Asian Journal of Andrology (2010) 12: 71–78

www.nature.com/aja

Perspectives

Tests to measure the quality of spermatozoa at spermiation

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Abstract

This commentary is to critique the revised World Health Organization (WHO) semen analysis manual as it pertains to characteristics of a spermatozoon at spermiation. The aims of the revised WHO manual include improving the 'quality of semen analysis' without any restriction to clinical use. Furthermore, the manual states that semen analysis may be useful for (a) 'investigating male fertility status' and (b) 'monitoring spermatogenesis during and following male fertility regulation.' However, if the analysis of ejaculated spermatozoa is intended for the purposes described in (b), then cells that are abnormal at spermiation must be identified. This paper takes the position that the manual does not identify methods to estimate the quality of spermatozoa at spermiation. Instead, it uses a 'gold standard' of sperm passing through the cervical mucus or arriving near the site of fertilization. Although this standard is appropriate for drawing conclusions regarding the probability that an individual could impregnate his partner, it is not appropriate for studying illness of the testes per se. Herein, the measures of sperm quality presented in the WHO manual are critiqued with respect to the detection of spermatozoa that were abnormal at spermiation *vs.* those that became abnormal subsequently. Quality assessments based on the percentage of motile or 'viable' spermatozoa are meaningless. Alternative quality attributes defining spermatozoa at spermiation are presented in this paper. In conclusion, assessment of spermatozoal quality at spermiation, on the basis of quality attributes of individual ejaculated spermatozoa, is best achieved through application of (a) a new paradigm for the morphological evaluation of sperm quality and (b) modern analytical techniques to evaluate, in an adequate sample, several appropriate independent attributes in each spermatozoon in order to more accurately identify the proportion of abnormal spermatozoa.

Asian Journal of Andrology (2010) 12: 71–78. doi: 10.1038/aja.2009.19

Keywords: attributes of spermatozoa, purpose of semen examination, semen analysis, sperm quality, spermiation

1 Introduction

Under 'Scope' (Part 1.3), the revised World Health Organization (WHO) manual on semen analysis [1] states, 'Semen analysis may be useful for investigating male fertility status as well as monitoring spermatogenesis during and following male fertility regulation.' The thesis underlying this paper is that the manual does not present or describe any method for estimating the quantity or quality of spermatozoa resulting from spermatogenesis. As a result, the phrase 'useful for … monitoring spermatogenesis' is misleading. For example, in Part 2.1, the manual states that the total number of spermatozoa per ejaculate reflects sperm production by the testes; however, this topic is not found in the Table of Contents. More important, to quantify sperm production by the testes one needs a 'rate function', such as the total number of spermatozoa in an ejaculate per hour of abstinence [2]. In addition, most of the methods described in the manual do not establish whether the quality of spermatozoa at spermiation differs among the individuals studied or might have been affected by exposure to a drug or an environmental agent.

Part 2.1 of the manual [1] correctly states that the total number of spermatozoa per ejaculate and total fluid volume are the two major quantitative attributes of semen, and that these are necessary measurements. However, there is no explicit statement that the quantity and quality of spermatozoa are not tightly linked biologically. The

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Received: 18 December 2008 Revised: 19 December 2008
Accepted: 17 February 2009

quality of each spermatozoon is at least as important as the number of spermatozoa per ejaculate. Indeed, the quality of each spermatozoon, in a representative sub-sample of semen, should be measured with respect to multiple independent attributes, which can be evaluated in either a subjective or an objective manner.

2 Why should one be interested in the quality of spermatozoa at spermiation?

Clinicians usually have semen evaluated to determine the number and quality of spermatozoa that an individual ejaculates, allowing estimation of the probability that he could impregnate his partner. The overarching question is whether there are sufficient spermatozoa capable of reaching the site of fertilization, entering an oocyte, and forming a zygote capable of developing into a blastula and fetus (blastula-producing spermatozoa).

On the other hand, collaborating andrologists and epidemiologists should recognize that semen cannot be 'ill', although it can reflect 'illness' of one or more organs affected by the factor(s) of interest. An andrologist–epidemiologist team should seek evidence to associate the presence or absence of illness of the testes (or epididymides, seminal vesicles, bulbourethral or prostate glands; the illness can have an intrinsic or extrinsic origin) in each individual with one or more factors of interest to the epidemiologist. This requires a study of one or more specific functions of the target organ(s) rather than a conventional semen analysis. A conventional semen analysis will not tell us whether the testes were functioning abnormally or normally 0–10 weeks earlier.

An andrologist–epidemiologist team planning a project to learn whether a particular 'factor' was associated with illness of the testes should consider a study of Leydig cells, Sertoli cells and spermatogenesis, looking at the selected quantitative or qualitative aspects of each. In this paper, I consider only spermatogenesis. The quantification of human spermatogenesis as daily sperm production [3] requires access to testis tissue, which would be inappropriate for an epidemiological study [4]. A non-invasive approximation of the number of spermatozoa produced daily can be made on the basis of total spermatozoa per ejaculate per hour of abstinence (i.e., 10^6 spermatozoa per hour), with the precision of the estimate depending on the details of sample acquisition and number of samples evaluated [2, 5]. With respect to the qualitative features of spermatogenesis, the difference in intent noted in the previous paragraphs requires that measures of sperm quality be restricted to sperm attributes that remain unchanged after spermiation. In the WHO manual, this topic has apparently been ignored. Regrettably, there are few studies comparing human spermatozoa in the rete testis fluid, efferent ducts or proximal epididymal duct with those in the distal cauda epididymidis or ejaculated semen. This paper is an attempt to provide targets for a long-overdue discussion of the measurement of quality of spermatozoa at spermiation using ejaculated semen.

3 Changes to spermatozoa within the epididymis

It is generally assumed that changes in spermatozoa during their epididymal transit are not under genomic control, because DNA condensation occurs before spermiation (in normal cells) and transcription stops [6]. Instead, any changes result from interaction of the spermatozoa with successive microenvironments in the lumen of the epididymal duct [7]. In humans, the secretion of novel molecules is less regionalized than in other mammals [8]. In addition, the epididymal microenvironment is abnormal in some individuals.

During their epididymal transit, human spermatozoa undergo a series of changes with respect to nuclear length, width or area [9–11]; disulfide cross-linking of chromatin and structural components of the tail [12, 13]; DNA compaction and heterogeneity [9]; negative charge of the plasma membrane surface [12]; and lipid composition [14]. At least five proteins on the surface of the plasma membrane in caput spermatozoa are absent from cauda spermatozoa, but there are at least four new surface proteins on cauda spermatozoa [8, 15]. All of these changes can be detected in direct comparisons between mature spermatids or caput epididymidal spermatozoa and cauda epididymidal spermatozoa. However, the size and shape of the acrosome [12, 13] and the length of the tail [10] are similar in spermatozoa from both the caput and cauda epididymidis. The head of the spermatozoa from the cauda epididymidis has an area or form factors similar to those for sperm ejaculated by donors [16], but the variation in the extent of nuclear compaction is greater for cauda epididymidal spermatozoa. Furthermore, during epididymal transit, human spermatozoa also acquire or develop the capacity for progressive forward motion when diluted into a physiological salt solution, and the capability to bind to a zona pellucida [13, 15–19].

An important question to ask is whether the failure of a spermatozoon to undergo these and other 'expected changes' during its transit through the epididymis is because of abnormal spermatogenesis or because of faulty epididymal function. To my knowledge, no study has directly addressed this important question. Nevertheless, in a global sense it is clear that epididymal maturation of spermatozoa is not completely an intrinsic process, and that the epididymal microenvironment is involved [6–8]. An unresolved question is whether the heterogeneity in a population of spermatozoa leaving the seminiferous

epithelium includes spermatozoon-to-spermatozoon differences in their potential to respond both to the changing epididymal microenvironment during transit and to proximity to spermatozoa that are somehow 'different'. Arguably, the failure of one or many spermatozoa to undergo the expected changes could result from abnormal formation during spermatogenesis, in which a given cell was partially or totally deprived of the ability to respond to the sequence of normal epididymal microenvironments. In this scenario, the 'fault' lies with the spermatozoon.

However, assigning the blame to defective spermatogenesis seemingly relies on the idea that each modification that a spermatozoon undergoes within the epididymis is an all-or-none change, resulting from a binomial action (i.e., enough or not enough) of a molecule(s) in the epididymal milieu. This idea is illogical; also see Amann and Hammerstedt [20] and discussion in the next section. Any failure to complete an expected change probably results from a complex interaction of sperm-based and milieu-based deficiencies, with certain failures leading to downstream deficiencies. It is likely that failure of the previously described changes could result from a combination of pre- and post-spermiation events. Thus, there is a need to describe a spermatozoon that is abnormal at spermiation.

A crucial concern is which changes occurring after spermiation might be left undetected in an evaluation performed by a typical technician following standard procedures to examine sperm quality, such as those described in the manual [1]. If the technician could not discern the change in a given spermatozoon, then its status in ejaculated semen would be assumed to be unchanged from its status at spermiation. In the absence of appropriate studies, we do not know what changes a technician or instrument might fail to detect. Although certain biochemical markers of immature spermatozoa released from the seminiferous epithelium can persist unaltered in ejaculated semen [21–23], the most reasonable conclusion would be that certain (but not all) attributes of sperm shape or size might be meaningful, whereas visual or computer-based measurements of sperm motion would be meaningless.

4 Important considerations

When using semen to evaluate the quality of spermatozoa leaving the testes, one needs to restrict the attributes of sperm quality to those unlikely to have been altered after spermiation. This is because human spermatozoa are modified during transit through the excurrent ducts (see previous section) and also during the interval between contact with seminal plasma and evaluation in the laboratory (e.g., initiation of progressive motion). This restriction eliminates the challenge of determining whether the atypical changes in spermatozoa, detected in ejaculated cells, are due to faulty programming of a subpopulation of maturing spermatids or an atypical microenvironment(s) within the excurrent ducts and/or seminal plasma. In theory, attributes altered in a consistent and well-defined manner could also contribute to the judgment of quality at spermiation, but the information to make valid extrapolations is lacking.

It is important to acknowledge that no one can identify a normal spermatozoon—not today, and probably not 25 years from today. During spermiogenesis, spermatozoal maturation, and the interval between the mixture of sperm with seminal fluids and laboratory evaluation, more than 100 processes must function properly at the right time and place. Therefore, one should evaluate multiple independent attributes for each spermatozoon and then identify cells that are detectably abnormal in one or more of those attributes thought to be important in blastula-producing spermatozoa [20]. Certain non-blastula-producing spermatozoa have one detectable abnormality, others have a different detectable problem, and some may have both abnormalities—but many spermatozoa have an undetected problem and are misclassified as non-abnormal (i.e., normal with respect to the attributes examined). If additional tests were conducted, these misclassified spermatozoa might be revealed to instead belong to another sub-population of abnormal spermatozoa. Any conclusion is complicated by knowledge that all blastula-producing spermatozoa are not identical, because of redundancy in the mechanisms necessary to complete certain steps. Defects in certain attributes might have no impact on the success of a given spermatozoon in producing a blastula.

In general, a spermatozoon might be abnormal because of defects precluding (a) completion of the steps up to and including entrance into an oocyte or (b) formation of a zygote, blastula and viable fetus. These two classes of defects have been termed [24] 'compensable' and 'uncompensable', respectively, because with respect to common animals the impact of the latter class is not diminished by the insemination of more spermatozoa. It is likely that in humans, as in animals [24], defects affecting sperm motion or sperm–zona binding are compensable and can be overcome by insemination of more spermatozoa, whereas chromosomal/DNA defects (not necessarily head shape) are uncompensable.

As 'normal at spermiation' cannot be described, it is necessary to define 'abnormal at spermiation' for each attribute to be measured. This provides the framework for deciding whether a given attribute is altered between spermiation and evaluation in the ejaculated semen. For certain studies, *a priori* establish criteria or methods to distinguish any detected difference in quality of spermatozoa at spermiation as a historic problem (e.g., prenatal in origin) *vs.* a recent or current problem. Such distinctions

are difficult and typically are not made, and they are beyond the scope of this paper.

It is important to recognize that it may be inappropriate to quantify many attributes as a binomial response. Some cells probably function normally with more or less of a certain attribute because other attributes compensate for the excess or deficiency (see Figure 5 in [20]). Furthermore, it is uncertain how much of an attribute is actually required. An excess or deficiency of one attribute could have a role anywhere from having no effect to causing complete failure of that cell to perform expected functions or even to survive. Thus, a matrix of continuous or binomial responses for attributes expressed in each spermatozoon, measured for multiple cells within a subsample, would be preferable to single or several concurrent bulk measures on a population of spermatozoa. This is consistent with observations that a given semen sample contains sub-populations of spermatozoa that can be isolated, characterized, and found to be demonstrably different in multiple attributes [22, 23, 25, 26].

5 Tests that might be useful for assessing the quality of spermatozoa at spermiation

The manual [1] details or mentions > 20 tests of sperm quality. Most of these tests are summarized in Table 1, together with a notation regarding the appropriateness of a given test for evaluating the quality of spermatozoa at spermiation. Others might group the tests differently from shown in Table 1.

5.1 Inappropriate tests

When evaluated as recommended in the manual, neither the percentage of motile spermatozoa nor the percentage of morphologically normal spermatozoa in ejaculated semen accurately portrays cells at spermiation. With respect to sperm motion, there are two main reasons. First, spermatozoa are normally immotile when shed from the seminiferous epithelium. Second, even if a spermatozoon is immotile when examined after ejaculation, or swims in a manner considered less than ideal, without a battery of other tests it is impossible to determine whether the problem was caused by malformation (e.g., axoneme component absent in most cells, atypical head shape), epididymal malfunction (e.g., microenvironment unsuitable for modifying spermatozoa to allow motion) or an abnormal milieu in seminal plasma (e.g., insufficient zinc or fructose) consequent to malfunction of one or more accessory sex glands.

The procedure for evaluating sperm morphology, as recommended in the manual [1] (Sections 2.13.1, 2.13.2, 2.15.1), is based on the concept that any spermatozoon that deviates from the appearance of the majority of spermatozoa present in endocervical mucus should be classified as abnormal (Section 2.13.2). This is an inadequate criterion when the purpose is to gauge the quality of spermatogenesis, even if it is appropriate when the aim is to provide counsel on potential fertility. The procedures recommended in the manual do not provide separate classifications for spermatozoa that are potentially abnormal at spermiation, abnormal as a result of events after spermiation or non-abnormal (i.e., normal using the terminology of the manual). Further, tailless heads are excluded from classification despite the fact that the sperm head must have originated in the seminiferous epithelium. Suggestions for evaluation of spermatozoal morphology are provided in a subsequent section.

Owing to documented changes in spermatozoa during epididymal transit (see above), tests of DNA compaction, cervical mucus penetration, induced acrosome reaction and zona pellucida or oocyte binding should be rejected as being unable to portray the status at spermiation. In addition, because there is circumstantial evidence that the properties of ‘mature’ spermatozoa can be altered by close contact with ‘immature’ spermatozoa when in the epididymal duct [26], measuring the production of reactive oxygen species by spermatozoa, lipid peroxidation or DNA structure (via a sperm chromatin structure assay) may also be inappropriate for judging the quality of spermatozoa leaving the seminiferous epithelium. These and other modern analytical techniques should be validated for cells at spermiation by studying populations of spermatozoa from the efferent ducts or proximal epididymis and from the cauda epididymidis or vas deferens. This could be ethically done by using tissues from consenting organ donors who die unexpectedly and for whom a medical history could be obtained later.

Observation of agglutinated spermatozoa, or tests for sperm-associated antibodies, would also be meaningless, because the adluminal compartment of the seminiferous tubule, where spermatids develop, is considered to be an immunologically privileged site.

Dye exclusion is not a meaningful test with respect to the quality of spermatozoa at spermiation. Furthermore, dye exclusion is improperly termed ‘sperm vitality’ in Section 2.6 of the manual [1]; this latter term should be abandoned. What characterizes a living spermatozoon—ATP production, mitochondrial activity, plasma membrane impermeability only to certain dyes? The tests described in the manual almost certainly evaluate the permeability of the plasma membrane (e.g., permeable or impermeable to eosin-Y under conditions used). The authors of the manual apparently recognized what is being measured (as discussed in paragraph 2 of Section 2.6), yet perpetuate the myth that this test distinguishes live and dead spermatozoa because the authors continue use of improper terminology.

Table 1. Usefulness of attributes of sperm quality presented in the manual [1], or elsewhere, to evaluate quality of sperm at spermiation.

Attribute or test	Reflective of sperm quality at spermiation	Why not appropriate or why appropriate to evaluate sperm quality at spermiation
Not appropriate to evaluate quality of sperm at spermiation		
Motility (% motile)	No	At spermiation, sperm typically are immotile. Motion is dependent on molecules in seminal plasma as well as sperm structure and function
Motion (various CASA motion measures)	No	At spermiation, sperm typically are immotile. Motion is dependent on molecules in seminal plasma as well as sperm structure and function
Morphology (% of total abnormalities)	No	Some tail abnormalities due to epididymal malfunction or abnormal seminal plasma
Acrosome status (% acrosome not intact)	No	Not established whether what is seen reflects the status at spermiation
Cytoplasmic droplet (% with proximal or distal droplet)	No	Retention can reflect epididymal and accessory sex gland function
Spermatogonia or spermatocytes (% of total germ cells)	No	Reflects abnormal spermatogenesis, not sperm quality
DNA compaction	No	Changes during epididymal transit in some sperm
Cervical mucus penetration	No	Requires sperm motion (see under motility)
Zona pellucida binding (number of sperm bound)	No	Capability acquired in epididymis
Acrosome reaction (% induced to acrosome react)	No	Requires capability to bind zona proteins, acquired in the epididymis
Zona-free hamster oocyte binding (number bound)	No	Capability acquired in the epididymis
Reactive oxygen species (luminol test)	No	Might reflect epididymal malfunction or seminal plasma
Lipid peroxidation status	No	Might reflect epididymal malfunction or seminal plasma
Immunobead (% motile sperm binding beads)	No	Antibodies not bound to sperm before leaving seminiferous epithelium, but can subsequently gain access to sperm
Mixed antiglobulin reaction	No	Antibodies not bound to sperm before leaving the seminiferous epithelium, but can subsequently gain access to sperm
Dye exclusion (% with permeable membrane)	No	Permeability changes during epididymal transit as plasma membrane is remodeled; possible influence of seminal plasma
Hypo-osmotic swelling (% with tail change)	No	Permeability changes during epididymal transit as plasma membrane is remodeled; possible influence of seminal plasma
Uncertain if appropriate to evaluate quality of sperm at spermiation		
DNA integrity (TUNEL or nick-translation)	Questionable	Possibly altered during epididymal transit in non-compact sperm
Chromatin integrity (SCSA)	Questionable	Possibly altered during epididymal transit in non-compact sperm
Appropriate to evaluate quality of sperm at spermiation		
Head morphology (% with certain head abnormalities)	Yes	Can be restricted to abnormalities of spermiogenesis (see list)
Analine blue staining (% of heads moderately or darkly stained)	Yes	Histones not replaced by protamines during spermiogenesis
Computer head morphology (% abnormal head shape)	In future	Establish no detected head defect arises from epididymal malfunction

(To be continued)

Table 1. Usefulness of attributes of sperm quality presented in the manual [1], or elsewhere, to evaluate quality of sperm at spermiation. (Continued)

Attribute or test	Reflective of sperm quality at spermiation	Why not appropriate or why appropriate to evaluate sperm quality at spermiation
Head morphometry (% abnormal size, etc.)	In future	Establish computer does not detect decrease in head size in epididymis
Tail length (% with markedly short tail)	Yes	Short tail and expression of abnormal spermiogenesis
Residual cytoplasm (% with notable residual cytoplasm)	Yes	Expression of abnormal spermiogenesis (not a cytoplasmic droplet)
Creatine phosphokinase B (% with CK-B)	Yes	High CK-B identifies immature sperm; faulty spermiogenesis
Caspase-3 (% with activated caspase-3)	Yes	High caspase-3 identifies cells surviving faulty spermiogenesis
Hyaluronic acid binding (% sperm failing to bind)	Probably	Show cause is failed membrane remodeling during spermiogenesis
Signal transduction (various tests)	Probably	Depends on the specific enzyme measured

Evidence against the binomial detection of 'live' *vs.* 'dead' spermatozoa includes the profound changes in cell status in response to dye, pH, temperature, duration of exposure to stain, and interval between staining and examination on percentage of stained spermatozoa [27]. It is illogical that the percentage of dead spermatozoa in paired aliquots would differ up to two-fold before mixing with staining solution(s), but logical that the differing compositions of two staining solutions could minimize or rapidly induce changes in permeability of the plasma membrane in many spermatozoa in one or the other aliquot. Alternatively, certain staining procedures must instantaneously cause the death of some spermatozoa in a suspension without affecting other spermatozoa in the same suspension.

Another argument that differential stains do not reveal 'live' *vs* 'dead' spermatozoa is that the percentage of unstained spermatozoa released from the bovine corpus epididymidis is ~30% lower ($P < 0.01$) than the percentages for the caput or cauda [28]. The change between the corpus and cauda cannot be a result of the elimination of dead spermatozoa because almost all spermatozoa entering a bull's epididymal duct leave via the urethra [2]. It was concluded [28] that the change in eosinophilia resulted from a transient change in membrane permeability. This could facilitate sperm maturation by allowing transmembrane movement of otherwise impeded molecules. Arguably, the difference was only ~30% because of the to-and-from mixing of spermatozoa within the epididymal duct [29] and slight variation in sampling site. (See the discussion of 'vitality restoration' of human spermatozoa in [30]). Unfortunately, the percentages of human spermatozoa excluding eosin-Y (or any other dye) have not been reported for either the efferent ducts or multiple sites within the epididymal duct. In any case, it is unlikely that the results of a dye-exclusion test or hypo-osmotic swelling test on ejaculated human spermatozoa will correctly portray their status at spermiation, because changes in surface properties or lipoproteins associated with the plasma membrane around human spermatozoa during epididymal transit (see above) likely alter the membrane's permeability to dye or water.

5.2 Uncertainty about the appropriateness of tests

It seems likely that the integrity of DNA or chromatin could be altered after spermiation in spermatozoa with incomplete nuclear compaction and/or incomplete replacement of histones by protamine during spermiogenesis. The proportion of such spermatozoa is substantial in many human epididymides. Hence, tests probing for single- or double-stranded DNA or integrity of DNA are placed in a separate category in Table 1. Future analyses of individual spermatozoa within populations from the proximal and distal epididymal duct might allow a better classification of these tests as appropriate or inappropriate for evaluating the quality of spermatozoa at spermiation.

5.3 Appropriate tests

Sperm morphology could be a meaningful measure of quality at spermiation if the scheme in the manual [1] was changed to separately tabulate spermatozoa as (a) abnormal at spermiation; (b) abnormal because of events probably occurring after spermiation; or (c) non-abnormal. On the basis of Figure 2.13 in the manual [1], the first category might consist of spermatozoa that are abnormal because the head shape was tapered, pyriform, round, amorphous or small; the head displayed an asymmetric im-

plantation fosa or an abnormally shaped acrosome; or the tail was short, had a thin midpiece or was associated with excess residual cytoplasm (not a 'cytoplasmic droplet'). A short tail or excess residual cytoplasm is indicative of an immature cell in which spermiogenesis was improper or incomplete [31].

The first category should also include spermatozoa with two heads, perhaps sharing a common acrosome, or those with two tails, provided the technician is confident that the 'cell' really is not two spermatozoa overlaid one on top of another. On the basis of published examinations of spermatozoa from several levels of the epididymis, it is likely that defects placed in the first category were established during spermiogenesis and not during transit through the epididymis [13]. The second category might include cells with a bent neck or tail, coiled tail, or retained proximal or distal cytoplasmic droplet. This is because the etiology of these defects is usually uncertain. Cytoplasmic droplets might be retained because of faulty programming during spermiogenesis, epididymal dysfunction or abnormal seminal plasma. Spermatozoa with an excessive number of nuclear vacuoles should probably also be in the second category. Non-abnormal cells would be those not placed in either of the first two categories.

It is recommended that each spermatozoon be entered into the database only once, categorized by the most important defect, in decreasing order of likely importance: short tail or residual cytoplasm; abnormal head shape or implantation fosa; abnormal acrosome; all other defects; and non-abnormal. This is contrary to suggestions in Section 3.1 of the manual [1], but it may provide better insight on illness within the testes. It is immaterial whether a spermatozoon had more than one defect at spermiation if each tallied defect prevents that cell from being a blastula-producing spermatozoon, as is likely for those in the first category described above. With respect to abnormal spermatogenesis, the simplest endpoint would be the percentage of spermatozoa abnormal at spermiation regardless of the nature of the defect, calculated as 100 times ([total number of cells abnormal at spermiation)/(total number of spermatozoa evaluated]). Similar calculations could give the percentages of spermatozoa that fall into each of the above four defect groups.

Although the replacement of histones by protamine is a part of normal spermiogenesis, in some spermatids this replacement is incomplete. Spermatozoa with incomplete replacement of histones are considered 'immature', and can be identified by strong/intermediate uptake of aniline blue *vs.* light/no stain uptake [23]. Most spermatozoa with an abnormal head shape (especially if amorphous with residual cytoplasm [25]) have inadequate replacement of histones, abnormal chromatin packing or structure, or defective nucleotide sequences. Such spermatozoa have a high content of creatine phosphokinase-B and a low content of heat shock protein HspA2 [21] (heat shock protein HspA2 in spermatozoa was formerly termed creatine phosphokinase-M [32]).

Approximately 85% of individual ejaculated spermatozoa display similar intensities of staining or reaction product with either aniline blue or creatine phosphokinase-B; aniline blue or caspase-3; and aniline blue or DNA nick translation [23]. It is thought that the underlying defect is the failure of programming to replace histones with protamine, and that this nuclear defect is accompanied by, or results in, the failure of cytoplasmic elements to mature prior to release of a spermatozoon from the seminiferous epithelium. Such immature spermatozoa are annexin positive [33] and have a high content of caspase-3. Cells marked with caspase-3 might survive because of anti-apoptotic BclXL [22]. However, some spermatozoa display a strong 'signal' with one probe but a weaker response with others, on both a within-ejaculate and an among-individual basis. Owing to this variety of defects in spermiogenesis, it would be imprudent to rely on a single measure of sperm immaturity [22, 23].

Incomplete remodeling of the plasma membrane can be demonstrated by the failure of ejaculated spermatozoa to bind to hyaluronic acid on a solid substrate [34, 35]. It is not known whether the capacity to bind to hyaluronic acid is present in spermatozoa entering the efferent ducts or is acquired by mature spermatozoa during remodeling of their plasma membrane within the epididymis. This attribute was arbitrarily placed in this category.

From the preceding paragraphs it is obvious that several semi-quantitative slide-based measures or quantitative flow cytometry-based assays could be used to detect spermatozoa that were immature at spermiation rather than rely on a subjective evaluation of sperm morphology. In addition to the citations herein, recent papers by Huszar's, Agarwal's, or Aitken's groups should be sought when selecting assays to perform. In an epidemiological study, samples could be gathered in the field, preserved with phenyl-methyl-sulfonyl-fluoride, and shipped overnight at 4ºC to a laboratory for processing, after which the spermatozoa could be evaluated for multiple attributes [36]. At present, spermatozoa abnormal at spermiation are best identified via the techniques noted above rather than the evaluations of sperm quality detailed in the manual [1]. Hopefully, the 1950s tests of sperm quality presented in the manual will soon be supplemented or replaced by proteomic analyses to clearly identify spermatozoa that are abnormal at spermiation.

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