Introduction

Sperm Function Testing: Historical Perspectives and Future Horizons

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In this impressive volume, the editors have pulled together an international cast of distinguished authors, who present a detailed account of sperm function testing in terms of rationale, methodologies and clinical significance. In this introduction, my intention is to give a historical perspective on the evolution of these techniques and a view of where this field will head in the future. For what it is worth, I should also mention that these reflections are presented from the standpoint of someone who has spent the best part of half-a-century considering how we can reliably and effectively monitor the functional quality of human spermatozoa.

0.1 In the Beginning . . .

When a male patient comes into a clinic asking for an assessment of his fertility, the first instrument we reach for is the conventional semen profile. The assumption that underpins such traditional diagnostic assessments is that fertility is only possible if a patient possesses more than a certain critical number of motile, morphologically normal spermatozoa in his ejaculate. It is, ultimately, a descriptive approach to infertility diagnosis designed to indicate whether the various parameters of semen quality are within the normal range. The World Health Organization has played a major role in both the standardization of the laboratory techniques used to create a conventional semen profile and the thresholds of normality that can be used for calibration purposes, via the publication of its laboratory manual [1]. This manual is now in its fifth edition, available online, and is a credit to the hard work and dedication displayed by a small group of andrologists over many years, to bring this project to fruition. Its publication has helped build a significant level of consensus around the laboratory techniques that we should use to analyse human semen and has provided the field with a careful, detailed analysis of what "normal" looks like [2]. Notwithstanding the amount of effort that has gone into refining the conventional semen analyses, there are still areas where disagreement abounds.

The first, and most fundamental, is whether the diagnosis of infertility can be approached on the basis of "thresholds of normality", no matter how carefully the latter are established. I am sure that most will agree that the binary classification of males as "fertile" or "infertile" is a gross oversimplification of the truth. In reality, fertility is a continuous variable; it cannot be depicted in terms of black and white but should acknowledge the existence of multiple shades of grey. This may mean that we have to educate patients not to expect a diagnosis of "fertile" or "infertile" but a "percentage probability of spontaneous conception as a function of time". In practice, we shall only be able to create the data bases needed to establish such prognoses if we contemplate establishing preconception cohort studies, which involve the collection of semen quality data and the long-term follow-up of couples to determine their reproductive fate.

Even if we did go to the expense and considerable effort of establishing such databases, what would we learn? Those limited prospective studies that have been conducted suggest that the conventional semen profile is of little diagnostic value outside of extreme oligo- or azoo- spermia [3, 4]. It is for this reason that we see a high (~30 percent) incidence of unexplained infertility in the general patient population, where the semen profile is normal and yet functional deficiencies exist [5]. Such poor prognostic value is a reflection of the obvious limitations of the semen profile since all it can provide is an assessment of "normality" relative to a spontaneously fertile population. It may generate an indication of the relative quality of the underlying spermatogenic process but the descriptive criteria at its core cannot supply accurate information on the fertilizing capacity of the ejaculate.

0.2 The Birth of Functional Assays

Realization that descriptive criteria were of little value in determining the fertilizing potential of human spermatozoa sparked interest in developing a more functional approach to male infertility diagnoses. One of the first aspects of sperm function to be considered during this phase in the evolution of diagnostic laboratory andrology was sperm motility. A quantum leap in the quality of sperm motility data was achieved with the development of Computer Aided Sperm Analysis (CASA) systems that permitted accurate quantification of the movement characteristics of human spermatozoa and research into which aspects of sperm movement are significant in defining the fertilizing potential of these cells.

One of the first areas to be addressed using this newly developed technology was the quality of sperm movement needed to achieve penetration of cervical mucus since failure to penetrate this barrier was, at the time, a well-established cause of infertility [6]. In these studies, the amplitude of the flagellar wave was shown to be critical for mucus penetration, as reflected in the amplitude of lateral sperm head displacement [7, 8]. Interestingly, this research revealed patients in whom inadequacies in the flagellar waveform was the only detectable defect in their ejaculate [8]. For those laboratories not possessing CASA systems, the identification of such patients was achieved using simple penetration tests based on human or bovine cervical mucus or artificial polymers possessing similar physico-chemical characteristics [9, 10].

Failure to penetrate the zona pellucida was another recognized cause of infertility related to the movement characteristics of the spermatozoa defined by CASA technology. In this case, the key attribute of sperm motility needed to effect zona penetration was their ability to establish a state of hyperactivation, characterized by large amplitude, high frequency, asymmetrical flagellar waves capable of generating significant propulsive force. Accordingly, CASA criteria were developed to enable the accurate classification of such cells [11]. Such measurements were shown to be helpful in positively identifying sperm populations capable of fertilizing human oocytes *in vitro*. However, they were much less effective in identifying cases of fertilization failure [12]. Clearly, not all aspects of sperm function are reflected in the capacity of these cells for movement.

0.3 Fertilization Bioassays

In order to address this deficiency, the field searched for an *in vitro* fertilization bioassay that could be used to address other aspects of sperm function aside from their motility including their ability to capacitate, acrosome react and achieve fusion with the oocyte. Of course, human oocytes could not be used for such diagnostic purposes for ethical reasons. However, an alternative was identified by one of the greatest gamete biologists the world has ever seen, Ryuzo Yanagimachi (Yana). Yana made the unexpected discovery that the vitelline membrane of hamster oocytes was able to fuse with human spermatozoa providing these cells had capacitated, acrosome reacted and generated a fusogenic equatorial segment, just as they must *in vivo* [13]. The ultrastructural details of sperm-oocyte fusion in this heterologous model exactly reflected the homologous situation, inviting speculation that this *in vitro* fertilization model might be used to generate diagnostic information of clinical significance. In the event, this test was found to be a very good diagnostic criterion for sperm function, accurately predicting the fertility of male patients in prospective studies, under circumstances where the conventional semen profile was shown to be of limited diagnostic value [3, 14–16]. When the results of this assay were combined with data describing sperm movement and the conventional semen profile, multivariate discriminant equations could be written that predicted the fertility of cryostored semen samples in a donor insemination program with more than 80 percent accuracy [17]. While the biochemical basis for this bioassay has recently been resolved (the hamster egg receptor, Juno, apparently binding to the human sperm ligand, Izumo1 [18]), the test is clearly not a key part of our current diagnostic armamentarium. Why is this?

The fundamental problem with the hamster oocyte penetration test is that it is a bioassay. It is complex, extremely labour intensive, involves the use of animals and is difficult, if not impossible, to standardize. The World Health Organization did everything it could to generate a standard method for performing the assay [19]. However, a survey of the relevant literature reveals a wide range of protocols used to conduct this assay and a correspondingly wide variation in the conclusions drawn as to its diagnostic validity. In truth, this assay was never intended to be a routine diagnostic test but rather a unique model that

might help us understand the biochemical basis of defective sperm function. Achievement of this goal would provide a logical basis for the development of standardize-able biochemical assays with which to generate the same diagnostic information as provided by the bioassay.

Exactly the same argument could be made for bioassays looking at sperm-zona interaction. In the right hands, assessments of sperm-zona interaction using, for example, the hemi-zona assay, are highly predictive of fertility [20]. However, the logistical complexities of running and standardizing the assay has meant that it has not been widely adopted as a front-line diagnostic procedure. As our understanding of the fundamental biochemical processes underpinning sperm-zona interaction improves [21] so we can anticipate opportunities arising to replace this bioassay with biochemical tests that will generate the same information at a fraction of the cost and time required to run the bioassay.

0.4 Oxidative Stress

One of the key findings secured with the hamster egg penetration assay was that in infertile males, levels of sperm-oocyte fusion were reduced even if a calcium signal was artificially generated in the cell using the ionophore, A23187 [22]. These data suggested that a common defect in human spermatozoa is an inability to complete membrane fusion events, such as the acrosome reaction and oocyte fusion, in response to a calcium stimulus. In looking for a membrane defect that might impair the responsiveness of the sperm plasma membrane to calcium, we established the importance of reactive oxygen species (ROS) generation and lipid peroxidation [23–25] in the pathophysiology of defective sperm function. Insertion of the terms "oxidative stress" and "male infertility" into PubMed currently generates 1465 hits. Several chapters of this book are devoted to this particular topic so I shall not elaborate here. Suffice it to say that exposure to ROS emanating from infiltrating leukocytes or the spermatozoa themselves and/or exposure to an environment low in extracellular antioxidant protection leads to a state of oxidative stress in these cells. A major consequence of this stress is to initiate lipid peroxidation cascades that culminate in the generation of toxic electrophilic aldehydes such as 4-hydroxynonenal and acrolein [26]. These aldehydes bind to the vulnerable nucleophilic centres of proteins in the immediate vicinity (cysteine, histidine and lysine residues in particular) stimulating increased levels of ROS generation by the mitochondria, the suppression of sperm motility and the impairment of sperm-egg recognition [27–29]. In addition, oxidative stress attacks the DNA in the sperm nucleus leading to oxidative DNA damage and strand breakage [30]. There are chapters in this book by internationally recognized pioneers in the field such as Don Evenson, who was among the first to understand that DNA damage to the male germ line is a determinant not just of fertility but also, potentially, the mutational load carried by the offspring [31].

0.5 The Future of Sperm Function Testing

We have moved a long way since the conventional semen profile was established as the foundation stone of male infertility assessments. It is now clear that defective sperm function can exist under conditions where the conventional semen profile is normal, due to biochemical lesions in the spermatozoa that impair both their fertilizing potential and their ability to establish a normal pregnancy. Unfortunately, research in this important area suffered a setback from the introduction of ICSI as a method of oocyte insemination. ICSI is very forgiving of defects in the functional competence and genetic integrity of spermatozoa and, as a result, the clinical demand for sperm function testing waned. Hopefully, we have now reached a point in the evolution of this field where the importance of assessing sperm quality is becoming more apparent. Such assessments may help us develop rational approaches towards the assignment of patients to IVF or ICSI, or even avoid assisted conception altogether. Furthermore, measurements of DNA damage may help us to understand potential paternal impacts on the health and wellbeing of offspring and open the door to therapies that will reduce levels of DNA damage prior to the initiation of assisted reproductive technology (ART).

It has been a long journey but the time for more extensive sperm function testing may have finally arrived. For anyone engaging with this complex field, this book will provide the reader with an authoritative snapshot of all that is current in assessing the functional qualities of these infinitely complex, fascinating cells.

References

1. World Health Organization. (2010). *WHO Laboratory Manual for the Examination and Processing of Human Semen*, 5th ed. Geneva: The WHO Press. www.who.int/reproductivehealth/publications/infertility/9789241547789/en/
2. Cooper TG, Noonan E, von Eckardstein S, Auger J, Baker HW, Behre HM, Haugen TB, Kruger T, Wang C, Mbizvo MT, Vogelsong KM. World Health Organization reference values for human semen characteristics. *Hum Reprod Update* 2010; **16**: 231–45.
3. Aitken RJ, Irvine DS, Wu FC. Prospective analysis of sperm-oocyte fusion and reactive oxygen species generation as criteria for the diagnosis of infertility. *Am J Obstet Gynecol* 1991; **164**: 542–51.
4. Aitken RJ, Best FS, Warner P, Templeton A. A prospective study of the relationship between semen quality and fertility in cases of unexplained infertility. *J Androl* 1984; **5**: 297–303.
5. Templeton A, Aitken J, Mortimer D, Best F. Sperm function in patients with unexplained infertility. *Br J Obstet Gynaecol* 1982; **89**: 550–4.
6. Kremer J, Jager S. The sperm-cervical mucus contact test: a preliminary report. *Fertil Steril* 1976; **27**: 335–40.
7. Aitken RJ, Sutton M, Warner P, Richardson DW. Relationship between the movement characteristics of human spermatozoa and their ability to penetrate cervical mucus and zona-free hamster oocytes. *J Reprod Fertil* 1985; **73**: 441–9.
8. Feneux D, Serres C, Jouannet P. Sliding spermatozoa: a dyskinesia responsible for human infertility? *Fertil Steril* 1985; **44**: 508–11.
9. Mortimer D, Mortimer ST, Shu MA, Swart R. A simplified approach to sperm-cervical mucus interaction testing using a hyaluronate migration test. *Hum Reprod* 1990; **5**: 835–41.
10. Aitken RJ, Bowie H, Buckingham D, Harkiss D, Richardson DW, West KM. Sperm penetration into a hyaluronic acid polymer as a means of monitoring functional competence. *J Androl* 1992; **13**: 44–54.
11. Sukcharoen N, Keith J, Irvine DS, Aitken RJ. Definition of the optimal criteria for identifying hyperactivated human spermatozoa at 25 Hz using in-vitro fertilization as a functional end-point. *Hum Reprod* 1995; **10**: 2928–37.
12. De Geyter C, De Geyter M, Koppers B, Nieschlag E. Diagnostic accuracy of computer-assisted sperm motion analysis. *Hum Reprod* 1998; **13**: 2512–20.
13. Yanagimachi R, Yanagimachi H, Rogers BJ. The use of zona-free animal ova as a test-system for the assessment of the fertilizing capacity of human spermatozoa. *Biol Reprod* 1976; **15**: 471–6.
14. Aitken RJ. Diagnostic value of the zona-free hamster oocyte penetration test and sperm movement characteristics in oligozoospermia. *Int J Androl* 1985; **8**: 348–56.
15. Gwatkin RB, Collins JA, Jarrell JF, Kohut J, Milner RA. The value of semen analysis and sperm function assays in predicting pregnancy among infertile couples. *Fertil Steril* 1990; 53: 693–9.
16. Margalioth EJ, Feinmesser M, Navot D, Mordel N, Bronson RA. The long-term predictive value of the zona-free hamster ova sperm penetration assay. *Fertil Steril* 1989; **52**: 490–4.
17. Irvine DS, Aitken RJ. Predictive value of in-vitro sperm function tests in the context of an AID service. *Hum Reprod* 1986; **1**: 539–45.
18. Bianchi E, Wright GJ. Cross-species fertilization: the hamster egg receptor, Juno, binds the human sperm ligand, Izumo1. *Philos Trans R Soc Lond B Biol Sci* 2015; **370**: 20140101.
19. Aitken RJ. (ed.) The zona-free hamster oocyte penetration test and the diagnosis of male fertility. World Health Organisation Symposium. *Int J Androl* 1986; Supplement 6.
20. Oehninger S, Morshedi M, Franken D. The hemizona assay for assessment of sperm function. *Methods Mol Biol* 2013; **927**: 91–102.
21. Redgrove KA, Nixon B, Baker MA, Hetherington L, Baker G, Liu DY, Aitken RJ. The molecular chaperone HSPA2 plays a key role in regulating the expression of sperm surface receptors that mediate sperm-egg recognition. *PLoS One* 2012; **7**: e50851.
22. Aitken RJ, Buckingham DW, Fang HG. Analysis of the responses of human spermatozoa to A23187 employing a novel technique for assessing the acrosome reaction. *J Androl* 1993; **14**: 132–41.
23. Aitken RJ, Clarkson JS. Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J Reprod Fertil* 1987; **81**: 459–69.
24. Aitken RJ, Clarkson JS, Fishel S. Generation of reactive oxygen species, lipid peroxidation, and human sperm function. *Biol Reprod* 1989; **41**: 183–97.
25. Aitken RJ, Clarkson JS, Hargreave TB, Irvine DS, Wu FC. Analysis of the relationship between defective sperm function and the generation of reactive oxygen species in cases of oligozoospermia. *J Androl* 1989; **10**: 214–20.

26. Moazamian R, Polhemus A, Connaughton H, Fraser B, Whiting S, Gharagozloo P, Aitken RJ. Oxidative stress and human spermatozoa: diagnostic and functional significance of aldehydes generated as a result of lipid peroxidation. *Mol Hum Reprod* 2015; **21**: 502–15.

27. Aitken RJ, Whiting S, De Iuliis GN, McClymont S, Mitchell LA, Baker MA. Electrophilic aldehydes generated by sperm metabolism activate mitochondrial reactive oxygen species generation and apoptosis by targeting succinate dehydrogenase. *J Biol Chem* 2012; **287**: 33048–60.

28. Baker MA, Weinberg A, Hetherington L, Villaverde AI, Velkov T, Baell J, Gordon CP. Defining the mechanisms by which the reactive oxygen species by-product, 4-hydroxynonenal, affects human sperm cell function. *Biol Reprod* 2015; **92**: 108.

29. Bromfield EG, Aitken RJ, Anderson AL, McLaughlin EA, Nixon B. The impact of oxidative stress on chaperone-mediated human sperm-egg interaction. *Hum Reprod* 2015; **30**: 2597–613.

30. Drevet JR, Aitken RJ. Oxidation of sperm nucleus in mammals: a physiological necessity to some extent with adverse impacts on oocyte and offspring. *Antioxidants* 2020; **9**(2): 95–109.

31. Wyrobek AJ, Eskenazi B, Young S, Arnheim N, Tiemann-Boege I, Jabs EW, Glaser RL, Pearson FS, Evenson D. Advancing age has differential effects on DNA damage, chromatin integrity, gene mutations, and aneuploidies in sperm. *Proc Natl Acad Sci U S A* 2006; **103**: 9601–6.

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].

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.

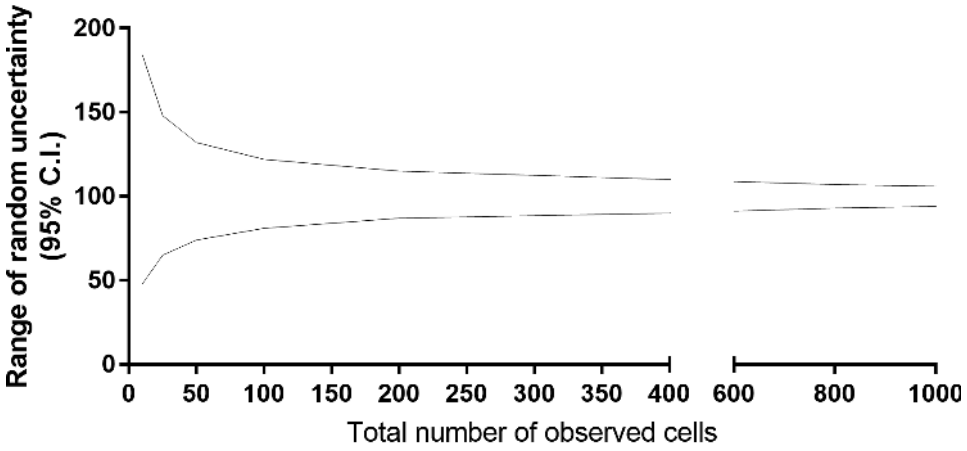

Figure 1.1 Range of uncertainty of sperm concentration results based on different numbers of observations (100 represents calculated value; lines show range of 95 percent Confidence Interval for the different numbers of observations).

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 pre-warmed 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,

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.

References

1. World Health Organization. (2010) *WHO Laboratory Manual for the Examination and Processing of Human Semen*, 5th ed. Cooper TG, Aitken J, Auger J, Baker HWG, Barratt CLR, Behre HM, et al., eds. Geneva: The WHO Press, p. 286.
2. Björndahl L, Mortimer D, Barratt CLR, Castilla JA, Menkveld R, Kvist U, et al. (2010) *A Practical Guide to Basic Laboratory Andrology*. Cambridge and New York: Cambridge University Press, pp. xii, 336.
3. Jedrzejczak P, Taszarek-Hauke G, Hauke J, Pawelczyk L, Duleba AJ. Prediction of spontaneous conception based on semen parameters. *Int J Androl* 2008; **31**(5): 499–507.
4. Guzick DS, Overstreet JW, Factor-Litvak P, Brazil CK, Nakajima ST, Coutifaris C, et al. Sperm morphology, motility, and concentration in fertile and infertile men. *N Engl J Med* 2001; **345**(19): 1388–93.
5. Levine H, Mohri H, Ekbom A, Ramos L, Parker G, Roldan E, et al. Male reproductive health statement. XIIIth International Symposium on Spermatology, May 9–12, 2018, Stockholm, Sweden. *Basic Clin Androl* 2018; **28**: 13.
6. Björndahl L, Kvist U. Sequence of ejaculation affects the spermatozoon as a carrier and its message. *Reprod Biomed Online* 2003; **7**(4): 440–8.
7. Björndahl L, Kvist U. Human sperm chromatin stabilization: a proposed model including zinc bridges. *MHR* 2010; **16**(1): 23–9.
8. MacLeod J, Gold RZ. The male factor in fertility and infertility. III. An analysis of motile activity in the spermatozoa of 1000 fertile

men and 1000 men in infertile marriage. *Fertil Steril* 1951; **2**(3): 187–204.

9. Holmes E, Björndahl L, Kvist U. Post-ejaculatory increase in human semen osmolality in vitro. *Andrologia* 2019: e13311.
10. Holmes E, Bjorndahl L, Kvist U. Possible factors influencing post-ejaculatory changes of the osmolality of human semen in vitro. *Andrologia* 2019: e13443.
11. Pound N, Javed MH, Ruberto C, Shaikh MA, Del Valle AP. Duration of sexual arousal predicts semen parameters for masturbatory ejaculates. *Physiol Behav* 2002; **76**(4–5): 685–9.
12. Cooper TG, Brazil C, Swan SH, Overstreet JW. Ejaculate volume is seriously underestimated when semen is pipetted or decanted into cylinders from the collection vessel. *J Androl* 2007; **28**(1): 1–4.
13. Woodward B, Gossen N, Meadows J, Tomlinson M. Uncertainty associated with assessing semen volume: are volumetric and gravimetric methods that different? *Hum Fertil* 2016; **19**(4): 249–53.
14. Björndahl L, Soderlund I, Johansson S, Mohammadieh M, Pourian MR, Kvist U. Why the WHO recommendations for eosin-nigrosin staining techniques for human sperm vitality assessment must change. *J Androl* 2004; **25**(5): 671–8.
15. Björndahl L, Soderlund I, Kvist U. Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment. *Hum Reprod* 2003; **18**(4): 813–16.
16. Barratt CL, Björndahl L, Menkveld R, Mortimer D. ESHRE special interest group for andrology basic semen analysis course: a continued focus on accuracy, quality, efficiency and clinical relevance. *Hum Reprod* 2011; **26**(12): 3207–12.
17. Menkveld R, Stander FS, Kotze TJ, Kruger TF, van Zyl JA. The evaluation of morphological characteristics of human spermatozoa according to stricter criteria. *Hum Reprod* 1990; **5**(5): 586–92.
18. Mortimer D, Menkveld R. Sperm morphology assessment: historical perspectives and current opinions. *J Androl* 2001; **22**(2): 192–205.
19. Ahlgren M. Sperm transport to and survival in the human fallopian tube. *Gynecol Invest* 1975; **6**(3–4): 206–14.
20. Jouannet P, Ducot B, Feneux D, Spira A. Male factors and the likelihood of pregnancy in infertile couples. I. Study of sperm characteristics. *Int J Androl* 1988; **11**(5): 379–94.
21. Menkveld R, Rhemrev JP, Franken DR, Vermeiden JP, Kruger TF. Acrosomal morphology as a novel criterion for male fertility diagnosis: relation with acrosin activity, morphology (strict criteria), and fertilization in vitro. *Fertil Steril* 1996; **65**(3): 637–44.
22. Björndahl L. The usefulness and significance of assessing rapidly progressive spermatozoa. *Asian J Androl* 2010; **12**(1): 33–5.