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Automated analysis of rat sperm morphometry and morphology: Moving towards cut-off values for normal sperm morphology and toxicology

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Abstract

This study investigated Automated Sperm Morphology Analysis to study rat sperm morphometry and morphology in two strains of rats (Sprague Dawley and Wistar) in three centers. The main aim was to develop normo-spermic baseline values for sperm morphometry and to quantify the percentage of morphologically normal sperm in healthy untreated rats. Standardizing this procedure will establish an important new measurement tool for studies in reproductive toxicology. The participating centers were (i) IRSN in Paris, France (Sprague Dawley rats), (ii) University of the Western Cape, South Africa (Wistar rats) and (iii) Stellenbosch University (Wistar rats), South Africa. All three centers used identical sperm isolation techniques from the cauda epididymis, the same staining protocols (SpermBlue), identical CASMA software (SCA RatTox system, version 5.4, Microptic SL, Barcelona, Spain) and microscopes (Nikon) with similar optics (x60 objective in brightfield mode). From the results it is evident that it is indeed possible to distinguish between Sprague Dawley and Wistar sperm morphometric characteristics. We were furthermore able to develop cut-off values for evaluating the percentage normal sperm in these two rat strains quantitatively in the automatic analysis mode. Normal sperm morphology varied between 67% and 74%, in contrast with previous findings suggesting values above 90%.

Key words

CASMA, rat sperm, morphology, morphometry, Sprague Dawley rats, Wistar rats

1. Introduction

There is consensus in the literature that the rat is a good model for reproductive toxicology studies (Perrault and Cancel, 2001). The question is which “reproductive” toxicology tests should be targeted? A conceptual framework and protocol, the Standard Multigenerational Test Protocol, has met the needs of many groups involved in toxicology testing/monitoring (US Environmental Protection Agency, 1998; Claudio et al., 1999). Perrault and Cancel (2001) indicated it was revised and updated to include sperm production, motility and morphology in an effort to harmonize the Multigenerational test and the Reproductive Assessment by Continuous Breeding (RACB) test (Chapin and Conner, 1999).

Moreover, studies on sperm production, morphology and motility proved useful in numerous reproductive studies employing rats and mice (Harris et al., 1992; Linder et al., 1992; Clegg et al., 2001; Hattingh and van der Horst, 2000; Mdhluli and van der Horst, 2002; Brooks and van der Horst, 2003). In this investigation it was accordingly appropriate to address at least one of these facets, sperm morphology, in more depth and detail.

Sperm morphology has always been considered an important parameter when evaluating semen/sperm quality. However, a major concern has always been the subjective nature of manual microscopic assessment and thus manual assessment of sperm morphology has similarly been viewed as problematic. Verstegen et al. (2002) showed large variations between technicians and laboratories due to the subjective evaluation of sperm morphology and this made it difficult to accurately interpret the results. Various studies alluded to methods employed for manual analysis of rat sperm for the determination of normal morphology. In numerous instances an increase in abnormal forms were reported when animals were exposed to a toxicant (Linder et al. 1992; Seed et al., 1996; Linder et al., 1997; Chapin and Conner, 1999; Kuo-Hsin Lee et al. 2014; Vashishat

et al. 2012), but simultaneously the deficiencies of manual subjective assessment of sperm were also highlighted (Seed et al. 1996; Perrault and Cancel 2001). The problem of classifying borderline sperm forms using subjective assessment were emphasized by Perrault and Cancel (2001) as one of the contributing factors.

A challenge with automated analysis of rat spermatozoa is the curious hook shape of the sperm head. Despite these challenges, various computer aided sperm morphology analysis (CASMA) systems have been developed to objectively evaluate rat sperm morphology (Davis et al., 1994 - Rat Morf system of HT, Rat Metrix system of Hamilton Thorne; RatTox morphology of the Sperm Class Analyzer, Microptic SL, etc.). Surprisingly, despite these sophisticated developments, it was still extremely difficult to find peer reviewed publications on sperm morphology using CASMA and to our knowledge there are no detailed research papers providing either comprehensive morphometric data of rat sperm or comparing sperm of different laboratory rat strains in terms of morphometry and morphology. Accordingly, while instruments such as the Hamilton Thorne RatMetrix and Sperm Class Analyzer RatTox systems provide guidelines for establishing normal versus abnormal sperm morphology, cut-off points are not available to objectively estimate the percentage of morphologically normal sperm.

The main aim of this investigation was to employ the morphology module of the Microptic SCA RatTox system in order to study and compare sperm morphometry in two strains of laboratory rats. The specific objectives were to determine standard/normo-spermic sperm morphometric values for these two rat breeds, Sprague Dawley and Wistar, as well as to establish cut-off points to allow for the determination of the percentage morphologically normal sperm.

Once CASMA is standardized it may provide relevant information for studies focused on evolutionary biology, sperm formation, sperm quality assessment, the prediction of fertility potential, semen cryopreservation, and the effect of reprotoxicants (Yániz et al. 2015).

2. Materials and Methods

2.1 Animals

Collectively, 49 male rats from three different research centers, i.e. Institute of Radioprotection and Nuclear Safety (IRNS), University of the Western Cape (UWC) and Stellenbosch University (SU), were included in this study. Table 1 lists the breeds (Sprague Dawley and Wistar), age, and origins of all rats used as well as the number of rats used for each research unit. All animals were maintained under standard conditions ($22^{\circ}\text{C} \pm 1^{\circ}\text{C}$ with a 12 hour day/night cycle, relative humidity of $\pm 40\%$), receiving food and water *ad libitum*. Body weights, food and water were measured weekly to maintain a record of food consumption and weight gain. All animals were treated according to the specific regulations and ethical principles set by the committees governing the respective research units; The University of the Western Cape's Senate Research committee, the Stellenbosch University Research Ethics Committee: Animal Care and Use, and the Animal Care Committee of the Institute of Radioprotection and Nuclear Safety , and respectively in accordance with the revised South African National Standard for the Care and Use of Animals for Scientific Purposes (South African Bureau of Standards, SANS 10386, 2008) and the French legislation and European legal requirements (Decree 86/609/EEC) concerning the protection of animals used for experimental purposes (Ministry of Agriculture Act No. 87-848, October 19, 1987, modified May 20, 2001).

The animals were sacrificed either by euthanasia with an overdose of sodium pentobarbital (intraperitoneal 100mg/kg) (UWC & US) or anaesthetized by an inhalation of 5 % isoflurane (Abbot France, Rungis, France) followed by intracardiac puncture euthanasia).

2.2 Sperm isolation from epididymis and staining of sperm

Directly after sacrifice the epididymides were harvested from each rat and placed in a petri dish containing a 5ml solution of Hams F-10 nutrient medium (Sigma-Aldrich, St Louise, MO, USA) or DMEM (Sigma Chemicals, St Louis, MO, USA) supplemented with 3% Bovine Serum Albumin (BSA) (Roche Diagnostics GmbH Mannheim, Germany) at 37°C. The proximal caudal portion of each epididymis was dissected free from blood vessels, fatty tissue and other connective tissue. A short length of “cleaned” cauda epididymal tubule was removed by using either iridectomy scissors or fine-point scissors and placed in 2ml of 3% Hams-BSA in a 30mm petri dish.

Sperm flowed freely from the ends of the transected tubule into the medium (no blood etc.) and the surrounding medium quickly became opaque as it filled with highly motile sperm. Media containing sperm were collected from the edges of this opaque area using a positive displacement pipette (10µl). Sperm concentrations at the edge of these opaque areas varied from 1 to 3 million sperm/mL and sperm motility was in excess of 80%. Both sperm concentration as well as the percentage sperm motility measurements were performed using the Motility module of the Rattox system of SCA 5.4 (Microptic SL, Barcelona, Spain). The 10µl sperm suspension was placed on a slide and a sperm smear was made by dragging the fluid behind another slide while spreading it at a 45° angle across the slide. After air drying at room temperature, slides were

fixed by immersing them vertically into SpermBlue® fixative (Microptic SL, Barcelona, Spain) for 15 to 20 minutes, and stained thereafter with the SpermBlue® stain for 15 minutes, following a similar immersion technique (van der Horst and Maree, 2009). Stained slides were then gently submerged in distilled water for 3 seconds to remove excess stain, and allowed to air dry at a 60-80° angle. Slides were finally mounted using DPX (Merck, Modderfontein, South Africa) or an equivalent mounting medium such as Eukitt. Standardization of the staining and automated analysis for rat sperm in this study conformed to the original protocols and techniques described by van der Horst and Maree (2009) and accordingly ensured uniformity of analysis.

2.3 Automated Sperm Morphometry and Morphology

Sperm morphology and morphometry were studied by means of CASMA using the Sperm Class Analyser system (SCA®; Microptic S.L., Barcelona, Spain) version 5.4.0.0 and 6.1 software. Bright field optics employing a 60 x objective (i.e. 600 x primary magnification) and blue filter were used on either a Nikon Eclipse E50i (UWC) or a Nikon E200 (IRSN and SU) microscope. Software settings were as follows: contrast and brightness were optimized for complete thresholding of the sperm head and midpiece (Figs 1 and 2). One hundred sperm per animal were analyzed. Sperm images were captured digitally using a Basler 312fc firewire camera (UWC and SU) or a Basler 780-75 giga - Ethernet camera (IRSN) and analysed automatically using the SCA system's (Microptic S.L., Barcelona, Spain) RatTox morphology module. The software automatically analyses morphometric dimensions of the sperm head and mid-piece. In terms of the head morphometry, head length (ARC – head length), width, perimeter, surface area, roughness, regularity, and number of vacuoles (or nuclear craters representative of

fragmentation) were measured. Measurements of the midpiece included width, area and angle of insertion of the flagellum to the head. Furthermore, the Chord length (distance from the anterior tip of acrosome to the posterior part of head) was measured and the Linearity calculated (a derivative expressed as a percentage and refers to $\text{ARC/Chord} \times 100$ was measured). All these measurements and derivatives, as well as the relevant formulas for determining them are depicted in Figure 1. The software also calculates the teratozoospermic index (TZI), which indicates if a sperm has more than one abnormality.

Eighteen sperm randomly analyzed by the SCA RatTox software are shown in Figure 2. Each of these sperm are shown as the actual image captured and then next to it the subsequent analysis (thresholding). This aspect of analysis is important to show correctness of thresholding/measurement and for quality control as used in this investigation.

All morphometric/morphology data for all three centers were saved as *.mrf files. When these files were opened in the SCA morphology module, it showed all the analyzed sperm with all the morphometric features. Accordingly to ensure that all rat sperm analyzed from the three centers could be compared without any bias in terms of software, all these mrf files were opened in one computer at UWC using the SCA 6.1 software. This approach ensured further standardization among the three centers and comparability. The data from these files were used for all subsequent statistical analysis.

2.4 Statistics and quantitative approach for determining provisional cut-off points for normal sperm morphology

The MedCalc 11.1 statistical software package (Schoonjans, Mariakerke, Belgium) was used to analyze the results. Data mostly displayed a parametric distribution and an ANOVA was used to

test the differences between means, while the Kruskal-Wallis test was employed when data showed a non-parametric distribution. A p- value of <0.05 was considered statistically significant.

Compared to humans and domestic animals it is problematic to determine morphologically normal *versus* morphologically abnormal sperm in animal species where relatively few guidelines and publications exist. A new approach was used to determine cut-off points for normal *versus* abnormal rat sperm in this study. This was based on the grouping of the different morphometric measurements for each percentile, for example 0 to 100%, 2.5 to 97.5 percentile, 5 to 95 percentile, 10 to 90 percentile for each of the different morphometric parameters (e.g head length, width, perimeter). Accordingly the minimum and maximum value for a specific morphometric parameter could be derived from the different percentile intervals as analyzed using routine statistical methods. The rat morphometric values for different percentile groupings were subsequently used in the RatTox configuration function for normal morphometry (Table 2). It is emphasized that selection of a particular percentile interval was correlated with normal types *versus* distinct abnormal types such as banana shaped (spoon shaped) head or any other obvious deviation. However, it soon became evident that subjective visual assessment was not always an accurate mode to consistently distinguish between macrocephalic and microcephalic sperm in rats (see results later).

3. Results

The morphometric data for the two murine strains from the three different centers are shown in Table 3. The two Wistar strains (of slightly different origin, breeding and age) differed

significantly from the Sprague Dawley's (SD) for most parameters. Despite being bred as separate colonies, the two Wistar cohorts only differed from each other by one morphometric parameter namely Head Angle. These comparisons are also graphically depicted in Fig. 3. It appears that the Sprague Dawley (SD) rat sperm are smaller than that of Wistar (W) rats, but both the Chord and Linearity are larger in Sprague Dawley than in the Wistar strains. It implies that the hook of the anterior part of the sperm head (which involves mostly the acrosome) is less bent in the Sprague Dawley breed. In this context one would expect the Head Angle to be larger in Sprague Dawley (SD) than Wistar (W) rats. It was significantly larger in SD compared to the SU Wistar cohort. A screenshot of a sperm that has been thresholded with all the morphometric values indicated is indicated in Fig. 4. Values in red indicate that a particular parameter is abnormal based on the percentile grouping selected. In this instance Head surface area is higher than the selected threshold value for normal.

Normal and abnormal sperm correctly thresholded and classified as either normal (green box) or abnormal (red box) is shown in Fig. 5. Two abnormal spermatozoa are shown in Figs. 5 A and B, and the top sperm can easily be recognized as “spoon-shaped” (our terminology) or “banana shaped” (terminology used in some publications). The bottom sperm appears normal by subjective visual assessment, however when objectively measured by CASMA it is classified as abnormal on the basis of an ARC (head length) longer than and a surface area larger than the cut-off values for normal (macrocephalic). The sperm in Figs. 5 C and D represent three sperm and the top sperm is microcephalic which is almost impossible to discern by subjective visual assessment. However, in this instance the ARC is below the cut off point for normal. The morphometric measurements of the two bottom sperm in Fig. 5 C and D (green boxes) fall within the cut-off values for normal sperm.

The above analysis measures morphometric parameters of the sperm head and anterior part of the tail (mainly midpiece). It was possible to measure tail length in those sperm where the entire tail was present in a microscopic field. However, very few tail abnormalities were observed in the rats under investigation and these abnormalities as well as those indicated in the literature are shown in Table 4. Where possible, tails were also scored subjectively and the SCA software allows for tails to be added as normal or abnormal.

The percentage normal sperm as well as the ranges for normal sperm morphology from the different centers based on the cut-off points shown in Fig. 2 is shown in Table 4. No significant differences were found between the three groups for percentage normal sperm as well as for the teratozoospermic index (TZI). However, in Sprague Dawley animals there appears to be only one abnormality per abnormal sperm ($TZI = 1$) compared to Wistar rats with very few abnormal sperm had more than one abnormality ($TZI >1$ and <1.1).

4. Discussion

This investigation is the first of its kind, providing detailed quantitative normospermic morphometric values for two rat strains as determined in three research centers using the same sperm isolation techniques, similar equipment and similar software. One of the significant findings is that there are apparent differences in sperm morphometric values among breeds. We could show that for most sperm head parameters and some midpiece parameters, Wistar rats from two centers differed significantly from that of Sprague Dawley rats. It also shows our sperm morphometric values to be highly repeatable and independent of the user or the

laboratory, emphasizing the need for future use of quantitative approaches for rat sperm morphology. This has also been emphasized by Beletti and Costa (2002), Maroto- Morales et al., (2010) and van der Horst (2015) for sperm morphology of domestic animals.

Unfortunately no detailed comparative data exists for the same or other laboratory rat breeds using accurate CASMA analysis. Some data on head length, midpiece length and tail length exists for studies using measurements by calibrated eye ocular or semi-automated image analysis systems. Cummins (1983) and Cummins and Woodall (1985) provided a wealth of information on manual measurements of mammalian sperm including several wildlife rodents but not for the laboratory rat.

In a recent investigation, Vashishat et al. (2012) used rat sperm stained with eosin-nigrosin in conjunction with Magnus Pro Image Analysis software (Magnus Analytics) to measure different components of rat sperm in Wistar and Sprague Dawley. They reported an average sperm length of more than 25 μ m, which is considerably larger than our findings which average approximately 21 μ m. Most other sperm measurements such as surface area also seem to be much higher in the Vashishat study than is the case in our investigation. The SpermBlue stain used in our study contains balanced salts and is nearly isosmotic/isotonic to semen and did not result in any changes due to swelling, also evident in previous human and animal studies including mice (van der Horst and Maree, 2009; Maree et al. 2010).

Sanchez et al. (2013) used the NIS-Elements image analysis system (Nikon) in conjunction with plug-in software to threshold very divergent shapes of many different muroid rodent sperm heads. This development focuses entirely on the sperm head and is predominantly used in terms of defining a species based on sperm head morphometry in/during sperm competition. In the

case of reproductive toxicology where the laboratory rat is used a system is needed that analyses large numbers of sperm relatively fast in an automated or semi-automated mode.

In the “normo-spermic” rats analyzed in this investigation very few morphologically abnormal forms have been observed apart from spoon shaped, macrocephalic, microcephalic and amorphous. However, many other abnormalities such as blunt acrosomes, pin heads, abnormal head, double heads have been described in many other studies (Table 5). In future studies involving toxicants these different abnormal sperm forms need to be quantified and further cut-off points can be developed on the basis of performing Receiver Operating characteristic curves (ROC) for recognizing a specific abnormal type.

Previous publications using manual assessment of sperm morphology indicated that the percentage abnormal sperm in control non treated rats only amounted to approximately 2 to 3% abnormal forms. The current study showed that the percentage normal sperm seems to be considerably lower and vary from around 48 to 88%. Averages of about 71 % and 68% normal sperm were recorded in normo-spermic Wistar and Sprague Dawley rats respectively. There are a number of possible reasons for the discrepancies in findings between previous manual assessments and the current study. We believe that the unusual thin hooked shaped form makes it more difficult to discern differences (abnormalities) among rat sperm than for example in humans. In humans the sperm head is partly conical and highly heterogeneous sperm populations are common. There are accordingly more abnormal forms and abnormal types in humans than compared to rats. Unless a micro- and macrocephalic rat sperm are in the same visual microscopic field, it will be almost impossible to discern these differences when manually analyzed individually. Furthermore, subtle differences that may exist in border line forms makes

subjective manual assessment nearly impossible as compared to CASMA with totally objective cut-off points. This notion is also supported by Perrault and Cancel (2001).

Our approach to establish and define a normal rat sperm is a good starting point to decipher normal from abnormal forms. Undoubtedly, care needs to be exercised when relating abnormal sperm forms that may not have fertilizing potential. In human sperm it has for example never been proven that amorphous abnormal sperm cannot fertilize the oocyte. Moreover, our current classification of normal *versus* abnormal sperm must be seen as preliminary and a basis to move towards cut-off points for toxicological studies.

There is also a need to relate the different abnormal sperm forms to where they originate from. For instance, abnormal forms typically relate to faulty spermatogenesis with minor occurrence during transit along the epididymis. This is particularly important in toxicology where rats have been exposed to specific substances to subsequently identify/establish if the effect was at testicular or post-testicular level.

The current CASMA software programmes focus on the sperm head, acrosome and midpiece (anterior part of tail), but in future it needs to also expand to include comprehensive tail analysis. It is possible to perform these measurements, but it is problematic when using either x60 or x40 objectives as the long tails tend to fall outside of the microscope/camera field. There may be a need to use the x20 objective, but the required resolution for detailed head morphometry may be lost. In the interim the manual addition of the relatively few tail abnormalities (between 2 and 6%; Vashishat et al., 2012) as part of the CASMA system will provide an intermediate means to consider all parts of the sperm.

Detailed CASMA of rat sperm must be seen as one of the building blocks to assess the effects of different experimental conditions or toxicants on sperm biology. Sperm morphometric

information needs to be combined with sperm motility measurement and with sperm functional testing such as vitality, DNA fragmentation and acrosome intactness, as described by Kuo-Hsin et al. (2015). The analyses of all these different sperm parameters need to be viewed in the framework of the Multigenerational Sperm Protocol and preferably analyzed with a system of model building such as principal components and other multi-factorial tests. Subjective analysis, which tend to be too variable need to be replaced with tests using a proper quantitative and objective basis.

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Declaration of interests:

G van der Horst is a senior consultant to Microptic SL. The three centers used their RatTox SCA CASA system of Microptic SL in this investigation, We declare that there was no financial benefit and use of the SCA system for this investigation was purely co-incidental.

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Captions to Figures:

Fig. 1: Stained rat sperm, thresholded for head (blue) and midpiece (green), showing the various morphometric features as well as the derivatives and angles of sperm head and midpiece (LIN = Linearity)

Fig. 2: Stained rat sperm, automatically analyzed (thresholded) for head (blue) and midpiece (green). Each image on left is the actual stained sperm and directly to the right is the subsequent SCA analysis with blue overlay showing the head and green overlay the midpiece (thresholding). In the CASMA report of each analysis these figures allows for quality control and correctness of thresholding for the different components of sperm.

Fig. 3: Box and Whisker plots of the most important sperm morphometric parameters as determined by CASMA. Except ARC or Head length (A), all other parameters (B to H) for Wistar rats (US and UWC) differ significantly from Sprague Dawley rats (IRSN) $p < 0.05$.

Fig.4: Abnormal spermatozoon is indicated in a red block while the failed parameter(s) is also highlighted in red on the left, e.g. surface area, exceeded the cut-off point for normal.

Fig. 5: Two pairs of micrographs depicting the actual sperm and the subsequent analysis as well as classification thereof. (normal - green box or abnormal - red box). A and B: top sperm abnormal - “spoon-shaped” (banana-shaped) and bottom sperm abnormal - macrocephalic sperm;

C and D: Top abnormal - microcephalic sperm and two bottom sperm normal; by definition of sperm morphometry cut-off points as indicated in Fig. 2 and Table 2.

Captions to Tables

Table 1: Centers involved, number, strain, origin and age of rats

Table 2: Normal head and midpiece properties selected to determine normal *versus* abnormal sperm under the Configuration setup of the SCA 5.4/6.1 system using 5% to 95% percentiles. (SCA = Sperm Class Analyser, H = Head; MP = Midpiece)

Table 3: Average (\pm Standard Deviation) and range (minimum-maximum values) for sperm morphometric parameters of the head and midpiece of Sprague Dawley and Wistar rats from three centers in France and South Africa. H = Head; MP = Midpiece; TZI = Teratozoospermic index; N refers to the number of rats per center. Letters in the same row that differ are statistically significantly different ($p < 0.05$)

Table 4: The percentage morphologically normal sperm, \pm SD, the minimum and maximum normal percentages and the Teratozoospermic Index (TZI) for the sperm of different rat strains from each center.

Table 5: Examples of sperm morphology abnormalities observed, breed, origin of abnormality and reference; W = Wistar; SD = Sprague Dawley.