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Short Research Article

Research on Freezability of Spermatozoa by Use of Various Concentrations of Glycerol in Mithun (*Bos frontalis*) Semen Cryopreservation

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Abstract

A study was conducted to investigate the various glycerol concentrations on freezability of mithun semen. The present study was conducted in semen production laboratory, ICAR-NRC on Mithun, Medziphema, Nagaland, India. A total of 20 ejaculates were collected from apparently healthy mithun bulls (n=6) by trans-rectal massage method. These ejaculates were extended with tris–egg yolk-citrate-glycerol diluent (TECG) and 5, 6 and 7% (v/v) glycerol, respectively were used as a cryoprotectant. Extended semen was equilibrated, cryopreserved and stored in liquid nitrogen with standard protocols. Semen quality parameters (SQPs) such as percentage of forward progressive motility, viability (Eosin Nigrosin staining), acrosomal integrity (Giemsa staining) and total morphological abnormality (Eosin Nigrosin staining) were estimated with standard procedure at different stage of preservation and with different concentration of the glycerol. The result revealed that these semen quality parameters were differed non-significantly among the three experimental groups at immediately after final dilution (Stage: 1) and differed significantly (p<0.05) at 4 hrs of equilibration (Stage: 2) and at post thawing stage (Stage: 3) of semen cryopreservation. Further, 5% glycerol treated group has shown significantly (p<0.05) higher values of motility and live with acrosomal integrity and lower value of total morphological abnormality than in 6% or 7% glycerol treated groups. Based on the result, it was concluded that 5% glycerol was best for the TECG diluent for preserving mithun semen in liquid nitrogen.

1. Introduction

The production and reproduction performance of mithun cows can be increased by breeding females with semen of the bulls of superior genetic merit. But due to non-availability of a system to replace bulls at regular interval to maintain the genetic quality, there is a need to focus on some alternative way for breeding in mithun species. This can be achieved easily through the artificial insemination (AI) with the semen of proven mithun bulls or by multiple ovulation and embryo transfer (MOET). AI has become one of the most important techniques ever devised for the genetic improvement of farm animals like cattle, buffalo etc. Fresh bovine semen can be preserved successfully at 5 °C for 6 days using tris-egg yolk based extender without glycerol (Verberckmoes et al., 2004) and bull spermatozoa can be preserved by using 4% to 8% glycerol (Fennessy et al., 1990). However, there is very limited information in mithun. In this context, it is very essential to standardize a suitable semen preservation protocol in mithun in order to adopt AI. Mithun semen preserved at refrigeration temperature using tris-egg yolk based extender or cryopreserved in liquid nitrogen using tris-egg yolk-glycerol diluents, coupled with artificial insemination would be a viable option for propagation and conservation of quality germplasm. Considering the importance of it, the present research programme was taken not only for conservation but also for propagation of mithun.

2. Materials and Methods

The present study was conducted in semen production laboratory, ICAR-NRC on Mithun, Medziphema, Nagaland, India. A total number of 20 semen samples were collected from the adult healthy mithun bulls (n=6) and the semen

samples exhibiting a mass activity score of 3 or more were only processed further for freezing. After collection, the samples were partially diluted by adding 1 ml pre-warmed (37 °C) TECG diluent (Tris-hydroxymethyl aminomethane: 24.3 mg/ml, sodium citrate: 13.4 mg ml^{-1}, fructose: 10 mg ml^{-1} and egg yolk: 20%, Penicillium G sodium salt: 1000 IU ml^{-1} and dihydrostreptomycin: 1 mg ml^{-1}). The partially diluted samples were maintained at 37 °C for further processing. Final dilution of the samples was done with pre-warmed (37 °C) tris–egg yolk diluent in such a way that after final dilution and glycerol addition each ml of sample contained 60×10^6 sperm. Following the final dilution, the samples were equilibrated at 4 °C for 4 hrs and glycerol was added into the samples in a single dose. After equilibration, the samples were loaded into pre-cooled (4 °C) 0.50 ml straws leaving a small air space at the end and heat sealed. The straws were then frozen in liquid nitrogen vapour (5 cm above the liquid nitrogen level) for 10 min and subsequently plunged into liquid nitrogen for storage.

Semen volume, mass activity in the fresh samples and sperm concentration in partially diluted samples were determined. The SQPs viz, forward progressive motility (Tomar, 1997), livability (Eosin Nigrosin Staining; Tomar, 1997), acrosomal integrity (Giemsa Staining; Watson, 1975) and morphological abnormalities ((Eosin Nigrosin Staining; Tomar, 1997) were determined with the standard protocol at stage 1, 2 and 3 of cryopreservation. The frozen samples were evaluated after minimum 7 days of storage in liquid nitrogen. Statistical analyses were performed using the SPSS 18.0.0 software package (SPSS/IBM, Chicago, IL, USA). Arcsine transformation was done for the results and expressed in percentages. In this experiment, variations in sperm quality parameters among different experimental groups at each stage of cryopreservation were analyzed by one way of analysis of variance (ANOVA). A probability value of less than 0.05 ($p<0.05$) was considered as a significant.

3. Results and Discussion

Semen samples were collected from mithun bulls and quality of the samples were evaluated at different stages of cryopreservation viz. stage 1, 2 and 3 (Table 1). The result of the present study revealed that these SQPs were non-significantly differed between the different glycerol concentrations at stage 1. At stage 2 and 3, these SQPs were differed significantly ($p<0.05$) and progressive motile, live and acrosomally intact sperms were significantly ($p<0.05$) higher in 5% than 6 and 7% glycerol treated group. Similarly, total morphological abnormality was significantly ($p<0.05$) differed among the experimental groups and was lower in 5% than in 6 and 7% glycerol treated group both at stage 2 and 3 of cryopreservation in mithun.

The cryoprotective or anti-freeze agents are included usually into the semen extender for cryopreservation to reduce the cryo-stress and minimize the ice crystal formation in sperm. Glycerol is considered as the standard cryoprotective agents in most of the mammals to preserve the semen in liquid nitrogen and dosage is depend upon the rate of freezing and method of addition. In the present study, we found that 5% glycerol is suitable and optimum concentration to preserve the mithun sperm in liquid nitrogen. This present study indicated that the concentration of glycerol in freezing extender affected the quality and longevity of cryopreserved semen in dose dependent manner.

In the present study, inclusion of 5% glycerol in the tris egg yolk treated extender had significantly improved the percentage of progressive motile, live and acrosome intacted sperm and minimised sperm with morphological abnormality both at stage 2 or 3, whereas the lowest sperm quality was observed in the extender containing 6 and 7% glycerol. The appropriate or correct amount of glycerol (5 ml) enhancing or

Table 1: Variations (Mean±S.E.) in sperm qualitative parameters at different stages of cryopreservation with different concentration of glycerol in tris citrate-egg yolk-glycerol diluent

Physico morphological attributes	Glycerol concentration (%)		
	5	6	7
After final dilution (Stage: 1)			
Progressive motility (%)	66.50±2.10	68.00±2.00	67.00±2.20
Live sperm with intact acrosome (%)	69.80±2.30	70.30±1.80	70.20±2.10
Total morphological abnormalities (%)	5.60±0.50	6.60±0.40	6.60±0.50
After 4 hrs of equilibration (Stage: 2)			
Progressive motility (%)	50.00 ± 2.70^{a}	46.00 ± 1.70^{a}	39.50 ± 2.20^{b}
Live sperm with intact acrosome (%)	54.00 ± 2.60^{a}	49.00 ± 1.70^{ab}	43.20 ± 2.20^{b}
Total morphological abnormalities (%)	13.60 ± 0.80^{a}	17.80 ± 1.10^{b}	19.40 ± 0.50^{b}
After freeze-thawing (Stage: 3)			
Progressive motility (%)	36.00 ± 2.10^{a}	27.5 ± 1.10^{b}	13.50 ± 1.30^{c}
Live sperm with intact acrosome (%)	39.50 ± 2.00^{a}	30.50 ± 1.70^{b}	16.60 ± 1.40^{c}
Total morphological abnormalities (%)	26.10 ± 1.10^{a}	35.60 ± 0.90^{b}	38.50 ± 1.00^{b}

Means with different superscripts in rows varied significantly ($p<0.05$)

maintain the membrane fluidity by rearranging the membrane phospholipid and protein composition, thus increasing the motility, viability and acrosomal intactness of spermatozoa during cryopreservation (Holt, 2000). The range of 4 to 8% glycerol is generally used in freezing extender in bovine species (Fennessy et al., 1990) while 6 and 7% glycerol are frequently reported for bovine species (Perumal et al., 2011). However, we found that the 6 and 7% glycerol protocol poorly protected the spermatozoa against cryoinjury during freezing and thawing procedure in mithun species. In this study, sperm motility, live and acrosomal integrity were reduced after thawing regardless of the final glycerol concentration tested. In addition, all frozen thawed groups showed a decrease in values of these SQPs when compared with samples at immediately after final dilution.

Improvement in post-thaw motility as a result of increasing glycerol concentration upto optimum has also been demonstrated in other species (Zambell et al., 2002; Rasul et al., 2007; Abbas and Andrabi, 2002; Hoffmann et al., 2011; Buhr et al., 2001; Fernandez-Santos et al., 2006; Okano et al., 2006). Moreover, there was a negative correlation between the percentage of morphologically abnormal spermatozoa in fresh samples and post thaw sperm motility was found in the 6% and 7% glycerol groups. Thus, it indicated that the beneficial effect of glycerol on post-thaw sperm motility is more pronounced on sperm cells presenting normal morphology. From this study, we can speculate that glycerol may exert a dose-dependent beneficial effect on frozen-thawed mithun sperm motility up to a concentration of 5%. It is important to note that the use of glycerol at a final concentration of 6 or 7% has shown to impair post-thaw motility mithun spermatozoa and this may be probably due to the toxic effects of glycerol, post-thaw motility may return to decrease if concentrations above 5% are used.

Sperm plasma membrane is considered as one of the main sites susceptible to cryodamage (Hammerstedt et al., 1990). Lower concentration of glycerol (5%) has protected plasma membrane from the cryo-damage and it was positively correlated with the percentage of morphologically normal sperm in immediately after final diluted samples. In contrast, this relationship was not found in the 6% and 7% glycerol groups. Similar to the plasma membrane, the acrosome membrane is also highly susceptible to cryodamage during freezing/thawing procedures (Holt, 2000; Bailey et al., 2000). In mithun species, reduction on acrosomal integrity following sperm cryopreservation is a common finding as in other species (Zambell et al., 2002; Rasul et al., 2007; Abbas and Andrabi, 2002; Hoffmann et al., 2011; Buhr et al., 2001; Fernandez-Santos et al., 2006; Okano et al., 2006).

In the present study, optimum (5%) in final glycerol concentration significantly improved the overall percentage of intact acrosomal membranes. The percentage of viable spermatozoa presenting reacted acrosomes showed to rise as glycerol concentration increased, possibly as a consequence of cryo-capacitation like changes in spermatozoa. In other species, cooling and freezing/thawing procedures have already shown to induce membrane changes resembling the ones associated with capacitation, probably ascribed to increased membrane permeability and calcium influx (Bailey et al., 2002). Additionally, 5% glycerol resulted in higher percentage of intact acrosomes in dead cells compared with 6% and 7% glycerol. Thus, a detrimental effect on acrosome integrity due to high glycerol concentrations seems to exist in mithun spermatozoa as already reported in other species (Zambell et al., 2002; Rasul et al., 2007; Abbas and Andrabi, 2002; Hoffmann et al., 2011; Buhr et al., 2001; Fernandez-Santos et al., 2006; Okano et al., 2006).

Abbas and Andrabi (2002) reported that wide range of glycerol concentrations (2−12%) for cryopreservation of buffalo semen that 6 or 7% glycerol provided successful cryopreservation. Similarly, Rasul et al. (2007) compared different concentrations of glycerol for freezability of buffalo semen and they observed similar results that obtained by Abbas and Andrabi (2002). Moreover, Rasul et al. (2007) determined that lower doses of glycerol (0 and 3%) adversely affected freezability of buffalo semen. Similarly, Buhr et al. (2001) compared different doses of glycerol (0, 2, 4 and 8%) for cryopreservation of boar semen. They found that 2 and 4% glycerol concentrations provided higher post-thaw motility and acrosomal integrity. In equine, Hoffmann et al. (2011) reported that different concentrations of glycerol (1−4%) for cryopreservation of equine semen extended with skim milk-based extender did not affect post-thaw semen quality.

4. Conclusion

5% glycerol was best for the TECG diluent for preserving mithun semen in liquid nitrogen.

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