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A comparative study of cryodamages of boar spermatozoa frozen with conventional liquid nitrogen vapour freezing method

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ABSTRACT

The objective of the present study was to quantify the various cryodamages that boar spermatozoa undergo following freezing with conventional liquid nitrogen vapour freezing method. Sperm-rich fractions of ejaculates (15) collected from 6 boars were utilized for the study. The sperm parameters included for investigating the cryodamages were motile spermatozoa, live spermatozoa, live intact acrosome, plasma membrane intact spermatozoa, HOST-reacted spermatozoa, live spermatozoa with high mitochondrial membrane potential, lipid peroxidised spermatozoa and DNA-damaged spermatozoa. The results showed that the process of freezing significantly decreased per cent motile spermatozoa, live spermatozoa, live intact acrosome, plasma membrane intact spermatozoa, HOST-reacted spermatozoa and mitochondrial membrane potential of live sperm population, while increased per cent lipid peroxidised spermatozoa and DNA-damaged spermatozoa. The extent of cryodamage recorded in respect of motile spermatozoa, live spermatozoa, live intact acrosome, plasma membrane intact spermatozoa, HOST-reacted spermatozoa, live spermatozoa with high mitochondrial membrane potential obtained after freezing was 40.33, 34.87, 35.02, 39.48, 39.17 and 6.72 % respectively. The level of cryodamage in the form of lipid peroxidised spermatozoa and DNA-damaged spermatozoa were 8.00 and 3.06 % respectively. In conclusion, the detrimental effect of cryodamage associated with conventional liquid nitrogen vapour freezing was more pronounced on sperm motility, viability, acrosome integrity and plasma membrane integrity than on sperm mitochondrial membrane potential and membrane lipid, while its effect was minimal on sperm DNA.

Key words: Boar spermatozoa, Conventional freezing, Cryodamage, Cryopreservation

Spermatozoa undergo various cryogenic stresses during the cryopreservation process which result in decreased viability of post-thaw sperm. An increased understanding of cryodamage is of utmost importance that could lead to improved cryopreservation protocol rendering superior quality frozen semen and resultant higher fertility. The *in vitro* evaluation of sperm damage during cryopreservation process primarily encompasses sperm motility, membrane integrity, acrosome status, DNA integrity and fertilizing ability. Additional methods have been developed to assess various freezing damages of sperm at molecular level using fluorescent probes (Guthrie and Welch 2006, Aitken *et al.* 2007). Combination of different tests, rather than employment of a single test, would give comprehensive information with regard to various sperm cryodamages. To the best of our knowledge comparative studies of different cryodamages reckoning different sperm parameters are very scanty. Therefore, the present study was conducted to identify and quantify different cryodamages of boar sperm emanated following conventional liquid nitrogen vapour freezing.

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MATERIALS AND METHODS

The present study was conducted at Artificial Insemination Laboratory of Livestock Production Division of the institute and AAU, Khanapara, Asom. 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), Government of India and Institutional Animal Ethics Committee (IAEC).

Semen collection, processing and freezing: Ejaculates (15) from 6 healthy and fertile boars consisting of 2 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 sperm motility and 80 % sperm with normal morphology with intact membrane were selected for freezing. Semen was processed and frozen according to the straw freezing method (Westendorf *et al.* 1975).

Evaluation of sperm cryodamage: The incidence of cryodamage of spermatozoa was recorded based on the levels of sperm motility, live sperm, live intact acrosome, plasma membrane integrity, mitochondrial membrane potential (MMP), lipid peroxidation (LPO) and DNA integrity after equilibration and after freezing. Frozen semen samples were evaluated for different sperm parameters after thawing at 50°C in water-bath for 12 sec. The extent of cryodamage as per the parameters was estimated in percentage by deducting the value of a particular sperm parameter after freezing from after equilibration in percentage.

Thawed semen (1 ml) 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 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 equipped with 35°C microscope stage and phase contrast optics at a magnification of 400×. Post thaw sperm also evaluated for live sperm by Eosin-Nigrosin staining (Beatty 1957), acrosomal status by Nigrosin-Eosin-Giemsa staining (Tamuli and Watson 1994), sperm plasma membrane integrity by Carboxyfluorescein Diacetate and Propidium Iodide staining (Ortman and Rodriguez-Martinez 1994), hypo-osmotic swelling test (HOST) of spermatozoa in 100 mOsm (Jeyendran *et al*. 1984), mitochondrial membrane potentiality of live spermatozoa by JC-1 staining (Cossarizza *et al*. 1993), lipid peroxidation in sperm by BODIPY C_{-11} staining (Aitken *et al*. 2007) and integrity of sperm DNA by Acridine Orange staining (Thuwanut *et al.* 2008).

Statistical analysis: Data on different parameters were analysed using SPSS, version 16, statistical analysis system. The paired t-test was used to compare the means of equilibrated semen and frozen semen for motility, live sperm, live intact acrosome, HOST-reacted sperm, live sperm with high MMP, lipid peroxidised sperm and DNA-damaged sperm. Differences were considered statistically significant at $P<0.05$. The extent of cryodamage of the parameters was found out in percentage by deducting the value of a particular sperm parameter after freezing from after equilibration in percentage.

RESULTS AND DISCUSSION

In the present study the mean percentage of motile spermatozoa, live spermatozoa, live intact acrosome, plasma membrane intact spermatozoa and HOST-reacted spermatozoa decreased significantly ($P<0.001$) in semen after freezing as compared to that after equilibration at 5°C (Tables 1, 2). The cryodamage of these sperm parameters associated with freezing and thawing process could be attributed to intra- and extra-cellular ice crystal formation and loss of ATP, potassium and enzymes from sperm cells (Hammerstedt *et al*. 1990). Cremades *et al*. (2005) stated that the phases of freezing and thawing induced structural and/or biochemical damage in boar spermatozoa, resulting in a drastic reduction of % motile spermatozoa. The drastic decline in proportion of live sperm could be attributed to cryo-injury following freezing-thawing processes that could cause increase in permeability of sperm plasma membrane

Table 1. Per cent sperm motility, live sperm, live intact acrosome and plasma membrane intact sperm after equilibration (at 5°C) and after freezing (post thaw) of boar semen

Stage of semen	Motility	Live sperm	Live intact acrosome	Plasma membrane intact sperm
After equilibration (a)	$85.33^{a}\pm1.03$	$85.07^{a}\pm1.70$	$81.12^{a}\pm2.07$	$69.67^{a}\pm2.17$
After freezing (b)	$45.00^{b}\pm0.69$	$50.20^{b}\pm1.80$	$46.10^{b}\pm1.65$	$30.19^{b}\pm0.86$
(a) – (b)	40.33	34.87	35.02	39.48

Nos. of observation (n) =15; results are expressed as mean± standard error of means; $^{a, b}$Means bearing different superscripts differ significantly at $P < 0.001$ within a column.

Table 2. Per cent HOST-reacted sperm, live sperm with high MMP, lipid peroxidised sperm and DNA damaged sperm after equilibration (at 5°C) and after freezing (post thaw) of boar semen

Stage of semen	HOST-reacted sperm	Live sperm with high MMP	Lipid peroxidised sperm	DNA damage sperm
After equilibration (a)	$69.20^{a}\pm1.48$	$88.40^{a}\pm0.96$	$8.90^{a}\pm1.11$	$2.88^{a}\pm0.46$
After freezing (b)	$30.03^{b}\pm0.95$	$81.68^{b}\pm1.21$	$16.90^{b}\pm1.88$	$5.94^{b}\pm0.85$
(a) – (b)	39.17	6.72	–8.00	–3.06

Nos. of observation (n) =15; HOST: hypo-osmotic swelling test; MMP: mitochondrial membrane potential.Results are expressed as mean± standard error of means. ^{a,b} Means bearing different superscripts differ significantly at $P < 0.001$ within a column.

leading to rise in the percentage of stained spermatozoa (dead sperm). The present finding of post-thaw live intact acrosome was consistent with the report of Kaeoket *et al*. (2008). Waterhouse *et al.* (2006) also observed that freezing and thawing processes induced a marked reduction in the percentage of live intact acrosome of boar spermatozoa as compared to that of equilibrated semen. The marked reduction in plasma membrane integrity (as evaluated by both CFDA plus PI staining and HOST test) encountered after freezing as compared to that of before freezing in the current study might have been brought about by membrane destabilisation and damage (Hernandez *et al*. 2007, Kim *et al.* 2011).

The process of freezing significantly ($P<0.001$) decreased per cent live sperm with high mitochondrial membrane potential (MMP), while it increased per cent lipid peroxidised spermatozoa and DNA-damaged spermatozoa as compared to that after equilibration (Table 2). This might be ascribed to oxidative damage arising from excessive or inappropriate formation of reactive oxygen species (ROS) during freezing (Kim *et al*. 2011). In the present investigation the reduction of MMP in live sperm subpopulation after freezing, to 81.68±1.21 % from the stage of equilibration (88.40±0.96%) was although significant statistically (Table 2), the difference in percentage was not high (6.72%). The formation of intracellular ROS in the mitochondria was presumably of low magnitude, which did not result in disruption of electron transport for oxidative phosphorylation and thus maintained high MMP required for mitochondrial ATP production (Guthrie and Welch 2006).The freezing process caused a significant increment in lipid peroxidised sperm as compared to equilibrated semen. Guthrie and Welch (2007) reported that the freezing-thawing process increased lipid peroxidation as compared to fresh semen in boar. The current study also revealed that the magnitude of sperm DNA damage after freezing was moderate when compared with other sperm quality parameters evaluated in this study. This could be attributed to the status of boar sperm DNA which remained highly condensed with nuclear proteins especially protamine-1 in the sperm nucleus (Chanapiwat *et al*. 2010). The extent of DNA-damaged sperm after freezing in the current study was higher than that reported by Chanapiwat *et al.* (2010) and lower than that reported by Hu *et al*. (2008).

It was also found in the present study that the percentage of cryodamage in respect of sperm motility (40.33) was the highest followed by plasma membrane integrity (39.48), HOST-reacted sperm (39.17), live intact acrosome (35.02), live sperm (34.87) and live sperm with high MMP (6.72). The increment in percentage of DNA damaged sperm (3.06) after freezing than after equilibration was less as compared to that of lipid peroxidised sperm (8.00). This indicated that the harmful effect of cryodamage in boar was more on sperm motility, viability, acrosome integrity and plasma membrane integrity as compared to sperm mitochondrial membrane potential and sperm membrane lipid, while its effect was minimal on sperm DNA.

It could be concluded from our results that the detrimental effect of cryodamage associated with conventional liquid nitrogen vapour freezing in boar was pronounced more on sperm motility, viability, acrosome integrity and plasma membrane integrity as compared to sperm mitochondrial membrane potential and sperm membrane lipid, while its effect was minimal on sperm DNA. A better insight and understanding of the process of cryodamage would help in further mitigating boar sperm cryodamages that could lead to evolve superior freezing protocol.

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