Asian Journal of Andrology (2016) 18, 895–897

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INVITED COMMENTARY

Semen Analysis

Afterword to *Sperm morphometrics today and tomorrow* special issue in *Asian Journal of Andrology*

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Asian Journal of Andrology (2016) **18**, 895–897; doi: 10.4103/1008-682X.188451; published online: 30 September 2016

The problems associated with the subjective assessment of human sperm morphology have been well aired in another *Asian Journal of Andrology* Special Issue[1] that marked the publication of the 5th edition of the WHO Semen analysis manual, and contrary views have subsequently been presented.[2] However, the vagaries of the eye-brain system in assessing whether a sperm head is large or small can be eliminated by objective assessment where definitive structures are defined by their dimensions. These can then be classified automatically into as many categories as the data permit, conventionally on the basis of preset upper and lower limits, but also by more comprehensive analysis as discussed here.

This Special Issue on computer-aided sperm morphology assessment comprises four reviews (on sperm transport in mammals,[3] the current status of sperm morphometry in mammals[4] and birds,[5] and the relevant statistical methods to assess the morphometric results[6]); three clinical research papers (on sperm subpopulations in split ejaculates from adult men with normozoospermia[7] and in ejaculates from adolescents with or without varicocoele,[8] including the use of computer-assisted sperm analysis in assessing sperm nuclear DNA fragmentation[9]); and six veterinary research papers on sperm populations in the epididymis (in normozoospermic and teratozoospermic domestic cats[10]) and in ejaculated spermatozoa (from an endangered puma species,[11] roosters and guinea fowls,[12] rams, bulls and boars,[13] and on cryopreserved bovine semen[14]).

Although most of these studies examined the dimensions of the sperm head on air-dried, fixed, and stained cells, others have taken advantage of the objective method to determine the extent of organelle-specific fluorescent dye binding. DNA-binding dyes were used to examine sperm nuclear morphology in fixed smears of split human ejaculate fractions,[7] and together with chromosome-specific probes to determine the morphometrics of X- and Y-bearing bovine spermatozoa.[15] A combination of a nuclear dye with a fluorescent dye specific for the acrosome-specific dye permitted the simultaneous assessment of the whole head as well as its nuclear and acrosomal components.[13] These are novel approaches that should lead to rapid descriptive and diagnostic advances in both veterinary and clinical fields.

That there were more submissions from the veterinary than clinical field probably indicates the financial importance given to improving sperm diagnosis and selection in commercial industries. In fact, from the scientific point of view, much more work on sperm morphology and morphometry significance has been developed on other animal species than the human. The irruption of ICSI could also explain this difference. The fact that only one spermatozoon is enough to achieve a pregnancy has delayed spermatology research in human. On the other hand, the high variability of human infertility cases may make it seem an unattractive investment whereas the opposite is the case: the papers presented here show that investment in the equipment, and the researchers, to correlate clinical data with the morphometric results, would generate a range of observations on sperm subpopulations in fertile and infertile men that could explain currently unexplained causes of infertility.

THE SIGNIFICANCE OF THE MORPHOMETRIC ANALYSIS OF SPERM CELLS

The application of principal component (PC) and discriminant analysis to reveal subpopulations of spermatozoa is a powerful tool to evaluate raw semen and processed sperm cell suspensions, but not many clinicians are aware of the technique. As described in several papers here, PC analysis is a multivariate statistical method that reduces the number of variables used in subsequent calculations used to describe the data. By integrating the original variables according to their coherence in a database into a new complex mathematical variable, clearly defined homogeneous subpopulations of spermatozoa can be defined. In support of the theory above, the papers presented here showed that most of the variance from up to 13 morphometric variables could be explained by only two or three PCs: two in bulls,[16] adolescent humans,[8] adult human sperm head DNA,[9] domestic cats,[10] puma,[11] roosters, and guinea fowls[12] and three PCs in adult human split ejaculate samples.[7]

From these PCs, discriminant analysis was used to generate clearly separable homogeneous subpopulations of morphological forms. Here, the number of subpopulations ranged from two to five: two (for the X-/Y-bearing bovine sperm heads,[15] for large+elongated/small+elongated sperm heads in human adolescents[8]), three (for large+round/elongated/small spermatozoa in human sperm heads in split ejaculate fractions,[7] for elongated+intermediate/large+high acrosome/short+small sperm heads in the puma,[11] for small, wide and slightly elliptical/average size, long, narrow and very elliptical/very large, wide and elliptical sperm heads in the rooster[12]), four (for large/high medium/low medium/small in human sperm head DNA,[9] for small/short/large/narrow sperm heads in the bull,[14] for shape-related sperm heads in both normo- and terato-zoospermic cats[10]) to five (for very small, wide, very short and slightly elliptical/small, very short, very wide and slightly elliptical/very large, very wide, short and slightly elliptical/average size, very long, very narrow and very elliptical/average size, long, narrow and elliptical sperm heads in the guinea fowl[12]).

These awkward, convoluted, and very subjective descriptions of the nature of the sperm clusters generated by this technique highlight very well the difficulty in getting agreement (be it intra- or inter-laboratory, national or international) between observers on the definitions of normal sperm morphology, let alone abnormal forms. In contrast, the ability not only to detect, but also unambiguously define, subpopulations of spermatozoa by objective measurements derived from CASA-Morph is an important advance in morphological analysis. From this first step, advantage has to be taken of this knowledge for diagnosis of infertility, or promotion of reproductive performance in conservation biology, animal husbandry, or in the clinic. In other

words, subpopulations generated by this method could, and in future should, replace the previous approach of an artificial "*a priori*" classification of spermatozoa based on subjective evaluations. For example, in future, a new named and objective subpopulation, based on three PCs (e.g., SP2), could replace the subjective term "small." This paper opens the door to an integrated and holistic approach to sperm function. Until now, all the sperm parameters have been evaluated independently, diminishing the global power prediction. The more integrated the different interactive variables become, the better the evaluation of semen quality will be.

The significance of the different numbers of sperm populations in the species examined and seminal fractions obtained in remains to be followed up by studies investigating whether the presence or extent of certain sperm populations is associated with indicators of fertility or infertility. For example, if the changes in sperm populations upon maturation in the cat epididymis[10] are indicative of the epididymal maturation process, aberrant populations in the ejaculate could be indicative of epididymal malfunction in endangered feline species; the rapid and automated assessment of human sperm DNA damage[9] could be useful in optimizing selection methods that enrich populations in the less damaged cells required for ART; likewise, it would be interesting to find out whether differences in bovine sperm subpopulations between bulls, ejaculates and thawed straws[16] are present in the native semen or introduced by the cryopreservation protocol, and to use these subpopulations to monitor the development of methods to select the sperm subpopulation (of elongated and tapered spermatozoa) previously associated with fertility for AI.

THE FUTURE

New techniques of sperm morphometry have recently been developed, e.g., for the analysis of sperm nuclear morphology by the use of fluorescent stains, providing additional information on cell function,[16,17] as presented here,[13] and similar developments with new dyes are to be expected. The effect of preparative interventions on the final morphology and morphometry of sperm cells is well documented,[18–23] but eliminating the problem is a better option than attempting to take into account the preparative artifacts produced in the cells examined.

In this regard, the novel Trumorph® method dispenses altogether with air-drying, fixation, and staining, together with their artifacts, and involves the direct morphological examination of living, immobilized cells in raw semen.[24,25] Rapid, automated morphometric evaluation of such cells will provide the first approach to real-time analysis of sperm morphology that could precede the selection and removal of an unadulterated sperm cell, or spermatozoa, for ART. Perhaps observations could be extended to three dimensions in scanning confocal microscopy.

A NOTE ON CASA TERMINOLOGY

In preparing this Special Issue, it became apparent that the conventional CASA terminology was inadequate to describe the different uses to which the technology is now being put. In the papers initially submitted to this Special Issue, authors used several acronyms to describe the method they were using, including CASMA (Computer-Aided Sperm Morphology Analysis), CASMA-F (when fluorescent dyes were assessed) and ASMA (Automated Sperm Morphology Analysis). With these terms, neither the nature of the automation (with ASMA) nor the morphology examined (with CASMA-F) is clear from the abbreviation. In this Special Issue, for example, for spatulate spermatozoa, the sperm head itself, its acrosome, or its nucleus can each be analyzed by the system,[13] and filiform spermatozoa permit additional values on the length of the head and tail.[5] Thus, a change in terminology to one that indicates which sperm feature the system is measuring is needed.

The acronym CASA itself (computer-aided/assisted sperm analysis) is uninformative since the analysis could refer to any aspect of spermatozoa: their concentration, motility, kinematic parameters or morphology, or combinations of these. Indeed, the early papers used this blanket term to cover them all although the term CASA today is generally used in association with sperm kinematics.[26] For this use, the acronym would be more informative by the simple addition of M for motility (CASMA), but this letter could also be taken to stand for morphology. Using K for kinematics (CASKA) would be an alternative although not signifying that the percentage of moving cells is also recorded.

Any abbreviation must be informative, not only as to whether motility or morphology is being assessed, but also for the latter which organelles (whole head, acrosome, nucleus, midpiece, tail), sperm status (DNA fragmentation) or other features, are being analyzed. We suggest the following hyphenated compound terminology: the generic use of CASA for any kind of sperm computer-aided sperm analysis, followed by an abbreviation indicating the analysis performed, i.e., CASA-Conc (for concentration), CASA-Mot (for motility, including kinematics), CASA-Morph (for morphology, including morphometry), and CASA-DNA (when DNA is being studied). These could be extended if necessary to indicate when fluorescent dyes are used for morphology (CASA-Morph-F) or when DNA fragmentation is being assessed (CASA-DNAf).

In the revised manuscripts presented in this Special Issue, all authors agreed to use this terminology in their papers, and we hope others will also find it more informative and useful both for the authors and the readers.

AUTHOR CONTRIBUTIONS

CS, TGC, AV, and JLY contributed to different aspects of the review; CS and TGC wrote the manuscript.

COMPETING INTERESTS

CS developed Trumorph®. Neither he nor the other authors have interests that influenced the views presented in this paper.

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