



Effect of equilibration period on post thaw semen quality of Indian yak

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Glycerol is reported to be cryoprotectant of choice by various workers for successful freezing of semen of various species. During initial days of freezing a long equilibration period of 12 to 20 h protocol was followed. Later on, it was reported that a long equilibration time results in aging of sperm cells (VanDemark *et al.* 2011). Hence, it is important to evolve a suitable equilibration period for cryopreservation of semen for different species to get a better post thaw semen quality. Therefore, an initial study was carried out to standardize the equilibration period while freezing yak semen to obtain a better post thaw semen quality.

The study was conducted in 4 apparently healthy yak bulls, aged 3 – 4.5 years and weighing 300 to 400 kg maintained at ICAR - National Research Centre on Yak, Dirang, Arunachal Pradesh, India in the eastern Himalayas.

Semen was collected at weekly intervals using standard artificial vagina method. Immediately after collection semen was evaluated for volume, mass activity (0 to 4 scales) and initial motility (0 to 100%) as per Zemjanis (2002). The ejaculates having minimum volume of 1.00 ml, mass activity 3 and initial sperm motility 70% were used for processing and freezing.

Ejaculates (20) comprising 5 ejaculates from each bulls found suitable were extended (1:10) with Tris extender containing 6.4% glycerol at 35°C and cooled gradually from 35°C to 5°C @ 1°C/3 min. The extended semen was then split into 3 parts and equilibrated at 4°–5°C for 3 different periods i.e. 3, 4 and 5 hours. Sperm motility, live sperm percentage, hypo-osmotic swelling test (HOST) and acrosomal changes were studied in the fresh semen, after equilibration and freezing. The ALT and AST activities were estimated after equilibration and freezing by enzymatic colorimetric method using commercial KIT for ALT or AST and was expressed in IU/L.

Analyses were performed using the Statistical System software package. Data on different parameters were analyzed using two-factorial analysis of variance (ANOVA) (GLM procedure).

The mean $\pm$ SE of sperm motility, live sperm and HOST-reacted sperm in fresh semen of yak bulls immediately after collection were 73.00 ± 0.84 , 80.00 ± 0.78 and $72.05 \pm 1.15\%$ respectively.

The mean percentage of sperm motility and HOST reacted sperm after equilibration for 3, 4 and 5 h at 4°–5°C at different stages of processing and freezing are presented in Table 1. The mean $\pm$ SE of live sperm percentage were 74.10 ± 1.18 , 75.15 ± 0.97 and 73.15 ± 1.05 after equilibration, and 51.15 ± 1.72 , 56.95 ± 1.68 and 55.45 ± 1.48 after freezing at 3, 4 and 5 h of equilibration periods respectively.

The sperm motility and HOST-reacted sperm varied significantly ($P < 0.01$) between equilibration periods, between stages and due to equilibration period $\times$ stage interaction whereas the % live sperm differed significantly ($P < 0.01$) between stages but did not differ significantly between equilibration periods and due to interaction. Critical difference test revealed that the sperm motility and HOST-reacted sperm in frozen semen were significantly ($P < 0.05$) higher for 4 and 5 h of equilibration period than in 3 h of equilibration period whereas the difference between 4 and 5 h of equilibration was not significant. The significant effect of equilibration period on post thaw sperm motility was also reported by earlier workers on buffalo bull (El-Gawad and Allah 2007) and bull (Leite *et al.* 2010) semen. However, some of the workers did not find effect of equilibration on semen on post thaw semen quality. The diversity of opinion of different workers could be attributed to differences in composition of the extender and the species involved, which is important in determining the effect of equilibration period in different species and duration of different equilibration periods studied.

The sperm motility and HOST-reacted sperm in frozen yak semen were significantly ($P < 0.05$) higher for 4 h of equilibration than 3 hours of equilibration, the difference between 4 and 5 h of equilibration being not significant. This supports the findings of Leite *et al.* (2010) that the

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Table 1. Per cent (mean $\pm$ SE) sperm motility and HOST-reacted sperm of yak semen in tris extender at different stages of processing and freezing using different equilibration periods

Stages	Equilibration period							
	% sperm motility				% HOST-reacted sperm			
	3 h	4 h	5 h	Overall mean	3 h	4 h	5 h	Overall mean
Fresh semen	73.00 ^a $\pm$ 0.84	73.00 ^a $\pm$ 0.84	73.00 ^a $\pm$ 0.84	73.00 ^A $\pm$ 0.48	72.05 ^a $\pm$ 1.15	72.05 ^a $\pm$ 1.15	72.05 ^a $\pm$ 1.15	72.05 ^A $\pm$ 0.65
After equilibration	66.25 ^b $\pm$ 1.57	68.00 ^b $\pm$ 1.28	66.00 ^b $\pm$ 1.29	66.75 ^B $\pm$ 0.79	65.80 ^b $\pm$ 1.31	67.45 ^b $\pm$ 1.15	65.65 ^b $\pm$ 1.14	66.30 ^B $\pm$ 0.69
After freezing	38.75 ^d $\pm$ 1.62	47.00 ^c $\pm$ 1.64	45.00 ^c $\pm$ 1.49	43.58 ^C $\pm$ 1.01	39.30 ^d $\pm$ 1.27	47.70 ^c $\pm$ 1.34	44.75 ^c $\pm$ 1.19	43.92 ^C $\pm$ 0.85
Overall mean	59.33 ^B $\pm$ 2.08	62.67 ^A $\pm$ 1.64	61.33 ^{AB} $\pm$ 1.70		59.05 ^B $\pm$ 1.98	62.40 ^A $\pm$ 1.54	60.82 ^{AB} $\pm$ 1.65	

a, b, c, d Means bearing different letter superscripts differ significantly ($P < 0.05$). A, B, C Means bearing different letter superscripts differ significantly ($P < 0.05$) within row and within column.

combination of Tris extender and 4 hours of equilibration was the most desirable semen cryopreservation method for bull semen, in respect of post thaw motility and sperm with intact plasma and acrosomal membranes. The percentage of live sperm did not differ significantly between equilibration periods in the present study. The percentage of live sperm in frozen semen was recorded to be apparently higher for 4 hours than for 3 and 5 hours of equilibration. This is in agreement with the findings of Shahverdi *et al.* (2014) in buffalo bull semen.

Acrosomal changes investigation revealed that mean incidences of swollen, detached, completely lost acrosomes and the total incidence of acrosomal changes in fresh yak semen immediately after collection were 2.00 ± 0.27 , 0.60 ± 0.15 , 0.60 ± 0.18 and $3.20 \pm 0.19\%$ respectively. After equilibration for 3, 4 and 5 h at 4°C - 5°C the incidences of swollen acrosome was 4.05 ± 0.31 , 3.45 ± 0.26 and $3.80 \pm 0.30\%$ respectively, and after freezing the values were 7.85 ± 0.44 , 6.95 ± 0.34 and $7.60 \pm 0.41\%$ respectively. The corresponding values for detached acrosome were 1.05 ± 0.14 , 0.95 ± 0.11 and $0.92 \pm 0.13\%$ after equilibration, and 2.60 ± 0.15 , 2.05 ± 0.15 and $2.40 \pm 0.31\%$ after freezing, and for completely lost acrosome were 0.95 ± 0.12 , 0.86 ± 0.11 and $0.83 \pm 0.12\%$ after equilibration, and 1.65 ± 0.167 , 1.55 ± 0.15 and $1.45 \pm 0.18\%$ after freezing. The corresponding values for total incidence of acrosomal changes were 6.05 ± 0.34 , 5.20 ± 0.27 and $5.55 \pm 0.34\%$ after equilibration, and 12.10 ± 0.52 , 10.55 ± 0.42 and $11.35 \pm 0.52\%$ after freezing.

The analysis of variance showed that the mean incidences of swollen, detached and completely lost acrosomes differed significantly ($P < 0.01$) between stages but did not differ significantly between equilibration periods and due to equilibration period $\times$ stage interaction. The incidence of total acrosomal changes differed significantly ($P < 0.05$) between equilibration periods ($P < 0.05$) and between stages ($P < 0.01$) but not due to interaction. Critical different test revealed that, irrespective of stages, the total incidence of acrosomal changes for 4 h of equilibration period was significantly ($P < 0.05$) lower than that of 3 hours of equilibration period. However, the total acrosomal changes did not differ significantly between 3 and 5 h or between 4

and 5 h of equilibration period. The significantly ($P < 0.05$) lower total incidence of acrosomal changes for 4 h of equilibration period in the study is in agreement with that of Shahverdi *et al.* (2014). On the other hand, percentage of intact acrosome was reported to be significantly lower in buck semen as equilibration time increased (Sinha *et al.* 1992).

The mean extracellular release of ALT (IU/L) in yak semen equilibrated for 3, 4 and 5 hours was 6.60 ± 0.46 , 8.16 ± 0.56 and 6.92 ± 0.57 respectively after equilibration, and 10.52 ± 0.66 , 11.10 ± 0.84 and 11.41 ± 0.62 respectively after freezing. The corresponding values for AST (IU/L) were 7.42 ± 0.41 , 5.98 ± 0.44 and 6.29 ± 0.53 after equilibration, and 9.36 ± 0.72 , 9.97 ± 0.82 and 10.25 ± 0.84 after freezing.

The mean extracellular release of ALT and AST increased significantly ($P < 0.01$) during freezing. However, there was no significant effect of equilibration period on extracellular release of ALT and AST. Although, there was no significant effect of equilibration period on extracellular release of ALT and AST, the post thaw values of ALT and AST decreased as equilibration period was reduced from 5 to 3 h. On the contrary, Leite *et al.* (2010) observed that the incidence of damaged plasma membrane decreased as the period of equilibration in bull semen increased from 0 to 4 hours. The incidence of damaged plasma membrane is positively correlated with extracellular release of intracellular enzymes. The diversity of opinion of different workers might be due to differences in the freezing process, extender composition, cooling rate, thawing rate, climatic condition and / or species of animals used in different studies.

In the present study, it was observed that when yak semen was extended with Tris extender, 4 h equilibration gave significantly better post thaw semen quality in terms of sperm motility, HOST – reacted sperm and acrosomal changes. However, the difference between 4 and 5 hours of equilibration was not significant for post thaw quality of yak semen.

SUMMARY

Application of artificial insemination in yak for genetic improvement is still at infant stage in India. Semen

cryopreservation is a critical procedure and the technique varies with species and breed. Therefore, present study was designed to evaluate the effect of different equilibration period on the post thaw semen viability, membrane integrity and acrosomal damage of yak bulls. Semen (20) ejaculates collected from 4 yak bulls using artificial vagina were processed for freezing using Tris egg yolk glycerol extender following 3, 4 and 5 h of equilibration period. The fresh semen samples were evaluated for motility, % live, hypo-osmotic swelling test and acrosomal abnormalities. Following equilibration and freezing all the parameters were again studied along with the extracellular release of enzymes. It was evident in the present study that 4 h of equilibration period significantly improves the post thaw semen quality in yaks.

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