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Electron microscopic studies of porcine sperm: changes during freezing and post-thawing

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ABSTRACT

The aim of the present study was to characterize the ultrastructural changes in boar spermatozoa at different stages during freezing (fresh undiluted semen, after holding at 24ºC, after cooling to 5ºC and after equilibration at 5ºC) and after thawing using transmission electon microscopy (TEM). The study was also aimed to compare the sperm characteristics observed through conventional staining procedure with that of electron microscopy. A total of 72 ejaculates were collected from 6 Hampshire boars. Pooled semen was extended in BTSLEYG (Beltsvelli thawing solution lactose egg yok glycerol) extender and frozen in 0.5 ml French straws with conventional vapor freezing technique. Results of routine semen evaluation using conventional staining procedures seemed to be excellent. Microscopically assessed post-thaw percentage of progressively motile spermatozoa, live sperm count, spermatozoa with intact acrosome and hypo-osmotic reacted spermatozoa after thawing were 51.38±0.87, 56.83±0.74, 56.83±0.74 and 43.79±0.83**,** respectively. TEM examination of frozen–thawed semen revealed major changes in the morphology of spermatozoa localized predominantly within the acrosome and post acrosomal region of a head. Acrosomal and membrane integrity as observed through conventional statining and TEM was found to be 59.83±0.74 vs 37.88±8.85 and 43.79±0.83 vs 31.42±6.58%, respectively. It may be concluded that freezing and thawing of boar semen resulted into maximum sperm damage and electron microscopy revealed major changes in acrosome and plasma membrane which could not be seen with conventional staining procedure.

Key words*:* Acrosome, Boar sperm, Cryopreservation, Electron microscopy, Ultrastructure

Artificial insemination (AI) in swine has increased exponentially during the past few years using liquid-stored semen. Globally, around 99% of AI was done with liquid extended semen stored for few days between 16 and 20ºC (Wagner and Thibier 2000). The reason that cryo-preserved boar semen is not being used commercially is sub-optimal fertility of frozen boar spermatozoa. Cryopreservation of boar semen is associated with different insults to the spermatozoa, such as cold shock, osmotic stress, cryoprotectant intoxication and intracellular ice crystal formation during freezing and thawing (Prinosilova *et al.* 2012). Boar spermatozoa are one of the most sensitive cell types with respect to sustaining viability during freezing and thawing, with a large proportion of the spermatozoa not surviving these procedures (Prinosilova *et al.* 2012).

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Spermatozoa which survive after freezing and thawing are usually a mixture of cells, some of which survive well while others show modified motility and a shortened lifespan. Insemination with such sperm lead to lowered pregnancy rates and fewer piglets born, compared with AI using liquid-stored semen (Johnson 1985, Johnson *et al.* 2000). However, most of the recent research work on freezing of boar semen reported post thaw motility in the range of 50–60% , but *in-vivo* fertility results have shown either poor conception rate and reduced litter size. Recent researches have shown that in general spermatozoa surviving a freezing-thawing process have an altered membrane structure or altered membrane properties, which may render them functionally similar to capacitated and/or acrosome reacted spermatozoa (Schembri *et al.* 2002, Rodriguez-Martinez and Margareta 2011, Watson 2000). The assessment of the sperm plasma membrane integrity is of major significance because only spermatozoa with a good function of the plasma membrane, allowing water movement in and out of the cell, can survive the preservation processes. It is difficult to identify all morphological changes of spermatozoa during the process of freezing and thawing using microscopic techniques of semen evaluation because it allows observing changes on

the surface and edges of the cells. Transmission electron microscopy (TEM) examination may be the more useful and reliable tool to observe changes in the cell membrane and other inner structure of the cells (Goodman 2001, Rodriguez-Martinez *et al.* 1993, Strom-Holst *et al.* 1998). Therefore, the present study has been conducted to assess the membranal changes in spermatozoa at different stages during the process of freezing and post thawing, both by conventional staining procedures and by transmission electron microscopy, to ascertain the stage which is most critical for freezing of boar semen**.**

MATERIALS AND METHODS

Collection and evaluation of semen: Hampshire boars (6) maintained at Livestock Research Farm, ICAR Research Complex, Barapani, Meghalaya, India, were taken for the study. The boars were fertile and used for breeding purpose. Age of the boars varied from 24 to 36 months. 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 putting gauze inside the Butchner funnel. Immediately after collection, semen was brought to the laboratory in a thermos flask (at 35ºC) for further processing. A total of 72 ejaculates (12 each from 6 boars) were collected and subjected to routine semen evaluation. The semen samples which had more than 75% of initial motility and concentration more than 100 million per ml were subjected for further processing and freezing. Before freezing, the 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 37ºC microscope stage and phase contrast optics at a magnification of 400×. The sperm motility was recorded from 0 to 100 based on percentage of progressively motile spermatozoa. The live sperm count was determined using eosin-nigrosin staining technique. Two hundred spermatozoa were counted in each smear under 1000× magnification to determine the percentage of live spermatozoa. Acrosomal integrity was studied using Giemsa staining (Watson 1975). HOST was performed as per Jayendran *et al.* (1984). The slide was examined under the high power objective (40×) of a phase contrast microscope.

Semen processing and freezing: Freezing was done with conventional vapor freezing technique using BTSLEYE (Beltsvilli Thawing solution lactose egg yolk extender) as described earlier (Khan *et al. 2*012). Briefly, Initial dilution was done with holding fraction of extender (BTS) and was kept in BOD incubator for 3 h at 24ºC. After 3 h, semen was centrifuged and freezing fraction-I (lactose egg yolk; 80 ml of 11% â lactose + 20 ml of egg yolk) was added to make the volume 50% of the final volume. Temperature was reduced gradually from 24ºC to 5ºC within 1-½ h and freezing fraction-II (76 ml of 11% â lactose + 20 ml egg yolk + 6 ml glycerol) was added at 5ºC. Semen was equilibrated for 1 h and filled in French medium straws (0.5 ml). 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 at different stages of processing *viz*; after holding, after cooling to 5ºC, after equilibration and post thawing.

Electron microscopy: The ultra structural changes of sperm plasma membrane and acrosome were studied with the help of transmission electron microscopy (TEM) at different stages of processing and freezing i.e. in fresh spermatozoa, after holding, after cooling, after equilibration and after thawing.

Protocol for transmission electron microscopy (TEM): Samples were processed according to a well-established procedure that has been applied for spermatozoa of humans and several animal species including boars (El-Gothamy and El-Samahy 1992, Strom-Holst *et al. 1*998, Boonkusol *et al. 2*010). An aliquots of spermatozoa of fresh semen, after each stages of processing (viz. undiluted fresh semen, after holding, after cooling and after equilibration and immediately after thawing) were prefixed in 3% glutaraldehyde in 0.1 M cacodylate buffer and postfixed with 1% osmium tetraoxide (OsO_4) in 0.1 M cacodylate buffer for 2 h at 4 ºC. The samples were centrifuged and pellets were dehydrated in increasing acetone concentration. Infiltration was done in the mixture of propidium oxide and embedding medium with increasing concentration of embedding medium and finally in pure embedding medium. Infiltrated samples were transferred into BEEM capsules or embedding moulds and pure embedding medium was poured in to the mould and kept in to the oven at 50 ºC overnight. The blocks were cut on an ultramicrotome and ultra thin serial sections picked up on copper grids. The sections were counterstained with uranyl acetate and lead citrate and examined in microscope at 60 KV. Longitudinal, oblique and transverse sections of spermatozoa were included in the evaluation. At least 100 spermatozoa were observed at each stage to find out the percentage of normal and damaged sperms.

Statistical analysis: Statistical analysis of the data was performed using SPSS software. Sperm characteristics like motility, live sperm count, acrosomal integrity and hypo-osmotic reacted spermatozoa were analyzed by one way analysis of variance (ANOVA) using General Linear Model. If significant difference were identified, Benferroni's adjustment multiple comparison test was used for *post hoc* analysis. Statistical significance was set at $P<0.05$ and all data were presented as the mean ± standard error of the mean (SEM).

RESULTS AND DISCUSSION

Sperm characteristics of fresh undiluted semen: Initial motility of spermatozoa in fresh semen ranged between 80

to 95% with a mean of 88.00 ± 0.72%. The mean live sperm count was 90.25 ± 0.47 which ranged from 84 to 95% (Table 1). The mean acrosomal integrity as measured by% intact acrosome in fresh semen was 90.42 ± 0.56 that ranged from 81 to 96%. The hypo-osmotic reacted spermatozoa ranged from 46.0 to 71.0% with a mean of 61.14 ± 1.03%. Similar results were also obtained in our earlier studies on boar semen (Khan *et al.* 2012). All the parameters are within the range of acceptable limit for a normal fertile boar.

Sperm characteristics during processing and post thawing: The overall mean motility after holding (24°C), cooling (5°C), equilibration (5°C) and post thawing (37°C) was 81.04, 75.0, 72.70 and 51.38% respectively. As expected, there was a gradual reduction in average sperm motility with decrease in temperature from holding to equilibration which further decreased after thawing. There was no significant difference in motility at holding and after cooling (81.04 vs 75.00) but difference in motility at 24 °C and after equilibration (P<0.05) was observed. However, after thawing, the motile sperm percentage was significantly lower than after holding, after cooling and after equilibration stages.

There was no significant difference in average live sperm count was observed between holding, cooling and equilibration stages. A sharp decrease in live sperm count was reported after thawing which was significantly lower than reported after holding, cooling and equilibration stages. Similarly, the average intact acrosome was also decreased as the temperature declines from holding to equilibration but there was no significant difference. However, after thawing, the acrosomal integrity was significantly lower (56.14; P<0.05) than reported after holding, cooling and equilibration.

Average hypo-osmotic reacted sperm was found to be 61.50, 53.04, 50.54 and 43.79% respectively after holding, cooling, equilibration and thawing. There was no significant difference after cooling and equilibration (both were significantly lower than that reported at holding stage). Minimum hypo-osmotic reacted spermatozoa were reported after thawing which was significantly lower than that reported during different stages of processing.

Maximum progressive motility, live sperm count and hypo-osmotic reacted spermatozoa were reported in freshly ejaculated semen. Reduction in all the parameters were reported as the temperature decreases from 24°C to 5°C. However, there was no significant variation in terms of motility, live sperms, acrosoaml integrity between 24°C and 5°C, but hypo-osmotic reacted spermatozoa were significantly reduced at 5°C compared to 24°C, which might be due to sensitivity of boar sperm plasma membrane to osmotic changes and cold shock. It is well documented that the boar spermatozoa are highly susceptible to temperatures below 15°C. The viability of the spermatozoa is dramatically reduced within a few hours after expose to cooling below 15°C (Gilmore *et al.* 1996). Cold shock caused the damage of plasma membranes and alterations in the metabolism of the spermatozoa. This is caused by changes in the arrangement of plasma membrane compositions especially phospholipids (Medeiros e*t al.* 2002). The sperm damage cause by cold shock is characterized by an irreversible loss of motility and the loss of sperm permeability.

After thawing, a significant reduction in all the parameters viz, sperm motility, live sperm count, acrosomal integrity and hypo-osmotic reacted spermatozoa were observed. Mazur (1970) reported that during the process of

Table 1. Sperm characteristics of freshly ejaculated, at different stages during processing for freezing and post thawed boar semen

Sperm semen (%)	Fresh holding	Stages of processing of semen			
		Cooling 24°C	Equilibratin 5°C	Thawing 5°C	37°C
Motility	88.00±0.72	81.04^{a} ± 1.08	75.00^{a}±1.09	72.70^{a}± 1.04	51.38^{b}±0.87
Live sperm count	90.25±0.47	88.29^{a} ± 0.49	82.37^{a}±0.44	80.00^{a} ± 0.50	56.83^{b}±0.74
Acrosomal integrity	90.42±0.56	88.87^{a} ± 0.57	82.25^{a}±0.48	80.00^{a} ± 0.50	56.04^{b}±0.74
HOST reacted spermatozoa	61.14±1.03	60.50^{a}±1.04	53.04^{b}±0.78	50.54^{b} ± 0.89	43.79^{c}±0.83

Table 2. Average (Mean±S.E) of acrosome and plasma membrane integrity of porcine spermatozoa during freezing and post thawing using conventional staining and TEM

Stages	Acrosome integrity		Plasma membrane integrity	
	Geimsa Stain	TEM	HOST test	TEM
Fresh semen	90.42 ± 3.03	86.57 ± 9.32	60.14 ± 5.56^{A}	$85.25 \pm 12.32^{B**}$
After holding	88.87 ± 0.57	77.32 ± 11.25	61.50 ± 1.04^{A}	$81.25 \pm 10.58^{B**}$
After cooling	82.25 ± 0.48^{A}	$72.25 \pm 6.58^{B*}$	53.04 ± 0.78^{A}	$63.22 \pm 5.65^{B*}$
After equilibration	80.00 ± 0.50^{A}	$57.12 \pm 11.32^{B*}$	50.54 ± 0.89^{A}	$60.46 \pm 9.65^{B*}$
Post thawing	59.83 ± 0.74^{A}	$37.88 \pm 8.85^{B*}$	43.79 ± 0.83^{A}	$31.42 \pm 6.58^{B*}$

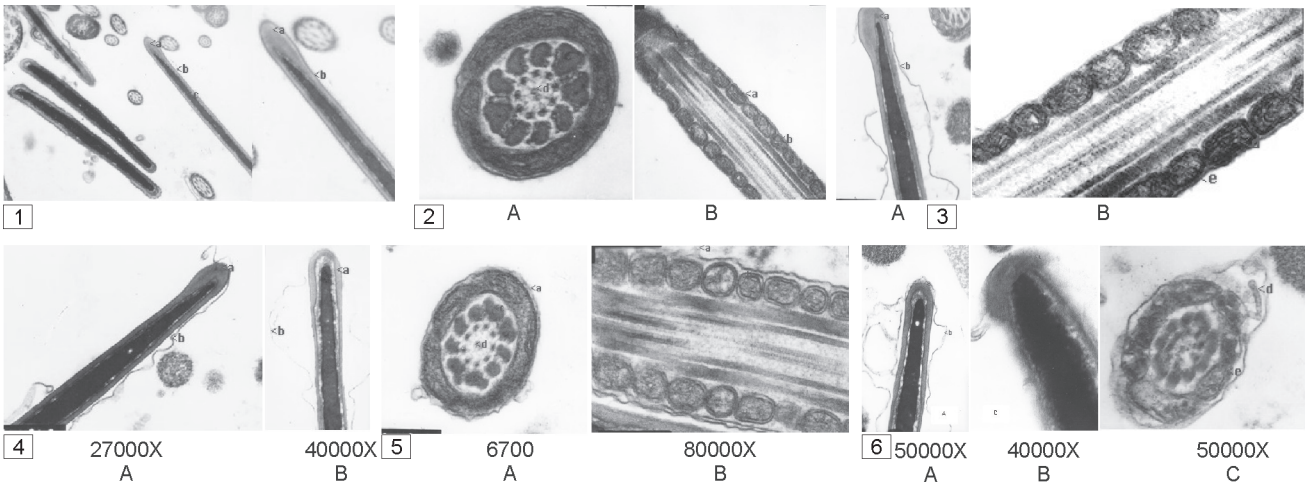
values having different superscripts in a row differ significantly for a particular traits (*P<0.05; **P<0.01).

freezing, the decrease of temperature from -15°C to -6°C causes sperm damage. This is caused by the intracellular ice formation and cellular dehydration (osmotic stress).

Comparative study of acrosome and membrane integrity (conventional staining vs TEM): Aliquots of spermatozoa were taken at various stages of processing and post thawing and acrosome and membrane integrity were assessed by Giemsa staining and HOST test. The results of the conventional staining were compared with TEM results. Significantly lower percentage of intact acrosome was observed with TEM than in conventional procedure as temperature drops to 5°C. However, no differences were noticed in fresh semen and after holding at 24°C (Table 2). Lower incidences of intact acrosome by conventional procedure reflect that sperms having slightly damaged acrosome or ruffled acrosomes were not detected by conventional staining procedure. On the contrary, the significantly higher percentage of spermatozoa showing intact plasma membrane was observed under TEM than the sperms showing HOST reaction under hypotonic solution. Lower percentage of HOST reacted sperms under HOST test might be due to use of 150 mOsm solution used for HOST test in this experiment instead of 100 mOsm.

Ultrastructural changes in spermatozoa: TEM of semen samples at different stages revealed a mixture of cells, some were normal and other showed various degree of cell damage including plasma membrane, acrosome and mitochondria. Degree and percentage of cell damage varied depending upon the stage of processing. Most of the spermatozoa (82.25%) in fresh semen were having intact plasma membrane over head and midpiece. Acrosome and acrosomal apical ridge were normal and intact in 86.57% spermatozoa (Table 2). Both outer and inner acrosomal membranes were clearly visible. Nucleus was electron opaque (Figs 1, 2). Intact plasma membrane over the head and middle piece, intact acrosome, acrosomal ridge and electron opaque nucleus were the common features found in majority of the spermatozoa in freshly collected semen. The present findings was in close agreement with Akiko *et al.* (2001) who reported that more than 90% of the fresh spermatozoa had intact acrosome, intact plasma membrane and outer acrosomal membrane and firm and clear matrix in cynomolgus monkeys. Prinosilová *et al*. (2012) also reported 98.1 ± 2.95% spermatozoa having intact acrosomal membranes in fresh boar semen.

After holding at 24°C, most of the cells (81.25%) showed intact plasma membrane over the head region but in few cells swollen and separating plasma membrane were also seen. Over the middle piece, the plasma membrane was intact and tightly attached. 77.32% spermatozoa showed normal intact acrosome. No change in acrosomal content was noticed. Both outer and inner acrosomal membranes were clearly visible. Mitochondria were intact and arranged linearly (Fig. 2). No significant changes in sperm head and middle piece was observed following holding. Intact plasma membrane over the head region, intact acrosome and acromal content observed in in freshly collected spermatozoa in the present study were also observed in spermatozoa held for 3 hours in BTS. Bwanga *et al*. (1990) also reported no alteration in the sperm plasma membrane, acrosome and acrosome apical ridge in boar spermatozoa after dilution. However, in few cells swollen and separating plasma membrane were noticed. This was in good agreement with the findings of Courtens and Paquignon



Figs 1–6. **1.** Boar spermatozoa with (a) normal acrosome (b) intact plasma membrane and (c) nucleus **2.** (A) Transverse section of middle piece of boar spermatozoa showing (a) intact plasma membrane (b) mitochondria (c) outer dense fibrils and (d) axoneme (B) Longitudinal section of middle piece of spermatozoa showing (a) intact plasma membrane (b) mitochondria **3.** Boar spermatozoa after cooling at 5 °C showing (a) intact acrosome (b) swollen and ruptured plasma membrane and (c) opaque nucleus (B) Middle piece of spermatozoa showing (d) intact mitochondria and (e) intact plasma membrane.**4** A, B. (a) swollen acrosome and loss of acrosomal contents and (b) swollen and ruptured plasma membrane. **5** A, B. Transverse and longitudinal sections of boar spermatozoa showing (a) damaged plasma membrane over middle piece and (b) dissolution of mitochondrial matrix (c) normal outer dense fibrils and (d) intact axoneme. **6** A,B,C. TEM of boar spermatozoa after thawing showing (a) loss of acrosomal contents (b) ruptured and swollen plasma membrane over sperm head (c) Damaged acrosomal apical ridge (d) Ruptured and swollen plasma membrane over mitochondria and (e) Thinning of mitochondrial matrix.

(1985) who reported abnormal acrosome (11.2% cells), elevated plasma membrane, loss of acrosomal membrane and acrosomal contents in boar spermatozoa after holding.

After cooling to 5°C, a sizable number of spermatozoa (63.22%) showed separating plasma membrane over the head region while in few the plasma membrane was found ruptured (Fig. 3) Acrosome, acrosome apical ridge and mitochondria were normal in most of the cells (72.25%). Few cells showed swollen acrosome. Plasma membrane was found intact over the middle piece. A sizable number of spermatozoa when examined after cooling showed separating or ruptured plasma membrane over the head but intact over middle piece. Swelling of acrosome as observed in some spermatozoa in the present study was comparable with the findings of Pursel *et al.* (1973) who reported 70–80% cells with intact acrosomes after exposure to 5°C.

In many spermatozoa examined after equilibration the plasma membrane over the head and middle piece was swollen and ruptured (Fig. 4). Only 60.46% spermatozoa showed intact plasma membrane over head and middle piece. 57.12% spermatozoa showed intact acrosome. However, few cells showed swelling of acrosome and loss of acrosomal contents. The mitochondria were normal and arranged linearly. Clear 9 + 2 pattern of axoneme was seen. Boar spermatozoa examined after equilibration showed swollen and ruptured plasma membrane in about 35–40% of the cells. Bwanga *et al*. (1990) reported swollen plasma membrane in most sectioned spermatozoa. On the contrary, Courtens and Paquignon (1985) reported majority of normal sperm after cooling and equilibration and very few swollen acrosomes. Higher incidence of swollen and ruptured plasma membrane observed after equilibration in the present study might be due to lower osmolarity (320 mOsm) of lactose egg yolk glycerol extender (Uzu *et al. 1*976). Rupture of plasma membrane over the middle piece recorded in the present study supported the view that plasma membrane over the middle piece and tail was less readily lost but frequently broken (Watson and Plumer 1985).

Ultrastructural changes of head and middle piece in boar spermatozoa after freezing and thawing are presented in Figs 5-6. Majority of the cells showed swollen, separating and ruptured plasma membrane over the head region. Only 31.42% spermatozoa showed intact plasma membrane after thawing. Fusion of plasma membrane with outer acrosomal membrane and formation of large spaces between plasma membrane and outer acrosomal membrane were commonly seen. 37.88% spermatozoa showed intact acrosome. Most of the cells showed swollen acrosome while, few cells showed loss of acrosomal contents and dissolution of acrosomal apical ridge. Thinned mitochondrial matrix was seen.

Similar acrosomal changes were also reported by Larsson *et al*. (1976) in almost all the frozen thawed spermatozoa. Similarly, Akiko *et al*. (2001) reported only 21.2% spermatozoa with normal acrosome, 36.4% with vesiculated acrosome and 28.8% with swollen acrosome following freezing and thawing in cynomolgus monkey spermatozoa. The biophysical changes brought about by the transition of liquid water to ice during the relatively slow cooling are the main causes for the sperm damage (Rodriguez-Martinez and Margareta 2011). During cryopreservation, sperm undergo successive and opposite changes in cell volume caused by osmotic changes during glycerolization, freezing and thawin (Hammertedt *et al. 1*990). Moreover, the fluidity and functional properties of the sperm membranes are modified as the temperature decreases during the cooling and freezing phases, as well as during thawing (Maxwel and Johnson 1997), reducing their selective permeability, and ultimately leading to a major influx of calcium (Bailey *et al. 2*003). The plasma membrane is thus considered to be the primary site of cold induced damage.

The percentage of intact plasma membrane over head and middle piece in TEM was higher than the hypo-osmotic reacted spermatozoa (HOST test) at all stages of processing including fresh semen might be due to higher osmolarity of HOST solution (150 mOsm) used in the present study.

Other ultrastructural changes of frozen thawed boar spermatozoa in the present study were formation of large spaces between plasma membrane and outer acrosomal membrane, presence of space between inner acrosomal membrane and nucleus, fuzzy appearance of acrosome and complete detachment of acrosome. These changes might be considered as indication of false acrosome reaction. Similar change were also observed by Akiko *et al*. (2001) in frozen thawed cynomolgus monkey spermatozoa and Nizanski and Kuropka (2005) in frozen thawed dog spermatozoa. Thinned mitochondrial matrix as observed in frozen thawed spermatozoa in the present study was also reported by Watson and Plummer (1985) in boar semen after freezing and thawing. Boar sperm plasma membrane are thought to be more sensitive to cold shock than those of other mammals because they have a lower cholesterol:phospholipid ratio that results in liquid to gel membrane phase transition at relatively higher temperatures during cooling, thereby rendering them more susceptible to damage earlier in the cooling process (Drobins *et al. 1*993). Accordingly, a temperature related loss of cholesterol should more readily induce such capacitation-like changes in pig sperm from other animals.

Results obtained in the electron microscopic study clearly indicated that the procedure of cryopreservation of boar semen exerts major ultrastructural changes especially in the region of the acrosome. It was difficult to identify that the acrosomal changes occurred due to false acrosome reaction or true acrosome reaction because of the presence of large number of dead sperm post thawing. Therefore, the exact condition of the acrosome in cryopreserved boar spermatozoa remains unclear. It may be concluded that the electron microscopy discovered a noticeably higher level of membrane damage than optical microscopy.

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