Review Report

Andrology laboratory review: evaluation of sperm morphology

Peter Chenoweth,[a] Leo Brito,[b] Augustine Peter,[c] Dagmar Waberski,[d] Gary Althouse,[b] Christine Aurich,[e] Gaia Luvoni,[f] Regina Turner,[b] Natalie Fraser,[g] Cheryl Lopate[h]
[a]School of Veterinary Sciences, College of Public Health Medical & Veterinary Sciences, James Cook University, Townsville, Queensland, Australia
[b]Department of Clinical Studies, New Bolton Center, School of Veterinary Medicine, University of Pennsylvania, Kennett Square, PA, USA
[c]Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Purdue University, West Lafayette, IN, USA
[d]Unit for Reproductive Medicine of Clinics/Clinic for Pigs and Small Ruminants, University of Veterinary Medicine, Hannover, Germany
[e]Centre for Artificial Insemination and Embryo Transfer, University of Veterinary Sciences, Vienna, Austria
[f]Department of Veterinary Medicine and Animal Science, Università degli Studi di Milano, Milan, Italy
[g]School of Veterinary Medicine, University of Queensland, Gatton, Australia
[h]Reproductive Revolutions, Case Road NE, Aurora, OR, USA

Abstract

Sperm morphology assessment has an important role in male fertility diagnosis and prognosis, both for humans and animals. Thus, it is important that relevant results are comparable and consistent. To achieve these aims, the following procedures are recommended: a. semen sample is suitably 'fixed' (e.g. in isotonic buffered formal-saline); b. sperm are examined at 1,000 x (phase or DIC microscopy); c.at least 200 sperm are counted; d. each sperm is placed into 1 category, only (e.g. normal, head, midpiece etc), and e. 70% 'normal' sperm is the threshold for a satisfactory sample. In addition, morphologists should be provided with relevant continuing education, upskilling, and monitoring programs. This review provides guidelines for the best performance of this assessment, as well as for avoiding pitfalls.

Keywords: Sperm, morphology, fertility, animal

Introduction

A taskforce, representing the Association of Applied Animal Andrology, American College of Theriogenologists, European College of Animal Reproduction, and Society for Theriogenology, was given the task of providing recommendations on best methods of evaluating domestic animal semen quality. This is the third publication in a series, being preceded by reviews on sperm concentration[1] and motility.[2] Initial evaluation of semen is generally performed macroscopically and usually includes volume, color, and consistency. Semen quality is a term that usually includes sperm motility, viability, morphology, concentration, and seminal fluid composition,[2] some of which were discussed.[1,2] Of these attributes, sperm morphology is widely recognized as the semen characteristic most directly associated with fertility,[3] despite the latter often being ill defined.[4] Indeed, "the assessment of sperm morphology is probably the most useful and important aspect of the semen examination."[5] The microscopic assessment of sperm morphology is based on the premise that sperm shape is linked with sperm function,[6] which is reinforced by evidence that abnormal sperm head shape is due to damaged DNA/chromatin.[7] and thus represents a 'useful tool' for assessing potential male fertility.[8] The procedure itself is simple to perform, and the results are considered to reflect sperm fertility, at least to a degree, and particularly where a large proportion of sperm are abnormal.[9] Despite such considerations, wider acceptance of this procedure is constrained by variations in results, leading to a lack of confidence in their accuracy and relevance and reduced ability to directly compare data. For accuracy to be consistently achieved, sample handling, fixation and/or staining must all be optimal[10] and standardized, as sperm morphology can be influenced by semen handling and observer variations,[11,12] thus challenging consistency and objectivity. Thus, this review aims to provide current guidelines for this procedure in order to help achieve greater consistency in its application, leading to improved acceptance of this important component of male fertility assessment.

CONTACT Peter Chenoweth **pchenoweth@hotmail.com**

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Equipment

To record sperm morphological defects, they must first be observed and recognized. The first consideration requires appropriate magnification. A variety of methods are available, with the decision to use a particular procedure often being influenced by its relative complexity, cost effectiveness, expedience and the degree of fine detail required. Equipment list for a basic andrology laboratory is provided.[13] Here, it is incontestable that optimal results depend upon using the best available equipment available in concert with appropriate semen handling and sample preparation. In turn, recognition of particular sperm defects, and an understanding of their relative significance, benefits from a good understanding of current knowledge and developments in physiology and cell biology.

Equipment used for the microscopic examination of sperm includes brightfield microscopy, ordinary phase-contrast microscopy, differential interference contrast phase microscopy (DIC), computer-assisted sperm analysis (CASA) configured for morphology, and electron microscopy. Brightfield microscopy has traditionally been used for routine andrological work as it has the advantages of relatively low cost and ease of use. It is, however, best suited for use with fixed, stained specimens, whereas phase microscopy and DIC are usually preferred for unstained specimens.[14] An earlier review[15] provided commonly used semen stains and a recommended equipment list for a basic andrology laboratory was suggested.[13]

Applications using CASA systems for sperm morphology assessment (i.e. automated sperm morphology analyses) are rapidly gaining traction.[11,16,17] These have advantages of high-speed, evaluation of large numbers of sperm, providing consistent, easily quantifiable results and thus reducing the significant variation that can occur between technicians and laboratories. However, the current lack of standardization methods makes comparisons difficult.[18] In addition, CASA employs negative phase-contrast microscopy that is less than ideal for the recognition of nuclear diadems and vacuoles.

Electron microscopy, although complex and expensive, is a most useful tool for depicting sperm ultrastructure, although this technique does not lend itself to quantitative applications.[19,20] Both transmission and scanning electron microscopy are now widely used in andrology.[6]

The generally recommended magnification of 1,000 x for the microscopical assessment of sperm morphology can be achieved using bright-field microscopy with an oil immersion objective and stained slides (e.g. using an eosin-nigrosin stain). However, it is considered preferable to use either ordinary phase or DIC microscopy in conjunction with a 'fixed' (i.e. 'wet') semen sample. Buffered formal saline,[21] is widely used as a fixative for this purpose. In routine clinical applications, it is often expedient to 'count' 100 sperm per sample, although this number is at the lower end of recommended estimates of sample size, especially when confidence limits are taken into account.[12,22,23] For example, World health organization (WHO) recommends counting at least 200 sperm for human semen assessment.[24] However, counting more sperm did not lead to a change in bull semen morphology classification, even when as many as 400 sperm were counted.[12] Thus, this review supports the conventional approach of characterizing 100 to 200 sperm for routine bull sperm morphology assessment.

Sperm morphology categorization

Following systems categorize animal sperm morphology:[25]

1. Origin of the defect (e.g. primary and secondary abnormalities[26])
2. Potential impact on fertility (e.g. major and minor abnormalities[27])
3. Localization of defect on sperm (e.g. head, midpiece, and tail defects[28,29])
4. Compensable and uncompensable sperm defects[30]
5. Systematic sperm defects[31,32]
6. Genetic sperm defects[33]

The most widely used system, at least in animal andrology laboratories, is considered to be number 3, above,[24,28,29] which is also the simplest and least ambiguous system of those above.

Interpreting and reporting sperm morphology

Sperm morphology reports should include such details as: a. the criteria used to categorize different defects;[25] b. the materials and methods used to prepare the samples for examination (e.g. fresh or frozen-thawed semen, fixatives, dilutions, and staining); c. relevant microscopic and/or imaging details; and d. the reference values used for final summation and conclusions.[34]

Standardization of sperm morphology assessment

It would be very useful to achieve consensus on sperm morphology techniques and interpretations to facilitate research and to reduce misunderstandings and differences that can result in economic loss, conflict, and personal distress.

Adoption of standardized procedures for semen analysis, including sperm morphology, would allow objective comparison of results, in turn improving confidence in the process.[35] Despite this, various attempts to standardize sperm morphology have not been widely adopted. This is probably due to difficulties in harmonizing differences in semen preparation and staining, microscope systems and optics and differences among technicians in their training, competence, and interpretation of results. This was illustrated that morphology evaluations of stallion sperm varied with both technician and methodology.[36] Here, wet-mount preparations examined by phase-microscopy produced better results than stained smears examined with bright-field microscopy. Two techniques ('wet' preparation using DIC phase-contrast microscopy and eosin nigrosin stained smears) were compared using microscopy and it was concluded that, although the results had some qualitative differences, the final breeding soundness examination classification of bulls did not differ.[37] It is a reassuring fact that veterinary practitioners were 92% in accordance when categorizing bull semen morphology[38] and there was little difference in the types of sperm defects observed in tropical *Bos indicus* bulls compared to temperate *Bos indicus* bulls.[39] Despite this, there remains a relative lack of confidence in sperm morphology results from both animal and human andrology laboratories.[18,40,41]

Sperm abnormality thresholds

An early observation was that there was a 'threshold' of observable sperm morphological abnormalities above which

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fertility became compromised. This threshold, ~ 30%, has remained remarkably consistent. In bulls, this was illustrated in natural mating trials in Texas,[42] where bulls preselected for good sperm morphology (> 70% normal sperm) achieved significantly more pregnancies than those that were unselected for sperm morphology. Fertility is compromised by morphological anomalies by themselves or due to the correlation of sperm morphology to other variables (e.g. DNA integrity in boars[43]). In felids, a threshold should be set lower than in other species, because domestic and wild are generally affected by teratozoospermia (< 40% morphologically normal sperm).[44] Since then, similar conclusions have been reached from trials in areas as diverse as in vitro fertilization and intrauterine insemination and studies on sperm DNA damage. The pathogenesis of this relationship has not yet been well elucidated. In pigs, most breeding organizations have defined thresholds between 15 and 30% for abnormal sperm, with or without specification of thresholds for sperm with cytoplasmic droplets (15-30%).[45] It is important to note that such thresholds only apply when the observed spermiogram is representative of normal, generalized spermatogenic stress. In some situations, such as immaturity or gossypol toxicity, the particular abnormalities encountered may be more indicative.

Stains and preparations

Various stains and preparations have been used in evaluating human and animal sperm microscopically and a representative list is provided (Table 1 in Appendix). There are useful references.[15,46] Supra-vital stains (e.g. eosin nigrosin) are commonly used for semen staining in the field as they are simple to use, and they can depict sperm morphology reasonably well in addition to providing an insight into sperm vitality.[47,48] However, differential-interference phase contrast microscopy of 'fixed' (i.e. unstained) samples at 1,000 x is regarded as the 'gold standard' for depicting certain types of sperm abnormalities, particularly acrosomal,[49,50] as well more subtle sperm head or midpiece defects. However, either stained or unstained methods produced similar results in terms of bull classification.[51] Here, it is considered that some approaches recommended for human semen assessment, such as using the Papanicolaou stain and strict criteria for morphology categorization,[3] are not easily adopted for animal semen assessment, due to problems of logistics and complexity. Despite this, morphological indices for canine sperm have been developed[52] based upon those described in the WHO laboratory manual for the examination and processing of human semen.[24] It is important to note that such thresholds only apply when the observed spermiogram is representative of normal, generalized spermatogenic stress. In some situations, such as immaturity or gossypol toxicity, the particular abnormalities encountered may be more indicative.

Semen handling and preparation

Sperm morphological defects may occur both pre- and postejaculation,[53] with the latter including collection, handling and cryopreservation procedures,[54] as well as the staining method employed.[10] Semen collection methods as well as collection frequency can influence sperm morphology, and this can vary with species.[55] In addition, sperm morphology can be influenced by environmental factors such as pH,[56] bacteria, and inflammatory products,[57–59] and age of the donor; sperm morphology declined in stallions after 11-14 years of age[60] and in dogs after 7 years.[61,62]

If a representative semen sample has been obtained, then care should be taken to avoid subsequent sperm damage by protecting sperm viability and/or integrity during handling and processing. Factors that can affect results include sample preparation, species, extender or medium, objective magnification and 'quality' as well as operator knowledge and experience.[12] Defects that can be attributed to poor semen handling and those linked with poor preparation of semen smears are listed (Tables 2 and 3, respectively, in Appendix). A checklist of factors that could adversely affect sperm morphology, as well as proposed solutions, is also available in the WHO laboratory manual for the examination and processing of human semen.[24]

Other cells in semen

Semen consists of a fluid medium in which sperm are suspended. However, other cells and organisms can be present in semen, with many of these able to be observed microscopically. Occasional sightings of other cells do not necessarily indicate that a problem exists, although some are more clinically relevant than others.[63] For example, increased numbers of leukocytes in semen can indicate infection, whilst being capable of directly causing sperm oxidative damage.[64] Round spermatids, spermatocytes, and spermatogonia may indicate stress or damage to the spermatogenic epithelium. Bacteria are commonly observed in both fresh and stained semen,[13,58] and are, in themselves, capable of causing alterations to sperm DNA and morphology.[59,65] Slide preparation, proper staining and appropriate microscopy are all important considerations for the recognition of nonsperm cells in semen.[24] The recognition of major nonsperm inclusions (i.e. epithelial cells, macrophages, red blood cells, white blood cells, spermatogenic precursors, 'round cells,' epididymal cells, bacteria, contaminants, and debris), as well as appreciating their relative significance, should be an essential part of the sperm morphologist's skillset.

Conclusion

Competent and accurate assessment of sperm morphology is an important component of male fertility diagnosis and prognosis. For sperm morphology assessment to be as useful as possible, it is important that results are comparable and consistent among and within veterinarians, technicians, and morphologists. This requires the combination of optimal technique with good equipment and its application within the context of relevant animal history, supported by appropriate knowledge of male physiology and pathology. To encourage greater consistency, the following procedures are suggested as a basis for sample comparisons, within and among species: a. semen sample in a suitable fixative; b. use of phase/DIC microscopy at 1,000 x; and c. count of 200 sperm, and d; a 30% threshold for 'abnormal' sperm. It is also important that animal sperm morphologists have ongoing access to relevant continuing education, upskilling, and monitoring programs.

Conflict of interest

None to report.

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Appendix

Table 1. Stains and preparations used for assessing sperm morphology in animals

Stain	Details and source	Uses, advantages, and disadvantages
Eosin nigrosin	Sources Lane Manufacturing, USA Minitube International *www.minitube.com* References[15,66]	A one-step differential membrane- dependent 'supra-vital' stain, easy to keep and simple to use. Employed for 'live/dead' estimation as well as sperm morphology, although finer sperm structures may not be obvious. Recommended by the Society for Theriogenology.
Modified Giemsa	Sources *www.sigmaaldrich.com* *www.fischersci.com* References[15,67]	Commonly used in hematology for cellular depiction. Also useful for sperm morphology, particularly for acrosome definition.
Williams stain	Reagents from *www.sigmaaldrich.com.* *www.fischersci.com* References[68,69]	Double-stain method (carbol-fuchsin eosin counterstained with methylene blue). Good sperm morphology stain, despite requiring several steps.
SpermBlue® SpermBlue® prestained slides	Sources Microptic SL, Barcelona, Spain. *www.micropticsl.com* Fertility Technology Resources. *www.fertilitystuff.com* YouNing Biotech Co. Ltd. *www.youning.com* Reference[10]	A one-step stain for human or animal sperm that can be used on fresh, frozen and extended semen, as well as for automated sperm morphology analyses.
Spermac®	Sources Spermac laboratories *www.spermac.com* FertiPro NV *www.fertipro.com*, Minitube International *www.minitube.com* References[70,71]	Versatile, rapid, dual stain allowing separate visualization of the nucleus and cytoplasm, as well as good acrosome definition.
Diff-Quik, Dip Quick®	Sources Microptic SL, Barcelona, Spain. *www.micropticsl.com* Proiser R&D SL *info@proiser.com* References[72,73]	A Romanowsky stain used widely in clinical cytology. Commonly available in clinical settings. Can be used for combined assessment of morphology (recommended by WHO), seminal cytology and sperm DNA/chromatin stability.
Aniline blue	Reagents from *www.sigmaaldrich.com* *www.fischersci.com* Reference[74]	Histology stain. Also differentiates histones and protamines, and is a simple procedure.

(Continued)

Table 1. *(Continued)*

Stain	Details and source	Uses, advantages, and disadvantages
Toluidine blue	Reagents from *www.sigmaaldrich.com* *www.fischersci.com* References[75,76]	Useful to detect sperm chromatin abnormalities, as well as to depict morphology.
Trypan blue	Reagents from *www.sigmaaldrich.com* *www.fischersci.com*	Membrane dependent 'vital' stain, used for 'live-dead' estimation as well as sperm morphology. Can be used with 'fixed' semen. Also useful for hematology and cell cultures; one-step procedure.
Farrelly stain	Source Minitube International *www.minitube.com*	A 2-step contrast stain, useful for sperm morphology in samples which do not contain glycerol.
Kovacs stain	Reagents (trypan blue, congo-red and Giemsa) from *www.sigmaaldrich.com* Reference[77]	Combined sperm viability and acrosome stain.
Casaretts stain	Reagents (aniline blue, eosin B, Phenol) from *www.sigmaaldrich.com.* Reference[78]	One step stain which is useful for depicting different structures in human and dog sperm.
Cell-Vu® Morphology slides	Source TekEvent Pty Ltd info@tekevent.com	Prestained slides, simple procedure. Can be used with undiluted semen. It provides good depiction of head, acrosome and tail.
Modified Papanicolaou stain	Reagents *www.sigmaaldrich.com* *www.fischersci.com* Reference[13]	A versatile cytology stain with a modified version used for sperm morphology, particularly human, and which is also useful for seminal cytology, including round cells in semen. Commonly available in clinics. The procedure is relatively complex and time consuming.
Sperm stain Ready to use	Source Microptic SL, Barcelona, Spain. www.micropticsl.com	A rapid, 2-step Romanowsky stain used in human andrology. It is also useful for differential blood cell staining.

Table 2. Sperm morphology problems associated with semen handling

Causes	Outcomes
Nonphysiologic temperatures	Reduced percent intact acrosomes (PIA)
Contamination	Increased numbers of bacteria, sperm clumping
Rough handling	Detached sperm heads
Inappropriate extender	Reduced PIA, increased crystal formation
Nonisotonic media	Reduced PIA, increased 'bent' midpieces and tails

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Table 3. Sperm morphology problems associated with the preparation of semen slides

Contributing factor	Causes
Sperm are disrupted and/or have signs of mechanical damage	Rough smearing technique (including mixing using the edge of a glass slide) or the coverslip was disturbed prematurely
Sperm are too sparse on the slide	Poor mixing of sample
	Over-dilution of sample
	Excess stain
The dried smear shows the appearance of 'cracking'	Over-thick smear
	Slide exposed to excess heat while drying
Areas of excessive stain accumulation occur on the slide	Aged and/or unmixed stain
	Poor technique in making the smear
Sperm are stained too darkly	Stain was too thick or strong
	Staining time was too long
Sperm are stained too lightly	Stain was too thin or weak
	Staining time was too short
A clear area resembling a 'halo' is observed above sperm heads	Sperm movement before stain dried