IS THE PHOSPHORYLATION STATUS OF TYROSINE PROTEINS A MARKER FOR THE CRYO-CAPACITATION OF BOAR SPERMATOZOA?

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Abstract

The phosphorylation of tyrosine protein residues in spermatozoa was dependent on the semen of individual boars at different stages of the cryopreservation technology. Sperm proteins in the fresh semen of a boar with poor semen freezability (boar K) exhibited a higher content of phosphotyrosine residues compared to boars with better semen freezability (boars F and J). In the semen samples extended in a Kortowo 3 extender (K3) and cooled at 16°C, there was a marked increase in protein phosphorylation in the sperm proteins of boars with good semen freezability. This was manifested in the appearance of phosphoproteins with molecular weights of 17, 32, 43, 52, 63 and 78 kDa. In the case of boar K, the cooled-storage of K3-extended semen at 5°C caused the extensive phosphorylation of sperm proteins, with molecular weights of 45, 65 i 100 kDa. A gradual reduction in sperm protein tyrosine phosphorylation was detected in the extender containing lipoprotein fraction isolated from ostrich egg yolk (LPFo) compared with the K3 extender, what has no protective substances. It might be suggested that seminal plasma acid phosphatases, especially the vesicular molecular form of acid phosphatase (PTAP), played a very important role in the regulation of tyrosine phosphorylation in boar sperm plasmalemma proteins. Differences were observed in the number of dephosphorylated proteins by different molecular forms of phosphatases. It was shown that phosphotyrosine residues in sperm proteins could be completely dephosphorylated by the vesicular PTAP. It seemed that only sperm phosphotyrosine proteins, with molecular weights of 17, 32, and 63 kDa, could be dephosphorylated by the epididymal molecular form of acid phosphatase.

Key words: boar, semen, phosphorylation, phosphotyrosine, cryopreservation.

Protein tyrosine phosphorylation in the sperm plasma membrane is associated with the capacitation of spermatozoa from several mammalian species (12). Cryopreservation of semen causes capacitation-like changes to spermatozoa, termed “cryo-capacitation” (2, 6). This phenomenon is associated with an increased sperm capacitation, reorganisation and destabilisation of plasmalemma, generation of reactive oxygen species (ROS), and phosphorylation of tyrosine residues in sperm proteins (1). Boar spermatozoa are very susceptible to cold shock, and there are individual differences in semen freezability. Furthermore, the applications of specific methods to protect the sperm structure and the effective assessment of the semen quality have enabled the detection of boar-to-boar variability in post-thaw semen quality. The supplementation of lipoprotein fractions isolated from ostrich egg yolk (LPFo) to boar semen during liquid storage has been shown to protect the sperm membranes, resulting in reduced phosphorylation of the tyrosine residues of sperm proteins (15).

The aim of this study was to analyse changes in the phosphorylation of tyrosine residues of boar sperm membrane proteins at different stages of the cryopreservation procedure. The effect of the molecular form of acid phosphatases, isolated from boar seminal plasma, on the phosphorylation of tyrosine residues of proteins of cryopreserved spermatozoa was also studied.

Material and Methods

Freezing of semen. Whole ejaculates (n=9) were collected from three sexually mature boars (F, J and K) using the gloved-hand technique. The boars were fed a commercial porcine ration and were kept in individual pens, under standard environmental conditions. Local ethics committee approval was obtained for this study.

The boar semen was frozen according to the standard freezing protocol (9), with modifications. After collection, the semen samples were extended in a boar semen extender (Kortowo 3, K3) and allowed to stand in a thermobox at 16°C for 3 h. Following storage, the samples were centrifuged at 800 x g for 10 min, and the sperm pellets were resuspended in an extender containing 11% lactose and 5% lyophilised LPFo (lactose-LPFo extender). The extraction procedure of the
LPFo fraction has been described elsewhere (10). The samples were cooled at 5°C over a 3 h period before further dilution with an extender consisting lactose, LPFo, glycerol, and Orvus Es Paste (OEP). The semen samples frozen in the K3 extender, without the addition of cryoprotective substances, were used as the control. The semen samples were packaged in aluminium tubes and frozen using a computer freezing machine (IceCube 1018, Sy-Lab, Austria). The analysis of sperm motility with and without caffeine (CASA), plasmalemma integrity (carboxylfluorescein diacetate/propidium iodide, CFDA/PI), and mitochondrial status (Rhodamine 123/propidium iodide, R123/PI) was conducted in fresh and post-thawed semen (3-5).

**Preparation of sperm extracts.** The sperm samples, collected at different stages of the cryopreservation procedure, were washed by centrifugation three times with 0.85% NaCl, and resuspended in a lysis buffer containing 10 mM Tris-HCl, 0.2 mM sodium orthovanadate, 1% (w/v) SDS, pH 7.4. The lysates were heated for 5 min at 95°C and centrifuged (10,000 x g for 10 min). The resulting supernatant was used for electrophoretic and Western blotting analyses.

**SDS-PAGE and immunoblotting.** The sperm extracts were subjected to SDS-PAGE electrophoresis on 12% polyacrylamide gels (7). The immunoblotting technique with biotinylated anti-phosphotyrosine monoclonal antibodies (Sigma) was used to identify tyrosine-phosphorylated proteins. After the SDS-PAGE electrophoresis and Western blotting, the Immobilon P membrane (Millipore) was treated according to a standard protocol provided by the manufacturer. The immunoreactive fractions were detected by a standard avidin-alkaline phosphatase/BCIP (5-bromo-4-chloro-3-indolyl phosphate)/NBT (nitro blue tetrazolium) detection system (Sigma). The protein tyrosine acid phosphatase (PTAP) and epididymal molecular form of phosphatase (PTAP) were isolated from the acid phosphatase were isolated from the boar seminal plasma according to a previous method (16, 17). The effect of the plasma according to a previous method (16, 17). The acid phosphatase were isolated from the boar seminal plasma according to a previous method (16, 17).

**Results**

There were differences in the semen freezability among the boars. Some selected parameters of semen quality, before and after freezing-thawing, are shown in Table 1. Among the boars, the lowest sperm motility in fresh and frozen-thawed semen was shown in boar K, with and without caffeine. Similar results were obtained for the percentage of spermatozoa with functional mitochondria. The highest percentage of spermatozoa with intact plasmalemma was obtained for boar F. According to the results presented, boars F and J were qualified as “good freezers”, whereas boar K, characterised by poor semen freezability, was qualified as a “bad freezer”.

The fresh semen of boars with good freezability (“good freezers”) was characterised by a low content of phosphotyrosine residues in the extracted sperm proteins (Fig. 1A, lane 1). Following pre-incubation at 16°C (Fig. 1A, lane 2) and extension in the K3 extender (Fig. 1A, lane 3), a marked increase in protein phosphorylation was manifested in the appearance of various phosphoproteins with different molecular weights (17, 32, 43, 52, 63 and 78 kDa). However, a gradual reduction in protein phosphorylation was detected in lactose-LPFo extender (Fig. 1A, lane 5), compared to the K3 extender (Fig. 1A, lane 4) during cooling at 5°C. The sperm proteins in the fresh semen of boar K (the “bad freezer”) exhibited a high content of phosphotyrosine residues (Fig. 1B, lane 1) compared with the “good freezers” (boars F and J). No marked changes in sperm protein tyrosine phosphorylation were observed after the cooling of the extended boar K semen in the K3 extender at 16°C (Fig. 1B, lane 2 and 3). The incubation of the extended semen at 5°C caused an increase in protein tyrosine phosphorylation of 45, 65 and 100 kDa proteins. There was no significant LPFo effect on the phosphorylation of tyrosine residues in the sperm extracts (Fig. 1B, lane 5).

### Table 1

<table>
<thead>
<tr>
<th>Boar</th>
<th>Sperm motility (%)</th>
<th>Spermatozoa with intact plasmalemma (%)</th>
<th>Spermatozoa with functional mitochondria (%)</th>
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<tr>
<td></td>
<td>without caffeine</td>
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<td></td>
<td>1</td>
<td>2</td>
<td>1</td>
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<tr>
<td>F</td>
<td>73.3&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>24.7</td>
<td>78.3&lt;sup&gt;abc&lt;/sup&gt;</td>
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<tr>
<td>J</td>
<td>78.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.5</td>
<td>83.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>K</td>
<td>71.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.3</td>
<td>76.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
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1 – fresh whole ejaculate; 2 – post-thawed, mean ± S.E.M.
<sup>abc</sup> - values within the same column with different letters are significant (P≤0.05)

**Statistical analysis.** The values were expressed as the mean ± standard error of the mean (S.E.M). All the results were analysed with a Student’s t-test, and a value of P≤0.05 was chosen as an indication of statistical significance.
Fig. 1. Effect of ejaculates of individual boars on the electrophoretic profile of tyrosine phosphoproteins in sperm extracts, in relation to different stages of the cryopreservation procedure.

std - biotinylated molecular weight standards (Sigma)
1 - fresh whole ejaculate
2 - pre-incubated and cooled whole ejaculate at 16°C
3 - extended in K-3 extender and cooled for 3 h at 16°C
4 - resuspended in K-3 extender after centrifugation and cooled for 3 h at 5°C
5 - resuspended in lactose-LPfo extender after centrifugation and cooled for 3 h at 5°C
6 - control (K-3 extender only) after post-thaw
7 - lactose- LPfo extender with glycerol and OEP after post-thaw

Fig. 2. Electrophoretic profiles of proteins containing phosphotyrosine residues in sperm extracts without incubation (A) and after incubation with epididymal acid phosphatase (B) and seminal vesicle PTAP (C), in relation to the different stages of the cryopreservation procedure.

std - biotinylated molecular weight standards (Sigma)
1 - fresh whole ejaculate
2 - pre-incubated and cooling of whole ejaculate at 16°C
3 - extended in K-3 extender and cooled for 3 h at 16°C
4 - resuspended in K-3 extender after centrifugation and cooled for 3 h at 5°C
5 - resuspended in lactose-LPfo extender after centrifugation and cooled for 3 h at 5°C
6 - control (K-3 extender only) after post-thaw
7 - lactose- LPfo extender with glycerol and OEP after post-thaw

This phenomenon might be associated with the impaired interaction of spermatozoa with the seminal plasma and LPfo at different stages of the cryopreservation procedure. The sperm proteins from boars with good semen freezeability exhibited a low level of tyrosine phosphorylation after freezing-thawing, compared with the boar showing poor semen freezeability (Figs 1A and B, lane 7).

It was demonstrated that phosphotyrosine proteins from sperm extracts can be dephosphorylated by the molecular form of acid phosphatase isolated from boar seminal plasma. Following the incubation of sperm extracts with the isolated epididymal molecular form of acid phosphatase, the 43, 52 and 63 kDa phosphoproteins were observed (Fig. 2 B). However, it was shown that phosphotyrosine residues in sperm proteins could be completely dephosphorylated by the vesicular protein tyrosine acid phosphatase (PTAP) (Fig. 2C). This underlined the significance of seminal plasma acid phosphatases, especially vesicular PTAP-ase, in the regulation of boar sperm function.

Discussion

Individual differences between boars facilitate the phosphorylation of tyrosine residues in sperm proteins during the process of semen cryopreservation.
The addition of ostrich egg yolk lipoprotein fractions to boar semen extender suppressed the phosphorylation of tyrosine residues in the sperm membrane proteins, as indicated by the boars with good semen freezability.

The results of this study reaffirmed the fact that sperm phosphotyrosine proteins could be a substrate for acid phosphatases of boar seminal plasma. The study of Vadnais et al. (13, 14) showed that the addition of 10% and 20% seminal plasma to post-thawed boar semen caused a marked decrease in the percentage of capacitated spermatozoa. It should be emphasised that such an effect was observed only after the removal of the egg yolk extender. The addition of seminal plasma to capacitated spermatozoa has been shown to decrease the levels of tyrosine phosphorylation in human and ram sperm proteins (8, 11). The results of the present study seem to suggest that the molecular forms of acid phosphatases, especially protein tyrosine acid phosphatase of the seminal vesicle fluid, may act as the major decapacitation factors in boar seminal plasma. Moreover, due to the large volume of boar semen, some cryopreservation protocols incorporate only the sperm rich fraction, thus reducing the presence of the seminal plasma at the first phase of the ejaculate. It can be suggested that the removal of acid phosphatases from the seminal plasma may lead to a disturbance in the phosphorylation-dephosphorylation status of the sperm proteins. This phenomenon is associated with an increased phosphorylation of sperm proteins, resulting in enhanced cryocapacitation. These findings indicate that retaining the full spectrum of enzymatic proteins in the whole ejaculate during cryopreservation could improve the post-thaw characteristics of boar spermatozoa. It is therefore possible that the phosphorylation level of tyrosine residues in sperm proteins could be used to detect cryocapacitation changes induced during the cryopreservation of boar semen. Moreover, it appears that tyrosine phosphorylation in freshly ejaculated spermatozoa may facilitate the freezability of boar semen. Further studies are required to understand the significant importance of protein tyrosine phosphorylation/dephosphorylation in boar semen following cryopreservation.

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