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Effect of Different Freezing Rates on Post-Thaw Semen Quality in Yak

B. K. D. Borah[1], B. C. Deka[2], R. K. Biswas[2], S. Deori[3*], P. Chakravarty[4], K. Ahmed[2] and S. Sinha[2]

[1]Krishi Vigyan Kendra, Namsai - 792103 (Arunachal Pradesh), India.
[2]College of Veterinary Science, Guwahati – 781022 (Assam), India.
[3]ICAR Research Complex for NEH Region, Umiam-793103 (Meghalaya), India.
[4]ICAR-National Research Centre on Yak, Dirang – 790101 (Arunachal Pradesh), India.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

A study was conducted on yak semen to evaluate the effect of different freezing rates on the quality of frozen semen. The semen was frozen following three freezing rates *viz.,* Freezing rate I (@ 5ºC/minute from +4^{o}C to -10^{o}C, @ 40ºC/minute from -10ºC to -100ºC and @ 20ºC/minute from -100ºC to -140ºC), II (@ 4ºC/minute from +4ºC to -12ºC, @ 40ºC/minute from -12ºC to -40ºC and @ 50ºC/minute from -40ºC to -140ºC and III (@ 5^{o}C/minute from +4ºC to -10ºC, @50ºC/minute from -10ºC to -100ºC and @ 20ºC/minute from -100^{o}C to -140ºC). The sperm motility, live sperm, HOST-reacted sperm, total incidence of acrosomal changes and extracellular release of Alanine aminotransferase and Aspartate aminotransferase of yak semen extended in Tris extender differed significantly (P <0.01) between stages of processing and freezing but did not differ significantly between freezing rates and due to stage × freezing rate interaction. Analysis of variance revealed that in frozen semen, a freezing rate significantly (P<0.05) influenced the percentage of live sperm with Mitochondrial Membrane Potential (MMP) but did not significantly influence the percentage of

**Corresponding author: E-mail: sourabhd1@rediffmail.com;*

sperm with DNA damage. The percentage of live sperm with MMP in frozen semen was significantly higher for freezing rate III than for freezing rate II but the difference between freezing rates I and III was not significant.

Keywords: Freezing rate; frozen semen; quality; yak.

1. INTRODUCTION

Yak (*Poephagus grunniens*) is an important genetic resource of the high altitude. They are socio-economically associated with the life of the highlanders and provide all the basic amenities to them like food in the form of meat and milk, clothing and tents from hide and fibers and the much needed transportation in the snow capped mountain regions. Yaks are reared under the pastoral system and migrate around the year in search of better pasture. Inbreeding is one of the major limitations in yak husbandry due to the geographical isolation of yak herds and continuous use of same breeding bull. Artificial insemination from semen of unrelated sires can be tried for overcoming inbreeding in yaks. Yak semen can be successfully cryopreserved in Tris-Egg Yolk- Glycerol extender [1]. This essentially not only preserves the genetic resources, but also supports the transportation of genetic materials in remote areas. Transporting the frozen semen is easier and economical comparing with moving the bulls for service. Semen cryopreservation is a complex process and depends on several factors like individual variations, the interaction between cryoprotectants and the extender time, period of cooling and equilibration and the speed of freezing and thawing [2]. During the freezing process, the freezing rate is one the important factor that controls the survivability of the cells. Every cell type has an optimal freezing rate for survival after the freezing process [3]. Watson [4] reported that the cell dehydration process through slow freezing is beneficial for survivability of cells, whereas rapid freezing rates are more likely to cause cell death. During the semen cryopreservation, the freezing rate affects the post-thaw quality of spermatozoa [5]. Several workers studied different freezing rates to improve the quality of frozen semen of different animals [5,6,7]. However, such reports for yak semen are not available. Hence, the present study was designed with an objective to evaluate the quality of frozen yak semen applying different freezing rates.

2. MATERIALS AND METHODS

The experiment was conducted on four healthy yak bulls aged between 3 and 4½ years. The bulls were maintained under uniform intensive management conditions at the institute farm of ICAR-National Research Centre on Yak, Dirang in the state of Arunachal Pradesh, India, at an altitude of above 2730 msl. Artificial vagina method was applied to collect the semen at weekly intervals. A total of 6 ejaculates per bull were collected. Immediately after collection, the collection tube containing semen was placed in a water bath maintained at 35°C. Ejaculate volume was recorded directly from the glass graduated semen collection tube and expressed in milliliter. Mass activity was estimated immediately after collection of semen. A drop of semen was placed on a prewarmed (37°C) glass slide and examined under low power objective at a magnification of 100X without cover glass. The scoring was done on the basis of wave pattern (0 to 4+). For initial sperm motility, a drop of diluted semen was placed on a prewarmed glass slide (37°C) and a cover glass was placed on it. Then it was examined under a phase contrast microscope at a magnification of 400X. Initial sperm motility was recorded from 0 to 100 based on the percentage of progressively motile sperm. The ejaculates having minimum volume 1.00ml, mass activity 3+ and initial sperm motility 70 percent were processed further for freezing.

Twenty four ejaculates were frozen using a programmable freezer (Mini-Digitcool, IMV Technologies) to study the effect of three freezing rates on quality of frozen semen by split sample technique. Semen was extended using a single fraction of Tris-egg yolk-fructose extender containing glycerol 5 percent and supplemented with taurine 50 mM, cooled @ 1°C/3 minutes and equilibrated for 4 hours. The filled straws were frozen following three freezing rates *viz.,* @ 5°C/minutes, from 4°C to -10°C, @40°C/minutes from -10°C to -100°C and @ 20°C/minutes from -100°C to -140°C (Freezing rate I), @ 4°C/minutes from 4°C to -12°C, @ 40°C/minutes from -12°C to -40°C and @ 50°C/minutes from -40°C to -140°C (Freezing rate II) and @ 5°C/minutes from 4°C to -10°C, @ 50°C/minutes -10°C to -100°C and @ 20°C/minutes from -100°C to -140°C (Freezing rate III).

The semen samples were evaluated for sperm motility, live sperm percent, plasma membrane

integrity by hypo-osmotic swelling test (HOST), acrosomal changes by Giemsa staining, and extracellular release of Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) both after equilibration and freezing. Mitochondrial Membrane Potential (MMP) and sperm DNA damage were evaluated under the fluorescence microscope only after cryopreservation. Data analyses were performed using the Statistical System software package (SAS, Cary, NC, USA, 2010). Data on different parameters were analyzed using a two-factorial analysis of variance (ANOVA) (GLM procedure).

3. RESULTS AND DISCUSSION

The mean percent sperm motility, live and HOST-reacted sperms, acrosomal change incidences, extracellular release of ALT (IU/l) and AST (IU/l) were 71.00 ± 2.14, 77.67 ± 2.39, 69.87 ± 2.13, 11.67 ± 0.26, 11.07 ± 0.43 and 11.07 ± 0.43 respectively after equilibration. The mean post-thaw sperm motility in semen samples that were cooled from +4°C to -140°C using three freezing rates *viz.,* Freezing rates I, II and III was 56.67 ± 2.32, 57.33 ± 2.88 and 60.00 ± 3.16 percent respectively. The corresponding values for live sperm were 62.67 ± 1.87, 62.40 ± 2.32 and 65.53 ± 2.52 percent, for HOST-reacted sperm were 55.20 ± 1.67, 56.33 ±1.97 and 58.33 ± 2.54 percent, for total incidence of acrosomal changes were 12.27 ± 0.37, 11.73 ± 0.43 and 11.00 ± 0.50 percent, for extracellular release of ALT (IU/l) were 11.69 ± 0.61, 11.11 ± 0.45 and 10.41 ± 1.06 and for AST (IU/l) were 11.69 ± 0.61, 11.11 ± 0.45 and 10.41 ± 1.06 respectively. The sperm motility, live sperm, HOST-reacted sperm, total incidence of acrosomal changes and extracellular release of ALT and AST of yak semen extended in Tris extender differed significantly ($P<0.01$) between stages of processing and freezing but did not differ significantly between freezing rates and due to stage X freezing rate interaction. The critical difference test revealed that sperm motility, live sperm, HOST-reacted sperm and extracellular release of ALT and AST dropped significantly ($P<0.05$) and total incidence of acrosomal changes, irrespective of freezing rates, increased significantly ($P<0.05$) at each stage of processing and freezing of semen.

The mean incidence of post-thaw MMP and DNA damage in yak semen samples using freezing rates are presented in Table 1.

Analysis of variance revealed that in frozen semen, a freezing rate significantly ($P<0.05$) affected the MMP percentage but did not significantly affect the percentage of sperm DNA damage. Critical difference test showed that percentage of MMP in frozen semen was significantly higher for freezing rate III than for freezing rate II but the difference between freezing rates I and III, and between freezing rates I and II was not significant. In the present study, although not significant, the percentages of post-thaw sperm motility, live sperm and HOST- reacted sperm of yak semen frozen in Tris extender were found to be higher for freezing rate III than for freezing rates I and II. Between freezing rate I and II, the latter one yielded slightly higher percentages of sperm motility and HOST-reacted sperm which supports the earlier findings in buck semen [6]. However, Ahmed [8] obtained significantly higher post-thaw sperm motility and live sperm in frozen swamp buffalo semen for freezing rate I than freezing rate II used in the present study. The freezing rate I used in the present study for freezing of yak semen represented the IMV Digit-cool standard curve recommended for freezing of bovine semen. This indicated that the cryopreservation protocol varied among species. The apparently higher values of different post-thaw semen parameters obtained with freezing rate III than with freezing rate II for freezing of yak semen revealed that relatively faster freezing rate from -10 to -100°C was beneficial for yak spermatozoa. The incidences of various acrosomal changes were apparently lower in frozen yak semen for freezing rate III than for freezing rates I and II but the difference was not statistically significant. This is in agreement with the earlier report of Ahmed [8] in buffalo semen, Nur et al. [5] in ram semen and Sarma et al. [6] in buck semen. Ustuner et al. [9] reported that the use of different freezing rates had no effect on post-thaw sperm motility and acrosome integrity but sperm DNA integrity was affected during goat sperm freezing. The freezing rate incriment from 10°C/min to 24°C/min between +5°C and -150°C resulted in higher post-thaw DNA damage. In an another study, Pugliesi et al. [10] found that fast freezing technique, when compared to conventional one efficiently, preserves the viability and fertilizing capacity of equine spermatozoa.

The extracellular release of ALT and AST in semen frozen with Freezing rate III was lower compared to that with freezing rates I or II. The lower extracellular release of ALT and AST in semen frozen with freezing rate III might be due to lower damage to the plasma membrane and

Table 1. Incidence of MMP and DNA damage (mean ± SE) of frozen yak using different freezing rates

Freezing rates	MMP (%)	DNA damage (%)
Freezing rate I	91.73^{AB} ± 0.80	1.80 ± 0.24
Freezing rate II	89.97^{B} ± 0.65	1.47 ± 0.21
Freezing rate III	92.70^{A} ± 0.74	1.33 ± 0.17

$^{A, B,}$ Means bearing different letter superscripts within column differ significantly (P < 0.05)

acrosome during freezing using freezing rate III.The percentage of live sperm with MMP in yak semen frozen in Tris extender using freezing rate III was significantly (P<0.05) higher than that using freezing rates I and II. Although statistically not significant, the incidence of sperm with DNA damage in yak frozen semen was found to be apparently lower for freezing rate III than for freezing rates 147 I and II. This revealed that maintenance of MMP and DNA integrity of yak sperm was relatively better in freezing rate III than freezing rates I and II. The post thaw percentage of live sperm with MMP recorded in the present study in yak semen was higher than that reported in bull semen by Garner et al. [11]. In the present study only the live sperm with MMP was counted leaving dead spermatozoa uncounted. The high MMP of spermatozoa might be due to capacitation like effect of freeze/thaw procedure which moved sperm toward hyperactivation and consequently rise in mitochondrial activity and low MMP of spermatozoa might be due to action of ROS produced mostly during freeze-thaw procedure, on sperm mitochondria causing oxidative stress [12]. The sperm DNA/chromatin damage is associated with the factors *viz.,* high body temperature, toxic agents, composition of extender, processing and freezing techniques in different species of animals and also due to oxidative stress [13]. However, the percentage of DNA damage in yak spermatozoa was found to be very less. Two main temperature ranges are there regarding the damages to spermatozoa during freezing: Theperiod of super cooling from 0ºC to -5ºC and the formation of ice crystals from -6ºC to -15ºC [14]. The primary cause of cell death during freezing is considered to be due to formation of intracellular ice crystals and increase solute concentrations both inside and outside the cells as water freezes. During slow freezing, increase in osmotic pressure of the suspension medium that accompanies freezing pulls water from within the cell, causing intracellular dehydration with an increase in intracellular electrolyte concentration. In rapid freezing, intracellular ice is formed because there is insufficient time for all freezable water to diffuse from the cell. During slow freezing the cells are exposed for longer duration to concentrations of solutes that are increased slowly and cellular dehydration may occur. With rapid freezing the length of exposure to increased solute concentration is reduced and intra cellar dehydration may not occur [15]. Sperm cells are usually frozen at faster rates (15 - 60°C per minute), which give rise to best results [16,17]. The inherent ability of the spermatozoa to withstand different freezing rates varies from species to species which may be due to differences in shape, volume, composition and size of organelles of spermatozoa. Byrne et al. [16] and Anel et al. [17] reported that cooling rates must be high (≥50°C per minute) between -10 and -60°C and then could be lower (20-30°C per minute) until freezing was completed.

The yak semen, in the present study, frozen with Freezing rates III resulted in significantly better quality in terms of MMP after freezing than that of freezing rates I and II. This could be accomplished because of lower total time taken to complete the entire freezing process in freezing rate III (6 min 36 sec) than in I (7 min 3 sec) and II (6 min 42 sec). The optimal cooling rate is most important which allows spermatozoa to be cooled slowly enough to allow water to leave the cells by osmosis, preventing formation of intracellular ice crystals but quick enough to prevent longer exposure to high concentrations of solutes. The curve appears with different freezing rates during freezing determines the post thaw quality of semen. Barbas and Mascarenhas [18] advised to freeze semen according to a parabola shaped curve and the most adequate freezing rate was the fastest one that allows extracellular water freezing without intracellular ice formation. In the present study more parabola shaped curved occurred in freezing rate III than in rates I and II and freezing point plateau was found increase with longer duration of freezing. Similar observation was made by Eriksson and Rodriguez-Martinez [19] in boar, Ahmed [8] in swamp buffalo and Sarma et al. [6] in buck semen. In the present study ice nucleation and latent heat of fusion occurred between -5 to -15°C. The approximate duration of the freezing point plateau was 2 minute 5

seconds in Freezing rate I, 2 minute 20 seconds in Freezing rate II and 1 minute 40 seconds in Freezing rate III. Thermal dynamics arising in the freezing process using programmable freezer in the present study showed only very little differences. However, latent heat release of fusion could make it difficult for controlling the cooling rate inside the straw in all the freezing rates. The lower duration of freezing point plateau in Freezing rate III could reduce the extent of uncontrolled cooling inside the straw which tended to maintain more symmetry with the programmed rate of cooling as compared to Freezing rate I and II. This might explain the resultant better semen quality with Freezing rate III than that with Freezing rate I and II.

4. CONCLUSION

In present study, it is observed that except MMP, all the post thaw parameters tested for evaluation of frozen yak semen showed non-significant difference between the freezing rates. This could be due to the narrow differences in the three freezing rates adopted in the present study. The percentage of live sperm with MMP in frozen semen was significantly higher for freezing rate III than for freezing rate II but the difference between freezing rates I and III was not significant. All other parameters studied in the post-thaw semen were also better in freezing rate III in comparison to freezing rates I and II, although the values were non-significant. Therefore, it may be concluded from the present study that freezing yak semen @ 5^{o}C/minute from +4^{o}C to -10^{o}C, @ 50^{o}C/minute from -10^{o}C to -100^{o}C and @ 20^{o}C/minute from -100^{o}C to -140^{o}C may be recommended for successful cryopreservation.

ETHICAL APPROVAL

The experiments have been examined and approved by the Institutional Animal Ethics Committee.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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