Chapter 1

Standard Semen Examination: Manual Semen Analysis

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1.1 Introduction

The aim of this chapter is not to provide all practical details necessary for proper semen examination. There already exist other sources for that type of information [1, 2]. The purpose of this chapter is therefore to focus on biological and physiological aspects that are relevant to the examination of human ejaculates. It is aimed to give the clinician a proper background to set requirements for a qualitative laboratory service needed to diagnose and treat men with disorders in the male reproductive organs contributing to couple infertility.

The investigation of the human ejaculate includes observations (e.g. color, odor, viscosity and liquefaction) as well as measurements and assessments (volume, concentration, motility, vitality and morphology). It is therefore adequate to refer to semen examination that is considered to be a concept that is wider than analysis, also including observations and not only measurements.

The examination of the ejaculate is an essential cornerstone in the evaluation of the reproductive functions of the human male. The ejaculate is different from all other body fluids possible to analyze in a modern medical laboratory, and many delusions still exist concerning what the ejaculate is and what it can tell about the man and his fertility. Since the rise of Assisted Reproductive Technologies (ART) with the introduction and the development of *In Vitro* Fertilization (IVF) and Intra Cytoplasmic Sperm Injection (ICSI), the main focus in studies of ejaculate examination has been on its usefulness to predict the probability for spontaneous and assisted conceptions, pregnancies and live births of children. However, since many factors affecting a couple's fertility are independent from semen examination results, most isolated male or female factors cannot be expected to solely be a major determinant of a couple's probability for a successful pregnancy. Still, if simplistic testing of isolated ejaculate parameters is abandoned in favor of multifactorial ejaculate evaluations, useful predictive information regarding spontaneous and assisted pregnancies can be achieved [3, 4]. Thus, although not completely without interest, this predominant focus on ejaculate examination as a pregnancy predictor has drawn the attention from the information that can be gained about the functions of the male reproductive organs. In the light of a possible decrease in general human male reproductive function [5] it is essential that ejaculate examination for the evaluation of the function of the male reproductive organs is in focus.

Another aspect that is essential to the understanding of semen examination is that the "semen sample" only exists in the laboratory. Semen does not even exist in the body: the ejaculate is formed instantly during the process of ejaculation: spermatozoa are transported from the cauda epididymides to the urethra where they are suspended in prostatic fluid concomitantly emptied from the small prostatic gland acini and expelled in the first ejaculate fractions. The seminal vesicles empty into the urethra after the bulk of spermatozoa has been ejaculated [6]. In general, the seminal vesicular fluid appears to be hostile to sperm function (motility), survival and DNA protection [7]. There is no evidence that the seminal vesicular fluid is in any way beneficial for sperm function *in vivo* and there is no evidence that exposure of spermatozoa to seminal vesicular fluid ever occurs *in vivo* [6, 8]. In contrast, examination of ejaculates *in vitro* is based on collection of all parts of the ejaculate in one device, where the entire ejaculate is included in the gel-like substance originating from the seminal vesicles. This structure is then subsequently decomposed by enzymes of prostatic origin. The effect of this process – called liquefaction – is not only that the ejaculate becomes more watery in appearance, but the process also means that the osmolality of ejaculates increase *in vitro* [9, 10].

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1.2 Pre-Examination Aspects

The composition and quantity of each ejaculate depends on several factors, among them the rate of sperm production but not limited to that. The procurement of an ejaculate for examination is highly dependent on the man himself. Frequency of ejaculation – and not only time of sexual abstinence before collection of the examination sample – will have influence on characteristics like sperm number, motility and morphology. Also the duration and quality of sexual arousal during sample collection is important [11]. In addition, truthful information on the completeness of sample collection is required for correct interpretation of ejaculate examination results.

Due to the continuous changes (osmolality, pH) occurring in the ejaculate *in vitro* leading to deterioration of sperm motility and changes in morphological appearance, standardized temperature and time to initiation of assessments is required.

1.3 Examination Aspects

1.3.1 Ejaculate Volume

The ejaculate volume is important to achieve reliable data on total sperm number as well as measures of secretory contributions from the epididymides, prostate and seminal vesicles.

There is a highly variable and significant volume loss when using pipettes for measuring ejaculate volume or transferring to reliable measuring devices [12]. Practically, a reliable and more correct volume is best obtained by weighing the collection device before and after sample collection. The specific weight (density) of human semen has been assessed to be 1.03–1.04 g/mL [13], indicating that the error in volume, based on ejaculate weight, would be less than 4 percent even when assuming a specific weight of 1.0 g/mL. A 4 percent error is much less than can be expected from measurements where the ejaculate must be removed from the collection device and an unknown volume is left in the collection device.

1.3.2 Sperm Concentration

The accuracy of sperm concentration assessment depends on several factors. The most basic is that the examined aliquots are representative of the entire ejaculate. Macroscopically well mixed ejaculates quite often still show considerable variation between different fields of vision in the microscope (10 µL aliquot). Based on this typical finding the recommendation is that aliquots of at least 50 µL should be used to reduce the risk for poor representation of the entire ejaculate. Furthermore, comparison of two separate aliquots should be examined and compared, to further reduce the risk for poor representation and other errors that may occur in the process of establishing the correct number of spermatozoa.

Another essential aspect is the use of dilutions. One important reason is that it is much easier to assess non-moving objects than live motile spermatozoa. Another equally important reason is that with adequate dilutions counting is easier when spermatozoa are evenly spread within and between the microscopic fields; densely packed spermatozoa or too diluted makes counting more difficult, time consuming, exhausting and less reliable.

A third important aspect is to reduce the influence of random factors that cause a spermatozoon to occur or not occur in the area of observation. If only a few spermatozoa are observed, the influence of random factors can influence the final result considerably. Therefore, the number of observations is crucial. As can be seen in Figure 1.1, the uncertainty of a cell counting result varies with the number of observations. The recommendation of assessing at least 400 spermatozoa is based on the fact that statistically a total of 400 observations reduces the random variability to ±10 percent of the observed value [2].

Further sources of possible causes of significant errors and variability lie in the accuracy of the volume assessed in the counting chamber. While the area examined can be very precisely determined, the depth can vary. A shallow chamber (10 µm) would cause a 10 percent volume error if the cover is only 1 µm wrong. However, in a 100-µm deep hemocytometer, the same absolute error would only cause a 1 percent error in the volume.

1.3.3 Sperm Motility

The only practical way of assessing sperm motility "manually" in the microscope means using wet preparations with a depth of approximately 10–20 µm, meaning that a 10 µL aliquot under a 22 mm × 22 mm cover slip is appropriate. It is necessary also for the representativity of the aliquots to examine at least two aliquots and compare results to minimize random error.

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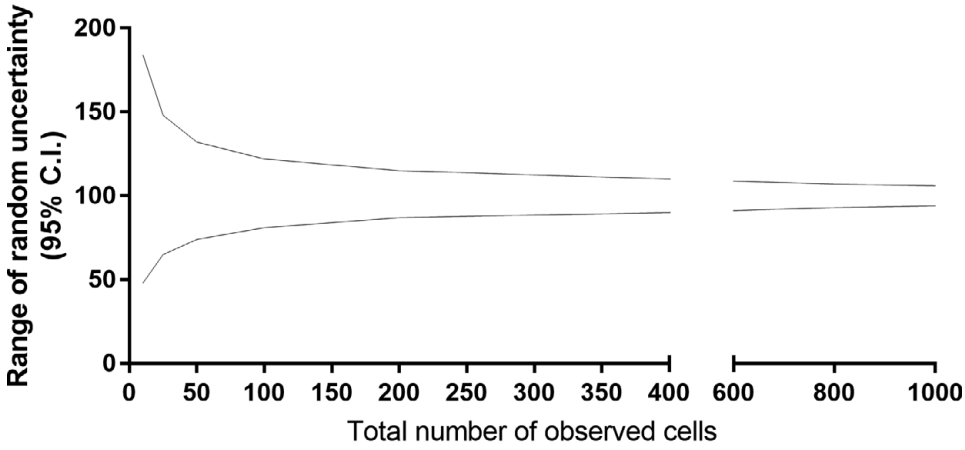

Figure 1.1 Range of uncertainty of sperm concentration results based on different numbers of observations (100 represents calculated value; lines show range of 95 percent Confidence Interval for the different numbers of observations).

Sperm velocity is very dependent on the temperature. While "room temperature" is not well defined and can vary significantly in most laboratories, the recommendation is to standardize microscope stage temperature to 37°C and preferably to use prewarmed slides.

The same statistical consideration as for sperm concentration assessment is relevant for the motility assessment. Thus, at least 400 spermatozoa should be included in the assessment of each sample to reduce the random error to ±10 percent of the observed value.

1.3.4 Sperm Vitality

Vitality assessment is only important when many spermatozoa are immotile. The investigation is essential to identify samples with many live but immotile spermatozoa to distinguish from disorders where reduced sperm motility is due to poor sperm survival.

Since the assessment of sperm vitality by eosin-nigrosine staining [14, 15] does not appear to be sensitive to aliquot representativity (compared to sperm concentration and sperm motility assessment), replicate assessment and comparison is not considered necessary for the purpose of distinguishing between samples with immotile dead spermatozoa and samples with immotile live spermatozoa [16]. It is more important to use adequate equipment (bright field, high magnification (x1000), high resolution microscopy) and count at least 200 spermatozoa in each ejaculate.

1.3.5 Sperm Morphology

The assessment of human sperm morphology may be the most controversial part of basic semen examination. The usefulness of the examination has mainly been assessed in relation to ART. There are, however, many reasons why this is a too simplistic argumentation. One important point is that investigations of spermatozoa that have been able to reach the site of fertilization are much more uniform in appearance than spermatozoa in the ejaculate [17, 18]. The definition of sperm morphology based on observations of spermatozoa passing through cervical mucus and binding to the zona pellucida (Tygerberg Strict Criteria) is the basis for the current World Health Organization recommendations [1]. One argument raised against the use of the Tygerberg Strict Criteria is that very few spermatozoa in the ejaculate have a morphology that fulfil the criteria. However, the number of spermatozoa that can reach the site of fertilization is very low, in the magnitude of 100–1000 spermatozoa from a normal ejaculate [19]. From a statistical point of view, sufficient numbers of spermatozoa with "normal" morphology are very likely to exist, although not possible to detect when 200 or 400 spermatozoa from the ejaculate are randomly chosen and assessed. The distribution of morphological abnormalities still gives information about the function of the spermatogenesis. Therefore, a very important use of sperm morphology is to understand the function of the testicles, rather than only predict the outcome of spontaneous or assisted fertilization. Together with data on sperm number and motility,

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morphology data provides information on qualitative and quantitative aspects of testicular function.

The general assessment of the morphology of each spermatozoon should include at least four aspects: head, neck/midpiece, tail and presence of cytoplasmic residues. Only recording head abnormalities excludes essential information. Presence of more than one type of abnormality appears to indicate more severe problems, often testicular problems [18, 20]. A specific assessment that is useful when evaluating fertilization failure is the acrosome index, measuring the presence of normal acrosomes [21].

To obtain useful data, the choice of staining is essential. Without staining, phase contrast is necessary to see spermatozoa, but the level of details possible to discern will be too low. Among the different staining procedures available, the sperm adapted Papanicolaou stain is considered the best overall staining of all parts of the spermatozoon [1]. Replicate assessments with comparisons appear to be less critical than correct equipment, as well as proper training to obtain reliable and consistent results [16].

1.4 Post-Examination Aspects

For the proper interpretation of data, it is essential that the laboratory not only presents the basic examination result. Critical information like time between ejaculate collection and initiation of assessments should always be included, as well as the abstinence time (days). Any aberrant macroscopic property (color, liquefaction, odor, viscosity) should also be reported. The total number of spermatozoa is more important than concentration, since the latter is largely dependent on the rate of secretion from the seminal vesicles and the prostate. For motility not only the proportion of motile spermatozoa is of interest, rather the proportion of progressively motile spermatozoa is important. In contrast to the 2010 edition of the World Health Organization recommendations [1], the proportion of rapidly progressive spermatozoa provides essential information: lack of rapid progressive spermatozoa is the strongest negative predictor of common IVF success [16, 22].

1.5 Conclusions

Examination of the human ejaculate is basic to the evaluation of the man in an infertile couple. Results primarily provide information about the functions of the male reproductive organs and can thereby give clues to essential investigations and treatments of the man. Ejaculate examination can also contribute to the choice of proper modalities for assisted fertilization for the couple.

As the case with all laboratory investigations, basic semen examination must be performed with insights of possible causes for errors and how systematic and random errors can be minimized. With proper training, and internal and external quality control, reliable results can be obtained, but also patients must be involved to provide essential information on abstinence time and sample collection.

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