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Cryopreservation of mithun semen: comparative study of conventional vs controlled freezing

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Mithun (*Bos frontalis*), a semi-wild, free-ranging bovine species present in Arunachal Pradesh, Nagaland, Manipur and Mizoram of India, is primarily used as a meat animal and it plays an important role in the socio-economic life of the tribal population of NEH region. Recent statistics publicized a decreasing trend in mithun population as per quinquennial All India Livestock Census (2012). In these circumstances, there is an utmost necessity for genetic resources conservation programs to overcome this crisis. Artificial insemination (AI) is the first generation biotechnological technique that has contributed intensely in genetic improvement. The advantages of AI could be maximized if spermatozoa were cryopreserved, since it is the best alternative for long range distribution of high quality genetic material (Roca *et al.* 2006)

However, the success of cryopreservation greatly depends on cryopreservation protocol (Johnson *et al.* 2000). Freezing and thawing phases of cryopreservation process induces structural and/or biochemical damage in boar spermatozoa resulting in a drastic reduction in sperm quality (Sancho *et al.* 2007). Sperm damages emanated during cryopreservation procedures could be mitigated to some extent through improvement in freezing strategies (Roca *et al*. 2006). The cooling rate prior to spermatozoan cryopreservation seems to be an important step to minimise the deleterious effects of injury during the freezing process (Kumar *et al.* 2003).

Different approaches for cooling semen commonly used are: conventional i.e., in different containers with liquid nitrogen maintaining temperature manually, and controlled method in which semen straws are maintained and cooled via automatic process in biofreezer. Controlled rate of freezing method yielded superior post thaw sperm quality as compared to conventional freezing method (Kaeokat *et al.* 2008). Hence, the aim of our study was to assess the effect of conventional and controlled freezing method on sperm parameters to develop a suitable freezing protocol for cryopreservation of mithun semen.

The experiment was carried out during March 2014 to September 2015. All experimental procedures involving animals were approved by the Institutional Animal Ethics Committee (IAEC). Adult fertile mithun bulls (6), 3–4 year-old (body weights 300–400kg) maintained at Mithun farm of the institute were used in this study. Each bull was maintained under uniform feeding and housing conditions.

Semen was collected once in a week by rectal message method in a sterilised graduated tube kept in thermoflask. Pooled ejaculates (10) from six (6) healthy fertile bulls were collected for the study.

Semen was diluted with tris egg yolk citrate glycerol (TEYG) extender. Briefly, 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 and the volume was adjusted to 73 ml with distilled water. To this, sterilized mixture of egg-yolk and glycerol was added @ 20% and 7%, respectively. The freshly collected semen was diluted with TEYG extender to make the final sperm concentration of 30 million/ml. Before freezing, the fresh semen samples were analyzed for initial progressive motility, live sperm count, acrosomal integrity and hypo-osmotic sperm swelling test (HOST).

The extended semen were packed and sealed into 0.5 ml straw and cooled up to 5°C by keeping them in cold handling cabinet for 90 min. Once the temperature reached to 5°C, the semen straws were equilibrated for 4 h. The straws were divided in 2 groups for the freezing viz. one half was frozen using controlled-rate freezing system (Biofreezer, IMV technologies, France) and the other half in the conventional freezing system. In control freezing system, the straws were placed evenly on racks inside the biofreezer. The biofreezer equipment was programmed with the following rate: 5°C/min from 5 to –10°C, 20°C/min from –10 to –100°C and 40°C/min. from –100 to –140°C. Conventional freezing was performed using an isothermal box filled with liquid nitrogen (N_2). Semen straws were placed horizontally 4 cm above the liquid N_2 level for 10–12 min. After freezing, the

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straws from both the freezing systems were immersed into liquid nitrogen for storage.

Thawing was done by putting the straw in water at 35°C for 20 sec. Each sample was evaluated for motility, liveability, acrosomal integrity and HOST test and abnormalities at 2 different stages of processing. Sperm motility was assessed in a microscope equipped with thermal stage and phase contrast optics (Nikon, Eclipse, 80i, Japan) at a magnification of 40×. The sperm motility was recorded from 0 to 100 based on percentage of progressively motile spermatozoa. The live sperm count was determined using eosin-nigrosin staining technique. Acrosomal integrity was studied using Giemsa staining (Watson 1975). HOST was performed as per the method described by Jayendran *et al.* (1984).

The different physico-morphological attributes of fresh as well as frozen semen with their mean values and standard errors are shown in Table 1. Significant (P<0.05) differences in motility, liveability, acrosomal integrity, HOST and abnormalities were recorded between conventional and controlled method (Table 2). Acrosomal integrity in fresh and frozen sperm is presented in Table 3.

The colour of the semen varied from white to milky white to yellowish white which comes in normal colour range for the mithun. Similar observation was also reported by Karunakaran *et al.* (2007). While semen volume was similar to those reported by Bhattacharya *et al.* (2005), it was lower than that reported by Mondal *et al.* (2010). Sperm concentration was also found to be almost similar to those reported by Mondal *et al.* (2010).

Individual motility of spermatozoa in fresh semen ranged between 76–84% with a mean of 81.44±2.07 which was supported by the studies of Karunakaran *et al.* (2007). Though the results of present study revealed slight decrease in per cent motility of spermatozoa after controlled freezing, yet the difference was nonsignificant.

The mean live sperm count in fresh semen was 84.65± 2.17 which ranged from 78–89%. In mithun sperm viability (%) was reported as 80.6±4.1 (Karunakaran *et al.* 2007), 80.7±2.2 (Bhattacharyya *et al.* 2009) and 98±9 (Mondal *et al.* 2010). The difference in liveability (%) of sperms between conventional and controlled freezing condition was found to be insignificant. The mean intact acrosome (%) in fresh semen was 88.68±2.25 ranging from 81–96% which was slightly lower than reported by Mondal *et al.* (2013). However, the difference in acrosomal integrity was found to be insignificant between conventional and controlled freezing.

In the present study, the acrosome status was categorised as intact, damaged and completely lost. The mean intact acrosome was significantly decreased in frozen semen (P<0.05). This observation is in agreement with the studies by Mondal *et al.* (2013). The hypo-osmotic swollen spermatozoa ranged from 82–88% having a mean of 86.66±2.25. A significantly (P<0.05) higher hypo-osmotic swollen spermatozoa was observed in conventionally frozen semen than in semen after controlled freezing. The results of our study showed significant influence of semen freezing method on plasma membrane integrity which is in agreement with Salazar *et al.* (2011) and in contradiction with the works done by Maziero *et al.* (2012) and Kumar *et al.* (2009). This difference may be due to different semen processing protocols.

The total abnormality in spermatozoa ranged from 6–11% with a mean of 7.15±1.13, though the difference in sperm abnormalities between conventional and controlled freezing was insignificant (P<0.05). In the present study,

Table 1. Evaluation of seminal parameters of mithun semen (n=10)

Physico-morphological attributes	Value
Volume (ml)	1.91±0.78
Colour	Milky to yellowish white
Concentration (×10^6/ml)	623.04±7.59
Mass activity (0-5 scale)	3.18±0.70
Individual motility (%)	81.44±2.07
Liveability (%)	84.65±2.17
Acrosomal integrity (%)	88.68±2.25
Total sperm abnormality (%)	7.15±1.13
Plasma membrane integrity (%)	86.66±2.25
Nuclear integrity of spermatozoa (%)	86.72±2.22

Table 3. Acrosome integrity of mithun semen after different freezing methods

Type of semen	Intact acrosome (IA)	Damaged acrosome (DA)	Completely lost acrosome (LA)
Fresh semen	65.44±1.89^a	23.88±1.92^a	10.66±1.53^a
Post-thaw semen (conventional freezing)	45.60±1.64^b	29.88±2.25^a	24.40±2.68^b

Different superscripts differ significantly.

Table 2. Seminal parameters of mithun semen after different freezing methods

Type of freezing	Motility (%)	Liveability (%)	Acrosome integrity (%I)	HOST (%)	Abnormalities (%)
Fresh semen	81.44±2.07^a	84.65±2.17^a	88.68±2.25^a	86.66±2.25^a	7.15±1.13^a
Frozen semen (Conventional freezing)	41.33±1.60^b	53.33±1.28^b	56.83±1.40^b	56.88±1.45^b	18.0±0.73^b
Frozen semen (Controlled freezing)	35.83±1.57^b	46.83±1.13^b	49.50±1.87^c	43.77±1.18^c	16.16±1.07^b

Different superscripts differ significantly (P<0.05).

percentage of total sperm abnormality was higher than earlier reports by Mondal *et al.* (2010) and Karunakaran *et al.* (2007). In our experiment, it was observed that the results of sperm parameter using both the conventional and controlled freezing method yielded significantly different results, where conventional freezing method proved to be beneficial. This finding demonstrates that conventional freezing method is simple and inexpensive and reduces the economical costs of mithun spermatozoa cryopreservation without causing significant losses of semen quality after thawing. This finding was in agreement with the works done by Clulow *et al.* (2008) and Maziero *et al.* (2012).

In conclusion, the results indicated that conventional freezing had significant effect on spermatozoa survivability and acrosomal integrity during post-thaw incubation, compared to controlled freezing. Further research efforts are needed to comparatively assess the freezing process of semen by both conventional and controlled cooling rate cryopreservation protocols considering differential rate of cooling rate.

SUMMARY

In this present study, 2 different semen freezing techniques (conventional and controlled freezing) for cryopreservation of mithun semen were compared with regard to post-thaw sperm characteristics. Pooled ejaculates (10) of mithun semen were utilized for the present study. Each ejaculate was aliquoted into 2 and extended with tris egg yolk citrate glycerol (TEYG) extender and freezing was done with conventional method as well as through programmed freezer using 0.5 ml straw. Semen samples were evaluated for sperm motility, liveability, acrosome integrity, plasma-membrane integrity and sperm abnormalities both in fresh and frozen semen. Though there was nonsignificant difference in post-thaw sperm motility and liveability; however, acrosome and plasma-membrane integrity were significantly higher in conventional freezing method as compared to that in controlled freezing method.

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