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INVITED EDITORIAL

Semen Analysis

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

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Early in his investigations, Leeuwenhoek (1670s)[1] deduced that spermatozoa were alive and an integral part of semen, rather than artifacts or parasites. He eventually observed spermatozoa in the semen of men, dogs, horses, birds, fishes, amphibians, molluscs, and many insects, and concluded that they must be a universal feature of male reproduction. The huge differences in sperm form among species have been discussed in relation to evolutionary changes dictated by the egg and its investments.[2] Spallanzani (1800s)[1] was the first scientist to develop successful methods for artificial insemination, first with amphibians and later with dogs. With these experiments, he showed that physical contact between intact spermatozoa and ova was necessary to achieve the fertilization. Some years later (1820s), Prévost and Dumas[1] performed the defining experiment to identify correctly the function of spermatozoa in reproduction.

These two aspects of sperm morphology, evolution and reproduction, have driven the advances in morphometry discussed in this special issue, which in turn should benefit the fields of basic biology, as well as the economically important areas of veterinary husbandry and that of human reproductive medicine.

SPERM MORPHOLOGY AND PHYLOGENY

The relationship between sperm morphology and phylogeny, examined first by Wagner (1837),[1] suggested that the former could provide a useful clue to the latter. The extension of observations to over 400 species was compiled by Retzius (1900s),[1] who defined the three parts of the spermatozoon, a short roundish nucleus, a short midpiece that usually contains four or five mitochondria, and a long thin tail. Retzius[3] was also the first to describe the sperm cell's cytoplasmic droplet, whose importance is only now being recognized.

HUMAN SPERM MORPHOLOGY AND FERTILITY

The first assessment of human sperm morphology and its relationship to fertility was by MacLeod and Gold.[4] In their classification, which defined only obviously abnormal cells as not normal cells and nonfunctional, <25% of the total spermatozoa were abnormal in fertile men. However, the authors were aware that the classification criteria were quite arbitrary and could vary with different observers. Subsequently, standards for assessing human semen, including sperm morphology, were proposed,[5–11] all of them based on so-called "normal values;" unstated but presumably lower cutoff values of a range related to fertility. This resulted in the situation that the male was regarded as subfertile or infertile when semen parameter values were below the given values.[12,13] Similar attempts to produce morphological correlates with fertility have been made in other species, but they suffered from the same limitations.[14]

Conversely, the accidental discovery of IntraCytoplasmic Sperm Injection (ICSI)[15] and its subsequent explosive uptake in infertility clinics from the 1990s was based on the premise that every spermatozoon was competent to fertilize oocytes with this technique. Indeed with this method, which bypasses the normal obstacles to potentially fertilizing spermatozoa of the approach to, adherence to and penetration of the oocyte, even immature germ cells (round and elongated spermatids and their nuclei) can initiate fertilization in the related techniques of ROSI, ROSNI, and ELSI,[16] although their immature cytoplasmic components may make the method unsafe.[17,18] These techniques heralded the slowdown in advances of fertility-related sperm morphology, since the morphology of the injected sperm cell, other than having a DNA-containing head (and sometimes a tail), was incompletely assessed during the selection at low magnification microscopy of viable (motile) sperm cells or germ cell preparations.

SPERM PREPARATION AND SPERM ARTIFACTS

It has been known since the 19th century that the microscopical structure of living cells can be modified by fixation and any postmortem change,[19] but inadequate attention has been paid to factors that may damage spermatozoa before fixation and microscopical examination. Any conclusion about the morphology of spermatozoa is therefore open to serious doubt, unless steps are taken to determine and control the viability of the material studied.[20] It is disturbing that this knowledge seems to have been forgotten for the long period when the definition of what a "morphologically normal spermatozoon" was being decided for many species. For example, air-drying of unfixed human ejaculated and monkey epididymal spermatozoa permits the expansion of sperm heads into macro-head forms in a process that is prevented by prior fixing of the cells;[21,22] these forms do not exist as living cells in semen and cannot contribute to male infertility.

The vexed question of the appearance and presence of cytoplasmic droplets, sperm organelles often considered abnormal by

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clinicians but normal by research scientists, was also highlighted by comparing sperm cells in semen, both unfixed live and fixed *in situ*, where droplets are present, and sperm cells fixed and stained after air-drying, where true droplets are rarely observed.[23] The morphological difference between the abnormally retained excess residual cytoplasm and the normally present cytoplasmic droplets, the different responses of each organelle to preparative techniques, and the possible avoidance of normal cells with droplets in ICSI were subsequently reported.[24,25]

INCONGRUITY OF STANDARDIZED MORPHOLOGICAL TECHNIQUES AND ANTICIPATED RESULTS

Although standards for assessing sperm morphology have been established, they depend on defined protocols; for instance, the WHO (2010)[26] criteria refer to the use of the Papanicolaou staining procedure on cells fixed in air-dried seminal smears; i.e., the very techniques that introduce morphological artifacts could seriously change the final assessment of the spermatozoa and the diagnostic interpretation of the semen sample. Can it really be that all the observations made on "normal cells" over more than 60 years were of cells prepared with the histological techniques of dehydration, fixation, and staining that induce the formation of artifacts, which are absent from the fertilizing cell?

In addition, many laboratories are using stain techniques other than those recommended by the WHO. This is a large, variable, and largely uncontrollable incongruity because it has been demonstrated in many species that different staining techniques could induce changes in sperm head size and shape.[27,28]

SUBJECTIVE ASSESSMENTS LEAVE A LOT TO BE DESIRED

In the subjective analysis of sperm morphology, not only the method needs to be taken into account, but also the technicians' inferences from the forms observed.[29] In the most recent edition of the WHO manual,[26] the criterion of "normality" was changed from single normal values to confidence intervals for each variable from a fertile population.[30] Unfortunately, many laboratories persist in using the former manual.[31] Even with well-defined standards, good internal and external quality control programs need to be designed and put into practice[32,33] but are more than often not introduced.

Some work has been done to consider the effect of preparative interventions on the final morphology and morphometry sperm cells,[28,34,35] but eliminating the problem is a better option than attempting to take into account artifacts. In this regard, a new technique (Trumorph®) that allows observation of sperm morphology of unfixed cells in raw semen without staining, thus preventing artifact production, has been developed.[36,37] Other techniques, for the analysis of sperm nuclear morphology by the use of fluorescent stains, have been recently developed, providing additional information on cell function.[38–40]

OBJECTIVE APPROACHES TO SPERM MORPHOLOGY ASSESSMENT

In an attempt to bypass the subjective evaluation of morphology, van Duijin (1972)[1] provided an accurate morphometric study of human sperm heads and their differences between normal and subfertile men, albeit on fixed and stained samples. During the 1980–1990s, many groups worked on the definition of sperm head morphometry with semi-automatic image analysis systems.[41–47] From the beginning, the introduction of commercial Computer-Assisted/Aided Semen Analysis (CASA) technology for morphological analysis proved useful in different species for determining sperm quality.[22,34,48–55] However, CASA use was frequently restricted to reproducing what a technician could do, i.e., looking for a classification reflecting the number of normal spermatozoa present in the sample.[56] This approach may make sense for general clinical purposes, and for laboratories following the criteria used until now, and is an improvement in that the evaluation is objective and based on metric measurements that provide greater importance to the data obtained. Other advantages of CASA are a reduction in the time required for analysis, and lower variability in data, increasing the repeatability and precision of the measurements, and the automatic and long-term storage of the information, making possible reanalysis and the possibility of comparisons of results between laboratories. All these improve male fertility diagnosis, and consequently the decision on what is the best assisted reproductive technique to apply.[57,58]

On the other hand, is there any sense in using this sophisticated technology merely to replace a technician's decision of what a normal spermatozoon is? Furthermore, what is a "normal spermatozoon"?

CURRENT AND FUTURE DIRECTIONS

A recent paper on these pressing problems[59] covered the definition of the morphology of a potentially fertilizing spermatozoon, possible changes in sperm characteristics as they migrate from the ejaculate toward the oocyte, and putative functions for all the abnormal spermatozoa observed in high proportions in species such as the human. These questions may well be answered by looking into the study of sperm heterogeneity both within and between ejaculates and species. With the information we have now, it is no longer valid to consider the entire seminal sperm population as one population, represented in calculations by central tendency statistics; the problem requires analysis of the complex subpopulation structure.[40,60,61] Although reviews on the use of CASA for morphological analysis have been published,[38,62,63] this special issue of the *Asian Journal of Andrology* is devoted to sperm morphometry. It presents an update of this objective approach in its various forms, in which advanced technology is applied for the comprehension of the real structure of the seminal sperm population, and discusses how this could be important in charting the evolution of species and be significant for the improvement of fertility treatment.

Although there are many commercially available CASA machines that can assess sperm dimensions, few research groups are utilising the proper statistical analyses that should be used to assess the large volumes of data generated. The mention of specific companies or of certain manufacturers' products does not imply that they are endorsed, supported or recommended by the *Asian Journal of Andrology*.

This issue comprises 15 papers; this introductory Foreword and the Afterword[64] summarize the contents of the remaining 13 papers, of which four are strictly reviews in clinical and veterinary science, and 9 are research papers, three in clinical science and six in the veterinary field, some with extensive literature coverage. They provide data on the morphometric measurements of spermatozoa, and statistical analysis of sperm populations, in epididymal and ejaculated semen from domestic cats, endangered species (pumas), birds (roosters, guinea fowl), domestic animals (cattle, sheep, and pigs); they also explore the role of CASA in determining and changes upon cryopreservation of bovine semen, in the prediction of the sex of progeny, and in the clinical conditions of DNA fragmentation, split ejaculates, adolescence and varicocoele.

COMPETING INTERESTS

CS is Professor at Valencia University and acts as Scientific Director of Proiser R+D S.L Research and Development Laboratory neither he nor TGC has interests that influenced the results presented in this paper.

AUTHOR CONTRIBUTIONS

CS started working on sperm morphology in the early 1980s and had the opportunity to work with TGC in 1993. From then on, they have collaborated on technical developments in this area that sought the best approaches for sperm observation and analysis, particularly in relation to CASA technology. This joint interest was the inspiration for the present AJA Special Issue, which provides an update of the knowledge obtained from the most advanced concepts on sperm subpopulation structure in different species.

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