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A comparative study of effects of different freezing methods on sperm quality, DNA integrity and membrane protein of cryopreserved boar semen*

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ABSTRACT

The present study determined the effects of conventional, and controlled freezing method adopting three freezing rates, viz. 20°C, 40°C and 60°C/min on quality of sperm (motility, viability and plasma membrane integrity), DNA integrity and plasma membrane protein profile of cryopreserved boar semen. Sixty sperm-rich fractions of ejaculates from six boars were utilized for freezing of semen with different freezing methods in lactose-egg yolk glycerol extender. Semen samples were evaluated for sperm motility, viability (Propidium Iodide assay), functional integrity of plasma membrane (HOST), DNA integrity (Acridine Orange stain) and plasma membrane protein profile (SDS-PAGE) after equilibration and after freezing. The results revealed that the post thaw sperm motility, sperm viability, and plasma membrane integrity (HOST-reacted) were significantly higher in all the three controlled freezing methods (20°C, 40°C and 60°C/min) as compared to that in conventional method. In addition, the number of sperm plasma membrane protein loss was less in controlled freezing methods as compared to that in conventional freezing. However, the post thaw sperm DNA integrity did not influence by difference in freezing methods. No significant difference on the post thaw sperm characteristics was recorded among the three controlled freezing rates. All the sperm parameters assessed declined significantly after freezing as compared to that after equilibration irrespective of freezing method employed. In conclusion, controlled freezing methods conferred better post thaw sperm quality as compared to conventional method, and thus the freezing rates of either 20, 40 or 60°C/min could provide better freezability of boar semen.

Key words: Boar semen, Cryopreservation, DNA integrity, Freezing method, Membrane protein, Sperm quality

Although frozen boar semen has been available since long, a very low proportion of the commercial artificial inseminations are carried out in the pig utilizing frozen semen due to its lower survival post-thaw that results in low farrowing rates and litter sizes (Roca *et al*. 2006, Hernandez *et al*. 2007). The low fertility of frozen thawed boar semen could result from the damage sustained by the sperm cells during cooling, freezing and thawing processes of cryopreservation (Holt 2000, Watson 2000). An increased understanding of effects of different freezing methods on sperm quality is of utmost importance that could lead to improved cryopreservation methods. The *in-vitro* evaluation of sperm motility, sperm viability, integrity of plasma membrane, DNA integrity and plasma membrane protein profile during cryopreservation process are good indicator of sperm quality and fertility status of spermatozoa.

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It has been documented that sperm damages emanated during cryopreservation procedures could be mitigated to some extent through improvement in freezing strategies (Fiser and Fairfull 1990, Woelders and Besten 1993, Roca *et al*. 2006). Adoption of suitable freezing protocol has resulted in improvement of cryopreservation technique in boar semen (Eriksson and Rodriguez-Martinez 2000). It has been reported that controlled rate of freezing method yielded superior post thaw sperm quality as compared to conventional freezing method (Petyim *et al*. 2007, Kaeoket *et al*. 2008). To the best of our knowledge, the comparative study of effects of different freezing methods on quality of cryopreserved boar semen has not been carried out in India. Therefore, a study was designed to assess the effect of conventional, and controlled freezing methods adopting freezing rates either of 20°, 40° or 60°C/min on sperm motility, viability, membrane integrity, DNA integrity and plasma membrane protein profile in order to find out a suitable freezing method for cryopreservation of boar semen.

MATERIALS AND METHODS

The present study was conducted at Artificial Insemination Laboratory of Livestock Production Division of the institute and College of Veterinary Science, AAU, Khanapara, Guwahati, Asom, India. All the animals were humanely treated and the study was designed as per the guidelines of the Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA), Govt. of India and Institutional Animal Ethics Committee (IAEC).

Semen collection, processing and freezing: Ejaculates (60) from 6 healthy and fertile boars consisting of two boars each of Hampshire (HS), Hampshire × Khasi local (HS × KL) with 87.5% exotic inheritance and HS × KL with 75% exotic inheritance were collected by gloved hand technique using dummy sow. Immediately after collection, semen was brought to the laboratory at 35°C and evaluated for semen volume, sperm motility, sperm concentration and live sperm using standard laboratory procedure. Semen ejaculate showing more than 70% progressive motility and 80% sperm with normal morphology were selected for freezing. Semen samples were processed and frozen following straw freezing procedure as described by Westendorf *et al.* (1975). The sperm-rich fraction was collected in an insulated thermos flask, separating the gel fraction through gauze during collection. Semen was transferred to measuring glass cylinder and diluted with Beltsville Thawing Solution (BTS) (Johnson *et al*. 1988) at 1:1 ratio, transferred to 50 ml centrifuge tube, held at 24°C for 2 h and then at 18°C for 1 h in a BOD incubator (NSW-152, Narang Scientific Works Pvt. Ltd., Mayapuri Ind. Area, New Delhi, India). After a total of 3 h of holding semen in 50 ml tubes was centrifuged (REMI C-24, Mumbai, India) for 10 min at 600 × g at 18°C. The supernatant was discarded and sperm pellet was re-suspended with fraction I of lactose-egg yolk extender (lactose 11% - 80 ml and egg yolk 20 ml) to a concentration of 1.5×10^9 sperm/ml. The extended semen was cooled to 5°C for 90 min in the BOD incubator. The extended semen cooled to 5°C was then transferred to cold handling cabinet (IMV Technologies, France) maintained at 5°C and mixed with fraction II of lactose-egg yolk- glycerol extender (lactose 11% - 74 ml, egg yolk 20 ml and 6 ml glycerol) to make the final concentration of semen to 1.0×10^9 sperm/ml. Then, semen was equilibrated for 60 min at 5°C at cold handling cabinet and during this period filling and sealing of straws were carried out. Medium straws (0.5 ml, Genuine Cassou straw, IMV Technologies, France) were used for loading of semen. All the equipments required i.e. straws, sealing powder, towel, filling comb and freezing rack were kept in a cold handling cabinet at 5°C. After 30 min of equilibration, the straws were filled with semen by filling comb and open ends were sealed with PVC powder (IMV Technologies, France). At the end of 60 min of equilibration period, the straws were placed on a pre-cooled (5°C) towel and were dried by rolling between the folds of the towel. The straws were put horizontally on the freezing rack for freezing. For conventional freezing method (freezing method 1), the straws were arranged horizontally 3 cm above the liquid nitrogen level in a thermocole box and exposed to liquid nitrogen vapours for 15 min. Immediately after vapour freezing, the straws were transferred into a goblet containing liquid nitrogen and placed in liquid nitrogen container for storage. The controlled rate of freezing was done in a programmable freezing machine (Cryo Med Freezer, Thermo Scientific Model 7452, controlled environment equipment 401, Mill creek road, Ohio, US) adopting three freezing rates, viz. cooling rate 3°C/min from 5 to –6°C, 1 min holding at –6°C and then freezing rate of 20°C/min from –6 to –140°C (freezing method 2); cooling rate 3°C/min from 5 to –6°C, 1 min holding at –6°C and then freezing rate of 40°C/min from –6 to –140°C (freezing method 3) and cooling rate 3°C/min from 5 to –6°C, 1 min holding at –6°C and then freezing rate of 60°C/min from –6 to –140°C (freezing method 4). In each case after freezing, the straws were plunged in a thermocole box containing liquid nitrogen and transferred into goblet containing liquid nitrogen and placed in liquid nitrogen container for storage.

Evaluation of semen: Semen was evaluated for sperm motility, viability, plasma membrane integrity (HOST-reacted), DNA integrity and plasma membrane protein profile at two stages of cryopreservation i.e. after equilibration and after freezing for each freezing method. Thawing of frozen semen was done by immersing straws in a circulating water bath at 50°C for 12 sec. One ml of thawed semen was diluted (1:2) with warm (35°C) Beltsville Thawing Solution (BTS) in 2 ml eppendorf tube and kept at 35°C in a dry bath for 10 min. After 5 min, a drop of semen was put on a glass slide and then a cover glass was put over the preparation. Sperm motility was assessed subjectively under a microscope (Olympus, BX 51 FT, Japan) equipped with 35°C microscope stage and phase contrast optics at a magnification of 400×. Post thaw sperm were also evaluated for viable sperm by Propidium Iodide stain (PI) (Harrison and Vickers 1990), sperm plasma membrane integrity by hypo-osmotic swelling test (HOST) (Jeyendran *et al.* 1984) and integrity of sperm DNA by Acridine Orange staining (AO) (Thuwanut *et al.* 2008).

Plasma membrane protein profiles were determined by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Laemmli 1970). Two ml semen sample containing 1×10^9 sperm/ml was taken in a 4ml tube and centrifuged twice at 600 × g for 10 min in phosphate buffer solution (PBS). The sperm pellet was then resuspended and sonicated for 30 to 60 sec in 1 ml ice cold cell lysis buffer (pH- 6.8). After 30 min in ice, the homogenised suspension was centrifuged at 10,000 × g for 15 min at 4°C and the supernatant was carefully removed and kept in a 2 ml plastic vial. The total amount of protein in the supernatant was estimated by a standard protein kit (BIURET method, Merck Specialities Pvt. Ltd., Goa). After quantifying, protein sample was mixed with 5 × sample buffer (Tris – HCl (1 M)- 6 ml, Glycerol (50%) - 5 ml, SDS (10%)- 2 ml, Mercaptoethanol- 0.5 ml, Bromophenol (1%)- 1 ml, Milli Q water- 0.9 ml) at 4:1 ratio and heated at 37°C for 30 min at dry bath. Finally, 15 µl of sample containing 20 µg protein was loaded into mini gel using 5%

polyacrylamide stacking gel and 15% running gel at 100–150 volt at room temperature over 150 min or until the dye front reached the bottom of the gel. Gel was then stained with Coomassie brilliant blue stain for overnight. After overnight staining, the gel was destained in destaining solution for 3 to 4 h and the gel was examined for the presence of protein band and it was compared with a standard protein marker (Serva unstained SDS-PAGE Protein Marker 6.5 – 200 KDa, Gel Company GmbH, Tuebingen, Germany).

Statistical analysis: Data on different parameters except membrane protein profile were expressed as the mean±SEM, and analysed using two-factorial analysis of variance (ANOVA) following General Linear Model (GLM) which included the effect of stages of semen cryopreservation and freezing methods, and interactions between them. The statistical model was used for analysing the samples after equilibration and after freezing of cryopreservation taking the effects of boar and breed as fixed. When ANOVA showed a significant effect, values were tested using Fisher's least significant difference test using Tukey test. Values were considered significant when P<0.05.

RESULTS AND DISCUSSION

Effect on sperm motility, viability, plasma membrane integrity and DNA integrity: The effect of different freezing methods on post thaw sperm motility, viability (live sperm), plasma membrane integrity (HOST-reacted) and DNA integrity are shown in Table 1. It was observed that the mean percentages of sperm motility, viable sperm, plasma membrane intact sperm and sperm with intact DNA were significantly reduced (P<0.05) in semen after freezing as compared to that in semen after equilibration irrespective of freezing method. This might be attributed to cold shock, osmotic and toxic stresses, and also by formation and dissolution of ice in the intra and extracellular environment (Holt 2000, Watson 2000). Freezing and thawing phases of cryopreservation process induced structural and/or biochemical damage in boar spermatozoa resulted in a drastic reduction of sperm quality (Sancho *et al*. 2007).

The result of present study demonstrated that there was a significant (P<0.05) improvement in post thaw sperm motility, viability and functional integrity of plasma membrane in controlled freezing methods as compared to that in conventional method. The higher post thaw sperm motility obtained with controlled rate of freezing methods in this study was thought to be less detrimental effect of it on sperm function, compared to conventional freezing method. This is consistence with the previous studies (Bwanga *et al*. 1991, Petyim *et al*. 2007, Kaeoket *et al*. 2008). The disruption of plasma membrane of spermatozoa resulting from rapid and uncontrolled rate of cooling in conventional freezing method led to substantial reduction of sperm with intact plasma membrane. However, there was no significant difference between the controlled freezing methods on these parameters. The non-significant difference in these post thaw sperm qualities with different controlled freezing rates could be due to the ability of boar spermatozoa to tolerate cooling rate in the range of –15° to –60°C/min through the critical temperature zone of –15° to –25°C without or with only marginal difference in cryosurvival (Medrano *et al*. 2009). Another reason might be due to that the range of freezing rates adopted in the present study was probably too narrow to demonstrate any major effect on integrity of plasma membrane of boar spermatozoa. Previous studies (Eriksson and Martinez 2000, Hernandez *et al*. 2007) also could not register significant difference in sperm viability post thaw using different cooling rates for freezing of boar semen. However, some of earlier studies (Fiser and Fairfull 1990, Woelders and Besten 1993) recorded significant difference in impairment of post thaw sperm plasma membrane when freezing rates of wider range were utilized.

The finding of present study revealed that the mean percentage of DNA-damaged sperm after freezing did not differ significantly between conventional and controlled rate of freezing (Table 1). This indicated that the extent of DNA damage in boar spermatozoa was similar in conventional and controlled freezing methods. The DNA integrity of boar

Table 1. Per cent sperm motility, viable sperm, plasma membrane intact sperm and DNA-damaged sperm in boar semen after equilibration and after freezing adopting different freezing methods

Cryopreservation stage	Freezing method	Motility	Viable sperm	Plasma membrane intact sperm	DNA-damaged sperm
After equilibration	1	85.33^{a}±1.03	78.26^{a}±1.57	69.20^{a}±1.48	2.88^{b}±0.46
	2	84.00^{a}±0.87	78.40^{a}±0.61	71.26^{a}±1.23	2.46^{b}±0.42
	3	85.33^{a}±0.76	78.20^{a}±0.94	73.03^{a}±1.38	2.70^{b}±0.45
	4	$85.00^{a\pm}$0.69	76.53^{a}±0.99	70.06^{a}±1.38	2.03^{b}±0.29
After freezing	1	45.00^{c}±0.69	32.21^{c}±1.20	30.03^{c}±0.94	5.93^{a}±0.84
	2	51.00^{b}±0.72	39.96^{b}±0.78	36.06^{b}±0.66	4.39^{a}±0.46
	3	52.00^{b}±0.65	38.11^{b}±1.79	36.80^{b}±1.27	6.99^{a}±1.33
	4	51.00^{b}±1.00	37.79^{b}±1.11	35.11^{b}±1.25	5.09^{a}±0.46

Data expressed as mean±S.E. 1, conventional method; 2, controlled method (20°C/min); 3, controlled method (40°C/min); 4, controlled method (60°C/min). [a,b,c]Means bearing different superscripts within column differ significantly (P<0.05).

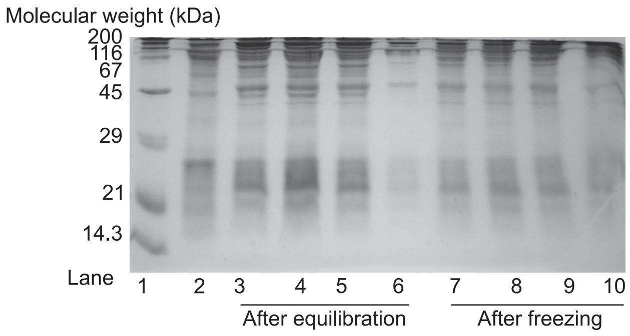
Table 2. Boar sperm plasma membrane protein profile* after equilibration and after freezing adopting different freezing methods

Stage	Sperm membrane protein in descending order of molecular weight (kDa)			
	Conventional freezing	Controlled freezing from –6 to –140°C		
		20°C/min	40°C/min	60°C/min
After equilibration	205, 124, 109, 95, 75, 65, 57, 50, 42, 25, 23, 21, 14 (13)	130, 125, 93, 80, 63, 56, 48, 24, 21 (9)	153, 130, 93, 85, 65, 53, 50, 44, 25, 24, 21 (11)	169, 101, 96, 66, 59, 52, 43, 38, 25, 24, 22, 20 (12)
After freezing	180, 167, 98, 90, 67, 53, 43, 25, 24, 21 (10)	166, 93, 89, 65, 53, 43, 34, 25, 24, 21 (10)	144, 86, 66, 53, 45, 41, 34, 28, 25, 23, 21 (11)	188, 134, 105, 76, 68, 53, 47, 43, 34, 26, 22 (11)

*8 observations. Figures in the parentheses indicate number of protein bands.

sperm was not influenced by difference in freezing methods. It is documented that the cryoprotectant present in the semen extender played an important role in maintaining DNA integrity and thus resulted in less DNA-damaged sperm in the present study (Hu *et al*. 2008).

Effect on plasma membrane protein profile: It was observed in the present study that the protein profiles of spermatozoon plasma membrane did not deviate widely after freezing as compared to that after equilibration (Fig. 1). A total of three proteins of equilibrated semen were not detected after conventional freezing. No protein was found lost after freezing with method 2 and 3, while one protein was absent after freezing following method 4 when analogy was made with the protein profile after equilibration (Table 2). Present finding could indicate that the quality of semen frozen adopting controlled freezing was superior to frozen semen obtained with conventional method of freezing with regard to presence of plasma membrane protein of spermatozoa. Although the loss of sperm plasma membrane protein was not marked, proteins appeared with altered molecular weights after freezing as compared to that after equilibration. Variation in the molecular weight of sperm plasma membrane protein after freezing was reported (Dhanju *et al.* 2001). The fast and uncontrolled rate of freezing in conventional freezing method could have exerted more harmful effects on sperm membrane protein than



1-Marker, 2- Extended semen, 3-Freezing method 1, 4- Freezing method 2, 5- Freezing method 3, 6- freezing method 4, 7- freezing method 1, 8-Freezing method 2, 9-Freezing method 3, 10-Freezing method 4

Fig. 1. Sperm plasma membrane protein profile at different stages of processing and freezing adopting different freezing methods.

controlled freezing method since alteration in the quality of proteins was observed (Dhanju *et al*. 2001) due to the deleterious effects of freezing and thawing injury on sperm membrane.

The present study revealed that the controlled freezing methods adopting cooling rate 3°C/min from 5 to –6°C, 1 min holding at –6°C and then freezing rates of either 20°C, 40°C or 60°C/min from –6°C to –140°C resulted in improvement of post thaw motility, viability, functional integrity of plasma membrane and plasma membrane protein profile as compared to conventional freezing method. The freezing protocol adopting freezing rates of either 20°, 40° or 60°C/min in lactose egg yolk glycerol extender using medium straw could be utilised for freezing of boar semen.

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REFERENCES

Bwanga C O, Einarsson S and Rodriguez-Martinez H. 1991. Cryopreservation of boar semen. II: Effect of cooling rate and duration of freezing point plateau on boar semen frozen in mini- and maxi-straws and plastic bags. *Acta Vetenaria Scandinavia* **32**: 455–61.

Dhanju C K, Cheema R S and Kaur S P. 2001. Effect of freezing on protein profiles of sperm membrane extracts and seminal plasma of buffalo bulls. *Asian Australasian Journal of Animal Science* **14**(12): 1678–82.

Eriksson B M and Rodriguez-Martinez H. 2000. Effect of freezing and thawing rate on the post-thaw viability of boar spermatozoa frozen in Flat Pack and Maxi-straws. *Animal Reproduction Science* **63**: 205–20.

Fiser P S and Fairfull R W. 1990. Combined effect of glycerol concentration and cooling velocity on motility and acrosomal integrity of boar spermatozoa frozen in 0.5 ml straws. *Molecular Reproduction and Development* **25**: 123–29.

Harrison R and Vickers S E. 1990. Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. *Journal of Reproduction and Fertility* **88**: 343–52.

Hernandez M, Roca J, Calvete J J, Sanz L, Muino-Blanco T, Cebrian-Perez J A, Vazquez J M and Martinez E A. 2007. Cryosurvival and *in vitro* fertilizing capacity post-thaw is improved when boar spermatozoa are frozen in the presence of seminal plasma from good freezer boars. *Journal of Andrology* **28**: 689–97.

Holt W V. 2000. Basic aspects of frozen storage of semen. *Animal Reproduction Science* **62**: 3–22.

Hu H J, Li Q W, Jiang Z L and Li W Y. 2008. Effects of different extenders on DNA integrity of boar spermatozoa following freezing-thawing. *Cryobiology* **57**: 257–62.

Jeyendran R S, Van der Ven H H, Perez-Pelaez M, Crabo B G and Zaneveld L J D. 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *Journal of Reproduction and Fertility* **70**: 219–28.

Johnson L A, Aalbers J G and Grooten H J G. 1988. Artificial insemination of swine: fecundity of boar semen stored in Beltsville TS (BTS), Modified Modena (MM), or MR-A and inseminated on one, three and four days after collection. *Zuchthygiene* **23**: 49–55.

Kaeoket K, Tantiparinyakul K, Kladkaew W, Chanapiwat P and Techakumphu M. 2008. Effect of different antioxidants on quality of cryopreserved boar semen in different breeds. *Thailand Journal of Agricultural Sciences* **41**(1–2): 1–9.

Laemmli U K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**: 680–85.

Medrano A, Holt W V and Watson P F. 2009. Controlled freezing studies on boar sperm cryopreservation. *Andrologia* **41**(4): 246–50.

Petyim S, Choavaratana R, Kunathikom S, Laokirkkiat P and Prechapanich J. 2007. Freezing effect on post-thawed sperm characteristics especially sperm DNA integrity comparing between liquid nitrogen vapour and computerized program freezer. *Siriraj Medical Journal* **59**: 298–302.

Roca J, Hernandez M, Carvajal G, Vazquez J and Martýnez E. 2006. Factors influencing boar sperm survival. *Journal of Animal Science* **84**: 2692–99.

Sancho S, Casas I, Ekwall H, Saravia F, Rodriguez-Martinez H, Rodriguez-Gil J E, Flores E, Pinart E M B, Garcia-Gil N, Bassols J, Pruneda A, Bussalleu E, Yeste M and Bonet S. 2007. Effects of cryopreservation on semen quality and the expression of sperm membrane hexose transporters in the spermatozoa of Iberian pigs. *Reproduction* **134**: 111–21.

Thuwanut P, Chatdarong K, Techakumphu M and Axner E. 2008. The effect of antioxidants on motility, viability, acrosome integrity and DNA integrity of frozen-thawed epididymal cat spermatozoa. *Theriogenology* **70**: 233–40.

Watson P F. 2000. The causes of reduced fertility with cryopreserved semen. *Animal Reproduction Science* **60–61**: 481–92.

Westendorf P, Richter L and Treu H. 1975. Zur Tiefgefrierung von Ebersperma. Labor-und Besamungsergebnisse mit dem Hiilsenberger Pailletten-verfahren. *Dtsch Tierarztl Wschr* **82***:* 261–67.

Woelders H and Den Besten M. 1993. Cryopreservation of boar semen with small between-boar variation of post-thaw sperm survival. *Cryobiology* **30**: 645.