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Morphometric analysis of mithun sperm from fresh and frozen-thawed semen

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ABSTRACT

The present study was conducted to compare various morphometric parameters of mithun sperm (*Bos frontalis*) in fresh and frozen-thawed semen. Ejaculates (36) from 6 mithun bulls were subjected for cryopreservation with commercial extender (optixel). The semen sample after dilution was cooled gradually from 35°C to 5°C and then frozen in biofreezer under controlled freezing rate. Both fresh and frozen-thawed semen were processed for staining using Eosin-Nigrosin and Giemsa stain and digitized for morphometric evaluation. Mean linear measurements of different parameters, viz. head-length, head-width, head-base, acrosome length, acrosome width, midpiece length, tail length, head shape, head area, head circumference and total sperm length, were taken. Results revealed no difference in various sperm morphometric parameters between fresh and frozen-thawed sperm except the head area which was significantly lower in frozen-thawed sperm. This is the first comprehensive report on mithun sperm morphometry, and it showed little variation in various morphometric parameters when compared to crossbred bull and Murrah buffalo bull sperm.

Key words: *Bos frontalis***,** Mithun semen, Nagaland, Sperm morphometry

Mithun (*Bos frontalis*), a domesticated rare bovine species, is reared under free range forest condition as well as under semi-intensive and intensive system. Mithun population in India is around 297, 289 as per 12th Livestock census. This massive build animal is believed to have originated more than 8000 years ago from wild Indian gaur (*Bos gaurus*) (Simoons 1984).

In order to conserve and genetic improvement of this rare bovine species and to increase their population, selective breeding programme has been initiated by this institute. Artificial insemination (AI) with superior bull semen is one of the best biotechnological tools for rapid upgradation of mithun population. Semen evaluation is the most important aspect in determining breeding soundness of a bull. Recent studies revealed that male fertility depends not only on sperm motility, viability or concentration but also on morphometry of sperm cells (Singh *et al.* 2011).

Traditionally, sperm heads have been analyzed manually using one-dimensional measurements of length, width, and area (Davis and Gravance 1994) which have gained in precision with computers and image analysis software (Katz *et al.* 1986). Accuracy of sperm morphometry depends on several factors (Maree *et al.* 2010) including potential variations between laboratories (Ridell *et al.* 2005). To further improve sperm morphometry assessments, automated sperm morphometry analysis (ASMA) systems were developed (Jagoe *et al.* 1986) which provide information on sperm head linear dimensions (i.e., size) and use a series of mathematical formulae to calculate dimensions-derived parameters (as an approximation to head shape).

Sperm head morphometry assessed by computer-assisted semen analysis (CASA) correlated with fertility in various species including horses (Casey *et al.* 1997), boar (Pena *et al.* 2005) and canines (Núñez-Martínez *et al.* 2007). Substantial differences in sperm head shape and size were found both within and between the breeds in stallions (Hidalgo *et al.* 2008), bulls (Boersma *et al.* 1999) and boar (Saravia *et al.* 2007).

In this context, determination of the normality of sperm morphometric parameters becomes particularly important in classifying sperm as having normal or abnormal morphological structure. Though a number of studies had been conducted on bull and other animals to evaluate sperm biometry, however restricted or no data is available in context of mithun. This is probably the first comprehensive study on mithun sperm morphometry with the objective to evaluate the morphometric features of mithun sperm both in fresh and frozen semen through computer image analysis.

MATERIALS AND METHODS

Study period and sample collection: The experiment was carried out during March 2016 to September 2016. All experimental procedures involving animals were approved

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by the Institutional Animal Ethics Committee (IAEC). Adult fertile mithun bulls (6), age between 3–4 years and body weight 300–400kg, maintained at Mithun farm of ICAR-National Research Centre on Mithun, Medziphema, Nagaland were used in this study for collection of semen. Each bull was maintained under homogeneous feeding and housing conditions. Semen was collected once in a week by rectal message and artificial vagina method in a sterilized graduated tube kept in thermoflask. Ejaculates (36) from 6 healthy fertile bulls were collected for the study.

Semen dilution and freezing: Freshly collected semen was immediately brought to the laboratory in a thermoflask and kept at 35°C in water-bath. Semen sample was diluted with commercially available Optixcell extender (IMV Technologies, France) to make final sperm concentration of 20 million/0.5ml straw.

The extended semen was packed and sealed into 0.5 ml straw using automatic filling and sealing machine (IMV Technologies, France) and cooled up to 5°C by keeping them in cold handling cabinet for 90 min. Semen straws were equilibrated for 4 h at 5°C. The straws were placed evenly on racks inside the controlled-rate freezing system (Biofreezer, IMV Technologies, France). The biofreezer was programmed with the following rate: 3°C/min from 5 to –10°C (ii) 20°C/min from –10 to –100°C (iii) 40°C/min from –100 to –140°C. After freezing, the straws from both the freezing systems were immersed into liquid nitrogen for storage. Thawing of straw was done at 35°C water for 20 sec. Each sample was evaluated for morphometrical analysis after staining.

Staining of sperm for morphometric analysis: Slides were prepared from each sample by placing 10 µl of semen on the clear end of a frosted microscope slide and mixed with 10µl Eosin-Nigrosin stain (0.67g Eosin, 0.9g NaCl and 5g Nigrosin dissolved in distilled water, heated and filtered) and dragging the drop across the slide to make a thin smear for microscopic observation. To differentiate acrosome, Giemsa stain (Watson 1975) [0.76g/100mL Giemsa dissolved in glycerol-methanol mixture (1: 3), ripened for 1 week at room temperature and filtered] was used. Briefly, a thin smear of diluted semen was prepared on dry, grease free slide and smear was dried in air. Smear was fixed by pouring Hancock fixative over the slide and keeping it for 20 min. After fixation, slide were washed in slow running tape water for 20 min and dipped in Giemsa working solution (7.5%; V/V) Giemsa stock and 5% Soresen's buffer (V/V) diluted with distilled water) for 8 h. The slides were washed in running tape water and examined under 1,000× magnification for morphometric evaluation.

Experimental design and image acquisition: Stained semen smear of both fresh and frozen sample were used to perform morphometry module using a commercially available microscopic system. The equipment consisted of a microscope (Meizi, Techino, Japan) equiped with a 10× bright-field objective and a ProgRes camera (C7, Jenoptik, Germany) connected to a Pentium processor. The illumination source and light intensity were centered and offset of the camera were optimized for all the slides. The computer system had an image analysis software installed in it and a high-resolution assistant monitor HP 23VX (HP, Singapore) were assembled with the computer system.

Spermatozoa (200) from both fresh as well as frozen semen were evaluated and digitized for image acquisition to calculate sperm morphometrics from an entire set of semen sample. Sperm morphology was measured in computer software in terms of nine basic parameters (Fig.1), all in micrometers (µm): sperm head length (longitudinal distance covering the head, AB), width (horizontal end to end distance of a sperm head, CD), base (minimal distance above the midpiece, EF), acrosomal cap length (the distance between the midpoint of the base line of the AC and its apex, GH) width (maximum scale reading of the line parallel to the base line, IJ), midpiece length (continuous distance below head base about as long as the head, KL), tail length (distance of narrowest part running below the midpiece, MN), total length (the overall length from head apex to tip of the tail, OP), head shape (the ratio of head width to the head length, CD/AB), head circumference (free line distance surrounding the whole head) and head area (Ostermeier *et al*. 2001). The measurements of each individual sperm were recorded for further data analysis. Descriptive stastistics (SPSS 16.0, USA, Inc) were performed on the available data to determine the average morphometric parameters.

All the sperm morphometric parameters (mean, SE etc.)

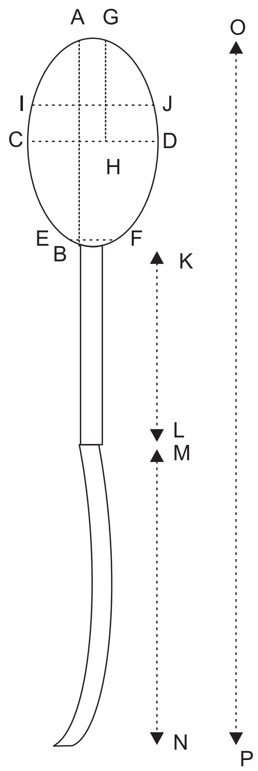

Fig. 1. Schematic representation of sperm cell to analyze various morphometric sperm parameters. AB, head length; CD, head width; EF, head base; GH, acrosomal cap length; IJ, acrosomal cap width; KL, midpiece length; MN, tail length; OP, total length.

were analysed using descriptive statistical test using SPSS 16 software.

RESULTS AND DISCUSSION

The sperm dimensions (mean±SE) of various parameters are summarized in Table 1. Of the 200 spermatozoa captured, 150 were correctly analysed for morphometric evaluation. Fig. 2a showed various dimensions of mithun sperm head while Fig. 2b revealed tail dimension components. The outline of the head was adequately lucid, even, and distinct enough to identify. The midpiece was little translucent pale pink while the end of the tail was again darker and opaque (Fig. 2b) in shade so the boundary between the midpiece and the tail could be detected accurately. The background was light and unstained and did not encumber the evaluation.

Sperm morphometry, in combination with other objective traits, can be useful for developing a fertility index. Associations of abnormal spermatozoa with bull fertility have yielded varying results. Abnormal bull sperm morphology has been correlated with reduced fertility (Correa *et al*. 1997). In particular, the occurrence of abnormal sperm head morphology is associated with lower fertility in the bull (Sekoni *et al*. 1987).

The present study revealed that the total dimensions were not uniform in all spermatozoa. The total length of mithun sperm in fresh semen varied from 62–84µm having a mean of 72.49±0.56 µm while the mean total length of frozen-thawed sperm was 70.14±0.6 µm which is slightly larger than the result reported in mithun bulls (67.9±0.6 µm) (Bhattacharyya *et al.* 2009). The total length of mithun sperm seemed to be similar with that of cattle bulls (Salisbury *et al.* 1985). Nevertheless, the tail of mithun sperm observed in this experiment is lengthier than the goat (Sundararaman *et al.* 2004), dog (Rijsselaere *et al.* 2004) and deer (Sundararaman *et al*. 2006) spermatozoa.

Table 1. Morphpometric parameters (mean±SE) of mithun sperm in both fresh and frozen semen (n=150)

Parameters	Fresh sperm (µm)	Frozen sperm (µm)
Head length	9.13±0.10^{a}	9.03±0.08^{a}
Head width	4.72±0.07^{a}	4.07±0.07^{a}
Head base	2.21±0.04^{a}	2.20±0.06^{a}
Acrosomal cap length	4.33±0.05^{a}	4.03±0.08^{a}
Acrosomal cap width	4.12±0.08^{a}	4.0±0.06^{a}
Midpiece length	15.64±0.28^{a}	15.33±0.21^{a}
Tail length	43.00±0.40^{a}	42.87±0.38^{a}
Head shape	0.52±0.008^{a}	0.50±0.01^{a}
Head area	46.49±1.64^{a}	40.89±1.08^{b}
Head circumference	23.71±0.37^{a}	18.94±0.41^{a}
Total length	72.49±0.56^{a}	70.14±0.6^{a}

*values with different superscript (a,b) differs significantly (P<0.05).

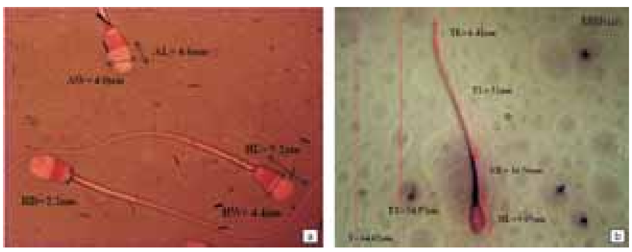

Fig. 2a. Morphometric dimensions of Mithun sperm head. (HL, Head length; HB, head base; HW, head width; AL, acrosomal cap length; AW, acrosomal cap width). 2b. overall tail dimensions (ML, Midpiece length; TL, tail length; TT, total tail length; T, total length).

No report on morphometric parameters of sperm head of mithun sperm could be traced to compare the results of our study and to the best of our knowledge; this is the first report on comparison of sperm head morphometry of mithun sperm in both fresh and frozen-thawed semen. In this study we found that the average size of the mithun sperm head length in both fresh and frozen-thawed semen was smaller than Jersey bulls (Beletti *et al*. 2005, Boersma *et al*. 2001, Gravance *et al*. 1996). The mean sperm head width of fresh (4.72±0.073 µm) semen as well as frozen-thawed (4.07±0.07 µm) semen was slightly less than crossbred (5.11±0.01 µm) and Murrah bulls (4.91±0.01µm) (Roy 2014). Present study revealed that the length (4.33±0.05µm) and width (4.12±0.08µm) of the sperm acrosomal cap in fresh mithun sperm was almost similar. In contrast, it was reported that sperm acrosomal cap width is slightly longer than acrosomal cap length in crossbred and Murrah bulls (Roy 2014). The sperm head shape of fresh mithun sperm (0.52±0.008µm) was almost similar with crossbred bulls (0.56±0.00µm) but considerably varied with Murrah bulls (0.65±0.00µm). The head area of mithun sperm ranged from 22–70µm^2 in fresh semen with an average of 46.4±1.63µm^2, which is significantly higher than average head area (40.89±1.18 µm^2) in frozen-thawed semen (P<0.05). However, in crossbred and Murrah bulls the average head area reported is much smaller (30.76± 0.07 and 24.41± 0.05µm^2 respectively) (Roy 2014).

The variations in the sperm biometric dimension in mithun sperm from that of cattle and buffalo sperm may be due to difference in breeds (Gravance *et al*. 1995). However, in our study we found the morphometric parameters of frozen-thawed semen were little lesser or almost similar with that of fresh semen which are statistically insignificant except the head area. However, bull to bull variations were observed suggesting that the morphometric characteristics vary between individual males. Earlier reports also suggested within species variation in the size of sperm head among stallions (Casey *et al.* 1997), goats (Sundararaman *et al*. 2004) dogs (Rijsselaere *et al*. 2004) 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 (Chandler *et al*. 2002) and incompletely condensed chromatin (Hingst *et al*. 1995).

The computational investigation of sperm morphology offers accurate evaluation of significant variations in morphological characterization which sometimes remain unobserved by conventional assessment. Therefore the simultaneous morphometrical assessment of both fresh and frozen-thawed ejaculates could be useful in evaluation of oocyte fertilizing capability. Moreover, this study on morphometrical analysis of sperm parameters through image analysis may provide baseline information for further studies related to mithun fertility.

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