overall functionality of the accessory sex glands. The measure of semen volume is a critical step as it determines the calculation of the total number of spermatozoa and other nongerminal cell counts. The WHO (2010) recommends weighing the sample in the same container in which it is collected in order to avoid the loss of sample during the transfer. Then, the volume would be calculated assuming a semen density equal to 1g/ml. Otherwise, the sample can be collected into a graduated cylinder and directly measured. The sample is considered to be normal if the volume of the ejaculate is about 1.5–6.0 ml (WHO, 2010). Incomplete collection, obstruction of ejaculatory ducts, congenital bilateral absence of the vas deferens (CBAVD), partial retrograde ejaculation or hypogonadism can result in low semen volume. On the other side, higher semen volume (hyperspermia) may indicate inflammation of the accessory glands.

3.3.5 | pH

The pH of semen is an indicator of balance between acidic prostatic secretion and alkaline seminal vesicular secretion. For evaluation of the pH, a drop of sample is placed on a pH paper and the resultant colour is compared against the calibration strip. According to the WHO manual, a pH of 7.2 is set as the lower threshold value. In case of CBAVD and obstruction of ejaculatory ducts, the loss of seminal vesicles’ fraction results into a more acidic pH (pH < 7), while a more alkaline pH is usually related to poor sample handling and delay in analysis.

3.4 | Microscopic evaluation of semen—Assessment and clinical interpretation

3.4.1 | Agglutination

Adherence of spermatozoa to each other forming clumps is known as agglutination. This phenomenon affects sperm motility and is graded according to the number of spermatozoa involved and the site of agglutination (head-to-head, tail-to-tail or mixed). Presence of agglutination interferes with the assessment of sperm motility and concentration. It is indicative of the presence of antisperm antibodies and further testing is required to deduce an immunological cause of infertility (Krapez, Hayden, Rutherford, & Balen, 1998).
3.4.2 | Presence of round cells

Besides spermatozoa, round cells can be observed in the ejaculated sample, which include leucocytes and immature germ cells. If the round cell concentration exceeds $1 \times 10^6$/ml, then a differential staining technique should be performed on the semen smear to distinguish leucocytes and immature germ cells (WHO, 2010). Leucocytospermia is reported when the seminal leucocyte concentration is higher than $1 \times 10^6$ cells/ml. Furthermore, round cells can be precisely identified and quantified by detecting peroxidase activity and the antigen CD45 (immunohistochemistry) (WHO, 2010). The presence of round cells in the ejaculated sample is a possible indication of spermatogenic insult and urogenital infection (Brunner, Demeter, & Sindhwani, 2019; Palermo et al., 2016).

3.4.3 | Sperm concentration

Sperm concentration is defined as the number of spermatozoa per unit volume of semen and is directly proportional to the number of sperm discharged and the amount of fluid diluting them. Therefore, sperm concentration is influenced by the functionality of reproductive organs. Sperm concentration is usually assessed by loading an aliquot of liquefied sample into a haemocytometer chamber. Depending on the chamber used, a specific pattern of gridlines is used to count at least 200 spermatozoa per replicate under the phase contrast microscope at ×200 or ×400 magnification.

Often the terms ‘sperm concentration’ and ‘total sperm number’ are confused or misunderstood as synonyms. The total sperm number is a measure of the testicular sperm productivity and is described as the total number of spermatozoa in the entire ejaculate. It is calculated by multiplying sperm concentration and semen volume. The total number of spermatozoa and progressively motile spermatozoa are useful parameters in recommending ART treatment as they indicate the total number of potentially functional spermatozoa available.

A semen sample is defined as oligozoospermic when the sperm concentration is lower than $15 \times 10^6$ spermatozoa per mL and as azoospermic when there are no spermatozoa in the ejaculate (WHO, 2010). In patients suspected for azoospermia, at least two samples should be collected on different days. The samples should be centrifuged, and the pellet examined for spermatozoa. Azoospermia can be classified as obstructive and nonobstructive azoospermia based on the evaluation of the other semen parameters, such as volume, pH and fructose, which are generally lower in case of obstruction (Wosnitzer, Goldstein, & Hardy, 2014).

3.4.4 | Sperm motility

Sperm motility should be assessed immediately following liquefaction in order to avoid dehydration or variation induced by changes in pH or temperature. The WHO 5th edition classifies motility into progressive, nonprogressive and immotile sperm, with the lower reference value of 40% and 32% for total and progressive motility, respectively (WHO, 2010). For the evaluation of motility, an aliquot of liquefied sample is loaded into the counting chamber and examined under phase contrast microscope at ×200 or ×400 magnification. A minimum of 200 spermatozoa in a total of at least 5 fields in each replicate is evaluated. The total number of progressive and nonprogressive motile spermatozoa as well as the total number of immotile sperm is counted to calculate the percentage of total motility.

Although it is not indicative of any specific condition, a reduction in sperm motility under the lower reference value (asthenozoospermia) could be due to infection/inflammation of accessory sex glands, testicular (e.g. varicocele, cryptorchidism and testicular cancer) or related to lifestyle and environmental risk factors, including pollution exposure, alcohol and drug consumption, smoking, psychological stress (Shahrokhi, Salehi, Alysain, Taghiyari, & Deemeh, 2019). These factors increase seminal oxidative stress resulting in the impairment of sperm motility due to peroxidative damage of the sperm plasma membrane or flagellar proteins. Complete absence of motility is observed in men with primary ciliary dyskinesia or antisperm antibodies (Cui et al., 2015; Munro et al., 1994).

3.4.5 | Sperm morphology

Sperm morphology is one of the most challenging sperm parameters to analyse and interpret as there is a significant variation in the shape of spermatozoa within an ejaculate as well as between different ejaculates of the same individual. Nevertheless, the evaluation of sperm morphology is a critical component in determining the sperm quality. The assessment of sperm morphology involves smearing a drop of a well-mixed sample on a slide followed by air-drying, fixing, staining and microscopic examination. The recommended staining solutions include Papanicolau, Shorr or Diff-Quick, which result in differential staining of sperm head, acrosome region, tail and cytoplasmic residue (Henkel et al., 2008; WHO, 2010). Atypical morphology is due to abnormal spermatogenesis or maturation, while specific morphological defects such as globozoospermia, multiple tail abnormalities, headless spermatozoa and fibrous sheath dysplasia are indicative of genetic abnormalities (Gatimel, Moreau, Parinaud, & Léandri, 2017). When samples are evaluated according to strict criteria (lower reference value equal to 4%), an association with reproductive outcomes has been reported, although results are still contradictory due to the high rate of inter-laboratory variations which makes it difficult to use sperm morphology in clinical investigation (Gatimel et al., 2017).

3.5 | Frequency of testing

Due to high biological variability, at least two semen samples should be evaluated to characterise semen quality, particularly...
in case of azoospermia. However, one semen analysis is recommended in case of normal parameters (Jungwirth et al., 2018; WHO, 2010).

4 | ADJUNCT TESTS

4.1 | Seminal fructose

4.1.1 | Fructose test

Under the influence of heat and low pH, fructose reacts with indole and forms a coloured complex, which absorbs light at a wavelength of 470 nm (Toragall, Satapathy, Kadadevaru, & Hiremath, 2019). For this test, 5 µl of seminal plasma is incubated with ZnSO₄ (63 µmol/L) and NaOH (0.1 mol/L) for 15 min at room temperature, to allow deproteinisation. Subsequently, the sample is centrifuged, and an aliquot of the supernatant is incubated with indole reagent and hydrochloric acid at 50°C for 20 min and then cooled on ice for 15 min. The absorbance of the coloured complex is measured spectrophotometrically at 470 nm. The fructose concentration is calculated by comparing the absorbance against a standard curve (WHO, 2010).

4.1.2 | Indications for testing and reference value

Fructose is mainly secreted by seminal vesicles and therefore considered a seminal marker of gland functionality. According to WHO (2010), the lower reference value is 13 µmol per ejaculate. Evaluation of seminal fructose is suggested when an obstruction is suspected. Low levels of seminal fructose along with low sample volume and pH is indicative of obstructive azoospermia. Furthermore, low concentration of seminal fructose can be noted in men with CBAVD or partial retrograde ejaculation (Daudin et al., 2000; WHO, 2010).

4.2 | Sperm vitality

Sperm vitality is determined based on the membrane integrity of the cells and can differentiate live from dead immotile sperm. Sperm vitality assessment is recommended for all samples with less than 40% progressive motile spermatozoa.

4.2.1 | Eosin-Nigrosin staining

Sperm vitality is most commonly assessed by the Eosin-Nigrosin staining (Björndahl, Söderlund, & Kvist, 2003). This method is based on the principle that damaged plasma membrane, as seen in dead spermatozoa, allows the entry of membrane-impermeant stain, while the vital sperms exclude the dye and remain unstained.

A drop of semen sample is mixed with an equal volume of Eosin-Nigrosin solution and smeared on a glass slide. The slides are examined with bright optics at ×1,000 magnification and oil immersion. Nonviable sperm stains light-to-dark pink in colour while the live cells appear white (Figure 2a). The use of nigrosin facilitates the microscopic evaluation by producing a dark-colour background, thus enabling the counting of lightly stained spermatozoa. The number of stained (dead) and unstained (vital) cells are counted in a total of 200 spermatozoa evaluated in each replicate and the percentage of viable cells calculated.

4.2.2 | Hypo-osmotic swelling (HOS) test

The HOS test is an alternative test used to assess sperm vitality. The test differentiates live and dead spermatozoa based on the occurrence of flagellar swelling when spermatozoa are incubated in a hypo-osmotic solution. The HOS test is based on the fact that...
only live cells having an intact, semipermeable membrane can swell under hypo-osmotic conditions. The swelling solution is prepared by mixing sodium citrate dihydrate (0.735 g) and D-fructose (1.351 g) in 100 ml of purified water. An aliquot of the sample is mixed with the hypo-osmotic solution and incubated for 30 min (in case of routine diagnostics) or 5 min (if the spermatozoa are processed for ART) at 37°C. Following incubation, a wet preparation is subjected to examination under phase contrast optics at ×200 or ×400 magnification. A total of 200 spermatozoa are evaluated in each replicate and the results are expressed as a percentage of viable cells. The swollen spermatozoa (viable) are identified by change in the shape of the sperm cells as reflected by coiling of the tail (Figure 2b).

**4.2.3 | Indications for testing and reference value**

Eosin-Nigrosin staining is indicated in case having high percentage of immotile sperm. It is clinically important to assess the vitality results in conjunction with motility results of the same sample. The presence of a high percentage of vital, but nonmotile spermatozoa, may be suggestive of structural defects in flagella, whereas a larger proportion of nonviable and immotile spermatozoa may indicate epididymal pathology (Chemes & Rawe, 2003; Correa-Pérez, Fernández-Pelegrina, Aslanis, & Zavos, 2004). Since the HOS test does not kill or damage spermatozoa, it is indicated when staining of spermatozoa must be avoided, for example when selecting spermatozoa for intracytoplasmic sperm injection (ICSI) (Cincik et al., 2007; WHO, 2010). The WHO manual reports 58% as the lower reference limit for sperm vitality and suggests to analyse the semen sample soon after liquefaction in order to avoid the adverse effects of dehydration or fluctuations in temperature or pH on vitality (WHO, 2010).

**4.3 | Endtz test**

**4.3.1 | Principle and methodology**

Polymorphonuclear leucocytes (PMN, neutrophils) are the predominant form of leucocytes in the human ejaculate. The Endtz test is a simple and inexpensive method used for screening granulocytes based on the histochemical identification of peroxidase enzyme, which is characteristic of granulocytes. This test involves ortho-toluidine staining of peroxidase-positive cells and is useful in distinguishing leucocytes from the other round cells, which are peroxidase free. Furthermore, this technique fails to detect activated polymorphs that have released their granules as well as other peroxidase-negative leucocytes, such as lymphocytes, macrophages and monocytes (WHO, 2010). In this method, an aliquot of semen sample (20 µl) is mixed with twice the volume of Endtz solution and incubated for 5 min at room temperature in the dark. Following incubation, an aliquot of the mixture is loaded onto a hemocytometer chamber and examined for dark brown-stained

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Kinematic parameter</th>
<th>Description</th>
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<tbody>
<tr>
<td>Sperm motion velocity</td>
<td>Curvilinear velocity (VCL)</td>
<td>The velocity along a curvilinear path, considering the movement of the head</td>
</tr>
<tr>
<td></td>
<td>Straight-line velocity (VSL)</td>
<td>The velocity along a straight-line path between the first detected position and the last one</td>
</tr>
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<td></td>
<td>Average path motility (VAP)</td>
<td>The average velocity of the sperm head along the entire path</td>
</tr>
<tr>
<td>Sperm velocity ratio</td>
<td>Linearity (LIN)</td>
<td>The linearity of a curvilinear path, calculated as VSL/VCL</td>
</tr>
<tr>
<td></td>
<td>Wobble (WOB)</td>
<td>A measure of oscillation of the actual path about the average path, calculated as VAP/VCL</td>
</tr>
<tr>
<td></td>
<td>Straightness (STR)</td>
<td>Linearity of the average path, calculated as VSL/VAP</td>
</tr>
<tr>
<td>Sperm wobble</td>
<td>Amplitude of lateral head displacement (ALH)</td>
<td>The magnitude of lateral displacement of the sperm head along its average path</td>
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<tr>
<td></td>
<td>Beat-cross frequency (BCF)</td>
<td>The average rate at which the curvilinear path crosses the average path</td>
</tr>
<tr>
<td></td>
<td>Mean angular displacement (MAD)</td>
<td>The time-averaged absolute values of the instantaneous turning angle of the sperm head along its curvilinear trajectory</td>
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</table>

*aAdapted from WHO guidelines (WHO, 2010).*
peroxidase-positive cells under a phase contrast optics at ×200 or ×400 magnification.

4.3.2 | Indications and reference value

The Endtz test is performed when the seminal concentration of round cells is higher than 1 × 10⁶ cells/ml (WHO, 2010). The condition of leucocytospermia is defined as a seminal leucocyte concentration that is higher than 1 × 10⁶ cells/ml and has been associated with genital infections/inflammatory conditions, high antisperm antibodies and elevated levels of ROS (Lackner et al., 2006; Mahfouz et al., 2010).

5 | COMPUTER-ASSISTED SPERM ANALYSIS (CASA)

5.1 | Manual assessment versus CASA

The CASA instruments were first introduced to the market in the 1980s with the objective of reducing subjectivity, labour and time associated with semen evaluation as well as to decrease the inter- and intra-observer variability (Mortimer, 2000). These systems are based on capturing the image of the microscopic field as frames (series of pictures), which are digitalised and subsequently analysed using an algorithm. Several manufacturers have produced semi- or fully automated CASA systems having different algorithms. Generally, these systems can assess sperm motility and kinematics. Until recently, measurement of sperm concentration using CASA was not possible due to difficulties in differentiating spermatozoa from particulate debris. However, recent advancements in CASA equipped with a fluorescent microscope can assess sperm concentration as well as concentration of progressively motile spermatozoa with the use of fluorescent DNA stain and a tail detection algorithm (Hu, Lu, Shao, Huang, & Lü, 2013; Zinaman, Uhler, Vertuno, Fisher, & Clegg, 1996). High precision and detailed analysis of sperm kinematic parameters are the two major advantages of CASA over manual methods.

5.2 | Parameters evaluated and their significance

The majority of the CASA system allows analysis of classic sperm characteristics (sperm concentration, motility, morphology and vitality) as well as sperm kinematics, which cannot be determined by microscopic evaluation. The characteristics of sperm kinematics are defined by sperm motion velocity, velocity ratios and wobble (Table 2). The assessment of these multiple parameters of sperm kinematics allows a detailed four grade categorisation [Grade 1—immotile; Grade 2—nonprogressive; Grade 3—slowly progressive; Grade 4—rapidly progressive] of sperm motility when compared to a three-grade classification proposed by the WHO for conventional assessment. Classifying progressive motility into rapid and slow by manual assessment is not possible without significant bias.

Generally, CASA systems classify the sperm head and midpiece as normal or abnormal based on the dimension of head and midpiece, head ellipticity and regularity, and the measurement of the acrosome area using different staining methods. The automated systems have greater precision and reproducibility than manual assessment. However, the accuracy of sperm morphometric assessment is compromised by methodological discrepancies and technical difficulties in distinguishing sperm head from debris, especially when sperm concentration is low. The pros and cons of CASA are depicted in Table 3.

6 | DIAGNOSTIC VALUE OF SEMEN ANALYSIS

6.1 | What can routine semen analysis offer in the investigation of male infertility?

A standard semen analysis is the first test prescribed by a clinician to assess male factor infertility in a couple. The lower reference values provided by the WHO are used to guide the diagnosis and treatment of the male partner. Diagnosis of normozoospermia may push the investigation towards the female partner as the primary cause

<table>
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<th>Pros</th>
<th>Cons</th>
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<tbody>
<tr>
<td>Rapid analysis of larger number of samples</td>
<td>Expensive instrumentation and requires well-trained personnel</td>
</tr>
<tr>
<td>Decreased subjectivity</td>
<td>Multiple biological factors (semen hyperviscosity, presence of round cells, debris and sperm agglutination) interfere with CASA analysis</td>
</tr>
<tr>
<td>Increased reproducibility</td>
<td>Precision of sperm concentration measurement decreases outside an optimal range (2–50 × 10⁶/ml)</td>
</tr>
<tr>
<td>Allows detailed evaluation of sperm motion characteristics</td>
<td>Low accuracy in sperm morphometric assessment</td>
</tr>
<tr>
<td>Allows re-analysis of recorded videos and pictures at a later time point</td>
<td>No comparability of results across instruments due to lack of standardisation of CASA systems</td>
</tr>
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</table>

TABLE 3 Pros and Cons of CASA
of infertility, while a semen sample with altered sperm parameters suggests male factor as a putative cause for infertility and guide towards more advanced investigations, genetic testing and treatments (Wang & Swerdloff, 2014). Furthermore, morphological abnormalities such as globozoospermia, macrocephaly, multiple tail defects and decapitated sperm syndrome are indicative of genetic disorders (Gatimel et al., 2017).

Semen analysis is also useful in monitoring spermatogenesis in men undergoing fertility regulation (WHO, 2010). Prospective studies conducted in couples who discontinued contraception and were trying to conceive revealed a significant association between sperm parameters (sperm count and percentage of morphologically normal sperm) and time to pregnancy, a marker of couple fecundity (Buck Louis et al., 2014; Slama et al., 2002). Sperm morphology can be highly predictive of fertilisation and pregnancy rates after intrauterine insemination (IUI) (Kruger & Coetzee, 1999; Van Waart, Kruger, Lombard, & Ombelet, 2001), although evidence is still contradictory (Lemmens et al., 2016; Thijsen et al., 2017). However, sperm abnormalities are not related to reproductive outcomes in case of ICSI, reducing the power of conventional semen analysis in predicting fertility (Nagy et al., 1995; Oehninger et al., 1995).

6.2 | Limitations of semen analysis

Since the semen characteristics that distinguish fertile and infertile men are poorly defined, semen analysis cannot accurately predict male infertility. About 15% of infertile men have semen parameters within the normal reference range and are categorised as unexplained infertility (Esteves, Sharma, Gosálvez, & Agarwal, 2014; Hamada, Esteves, Nizza, & Agarwal, 2012). Normally, spermatozoa undergo several changes during their transit through the female reproductive tract before fertilising the oocyte. However, standard semen analysis cannot assess the ability of spermatozoa to undergo capacitation, acrosome reaction and hyperactivation as well as its potential to fertilise the oocyte (Cho, 2012; Inoue, Ikawa, & Okabe, 2011). Therefore, conventional semen analysis has a limited predictive value for reproductive outcomes. Furthermore, the conventional semen analysis suffers from subjectivity and poor standardisation of human manual analysis. A large number of laboratories do not strictly adhere to guidelines, reducing the diagnostic power of semen analysis (Filimberti et al., 2013; Mallidis et al., 2012; Punjabi et al., 2016). Improper sample collection as well as lack of adherence to the recommended length of abstinence and time of delivery to the laboratory may cause bias in semen evaluation.

The validity of the 2010 WHO reference values are extensively debated. Several clinicians and researchers have criticised the lower cut-off values as being too low, with reference values being derived based on the semen characteristics of only fertile men, lack of consideration of a broader geographical diversity and ethnic differences, all of which could influence semen parameters (Cooper et al., 2010; Esteves, 2014; Esteves et al., 2012; Murray et al., 2012). Finally, diagnosis of infertility based on semen analysis can be challenging due to inherent biological variability between semen ejaculates (Leushuis et al., 2010). Hence, the WHO guidelines (2010) recommend the examination of at least two semen samples to obtain a representative evaluation of semen quality (WHO, 2010).

7 | TECHNOLOGICAL ADVANCES IN SEMEN ANALYSIS

Technological advancements have been facilitated by the development of several home-based semen analysis systems, which allow the in-house evaluation of semen (Yu et al., 2018). The home-testing systems are based on antibody reaction, microfluidics or smartphone apps (e.g. Sandstone Diagnostics’ Trak device and the Yo Sperm Test) (Vij & Agarwal, 2017; Yu et al., 2018). Although these systems are far from being perfect, they can minimise possible errors due to sample transportation and handling. Other benefits include cost-effectiveness, privacy and quick response to fertility-related queries. Furthermore, home-based tests provide a preliminary idea on the fertility status and indicate whether a user should or need not pursue further testing, which is particularly useful for men who are hesitant to seek medical evaluation as well as post-vasectomy patients. However, most of these systems provide information about one semen parameter, such as sperm concentration (e.g. SpermCheck Fertility Test) or motile sperm concentration (e.g. Fertell Male Fertility Home Test, SwimCount Sperm Quality Test) or a few (e.g. Micra Sperm Test, paper-based devices or microfluidic devices), but not all sperm functional parameters pertinent to fertility testing (Yu et al., 2018). Therefore, home-based semen analysis systems are not a replacement of conventional semen analysis as home-based tests provide only rudimentary results based on a few parameters and are prone to false-negative results (Coppola et al., 2010; Yu et al., 2018).

8 | CONCLUSION

The conventional semen analysis is the first and key step in the evaluation of the male fertility potential. However, it is a poor indicator of reproductive outcomes. Alterations in multiple semen parameters are indicative of putative sperm dysfunction and associated impairment of male fertility. Although several CASA systems have been developed with the objective of reducing time and subjectivity related to manual semen analysis, lack of standard guidelines and high cost associated with CASA limits its application in clinical practice. Technological advancements have resulted in the development of several home semen testing systems that serve as a quick and cost-effective self-screening tool for male fertility.

9 | TAKE-HOME MESSAGE

- Semen analysis is the initial and fundamental step in the evaluation of the male fertility potential.
• Semen analysis reflects the testicular production of spermatozoa as well as the patency of the duct system and glandular secretory activity.

• Macro- and microscopic evaluation of semen is conducted according to the most recent guidelines of WHO (5th edition, 2010)

• The pitfalls of conventional semen analysis limit its diagnostic power in the clinical setting.

REFERENCES


injection is not related to any of the three basic sperm parameters. *Human Reproduction*, 10(5), 1123–1129.


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