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Review

Comparison of different methods for assessing sperm concentration in infertility workup: A review

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Sperm count assessments form the essential component of the diagnostic and prognostic evaluation of male fertility, according to guidelines of WHO (1992). The problem of subjective bias, inter and intra operator variability of reporting is discussed in this paper. The problem of inter operator variability has been improved and reproducibility has been made more objective with the introduction of computer-assisted semen analysis (CASA) protocols. To overcome the stated limitations and achieve objective assessment with a high precision, a new technique called flow cytometry was developed. Different methods for the estimation of sperm concentrations like hemocytometry, spectrophotometry, microcells, plate reader, image analysis and finally flow cytometry are compared and contrasted. Their relative merits and demerits are discussed with a detailed review of literature. Methods for estimation of sperm concentration are discussed in this paper.

Key words: Sperm counts, semen analysis, flow cytometry.

INTRODUCTION

Reproductive biology needs accurate and precise determination of sperm counts to achieve any success rate (Foote et al., 1978; Fenton et al., 1990; Woelders, 1991; Evenson et al., 1993; Donoghue et al., 1996). Microscopic estimation of sperm counts is the oldest and the simplest procedure of the semen analysis. This analysis is performed routinely by toxicology laboratories and by veterinary insemination centers, in addition to human infertility clinics (Graham, 1994; Vetter et al., 1998; Auger et al., 2000). This method of estimation of human spermatozoa suffers from many drawbacks like subjective bias. The subjective aspect when compounded by low sperm counts leads to variability in intra and inter laboratory results (Auger et al., 2000). This problem is discussed in detail in this paper. The problem of inter operator variability has been improved and reproducibility has been made more objective with the introduction of computer-assisted semen analysis (CASA) protocols (Davis and Katz, 1992; Krause et al., 1993; Holt et al., 1994). But here, small changes in instrument settings and assay conditions were used to affect significantly the objectivity of these measurements. The present review discusses the methods for estimation of sperm concentration.

METHODS FOR ESTIMATION OF SPERM CONCENTRATION

The initial method used in most centers is to estimate the sperm concentration by a hemocytometric count or by a spectrophotometric determination of turbidity of a measured dilution of a sample of semen (Foote et al., 1978; Woelders, 1991; Donoghue et al., 1996). Evenson et al. (1982) have pointed out that many artifacts such as cytoplasmic droplets and other debris can adversely affect

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the accuracy of these measurements. Although electronic sperm cell counters can be used for a rapid estimation of sperm counts, Evenson et al. (1993) have demonstrated that any cellular debris in the same range as the size of a spermatozoon can interfere with the readings. This problem is particularly accentuated in sperm counts made on freeze-thawed semen samples as they contain egg yolk particles, fat droplets and other particulate matter (Parks, 1992; Evenson et al., 1993). Use of hemocytometers for estimation of sperm counts in freeze-thawed samples of semen has not gained much acceptance because of many technical reasons (Freund and Carol, 1964). Evenson et al. (1993) have developed a flow cytometric method of semen analysis in which beads composed of fluorescent microspheres were used. This method met with limited success because of the laborious process of preparation of these beads and the need for highly skilled personnel. Studies have reported (Carlsen et al., 1992; Auger et al., 1995; Irvine et al., 1996; Aitken, 1999) that the quality and counts of spermatozoa is showing downward trend in human semen analysis reports. Sperm count assessments form the essential component of the diagnostic and prognostic evaluation of male fertility, according to guidelines of WHO (1992). Although the report of WHO (1992) had laid down clear cut guidelines for the hemocytometric estimation of sperm counts, Auger et al. (2000) have demonstrated vividly that the results are difficult to compare because of variations between laboratories and between technicians. All these had led to the increased use of flow cytometry to estimate sperm concentrations and to bring concurrent agreement between different laboratories (Eustache et al., 2001; Tsuji et al., 2002).

To overcome the stated limitations and achieve objective assessment with a high precision, a new technique called flow cytometry is utilized (Gledhill et al., 1976; Garner et al., 1986; Morrell, 1991; Parks, 1992; Graham, 1994, 2001). This technique also allows the researcher to examine several different other parameters like plasma membrane integrity (Evenson et al., 1982; Garner and Johnson, 1994, 1995), mitochondrial function (Evenson et al., 1982; Graham et al, 1990; Garner et al, 1997), acrosomal status (Graham et al., 1990; Thomas et al., 1997) and chromatin structure (Evenson et al., 1980). A flow cytometer is an easy instrument to operate now in clinical use for the estimation of sub populations of lymphocytes and in the stem cell laboratories world wide. Garner et al. (1994) reported a protocol to assess sperm concentrations with fluorescent microspheres popularly called beads. They assessed sperm viability by flow cytometry using a modified SYBR 14 and propidium iodide (PI) method. This protocol can differentiate live, dead and moribund spermatozoa of different species of mammalian and avian semen.

Efforts are being made to correlate the fertility success ratio in bulls and bears with that of sperm viability. Kroetsch et al. (2009) have shown that there is significant variation in the fertility ratio of semen samples obtained from the same male animal but in different ejaculates. Matson (1997) argued that the chance of selecting the best ejaculate (from the same animal) depends first and foremost on the precision of the semen analysis. He therefore concluded that simultaneous estimation of sperm concentration and viability results in more accurate prediction of success rates. If these two estimations were performed on different samples or on different instruments or by different personnel, the reports and the results may not be that accurate.

RESULTS AND DISCUSSION

We now make a comparative analysis of different methods to estimate the sperm concentrations that were used in many infertility centers worldwide. Of these, the first three are conventional methods and the remaining novel methods are as follows:

1) Hemocytometry method
2) Spectrophotometry method
3) Microcell analysis
4) Fluorescent plate reader
5) Image analysis
6) Flow cytometer

Hemocytometry method

Hemocytometry is the oldest, well established ‘gold standard’ in all cell count estimations including sperm counts. Seman et al. (1996) had exposed threadbare that hemocytometer readings are prone to wide variations and imprecise readings. Mahmoud et al. (1997) have showed that different models of hemocytometers also contribute to observer variation. Prathalingam et al. (2006) have used Thoma hemocytometrs since they observed in the previous studies of having less cyclic voltametric (CV) compared to other models (Christensen et al., 2005). Although their estimated CV is less than that reported by others, Prathalingam et al. (2006) had reported that the hemocytometer turned out to be the third most imprecise method to estimate sperm concentrations. As hemocytometry is laborious for routine use and prone to observer bias, Cooper et al. (1992) made an attempt to automate and at the same time capture images from the hemocytometer loaded with fluorescent labeled spermatozoa.

Spectrophotometric method

It is routinely used in many of the artificial insemination laboratories throughout the world in estimating sperm counts. The results obtained from this method are very well verifiable. Hansen et al. (2002) have reported that the CV by spectrophotometric method is 6.3%, whereas Prathalingam et al. (2006) reported the results as 4.1%. This method has the advantage of completing the estimation quite rapidly but the problem is that the equipment needs frequent calibration and maintenance. Unlike other methods that estimate particulate matter, spectrophotometry uses a procedure of colour estimation of the given solutions. So the absorption reading of the cell suspension varies with time and so the time frame within which the estimation is performed is critical for this procedure. This element reduces the objective value of this test, particularly when repeat estimations are performed on the same sample. Prathalingam et al. (2006) had suspected that this element could have influenced the CV value of this test in their study.

Lu et al. (2007) had given the data shown in Table 1 comparing to methods of sperm cell counting. They had used 60 semen samples to load the upper and lower chambers of 3 hemocytometers and the right and left

Table 1. Sperm counts obtained by hemocytometer and cell – VU devices.

Chamber No.	Hemocytometer No.			Cell – VU No.		
	1	2	3	1	2	3
Lower/Left	50.8±18.8	58.2±37.4	50.5±33.7	52.8±35.9	61.5±50.2	54.5±49.6
Upper/Right	49.0±18.2	56.0+34.8	50.6±32.5	50.4±33.8	59.4±46.3	50.5±43.0
CV	0.925	0.969	0.988	0.968	0.994	0.996

Table 2. A correlation matrix indicating the r value of each method compared.

Method	Flow cytometry	Hemocytometry	Image analysis	Microcells	Plate reader	Spectrophotometry
Flow cytometry	*					
Hemocytometry	0.99	*				
Image analysis	0.90	0.90	*			
Microcells	0.96	0.96	0.86	*		
Plate reader	0.91	0.91	0.86	0.83	*	
Spectrophotometry	0.99	0.99	0.90	0.97	0.88	*

sides of 3 cell-VU counting slides.

Fluorescent plate reader method

Spermatozoa were labeled with a fluorescent dye and loaded on a hemocytometer. The resultant image was analyzed with the help of software. Gravance et al. (2000) had performed some pioneering work on this method and reported that the use of the image analysis program has generated a higher CV than that obtained with hemocytometers. Though these results surprised many researchers, it was surmised that it could be because of the lower number of spermatozoa that were counted in these initial studies. In view of the above discussion, Prathalingam et al. (2002) had optimized the protocol before initiating the study. The optimal concentration of spermatozoa was initially estimated to be 2.5×10^6 cells/ml. When the same concentrations of spermatozoa were used for the hemocytometer and for this method, the software program was unable to distinguish cells within clusters. This probably led to errors in calculations. But the use of this software made the analysis much more rapid. It had become a well known fact that the use of plate reader and a software program made the simultaneous measurements of several ejaculates possible. Prathalingam et al. (2006) have opined that the higher CV could be improved by increasing the area under analysis instead of using specimens with higher sperm concentrations. Moreover, a fluorescence plate reader could be used as a low cost alternative to flow cytometry by allowing large number of ejaculates to be processed. Prathalingm et al. (2002) had concluded that further refinement of the protocols are needed for this method as this procedure had the double advantage of counting the number of spermatozoa as well as assessing the viability of the sperms.

Pranthalingam et al. (2006) made a comparative analysis of different methods. They had estimated 100 samples of semen of different species and gave the data comparing the results from different methods of estimation of sperm concentrations as shown in Table 2.

Microcells analysis

Microcells have the added advantage of assessing the motility of spermatozoa simultaneously with the measurements of cell counts. Tomlinsen et al. (2001) have reported significantly low sperm counts with microcell method as compared to those obtained by hemocytometers ($p = 0.11$). Sokol et al. (2000) reported a close correspondence between the readings obtained by hemocytometer and with microcells ($r = 0.88$). Prathalingam et al. (2002) have made a study on bull semen and cautioned while extrapolating the results to humans because human semen contains more debris and less sperm concentrations and lower rates of motility per ejaculates. They also have reported a higher CV for microcells as compared to hemocytometers, a finding that is in correspondence with Tomlinson et al. (2001) and Brazil et al. (2004b).

A common problem encountered by many researchers doing comparative studies of estimates of sperm counts is that they were dealing with an unknown number of spermatozoa in each sample, so there was no standard sample to compare with the test sample. Latex beads of known numbers were used by some researchers (Seaman et al., 1996; Brazil et al., 2004a) as a control to measure the concentrations of spermatozoa in test semen samples. When combined with an unknown number of spermatozoa, the advantage of these latex beads is that their numbers can be estimated accurately by

gravimetric method as long as their properties are constant and uniform. These latex beads can be stored and used repeatedly for an extended period of time and this allows comparison of sperm counts against a given reference of consistent bead numbers (Accubead, Hamilton Thorne, Beverly, Mass). Although their use has improved readings, variability across studies persisted as the number of beads did not match in different batches ($18 \times 10^6 \pm 2.5 \times 10^6$ beads/ml; $35 \times 10^6 \pm 5 \times 10^6$ beads/ml; Accubead). Mahmoud et al. (1997) have reported an increased CV for bead and sperm counts when the semen was mixed with latex beads and estimated with an improved Neubar Chamber.

CONCLUSION

This review had examined the merits and demerits of several common methods of estimation of sperm concentrations in current use. The oldest, the commonest and the one considered as the 'gold standard' for long is the hemocytometric method. The flow cytometer is the latest, sophisticated and the most precise method developed till date. The spectrophotometer is the second most precise method and is very commonly used for the estimation of sperm counts of several non-human species. But it may not be the ideal procedure for a clinical laboratory as the volume of semen and sperm concentration is low for humans. Although flow cytometric procedure is gaining ground in many research laboratories, a preliminary sperm count assessment with a different procedure is recommended by many researchers to ensure that an adequate dilution of semen for the flow cytometer is achieved. The optimal sperm concentration is considered to be about 250×10^3 sperms/ml for the flow cytometer to give the best results. It is also considered to be one of the essential precaution that the sperm concentration and flow rate are adjusted to ensure that there will be no 'missed events'.

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