

IMPACT OF CRYOPRESERVATION ON THE STRUCTURAL DIMENSIONS OF MURRAH BULL SPERMATOZOA

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ABSTRACT

The present study was conducted to evaluate the effect of cryopreservation on sperm morphometry of Murrah bull spermatozoa. A total of 16 ejaculates were collected from Murrah bulls for this study. Semen was collected with Artificial Vagina method at twice a week interval. Semen was evaluated for normal sperm parameters and sperm morphometry was done for normal samples of fresh semen. Semen was extended in glycerolated egg yolk tris extender and equilibrated for 4 h at 5°C, followed by cryopreservation in automatic biological freezer. Semen samples were

evaluated after thawing at 37°C for 45 seconds for normal parameters and sperm morphometry. Findings of the present study revealed that there was significant difference was observed in sperm head morphometry after cryopreservation. Therefore, the study was concluded with that the cryopreservation alters the sperm architecture which might be responsible for reduced fertility after cryopreservation.

Keywords: *Bubalus bubalis*, buffaloes, cryopreservation, fertility, Murrah bull, morphometry, sperm

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INTRODUCTION

Assessment of semen quality often relies on measuring sperm concentration and motility; however, these parameters offer only limited insight into the actual fertility potential of sires (Gangwar *et al.*, 2023; Zhang *et al.*, 1998). Semen quality has also been evaluated using other parameters, such as computerized examination of motility and acrosomal integrity of spermatozoa; nevertheless, yet their correlations with bulls non -returns rate are generally low and inconsistent (Januskauskas *et al.*, 2003). Subfertile bulls with visually normal semen serve as a stimulus for further investigations focused on understanding other fertility parameters. Among these, sperm head morphology has emerged as a potential indicator of fertility (Casey *et al.*, 1997). Integrating sperm head shape, morphometric parameters, and other measurable traits into a comprehensive fertility index could enhance the ranking of sires based on their fertilizing potential. The relationship between sperm size and motility may influence how sperm move through the female genital tract. However, it remains unclear how sperm structure directly affects fertilization. It is also uncertain whether factors other than size contribute to this process (Francisco *et al.*, 2016). Morphometric analysis is especially significant, as sperm head shape has been linked to fertility in various species (Ostermeier *et al.*, 2001; Januskauskas *et al.*, 2003) and to the ability to withstand freezing (Thurston *et al.*, 2001; Pena *et al.*, 2005). Sperm morphology analysis is especially important when it identifies defects that strongly affect fertility and cannot be compensated for by having more spermatozoa. One such defect is the pyriform-shaped sperm head, which reflects problems with chromatin condensation during the development of sperm cells, a critical process for

fertility (Saravia *et al.*, 2007).

Some sires show normal semen characteristics but still have reduced fertility, which motivates further research to identify other reliable fertility markers. Combining measurements of sperm head shape and size along with other measurable traits into a single fertility index could potentially help rank sires based on their ability to fertilize. This study aimed to investigate how the freeze-thawing process affects the size and shape of spermatozoa of Murrah bulls and to determine if these changes, together with traditional semen evaluations, can serve as useful indicators of sperm ability to withstand freezing and thawing may influence their potential fertility after cryopreservation.

MATERIALS AND METHODS

Experimental animal

The study involved two Murrah buffalo bulls. They were between 5 and 6 years old. The bulls were kept under similar feeding and managemental conditions. These conditions followed the minimum standard protocol (MSP) set by the Indian Government. The study took place at the University Instructional Livestock Farm Complex. This complex is part of the College of Veterinary Sciences and Animal Husbandry at Uttar Pradesh Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan (DUVASU), Mathura. Mathura is in the semi-arid region of northern India, in the state of Uttar Pradesh.

Semen collection

Semen was collected twice a week throughout the study period, which lasted from

October to March during 2016 to 2017. Semen collections were performed in the morning between 8:00 and 8:30 A.M., before the animals were fed. The semen was collected using an Artificial Vagina method. This schedule and method ensured consistent and standardized sample collection for the study. A male of the same species was used as a teaser animal for semen collection. Each bull was allowed two false mounts prior to collection. In total, 16 ejaculates were obtained from two bulls, with eight samples collected from each individual. Semen was collected in a clean, dry, graduated centrifuge tube fitted to the latex cone of the artificial vagina. Immediately after collection, the tubes were labelled, transported to the laboratory, and maintained in a water bath at 32 to 34°C for physico-morphological evaluation.

Semen evaluation

Semen samples were assessed for various parameters including volume and mass motility, following the methods described by Salisbury *et al.* (1978). Initial progressive motility was evaluated as per Ahmad (1994). Sperm concentration measurements were conducted following Mishra *et al.* (2012). The total sperm count per ejaculate was determined using the approach of Bane (1954). Additionally, the percentage of live spermatozoa was assessed according to Campbell *et al.* (1953). These evaluations provided a comprehensive analysis of semen quality.

Semen cryopreservation

The extended semen in glycerolated egg yolk tris extender was filled in french mini straws and kept for equilibration for 4 h at 5°C. The straws were then subjected to cryopreservation following the standardized freezing protocol of the semen biology laboratory, DUVASU, Mathura, involving

a gradual temperature reduction from 4°C to -10°C at a rate of 5°C per minute, from -10°C to -100°C at 40°C per minute, and from -100°C to -140°C at 20°C per minute.

Sperm morphometry

Two semen straws from each bull, originating from the same ejaculate, were thawed in a water bath at 37°C for 45 seconds and mixed. A small drop of the pooled semen was mixed with 2 to 3 drops of eosin-nigrosin stain (prepared by dissolving 1 g eosin and 5 g nigrosin in 100 mL of 2.9% sodium citrate buffer, pH 6.9) and placed on a pre-warmed glass slide at 37°C. The mixture was homogenized gently, and four thin smears were prepared per semen sample. The smears were air-dried, and from each slide, four morphologically normal and unstained spermatozoa were selected for morphometric evaluation following the method described by Hancock (1951). Sperm cells exhibiting eosin uptake were considered non-viable (dead), whereas unstained spermatozoa were categorized as viable (live).

A total of 16 live sperm cells were assessed from different fields across the 4 slides were prepared for each ejaculate from every bull, accounting for 128 spermatozoa per bull (16x4x2). Observations were carried out at 1000× magnification using a computerized phase-contrast microscope (Leica DM750). Prior to analysis, the system was calibrated using standard reference images at known magnifications, ensuring a measurement precision of 0.1 µm.

Each sperm head was measured for four primary parameters-head area ($A = 1.05 - 0.081 \times B^2 + 0.64 \times W \times L$, in µm²), head length (L, µm), head width (W, µm), and head base width (B, µm)-along with three derived shape indices: ellipticity (L/W), elongation ((L - W)/(L + W)), and

regularity ($\pi LW/4A$). Additional morphometric traits, including length of midpiece and tail, and total sperm length, were also recorded. The head shape index was measured as the ratio of head length to head breadth, as described by Gravance *et al.* (1998).

Statistical analysis

Statistical evaluation was conducted utilizing the SPSS software (version 22.0 for Windows, SPSS Inc., Chicago, USA). The findings are expressed as the average values with their accompanying standard errors (mean $\pm$ standard error of the mean). Sperm morphometry was compared by paired “t”-test at both the stages.

RESULTS

In the present study the overall mean of head length, width, base width, head area, midpiece length, tail length, total length, head regularity, head ellipticity and head elongation of spermatozoa in fresh semen of Murrah bull were $8.06 \pm 0.04 \mu\text{m}$, $4.62 \pm 0.02 \mu\text{m}$, $2.54 \pm 0.04 \mu\text{m}$, $24.34 \pm 0.18 \mu\text{m}^2$, $12.47 \pm 0.07 \mu\text{m}$, $42.24 \pm 0.19 \mu\text{m}$, $62.40 \pm 0.26 \mu\text{m}$, 1.20 ± 0.0018 , 1.75 ± 0.01 and 0.27 ± 0.0035 while the respective value for post thaw semen were $7.63 \pm 0.02 \mu\text{m}$, $4.49 \pm 0.03 \mu\text{m}$, $2.36 \pm 0.03 \mu\text{m}$, $22.53 \pm 0.17 \mu\text{m}^2$, $12.27 \pm 0.07 \mu\text{m}$, $42.22 \pm 0.20 \mu\text{m}$, $62.12 \pm 0.20 \mu\text{m}$, 1.194 ± 0.0007 , 1.71 ± 0.01 and 0.259 ± 0.003 (Table 1).

The mean head size of sperm decreased significantly ($P < 0.05$) after thawing as compared to fresh semen. The mean mid piece length, decreased significantly ($P < 0.05$) after thawing, while tail length and total length of sperm did not differ significantly as compared to fresh semen. After thawing, the average head ellipticity and

elongation of sperm showed a significant decrease ($P < 0.05$) compared to fresh semen. However, there was no significant change observed in head regularity between thawed and fresh samples. This suggests that while some shape parameters of the sperm head are affected by the freeze-thaw process, others remain stable.

DISCUSSION

Sperm morphometric traits have been linked to fertility in stallions (Gravance *et al.*, 1996) and boars (Hirai *et al.*, 2001). There is previous study in cattle, also indicates that cryopreservation alters the sperm morphology in cattle (Gangwar *et al.*, 2019). It is suggested that the size and shape of the sperm head can influence total sperm volume, which subsequently affects freezability by altering the sperm's resistance to cryodamage (Esteso *et al.*, 2006). The sperm biometry data obtained from Murrah buffalo bulls in this study may serve as a basis for assessing their fertility status. Sperm morphology and morphometry, along with other objective parameters, may serve as valuable tools for developing a fertility index and assessing cryoresistance

Harapanhalli and Mukherjee (1973) investigated the influence of various diluents on sperm morphology in Murrah buffalo and found that storing semen in Egg-Yolk-Glucose-Sodium Citrate (EYGC) diluent for 72 h did not affect the head width when compared with fresh (neat) semen. In fresh semen, the average values for head length, head width, head area, midpiece length, and midpiece width were $7.323 \mu\text{m}$, $4.903 \mu\text{m}$, $28.960 \mu\text{m}^2$, $11.288 \mu\text{m}$, and $0.551 \mu\text{m}$, respectively. Earlier findings by Venkataswami and Vedanayagam (1962) recorded the head length and

Table 1. A comparison of sperm morphometric measurements (μm) between fresh and post-thaw semen of Murrah bulls (Mean $\pm$ SEM = 256).

Parameters	Fresh	Post-thaw	't' value
Head length	8.06 ^b $\pm$ 0.04	7.63 ^a $\pm$ 0.02	8.21**
Head width	4.62 ^b $\pm$ 0.02	4.49 ^a $\pm$ 0.03	3.65**
Base diameter	2.54 ^b $\pm$ 0.04	2.36 ^a $\pm$ 0.03	3.17**
Head area (μm^2)	24.34 ^b $\pm$ 0.18	22.53 ^a $\pm$ 0.17	7.54**
Mid Piece length	12.47 $\pm$ 0.07	12.27 $\pm$ 0.07	1.96 ^{NS}
Tail length	42.24 $\pm$ 0.19	42.22 $\pm$ 0.20	0.08 ^{NS}
Total length	62.40 $\pm$ 0.26	62.12 $\pm$ 0.20	1.86 ^{NS}
Head regularity	1.20 $\pm$ 0.0018	1.194 $\pm$ 0.0007	1.25 ^{NS}
Head ellipticity	1.75 ^b $\pm$ 0.01	1.71 ^a $\pm$ 0.01	2.15*
Head elongation	0.27 ^b $\pm$ 0.0035	0.259 ^a $\pm$ 0.003	2.12*

* $P < 0.05$; ** $P < 0.01$.

Means with different superscript differ significantly within a row.

width of Murrah buffalo sperm as $7.40 \pm 1.07 \mu\text{m}$ and $4.48 \pm 1.29 \mu\text{m}$, respectively, while the midpiece and tail lengths were reported as $12.41 \pm 0.85 \mu\text{m}$ and $43.61 \pm 5.56 \mu\text{m}$. Hameed *et al.* (2014) reported the sperm head length $7.63 \pm 0.02 \mu\text{m}$, head breadth $4.80 \pm 0.02 \mu\text{m}$, midpiece length $11.53 \pm 0.04 \mu\text{m}$, tail length $46.01 \pm 0.93 \mu\text{m}$ and total length $65.17 \pm 0.92 \mu\text{m}$ in *Nili Ravi* bulls.

Biswajit (2014) reported the average measurements of spermatozoa in Murrah buffalo bulls as follows: head length $7.59 \pm 0.01 \mu\text{m}$, head width $4.91 \pm 0.01 \mu\text{m}$, head base $2.47 \pm 0.01 \mu\text{m}$, acrosomal cap length $3.46 \pm 0.01 \mu\text{m}$, acrosomal cap width $4.51 \pm 0.01 \mu\text{m}$, and tail length $56.14 \pm 0.01 \mu\text{m}$. Similarly, Tapaloaga and Tapaloaga (2015) observed that the sperm head length in buffalo bulls ranged from 6.95 to $7.89 \mu\text{m}$, with an overall mean head width of $4.97 \mu\text{m}$. They also reported tail length values between 56.24 and $56.96 \mu\text{m}$ and an average head area of $24.35 \mu\text{m}^2$.

The findings of the present study on various buffalo sperm parameters are consistent with those reported earlier by Agrawal *et al.* (2007) and Tapaloaga and Tapaloaga (2015). Ultrastructural evaluations have revealed that cryopreservation adversely affects several sperm organelles, causing irreversible damage to the acrosome, mitochondria, and tail structures. The observed reduction in motility post-thaw is likely due to mitochondrial and tail impairments. Additionally, acrosomal swelling resulting from cold shock indicates compromised membrane integrity (Watson, 1990).

Gravance *et al.* (2009) reported that among all measured parameters, only the pre-freeze sperm head width showed a significant correlation ($r = -0.53$, $P < 0.05$) with the non-return rate. None of the other pre-freeze or post-thaw morphometric traits had a significant relationship ($P > 0.10$) with post-thaw fertility in individual bulls. Furthermore, the percentage change in the width-to-length (W/L)

ratio before and after freezing was significantly associated with the post-thaw non-return rate ($r = 0.61$, $P < 0.05$). The notable difference in this ratio was primarily due to a pronounced reduction in sperm head length, while the width decreased only slightly during cryopreservation. These results indicate that alterations in sperm head morphometry may serve as an indicator of cryoinjury sustained by spermatozoa during the freezing process.

The findings of the present study are consistent with earlier reports by Aggarwal *et al.* (2007); Biswajit (2014) on Murrah bulls. Buffalo spermatozoa have distinct morphological features than cattle counterparts. A typical spermatozoon has suffered more cryodamages during cryopreservation than cattle. Therefore, further investigating studies must be required to know the exact time of cryodamages occurred during cryopreservation.

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