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Limitations of Semen Analysis as a Test of Male Fertility and Anticipated Needs from Newer Tests

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Abstract

Semen analysis is the first step to identify male factor infertility. Standardized methods of semen analysis are available allowing accurate assessment of sperm quality and comparison amongst laboratories. Population based references ranges are available for standard semen and sperm parameters. Sperm numbers and morphology are associated with time to natural pregnancy whereas sperm motility may be less predictive. Routine semen analysis does not measure the fertilizing potential of spermatozoa and the complex changes that occur in the female reproductive tract before fertilization. Whether assisted reproduction technology is required depends not only on male factors but female fecundity. Newer tests should predict the success of fertilization *in vitro* and the outcome of the progeny.

Keywords

Sperm concentration; sperm motility; sperm morphology; reference ranges; female fecundity

Introduction

Semen analysis is the cornerstone for the assessment of the male partner in a subfertile couple. Compared to many other tests used in the assessment of the infertile couple, semen analysis has been standardized throughout the world. This was made possible through the efforts of the World Health Organization (WHO) since the1970s by producing, editing, updating, and disseminating a semen analysis manual (1). The manual provides step by step methods on how to perform a routine semen analysis, guidance on establishing internal and external quality control for these measures, and recommendations on more commonly used

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tests to assess sperm function. The goal of the manual is to improve the standards of semen analysis and to ensure that the semen and sperm parameters assessed in one laboratory using this manual will be the same as the analysis done in another laboratory using the same manual. International and national societies of andrology, reproductive medicine, human reproduction and pathology contributed by providing hands on training to ensure that the technologists are using these standardized methods to assess semen and sperm quality. This allows comparative studies and pooling of data from across the globe for epidemiology studies to assess semen quality (2, 3) and to develop reference ranges for semen and sperm parameters (4). Semen analysis should be performed in laboratories with experienced technologists who have been trained in these standardized methods for routine clinical examination of semen. Despite our ability to assess sperm quality through a semen analysis methodology harmonized across laboratories, the use of these parameters cannot precisely and accurately predict the fertility of a man presenting to a clinician. This is because there are many factors in addition to sperm and semen quality that contribute to the ability of spermatozoa to fertilize an oocyte. To reach and fertilize the oocyte ejaculated spermatozoa have to traverse the female reproductive tract, hyperactivate and undergo acrosome reaction at the correct time and site, penetrate the cumulus and zona pellucida and ultimately fuse with and fertilize the oocyte. The assessment of some of these changes in the spermatozoa will be discussed in other manuscripts in this series. In addition to sperm function, female factors are extremely important to ensure optimization of the condition of the oocyte to allow for fertilization (5).

Development of Reference Ranges for Semen Quality and Sperm Parameters

Many studies have been criticized for the selection of subjects and methods used to develop reference ranges for semen and sperm quality in particular the thresholds defining male factor subfertility using sperm concentration, motility and morphology, the three classical sperm parameters measured by all laboratories. The WHO initially adopted a sperm concentration of <20 million/ml, > 50 % motile and normal sperm morphology of > 50% as thresholds below which sub-fertility may be present. This was based on studies done in the 1950s by Macleod and colleagues in 1000 men of known fertility and 1000 couples with subfertility (6–9). More recent studies in 2001 evaluated male partners of fertile and infertile couples suggested that lower thresholds of sperm concentration <13.6 million/ml, motility <32 % and normal morphology <9 % should be used to define possible male factor infertility (10). The WHO collected data from >4500 men in 14 countries including prospective and retrospective studies on fertile men and men of unknown fertility. It is important to note that all the centers used the WHO manual for semen and sperm analyses. Data from men with proven fertility whose partners had a time to pregnancy of < 12 months were then chosen to provide reference ranges for semen parameters (4). Using a one-sided lower reference limit of the 5^{th} percentile (95^{th} percent confidence intervals) the lower thresholds for semen parameters are as follows: semen volume 1.5 ml (1.4–1.7); sperm concentration 15 million/ml (12–16); total sperm number per ejaculate 39 million (33–46); sperm motility 40% (39–42); sperm morphology using strict criteria 4% normal forms (3–4); and vitality 58% (55 – 63). The semen quality from the general population was lower than

that of fertile men. The WHO recommends using these reference limits in conjunction with clinical assessment including the female partner's fecundity to determine the fertility prospects for the couple.

Seminal Fluid: Collection and Assessment

The seminal fluid is made up a mixture of secretions from the testis, epididymis, prostate and seminal vesicles and the contribution from each of these glands varies by the interval of abstinence and the method used to obtain the semen samples. Though sexual abstience of 2 to 7 days is generally advised before submission of a sample for analysis (1), a recent study suggests that in subfertile men, the samples should be collected after 1 day of sexual abstinence for optimal semen quality (11). In men, semen samples collected by masturbation in the clinic may be of a lower quality than those collected at home (12) however erotic materials or isotonic lubricants do not appear to influence the quality of the sample (13, 14).

When seminal fluid volume is markedly reduced, the clinician should suspect incomplete collection, severe androgen deficiency and obstruction in ejaculatory ducts or bilateral absence of the vas deference. In the latter two conditions, the seminal fluid will have acidic pH, very low fructoise levels and no spermatozoa and the diagnosis can be confirmed by physical examination confirming bilateral absence of vas deferens or by transrectal ultrasound showing dilated seminal vesicles in ejaculatory duct obstruction (15). There are a number of biochemical tests to measure functions of the accessory gland including zinc and acid phosphatase (prostate), fructose (seminal vesicle), carnitine and alpha-glucosidase (epididymis) (1). These biochemical tests are not rountinely performed and are of rare clinical usefulness as biomarkers of male infertility.

Sperm Concentration and Total Sperm Number in the Ejaculate

The standardization of measurement of sperm concentration and semen volume allows for more accurate calculation of sperm output. Despite many comments and discussions about using sperm concentration as a biomarker of male infertility, the accurate assessment of number of spermatozoa in an ejaculate remained the standard practice for evaluation of the infertile couple. A single parameter fundamentally cannot be employed as a valid biomarker of ferility because a multitude of factors contributes to infertiliy including the inherent biological variability of sperm concentration, the methods of fertilziation (*in vitro* versus *in vivo*), the health of the man at time of collection, and female factors amongst many others. Sperm concentration in a man showed considerable variation and at least two semen samples should be examined for sperm concentration before providing a conclusion that the sperm concentration or total sperm count is below the reference range (16). Retrospective data analyses from cryobanks on 18 to 20 consecutive semen samples from 48 semen donors showed that an optimal duration of abstinence to distinguish high or low sperm production may be between 42 to 54 hours and collection of three samples may provide results closer to the true value (17). The author also suggested that the rate of daily sperm production may better reflect altered spermatogenesis and that assessment of total number of spermatozoa per ejaculate is reflective of sperm production provided the abstinence interval is appropriate (18, 19).

The lower limits of sperm concentration and total number of sperm per ejaculate that reflects male subfertility is not known. The 5th percentile of WHO reference value for sperm concentration is 15 million/ml (95th confidence interval 12 to 16) and for total sperm number per ejaculate is 39 million (33 to 46). This is based on data generated from 1859 fertile men with a time-to-pregnancy of less than 12 month (4) using a one-side distribution as there is no upper limit of sperm concentration that is associated with infertility. It is now recognized that there are geographic differences in sperm concentration across countries. Epidemiological studies suggest that this may be related to the environmental toxicants though the association is controversial (3, 20–22). There are also differences in sperm concentration across racial/ethnic groups (23). More recent studies suggest that obesity is associated with suppression of the hypothalamic-pituitary-testis axis (24) and morbid obesity may be associated with decreased sperm concentration and reduced fertility (25–29). Life style modifications and bariatric surgery reduce BMI which may be associated with improvement in semen quality and fertility potential (30, 31).

What is the predictive value of sperm concentation for fertility in men? Data from an observational study of pregnant women from four countries in Europe showed increasing sperm concentration up to 55 million/ml affected time-to-pregnancy (a measure of fecundity). Similar to sperm concentration, the total sperm number per ejaculate was also associated with the probability of conception. There was also a direct correlation between normal sperm morphology (up to 19 % normal using strict criteria) and time to pregnancy (32). A prospective observation study of 430 healthy couples who discontinued their contraceptive use for 6 months showed that the probablity of conception increased linearly with sperm concentration up to 40 million/ml above which the liklihood of conception was not further increased (Bonde et al,1998). When 200 couples who had discontinued contraception were followed for 12 months, total sperm numbers and sperm concentration were signifcanlty related to time to pregnancy (33). In a more recent study of 501 couples who discontinued contraception in the Longitudinal Investigation of Fertility and the Environment (LIFE) study, sperm concentration and total sperm number were related to time to conception, which became not significant in combined statistical models accounting for simultaneous effects of semen parametes (34). In summary the literature suggests that when sperm concentration or total sperm concentration is low, the fecundity of the men is probably decreased.

Sperm Motility

Assessment of sperm motility is performed in most laboratories by visual assessment under the microscope and quantified as percent total motility, progressive motility and spermatozoa with no motility. It is Important to determine sperm viablity if a large proportion of spermatozoa in a semen sample is non-motile because dead cells will not fertilize an oocyte. Some laboratories use computer assisted sperm analysis (CASA) where different sperm motility parameters have been shown to be associated with fertility (35, 36). CASA is especially useful for research and epidemiological studies where objective assessment is necessary to detect small changes in sperm motility characteristics such as sperm curvilinear and straight line velocity, amount of head and tail movement, and many others. CASA can be used to assess hyperactivated motility (required for penetration of the

cumulus and zona pellucida) which may be a biomarker for the success of human fertilization. Unfortunately, assessment of hyperactivcated motility by CASA is not standardized and inadequate data are available to support its routine use (35).

Sperm motility has no role when In-vitro fertilziation and intra-cytoplasmic sperm injection are used unless no motility is present. Sperm motility was not found to be an important factor associated with the probability of conception in couples who disontinued contraception for 6 months (37) or 12 months (33). In the LIFE when 501 couples discontinued contraception and were followed for 12 months, time to pregnancy was significantly associated with percent sperm motility, straight line velocity, percent linearity and straightness but all these motility parameters became not significant when mulitple semen parameters and covariates were simultaneouly assessed in the model (34).

Sperm Morphology

Manual assessment of sperm morphology is an integral procedure in routine semen analyses. In most laboratories specializing in asessment of infertile couples, sperm morphology is assessed by the “strict” criteria (38, 39). The strict criteria are based on characteristics derived from spermatozoa recovered from the post-coital cervical mucus (39) and from the surface of the zona pellucida. This criteria incorporates histomorphometric measurement of the sperm head and pattern recognition for the various defects of sperm head, neck, body and tail. The technologist assessing seminal fluid smears needs training and re-training to be able to read the slides reproducibly and accurately. Any slight abnormality of the spermatozoa will classfy the spermatozoa to have abnormal morphology using the strict criteria. The strict criteria gained popularity in the assisted reproduction practices because initial studies indicated that poor sperm morphlogy predicted the failure of intra-uterine insemination and *in vitro* fertilization, but not all studies have confirmed this(40–44). This strict criteria is also used by the WHO for the assessment of normal sperm morphology in the latest version of the manual (1). If the percent of sperm with normal sperm morphology is over 4 percent of sperm cells, this is generally regarded as within the 95% fertile reference range (4).

Is assessment of sperm morphology useful to predict *in vivo* fertility without assisted reproductive techniques? In partners of pregnant women, the percent of morphologically normal spermatozoa assessed by strict or other criteria influenced time to prenancy in these couples (32). In couples where contraception was withdrawn for 6 to 12 months to study the relationship between sperm parameters and time to pregnancy, the percent spermatozoa with normal morphology and the number of morphologically normal spermatozoa were important and signficant predictors of probability of conception independent of sperm concentration (33, 37). In the LIFE study of couples who discontinued contraceptive use in the United States, normal sperm morphology (using either strict or traditional methods), amorphous, round and pyriform heads, neck and midpeice abnormalities, and coiled tails were significant predictors of time to pregnancy. In this most recent study, sperm head morphometry (width, elongation factor, and acrosome area of head) were also significantly related to fecundity rate. Again as with sperm concentration and motility, when

simultaneous adjustment of multiple semen parameters was used in the statistics model, only percent of spermatozoa with coiled tail was significantly related to fecundity (34).

What Rotuine Semen Analyses Can and Cannot Assess?

Semen analysis remains the first laboratroy test a clinician will order after completing a detailed history and physical examination for the male partner of an infertile couple. The standarization of the rountine semen analyses (semen volume, sperm count, motlity and morphology) allows the comparison across laboratories. Reference range based on fertile men has been developed and generally adopted by most clinicians working with an infertile couple. The lower limit thresholds may not be applicable to every man but can be used as guidance for determining the next step of diagnosis and treatment. A semen analysis that is within the reference range (e.g. above the 5th percentile of the WHO recommended values) indicates that the male partner may not be the primary problem for the infertile couple. Focus should be first on the female partner. Whereas a semen sample that has triple defects: low sperm count, poor motility and abundance of abnomral sperm morphology indicates that male factor infertility is likely. Though specific approaches to the treatment of male infertility are very few, they need to be investigated while workup of the female is completed. Prospective studies in couple who stopped contracpetive use showed that sperm count/total sperm number and percent morphologically normal spermatozoa can predict time to pregnancy which is a surrogate marker for fecundity (33, 34, 37).

Spermatozoa have to undergo many changes before fertilziation can occur. For i*n vivo* fertilization, spermatozoa must have adequate motility propelled mainly by ATP generated from glycolysis and not from the mitochondria. But sperm mitochondria are important for calcium homeostasis and for generation of contolled levels of reactive oxygen species necessary for normal sperm function (45, 46). During the transit of the female reproductive tract spermatozoa undergo capacitation. The process of capacitation involves generation of adenyl cyclase which activates protein kinase A resulting in sperm protein tyrosine phosphorylation enabling spermatozoa to acquire fertilizing capacity. Spermatozoa then undergo acrosome reaction and hyperactivation when in contact with the zona pellucida (47, 48). Recent studies using advanced technology showed that spermatozoa can penetrate the cumulus without undergoing the acrosome reaction. Spermatozoa must be coated with the sperm surface protein ADAM3 to allow passage through the cumulus and binding to the zona (49). These processes enable a sperm cell to penetrate the zona and begin the process of fertilization of the oocyte. The fertilization of the ooctye requires at least the presence on IZUMO 1 on the spermatozoa and CD 9 on the ooctye (50, 51). Thus it is clear that examination of the semen and the spermatozoa in the ejaculate cannot assess: 1) the process of capacitaion of the spermatozoa in the female reproductive tract, 2) the acquisition of sperm surface proteins that are requried for zona binding and penetration, and 3) the ability to fertilize the egg. Some of these sperm function tests are described in later manuscripts serving as biomarkers of spermaotozoal fertilizing capacity.

What may be the Requirment for the Next Generation of Biomarkers/Tests of Male Fertility?

There are a large number of sperm function tests including the sperm oocyte penetration test, hemizona assay, stimulation of acrosome reaction, hyperacticated motility assessment using CASA, and *in vitro* capacitation tests that may assess each step that spermatozoa must undergo before fertilization occurs. These sperm function tests have been shown to be associated with fertilization *in vitro* but none of these *in vitro* tests have consistently predict the time to pregnancy better than sperm concentration and morphology. Sperm DNA integrity was not associated with fecundity of the couple (34). Because infertility is a complex process involving both the male and female factors, it will not be possible to predict fertility using parameters from either partner alone unless there is azoospermia in the male or premature ovarian failure in the female. Female fecundity contributes significantly to the fertility potential of the couple (5). In a propsective study based on 3917 couples presenting with unexplained infertiliy, a mixed model was used to distingush couples who may have chance of natural conception and those who would be infertile without utilizing assisted reproductive technology. The statistical mixed model estimated that 47% of the couple were infertile, only female age (odds ratio 1.11, 95% confidence interval 1.03–1.19) and previous pregnancy (odds ratio 0.22, 95% confidence interval 0.07–0.67) were significantly related to infertility and semen quality was not a statistically important factor for unexplained infertility (52).

For a clinician who treats infertile couples, the questions are 1) Is there a problem with the male partner? 2) How significant is the abnormality? 3) Is there a cause of this abnormality? 4) Can the abnormality be treated? 5) Should the couple be referred for intracytoplamic sperm injection (ICSI) or IVF? 6) Can sperm biomarkers predict the success of ICSI and IVF? and 7) Will the defect in the male factor affect the progeny? Performing a routine semen analyses will provide leads to whether the problem may be present in the male partner and an estimate of the severity of the problem. To find out the cause of the abnormality will require further testing that may include assessing the general health of the male partner (smoking, obesity, hypogonadims, chronic diseases), genetic testing to exclude Y chromosome microdeletions and other common genetic defects, and excluding obstructive causes that can be amenable to treatment. Sperm function testing is not usually done as the couple with moderate to severe non-obstructive oligozoospermia are referred for ICSI and IVF. At this stage, genetic testing should have been done and counseling if necessary should have been provided to the coupleFuture sperm function tests need to accurately predict the success of fertilization *in vitro* and whether the progeny will be healthy. This may include using epigenetics and deep sequencing studies for clinical diagnosis of male infertility to discover spermatozoal epigenetic disorders (53–55), spermatozoal small noncoding RNA defects (56, 57) and other subtle genetic abnormalities that may impact fertilizing potential and the outcome of the progeny (58).

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