

## CRYOPRESERVATION OF SEMEN OF MITHUN AND SIRI BULLS

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### ABSTRACT

The objective of the study was to assess the semen characteristics of Jersey, Mithun and Siri breeds of bulls and to compare the suitability of Tris Egg yolk Citrate and commercial soya lecithin based BIOXcell extenders for cryopreservation of semen from these breeds of bulls. The mean semen volume was  $4.55 \pm 1.57$ ,  $4.86 \pm 1.33$  and  $4.83 \pm 1.72$  ml in Jersey, Mithun and Siri bulls, respectively. Mean mass activity of 3, mean initial motility of  $78.0 \pm 4.22$ ,  $80 \pm 4.47$  and  $78.33 \pm 4.08\%$ , mean sperm concentration of  $1416 \pm 484.19$ ,  $1530.91 \pm 517.41$ ,  $1122.83 \pm 293.68 \times 10^6$  and mean livability of  $69.20 \pm 6.81$ ,  $70.45 \pm 8.42$ ,  $76.67 \pm 8.17\%$  was found in Jersey, Mithun and Siri bulls, respectively. There is no significant difference in all above mentioned variables among the breeds. The post-thaw motility of semen cryopreserved in Tris Egg yolk Citrate extender was found to be  $45.50 \pm 4.38$ ,  $46.82 \pm 6.81$  and  $47.50 \pm 8.22\%$  in Jersey, Mithun and Siri bulls, respectively. Whereas in BIOXcell extender, it was  $45.50 \pm 5.99$ ,  $46.36 \pm 5.05$  and  $49.17 \pm 5.85\%$  in Jersey, Mithun and Siri bulls, respectively. Similarly, the plasma membrane integrity of thawed spermatozoa in Tris egg yolk extender was  $17.50 \pm 2.32$ ,  $21.00 \pm 3.12$  and  $21.58 \pm 3.83\%$  in Jersey, Mithun and Siri bulls, respectively. Whereas in BIOXcell extender, it was found to be  $20.45 \pm 2.86$ ,  $20.86 \pm 2.281$  and  $22.33 \pm 3.24\%$  in Jersey, Mithun and Siri bulls, respectively. No significant difference ( $p > 0.05$ ) was observed in post-thaw motility as well as in plasma membrane integrity between extenders and between breeds, and there was no significant effect ( $p > 0.05$ ) of breed x extender interaction. It can be concluded that the semen characteristics of Siri and Mithun bulls fall with the range established for other breeds of bulls. Furthermore, both Tris egg yolk and BIOXcell extenders are equally suitable for the cryopreservation of semen from these three breeds of Bulls.

**Key words:** Mithun bull, Semen characteristics, Semen cryopreservation, Siri bull

### INTRODUCTION

The advent of technology such as artificial insemination (AI) and semen cryo-preservation has vastly increased the utility and the contribution of the bulls with high genetic merits. For AI, production of quality semen is of primary importance. The major cause of variation in semen quality is the environment (Foote, 1978). Quality of semen is determined by a combination of factors operating at two levels; at the level of the bull, conditions such as nutrition and temperature are considered to exert important influence on bull performance and semen quality while at the level of the spermatozoa, environment relates to those conditions to which the spermatozoa are exposed after ejaculation and in the processes of cryopreservation.

The series of drastic changes in their physical and chemical environment during the process of cooling, freezing and thawing can cause damage to up to 50% of the spermatozoa (Watson, 2000). The primary site of cryo-injury to spermatozoa is in the plasma membrane (Parks and Graham, 1992), which are attributed to membrane alterations induced by phase transitions that occur when membranes are cooled (Hammerstedt *et al.*, 1990; Woelders, 1997; Watson, 2000; Medeiros *et al.*, 2002), mechanical stress on cell membranes due to osmotic stress and temperature changes during freezing and thawing (Curry and Watson, 1994; Holt, 2000; Morris *et al.*, 2007), increases in lipid peroxidation of the membrane induced by reactive oxygen species (ROS) (Alvarez and Storey, 1992; Meyers, 2005) and intracellular ice crystallization during cryopreservation (Mazur, 1984). The processes induce certain detrimental effects, in terms of sperm structure, biochemical and functional damage, resulting in a reduction of sperm motility, membrane integrity and fertilizing ability (Salamon and Maxwell, 2000). Therefore, the composition of extender and suitable cryoprotectants are important factors for successful semen cryopreservation (Hammerstedt *et al.*, 1990; Curry *et al.*, 1994).

Currently, egg yolk is an important constituent of extenders for cryopreservation of semen of domestic animals. This cryoprotective property of egg yolk is attributed to the phospholipids, cholesterol and low density

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lipoproteins in egg yolk (Pace and Graham, 1974; Watson, 1976). The most commonly used egg yolk in extenders for cryopreservation of sperm is from chicken because of its wide availability. However, three main disadvantages work against the use of egg yolk in semen extenders; the wide variability in their composition, the risk of microbiological contamination and disease transmission, and interference in the microscopic examination attributed to the greater viscosity and the presence of particulate debris in extenders (Vishwanath and Shannon (2000). This has led to the search for extenders free of ingredients of animal origin. Lecithin from soy beans has been used successfully for semen cryopreservation of bull (Thun *et al.*, 2002; Amirat *et al.*, 2005; Muiño *et al.*, 2007), ram (Forouzanfar *et al.*, 2010) and dog semen (Beccaglia *et al.*, 2009). BIOXcell (Gil *et al.*, 2003a,b; Hansen *et al.*, 2005; Stradaoli *et al.*, 2007; Celeghini *et al.*, 2008) has been used for the cryopreservation of bovine, caprine and ovine semen elsewhere.

Furthermore, semen from different breeds and species has different characteristics and physiology and therefore may respond differently to different cryopreservation methods including extenders. Moreover, there have been no studies on cryopreservation of semen of Siri and Mithun bulls. Thus, the objective of this study was to compare the effect of Tris egg yolk extender and commercial soya lecithin based extender (BIOXcell) on the freezability and post-thaw sperm quality of semen from these bulls.

## **MATERIALS AND METHODS**

### **Animal**

The experiment was conducted during the months of June to September, 2013. Bulls (n=2) of three breeds, namely Siri, Mithun and Jersey of 5-6 years of age maintained at the National Dairy Development Centre used for semen collection for AI were used for the study. The bulls were offered a daily diet consisting of 3kg concentrate mixture, 100 g of gram and ad libitum green grasses. The Semen laboratory is located at an altitude of around 2500 m above msl with an average ambient temperature of around 21<sup>0</sup>C.

### **Experimental Design and treatments**

The experiment was conducted with a Completely Randomized Block Design with breed as block and two animals in each block. Five ejaculates were collected from each bull of the 3 breeds and each ejaculate was divided into 2 aliquots, and assigned to two treatments. Treatment 1 and Treatment 2 consist of extending the semen with Tris-yolk citrate and commercial soya lecithin-BIOXcell extenders, respectively.

### **Semen Collection and evaluation**

Semen was collected once a week from each bull using artificial vagina. The collection tube containing the semen is transferred to a water bath maintained at 35-37<sup>0</sup>C and subjected to macroscopic and microscopic evaluation. The volume, colour and consistency were recorded. Mass activity was graded according to Roberts (1971). Initial progressive motility was recorded as an aggregate of the visual assessment by two experienced persons. Semen was stained with Eosin-Nigrosin stain to assess sperm viability and morphological abnormality. Sperm concentration was measured using a haemocytometer as described by Henery (1991).

### **Preparation of Extender and semen dilution**

The extenders were freshly prepared just before dilution. Tris-egg yolk extender was prepared by dissolving 3.025 g Tris buffer, 1.67 g citric acid and 1.25 g fructose in 50 ml of distilled water by stirring after which the volume was adjusted to 73 ml with distilled water. To this, 20 ml of sterilized egg yolk was added and the solution is then centrifuged and divided into two parts; Part A (50 ml) and Part B (43 ml). Part A was warmed to 37<sup>0</sup>C while to Part B, 7 ml Glycerol was added, thoroughly mixed by heating and stirring, and cooled to 5<sup>0</sup>C. BIOXcell<sup>TM</sup> extender was prepared by warming the BIOXcell solution to 34<sup>0</sup>C for 10 min and diluted 4 times the volume of the BIOXcell solution with sterile distilled water kept at 34<sup>0</sup>C. The extender was then maintained at 34<sup>0</sup>C. Semen samples showing normal characteristics of motility (>70%), morphology and concentration (>500x10<sup>6</sup>/ml) were divided into 2 equal aliquots and diluted to obtain spermatozoa concentration of 20 x 10<sup>6</sup> per straw. One aliquot was extended with Tris-citrate egg yolk citrate extender in two steps. First dilution was carried out using solution A at 37<sup>0</sup>C and gradually cooled from 37<sup>0</sup>C to 5<sup>0</sup>C over 120 min in a biological freezer. The second dilution was made when the semen was cooled to 5<sup>0</sup>C. Solution B maintained at 5<sup>0</sup>C was added. The

other aliquot was extended with BIOXcell extender in a single step within 10 min of collection. Then the diluted semen was kept at 5°C for about 4 h for glycerol equilibration.

### Semen freezing and thawing

After equilibration, the semen was loaded into 0.25 ml straw with the help of straw filling and sealing machine after which they were placed in a liquid nitrogen (LN) cooling tank to vapour cool to -85°C in 10 min. When the temperature reached to -85°C, the straws were plunged into LN and stored. After storage for a week in LN 10 semen straws were thawed at 37°C for 30-60 seconds and examined under microscope at x40 magnification for post-thaw motility.

### Assessment of membrane integrity

The combination of hypo osmotic swelling (HOS) test and Eosin Nigrosin (EN) staining method described by Moreno *et al.* (2011) was used to assess the membrane integrity of spermatozoa. For this, 0.1 ml of thawed semen was mixed with 1.0 ml hyposmotic solution (150 mOsm/mL) consisting of sodium citrate (1.47 g/ 100 mL) and fructose (2.7 g/ 100 mL), and incubated at 37°C for 30 min. After incubation, slides were prepared from each sample by placing 10 µl of semen on a glass slide followed by mixing with 10 µl of EN staining solution. The staining of sperm head and the swelling response of sperm tail irrespective of the types of tail coiling from type b to g (presence of a swollen area at the tip of the tail (b-d), a hairpin curvature of the tail (c-e), a shortened and thickened tail (f), or a swollen area that partly or completely enveloped the curved tail of the spermatozoon (d, e & g)) as described by Jeyendran *et al.* (1984) was determined. The type IV spermatozoa (head white (EN -ve) and Tail-swollen (HOS +ve)) as described by Zhu and Liu (2000) representing the spermatozoa with intact membrane in both the head and tail was recorded.

### Statistical analysis

Data were analyzed using the SAS 9.1 statistical software package and expressed as the mean and standard deviation. The GLM procedure was used to test the difference in the mean semen characteristics between breeds and post-thaw semen quality between different treatments at a significance level of P<0.05. Pearson's correlation coefficient test was used to investigate the correlation between initial motility and livability with post thaw motility and plasma membrane integrity.

## RESULTS

### Semen Characteristics of different breeds of Bulls

The semen characteristics of three different breeds of bulls, namely Jersey, Mithun and Siri were compared. The colour of semen was white and creamy with normal appearance and mean morphological abnormalities was 12.02±6.71% in all the bulls. The mean semen volume, initial motility, concentration and livability of the three breeds of bull are shown in Table 1. Mithun and Siri bulls had slightly higher volume of semen while Mithun had slightly higher initial motility and concentration, and Siri had slightly higher livability than Mithun and Jersey. However, there was no significant difference (p>0.05) in all above mentioned variables among the breeds.

Table 1. Semen Characteristics of different breeds of Bulls

Breed of Bull	Volume ( ml)	Initial Motility (%)	Concentration (x10 <sup>6</sup> )	Livability (%)
Jersey ( n=2)	4.55± 1.57 <sup>a</sup> (2.5-7.0)	78.0±4.22 <sup>b</sup> (70-80)	1416±484.19 <sup>c</sup> (840-2540)	69.20±6.81 <sup>d</sup> (55-77)
Mithun( n=2)	4.86±1.33 <sup>a</sup> (3.0-7.0)	80±4.47 <sup>b</sup> (70.0-90.0)	1530.91±517.41 <sup>c</sup> (1030-2680)	70.45±8.42 <sup>d</sup> (50-80)
Siri ( n=2)	4.83±1.72 <sup>a</sup> (3-7)	78.33±4.08 <sup>b</sup> (70-80)	1122.83±293.68 <sup>c</sup> (870-1667)	76.67±8.17 <sup>d</sup> (70-90)

Figures in main column with similar superscript are not significantly different at p>0.05.

**Post-thaw motility**

The same semen samples from above were frozen using two types of semen extenders, namely Tris Egg yolk citrate and a soy lecithin based commercial extender BIOXcell to assess the post thaw semen quality. The post thaw motility and plasma membrane integrity in the two extenders in different breeds are shown in Table 2. The aggregate mean post-thaw motility and plasma membrane integrity for all the semen samples from all the breeds were 46.57±5.99% and 20.93± 2.88% respectively. While these two variables of semen cryopreserved in both the extenders were found to be slightly higher in Siri bulls than Mithun and Jersey, there was no significant difference (p>0.05) between extenders and between breeds, and there was no significant effect (p>0.05) of breed x extender interaction.

Table 2. Post-thaw motility and plasma membrane integrity of semen of different breeds of bulls cryopreserved in Tris Egg yolk Citrate and BIOXcell Extenders

	Post-thaw motility (%)		Intact membrane (%)	
	Tris Egg yolk Citrate	BIOXcell	Tris Egg yolk Citrate	BIOXcell
Jersey	45.50 ± 4.38 <sup>a</sup>	45.50 ± 5.99 <sup>a</sup>	20.15 ± 2.32 <sup>b</sup>	20.45 ± 2.86 <sup>b</sup>
Mithun	46.82 ± 6.81 <sup>a</sup>	46.36 ± 5.05 <sup>a</sup>	21.00 ± 3.12 <sup>b</sup>	20.86 ± 2.28 <sup>b</sup>
Siri	47.50 ± 8.22 <sup>a</sup>	49.17 ± 5.85 <sup>a</sup>	21.58 ± 3.83 <sup>b</sup>	22.33 ± 3.24 <sup>b</sup>

Figures in main column with similar superscript show no significant difference at p>0.05.

There was a very high correlation between post-thaw motility and plasma membrane integrity (r=0.93), p<0.01. Although positive correlation was found between post-thaw motility and plasma membrane integrity with initial motility and livability, it was not significant (p>0.05).

**DISCUSSION**

*Bos taurus* and *Bos indicus* have several markedly different physiological and anatomical features (Felius, 1985) which could result in difference in sperm characteristics. Generally, *Bos indicus* bulls have a higher sperm concentration and higher sperm morphologic defects than *Bos taurus* (Brito *et al.*, 2002). However, such difference is not evident in this experiment. The colour, consistency, volume, mass activity, initial motility, livability and percent of morphological abnormalities fall within the range described by Roberts (1971) and Hafez (1993). In the case of Mithun breed, while semen volume was similar to those reported by Mondal *et al.* (2010), it was higher than that reported by Bhattacharya *et al.* (2005). Sperm concentration was also found to be much higher than those reported by both Mondal *et al.* (2010) and Bhattacharya *et al.* (2005). This discrepancy could be due to the collection method employed. In regards to the semen characteristics of Siri bulls, to the knowledge of the authors, this is the first published information which should be further investigated using larger sample size and extending over different seasons.

The composition of extender and suitable cryoprotectants are important factors for successful semen cryopreservation (Hammerstedt *et al.*, 1990; Curry *et al.*, 1994). In this experiment, two extenders, namely, Tris egg yolk citrate and BIOXcell (a commercial soybean based extender ) were compared for the cryopreservation of semen from Jersey, Mithun Bulls and Siri bulls. Viviana *et al.* (2003) have reported higher sperm motility and significantly higher non return rate with Soya Lecithin based extender as compared with TRIS-EY extender. On the contrary, Thun *et al.* (2002) found Tris-egg yolk extender to produce the best semen quality and field fertility than Biociphos. Although, BIOXcell is fortified with antioxidants, it appears that it has no advantage over the egg yolk based extender as the natural constituent of egg yolk such as phospholipids, cholesterol and low density lipoproteins in egg yolk are equally effective in protecting the spermatozoa against oxidative stress (Pace and Graham, 1974; Watson, 1976). In this study, no significant differences were found between Tris Egg yolk Citrate and BIOXcell extenders and no breed x extender interaction effects was also observed. This is in agreement with Hinsch *et al.* (1997) who found no significant differences in motility, viability, and acrosomal status of spermatozoa between soya based extenders and egg yolk-containing extenders. However, the risk of microbiological contamination and disease transmission in using egg yolk in Tris-egg yolk coupled with the

clarity of diluted and frozen semen in BIOXcell during examination, and convenience of preparation and handling may increasingly favour the use of BIOXcell in the future.

Post-thaw motility of spermatozoa is the most widely used parameter for judging the quality of frozen semen and the potential fertility of the semen. Good progressive motility of spermatozoa is an indicator of both unimpaired metabolism and intactness of membranes (Johnson *et al.*, 2000). An intact and functionally active membrane is essential for the spermatozoon to sustain metabolism, undergo capacitation and acrosome reaction and, further, attach to and penetrate the oocyte zona pellucida (Jeyendran *et al.*, 1984). Positive correlation between membrane integrity assessed by fluorometric methods and fertility has been reported by Januskauskas *et al.* (2003). Moreover, Moreno *et al.* (2011) reported significant correlation between sperm with type IV and field fertility elsewhere. The HOS-EN which is a combination of the HOS test and the EN staining method described by Zhu and Liu (2000) was used for this study to categorize spermatozoa into 4 types of which type IV (White head with swollen tail) representing the viable spermatozoa with intact membrane (Moreno *et al.*, 2011). Obtaining lower percent type IV than the post thaw motility in the present study is consistent with the finding of Zhu and Liu (2000). This could be attributed to the retention of motility by the spermatozoa with slight membrane damage. The high correlation between post-thaw motility and percent type IV spermatozoa and between membrane integrity (Januskauskas *et al.*, 2003) and percent type IV spermatozoa (Moreno *et al.*, 2011) and field fertility indicate the usefulness of post-thaw motility of spermatozoa as a measure of the potential fertility of the frozen semen.

The semen characteristics of Siri and Mithun bulls fall with the range established for other breeds of bulls. The semen characteristics of Siri bulls studied in this study is the first published information and should be validated with larger number of ejaculates. Since there was no significant differences between the extenders in the post-thaw motility and membrane integrity, both Tris egg yolk and BIOXcell extenders could be used based on affordability. However, the conception rates after AI and fertility after long term storage in the two extenders are not known and need to be studied. There is also need to investigate and establish the optimal amount rate of glycerol addition for the cryopreservation of semen of Mithun and Siri breeds of bulls.

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