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Asian Journal of Animal and Veterinary Advances

Academic
Journals Inc.

www.academicjournals.com

Asian Journal of Animal and Veterinary Advances 2 (4): 196-204, 2007
ISSN 1683-9919

Analyses of Morphological and Morphometrical Deviations of Bull Spermatozoa by Computer Assisted Semen Analysis Technique

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Abstract: To eliminate the subjectivity inherent in conventional methods, morphology evaluation of spermatozoa by morphometry using CASA technique was undertaken in bulls. In this study, 1219 properly digitized spermatozoa, 616 and 603 sperm for 2 clinically normal Jersey bulls identified by scanning the sperm slides prepared from 19 ejaculates were used for morphological and morphometric analysis by CASA technique. The total percentage of sperm abnormalities recorded was 24.4%, of which, the occurrence of tail abnormalities was more often than head and head and tail (in combination). The total and head abnormalities showed significant ($p \leq 0.05$) differences between bulls. Among the tail abnormalities the incidence of coiled tail was most frequent followed by bent tail and absent tail. The morphometric characteristics viz. major axis (μm), minor axis (μm), elongation (%), head area (μm^2), perimeter (μm) and tail length (μm) were recorded as 9.53, 4.73, 49.5, 35.7, 23.7 and 61.14, respectively. The between bull variations for major axis, elongation, head area, perimeter and tail length were reflected in the morphological differences as well. The incidence of head abnormalities due to higher morphometric values was significantly ($p \leq 0.05$) more than abnormalities due to lower and/or higher and lower morphometric values. CASA technique facilitates identification of even small but significant variations in sperm head morphomtrey and certain types of morphological deviations namely total and head abnormalities between clinically normal bulls, which encourages further studies to explore the difference in fertility if any, between bulls.

Key words: Bull sperm, Morphometry, Morphology, CASA

INTRODUCTION

The shape and size of spermatozoa are species-specific and differ between various wild animals such as deer (Soler *et al*., 2005; Esteso *et al*., 2006; Sundararaman *et al*., 2006), alpaca (Buendia *et al*., 2002) and farm animals like cattle (Gravance *et al*., 1996; Boersma *et al*., 2001; Beletti *et al*., 2005), sheep (Gravance *et al*., 1998; Sancho *et al*., 1998), goat (Gravance *et al*., 1995; Sundararaman and Edwin, 2004; Hidalgo *et al*., 2006), horse (Davis *et al*., 1993; Casey *et al*., 1997; Arruda *et al*., 2002) and companion animal, dog (Dahlbom *et al*., 1997; Rijsselaere *et al*., 2004). Existence of significant differences in sperm morphology between species suggests that dimensional characteristics of spermatozoa are genetically controlled.

The role of percentage of morphologically normal sperm of man and animals in fertility has been widely published. Normal sperm morphology may be an indicator of the fertility potential of a given male (Padrik and Jaakma, 2002; Esteso *et al*., 2006). The association of increased morphological abnormalities of spermatozoa with reduced reproductive efficiency has been reported in humans

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(Kruger *et al*., 1988; Davis *et al*., 1993), stallions (Bielanski and Kaczmarski, 1979; Jasko *et al*., 1990), goats (Skalet *et al*., 1988; Gravance *et al*., 1998) rams (Osinowo *et al*., 1988), pigs (Alm *et al*., 2006) and bulls (Saacke, 1970; Fitzpatrick *et al*., 2002; Walters *et al*., 2005).

In bulls, higher proportion of abnormal spermatozoa could be an indicator of genetically heritable fertility (Hafez, 1987). Occurrence of a specific structural abnormality of sperm affecting the whole population or a high percentage of the populations might be associated with infertility (Perotti *et al*., 1981). Morphometric analysis of sperm heads has been shown to be an indicator of *in vitro* fertility (Kruger *et al*., 1993; Thompson *et al*., 1994).

Despite spermatozoa are terminal, highly differentiated cells, whose multitude of attributes that are of relevance for fertilization cannot be easily assayed by one single test (Rodriguez-Martinez, 2003), spermiogrammic analysis is currently considered as the most desirable approach to evaluate the reproductive ability of bulls (Hafez and Hafez, 2000). Comprehensive semen evaluation tests include morphology estimation by visual approximation of shape and size of spermatozoa, which is often subjective. However, CASA systems have been shown to correctly recognize and digitize 91% of the spermatozoa encountered (Davis *et al*., 1992) and to classify sperm head morphology with 95% accuracy (Morruzzi *et al*., 1988) compared to manual video-microscopic evaluation (Casey *et al*., 1997). Therefore, morphological assessment of spermatozoa by objective methods could add more value to semen evaluation protocols. With this background, a study was conducted to assess the morphology of bull spermatozoa by morphometry using CASA and to analyze the incidence of different types of abnormalities and deviations in morphomtric characteristics.

MATERIALS AND METHODS

Semen Collection, Evaluation and Processing

Semen samples were collected from 2 clinically normal Jersey bulls, aged 2 years, by artificial vagina. The bulls were in regular semen collection schedule of twice a week. Two consecutive ejaculates were harvested from each bull in every collection schedule. A total of 19 ejaculates, 9 and 10, respectively from 2 bulls were used for CASA analysis. This study was carried out during the July-August-September 2006.

The semen samples were evaluated for ejaculate volume, sperm concentration and sperm motility by conventional methods. After evaluation equal volume of Tris-egg yolk-glycerol based diluent was added to the semen samples. The sperm motility was assessed by phase contrast microscope at 37°C. Only samples containing progressive sperm motility of 70% and above were included for the study. The semen samples were further extended so as to fix the sperm number as 30 million per insemination dose.

Preparation Sperm Slides

After the final dilution, one aliquot (1 mL) of extended semen was further diluted with tris buffer so as to reduce the sperm concentration to approximately 20 million mL^{-1}. Sperm slides were prepared by placing a 4 μL drop on a clean grease free glass slide and dragging the drop across the slide. The slides were air dried and stained by using, STAT III Andrology Stain (Mid-Atlantic Diagnostics Inc. NJ). Dried smears were fixed by immersing in methanol for 30 sec. After air-drying, the slides were immersed in Xanthene dye solution for 60 sec. Again after air-drying, the slides were immersed in Thiazine dye mixture for 60 sec. The excess stain on edges were blotted during the staining operation and finally washed with distilled water and air-dried. Several such slides were prepared from each sample for CASA analysis.

Table 1: Category gates used in HT-IVOS metrix software for evaluation of bull spermatozoa

	Low		High	
Parameters	Abnormal	Normal	Abnormal	Normal
Major axis (μm)	8.0	8.5	10.0	11.0
Minor axis (μm)	3.5	4.0	5.0	5.5
Elongation (%)	40.0	45.0	55.0	60.0
Head area (μm^2)	25.0	30.0	40.0	45.0
Perimeter (μm)	19.0	20.0	30.0	31.0

CASA Analysis

Hamilton Thorne integrated visual optical system (HTM-IVOS) version 10.9 was the Computer Assisted Semen Analyzer (CASA) used in this experiment. The metrix software option for multi-species sperm morphology analysis was chosen for morphological and morphometrical analyses of bull sperm (Table 1). The sperm slide was loaded in the CASA system. The 60X objective was chosen (Gravance *et al*., 1996). After focusing, an image containing a spermatozoon was selected and the illumination was adjusted using adjust image option in order to obtain optimal illumination intensity (Rijsseaere *et al*., 2004). From each slide only properly digitized sperm heads were considered for morphometric measurement. Sperm heads were considered properly digitized when the computer-generated outline of the sperm head corresponded to the actual head outline. Using the reject option of the software, the non-sperm images scanned by the systems were eliminated from the analysis (Gravance *et al*., 1996). A total of 1219 properly digitized spermatozoa, 616 and 603, respectively for the 2 Jersey bulls identified by scanning from the sperm slides prepared from 19 ejaculates were used for morphometric analysis.

Major axis: the length of sperm head (μm); minor axis: the width of the sperm head (μm); elongation: the ratio of minor/major axis X 100 (%); head area: the total area of the sperm head (μm^2); perimeter: the length of the sperm head perimeter (μm); tail length: the measured length of the tail (μm) were the morphometric characteristics measured.

Morphologically, a sperm cell was classified as normal or abnormal depending on the measured value for all the morphometric characteristics. If all the values were within the normal limits as indicated in the category gates of the CASA system, the spermatozoon was classified as normal provided the tail morphology of the sperm cell was also normal. If the morphometric values were in the abnormal range, the spermatozoon was designated as abnormal. The ranges of values used in metrix software to define bull spermatozoa as normal, abnormal and rejected are given in Table 1.

Classification of Data and Statistical Analyses

Based on the involvement of the part of the spermatozoa, the abnormalities were classified as head, tail and head and tail types. The abnormalities of tail as identified by the CASA system were classified as bent, coiled and absent tail. Further, the head abnormalities were sub-divided as high, low and high and low. If a sperm cell was identified as abnormal by the system because of higher head morphometric value(s), the head abnormality was classified as high and as low if the morphometric value was lower than the minimal value. If the abnormality was due to higher morphometric value in one parameter and lower in another in the same sperm cell, the head abnormality was classified as high and low. The data are presented as mean±SE. The values in percentages were subjected to analysis after arcsine transformation. The data were analyzed by one-way analysis of variance using microstat software (Ecosof Inc., 1984 Baltimore, USA). Significance of difference between means were determined at $p \leq 0.05$ and at $p \leq 0.01$ and the variations were designated as significant and highly significant, respectively.

RESULTS

The overall abnormality of spermatozoa was 24.4% (Table 2). The variations found between bulls for total sperm abnormalities in general and head abnormalities in particular were significant ($p \leq 0.05$). However, the variations between bulls for different types of tail abnormalities were not significant (Table 3).

Highly significant differences ($p \leq 0.01$) were noticed between bulls for major axis, elongation, head area, perimeter and tail length (Table 4).

Highly significant ($p \leq 0.01$) differences were observed between the incidences of types of sperm abnormalities in terms of location (Table 5). The proportion of abnormalities involving tail alone was the most, whereas the involvement of both head and tail in combination was the least. Table 6 shows the significant ($p \leq 0.05$) differences between the incidences of types of head abnormalities in terms of morphometric values. The head abnormalities due to higher morphometrics were more than for abnormalities because of other types of deviations.

Table 2: Mean percentages (±SE) for sperm abnormalities in Jersey bulls

Bull ID	Sperm abnormalities (All types)	Proportion of types of abnormalities		
		Head	Tail	Head and Tail
J 4049 (n: 9)	27.50±0.06[a]	11.10±0.00[a]	11.50±0.00	4.00±0.00
J 4059 (n: 10)	21.60±0.15[b]	5.30±0.13[b]	11.90±0.15	2.20±0.13
Overall (n: 19)	24.40±0.06	7.80±0.00	11.70±0.00	3.00±0.00

Means bearing different superscripts in a column differ significantly ($p \leq 0.05$), n: No. of ejaculates

Table 3: Mean percentages (±SE) for types of tail abnormalities in spermatozoa of Jersey bulls

Bull ID	Types of sperm abnormalities		
	Bent tail	Coiled tail	Absent tail
J 4049 (n: 9)	9.90±0.96	68.90±0.91	13.70±0.96
J 4059 (n: 10)	16.00±0.87	66.30±0.19	7.70±0.62
Overall (n: 19)	12.90±0.44	67.60±0.24	10.40±0.38

n: No. of ejaculates

Table 4: Means (±SE) for morphometric characteristics of bull spermatozoa of Jersey bulls

Bull ID	Major axis (μm)	Minor axis (μm)	Elongation (%)	Head area (μm^2)	Perimeter (μm)	Tail length (μm)
J 4049 (n: 616)	9.71±1.30[a]	4.73±9.16	48.60±0.12[b]	37.00±8.87[a]	24.2±2.70[a]	61.78±0.19[a] (143*)
J 4059 (n: 603)	9.34±1.52[b]	4.73±9.55	50.50±0.11[a]	34.30±0.11[b]	23.2±3.57[b]	60.26±0.23[b] (103*)
Overall (n: 1219)	9.53±1.14	4.73±6.61	49.50±8.42	35.70±0.08	23.7±2.61	61.14±0.15 (246*)

Means bearing different superscripts in a column differ significantly ($p \leq 0.01$), n: No. of spermatozoa analyzed for all morphometric characteristics except tail length, *: No. of spermatozoa analyzed for tail length

Table 5: Mean±SE for incidence of sperm abnormalities in terms of location, in Jersey bulls

Location of abnormality	Incidence (%)
Head	16.22±1.34[b]
Tail	20.01±1.38[ab]
Head and tail	9.96±1.23[c]

Means bearing different superscripts differ significantly ($p \leq 0.01$), No. of ejaculates: 19

Table 6: Means±SE for incidence of head abnormalities in terms of morphometric values of spermatozoa in Jersey bulls

Classification of head abnormality based on morphometry	Incidence (%)
Sperm with higher morphometric value	45.40±5.78[a]
Sperm with lower morphometric value	27.24±5.05[ab]
Sperm with both higher and lower morphometric value	17.52±4.34[b]

Means bearing different superscripts differ significantly ($p \leq 0.05$), No. of ejaculates: 19

Table 7: Means±SE for proportion of types tail abnormalities in spermatozoa of Jersey bulls

Type of tail abnormality	Proportion (%)
Bent tail	21.05±3.81[a]
Coiled tail	55.27±2.82[a]
Absent tail	18.77±3.53[c]

Means bearing different superscripts differ significantly ($p \leq 0.01$), No. of ejaculates: 19

Regarding the abnormalities of tail (Table 7), the incidence of coiled tail was more than bent tail and absent tail. The variations between the occurrences of different tail abnormalities were highly significant ($p \leq 0.01$).

DISCUSSION

In this study a little more than 75% of spermatozoa analyzed were morphologically normal. These findings were obviously due to the fact that the bulls involved in the trail were selected and reared as Artificial Insemination (AI) sires and are in use for frozen semen production. Higher percentages of sperm with normal morphology recorded may suggest normal fertility as it appeared in an electron microscope study that a higher proportion of sperm bound to the zona pellucida were of normal morphology (Thundathil *et al*., 2001).

In fertile bulls the incidence of abnormal morphology of spermatozoa was found to be 10 to 18% (Lagerlof, 1934, 1936; Saacke *et al*., 1968; Rao *et al*., 1980). However, the higher sperm abnormalities (24.4%) observed in this trial may be because of different methods employed for morphological assessment. The computer automated system used in this trial, have been shown to classify sperm heads as normal or abnormal with 95% accuracy (Moruzzi *et al*., 1988) compared to manual video-microscopic evaluation (Casey *et al*., 1997).

The size of the sperm in terms of head length recorded in this trial for Jersey bulls is the largest (9.53 μm) when compared with the reports of Gravance *et al*. (1996), Boersma *et al*. (2001) and Beletti *et al*. (2005). The head area reported by Gravance *et al*. (1996) was smaller than that observed in this study and by other workers (Boersma *et al*., 2001; Beletti *et al*., 2005). The variations in the morphometric characteristics may be due to different breeds involved in the studies (Gravance *et al*., 1995). No report on bull sperm tail morphology could be traced to compare the results of this study. Nevertheless, the tail of bull sperm observed in this experiment is lengthier than the goat (Sundararaman and Edwin, 2004), dog (Rijsseleare *et al*., 2004) and deer (Sundararaman *et al*., 2006) spermatozoa.

Highly significant differences between bulls in major axis, elongation, head area, perimeter and tail length suggest that the morphometric characteristics varies between individual males. Within a species, estimates of the size of sperm heads vary greatly among stallions (Davis *et al*., 1993; Gravance *et al*., 1996; Casey *et al*., 1997) goats (Gravance *et al*., 1995; Sundararaman and Edwin, 2004) dogs (Dahlbom *et al*., 1997; Rijsseaere *et al*., 2004) rabbits (Gravance and Davis, 1995) alpaca (Buendia *et al*., 2002) and bulls (Gravance *et al*., 1996). The variation may be due to biological, such as inherited differences (Beatty, 1970) and stress factors influencing the condition of the male (Foote, 2003), DNA content associated with the sex chromosomes (Van Munster *et al*., 1999; Chandler *et al*., 2002) and incompletely condensed chromatin (Hingst *et al*., 1995). Despite the fact that overall head abnormalities in general was lower, it was more frequent in one of the bulls. The sperm head of the same bull was significantly larger. Further, most of the head abnormality in this study was due to larger sperm size in terms of morphometrics, which was significantly higher than the head abnormalities due to incidence of lower morphometrics and both higher and lower morphometrics in combination. Larger sperm heads are associated with subfertility (Boyle *et al*., 1992; Casey *et al*., 1997) since sperm with head abnormality have reduced capacity to bind ovum (Kot and Handel, 1987) and may lead to early embryonic loss, lowered fertility and embryo quality (Krzanowska and Lorenc, 1983; De Jarnette *et al*., 1992).

The incidence of tail abnormality was most frequent in this experiment and did show similarity in both the bulls. The occurrence of bent tail was similar to the earlier findings of Zimjanis (1969). This abnormality is often due to translocating protoplasmic droplet (Saacke, 1970; Gosch *et al*., 1989). Bent tail with entrapped protoplasmic droplet was reported to be the most common abnormality in bulls (Wildeus and Entwistle, 1984; Chacon, 2001). However, in the present experiment coiled tail was the most frequently encountered tail abnormality. This variation may be due to sub-classification of different tail abnormalities on microscopic studies by different workers. A fraction over 18% of tail abnormalities was identified as absent tail. The tail may be detached following ejaculation (Jasko *et al*., 1990; Rao, 1998) or prior to ejaculation in certain individual males. If the detachment occurs prior to ejaculation it may cause infertility (Jasko *et al*., 1990).

To conclude, the proportion of sperm abnormalities in this study is slightly higher than the threshold value of 20% (Hafez, 1987). Nevertheless, it is well within the permissible levels recommended by Society for Theriogenology (Chenoweth, 1997). The variation in sperm head size between bulls is to be viewed with caution, as it could be a sensitive biomarker related to fertility (Gravance *et al*., 1996; Sailor *et al*., 1996). Furthermore, the morphometrical differences are also reflected in the significant morphological differences between bulls. Little is known on the impact of small morphological differences on male fertility (Wijchman *et al*., 2001; Suzuki *et al*., 2002). Since CASA allows identification of minute differences between spermatozoa in morphometric measurements which would otherwise be unnoticed by traditional manual evaluation, the results obtained in this study on head morphometry can be utilized to explore differences in fertility if any, among bulls. Strict objective guidelines for evaluation sperm head morphology have to be evolved for bulls as in humans. Further research on CASA technique can contribute significantly towards achieving this goal.

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