Effect of sample size and staining methods on stallion sperm morphometry by the Sperm Class Analyzer

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ABSTRACT: Computer-assisted sperm morphometry analysis has improved the assessment of sperm morphology, but the results depend on the use of adequate evaluation and staining procedures of spermatozoa from individual species. In this study, the morphological module of the Sperm Class Analyzer® was used for the morphometric analysis of stallion sperm heads and midpieces. Semen samples were obtained from six fertile stallions in order to evaluate the influence of three staining procedures (Diff-Quik, Hemacolor and Harris' Haematoxylin) on the accuracy of image processing and sperm morphometry, and the effect of the sample size on sperm morphometric measurements. Harris' Haematoxylin was the staining technique of choice on the accuracy of the image processing with an optimum contrast of sperm cells with the surrounding background that allows an efficient boundary detection and segmentation which results in the highest proportion of sperm heads and midpieces assessed (80.47%). The results indicate that the staining methods affected significantly the sperm dimensions with increased values from Diff-Quik than Hemacolor and Harris' Haematoxylin respectively (Diff-Quik > Hemacolor > Harris' Haematoxylin). No differences in morphometric parameters were found when 100, 150, 175 or 200 spermatozoa were analysed. In conclusion, to obtain objective and accurate sperm morphometric measurements by the Sperm Class Analyzer®system in the stallion, it's recommended the analysis of 100 spermatozoa from slides which have been previously stained with Harris' Haematoxylin.

Keywords: ASMA; sperm head; midpiece; morphometric anlaysis; validation

The evaluation of sperm quality is useful in predicting the fertility of sperm donors (Colenbrander et al., 2003) and is of great importance in maximizing reproductive efficiency, either under natural breeding conditions (Jasko et al., 1990) or in programs of assisted reproduction (Rodriguez et al., 2001). Furthermore, it is a useful tool in the clinical diagnosis of subfertile animals. Conventional evaluation techniques have been based on the subjective assessment of semen parameters such as motility, morphology and semen volume or concentration (Verstegen et al., 2002). Abnormalities in sperm morphology are an important indicator of decreased fertility in humans (Kruger et al., 1988) and some animal species (Voss et al., 1981; Chandler et al., 1988; Sekoni and Gustafsson, 1987) and have also been used as an indicator of the effect of various toxicants on sperm production (Foote et al., 1986).

However, the subjective assessment of sperm morphology based on visual observation has led to widely varying results due to numerous factors such as the use of different staining procedures or the experience of technicians, among others. According to Zaini et al. (1985), the variability in results can range from 40–60%, demonstrating the low repeatability of these methods (Jequier and Ukombe, 1983; Ombelet et al., 1997; Cooper et al., 1999). These variations make it difficult to accurately interpret data, underscoring the need for techniques which are objective, precise and repeatable.

In the 1990's, the introduction of automated sperm morphometry analysis systems (ASMA) attempted to overcome the problem of the subjectivity of visually based methods of assessment. Although this technology was originally designed for human sperm (Davis et al., 1992; Kruger et al., 1993; de Monserrat et al., 1995), it has been progressively adapted to some animal species (Gravance et al., 1996; Sancho et al., 1998; Iguer-Ouada and Verstegen, 2001). These systems are capable of detecting subtle differences that conventional methods were unable to identify (Jagoe et al., 1987), such as the relationship between sperm morphometry and fertility (Casey et al., 1997).

For accurate sperm morphometry analysis, a number of analytical variables are used for each species. Currently, the precision of ASMA systems depends upon the standardization of these variables (Davis and Gravance, 1993; Gago et al., 1998; Gravance et al., 1995; Hidalgo et al., 2004), namely appropriate sample preparation (washing, fixation and staining) and correct microscopic image analysis. In addition to the variations inherent to the evaluation process, errors are often the result of differences between ASMA systems or the fact that an insufficient number of spermatozoa are analysed which are not representative of the sample.

The aims of the present study were to evaluate the effect of three different staining procedures on the accuracy of image processing and sperm morphometry, and the effect of the number of spermatozoa analysed to obtain a representative assessment of a stallion semen sample for sperm head and midpiece morphometry using the Sperm Class Analyzer® ASMA system.

MATERIAL AND METHOD

Semen collection and sample preparation

Semen samples were collected from six adult Spanish Thoroughbred stallions using an artificial vagina (Missouri model). All stallions were actively being used for natural service breeding with physiological fertility and semen parameters (motility, sperm concentration and subjectively assessed sperm morphology). One representative ejaculate per stallion was assessed in the experimental design.

After semen was collected, the volume of each gel-free ejaculate was recorded. The semen samples were extended in a skim milk diluent and placed in an incubator at 37°C. Motility was evaluated using the Sperm Class Analyzer® (SCA) motility module (the features are described in the section on morphometric analysis). Sperm concentration was calculated with a haemocytometer and a slide was prepared for subjective analysis of sperm morphology.

For morphometric analysis, 200 µl of the diluted sperm were deposited in the same volume of Dulbecco's Phosphate Buffered Saline (DPBS) in an eppendorf tube and centrifuged at 600 g for 10 min after removing the supernatant, the sperm pellets were resuspended in DPBS to a concentration of 50 million sperm/ml. One drop of 7 µl of the final dilution was placed on a microscopic slide and allowed to air dry.

Staining methods

Three semen smears per ejaculate and per stallion were stained with each of the three following staining techniques: Diff-Quik (DQ) (Baxter DADE AG 3186, Düdingen, Switzerland), Hemacolor (HC) (Merck, Darmstadt, Germany, Cat. no. 11661) and Harris' Haematoxylin (HH) (Papanicolau solution 1a, Merck Cat. no. 9253, Darmstad, Germany). Manufacturers' instructions were followed for the first and second method, although 1 and 2 minutes increased the time proposed for each step, respectively. The third semen smears were stained with Harris' Haematoxylin by leaving the slide in the stain for 40 minutes.

Once stained, all the slides were identified and permanently sealed with Eukitt mounting medium (Kindler & Co, Freiburg, Germany) and a coverslip.

Morphometric analysis

Morphometric analysis of the sperm head and midpiece was performed using the morphological module of the SCA version 2002 (Microptic SL, Barcelona, Spain). The equipment consisted of a microscope (Olympus BH-2; Tokyo, Japan) equipped with a bright-field 100× objective and a 3.3× photo-ocular. A video camera (Sony CCD-IRIS SSC-M370CE; Sony Corporation, Tokyo, Japan) was mounted on the microscope to capture the images and transmit them to the video digitizer board (Meteor II; Matrox Electronic Systems Ltd,

Quebec, Canada) located in a Pentium processor. The SCA computer system included a high-resolution principal monitor (Sony Multiscan 200 SX; Sony Corporation, Tokyo, Japan) and sperm image analysis software. The array size of the video frame grabber was 512 × 512 × 8 bit providing digitised images of 262 144 pixels and 256 grey levels. Resolution of images was 0.08 μm per pixel in the horizontal and vertical axes.

The spermatozoa were captured randomly in different fields with a 100× oil immersion objective rejecting only those that overlapped. This process was performed manually by interactive selection of cells to avoid the inclusion of foreign particles that interfered in the way of the posterior image processing (Figure 1a). The digitised cells were automatically segmented with a range of grey-level values predetermined by the analysis factor (the automatic algorithm to define the contrast between cell and field). The system detected the boundary of sperm heads and midpieces and their outlines were displayed as white overlays superimposed on the microscopic video image (Figure 1b). When the boundary did not match the microscopic image profile, the analysis factor was modified. When it was not possible to obtain a correct boundary, the cells were eliminated.

Thirteen morphometric parameters were calculated automatically: four for head size: length (L, in μm), width (W, in μm), area (A, in μm^2) and perimeter (P, in μm); four for head shape calculated from the previous parameters: ellipticity (L/W), rugosity ($4\pi A/P^2$), elongation ((L – W)/(L + W)), regularity ($\pi LW/4A$); and four for the midpiece: width (w, in μm), area (a, in μm^2), distance (d, in μm) (between the major head axis and the midpiece) and angle (o) (the angle of divergence of the midpiece and the head axis) (Figure 2). The measurements of each individual sperm cell were saved in an Excel® (Microsoft Corporation, Redmon, Washinton, USA) compatible database by the software for further analysis.

Experimental design

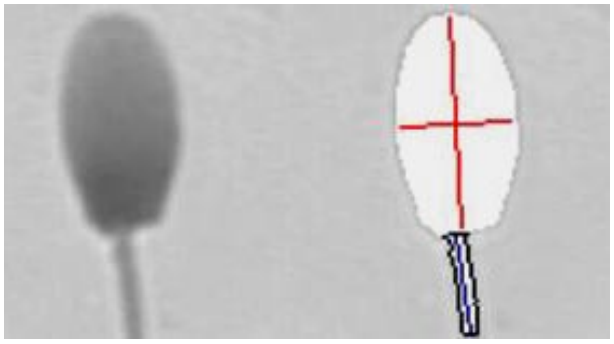
Effect of the staining technique on the accuracy of image processing. In order to determine the adequacy of the three staining techniques for capture and subsequent digitisation and binarization of images, at least 100 spermatozoa from each slide, 1 per staining and per animal, were captured and subsequently analysed totalising 3 700 spermatozoa over the entire semen samples. The percentage of sperm heads and midpieces which had been converted into correct binary images was determined visually by checking if the boundary assigned by the SCA to the spermatozoa matched its microscopic image profile and correctly delineated the sperm head and midpiece.

Effect of the staining technique on sperm morphometry. A minimum of 100 spermatozoa was analysed per slide using each of the three staining techniques, for all six animals. The morphometric parameters obtained with each method were then compared.

Effect of the number of spermatozoa analysed. To determine the minimum sample size needed to characterize the whole population, 200 sperm cells were analysed on each of the slides stained with HH for all six animals. Subsets of 100, 150, 175 and 200 spermatozoa were randomly selected from the initial reference group of 200 and compared.

Statistical analysis

For each morphometric parameter, normality of the data distributions and variance homogeneity were checked by the test of Kolmogorov-Smirnov



a b

Figure 1. Frame grabber and boundary of a sperm cell which has been properly digitised (a) and analysed (b) by the Sperm Class Analyzer®

and Cochran, respectively. For data that adjusted to a normal distribution, one way ANOVA producing significant *F*-values was followed by Tukey test for multiple comparisons. For data that did not adjust to a normal distribution, the Kruskal-Wallis non-parametric test was used followed by the Mann-Whitney *U*-test.

RESULTS

The semen parameters of the ejaculates used in the present study were within the physiological values for fertile adult stallions: a mean gel-free volume of 43 ml, 169 million sperm/ml for sperm concentration, 77% for sperm motility and 72% for normal sperm morphology (estimated by subjective analysis).

Effect of the staining technique on the accuracy of image processing. Of the 3 700 spermatozoa captured, 2 420 were correctly analysed using the three staining procedures (Table 1). No significant differences were found between the DQ (55.27%) and HC (61.76%) staining techniques. However, HH was by far the most accurate method ($P < 0.05$) with 80.47% of correctly analysed spermatozoa. The coefficients of variation obtained with HH were lower than those obtained with DQ and HC.

Effect of the staining technique on sperm morphometry. The morphometric values for the sperm head and midpiece are shown in Table 2 according to the three staining procedures. Sperm morphometric parameters were influenced by the staining method. The DQ and HC staining methods obtained significantly increased sperm dimensions than HH ($P < 0.05$).

Effect of the number of spermatozoa analysed. No Statistical differences were found among the subsets of 100, 150, 175 and 200 sperm cells for any of the sperm head or midpiece morphometric parameters of the spermatozoa stained with HH (Table 3). It suggests that the analysis of 100 spermatozoa is sufficient for the morphometric characterization of stallion semen sample under these conditions.

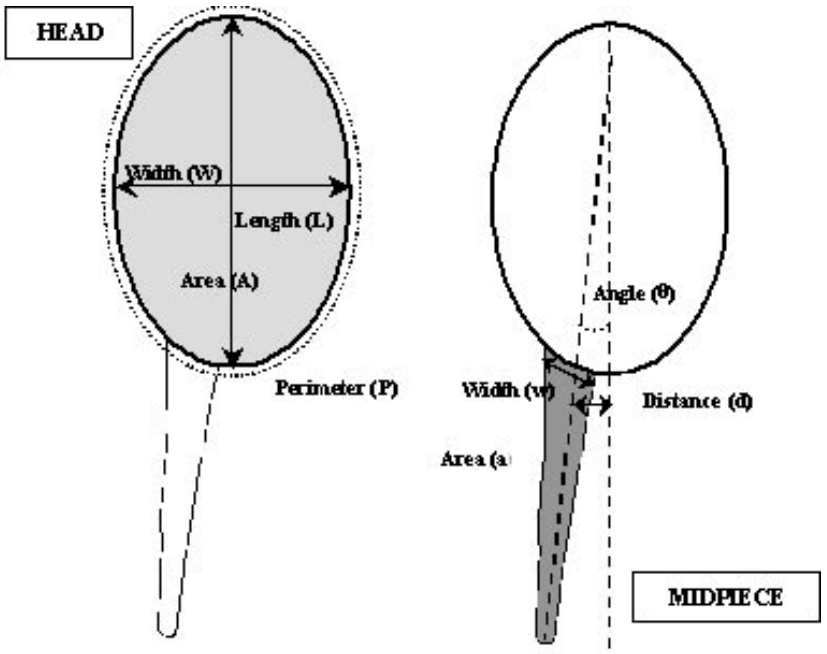


Figure 2. Morphometric parameters examined in this study (modified from Soler et al., 2003)

The morphometric parameters described for the sperm head are as follows L = Length (along the main axis), W = Width (along the smaller axis), A = Area, P = Perimeter .Derived parameters were automatically calculated for head shape: Ellipticity (L/W), Rugosity ($4\pi A/P^2$), Elongation (L – W)/(L + W), Regularity ($\pi LW/4A$). The morphometric parameters described for the midpiece are as follows: w = width (at the intersection of the midpiece with the sperm head), d = distance (between the main axis of the sperm head and the intersection with the midpiece), 0 = angle (formed by the axis of the midpiece and the main axis of the sperm head), and a = area (the area occupied by the entire midpiece)

Table 1. Percentage of correctly analysed spermatozoa (n = 2 420) from each animal stained with Diff-Quik, Hemacolor and Harris' Haematoxylin

Animal	Staining Method (% correctly analysed)		
	Diff-Quik	Hemacolor	Harris' Haematoxylin
1	43.77	72.85	88.10
2	43.72	89.47	74.62
3	60.79	37.82	75.28
4	57.71	52.63	83.68
5	50.25	54.30	81.70
6	75.38	63.52	79.44
CV	21.88	29.01	6.39
Mean	55.27[a]	61.76[a]	80.47[b]

The superscripts indicate significant differences ($P < 0.05$)
CV = coefficient of variation (%)

Table 2. Effect of the three different staining procedures on morphometric parameters of the sperm head and midpiece for all six animals

Stain	Diff-Quik	Hemacolor	Harris' Haematoxylin
n	612	603	1 205
Head Parameters			
Length (μm)	5.87 ± 0.39[a]	5.90± 0.41[a]	5.67 ± 0.36[b]
Width (μm)	3.07 ± 0.27[a]	2.97-± 0.30[b]	2.85 ± 0.31[c]
Area (μm^2)	14.72 ± 1.72[a]	14.29 ± 1.85[b]	13.42 ± 1.72[c]
Perimeter (μm)	15.64 ± 0.92[a]	15.61 ± 1.00[a]	15.00 ± 0.89[b]
Ellipticity	1.92 ± 0.18[a]	2.00 ± 0.19[b]	2.00 ± 0.20[b]
Rugosity	0.75 ± 0.04[a]	0.73 ± 0.04[b]	0.76 ± 0.04[c]
Elongation	0.31 ± 0.04[a]	0.33 ± 0.04[b]	0.33 ± 0.05[b]
Regularity	0.96 ± 0.03[a]	0.96 ± 0.03[a]	0.95 ± 0.03[b]
Midpiece Parameters			
Width (μm)	0.96 ± 0.23[a]	0.82 ± 0.23[b]	0.81 ± 0.27[b]
Area (μm^2)	2.09 ± 0.50[a]	1.72 ± 0.48[b]	1.63 ± 0.56[c]
Distance (μm)	0.27 ± 0.13[a]	0.26 ± 0.13[a]	0.26 ± 0.12[a]
Angle (°)	5.63 ± 6.02[a]	6.83 ± 7.76[b]	6.28 ± 6.34[a,b]

Values are mean ± standard deviation; n = number of spermatozoa analysed; different superscripts indicate significant differences ($P < 0.05$)
Ellipticity = L/W; Rugosity = $4\pi A/P^2$; Elongation = (L – W)/(L + W); Regularity = $\pi LW/4A$; L = Head Length; W = Head Width; A = Head Area; P = Head Perimeter

DISCUSSION

The use of SCA has been previously standardized in humans (de Monserrat et al., 1995) and other species (Gago et al., 1998; Buendia et al., 2002) with intrinsically low coefficients of variation that demonstrate the precision and accuracy of the system, as well as its high repeatability as no differences are found when analyzing the same sample several times. To apply the precision and repeatability of

Table 3. Comparison of morphometric parameters between different sample sizes from spermatozoa stained with Harris´ Haematoxylin for all six animals

Spermatozoa sampled	**Head Parameters**			
	Length (μm)	Width (μm)	Area (μm^2)	Perimeter (μm)
100	5.64 ± 0.01	2.84 ± 0.01	13.33 ± 0.07	14.95 ± 0.04
150	5.66 ± 0.01	2.86 ± 0.01	13.42 ± 0.06	15.00 ± 0.03
175	5.66 ± 0.01	2.86 ± 0.01	13.44 ± 0.05	15.01 ± 0.03
200	5.66 ± 0.01	2.85 ± 0.01	13.42 ± 0.05	15.00 ± 0.03
	Ellipticity	Rugosity	Elongation	Regularity
100	2.00 ± 0.01	0.75 ± 0.001	0.33 ± 0.002	0.95 ± 0.001
150	2.00 ± 0.01	0.75 ± 0.001	0.33 ± 0.002	0.95 ± 0.001
175	2.00 ± 0.01	0.75 ± 0.001	0.33 ± 0.002	0.95 ± 0.001
200	2.00 ± 0.01	0.75 ± 0.001	0.33 ± 0.001	0.95 ± 0.001
Spermatozoa sampled	**Midpiece Parameters**			
	Width (μm)	Area (μm^2)	Distance (μm)	Angle (°)
100	0.81 ± 0.01	1.61 ± 0.02	0.25 ± 0.005	6.22 ± 0.26
150	0.81 ± 0.01	1.63 ± 0.02	0.26 ± 0.004	6.23 ± 0.21
175	0.81 ± 0.01	1.63 ± 0.02	0.26 ± 0.004	6.28 ± 0.19
200	0.81 ± 0.01	1.63 ± 0.02	0.26 ± 0.004	6.28 ± 0.18

Values are mean ± standard error; no significant sample size effects were found

Ellipticity = L/W; Rugosity = $4\pi A/P^2$; Elongation = (L – W)/(L + W); Regularity = $\pi LW/4A$; L = Head Length; W = Head Width; A = Head Area; P = Head Perimeter

this technology to animal species, species-specific methods for sample preparation and staining are needed (Davis and Gravance, 1993; Boersma et al., 2001).

In accordance with previous studies on equine species regarding the most suitable method of sample preparation in this type of analysis (Davis et al., 1993), we have used washed samples. The performance of the SCA has been evaluated using three staining methods. Among the methods tested for stallions, Trypan blue and Giemsa (Kusunoki et al., 1988), Papanicolau (Hafez, 1987) and Spermac (Oettle, 1986) are not suitable for ASMA systems as they result in poorly stained cells, which do not permit digitisation (Gravance et al., 1995). The staining methods compared in this study (DQ, HC, HH) were chosen based on the positive results obtained in humans and in several animal species for different ASMA systems (Lacquet et al., 1996; Gago et al., 1998; Soler et al., 2000).

According to the results, all three staining procedures permitted the digitisation of stallion spermatozoa, although some differences are seen in the number of recognition and digitisation errors, showing the best results with the use of HH stain technique. The criteria followed in this study to evaluate the accuracy of the staining technique on image processing included the percentage of correctly analysed spermatozoa with the three staining techniques, and the coefficients of variation obtained with each procedure (Sancho et al., 1998). The SCA analyzes the images captured by creating a boundary that matches the external outline of the microscopic image of the spermatozoa and delineating the sperm head and midpiece. In order to obtain a correct image, the spermatozoa must contrast with the sample preparation background and there can be no particles that interfere in the delineation of the sperm cells. The accuracy of SCA in capturing and segmenting the spermatozoa stained with HH was higher than those stained with DQ and HC. These findings resemble those obtained by Gago et al. (1998) in the cynomolgus monkey. Spermatozoa stained with DQ and HC obtain more intense grey-level values, thus enhancing the contrast of images. However, the same thing occurs with other particles found in the sample, making it necessary to eliminate a larger number of cells, con-

sequently slowing down the process. On the other hand, the HH staining technique obtains suitable grey-level values for the correct digitisation of both sperm heads and midpieces, reduces the number of stained foreign particles and the boundaries correctly delineate the original microscopic image. This is supported by the fact that the coefficients of variation calculated for each animal were lower with HH, thus demonstrating the lower variability and higher precision of this staining method.

Morphometric values obtained with the SCA system was affected by the staining method used and should be interpreted accordingly. DQ and HC provide more intense grey-level values, resulting in enlarged cells, which influence the size morphometric parameters as length, width, area and perimeter of the head and the width and area of the midpiece. In general terms, the relationship between the three staining techniques for the sperm dimensions can be described as follows: DQ > HC > HH. The impact of the staining procedure on sperm dimensions had also been tested by comparing these three staining techniques in human (Soler et al., 2003) and monkey spermatozoa (Gago et al., 1998). These morphometric results are in accordance with other authors who have found increased dimensions in semen samples stained with DQ as compared to Papanicolau (Menkveld et al., 1990; Gago et al., 1998). In his study with the cynomolgus monkey, Gago et al. (1998) found intermediate values for HH, which were lower than HC when comparing the DQ, HC and HH techniques. The previous results resemble those found in this study, although we increased the HC staining time to enhance the intensity and contrast of the images. As observed with DQ, this process consequently increased the sperm dimensions.

The size of the sample is also an important factor to take into consideration. We expected that a higher numbers of spermatozoa analysed achieving a more accurate assessment of sperm morphometry. However, the results indicate that 100 properly digitised sperm cells appeared sufficient for the morphometric characterization of a stallion semen sample under these conditions, as it produced similar measurements as analysing 150, 175 or 200 spermatozoa. The analysis of 100 spermatozoa obtain accurate measurements and greatly reduces the time to perform an analysis, which was in agreement with results obtained in dog (Rijsselaere et al., 2004) and goat (Gravance et al., 1995). However, because heterogeneous abnormal sperm head morphology of equine species had been described previously, it is possible that to overcome possible problems associated with the evaluation of infertile samples, in these semen specimens a high number of spermatozoa should be analysed. The animals used in the present study were considered to be fertile on the basis of their use for breeding. Whether infertile samples are associated with larger number of spermatozoa analysed warrants further investigations.

In conclusion, the morphometric analysis of the stallion spermatozoa was influenced by the staining procedure. Harris' Haematoxylin could be considered the most accurate staining method with the SCA, based on the greater percentage of analysable cell. 100 properly digitised spermatozoa per slide should be analysed to morphometrically characterize the whole population in a stallion semen sample. In short, to obtain and objective and accurate evaluation of stallion sperm heads and midpieces with SCA, the analysis of 100 spermatozoa per slide is recommended in samples, which have been previously stained with Harris' Haematoxylin.

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