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Effect of equilibration period on post thaw viability and membrane integrity of boar spermatozoa during conventional method of freezing

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ABSTRACT

The study was conducted to find out the effect of equilibration period on post thaw viability and membrane integrity of Hampshire (HS) and crossbred (CB) boar semen frozen with conventional method of freezing. Ejaculates (72) were collected (12 each from 3 Hampshire and 3 crossbred boars) during the study period. The semen samples having more than 75% initial motility and concentration above 100 million/ml were subjected to freezing. Before freezing, each semen sample was divided into 4 aliquots and kept for 0, 1, 3 and 5 h at 5°C for equilibration. Freezing was done by putting the filled and sealed straws on liquid nitrogen vapours and then plunged into liquid nitrogen. Results showed that overall mean motility and live sperm count of spermatozoa after equilibration and after thawing was significantly higher than at 3 and 5 h of equilibration. Mean acrosomal integrity and hypo-osmotic reacted spermatozoa, irrespective of breeds, recorded after equilibration was significantly higher in 0 and 1 h equilibration than in 3 and 5 h. However, after thawing, overall mean acrosomal integrity remained significantly higher in 1 h equilibration. No significant difference between the genetic groups was recorded in terms of motility, live sperm count and hypo-osmotic reacted spermatozoa after equilibration as well as after thawing. But, the acrosomal integrity was significantly higher in HS than in CB boars (66.25±1.05 vs 63.41±1.43) in semen samples equilibrated for 5 h. It may be concluded from the present study that shorter exposure of spermatozoa at 5 °C is beneficial for viability and membrane integrity whereas longer exposure tended to deteriorate the post thaw semen quality.

Key words: Boar semen freezing, Cryopreservation, Equilibration, Hampshire, Semen

Equilibration prior to freezing plays an important role in sperm survival during cryopreservation. Determining the effective period of equilibration had been considered as one of the most important tasks in optimizing protocol for processing and freezing of semen in different species of animals. Addition of glycerolated diluent to cooled semen just at the beginning of equilibration is a common practice. However, boar spermatozoa showed greater sensitivity than spermatozoa of other species to glycerol levels adequate for optimum cyopreservation (Almid and Jhonson 1988). Sperm cryopreservation involves a series of sequential steps, and each of these steps may contribute to poor sperm quality after thawing (Dong *et al*. 2008). Despite this, studies often tend to focus on a few major steps, such as the selection of cryoprotectant, holding time, glycerol concentration and freezing and thawing rates. Equilibration was commonly carried out at 4° to 5°C or with a gradual cooling process to 4°C. However, equilibration time used in boar sperm cryopreservation has varied from 0.5 to 75 min (Almid and Jhonson 1988). Fiser and Fairfull (1996) reported that longer times of exposure at 5°C were beneficial; 4 h equilibration producing the highest percentage of motile spermatozoa with normal apical ridges. No systematic study has been done so far on duration of equilibration to which the boar spermatozoa should be exposed before freezing to minimize sperm damage and to increase the motility and viability post thawing.

The goal of our studies was to develop an optimized cryopreservation protocol for boar semen, with the intention of concurrently developing a better protocol for boar semen freezing. The present study was part of our systematic approach for the optimization process where effect of exposure of different equilibration period on post thaw sperm characteristics were evaluated in Hampshire as well as crossbred boar semen.

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MATERIALS AND METHODS

Semen collection and evaluation

Boars (6), 3 each from Hampshire (HS) and crossbred (CB; Hampshire × Khasi local with 75% of exotic inheritance) boars maintained at Livestock Research Farm, Division of Livestock Production, ICAR Research Complex, Barapani, Meghalaya, were taken for the study. The boars were in use for breeding and the age varied from 15 to 36 months. All the boars were clinically examined for sexual as well as for general health and were selected on the basis of clinical normality. Semen was collected twice weekly, by gloved hand technique using dummy sow, in a sterilized plastic bottle inside an insulated thermos flask. The gel portion of semen was removed at the time of collection by using gauze inside the Butchner funnel. Immediately after collection, semen was brought to the laboratory in a thermos flask (at 35°C) for further processing. A total of 72 ejaculates (12 each from 6 boars) were collected and subjected to routine semen evaluation (RSE). The semen samples which had more than 75% of initial motility and concentration more than 100 million/ml were subjected for further processing and freezing. Before freezing, fresh semen samples were analyzed for initial progressive motility, live sperm count, acrosomal integrity and hypo-osmotic sperm swelling test (HOST).

Sperm motility was assessed in a microscope equipped with 37°C microscope stage and phase contrast optics at a magnification of 400×. The live sperm count was determined using eosin-nigrosin staining technique. Two hundred spermatozoa were counted in each smear at a magnification of 1,000× to determine the percentage of live spermatozoa. Acrosomal integrity was studied using Giemsa staining (Watson 1975). HOST was performed as per Jeyendran *et al*. (1984).

Semen processing: Initial dilution was made by adding BTS (Beltsville thawing solution) in the ratio of 1: 1 and kept in BOD incubator for 3 h at 24°C. After 3 h of holding time, semen was centrifuged, supernatant was discarded and freezing fraction-I (11% lactose; 80 ml + egg yolk; 20 ml) was added to make the volume 50% of the final volume. Temperature was reduced gradually from 24 °C to 5 °C within 1–½ h by keeping the samples in BOD incubator. Then freezing fraction-II (11% lactose; 74 ml, egg yolk; 20 ml and glycerol; 6 ml) was added at 5°C.

Equilibration and freezing: Each ejaculate was divided into 4 aliquots and kept for 0, 1, 3 and 5 h equilibration period. French medium straws with 4 different colors of PVA powder (polyvinyl alcohol powder) were taken for 4 equilibration periods. All the equipment related to the filling and sealing of straws were kept in cold handling cabinet at 5°C. After equilibration, the straws were arranged horizontally 5 cm above the liquid nitrogen level in a thermocole box and exposed for 12 min to liquid nitrogen vapors. Immediately after vapour freezing, the straws were transferred into the goblet containing liquid nitrogen and transferred to liquid nitrogen container for freezing and storage. Each sample was evaluated for initial motility, live sperm count, acrosomal integrity and HOST test just after equilibration and after thawing.

Semen evaluation: Thawing was done by putting the straw in 50 °C water for 20 sec. One end of the straw was cut and the semen was diluted in 5 ml BTS. The diluted semen was kept for 30 min before evaluation. Each sample was evaluated for initial motility, live sperm count, acrosomal integrity, and HOST test.

The data were analyzed using analysis of variance (ANOVA) and critical difference test as per Snedecor and Cochran (1967).

RESULTS AND DISCUSSION

Sperm characteristics in fresh semen: Mean values of initial motility, live sperm count, acrosomal integrity and hypo-osmotic reacted spermatozoa are presented in Table 1.

Initial sperm motility: Initial motility of spermatozoa in fresh semen ranged between 80 and 95% with a mean of 88.00±0.72% in HS, and 75 and 95 with a mean of 86.25±0.81% in CB boars. There was no significant difference in initial motility of spermatozoa between genetic groups as well as between boars. The mean initial motility of spermatozoa in HS and CB boars was in close proximity with that reported in HS (Khan *et al.* 2011, Naskar *et al.* 2006) and CB (Naskar *et al*. 2006) boars. The mean values obtained in the present study were higher than the findings of Bhuyan *et al.* (1991) in Hampshire and crossbred boars, Nath (1992) in crossbred boars. These variations might be attributed to differences in breed, age, season and method of examination of sperm motility.

Live sperm count: The mean live sperm count in HS and CB boar semen was 90.25±0.47 and 88.16±0.45% respectively, which ranged from 84 to 95% in HS and 83 to 93% in CB boars. The mean live sperm count in HS was significantly (P<0.01) higher than in CB boars. The present findings were close to the values reported by workers in Hampshire and crossbred boars (Khan *et al*. 2011). This variation might be due to the differences in breed, method of semen collection and technique of estimation. In the present investigation, a significantly (P<0.01) higher live sperm count was observed in Hampshire than in CB boars. This indicated

Table 1. Sperm characteristics of freshly ejaculated Hampshire and crossbred boar semen

Parameters	Hampshire	Crossbred
Initial motility (%)	88.00±0.72	86.25±0.81
Live sperm count (%)	90.25^{a}±0.47	88.16^{b}±0.45
Acrosomal integrity (%)	90.42^{a}±0.56	88.00^{b}±0.47
Hypo-osmotic reacted sperm (%)	60.14^{a}±1.03	56.47^{b}±0.85

*Means bearing different superscripts in a row differ significantly (P<0.05).

a significant effect of genotype.

Acrosomal integrity: The mean acrosomal integrity as measured by per cent intact acrosome in fresh semen was 90.42±0.56 and 88.00±0.47 in HS and CB boars respectively, which ranged from 81 to 96% in HS and 80 to 94% in CB boars. The mean intact acrosome was significantly (P<0.01) higher in Hampshire than in CB boar. Our findings were in close proximity with that reported by Khan *et al.* (2010, 2011) in Hampshire boars but higher than that reported by Schilling and Vengust (1987). On the other hand, Bamba (1988) and Nath (1992) reported higher percentage age of normal acrosomes (94.2, 95.0 and 96.05%) in crossbred boars.

The mean percentage of intact acrosome observed in the present study was significantly (P<0.01) higher in Hampshire than in CB boars. These variations might be due to genetic variation and variation between individual boars.

Hypo-osmotic swollen spermatozoa: The hypo-osmotic swollen spermatozoa ranged from 46.0 to 71.0% with a mean of 60.14±1.03% in HS and from 46.0 to 65.0% with a mean of 56.47±0.85% in CB boars. A significantly (P<0.01) higher hypo-osmotic swollen spermatozoa was observed in HS than in CB boars which might be due to genetic variation. The mean hypo-osmotic swollen sperm% observed in the present investigation was comparable with that reported by Das *et al.* (2005) in Hampshire and crossbred boars and Khan *et al.* (2010) in Hampshire boars.

Sperm characteristics after equilibration and thawing: The mean values of sperm motility, live sperm count, acrosomal integrity and hypo-osmotic swollen spermatozoa in semen of Hampshire and crossbred boars after different periods of equilibration, after thawing are presented in Tables 2 –5.

Sperm motility: The overall mean motility of spermatozoa was recorded after 0, 1, 3 and 5 h of equilibration and after thawing (Table 2). Analysis of variance revealed no significant difference in sperm motility between the 2 genetic groups both after equilibration and after thawing. Semen processed with 0 or 1 h equilibration period showed significantly higher overall sperm motility after equilibration than that processed with 3 or 5 h equilibration. After thawing, a significant variation in overall sperm motility was recorded among all equilibration periods. Maximum post thawing sperm motility was observed in semen processed with 1 h equilibration period than with 0, 3 and 5 h of equilibration. Present study was in agreement with Guthrie and Welch (2005) who reported 73.0 and 67.1% sperm motility respectively, after equilibration. After thawing, a significant (P<0.01) difference in sperm motility was obtained between equilibration periods. Maximum post thaw motility was found in 1 h equilibration followed by 0 h and was comparable with that reported by Khan *et al*. (2012a,b)

Live sperm count: The overall mean live sperm count obtained after equilibration and post thawing in 0 and 1 h equilibration period was significantly higher (P<0.05) than that processed with 3 and 5 h of equilibration (Table 3). No significant difference in live sperm count was obtained

Table 2. Sperm motility (%) after equilibration and thawing in Hampshire and crossbred boar semen

Stages of processing and freezing	Genetic group	Equilibration period			
		0 h	1h	3 h	5 h
After equilibration	HS	66.25^{a}±1.39	69.58^{a}±0.96	60.83^{b}±1.03	62.08^{b}±1.14
	CB	72.08^{a}±1.56	70.00^{a}±1.56	60.41^{b}±2.25	62.08^{b}±1.29
	Overall	69.16^{a}±1.19	69.79^{a}±0.82	60.62^{b}±1.21	62.08^{b}±0.84
Post-thawing	HS	45.83^{a}±1.20	49.58^{b}±1.29	37.50^{c}±1.44	30.00^{d}±1.50
	CB	44.16^{a}±1.60	49.16^{b}±1.35	39.16^{c}±1.72	32.08^{d}±1.43
	Overall	45.00^{a}±0.99	49.37^{b}±0.91	38.33^{c}±1.11	31.04^{d}±1.04

Means bearing different superscripts (a,b,c,d) in a row differ significantly.

Table 3. Live sperm count (%) after equilibration and thawing in Hampshire and crossbred boar semen

Stages of processing and freezing	Genetic group	Equilibration period			
		0 h	1h	3 h	5 h
After equilibration	HS	81.41^{a}±0.64	81.33^{a}±0.81	63.08^{b}±1.18	64.00^{b}±1.32
	CB	81.00^{a}±0.88	80.75^{a}±0.86	61.08^{b}±1.89	64.33^{b}±1.29
	Overall	81.20^{a}±0.53	$81.0\ 4^{a}$±0.58	62.08^{b}±1.11	64.16^{b}±0.90
Post-thawing	HS	54.08^{a}±1.03	56.00^{a}±1.08	39.83^{b}±1.48	32.08^{c}±1.57
	CB	53.08^{a}±1.41	55.16^{a}±1.36	43.91^{b}±2.90	34.75^{c}±1.20
	Overall	53.58^{a}±0.85	55.58^{a}±0.84	41.87^{b}±1.65	33.41^{c}±1.00

Means bearing different superscripts (a,b,c,d) in a row differ significantly.

between the genetic groups at both stage of processing.

Present findings were in agreement with Wegmann (1990) who reported that longer equilibration time deteriorated the semen quality. Higher live sperm count obtained in the present study with comparatively shorter equilibration period was in agreement with Flores *et al.* (2008) who also recorded higher live sperm count with 0 and 1 h equilibration period than that with other equilibration periods.

Acrosomal integrity: Acrosomal integrity varied significantly (P<0.01) between equilibration periods at both stages of processing. Breed difference was significant (P<0.05) only after equilibration.

Mean acrosomal integrity, irrespective of breeds recorded after equilibration was significantly higher in 0 and 1 h equilibration group than in 3 and 5 h group. The values were in close proximity with that reported by Guthrie and Welch (2005) who reported intact acrosome percentage as 74.0 and 76.2 at equilibration and after 3 h holding at 15°C respectively in BTS extender. However, after thawing, overall mean acrosomal integrity remained significantly higher in 1 h equilibration group than in other equilibration groups which was in agreement with Waterhouse *et al.* (2006) and Flores *et al.* (2008). However, lower post thaw acrosomal integrity was reported by Gosolvez *et al.* (2004) and Guthrie and Welch (2005). Acrosomal integrity was significantly higher in Hampshire than in crossbred boars (66.25±1.05 vs. 63.41±1.43) only after 5 h equilibration period.

Hypo-osmotic swollen spermatozoa: Variation in % hypo-osmotic swollen spermatozoa due to equilibration period was highly significant after equilibration as well as after thawing (Table 5). No significant variation between the genetic groups were recorded. Results showed that significantly higher (P<0.01) percentage of hypo-osmotic swollen spermatozoa after equilibration in semen processed with 0 and 1 h equilibration period than with 3 and 5 h. When examined after thawing % hypo-osmotic swollen spermatozoa was found higher in 1 h equilibration group than in other equilibration periods.

The reduction in incidence of hypo-osmotic swollen spermatozoa percentage with the increase of equilibration period was in agreement with Wegmann (1990). Maximum hypo-osmotic swollen spermatozoa (43.62%) obtained in the present study with 1 h equilibration period was similar to the findings of Guthrie and Welch (2005) who reported the % hypo-osmotic swollen spermatozoa as 41.2 and 43.4%, respectively, when frozen in LEYG extender.

Higher sperm motility, live sperm count, acrosomal integrity and % hypo-osmotic swollen spermatozoa obtained in the present study with 1 h of equilibration as compared with other equilibration periods was similar to the findings of Almid and Jhonson (1988) who reported higher post thaw sperm motility after 5 min of equilibration than after 90 min. Wegmann (1990) observed no significant difference in the sperm characteristics when equilibrated up to 75 min and

Table 4. Acrosmal integrity (%) after equilibration and thawing in Hampshire and crossbred boar semen

Stages of processing and freezing	Genetic group	Equilibration period			
		0 h	1h	3 h	5 h
After equilibration	HS	$82.66^{a}\pm0.60$	$83.08^{a}\pm0.62$	$76.16^{b}\pm0.95$	$66.25^{aA}\pm1.05$
	CB	$81.25^{a}\pm0.70$	$82.66^{a}\pm0.83$	$74.08^{b}\pm0.98$	$63.41^{cB}\pm1.43$
	Overall	$81.95^{a}\pm0.47$	$82.87^{a}\pm0.51$	$75.12^{b}\pm0.70$	$64.33^{c}\pm0.89$
Post-thawing	HS	$53.66^{a}\pm1.01$	$57.00^{b}\pm1.08$	$51.25^{b}\pm0.89$	$43.50^{c}\pm0.89$
	CB	$52.75^{a}\pm0.98$	$56.4^{b}\pm1.25$	$48.75^{c}\pm0.69$	$45.25^{d}\pm0.97$
	Overall	$53.20^{a}\pm0.69$	$56.70^{b}\pm0.81$	$50.00^{c}\pm0.61$	$44.37^{d}\pm0.67$

Means bearing different superscripts (a,b,c,d) in a row differ significantly. Means bearing different superscripts (A, B) in a column within a particular stage of processing differ significantly.

Table 5. Hypo-osmotic reacted sperm (%) after equilibration and thawing in Hampshire and crossbred boar semen

Stages of processing and freezing	Genetic group	Equilibration period			
		0 h	1h	3 h	5 h
After equilibration	HS	$53.16^{a}\pm1.71$	$54.25^{a}\pm1.94$	$48.91^{a}\pm1.68$	$43.75^{b}\pm1.30$
	CB	$51.25^{a}\pm1.48$	$52.83^{a}\pm1.67$	$46.83^{b}\pm1.33$	$44.00^{b}\pm1.43$
	Overall	$52.20^{a}\pm1.12$	$53.54^{a}\pm1.26$	$47.87^{b}\pm1.07$	$43.87^{b}\pm0.94$
Post-thawing	HS	$40.33^{a}\pm1.02$	$43.91^{a}\pm1.65$	$35.08^{b}\pm1.37$	$31.16^{c}\pm1.20$
	CB	$41.25^{a}\pm1.58$	$43.33^{a}\pm1.51$	$35.58^{b}\pm0.87$	$31.66^{c}\pm1.25$
	Overall	$40.79^{a}\pm0.92$	$43.62^{b}\pm1.09$	$35.33^{c}\pm0.79$	$31.41^{d}\pm0.85$

Means bearing different superscripts (a,b,c,d) in a row differ significantly.

further opined that longer equilibration tended to deteriorate the semen quality. Absence of significant deterioration of sperm quality up to 1 h equilibration period but its deterioration after freezing and thawing after 1 h equilibration as observed in the present study justified the beneficial effect of 1 h equilibration period. The exact mechanism why the shorter exposure of glycerol is beneficial for spermatozoa is not yet clear; but it may be hypothesized that the longer exposure of spermatozoa to glycerol causes biophysical changes in plasma membrane due to which the selective permeability of plasma membrane is lost leading to the influx of calcium ion. As a result of influx of calcium ion, spermatozoa undergoes capacitation followed by acrosome reaction which is also evident in the present study as there is more damage in the acrosome and plasma membrane when the semen samples are exposed to 3 and 5 h of equilibration period.

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