Research Article

Semen Quality Parameters of Freezable and Non-Freezable Ejaculates of Mithun (*Bos frontalis*) Bulls

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Abstract | A study was designed to measure the semen quality parameters (SQP) such as volume, colour, concentration, mass activity, percent of sperm motility, viability, total morphological abnormality, intactness of acrosome, plasma membrane, nucleus, vanguard distance travelled by sperm in bovine cervical mucus and hydrogen ion concentration in freezable and non-freezable ejaculates of Mithun. Fifty ejaculates were collected from ten matured Mithun bulls and split to freezable and non-freezable ejaculates based on the post thaw motility (40% or more considered as freezable ejaculates). The result has shown that SQP differed significantly (P< 0.05) between the freezable and non-freezable ejaculates and freezable ejaculates have significantly (P< 0.05) higher value than the non-freezable ejaculates. However, morphological abnormality and pH were significantly (P< 0.05) higher in non-freezable than in freezable ejaculates. Mass activity was positively (P< 0.05) correlated with motility, liveability, integrity of acrosome, plasma membrane, nucleus and BCMPT and negatively correlated (P< 0.05) with morphological abnormality. Concluded that SQPs were significantly higher in freezable than in non-freezable ejaculates and indicates freezable sperm has higher structural stability than the non-freezable sperm caused freezable sperm.

Keywords | Mithun, Semen quality parameters, Freezability

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INTRODUCTION

Mithun (*Bos frontalis*) is a "mountain cattle" living as a semi-wild free-range in the North-Eastern Hill (NEH) region of India and responsible for the socio-cultural and economic status of the tribal population (Simoons, 1984). Latest livestock census of India (2007) indicated Mithun density and population is decreasing and is mainly due to lack of proper breeding management in the tribal regions. So proper and scientific interventions are needed from all quarters to conserve and preserve the Mithun population to enhance the socio-economic-cultural status of the tribes. Natural breeding is followed in Mithun,

though with limitations such as cost, disease transmission and infertility. Thus, use of artificial breeding and insemination are essential to preserve the valuable germplasm of Mithun in NEH region. The objective of this experiment on SQPs is to use in breeding soundness evaluation of bulls, predict the sperm fertilizing ability and application in the form of AI in frozen semen bank and semen biology laboratory. It has been observed that SQPs are differed widely between bulls, between ejaculates, between time of collection and between seasons (Raval and Dhami, 2010). SQPs are considered as important indices of semen quality and are significantly correlated with freezability and/or fertility in bovine semen (Fiaz et al., 2010). Moreover, there is a correlated relationship among the SQPs was highly significant and positive correlation was observed among the motility, liveability and acrosomal integrity and thus these SQPs could be applied for practical utility in routine semen evaluation to predict freezability, preservability and fertility of spermatozoa (Bhoite et al., 2005). Moreover, perusal of literatures showed that there was scanty of information on SQPs of freezable and non- freezable ejaculates in Mithun species. Therefore, the present study was designed to measure the SQPs of freezable and non-freezable ejaculates in Mithun species to pursuit future sperm preservation in this precious species.

MATERIAL AND METHODS

Experimental Animals

The present experiment was conducted at National Research Centre on Mithun, Jharnapani, Nagaland, India, which lies at 25°54'30" North Latitude and 93°44'15" East Longitude at an altitude range of 250-300 MSL. Apparently healthy Mithun bulls (n=10) of 3 to 5yrs of age (493 to 507 kg) with good body condition (score 5-6) were selected and maintained under similar feeding, housing and managemental conditions. Each breeding bull was fed as per the farm schedule in the present experiment. All the experimental procedures and protocols were followed as per the Institutional Animal Care and ethical Committee regulations.

Semen Collection and Evaluation

A total of 50 ejaculates were collected from ten bulls through rectal massage method. In shortly, massage was done by forth and back hand movement over the seminal vesicles, prostate and ampulla and then rhythmically strokes on the urethralis muscles subsequently gentle milking of ampullae one by one, which leads to erection and ejaculation. During collection, the initial transparent watery secretions were discarded and creamy white neat semen drops were collected in a graduated collection tube with the help of a broad funnel.

These ejaculates were split into freezable and non–freezable based on the post thaw motility (Rao and Rao, 1996; Prasad et al., 2000). Ejaculates having post thaw motility 40% and above were considered as freezable whereas non–freezable ejaculates were had less than 40% post thaw motility.

The percentage of sperm motility (Nikon, Eclipse 80i; magnification 400×), viability and morphological abnormality by Eosin Nigrosin staining (Tomar, 1997), intactness of acrosome by Giemsa staining (Watson, 1975), integrity of plasma membrane by hypo-osmotic swelling test (HOST) (Jeyendran et al., 1984), nuclear integrity by Fuelgen's staining technique (Barth and Oko, 1989) and vanguard distance travelled by sperm in bovine cervical mucus by cervical mucus penetration test (BCMPT) (Matouseket et al. (1989) were determined as per standard procedure.

Statistical Analysis

The results were analysed statistically with the student't' test using the SPSS (version 15.0; SPSS, Chicago, IL) between the freezable and non-freezable ejaculates and expressed as the mean ± S.E.M. Significant difference ($p < 0.05$) was considered after arcsine transformation of percentage data. Correlation coefficient was calculated using Pearson's method among the SQPs. Differences at ($p < 0.05$) was considered to be statistically significant.

RESULT AND DISCUSSION

In the present experiment, the results clearly indicated that volume, concentration, mass activity, forward progressive motility, viability, plasma membrane integrity, intactness of acrosome and nucleus and vanguard distance travelled by sperm in bovine cervical mucus were significantly ($P < 0.05$) higher in freezable ejaculates in comparison to the non-freezable ejaculates whereas the total morphological abnormality and pH

Table 1: Mean (±S.E.) semen quality parameters of freezable and non-freezable Mithun semen

Physico-morphological attributes	Freezable semen (n=25)	Non-freezable semen (n=25)
Volume (mL)	1.97 ± 0.97	1.53 ± 0.78
Colour	Creamy White	Milky Watery
Concentration (x10^6/ml)	606.41 ± 8.44^a	542.01 ± 8.30^b
Mass activity (0-5 Scale)	3.18 ± 0.80^a	2.35 ± 0.61^b
Individual motility (%)	79.68 ± 3.21^a	63.03 ± 3.16^b
Livability (%)	83.02 ± 3.18^a	68.49 ± 2.92^b
Acrosomal integrity (%)	87.04 ± 3.26^a	73.69 ± 2.87^b
Total sperm abnormality (%)	7.33 ± 2.16^a	13.47 ± 2.24^b
HOST (%)	84.99 ± 3.22^a	70.46 ± 2.93^b
CMPT (mm/h)	24.09 ± 1.83^a	19.18 ± 1.39^b
Nuclear Integrity of spermatozoa (%)	84.96 ± 3.15^a	71.20 ± 2.89^b
pH	6.89 ± 0.37^a	7.09 ± 0.40^b

Means with different superscript within rows differ significantly (P < 0.05); n= Number of ejaculates

were significantly (P< 0.05) higher in latter than in former in Mithun species. Thus, it may enhance to select the freezable ejaculates for preservation efficiently during cryopreservation and improve the fertilization rate in artificial insemination.

The colour of the semen in the present experiment varies from white to creamy white and which occur in normal colour range of semen for the Mithun species and the consistency varies from thin milky to creamy. The colour of semen was creamy white in freezable ejaculates and watery white in non-freezable ejaculates (Table 1). Similar observation was reported in earlier report in Mithun species (Karunakaran et al., 2007). The volume of ejaculate was significantly (p< 0.05) higher in freezable than in non-freezable ejaculates (Table 1). Volume of ejaculate in the present study was higher than earlier reports by Bhattacharyya et al. (2005) and Karunakaran et al. (2007). But volume was lower than semen was collected through artificial vagina method of semen collection in Mithun (Bhattacharyya et al., 2009; Kumar and Bhattacharyya, 2009; Mondal et al., 2010). The results of present study were lower than to those reported by Belorkar et al. (1988), Verma (1997), Mohanty (1999) and Loyi (2008) in cattle. In massage method, the bulls exhibiting more response yielded more volume of freezable ejaculates, but those bulls not responding and had longer protrusion and ejaculation time led to less quality and less volume of semen. In the present study, some bulls consistently ejaculate non-freezable ejaculates. Volume of the semen mainly depends upon the secretory function of the accessory sex glands and its functional as well as individual response. Variation in secretion of testosterone also leads to variation in semen volume. The mass activity was significantly (p< 0.05) higher in freezable ejaculates than in non-freezable ejaculates (Table 1). Mass activity of the ejaculates was positively correlated (p< 0.05) with sperm motility, liveability, intactness of acrosome, nuclear integrity and vanguard distance travelled by sperm and negatively correlated (p< 0.05) with total morphological abnormality in freezable ejaculates (Table 2) whereas in non-freezable ejaculates it was positively correlated (p< 0.05) with sperm viability and integrity of plasma membrane (Table 3). The present observation was similar with report submitted by Karunakaran et al. (2007) and Bhattacharyya et al. (2009). However, Bhattacharyya et al. (2005) reported lower than present value. But mass activity was lower in semen collected through massage method than collected through AV method in Mithun bulls (Mondal et al., 2010). Similar observation was reported in cattle by Belorkar et al. (1988) and Gebreselassie et al. (2012). Sethi et al. (1989) concluded that bulls donating larger volume of neat semen with higher mass activity are supposed to produce freezable ejaculates. Lower mass activity in present

Table 2: Correlation coefficient among the semen quality parameters of freezable ejaculates in Mithun (*Bos frontalis*) bulls

No	Attributes	1	2	3	4	5	6	7	8	9	10	11
1	Volume	1.00	0.20	0.61*	0.54	0.58	0.57	-0.46	0.54	0.71*	0.58	-0.19
2	Concentration		1.00	0.57	0.67*	0.72*	0.67*	-0.71*	0.67*	0.54	0.66*	-0.22
3	Mass activity			1.00	0.86*	0.87*	0.89*	-0.76*	0.76*	0.89*	0.89*	-0.12
4	Individual motility				1.00	0.96*	0.95*	-0.95*	0.94*	0.90*	0.96*	-0.40
5	Livability					1.00	0.97*	-0.94*	0.93*	0.92*	0.95*	-0.42
6	Acrosomal integrity						1.00	-0.93*	0.95*	0.90*	0.96*	-0.42
7	Abnormality							1.00	-0.92*	-0.83*	-0.90*	0.35
8	Plasma membrane integrity								1.00	0.90*	0.91*	-0.51
9	Cervical mucus penetration test									1.00	0.92*	-0.30
10	Nuclear Integrity										1.00	-0.41
11	pH											1.00

* Correlation coefficient were significant, p< 0.05

Table 3: Correlation coefficient among the semen quality parameters of non-freezable ejaculates in Mithun (*Bos frontalis*) bulls

No	Attributes	1	2	3	4	5	6	7	8	9	10	11
1	Volume	1.00	0.06	0.13	0.42	0.30	0.32	-0.26	0.32	0.31	0.35	0.11
2	Concentration		1.00	0.76*	0.71*	0.76*	0.78*	-0.64	0.76*	0.70*	0.77*	-0.44
3	Mass activity			1.00	0.86*	0.93*	0.90*	-0.84*	0.90*	0.91*	0.90*	-0.44
4	Individual motility				1.00	0.95*	0.91*	-0.85*	0.92*	0.84*	0.94*	-0.26
5	Livability					1.00	0.95*	-0.88*	0.95*	0.88*	0.95*	-0.42
6	Acrosomal integrity						1.00	-0.86*	0.93*	0.84*	0.93*	-0.35
7	Abnormality							1.00	-0.86*	-0.83*	-0.88*	0.28
8	Plasma membrane integrity								1.00	0.86*	0.92*	-0.43
9	Cervical mucus penetration test									1.00	0.87*	-0.43
10	Nuclear Integrity										1.00	-0.34
11	pH											1.00

* Correlation coefficient were significant, p< 0.05

experiment may be variation in semen collection through massage method, individual bull responses, concentration of live spermatozoa as well as effect of climatic variation.

Concentration of ejaculate has been considered as an important criterion for the production of maximum number of semen straw for getting maximum number of artificial insemination covered. In our experiment, sperm concentration of ejaculate was significantly ($p< 0.05$) higher in freezable than in non-freezable ejaculates (Table 1). However, the difference between these two groups has so far not been studied in this Mithun species. But average values of concentrations in both the groups were well within the range of overall sperm concentration in the present experiment. Similar observation was also reported by Bhattacharyya et al. (2005) and Karunakaran et al. (2007) in massage method of semen collection, but higher value was observed in artificial vagina method of semen collection (Bhattacharyya et al., 2009; Kumar and Bhattacharyya, 2009; Mondal et al., 2010) in the Mithun bulls. Similar reports as freezable ejaculates have higher sperm concentration than non- freezable ejaculates as reported in bovine species (Loyi, 2008; Gebreselassie, 2009; Gebreselassie et al., 2012). Total sperm concentration of ejaculates is greatly influenced by various factors such as individual bull, breed of bull, season of year, micro and macro environmental factors (Swain and Singh, 2004) and age of bull (Mathew et al., 1982). The variation in sperm concentration may also be varied due to managemental practices such as exercise, body massage, brushing, restraint before collections (Collins, 1951), frequency of semen collection (Singh and Prabhu, 1983) and variation in seasons (Gupta et al., 1978). There was significant ($p< 0.05$) difference between the mean sperm concentration between the two groups in the present study that could be attributed to the of the individual bulls response besides above factors.

The per cent individual motility of spermatozoa was significantly ($p< 0.05$) higher in freezable than in non-freezable ejaculates (Table 1). In our study, motility was positively correlated ($p< 0.05$) with sperm viability, intactness of acrosome, integrity of plasma membrane and nucleus and vanguard distance travelled by sperm in bovine cervical mucus and negative correlation ($p< 0.05$) was observed with morphological abnormality in freezable ejaculates (Table 2). However, the motility difference between these two groups has so far not been reported in this mountain cattle species. But some reports were available in Mithun, as forward progressive motility of sperm was 67.74 ± 2.44 (Bhattacharyya et al., 2005) and 75.30 ± 3.50 (Karunakaran et al., 2007) through rectal massage method and through artificial vagina method, it was 78.60 ± 2.60%, (Bhattacharyya et al., 2009), 55.00 ± 8.83% (Kumar and Bhattacharyya, 2009), 82% (Mondal et al., 2010). Similar observation was reported in cattle as freezable ejaculates have higher individual motility than non-freezable ejaculates (Mohanty, 1999; Gebreselassie et al., 2012). Ejaculates having post thaw motility 40% or more were considered as freezable ejaculates. Similar condition was reported in cattle, buffaloes, sheep, goat and boar. The poor semen quality in crossbred and Murrah buffaloes (Suryaprakasam and Rao, 1993) and the poor semen freezability in case of exotic and crossbred bulls (Mathew et al., 1982; Suryaprakasam and Rao, 1993) are the major reasons for disposal of breeding bulls.

Viability of spermatozoa is important criterion while selecting ejaculates for freezing to get more number of post thaw live spermatozoa. In our present experiment, live sperm percentage of ejaculate was significantly ($p< 0.05$) higher in freezable ejaculates than in non-freezable ejaculates (Table 1). The viability of spermatozoa was positively correlated ($p< 0.05$) with mass activity, individual motility, intactness of acrosome, integrity of plasma membrane and nucleus and vanguard distance travelled by sperm in cervical mucus and negative correlation ($p< 0.05$) was observed with total morphological abnormality in freezable ejaculates (Table 2) and similar results was observed in non-freezable ejaculates in the present study (Table 3). But the viability percent difference between these two groups has so far not been studied in this bovine species. In Mithun species, percentage of sperm viability through rectal massage method was reported as 68.53 ± 1.85 (Bhattacharyya et al., 2005), 80.6 ± 4.1 (Karunakaran et al., 2007) and through artificial vagina method, it was 80.7 ± 2.2, (Bhattacharyya et al., 2009), 71.73 ± 3.45 (Kumar and Bhattacharyya, 2009) and 98 ± 9 (Mondal et al., 2010). Similar observation as freezable ejaculates has significantly higher live sperm percentage than in non-freezable ejaculates as reported in cattle (Gebreselassie et al., 2012; Loyi, 2008). The variation in the viability could be due to breed difference, age and technical skill of the observ-

er (Tomar et al., 1985).

The abnormal sperm percentage of spermatozoa was significantly ($p< 0.05$) lower in freezable ejaculates than in non-freezable ejaculates (Table 1). It was negatively correlated ($p< 0.05$) with mass activity, individual motility, acrosomal integrity, liveability, plasma membrane integrity in freezable ejaculates (Table 2). In the present study, percentage of total sperm abnormality was higher than earlier reports as semen through rectal massage method had 5.7 ± 0.2 percent (Karunakaran et al., 2007) and through artificial vagina method had 4.8 ± 0.6 per cent (Mondal et al., 2010). Roberts (1982) opined that in fertile semen sample, the total sperm abnormality should not exceed more than 20 per cent. In the present study, the overall sperm abnormality includes caput, mid-piece and tail abnormalities. Moreover, the sperm abnormality may vary due to method of collection, temperature shock and technique employed (Bishop et al., 1954). Hence, the difference between the freezable and non-freezable ejaculates may be attributed to these factors.

The percentage of acrosomal integrity of spermatozoa was significantly higher in freezable than in non-freezable ejaculates (Table 1). Acrosomal integrity was positively correlated with mass activity, liveability, nuclear integrity, plasma membrane integrity and BCMPT and negatively correlated ($p< 0.05$) with total sperm abnormality in ejaculates (Table 2). No similar report was available in Mithun species. But in cattle Gebreselassie et al. (2012) recorded the percent of intact acrosome of crossbred bull sperm was 79.43 ± 1.10% and 64.17 ± 1.42% in freezable and non-freezable ejaculates, respectively and it was significant between two qualities of ejaculates. The acrosomal integrity depends on the factors like age of the bulls (Javed et al., 2000); temperature (Chandra et al., 1999), frequency of semen collections, sexual excitement before collection (Badaway et al., 1973) and due to variations in any of the above factors might have caused the differences with other earlier reports. Acrosome can be detached from the sperm head under the influence of different physical and chemical factors (Hartree and Srivastava, 1965). Optimum fertility depends on the acrosome being structurally and functionally intact (Gebreselassie et al., 2012).

The study of sperm membrane functional status is of particular importance since an intact and functionally active membrane is required for maintenance of sperm motility, metabolism, capacitation, acrosome reaction and hence for successful fertilization. During the HOST, the biochemically active spermatozoa when exposed to hypo-osmotic stress due to influx of water, undergoes swelling and subsequently increase in volume to establish equilibrium between the fluid compartment within the spermatozoa and extracellular environment. The osmotic changes induce typical morphological alteration characterized by presence of swollen area in the tail region. These changes have been considered as an indicator of the membrane integrity and normal functional activity of spermatozoa (Jeyendran et al., 1984).

The HOST positive sperm per cent of ejaculate was significantly ($p< 0.05$) higher in freezable ejaculates than in non-freezable ejaculates (Table 1). Plasma membrane integrity was positively correlated ($p< 0.05$) with mass activity, liveability, acrosomal integrity, nuclear integrity and BCMPT and negatively correlated ($p< 0.05$) with total sperm abnormality in freezable (Table 2) and non-freezable ejaculates (Table 3). No similar report is available in Mithun species. In cattle, it was reported by Gebreselassie et al. (2012) and Perumal (2008) that freezable ejaculates has higher plasma integrity value than non-freezable ejaculates. Routine semen evaluation has certain limitations for comprehensive prediction of fertility of bull semen. The HOST highlights the permeability of sperm membrane to hypo-osmotic solution and the projection of higher value is a valid indication of intact membrane and sample with higher value is regarded as potent for establishing pregnancy.

In the present study, per cent nuclear integrity of spermatozoa was significantly ($p< 0.05$) higher in freezable ejaculates than in non-freezable ejaculates (Table 1). Nuclear integrity was positively correlated ($p< 0.05$) with mass activity, liveability, acrosomal integrity, plasma membrane integrity and BCMPT and negatively correlated with total sperm abnormality in freezable (Table 2) and non-freezable ejaculates (Table 3). Perusal of literature did not reveal any study on nuclear integrity / DNA fragmentation in good and poor quality ejaculates in Mithun species. So the appropriate comparison could not be substituted by this study.

The *in-vitro* sperm penetration test is considered as a

sperm function test that measures the ability of sperm in the semen to swim up into a column of cervical mucus or its substitute. The sperm migration into the cervical mucus or its substitute is based on the same principle as per the test proposed by Kremer and Kroeks (1975). The vanguard distance travelled by sperm was significantly ($p< 0.05$) higher in freezable ejaculates than non- freezable ejaculates (Table 1). BCMPT was positively correlated ($p< 0.05$) with mass activity, individual motility, liveability, acrosomal integrity, nuclear integrity and plasma membrane integrity and negatively correlated ($p< 0.05$) with total sperm abnormality in freezable (Table 2) and non-freezable ejaculates (Table 3). Perusal of literature did not reveal any study on CMPT in freezable and non-freezable ejaculates in Mithun species. So the appropriate comparison could not be substituted by this study. But similar study was conducted in cattle and report revealed that freezable ejaculates have higher vanguard distance travelled by sperm than non-freezable ejaculates (Perumal, 2008; Perumal et al., 2011) in crossbred cattle. The difference of cervical mucus penetration with regard to freezable and non-freezable ejaculates would be possible due to the difference in the forward progressive motility, abnormality and loss of mitochondrial membrane potential, which could otherwise, has resulted in lowering the penetration rate.

It was concluded from the study that most of the seminal attributes were significantly higher in freezable ejaculates in comparison to the non-freezable ejaculates in Mithun. This indicates that freezable sperm has higher structural stability than the non-freezable sperm that leads to freezable ejaculates has higher functional sperm structures to move faster and forward direction to fertilize.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare

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