Indian Journal of Animal Sciences **85** (12): 1322–1327, December 2015/Article

Freezability of mithun (*Bos frontalis)* semen

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Received: 19 March 2015; Accepted: 26 May 2015

ABSTRACT

The present study was conducted to assess the freezability of the mithun semen. Post-thaw physicomorphological parameters (PMPs) such as post-thaw motility, viability, total sperm abnormality, integrity of acrosome, plasma membrane and nucleus and vanguard distance travelled in bovine cervical mucus by sperm (BCMPT) of ejaculates, biochemical attributes such as, aspartate amino transaminase (AST), alanine amino transaminase (ALT) and lactate dehydrogenase (LDH) and *in vitro* zona binding ability of spermatozoa with heterologous (buffalo) oocyte were studied in freezable and nonfreezable ejaculates. Ejaculates (50) were collected from healthy matured mithun bulls and grouped based on the post-thaw motility into freezable and nonfreezable. These selected ejaculates were extended with standard Tris egg yolk citrate glycerol extender and stored at ultra low temperature (–196°C). Post-thaw PMPs, binding index (BI) and binding % (BP) and biochemical profiles were examined with standard protocols. The results revealed that the PMPs, biochemical profiles, BI and BP differed significantly between freezable and nonfreezable ejaculates. The post-thaw motility was positively correlated with livability, intactness of acrosome, plasma membrane, nucleus and vanguard distance travelled by sperm and negatively correlated with post-thaw total sperm abnormality. It was concluded that the post-thaw PMPs and BI and BP were significantly higher, biochemical intracellular enzymatic profiles were significantly lower in freezable than in nonfreezable ejaculates indicates freezable sperm has functionally stable sperm structures and higher intactness of plasma and acrosomal membrane to motile faster and in forward direction to reach the fertilization site and successful conception.

Key words: Freezability, Mithun, Physicomorphological parameters, Post-thaw semen

According to livestock survey (2007), the mithun population has decreased in Nagaland, Manipur and Mizoram. This is due to the reasons such as lack of knowledge about breeding programme, intensive increasing inbreeding practices and breeding with inferior bulls. To overcome these problems and to conserve and preserve the mithun population in its home tracts, greater efforts are required urgently on appropriate scientific breeding management and other livestock management protocols from all the quarters. The main target of the present investigation is to diagnose the fertility capability and effective utilization of mithun bulls and its semen in artificial insemination and multiple ovulation and embryo transfer technology in this precious animal species. The values of physicomorphological parameters (PMPs) differ between bulls, ejaculates and also between seasons (Raval and Dhami 2010). Similarly PMPs, post-thaw motility, total morphological abnormality, integrity of acrosome, plasma membrane and nucleus of spermatozoa were found significantly correlated with fertilizing potential, freezability and preservability of bovine spermatozoa (Fiaz *et al*. 2010). Reports revealed that there was highly significant and positive relationship among PMPs and these PMPs could be applied efficiently in routine, regular semen evaluation to predict preservability, freezability and fertilizing potential of spermatozoa in mithun (Bhoite *et al.* 2005). There is scanty information on post-thaw PMPs and freezability of mithun semen, therefore, the present study was designed to assess the post-thaw PMPs and freezability and *in vitro* fertilizing potential by zona binding assay of freezable and nonfreezable ejaculates to pursuit future semen preservation protocols in mithun.

MATERIALS AND METHODS

Apparently healthy mithun bulls (10), approximately 3– to 5- year -old, weighing 500 kg with good body condition (score 5–6) were selected from mithun farm of the institute and maintained under uniform feeding, housing and management conditions. All the experimental protocols in

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the present experiments were met the Institutional Animal Care and Use Committee regulations. Ejaculates (50) were collected through trans-rectal massage method and divided into group 1: freezable and group 2: nonfreezable, based on the post-thaw motility (Prasad *et al*. 2000). Freezable ejaculates having post-thaw motility 40% and above whereas nonfreezable ejaculates having less than 40%. The selected semen samples were extended with standard Tris egg yolk citrate glycerol extender (@ 30 million spermatozoa/straw), frozen with biological freezer and stored at ultra low temperature in liquid nitrogen (–196°C). The stored semen straw was thawed at 37°C for 30 sec. Following parameters were estimated: percentage of post-thaw sperm motility (magnification 400×), viability and total sperm morphological abnormality (eosin nigrosin staining, Tomar 1977), acrosomal integrity (Giemsa staining, Watson 1975), plasma membrane integrity (hypo-osmotic swelling test (HOST), Jeyendran *et al*. 1984), nuclear integrity (Feulgen's staining technique, Barth and Oko 1989) and vanguard distance travelled by sperm in the bovine cervical mucus (BCMPT) (Matouseket *et al*. 1989). Spermatozoa of both groups were subjected to heterologous (buffalo oocyte) zona binding assay after freezing-thawing (post-thaw). The zona binding ability of the mithun spermatozoa was assessed in terms of binding index (BI) and binding % (BP) (Fazeli *et al*. 1993). The leakage of intra cellular enzymatic profiles such as AST, ALT and LDH activity were estimated with commercially available diagnostic kits. The results were analysed statistically with the student 't' test using the SPSS and expressed as the mean ± SEM. Differences with values of ($P<0.05$) were considered to be statistically significant after arcsine transformation of percentage data. Correlation among the PMPs and biochemical profiles was established with Pearson's correlation coefficient. Differences at $P<0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

Most of the post-thaw PMPs and zona BI and BP significantly differed and were higher in freezable than in nonfreezable ejaculates. Inversely, the post-thaw biochemical enzymatic profiles (AST, ALT and LDH) were significantly higher in nonfreezable than in freezable ejaculates. In this study, 40% of the freezable semen turned out to be nonfreezable and 15% of the nonfreezable semen gave a PTM of 40% or more. This is much lesser than reported by Ghosh *et al.* (2007) in bovine species, who reported a discard rate of 29.01% of frozen semen based on post-thaw motility. Similarly, Mishra and Tyagi (2006) observed that 5 – 15% post-thawed semen was discarded due to poor post-thaw sperm motility. Similarly, Gebreselassie *et al*. (2012) and Singh *et al*. (2013) reported that freezable sperm had higher post-thaw motility than the nonfreezable sperm. Moreover, Sharma *et al*. (1992) found around 14.5 % reduction in post-thaw motility from fresh semen, which could be due to cryo damage of the sperm during the cryopreservation process. Post-thaw motility of spermatozoa of various breeds of bovine species found ranging from 19.17 (Prasad 1997) to 53 % (Mohanty 1999). Similarly, average post-thaw motility was 55.83 ± 8.28% in crossbred bulls (Loyi 2008) and 42.29 ± 1.12% in buffalo (Mayuri 2006). During *in vitro* preservation, the spermatozoa are exposed to a foreign diluting media as well as to low and ultra low temperature. Death might occur due to release of toxic substances, ultra low exposure, enzymatic leakage, medium of preservation, degree of sperm permeability, aging effect of sperm and individual variation (Salisbury *et al*. 1978). During liquid nitrogen storage, there is a greater chance for death of sperm due to extreme fluctuation in temperature. Moreover, there is an inherent limit that many of the bull spermatozoa could not resist exposure to ultra low temperature. Poor post-thaw motility and freezability in some of the semen samples may be due to method of semen collection (massage method) and crossbred strains of mithun as pure strains of mithun have better PMPs, BI and BP and intactness of sperm membrane than crossbred mithuns as similar in crossbred cattle (Prasad *et al*. 2000). Decreased post-thaw sperm motility and reduced sperm metabolism were reported for bovine frozen in tris egg yolk based semen extender (Pace and Graham 1970). Post-thaw motility has a significant correlation with live and dead, abnormality and HOST (Tables 4, 5). Ramachandran *et al.* (2007) reported a similar positive correlation between the post-thaw motility and HOST reacted sperm in Indian cattle bulls (*Bos indicus*). A probable explanation for the poor freezability of the crossbred bull semen based on the post-thaw motility is the genetic combination, because both the exotic and indigenous pure breeds have better seminal attributes than their crosses (Prasad *et al*. 2000, Tyagi *et al*. 2000). The ability to survive at ultra low temperatures is an individual bull feature (Sagdeo *et al.* 1990).

Post-thaw livability in the present study revealed that freezable sperm has higher per cent of livability than sperm from nonfreezable ejaculates (Table 1). Similarly, Singh *et al*. (2013) and Gebreselassie *et al*. (2012) reported that

Table 1. Mean (±SE) post-thaw physico morphological parameters of freezable and non-freezable ejaculates of mithun

Physicomorphological parameters	Freezable ejaculates	Nonfreezable ejaculates
Post-thaw motility (%)	44.25 ± 1.76[a]	32.47 ± 2.12[b]
Livability	57.63 ± 2.24[a]	40.53 ± 2.61[b]
Acrosomal integrity (%)	61.69 ± 2.12[a]	48.59 ± 1.56[b]
Total sperm abnormality (%)	20.14 ± 1.13[a]	26.15 ± 1.65[b]
Plasma membrane integrity (%)	56.75 ± 1.87[a]	43.18 ± 2.34[b]
Vanguard distance by CMPT (mm/h)	21.25 ± 1.26[a]	18.12 ± 1.23[b]
Nuclear integrity of spermatozoa (%)	68.16 ± 1.42[a]	50.43 ± 3.18[b]

Means with different superscript within rows differ significantly ($P < 0.05$).

freezable ejaculates had significantly higher viability than nonfreezable ejaculates. The viability of spermatozoa was positively and significantly correlated (P<0.05) with post-thaw motility, acrosomal integrity, plasma membrane, nuclear integrity and BCMPT and negatively correlated (P<0.05) with total sperm abnormality as reported earlier in cattle (Perumal *et al*. 2011a, Srivastava and Kumar 2006). Ramachandran *et al.* (2007) reported a similar positive correlation between the post-thaw viability, motility and HOST reacted sperm in cattle bulls. Mishra *et al.* (1988) reported 20–25% drop in the viability following cryopreservation. The variation in the present study could be due to different strains of mithun, age, semen collection method (massage method), season and technical skill of the observer (Tomar *et al.* 1985). Similarly, post-thaw morphological abnormality of spermatozoa of the present study revealed that freezable ejaculates have significantly (P<0.05) lower value than in nonfreezable ejaculates (Table 1). It was negatively correlated (P<0.05) with post-thaw motility, acrosomal integrity, livability, plasma membrane integrity in freezable (Table 4) and nonfreezable ejaculates (Table 5). Roberts (1982) also suggested that in freezable quality semen, the total morphological sperm abnormality should not exceed more than 20%. In our study, overall total morphological abnormality was estimated as the sum of head, mid-piece and tail abnormalities. Further, sperm abnormality may be varied due to method of semen collection, technique employed in processing and temperature shock (Bishop *et al.* 1954).

Disintegration of acrosome or loss of acrosomal intactness may result into reduced ATP production and leakage of intracellular enzymes and proteins from spermatozoa. Sperm with defective acrosome may be highly motile but not able to fertilize the oocyte. Therefore, the acrosomal integrity should always be a major part of assessment of quality of spermatozoa in routine semen quality examination (Kumar 2006). Analysis of this parameter revealed that freezable group of ejaculates has higher percentage of spermatozoa with intact acrosome than in the nonfreezable group of ejaculates (Table 1). The difference in the intact acrosome percentage between freezable and nonfreezable ejaculates might be due to higher percentage of intact acrosome spermatozoa at fresh stage and often have higher sperm resistance to cryodamage in freezable quality in comparison to nonfreezable quality (Singh *et al.* 2013, Gebreselassie 2009, Gebreselassie *et al*. 2012). Acrosomal integrity was positively and significantly correlated with other post-thaw PMPs such as post-thaw livability, nuclear, plasma membrane integrity and BCMPT and negatively correlated (P<0.05) with total sperm morphological abnormality in freezable (Table 4) and nonfreezable ejaculates (Table 5). Moreover, acrosomal intactness depends on factors like age of bulls, frequency of semen collections, temperature and sexual excitement before collection, semen processing and evaluation (Javed *et al.* 2000). 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 finally successful fertilization.

Integrity of sperm plasma membrane, acrosomal membrane and progressive motility are essential to assess fertilizing capacity of spermatozoa in *in vitro*. A hypo-osmotic swelling test can assess functional and physiological and biochemical aspects of membrane potentiality (Perumal *et al*. 2011b). Functionally and biochemically integrity of plasma membrane of spermatozoa was evaluated using simple, quick and inexpensive method (HOST). In HOST, the biochemically active spermatozoa when exposed to hypoosmotic stress due to influx of water, leading to swelling and followed by increasing the volume to establish equilibrium between fluid compartment of internal and external environment of spermatozoa. The osmotic changes induce typical morphological alteration characterized by presence of swollen area in the tail region. These changes have been recognized as an index of plasma membrane integrity and normal functional and biochemical activity of spermatozoa (Jeyendran *et al.* 1984). In the present study, analysis (Table 1) of this parameter revealed that post-thawed spermatozoa of freezable ejaculates have higher plasma membrane integrity than nonfreezable ejaculates (Gebreselassie 2009). Plasma membrane integrity was positively correlated (P<0.05) with post-thaw acrosomal integrity, livability, nuclear integrity and vanguard distance travelled by sperm in cervical mucus and negatively correlated (P<0.05) with total sperm morphological abnormality in freezable (Table 4) and nonfreezable ejaculates (Table 5). Regular semen evaluation in andrological laboratory has certain short comes to predict the bull fertility. Testing of plasma membrane integrity is ideal and suitable to measure permeability of sperm membrane to hypoosmotic solution and result of higher value is a valid indication of intactness of membrane and livability and is recognized as capacity of sperm to establish successful pregnancy in animal species (Jeyendran *et al*. 1984).

Post-thaw nuclear integrity of spermatozoa revealed that freezable ejaculates have higher % than nonfreezable ejaculates in the present study (Table 1). This may be due to the intactness of nucleus and/or DNA in the freezable sperm was significantly higher and its biochemical composition was more superior to the nonfreezable sperm (Perumal *et al*. 2011a). This factor is influenced by inheritance in some bulls and is also affected by species variation, semen collection techniques, hormonal influences, contamination of other body fluids like urine, semen evaluation and processing techniques, feeding and breeding management of bulls, skill of laboratory technician and other management procedures. Further, post-thaw nuclear integrity was significantly positively correlated (P<0.05) with post-thaw motility, viability, plasma membrane, acrosomal integrity and vanguard distance travelled by sperm and significantly (P<0.05) negatively correlated with total sperm morphological abnormality as

similar report was observed in bovine species (Perumal *et al*. 2011a). Higher post-thaw nuclear integrity with higher PMPs caused higher BI and BP in the present study indicates intactness is very much essential in successful fertilization in mammalian species.

Sperm penetration *in vitro* into a column of cervical mucus is considered as a sperm function test that measures the ability of sperm to swim up into the cervical mucus. The post-thaw vanguard distance travelled by sperm was significantly (P<0.05) higher in freezable than in non-freezable ejaculates (Table 1). BCMPT was significantly positively correlated (P<0.05) with post-thaw forward progressive motility, viability, acrosomal, plasma membrane and nuclear integrity and negatively correlated (P<0.05) with total morphological abnormality of spermatozoa. The significant difference between the freezable and non-freezable ejaculates due to the forward migration of the sperm and swim up of higher sperm velocity were enhanced straight forward motility as adequate motility has been ascribed to penetrate mucus efficiently (Takemota *et al*. 1985). Further, capability of spermatozoa to penetrate bovine cervical mucus was found to be influenced by various semen parameters which could not be accurately predicted based on the single examination. Literature study did not reveal any report on BCMPT in freezable and non-freezable ejaculates in mithun species, but similar report was found in bovine species (crossbred) that freezable ejaculates have significantly higher vanguard distance than by nonfreezable sperm (Perumal *et al.* 2011b). The present result was suggested that sperm with higher penetration distance, there is an increase chance of more percent of the animal to become pregnant for the particular selected bull semen. Moreover, the individual differences between the bulls in freezable and freezable semen could be attributed to the fact that the sperm penetration velocity was significantly varied between the bulls and between the mucus (Anil Kumar and Devanathan 1995). Further, The different distance travelled by the sperm between freezable and nonfreezable ejaculates would be due to the difference in the speed of forward progressive motility, degree of morphological abnormality and efficiency of generating ATP by mitochondrial (mitochondrial membrane potential) (Perumal *et al.* 2011a).

Zona binding assay was carried out to investigate the ability of frozen-thawed mithun bull spermatozoa to bind with ova from heterologous animal species (buffaloes). Zona binding assay was consisted of determination of binding percent (BP) and binding index (BI) of post-thaw spermatozoa. The results indicated a positive and significant (P<0.05) difference in BP and BI between freezable and nonfreezable ejaculates (Table 3) and freezable ejaculates had higher BP and BI than nonfreezable ejaculates. This is because that the improved level of the post-thaw motility, viability, plasma membrane, acrosomal membrane and nuclear integrity intrinsically and lower level of total morphological abnormality. Thus, it ultimately improved the BP and BI parameters of sperm in freezable ejaculates.

In the present study, the metabolic enzymes such as dehydrogenase and transaminase enzyme activities were measured to determine the protective action as well as leakage of intracellular enzymes during ultra low temperature preservation of semen. AST and ALT are essential for metabolic processes, which provide energy for survival, motility and fertility of spermatozoa and these transaminase activities in semen are good and suitable indicators of semen quality as they measure sperm membrane stability and integrity (Lopez *et al.* 1989). Moreover, increasing percentage of morphological abnormal spermatozoa in semen ejaculate causes high concentration of transaminase enzyme in the extra cellular fluid due to sperm membrane damage and ease of leakage of enzymes from spermatozoa (Dogan *et al.* 2009). In the present study, post-thaw analysis of these intracellular enzymatic profiles revealed that freezable ejaculates had significantly lower AST and ALT than in non- freezable ejaculates (Tables 2, 6). Similarly, higher activity of AST and ALT was observed at post-thaw clearly indicated that the enzyme was leaked out into the extracellular fluid following cryopreservation of semen due to structural damage and increased cell membrane permeability. Moreover, AST and ALT levels were higher in non-freezable ejaculates as it has higher destabilized membrane integrity of acrosome, plasma membrane, mitochondria and flagella of the sperm. Similarly, post-thaw LDH activity revealed that freezable ejaculates have lower activity than in nonfreezable ejaculates (Tables 2, 6) and it may be due to higher damage of sperm plasma membrane or fragile nature in nonfreezable than in freezable ejaculates and leads to leakage of LDH from the cell.

Table 2. Mean (±SE) post-thaw biochemical profiles of freezable and nonfreezable ejaculates of mithun

Biochemical profiles	Freezable ejaculates	Nonfreezable ejaculates
AST (μ mole/litre)	272.15 ± 6.31[a]	363.32 ± 7.45[b]
ALT (μ mole/litre)	49.61 ± 1.88[a]	70.40 ± 3.24[b]
LDH (IU/Litre)	823.84 ± 3.98[a]	898.29 ± 6.29[b]

Means with different superscript within rows differ significantly (P < 0.05).

Table 3. Mean (±SE) heterologous zona binding assay of freezable and nonfreezable ejaculates at post-thaw stage of preservation

Parameters	Freezable ejaculates	Nonfreezable ejaculates
No of oocyte examined	54	52
No. of sperm bound oocytes	26	20
No. of zona bound sperm	1626	1446
Binding per cent	48.62±2.71[a]	38.07±2.52[b]
Binding Index	30.40±1.75[a]	26.74±2.20[b]

Means with different superscript within rows differ significantly (P < 0.05).

Table 4. Correlation coefficient among the post-thaw physicomorphological parameters of freezable ejaculates of mithun bulls

No	Attributes	1	2	3	4	5	6	7
1	Post-thaw motility	1.00	0.92*	0.76*	–0.92*	0.61*	0.89*	0.79*
2	Livability		1.00	0.80*	–0.93*	0.72*	0.93*	0.76*
3	Acrosomal integrity			1.00	–0.78*	0.46	0.78*	0.37
4	Abnormality				1.00	–0.60*	–0.92*	–0.70*
5	Plasma membrane integrity					1.00	0.65	0.61*
6	Cervical mucus penetration test					1.00	0.80*	
7	Nuclear Integrity							1.00

*Correlation coefficient were significant, P< 0.05.

Table 5. Correlation coefficient among the post-thaw physicomorphological parameters of nonfreezable ejaculates of mithun bulls

No	Attributes	1	2	3	4	5	6	7
1	Post-thaw motility	1.00	0.94*	0.92*	–0.90*	0.93*	0.80*	0.92*
2	Livability		1.00	0.91*	–0.93*	0.89*	0.72*	0.93*
3	Acrosomal integrity			1.00	–0.92*	0.90*	0.76*	0.88*
4	Abnormality				1.00	–0.93*	–0.74*	–0.94*
5	Plasma membrane integrity					1.00	0.76*	0.91*
6	Cervical mucus penetration test						1.00	0.58
7	Nuclear Integrity							1.00

* Correlation coefficient were significant, P< 0.05.

Table 6. Correlation coefficient among the post-thaw biochemical profiles of freezable and nonfreezable ejaculates of mithun

	Freezable ejaculates				Nonfreezable ejaculates			
No	Attributes	1	2	3	Attributes	1	2	3
1	AST	1	0.78*	0.77*	AST	1	0.92*	0.89*
2	ALT		1	0.96*	ALT		1	0.95*
3	LDH			1	LDH			1

* Correlation coefficient were significant, P< 0.05.

It was concluded that most of the post-thaw PMPs and BI & BP were significantly higher in freezable as compared to nonfreezable sperm indicates former has higher stable and intact sperm structures to mobile faster and in forward direction to reach the site of fertilization and successful fertilization.

ACKNOWLEDGMENT

The authors were thankful to the Director, ICAR-National Research Centre on Mithun, Jharnapani and the Director, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly for their kind help to complete the research work.

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