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Effect of additives in semen extender on post thaw sperm quality and plasma membrane protein profile of boar semen

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ABSTRACT

The objective of the study was to evaluate the effect of butylated hydroxytoluene (BHT), cholesterol-loaded methyl-ß-cyclodextrin (CLC), and their combination on sperm motility, viability, plasma membrane integrity and membrane protein profile of frozen-thawed boar semen. Ejaculates (16) from 6 boars were utilized. Each ejaculate was split into 4 fractions and cryopreserved in semen extender containing BHT (0.2 mM), CLC at 5 mg/ 200–240 × 10^6 sperm, BHT (0.2 mM) plus CLC at 5 mg/ 200–240 × 10^6 sperm or without BHT and CLC. Semen samples were evaluated for sperm motility, viability (propidium iodide assay), plasma membrane integrity (HOST) and membrane protein profile (SDS-PAGE) after equilibration and after freezing. The supplementation of BHT, CLC, and BHT plus CLC in semen extender significantly improved the viability and plasma membrane integrity after freezing as compared to control. Post thaw sperm motility was also significantly higher in BHT and BHT plus CLC. In addition, the number of sperm plasma membrane protein loss was less in additive treated sperm as compared to that in non-additive treated sperm. Among the additives, BHT plus CLC showed a significant improvement on post thaw sperm motility along with a non-significant improvement on other sperm parameters as compared to BHT and CLC alone. In conclusion, the supplementation of BHT, and CLC in extender before freezing improved the quality of cryopreserved boar semen and, the beneficial effect was more pronounced when BHT was combined with CLC than with BHT and CLC alone.

Key words: Additive, Boar semen, Butylated hydroxytoluene, Cholesterol-loaded methyl-ß-cyclodextrin, Semen, Sperm quality

Cryopreservation of boar semen is a multistep and lengthy process and takes about 8–10 h to complete the process. During this process substantial damage occurs to sperm which results in reduced fertility of post-thaw boar spermatozoa. The boar sperm damage during freezing could be related to the lipid composition of sperm plasma membrane, oxidative stress and reactive oxygen species (ROS) formation. High levels of ROS impair sperm motility, viability and sperm function by interacting with membrane lipids, proteins and nuclear and mitochondrial DNA (Anghel *et al*. 2010). The supplementation of antioxidant compounds to the semen extender have been used in many species to minimize ROS formation and mitigate sperm cryodamage (Roca *et al*. 2004, Yeste *et al*. 2014). Among them, butylated hydroxytoluene (BHT), a synthetic phenolic antioxidant, has been successfully tested to minimize cold shock damage and to improve cryosurvival of boar spermatozoa (Roca *et al*. 2004).

Tomas *et al*. (2011) and Blanch *et al*. (2012) demonstrated that the supplementation of cyclodextrin preloaded with cholesterol to the cooling media could improve the resistance of sperm to cryodamage due to cold shock and freezing damage. Methyl-ß-cyclodextrin is the most commonly utilized cyclodextrin to preload cholesterol for treating sperm (Moce *et al*. 2010). To the best of our knowledge a comparative study of effects of supplementation of BHT, CLC, and BHT plus CLC in semen extender before freezing on quality of cryopreserved boar spermatozoa has not been reported earlier. The aim of the present study was, therefore, to evaluate the effect of BHT, CLC and their combination on the quality of frozen-thawed boar semen.

MATERIALS AND METHODS

The present study was conducted at Artificial Insemination Laboratory of Livestock Production Division, ICAR Research Complex for NEH Region, Umiam, Meghalaya, India and College of Veterinary Science, AAU, Khanapara, Guwahati, Assam, India. All the animals were humanely treated and the study was designed as per the

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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: Semen samples were processed and frozen following the straw freezing procedure (Westendorf *et al*. 1975). Semen sample was transferred to glass measuring cylinder and diluted with isothermal Beltsville thawing solution (BTS) (Johnson *et al*. 1988) at 1:1 ratio. The diluted semen was split into four aliquots (A, B, C and D) and transferred to 50 ml centrifuge tubes, held at 24°C for 2 h and then at 18°C for 1 h in a BOD incubator. During this holding period cholesterol-loaded Methyl-ß-cyclodextrin (CLC) at 5 mg/ 200–240 × 10^6 sperm was added at 18°C in BTS diluted semen for aliquot B and incubated for 30 to 60 min during holding. For aliquot C, CLC (like in aliquot B) plus butylated hydroxytoluene (0.2 mM) was added. After a total of 3 h of holding semen in 50 ml tubes was centrifuged at 600 × g for 10 min 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) containing BHT (0.2 mM) to a concentration of 1.5 × 10^9 sperm/ml for aliquot A and C. No CLC and no antioxidant were added to the extender for aliquot D which served as control. The extended semen was cooled to 5°C in 90 min in a BOD incubator and transferred to a cold handling cabinet maintained at 5° C and mixed with fraction II of lactose-egg yolk-glycerol extender (lactose 11% –74 ml, egg yolk-20 ml and glycerol–6 ml) to make final concentration of 1.0 × 10^9 sperm/ml of semen. For aliquot A and C, the fraction II of lactose-egg yolk-glycerol extender was supplemented with 0.2 mM BHT. The extended semen was then equilibrated for 60 min at 5°C in the cold handling cabinet and filling and sealing of straws were carried out during this period. French medium straws (0.5 ml, Genuine Cassou straw, IMV Technologies, France) were used for loading of semen. All the equipments required i.e., straws, PVC powder, towel, filling comb and freezing rack were kept in the cold handling cabinet at 5°C. After 30 min of equilibration, the straws were filled with semen by a filling comb and open ends were sealed with PVC powder. At the end of 60 min of equilibration period, the straws were placed on a pre-cooled (5°C) towel and dried by rolling between the folds of the towel. The straws were then put horizontally on a freezing rack for freezing in a programmable freezing machine using cooling rate 3°C/ min from 5 to –6°C, 1 min holding at –6°C and then freezing @ 40°C/min from –6 to –140°C. Immediately 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) and plasma membrane protein profile at two stages of cryopreservation i.e. after equilibration and after freezing for each additive and control group. 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) BTS in 2 ml eppendorf tube and kept at 35°C in a dry-bath for 10 min. After 10 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 equipped with 35°C microscope stage and phase contrast optics at a magnification of 400X. Post thaw sperm were also evaluated for viable sperm (live sperm) by propidium iodide stain (PI) (Harrison and Vickers 1990) and sperm plasma membrane integrity by hypo-osmotic swelling test (HOST) (Jeyendran *et al.* 1984). 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 4 ml 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. After quantifying, protein sample was mixed with 5X 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 (unstained SDS-PAGE Protein Marker 6.5 – 200 KDa).

Statistical analysis: Analyses of the data were performed using the Statistical Analysis System software package. Data on different sperm parameters barring 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 additives, and interactions between them. 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 and plasma membrane integrity: The effect of different additives on post thaw sperm motility, viability (live sperm) and plasma membrane integrity (HOST-reacted) are shown in Table 1. It was

Table 1. Per cent motility, viability and intact plasma membrane of boar sperm after equilibration and after freezing supplemented with BHT, CLC, BHT plus CLC or without (control) in semen extender

Cryopreservation stage	Additive	Motility	Viable sperm	Plasma membrane intact sperm
After equilibration (at 5°C)	BHT	87.50$^{a\pm}$0.65	78.69^{a}±0.83	74.88^{a}±1.14
	CLC	87.25$^{a\pm}$0.66	78.56^{a}±0.71	74.19^{a}±0.85
	BHT plus CLC	88.13$^{a\pm}$0.63	75.06$^{a\pm}$1.15	75.75^{a}±1.00
	Control	86.25$^{a\pm}$0.72	76.06^{a}±1.12	73.50^{a}±0.59
After freezing (post thaw)	BHT	55.00^{c}±0.79	41.47^{b}±0.69	38.94^{b}±0.71
	CLC	53.13^{cd}±0.63	41.44$^{b\pm}$0.70	38.81^{b}±0.69
	BHT plus CLC	58.19^{b}±0.79	44.09^{b}±0.98	41.23^{b}±0.88
	Control	51.25$^{d\pm}$0.72	36.29^{c}±1.49	34.04^{c}±1.33

Data shown all mean (n=16)±S.E. $^{a, b, c, d}$ Means bearing different superscripts within row differ significantly ($P < 0.05$). BHT: butylated hydroxytoluene; CLC: cholesterol loaded methyl-ß- cyclodextrin.

Table 2. Plasma membrane protein profile* of boar spermatozoa after equilibration and after freezing supplemented with BHT, CLC, BHT plus CLC or without (control) in semen extender

	Butylated hydroxytoluene (BHT)	Cholesterol loaded methyl-ß -cyclodextrin (CLC)	Butylated hydroxytoluene plus cholesterol loaded methyl-ß-cyclodextrin (BHT plus CLC)	Control
After equilibration (at 5°C)	176, 158, 141, 100, 93, 66, 60, 56, 50, 44, 34, 27, 24, 22, 18(15)	200, 188, 158, 138, 101, 85, 65, 60, 53, 49, 44, 34, 27, 25, 22, 20(16)	194, 142, 101, 97, 65, 61, 56, 51,46, 30, 27, 24, 22, 21(14)	135, 116, 100, 96, 89, 65, 60, 53, 50, 44, 34, 30, 26, 24, 23, 21, 18, 15(18)
After freezing (post thaw)	174, 113, 100, 98, 66, 61, 50, 47, 42, 26, 24, 21, 18(13)	165,116, 100, 96, 71, 66, 61, 49, 41, 25, 23, 21, 20(13)	176, 108, 97, 93, 66, 62, 58, 52, 49, 42, 26, 23, 22, 21(14)	158, 115, 101, 86,77, 66, 61, 50, 42, 29, 26, 24, 21, 18(14)

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

observed in the present study that the mean percentage of sperm motility after equilibration did not differ significantly between additive supplemented and control groups but after freezing it was significantly ($P < 0.05$) higher in BHT plus CLC treated group than in other groups. Post thaw sperm motility in BHT treated group although did not differ significantly from that of CLC added group, it was significantly ($P<0.05$) higher as compared to that of control group. The significant improvement in post thaw sperm motility in the present study after addition of BHT in combination with CLC could be due to the synergistic effect of BHT and CLC on sperm plasma membrane. BHT was recognized as a protector of spermatozoa against oxidative stress caused by reactive oxygen species (ROS) resulting from the freezing and thawing processes (Roca *et al*. 2004). The exact mechanism by which CLC improved the sperm cryosurvival remained obscure. However, its beneficial effect could be exerted by preventing cold shock through compensating cholesterol depletion of sperm plasm membranes during cooling and freezing processes (Galantino-Homer *et al.* 2006, Moce *et al*. 2010).

In the current investigation the mean percentage of viable sperm (live sperm) and sperm with functional plasma membrane integrity (HOST-reacted) after freezing was significantly ($P < 0.05$) higher in additive treated group as compared to that in control group (Table 1). These findings brought to light the protective action of the additives on plasma membrane of boar spermatozoa against cryodamage as compared to control. BHT being lipid perturbant could prevent the increased membrane permeability that accompanied the cryodamage due to cold shock during freezing. Cholesterol was reported to exert multiple effects on sperm membrane that included stabilization of membrane, reduction in membrane permeability, facilitating morphological membrane characteristics and serving as a membrane antioxidant (Crockett 1998). This might explain the beneficial effect of CLC supplementation in the extender either alone or in combination with BHT.

The present investigation also revealed that the mean percentages of sperm motility, viability and plasma membrane intact sperm declined significantly ($P < 0.05$) after freezing as compared to that after equilibration irrespective of additive supplementation (Table 1). 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 (Cremades *et al*. 2005, Sancho *et al*. 2007).

Effect on plasma membrane protein profile

The result of plasma membrane protein profile indicated that the number of protein bands reduced to 13–14 after freezing from 14–18 after equilibration in different groups (Table 2). Two proteins and three proteins of equilibrated semen were not detected after freezing with BHT and CLC supplementation respectively. There was no loss of protein band in BHT plus CLC supplemented group between the stage of after equilibration and after freezing. Likewise, four proteins in equilibrated semen were not detected after freezing in the control group.

It was also observed in the current study that the number of appearance of proteins in altered molecular weight after freezing were 10, 13, 11 and 14 in BHT, CLC, BHT plus CLC and control respectively, when analogy was made with the protein profile after equilibration. Ollero *et al.* (1998) observed that membrane proteins were lost during the freezing-thawing process. Loss of some proteins as well as alteration of molecular weight in some proteins could have occurred during freezing. Dhanju *et al.* (2001) also made similar observations. The study indicated that the number of proteins lost and alteration in molecular weight of protein after freezing was less in BHT, and BHT plus CLC treated group as compared to CLC and control group which indicated the superiority of BHT plus CLC, and BHT supplementation in the extender over CLC in providing protection of sperm plasma membrane protein during cryodamage.

It could be concluded from the present findings that the addition of BHT, CLC, and BHT plus CLC to semen extender before freezing resulted in an improvement of sperm motility, viability, plasma membrane integrity and membrane protein profile. These beneficial effects on sperm quality were more pronounced with BHT plus CLC than with supplementation of BHT and CLC alone. Thus, BHT, CLC, and BHT plus CLC could be supplemented in semen extender for better freezability of boar semen.

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