BOAR SPERM HEAD MEMBRANE DAMAGE DURING CRYOPRESERVATION EVALUATED BY ELECTRON MICROSCOPY

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Abstract

As electron microscopy can reveal much smaller changes in plasma membrane integrity than optical methods, it appears to be a useful tool for evaluating semen quality or the damage inflicted on spermatozoa during preservation. By evaluating membrane integrity of the sperm head in 16 boars by transmission electron microscopy (TEM), it was found that the plasma membrane is very sensitive and tends to break in the acrosomal area but not in the rest of the sperm head. The earliest signs of injury to spermatozoa in the acrosomal part of the sperm head were observed in the plasma membrane that became swollen, broken or lost. The next damaged part of spermatozoa which is less sensitive than the plasma membrane is the acrosome and its external membrane. The freezing process caused a substantial increase in the degree of cell damage manifested as disturbed or missing plasma membrane, acrosomal reaction-like changes and unevenly distributed or lost acrosomal contents. The latter was not found in fresh semen. Using an appropriate buffer concentration for the fixatives applied during preparation of semen samples for TEM appeared as a very important factor due to the impact of their osmolality on plasma membrane integrity of sperm cells.

Key Words: Boar, sperm, membrane, TEM, ultrastructure

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. Therefore, the cryopreservation process results in reduced fertility compared with fresh semen. It has been known for many years that boar spermatozoa are extremely sensitive to cold shock but become more resistant during incubation, possibly as a result of membrane modification (Pursel et al, 1973).

For the prediction of semen quality from an aspect of functionality, 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. Boar spermatozoa are characterized by marked interindividual differences in their resistance to freezing. However, routine methods for the evaluation of sperm plasma membrane integrity are considered insufficient to determine the sensitivity of sperm cells to cold shock. Accordingly, finding of a method showing whether sperm can or cannot sustain cryopreservation would help develop novel preservation procedures. As it is relatively simple to estimate the proportions of sperm with the plasma membrane breakage over the acrosome and these vary from ejaculate to ejaculate, it has been proposed that electron microscopy may be a useful tool for evaluating semen quality or the damage occurring to spermatozoa during storage for artificial insemination (Jones, 1971).

The aim of our study was to evaluate the sperm head membrane integrity in fresh boar semen and the level of its damage during cryopreservation by transmission electron microscopy in relation to other parameters of semen analysis.

Materials and methods

Ejaculates from 16 boars were used in the experiment. Fresh ejaculates were evaluated by routine sperm analysis and electron microscopy and then were frozen in straws using the procedure described by Westendorf et al. (1975), as modified by Thurston et al. (1999). The standard semen analysis included semen volume, sperm concentration, total sperm motility and progressive motility, and plasma membrane integrity (viability) – evaluated by eosin nigrosin staining (World Health Organization 2010). Sperm concentration was measured in a Bürker chamber, sperm motility was analysed under an optical microscope at 200x magnification. Total sperm count was calculated by multiplying volume by sperm concentration. The acrosome-intact sperm rates were established by lectin *Pisum sativum* staining (Mortimer 1994). Sperm morphology was evaluated according to Tygerberg's strict criteria (Kruger et al. 1986). Samples were stained for sperm morphology analysis according to Farelly (smears were fixed in 3.5% formalin and stained with 5% aniline blue for 10 s and 0.5% crystal violet for 6 s) and evaluated with the use of the SASMO computer program (Strict Analysis of Sperm Morphology; Veznik et al., 2001). The analysis of sperm motility, viability, acrosomal integrity and

sperm morphology and the assessment by electron microscopy was done also after thawing of the semen samples.

Sperm cryosurvivability was determined as a percentage of motile spermatozoa that survived the freezing process: % of frozen/thawed motile sperm/% of fresh motile sperm * 100.

High cryosurvival rate (good freezers): ≥ 50%. Low cryosurvival rate (bad freezers): < 50%.

Methods of sample preparation for transmission electron microscopy

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., 1998; Boonkusol, 2012). Spermatozoa were centrifuged, fixed in 0.3 M glutaraldehyde in 0.1 M cacodylate buffer for 2 h and postfixed in 0.04 M osmium tetroxide in 0.1 M cacodylate buffer. Afterwards, the samples were embedded in agar blocks, dehydrated in graded ethanol series and embedded in araldite resin (Durcupan ACM, Fluka). Ultrathin sections (60 nm thick) were cut using the Leica EM UC6 ultramicrotome and stained with uranylacetate and lead citrate. The sections were examined under the FEI Morgagni 268D electron microscope (FEI Company). For quality assessment, the morphology of at least 100 spermatozoa was evaluated for each sample.

Longitudinal, oblique and transverse sections of sperm heads were examined to analyze the plasma membrane (PM) and acrosomal contents. Only acrosomal part of the sperm head was thoroughly evaluated, the rest of the sperm head appeared intact in all cases.

Examined categories: (1) spermatozoa with intact membranes (including swollen PM); (2) spermatozoa with defects of PM; (3) spermatozoa with bare acrosome (without PM); (4) spermatozoa with acrosome reaction-like changes (including spermatozoa with acrosome loss - after finishing the acrosome reaction) and (5) spermatozoa with acrosomal material unevenly distributed or lost, but covered with the outer acrosomal membrane.

All statistical analyses were performed with SPSS software (Version 18.0 for Windows, SPSS Inc., Chicago, IL, USA). If the data were normally distributed according to the Kolmogorov-Smirnov test, the Student's *t*-test and the paired *t*-test were used for comparison between groups. The nonparametric Mann-Whitney U test and Wilcoxon signed rank test were used in the case of violation of normal distribution. Spearman's correlation was used to assess the relationship between sperm parameters. The p-values of < 0.05 and < 0.01 were considered statistically significant.

Results and Discussion

The plasma membrane is very sensitive and tends to break in the acrosomal area but not in the rest of the sperm head. The earliest signs of injury to acrosomal part of the sperm head were observed on the plasma membrane which became swollen, broken or lost. The next damaged part of spermatozoa which is less sensitive than the plasma membrane is the external acrosomal membrane. That manifested itself as vesiculation of the acrosomal and plasma membranes (acrosome reaction-like changes) and uneven distribution and loss of acrosomal contents. In fresh ejaculates, 98.1 ± 2.95% spermatozoa were found to have intact acrosomal membranes. After freezing, the number of spermatozoa presenting a normal acrosome was significantly decreased (67.8 ± 8.94%). These values corresponded to the numbers of intact acrosomes assessed by *Pisum sativum* staining (91.2 ± 5.56% and 68.9 ± 5.37% in fresh and frozen/thawed samples, respectively). Considerable numbers of spermatozoa with unevenly distributed acrosomal contents but covered with a dilated acrosomal membrane were only found in frozen/thawed semen samples (25.4 ± 7.43%). There were no spermatozoa showing this defect in fresh ejaculates. Similar patterns of membrane damage appearing during preservation of semen from different animal species were also found by other authors (Jones and Steward, 1979; Plummer and Watson, 1988; Hofmo and Andersen Berg, 1989; Ström Holst et al., 1998).

All the evaluated parameters measured in both routine semen analysis and obtained by the assessment of sperm head membranes in good and bad freezers by electron microscopy are shown in Tables 1 and 2. The average cryosurvival rates of good and bad freezers were 63.8 ± 12.13% and 33.2 ± 14.41%, respectively. As we probed for relationships between sperm characteristics of fresh ejaculates, we found that neither most parameters of routine sperm analysis (except for sperm viability) nor the percentage of membrane-intact spermatozoa assessed by electron microscopy correlated with sperm cryosurvivability. No differences were found between good and bad freezers in any of the sperm membrane characteristics evaluated by TEM. Moreover, the electron microscopy evaluation of plasma membrane integrity did not correlate with its optical evaluation either in fresh or frozen semen samples. Only 13.4 ± 4.08% of spermatozoa did not show any ultrastructural defects of the membrane after freezing. A high percentage of spermatozoa with damaged membrane was seen even in fresh semen (49.0 ± 14.93%) compared to 69.8 ± 6.26% of viable spermatozoa assessed by eosin-nigrosin staining. A possible reason, besides the fact that electron microscopy can reveal smaller changes in plasma membrane integrity than optical methods, could be the use of a fixation solution with unsuitable osmolality during semen processing for electron microscopy. Due to the excessively low osmolality of the fixative, water entered the cells and subsequently caused swelling and rupture of the plasma membrane. The idea could be supported by the presence of a high number of spermatozoa with intact plasma membrane which was found to be swollen. This explanation is in accordance with the results of Jones (1971) who tested the effects of buffer concentrations in fixatives for boar spermatozoa and found that reduction in the cacodylate buffer concentration (related to reduction of its osmolality) caused the membrane to separate and break.

Figure 1. Fresh boar spermatozoa
A/ Intact PM (including spermatozoa with intact but swollen PM)
B/ Plasma membrane defects

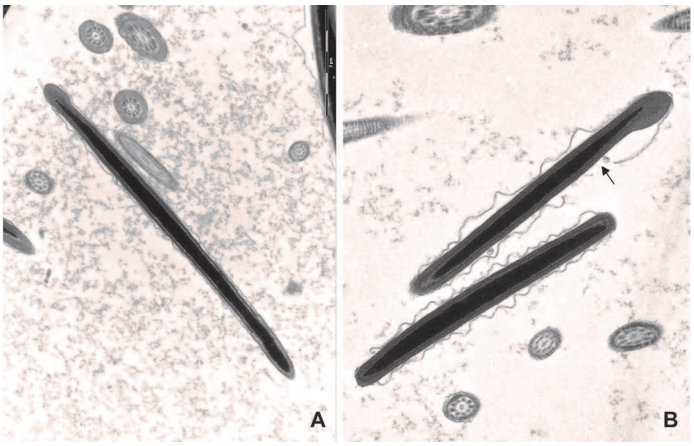

Figure 2. Boar spermatozoa after thawing
A/ A high number of bare acrosomes (without PM
B/ Vesiculation of the outer part of the acrosomal membrane and plasma membrane
C/ A complete loss of the acrosome, bare nucleus (black arrow)
D/ Acrosomal membrane is dilated (white arrow) and the acrosomal material unevenly distributed or lost (black arrow)

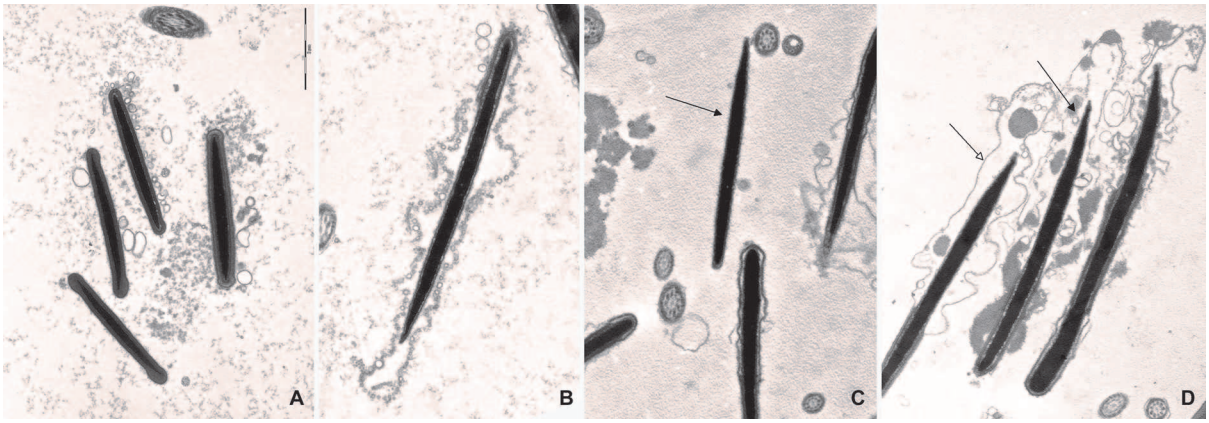

Table 1. Parameters of routine semen analysis for fresh and frozen boar semen varying in cryosurvivability (n=16)

		Motility [%]	Progressive motility [%]	Viability [%]	Morph. normal sperm [%]	Acrosomal integrity [%]
Good freezers	Fresh semen	63.8 ±8.58^{A} (50.0-75.0)	51.6 ± 8.07^{A} (40.0-63.0)	73.4 ± 4.85^{A,d} (66.5-81.5)	76.1 ± 7.22^{A} (66.5-87.5)	91.8 ± 4.17^{A} (84.0-97.0)
	Frozen semen	40.3 ± 6.80^{A,C} (30.0-53.0)	36.9 ± 7.14^{A,C} (26.0-50.0)	52.5 ± 9.04^{A,C} (42.0-66.5)	47.6 ± 9.00^{A} (34.0-55.5)	72.4 ± 4.22^{A,C} (65.5-78.5)
Bad freezers	Fresh semen	62.3 ± 12.36^{B} (40.0-76.0)	51.3 ± 18.50^{B} (15.0-73.0)	66.3 ± 5.61^{B,d} (58.5-75.5)	74.6 ± 11.42^{B} (48.5-84.0)	90.6 ± 6.95^{B} (75-96)
	Frozen semen	20.3 ± 9.91^{B,C} (3.0-35.0)	16.0 ± 9.97^{B,C} (1.0-30.0)	39.6 ± 4.43^{B,C} (33.0-47.0)	49.1 ± 10.64^{B} (32.0-60.5)	65.5 ± 4.12^{B,C} (60.5-72.5)

Within columns, values marked with the same letter differ significantly (^{A} $p < 0.01$, ^{a} $p < 0.05$)

Table 2. Signs of sperm injury, observable by TEM, that occurred in the plasma membrane and acrosome in fresh and frozen/thawed semen of boars varying in cryosurvivability (mean ± SD and range, n=16)

		Intact membranes	Defects of PM	Loss of PM (bare acrosome)	Intact acrosome	Acrosome reaction-like changes	Disruption of acrosomal material
Good freezers	Fresh semen	42.8 ± 15.31^{A} (18.0-63.0)	53.1 ± 15.99^{A} (33.0-80.0)	2.8 ± 2.04^{A} (0-6.0)	99.0 ± 1.93^{A} (95.0-100.0)	1.0 ± 1.93^{A} (0-5.0)	0^{A}
	Frozen semen	11.9 ± 2.36^{A} (9.0-16.0)	38.1 ± 8.39^{A} (25.0-47.0)	21.0 ± 5.66^{A} (13.0-28.0)	70.9 ± 10.13^{A} (54.0-83.0)	5.9 ± 4.12^{A} (1.0-12.0)	23.3 ± 8.60^{A} (13.0-34.0)
Bad freezers	Fresh semen	55.3 ± 12.44^{B} (32.0-69.0)	36.8 ± 10.85^{B} (24.0-53.0)	3.8 ± 3.68^{B} (0-10.0)	97.1 ± 3.60^{B} (90.0-100.0)	2.9 ± 3.60^{B} (0-10.0)	0^{B}
	Frozen semen	15.0 ± 4.96^{B} (8.0-21.0)	27.9 ± 6.60^{B} (19.0-36.0)	21.9 ± 7.61^{B} (15.0-37.0)	64.8 ± 6.86^{B} (54.0-72.0)	7.8 ± 2.66^{B} (5.0-13.0)	27.5 ± 5.83^{B} (21.0-38.0)

Within columns, values marked with the same letter differ significantly (^{A} $p < 0.01$, ^{a} $p < 0.05$)

Conclusion

Electron microscopy discovered a noticeably higher level of membrane damage than optical microscopy. The freezing process caused an extensive increase in the degree of damage to the cells manifested as disturbed or missing plasma membrane, acrosome reaction-like changes and unevenly distributed or lost acrosomal contents. Due to the fact that even fresh ejaculates displayed a very high percentage of cells with damaged plasma membrane, the probable reason of such a high damage could be the use of fixatives with osmolality that is suitable for human but extremely low for boar spermatozoa.

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