<u>**Review paper**</u>

FROM BASIC TO CONTEMPORARY SEMEN ANALYSIS: LIMITATIONS AND VARIABILITY

A. Moazzam [1,2], M. N. Choudhary[1], I. Muhammad[2], J. Sarwat[2] and A. Ijaz[1]

[1]Center for Research in Molecular Medicine, University of Lahore, Pakistan
[2] Department of Animal Sciences, Quaid-i-Azam University, Islamabad, Pakistan
Corresponding author E-mail: drarozia@yahoo.com

ABSTRACT

Fertility has been a core issue for the survival of human race since pre-historic ancient times. A couple who has failed to conceive or has been unable to induce pregnancy within one year of regular unprotected intercourse in the fertile phase of menstrual cycle are said to be infertile. Of the total infertile couples who seek evaluation for infertility, a large part of infertility is accounted by the male factor A general and obvious cause of male infertility is impaired spermatozoal function. Semen analysis is the first step towards assessment of a male's reproductive potential. Analysis of seminal parameters help in providing important clinical information regarding spermatogenesis, the functional competence of spermatozoa and also the secretory pattern of accessory genital glands. Although the clinicians base their initial diagnosis upon the fundamental information provided by analysis of these sperm parameters, yet more advanced sperm function tests are required for an exact diagnosis of the root cause of infertility in the male.

Key words: Semen Analysis, Leukocytospermia, Computer Aided Sperm Analyzer, World Health Organization.

SPERM FUNCTION TESTING: Semen analysis, the first step in diagnosis of male factor infertility, not only evaluates the spermatozoa but also the seminal plasma and non-sperm cells (Samplaski *et al*, 2010). An ideal sperm helps in (i) diagnosis of a specific spermatozoal dysfunction; (ii) prediction of fertilization or pregnancy rates and (iii) indication of specific therapies for alleviation of the identified dysfunctional spermatozoa (Muller, 2000). Assessment of seminal parameters is helpful in investigation of male factor infertility, genital tract infections and pathologies (Comhaire and Vermeulen, 1995; Silber, 2000). Semen analysis is useful in evaluation of adverse effects of drugs, environmental pollutants and chemical products affecting fertility of males (Sharpe, 2000; Bonde and Storgaard, 2002). A wide variety of semen parameters measured by the semen analysis, are an indicator of the semen quality and can reduce the number of variables evaluated (Aitken *et al*, 1982; Carrell, 2000; Krause 1995; De Jonge, 1999). Considering that a history of infertility is the major reason for semen analysis, it is necessary that methodologies employed for semen analysis should be standardized. Practicing a routine semen analysis is the first step towards determination of the influence of genital pathophysiology on the reproductive capacity of male, even then certain parameters might not be of any clinical significance (World Health Organization, 1999).

It is well-documented that accuracy of semen analysis, standardization of proper methodologies and procedures and reference values, all contribute towards the quality control in laboratory practice. Currently, the World Health Organization (WHO) has standardized basic requirements of semen analysis providing guidelines that describe several diagnostic procedures and andrology techniques for evaluation of semen quality that have gained worldwide acceptance as guidance to standardized methodology for human semen analysis and help in assessment of semen parameters, like, concentration, motility and morphology (WHO, 1987; 1992; 1999). Therefore, a basic semen analysis takes help of semen parameters towards formulation of a diagnostic work-up in accordance with the clinical evaluation of a male presenting for subfertility (Andrade-Rocha, 2003; WHO, 1999).

SEMEN PARAMETERS

Sperm Density: Total sperm number is defined as the total number of spermatozoa in the entire ejaculate and is obtained by multiplying the sperm concentration by the seminal volume (WHO, 2010).

Sperm Concentration: Sperm concentration is referred to as the number of spermatozoa per unit volume of the semen and is an indicator of the number of the spermatozoa ejaculated and the volume of the fluid that dilutes them. This determines the amount of spermatozoa present in the semen specimen and is expressed in sperms/milliliter (mL). It is further sub-divided as follows: (Andrade-Rocha, 2003).

Polyzoospermia: is defined as the presence of a sperm concentration between 250-350 x 10^6 /mL but with normal sperm parameters - motility, morphology and viability. In sperm counts greater than 350 x 10^6 /mL, polyzoospermia may be associated with asthenozoospermia and/or teratozoospermia (Andrade-Rocha, 1994). Polyazoospermia is considered a pathological finding not only because of an overproduction of spermatozoa, but also for its association with decreased reproductive performance as a result of dysfunctional acrosomal membrane (To¨pfer-Petersen *et al*, 1987), chromosomal abnormalities (Chan *et al*, 1986) and decreased ATP content (Calamera *et al*, 1987).

Normozoospermia: Although thesperm count is between 20-250 x 10^6 /mL, several disorders, e.g, leukocytospermia, antisperm antibodies, abnormal functional activity of the seminal vesicles and the prostate gland, genital tract infections varicocele, can cause impairment of semen quality in normozoospermic males, leading to asthenozoospermia and /or teratozoospermia (Andrade-Rocha, 2003).

Oligozoopsermia: Sperm counts may vary between 10-20x 10^6 /mL in mild, 5-10 x 10^6 /mL in moderate and < 5 x 10^6 /mL in severe oligozoospermia (Andrade-Rocha, 2003). Functional disturbances of the testis, e.g endocrine disorders varicocele and as well as factors of non-testicular origin, e.g drug toxicity, environmental pollutants, mumps orchitis, radiation and exposure to chemical products all are involved in the causation of mild and moderate oligozoospermia (Merino *et al*,1995). Severe oligozoospermia is associated with genetic abnormalities, such as Y chromosome microdeletions (Dohle *et al*, 2002). Oligozoospermia is associated with abnormal sperm morphology and decreased sperm motility, hence deteriorating semen quality and its fertilization capacity. However, these males have the natural ability to fertilize naturally, even in severe oligozoopsermic conditions (Matorras *et al*, 1996).

Azoospermia: Differential diagnosis of azoospermia is based on physical examination of the male, testicular biopsy, endocrine evaluation and genetic screening. Azoospermia is classified into two types, for diagnostic purposes, (i) non-obstructive or secretory resulting as a cause of extreme testicular failure and (ii) obstructive or excretory caused by occlusion of the testis, epididymis and excretory ducts, hence preventing the release of spermatozoa in the seminal ejaculate (Kolettis, 2002). Microdeletions of the Y chromosome may also be involved in the pathogenesis of azoopsermia (Dohle *et al*, 2002). Congenital bilateral absence of the vas deferens and the seminal vesicles as a result of cystic fibrosis gene mutation is a special case of azoospermia. Inspite of its rarity, this pathology is easily identifiable by the presence

of elevated levels of prostatic biomarkers, absence of seminal vesicle markers, a seminal ph<7.0 and a seminal volume of 1.0mL (Daudin *et al*, 2000).

Sperm Motility: Spermatozoa do not exhibit progressive motility, on reaching the caput of the epididymis from the seminiferous tubules and the rete testis. Once exposed to the microenvironment of epidiymis, occurance of molecular changes in the spermatozoa lead to increased capacity of forward progressive and sustained motility. Mature and motile sperm remain stored within the cauda epididymis, in a quiescent phase and release at ejaculation after acquiring an instantaneous burst of vigorous activity (Cooper, 1996; Moore, 1998). Analysis of sperm motility gives information on epididymal function. Sperm motility depends on the quality of the spermatozoa produced and hence related directly with testicular function. The secretions by the prostate also influence the sperm and seminal vesicles. Therefore, sperm motility is affected by functional disorders of the genitalia (Andrade-Rocha, 1994).

Sperm Morphology: This important parameter of semen evaluates the quality of the sperm and is divided into the following criteria:

(i)The WHO Criteria: World Health Organization, (1999), Criteria describes the percentage of normal oval sperm heads, as well as a variety of sperm defects present in the semen. Assessment of sperm morphology using this criterion thoroughly evaluates the sperm head, midpiece and tail defects, indicating abnormal spermatogenesis and associated seminal pathologies (Moench and Holt, 1931; Hartman *et al*, 1964; Zamboni, 1987; Bartoov *et al*, 1980).

(ii)The Tygerberg Strict Criteria: The Tygerberg Strict Criteria (Kruger *et al*, 1986) defines the sperm morphology by evaluation of the acrosomal status of the sperm membrane. According to this criterion, spermatozoa having an oval head and a well-defined acrosome covering 40-70% of the sperm head are classified as being normal. Semen having > 14% spermatozoa with normal morphology present a good prognosis for in vivo as well as invitro fertilization. Values ranging from 4-14% also indicate good prognosis but a decreased rate of fertilization than semen with more number of normal spermatozoa. Value of normal spermatozoa <4% indicates poor prognosis (Andrade-Rocha, 2003).

Leukocytospermia: The epididymis, prostate, seminal vesicles and bulbourethral glands, together, contribute to the formation of the seminal fluid (Vivas-Acevedo *et al*, 2010). Prostate and epididymis are considered as the major sources of seminal leukocytes (Simbini *et al*, 1998). Hughes *et al*, (1981), identified three different types of leukocytes, capable of phagocytizing

spermatozoa: (i) polymorphonuclear cells about 10-12 um in diameter, (ii) large macrophages, about 30um which are capable of engulfing numerous spermatozoa and (iii) smaller macrophages/monocytes having a 10-12um diameter. Leukocytes present generally in most ejaculates, play an important role in phagocytic clearance and immunosurveillance of abnormal spermatozoa (Tomlinson *et al*, 1992). Genital tract infection is confirmed by the presence of an increased concentration of leukocytes in the semen and has an association with an increased immature germ cell concentration (Sigman and Lopes, 1993).

Leukocytospermia, defined as >1 x 10 millionWBC/mL is correlated negatively with different parameters of sperm function, especially with impaired sperm motility and morphology, acrosomal membrane damage and sperm tail defects. Presence of leukocytes in the epididymis, seminal vesicles, urethra and prostate is a physiological process required for elimination of abnormal germ cells from the seminal ejaculate (Aziz *et al*, 2004; Wolff, 1995). High leukocyte content causes an increased generation of toxic metabolites exceeding the neutralizing capacity of antioxidants present in the seminal plasma, leading to generation of oxidative stress (Andrade-Rocha, 2003). Quantification of seminal leukocytes constitutes an important part of the standard semen analysis, but it may be difficult to see them under the light microscope. The Endtz test stains for peroxidase within the polymorphonuclear granulocytes, distinguishing them from immature germ cells (Shekarriz *et al*, 1995).

The diagnosis of leukocytospermia has been done earlier by immunohistochemical, cytochemical and morphological techniques (Wolff *et al*, 1992; Jochum *et al*, 1986). The Endtz Test has been recommended by the WHO for determination of WBCs in the semen and is based on the peroxidase activity of polymorphonuclear leukocytes (WHO, 1987). It is a simple, cost-effective test, but the only limitation is the lack of lymphocyte detection in the semen. Large numbers of contaminating leukocytes are indicative of poor semen quality and have been implicated as a possible cause of male infertility (Van der Ven *et al*, 1987; Wolff *et al*, 1990). However, neutrophils and macrophages are the main peroxidase positive cells which are important in diagnosis as they are the source of reactive oxygen species by phagocytosis (Wolff *et al*, 1992; Agarwal *et al*, 1994).

Contemporary methods of semen analysis: Currently methods employed for evaluation of human semen vary substantially, ranging from those recommended by the World Health Organization (WHO), to advanced automated technology, for example, Computer Aided Sperm Analyzer (CASA), for characteristics of sperm motility, morphology and analysis of other physical and biochemical parameters (Boyle *et al*, 1992; Barratt *et al*,

1993; Macleod and Irvine, 1995). Computer aided sperm analyzers (CASA) has been developed which uses digital image analysis for automated analysis of the semen (Mortimer, 2000). CASA provides a rapid measurement of individual "classical" sperm parameters, for example, sperm count and motility (Krause, 1995) and allowing determination of sperm motion characteristics called "kinematics" that cannot be determined under light microscopy (Davis and Katz, 1993; Boyle *et al*, 1992). The application of this technology has been challenged due to errors in its setup and detection of specific objects (Davis and Katz, 1993). The only advantage of CASA is that this system, through serial digital images, plots the movements of the sperm head, showing motion kinetics of the sperm that are not possibly assessed through routine microscopy (Kay and Robertson, 1998). Several studies have emphasized the clinical value of sperm kinetics in diagnosis of males with unexplained infertility and prediction of in vivo and in vitro fertilization rates (Consensus Workshop on Advanced Diagnostic Andrology Techniques, 1996; Peedicayil *et al*, 1997; Shibahara *et al*, 2004). Despite the clinical importance of assessment of sperm kinetics, it is still believed that individual sperm motility parameters hold little importance (Consensus Workshop on Advanced Diagnostic Andrology Techniques, 1996). The difference in various models of CASA instruments and difference in their setup have made it impossible to reach to a conclusion (Sukcharoen *et al*, 1995). A comparison of the measurements obtained by using CASA with those obtained by manual semen analysis may show discrepancy because of the difference in methodologies (Capri Workshop Group ESHRE, 2000). The use of CASA has been criticized because of difficulties in operating the equipment (Boone *et al*, 2000; Carrell, 2000; Oehninger *et al*, 2000) and because of difficulties in achieving optimum set-up procedures (Davis and Katz, 1993; Mortimer, 1994; Clements *et al*, 1995). Semen analysis with automated equipment may help in avoiding biases and intra- and inter- laboratory variability using the manual methodologies (Barroso *et al*, 1999). However, these computerized systems still have problems in their development and hence not recommended for routine use (Wang *et al*, 1991; Kruger *et al*, 1995; Davis *et al*, 1992; Davis and Gravance, 1994).

The most widely used semen parameter is sperm count. Males having a sperm count less than 20 million spermatozoa/mL are categorized as "subfertile", while males with a sperm count less than 5 million spermatozoa/mL are considered "infertile". Similarly, semen samples having less than 14% of normal sperm morphology, according to Strict criteria are "subfertile" while, males containing less than 5% sperms with normal morphology are categorized as "severely impaired" and recommended for donor insemination (Agarwal *et al*, 2003). Criticism on the reported predicted values of

sperm count is a result of day-to-day variation in the sperm concentration (Huszar *et al*, 1988a, b).

Sperm function testing seems to have lost significance in the era of assisted reproductive technology. Most couples seeking infertility treatment are opting for inexpensive and less invasive solutions that would increase the success rate for a spontaneous pregnancy or pregnancy through assisted reproductive treatment strategy (Muller, 2000). Despite the development of several sperm function tests, very few are being adopted in the clinical settings as none of them has been proved to be a reliable predictor of the fertility status of the male (Agarwal *et al*, 2008a).

The sperm-cervical mucus penetration test (SMTP), which measures the ability of the spermatozoa to swim in the cervical mucus has a different reference range, hence creating a discrepancy in interpretation of results (Kremer and Jager, 1992).

The presence of anti-sperm antibodies (ASA) has a negative effect on human fertility (Naz and Menge, 1994). However, the testing for anti-sperm antibodies still remains controversial because of the variability in application of different techniques as well as interpretation of results. Previously used methods are all obsolete, with only the mixed antiglobulin reaction (MAR) test and the immunobead test (IBT) being used to detect the presence of ASA (World Health Organization, 1999). Hence, it can be said that ASA testing plays a limited role in diagnosis of cases of unexplained fertility or severe asthenozoospermia (Agarwal and Said, 2011).

The European Society of Human Reproduction and Embryology (ESHRE) and World Health Organization (WHO), have included Sperm-Binding Assays and Induced Acrosome Reaction as additive tests helping in prediction of fertilization outcomes as well as diagnostic applicability in the clinical settings (ESHRE, 1996; Oehninger *et al*, (2000). Esterhuizan *et al*, (2000), pointed out that evaluation of the sperm morphology; especially the acrosomal configuration gives a good estimate of the fertilizing ability of the sperm. (Menkveld *et al*, 2001), concluded that the acrosomal status is a reflection of the fertilizing ability of the sperm. Determining the acrosome reaction is important for the diagnostic and therapeutic strategies in infertile couples opting treatment through assisted reproductive technology (Franken *et al*, 1997).The human sperm-oocyte interaction in vitro assay, first described by Overstreet and Hembree, (1976), was developed for evaluation of zona penetration and outlined procedures for the hemi-zona assay (Burkman *et al*, 1988) and intact zona pellucida binding test (Liu and Baker, 1992).

The Hypo-Osmotic Swelling Test determines the function of the sperm plasma membrane and is indicated in infertile patients having very few or no motile sperm in the semen (Jeyendran *et al*, 1984). This assay is generally used for assessment of sperm viability,

immotile cilia syndrome (Peeraer *et al*, 2004).or severe asthenozoospermia (Franken and Oehinger, 2012).

The results of zona-free hamster oocyte sperm penetration (SPA) assay, which examines the ability of spermatozoa for capacitation and used for prediction of the likelihood of spontaneous pregnancy in vivo as well as successful fertilization using IVF, are not considered meaningful because of the false positive and false negative rates (Consensus Workshop on Advanced Diagnostic Andrology Techniques, 1996).

Variability in semen analysis: According to a consensus, the most important step in the investigation of male infertility is the basic semen analysis that has been interpreted using the World Health Organization (WHO) Criteria (Agarwal and Said, 2011). For standardization and consistency in the laboratory procedures, WHO has been publishing manuals for the examination of human semen and semen-cervical mucus interaction. These manuals identify exclusion criteria, such as time of spermatogenic cycle, temperature, abstinence and patient health corresponding with the spermatogenic cycles. The manuals have been regularly updated since, 1980, 1987, 1992 and 1999, (Lewis, 2007). The consistency in results in different laboratories is because of addition of normal reference values from these WHO manuals. However, it is important to note that values were identified in healthy fertile men rather than men who were at the verge of subfertility. However, the data was obtained from laboratories who used different methodologies for semen analysis. This heterogeneity further decreased the clinical significance of the standard reference values established by the WHO (Alvarez *et al*, 2003, Jorgensen *et al*, 2001) because although these reference values were from men who had fathered children, yet these studies lacked actual reference ranges and limits. The lack of consensus between different laboratories, these reference values were considered either too high or too low, further subdividing a group of fertile men as subfertile (Barratt *et al*, 1988, Chia *et al*, 1998, Gao *et al*, 2007, 2008). In addition to treating the subfertile men, fertile men with a low semen quality may also be investigated and treated subsequently (Lemcke *et al*, 1997).

The data has recently been modified in men, based on the evaluation of one thousand nine hundred and fifty three men whose partners conceived within a period of one year. These men had 1.5mL semen volume, 39 million/ejaculate total sperm count, 15million/mL sperm concentration, 58% vitality, 40% total motility with 32% total progressive motility and 4% normal sperm morphology (Cooper *et al*, 2010). Previous data indicates subtle variations existing in semen characteristics from different geographic areas as well as between samples from the same individual (Alvarez *et al*, 2003, Jorgensen *et al*, 2001). However, there is still controversy about certain aspects of the 2010 WHO manual. Eliasson *et al*,

(2010), has recommended evaluation of sperm morphology and progressive motility. This evaluation of sperm count, motility and morphology could be improved by applying standardized procedures training workshops and thorough quality control schemes (Franken *et al*, 2003, 2006, 2007). Assessment of sperm membrane integrity is important for dead spermatozoa or alive spermatozoa with <40% progressive motility. Additionally a one-step eosin-nigrosin staining may also be required, especially in cases where ICSI is recommended (Bjorndahl, 2003).

Limitations of semen analysis: Although, manual semen analysis, using light microscopy, is an easy test to perform, accuracy in technique is important for accurate interpretation of results (Keel and Webster, 1990; Mortimer, 1990). However, manual analysis is prone to inter and intra-observer technical variations (Keel and Webster, 1990). International standardization of the essential sperm parameters – count, motility and normal sperm morphology, organization of international training workshops and establishment of external quality control plans will improve semen analysis (Franken *et al*, 2003; Franken and Kruger, 2006; Franken and Dada, 2007). Adequate training of technicians is necessary for consistency of results within a given laboratory (Barosso *et al*, 1999). This inter-laboratory variation maybe the result of different factors (i) different methodology of preparing the semen and seminal smears (ii) difference in interpretation of results (iii) experience of the technician (Coetzee *et al*, 1999).

Semen analysis has several limitations for epidemiological studies of male fertility. More importantly, the selection criteria, recruitment and sample collection, preparation and processing of the samples, uniformity in protocols, quality assurance and well-trained personnel, should be the same for the study group as well as for the reference group. Nevertheless, there are advantages also, for example, such studies allow the study of male fertility independent of any attempts made for obtaining a pregnancy, establishing a relationship between semen quality and fertility (Macleod, 1979; Bonde, 1996; Jørgensen *et al*, 2001; Andersen *et al*, 2000).

Conclusion: Manual semen analysis is still considered the most reliable method for assessment of sperm parameters-count, motility and morphology to date. An ideal sperm function test should be able to diagnose the exact cause of spermatozoal dysfunction, should be able to predict the fertilization and pregnancy rate and guide the clinicians in designing therapeutic strategies for treatment of these infertile males. Addition of advanced sperm function tests to the conventional semen analysis might eventually be useful in the clinical settings. However, more information is still required to determine the extent of predicting the fertility potential by these tests.

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