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Effect of cooling rate on quality of frozen yak semen during cryopreservation

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Abstract
A study was conducted to record the suitable cooling rate during yak semen cryopreservation. Both fast and slow cooling rates have some adverse effect during semen processing, which may vary with species also. In the present study, the yak semen was cooled using three cooling rates *viz.*, @ 1 °C/2 minutes, @1 °C/3 minutes and @1 °C/4 minutes. The post-thaw values for these cooling rates obtained were 42.50 ± 2.68, 51.50 ± 2.15 and 47.00 ± 2.67; 57.40 ± 1.79, 60.55 ± 1.98 and 57.35 ± 1.82; 46.10 ± 1.21, 52.35 ± 1.48 and 50.55 ± 1.50; 14.00 ± 0.29, 12.30 ± 0.25 and 13.30 ± 0.36 per cent for sperm motility, live sperm, host-reacted sperm and total incidence of acrosomal changes respectively and 14.24 ± 0.80, 13.86 ± 1.03 and 10.47 ± 0.70 IU/l and 13.54± 0.70, 11.68 ± 0.96 and 11.33 ± 1.05 IU/l for extra cellular release of ALT and AST activity respectively. In conclusion, a cooling rate of 1 °C/3 minutes (in 1.5 hrs) may be more effective for cryopreservation of yak semen.

Keywords: Cooling rate, cryopreservation, frozen semen, yak

1. Introduction
Semen cryopreservation is not only essential technique to preserve genetic resources, but also for transporting species between remote destinations. Transportation of frozen semen is easier and economical in comparison to moving the bulls for natural service. Especially, species like yak, which are reared in snow bound hilly terrains cryopreservation of semen, may play an important role for conserving its declining population. During semen cryopreservation in domestic animals a 2-step protocol (primary cooling and freezing) is followed in which samples are suspended in an appropriate extender and gradually cooled from room temperature to 5 °C, followed by freezing. Cooling reduces the metabolic rate of the spermatozoa and subsequently increases its fertile life. However, several studies have revealed that different cooling rate influences the quality of frozen semen in different species (Januskauskas *et al*., 1999; Memon *et al.*, 2013) [1, 2]. Rapid cooling affects the acrosomal morphology and percentage of live sperm and motility in frozen thawed samples (Blackshow, 1954; Gilmore *et al*., 1998; Watson, 2000) [3-5]. Semen cryopreservation protocols vary among species due to their inherent characteristics. Therefore, the present study was carried out to standardize the cooling rate of yak semen during cryopreservation process.

2. Materials and Methods
The study was conducted in four healthy yak bulls aged between 3 to 4½ years maintained under uniform managemental condition in the farm of ICAR-National Research Centre on Yak, Dirang, Arunachal Pradesh, India. The animals were fed concentrate mixture comprising maize grain, wheat bran, ground nut cake, mineral mixture and salt @ 2 – 2.5 kg / bull / day along with green grasses and tree leaves. The farm is located at 2730 m above mean sea level in the Nyukmadung area of eastern Himalaya in the state of Arunachal Pradesh in India. Semen was collected using standard artificial vagina method at weekly intervals. Immediately after collection, semen samples were evaluated as per standard methods. The ejaculates having minimum volume of 1.00 ml, mass activity 3^{+} and initial sperm motility 70 per cent were selected for freezing and diluted in Tris extender containing 6.4 per cent glycerol at 35 °C (Single fraction). The diluted semen then split into three equal parts and cooled gradually from 35 °C to 5 °C using three different cooling rates @ 1 °C/2 minutes (1 hr), @1 °C/3 minutes (1.5 hrs) and @1 °C/4 minutes (2 hrs). Following cooling the semen samples was equilibrated at 5 °C for 4 hours in the cold handling cabinet.

The semen was evaluated for sperm motility, live sperm per cent, Hypo-osmotic swelling test (HOST)-reacted spermatozoa, acrosomal changes, Alanine Transferase (ALT) and Aspartate Transferase (AST) levels both after equilibration period and freezing. The ALT and AST activities were estimated by enzymatic colorimetric method using commercial Kits for ALT or AST and was expressed in IU/L. The statistical difference between the groups was analysed as per Snedecor and Cochran (1994) [6].

3. Results and Discussion

The mean per cent of sperm motility, live sperm and HOST-reacted sperm in fresh semen immediately after collection were 76.25 ± 1.02, 83.15 ± 1.13 and 75.85 ± 1.08 per cent respectively.

The mean sperm motility in semen samples that were cooled from 35 °C to 5 °C using three cooling rates *viz.*, @ 1 °C/2minutes, @ 1 °C/3minutes and @ 1 °C/4minutes was 65.75 ± 1.42, 69.25 ± 1.16 and 68.25 ± 1.22 per cent respectively after equilibration, and 42.50 ± 2.68, 51.50 ± 2.15 and 47.00 ± 2.67 per cent respectively after freezing. The corresponding values for percentage of live sperm were 72.80 ±1.50, 77.05 ± 1.28 and 75.25 ± 1.44 after equilibration and 57.40 ± 1.79, 60.55 ± 1.98 and 57.35 ± 1.82 after freezing. The percentage of HOST-reacted sperm was 65.20 ± 1.04, 68.70 ± 0.66 and 67.55 ± 0.63 after equilibration and 46.10 ± 1.21, 52.35 ± 1.48 and 50.55 ± 1.50 after freezing for cooling rates of 1 °C/ 2 minutes, 1 °C/3 minutes and 1 °C/4 minutes respectively.

The sperm motility and HOST-reacted sperm differed significantly (P<0.01) between cooling rates and between stages but not due to interaction while the percentage of live sperm differed significantly (P<0.01) between stages. The critical difference test revealed that sperm motility, live sperm and HOST-reacted sperm dropped significantly (P<0.05) at each stage of processing and freezing of semen. The overall per cent sperm motility and HOST-reacted sperm were significantly (P<0.05) higher for cooling rate of 1 °C/3minutes than for 1 °C/2 minutes. However, the difference was not significant (P<0.05) between 1 °C/3 minutes and 1 °C/4 minutes for sperm motility and HOST-reacted sperm and between 1 °C/2 minutes and 1 °C/4 minutes for sperm motility. The per cent sperm motility and host-reacted sperm were significantly (P<0.05) higher for cooling rate completing in 1.5 hrs than for 1 hr. However the difference was not significant (P<0.05) between 1.5 hrs and 2 hrs for sperm motility and HOST-reacted sperm and between 1hr and 2 hrs for sperm motility.

The mean incidences of swollen, separating, entirely lost acrosomes and total incidence of acrosomal changes in fresh yak semen immediately after collection were 1.35 ± 0.17, 0.95 ± 0.13, 1.15 ± 0.17 and 3.40 ± 0.15 per cent respectively.

The mean incidence of swollen acrosome in yak semen that had been cooled from 35 °C to 5 °C using three cooling rates *viz.*, @ 1 °C/2minutes, @ 1 °C/3 minutes and @ 1 °C/4 minutes was 3.90 ± 0.22, 2.85 ± 0.22 and 3.05 ± 0.30 per cent respectively after equilibration, and 6.80 ± 0.15, 6.75 ± 0.26 and 6.90 ± 0.34 per cent respectively after freezing. The corresponding values for separating acrosomes were 2.10 ± 0.20, 1.50 ± 0.22 and 1.70 ± 0.24 per cent after equilibration and 2.90 ± 0.29, 2.25 ± 0.17 and 2.50 ± 0.26 per cent after freezing. The percentage of entirely lost acrosomes was 2.30 ± 0.19, 2.45 ± 0.18 and 2.65 ± 0.17 after equilibration and 4.25 ± 0.22, 3.55 ± 0.28 and 3.80 ± 0.19 after freezing for cooling rates of 1°/2minutes, 1 °C/3minutes and 1 °C/4 minutes. The corresponding values for total incidence of acrosomal changes were 7.90 ± 0.30, 6.20 ± 0.29 and 6.70 ± 0.35 per cent after equilibration and 14.00 ± 0.29, 12.30 ± 0.25 and 13.30 ± 0.36 per cent after freezing.

The total incidence of acrosomal changes differed significantly (P<0.01) between cooling rates, stages and due to interaction. Critical difference test showed that total incidence of acrosomal changes was significantly (P<0.05) lower for 1.5 hrs of cooling than 2 hrs and 1 hr while extra cellular release of ALT activity was significantly (P<0.05) higher for cooling times 1 hr and 1.5 hrs than 2 hrs.

Analysis of variance revealed that the incidence of swollen and entirely lost acrosomes differed significantly (P<0.01) between stages but did not differ significantly between cooling rates and due to cooling rate × stage interaction whereas the incidence of separating acrosome differed significantly between cooling rates (P<0.05) and between stages (P<0.01) but not due to interaction. The total incidence of acrosomal changes differed significantly (P<0.01) between cooling rates, stages and due to cooling rate × stage interaction. Critical difference test showed that total incidence of acrosomal changes was significantly (P<0.05) lower for cooling rate 1 °C/3 minutes than that for cooling rate 1 °C/4 minutes and for cooling rate 1 °C/4 minutes than for cooling rate 1 °C/2 minutes. The incidences of swollen, separating and entirely lost acrosomes and total incidence of acrosomal changes, irrespective of cooling rates, increased significantly (P<0.05) during equilibration and during freezing.

The mean extracellular release of ALT (IU/l) in cooling rates *viz.*, @ 1 °C/2 minutes, @ 1 °C/3 minutes and @ 1 °C/4 minutes was recorded to be 8.38 ± 0.83, 9.35 ± 0.76 and 7.41 ± 0.84 respectively after equilibration, and 14.24 ± 0.80, 13.86 ± 1.03 and 10.47 ± 0.70 respectively after freezing. The corresponding values for AST (IU/l) were 5.97 ± 0.94, 7.26 ± 1.18 and 6.40 ± 1.19 after equilibration, and 13.54± 0.70, 11.68 ± 0.96 and 11.33 ± 1.05 after freezing.

Statistical analysis showed that extra cellular release of ALT differed significantly (P<0.01) between cooling rates and between stages but not due to interaction whereas AST value differed significantly (P<0.01) between stages only. Critical difference test showed that extracellular release of ALT was significantly (P<0.05) higher for cooling rates 1 °C/3 minutes and 1 °C/2 minutes than that for 1 °C/4 minutes

While freezing yak semen in the present study, the effect of cooling was found to be significant (P<0.01) for sperm motility, HOST-reacted sperm, total incidences of acrosomal changes and extracellular release of ALT. The present finding is in agreement with that of earlier workers in bull (Ennen *et al.*, 1976; Dhami and Sahni, 1993) [7, 8], buffalo bull (Dhami and Sahni, 1994; Dhami *et al*., 1996; El-Gawad and Allah, 2007) [9-11] ram (Anel *et al*., 2003; Kumar *et al*., 2009) and buck (Memon *et al*., 2013) [12-14] semen. On the contrary, no significant effect of cooling rate on post thaw sperm motility, membrane integrity and acrosomal status were observed in bull (Januskauskas *et al*., 1999) [1], buffalo bull (Ramakrishnan and Ariff, 1994) [15] and buck (Jondet *et al.* 1972; Deka and Rao, 1987) [16, 17] semen. This variation might be due to differences in extender composition, rate of cooling used and species of animal in different studies.

The post thaw sperm motility and HOST-reacted sperm were found to be significantly (P<0.05) lower when yak semen was cooled from 35 °C to 5 °C in 1.5 hrs (@1 °C/3mins) than in 1 hr (1 °C/2mins). The difference between 1.5 hrs (@1 °C/3mins) and 2 hrs (@1 °C/4mins) was not significant except for total acrosomal changes. Similarly, significantly

higher post thaw sperm motility was observed in bull semen for cooling time 2 or 4 hrs than for 0.5 hr (Ennen *et al.,* 1976) [7]. In buffalo semen post thaw sperm motility was reported to be significantly higher and acrosomal defect was significantly lower when cooling from 35 °C to 5 °C was done in 2 hrs than in 1 hr (El-Gawad and Allah, 2007) [11].

In the present study the extracellular release of ALT was significantly (P<0.05) lower for cooling rate 1 °C/4 minutes than 1 °C/2 minutes and 1 °C/3 minutes, but the difference between cooling rates was not significant for AST release. However, El-Gawad and Allah (2007) [11] recorded significantly lower GOT (AST) in frozen thawed buffalo semen for 2 hrs of cooling than 1 hr of cooling time from 35 °C to 5 °C.

4. Conclusions

Thereby, it may be concluded from the present study that, a cooling rate of 1 °C/3 minutes over a period of 1.5 hrs is more effective during cryopreservation of yak semen based on the post thaw semen parameters.

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