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Original article

Interactions of ostrich egg yolk lipoproteins with seminal plasma of fractionated ejaculates and their effects on post-thaw boar semen quality

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Abstract

Electrophoretic methods were used to identify protein complexes formed between ostrich egg yolk lipoprotein fractions (LPFo) with seminal plasma (SP) of fractionated ejaculates, and to investigate the effect of these complexes on boar semen quality after cryopreservation. Chromatographic SP fractions (F1, F2 and F3), with or without LPFo solution, were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Comparative electrophoretic analyses of the SP revealed marked differences in the SDS-PAGE protein profiles among boars. Electrophoretic analyses showed that the interactions of LPFo with SP resulted in the appearance of high-intensity protein bands. Spermatozoa were exposed to SP chromatographic fractions originating from F1, F2 and F3, and the whole SP (wSP) before being frozen. Spermatozoa exposed to F1 and F2 exhibited significantly higher post-thaw motility compared to those treated with either F3 or wSP. In most of the boars the proportions of membrane-intact frozen-thawed spermatozoa differed among the treatments, being significantly lower in the wSP-treated samples. The incidence of frozen-thawed spermatozoa with DNA fragmentation was less prevalent in samples exposed to F3 or the wSP. The results of this study confirmed that the interactions of LPFo with fractionated SP during the cooling period contributed to alterations in the sperm membranes, rendering them less susceptible to temperature-related injury.

Key words: boar semen, ejaculate fractions, egg yolk lipoproteins, cryopreservation

Introduction

It is well known that the specific sensitivity of boar spermatozoa to cold shock damage is one of the main factors responsible for the compromised fertility of frozen-thawed semen (Yeste 2016, Fraser et al. 2018). Studies have shown the performance of frozen-thawed semen is highly variable among boars, and such variability has been associated with several factors, such as the treatment of pre-freeze (PF) semen and source of spermatozoa (Fraser and Strzeżek 2007, Hernández et al. 2007, Saravia et al. 2008). It is important to note that there has been growing interest in the incorporation of seminal plasma (SP) in the freezing protocol of boar semen (Alkmin et al. 2014, Perez-Patiño et al. 2016). Moreover, boar SP comprises

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a diverse cohort of protein with various binding affinity for numerous ligands, which might explain the multi-functionality of the SP (Rodríguez-Martínez et al. 2011). Although a comprehensive study of the proteome of boar SP has been provided by several authors (Perez-Patiño et al. 2016), available information still remains obscure about the role of SP components in sperm cryo-tolerance.

The cryopreservation protocol of boar semen has been established using whole hen egg yolk (HEY) in the freezing extender, even though it comprises several deleterious components that compromise sperm functions (Strzeżek and Hopfer 1987, Manjunath et al. 2002, Bergeron et al. 2004, Plante et al. 2015). The beneficial effect of HEY on sperm function is attributed mainly to low-density lipoproteins (LDL), which interact with the sperm membranes during semen preservation (Manjunath et al. 2002, Bergeron et al. 2004). In our laboratory we have isolated lipoprotein fractions from ostrich egg yolk (LPFo) and have confirmed that LPFo is a suitable substitute for HEY in the freezing extender for boar semen because of its beneficial cryo-protective effects on sperm function (Strzeżek et al. 2005, Fraser and Strzeżek 2007, Fraser et al. 2007, 2011, Wasilewska and Fraser 2017), and has been shown to give optimal fertility results (Fraser et al. 2018). Even though the mechanism of the cryo-protective action of LPFo on sperm function is unclear, it has been demonstrated that the interactions of LPFo with spermatozoa stabilize their membrane structures during the cooling procedure (Strzeżek et al. 2005, Fraser et al. 2010). The aims of the study were to i) employ comparative electrophoretic analysis to identify proteins complexes formed between LPFo and SP of the chromatographic fractions (LPFo-SP), and ii) to assess the effects of the LPFo-SP complexes on post-thaw (PT) quality of boar semen.

Materials and Methods

Animals and collections of ejaculate

Five Polish large white (PLW) boars (average age 1.5 years) were used in this study. Ejaculates (fractionated or whole), or the sperm-rich fractions (SRFs) were collected from the boars (Boars 1, 2, 3, 4 and 5) during the autumn-winter period (October to March). Experiments were performed in accordance with the guidelines of the Local Ethics Committee. We divided the study into two experiments: Experiment 1 comprised the chromatographic analysis of the LPFo solution, fractionated SP and LPFo-SP mixture, and comparative electrophoretic analyses of their profiles, while Experiment 2 included the assessment of post-thaw (PT) semen quality following pre-freeze treatment of spermatozoa with the selected SP chromatographic fractions (F1, F2 and F3), which contributed to the interactions between LPFo and SP.

Experiment 1

Seminal plasma collection and gel filtration chromatography

Fractionation of boar ejaculates was performed according to a previously described procedure (Zasiadczyk et al. 2015). Ejaculates were split into 3 fractions: F1, the first 8 ml of the sperm-rich fraction (SRF); F2, the remaining SRF and approximately 8 ml of the post-sperm rich fraction (PSRF); and F3, the remaining portion of the PSRF. The samples of F1, F2, F3, and the whole seminal plasma (wSP) were centrifuged (900×*g*, 10 min at room temperature), and the SP was collected and re-centrifuged (7800×*g*, 10 min). The obtained SP was stored at -80°C, until required. The content of total protein was determined in the fractionated SP and wSP (Lowry et al. 1951). Aliquots of the LPFo solution (5% LPFo in distilled water) and the SP harvested from F1, F2 and F3 were subjected to gel filtration chromatography (Fast Protein Liquid Chromatography, FPLC) on Superose 6 HR 10/30 column (Amersham-Pharmacia, Biotech). Thirty chromatographic fractions of 1ml/tube each were collected and the protein content was determined in each fraction (Lowry et al. 1951). Also, the LPFo solution was mixed with the SP from F1 (LPFo-F1), F2 (LPFo-F2), or F3 (LPFo-F2) at 1:15 (1ml LPFo solution/15 mg proteins in the SP) and incubated for 1h at room temperature before being subjected to gel filtration chromatography.

Electrophoretic analysis of chromatographic fractions of LPFo solution and SP

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed in only 14 chromatographic fractions (numbered 6 to 19) obtained from the LPFo solution, SP of fractionated ejaculates and LPFo-SP mixture, as described by Laemmli (1970), with some modifications (Zasiadczyk et al. 2015). Due to the absence of proteins or negligible amounts of them in the SP chromatographic fractions numbered 1 to 5, and 20 to 30, SDS-PAGE analysis was performed only in the 14 chromatographic fractions. Densitometry analysis was performed to quantify the protein bands.

Table 1. Chromatographic fractions of seminal plasma (SP) from fractionated ejaculates selected for the cryopreservation procedure in individual boars.

	Chromatographic fractions showing the appearance of new protein bands		
Boars	LPFo-F1	LPFo-F2	LPFo-F3
1	14 –16	13 – 16	14 – 15
2	15 – 17	7, 14 – 17	7, 14 – 16
3	14 – 16	13 – 16	16 – 18
4	15 – 18	15 – 18	16 – 17
5	16 –18	15 – 16	14 – 15

LPFo-F represents the interactions between ostrich egg yolk lipoprotein fractions (LPFo) and SP chromatographic fractions (F). The fractions, identified by SDS-PAGE analysis of LPFo-F1, LPFo-F2 or LPFo-F3 were pooled and used in the cryopreservation procedure.

Experiment 2

Pre-freeze sperm treatment and cryopreservation

Seven SRFs were collected from each boar and only SRFs that showed >70% motility and >85% morphologically normal spermatozoa were used in this study. At collections the SRFs were split into four parts ($1.5x10^9$ spermatozoa/ml), each portion was suspended in the selected SP chromatographic fractions originating from F1, F2, F3 and the wSP. Comparative electrophoretic analysis was used to select LPFo-F1, LPFo-F2 or LPFo-F3 complexes, which were identified by the appearance of high-intensity protein bands. The SP of two to five chromatographic fractions showing high-intensity protein bands with LPFo-F1, LPFo-F2 or LPFo-F3 complexes was selected, pooled and used in the cryopreservation procedure (Table 1). Briefly, sperm samples ($1.5x10^9$ spermatozoa/ml) were incubated with the selected SP (2 mg/ml) of the pooled chromatographic fractions of F1, F2 and F3, or wSP for 1h at room temperature. Following incubation, the samples were mixed with a LPFo-containing extender and then stored at 5°C for 2h before being frozen (Fraser and Strzeżek 2007). The cooled semen samples were diluted with another extender comprising lactose-LPFo extender, glycerol and Orvus Es Paste (OEP), loaded into aluminum tubes and frozen using a programmable computer freezing machine (Ice Cube 1810, SY-LAB, Austria). Post-thaw (PT) assessment was performed by thawing the frozen semen samples in a water bath (50°C for 60 sec). The thawed samples were diluted (1:10) with the Beltsville Thawing Solution (BTS and stored for 15 min at 37°C (0 min PT).

Assessment of semen quality

The sperm parameters were used to assess the quality of the pre-freeze SRFs and PT semen. Sperm motility was subjectively evaluated under a light microscope (Olympus BX 40, Tokyo, Japan) with a heated stage (38°C) at 200x magnification. Five randomly selected fields were used for sperm motility assessments at 15 (0 min PT), 60, 120 and 180 min incubation after thawing.

The sperm plasma membrane integrity (PMI) was evaluated with the SYBR-14 and propidium iodide (PI) assay (Garner and Johnson 1995) and hypo-osomotic swelling (HOS) test according to a previously described method (Jeyendran et al. 1984), with some modifications (Fraser et al. 2011). Sperm samples (10 µl) were mixed with 1 ml of hypo-osmotic solution (150 mOsm/kg) prepared with 75 mmol/l fructose and 25 mmol/l sodium citrate and incubated at 37°C for 60 min. Following incubation, samples were evaluated at 400× magnification under a phase-contrast microscope. Spermatozoa with intact plasma membranes displayed swollen tails in response to the treatment. Slides were prepared in duplicate and at least 100 sperm cells were examined per slide.

Lipophilic cation JC-1 and PI double fluorescent staining (Molecular Probes, Eugene, USA) were used to determine the percentage of spermatozoa with functional MMP (Thomas et al. 1998, Dziekońska et al. 2009). Stained slides were examined under a fluorescence microscope (Olympus CH 30, Tokyo, Japan) at 600× magnification, and at least 100 spermatozoa were counted in each duplicate.

The Giemsa staining technique was used to evaluate the percentage of spermatozoa with NAR acrosome integrity (Fraser et al. 2007). Spermatozoa with normal apical ridges were considered as acrosome-intact (NAR) and those with damaged apical ridges were considered as non-intact acrosome. Slides were prepared in duplicate and at least 100 sperm cells were examined per slide.

The Neutral Comet Assay (NCA) was used to determine the percentage of spermatozoa with fragmented DNA (Fraser and Strzeżek 2007). Slides stained with ethidium bromide were evaluated under a fluorescence microscope (Olympus BX 41) at 400× magnification. Slides were prepared in duplicate and at least 100 spermatozoa were examined per slide.

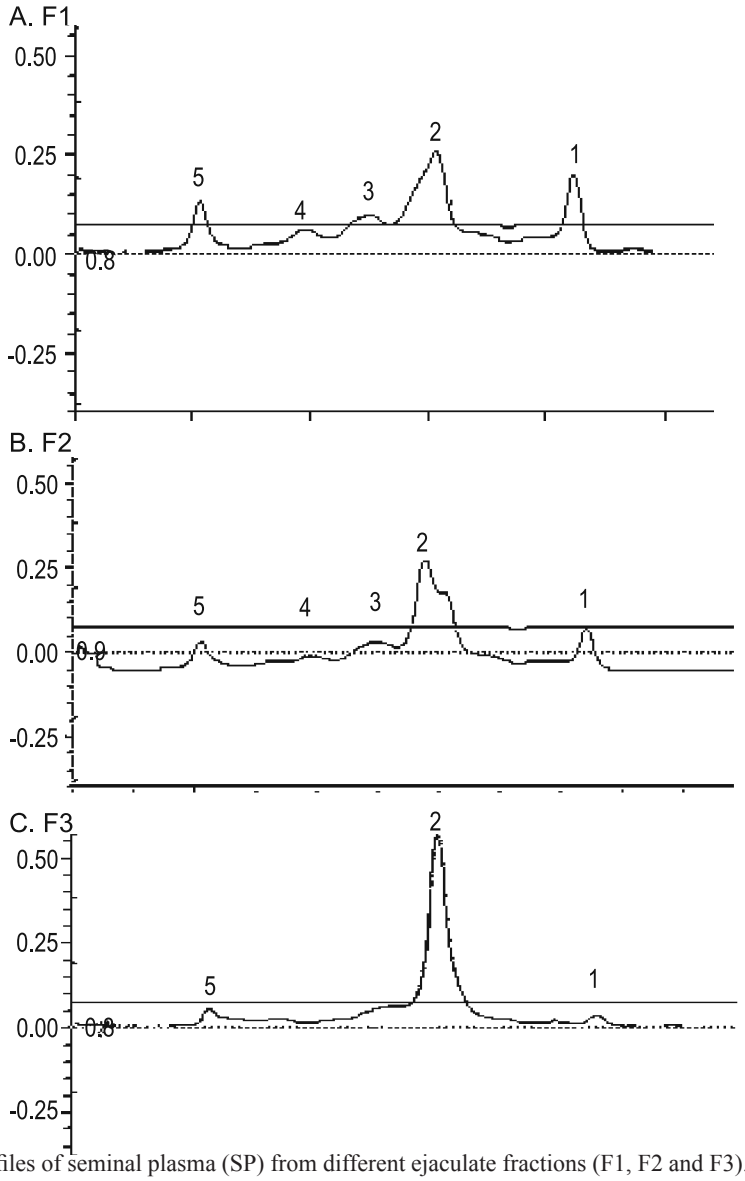

Fig. 1. Chromatographic profiles of seminal plasma (SP) from different ejaculate fractions (F1, F2 and F3). Peaks 1, 2, 3, 4 and 5 represent proteins with molecular weights in the range from 6 to 15kDa, 15kDa, 40 kDa, 160 kDa and 250 kDa, respectively. AU – absorbance units.

Statistical analysis

The main effects of boar (5), treatment (wSP, F1, F2 and F3) storage time (0 min, 60 min, 120 min and 180 min) and their interactions on the PT motility were evaluated using a 3-way ANOVA statistical model (5 × 4 × 4). A 2-way ANOVA, with boar and treatment as the main effects (5 × 4 factorial design), was used to assess PT sperm membrane integrity and DNA fragmentation. Analyses were performed with the general linear model (Statistica software package, StatSoft Incorporation, Tulsa, OK, USA) using the Neuman-Keuls test ($p<0.05$).

Results

Experiment 1

Chromatographic profiles of SP ejaculated fractions

Gel filtration chromatography resulted in the appearance of five peaks (6-5 kDa, 15kDa, 40 kDa, 160 kDa and 250 kDa) in F1 and F2 (Fig. 1A and Fig. 1B, respectively), whereas only three peaks (6 to15 kDa, 15kDa and 250 kDa) were observed in F3 (Fig. 1C).

LPFo and SP electrophoretic profiles

SDS-PAGE and densitometric analyses showed the appearance of different protein bands in the chromatographic fractions of the LPFo solution (Fig. 2). Proteins with molecular weights in the range of 20 to 35 kDa

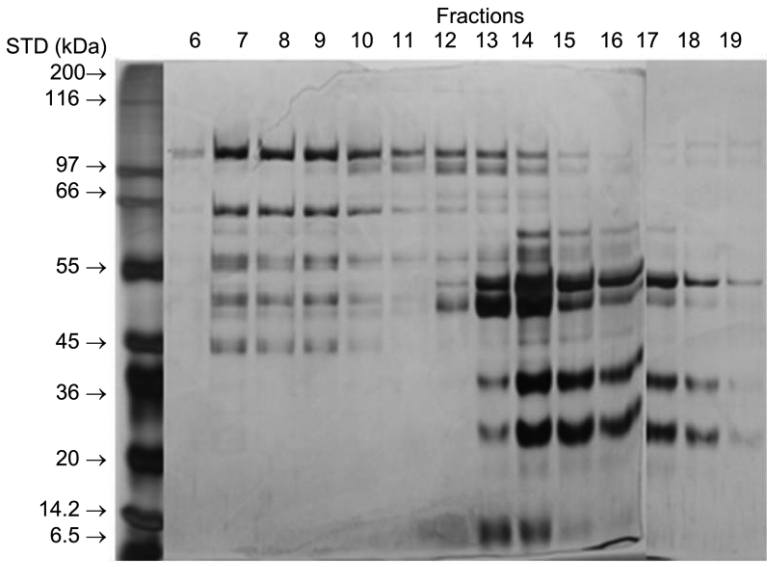

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) profiles of chromatographic fractions of ostrich egg yolk lipoprotein fractions (LPFo). Lane represents the chromatographic fraction STD – standard

and 50 to105 kDa were more prominent in fractions 13 to 15 (Fig. 2).

Under the experimental conditions, SDS-PAGE and densitometric analyses confirmed that the appearance of protein bands with high intensity represented the LPFo-SP complexes from F1, F2 or F3 in each boar. In this study we used Boar 2 to present the representative electrophoretic profiles of fractionated SP (Fig. 3A-C) and the LPFo-SP complexes formed in F1, F2 and F3, as indicated in the lanes marked in red (Fig. 3D-F). Densitometric analysis of the chromatographic fractions of F1, F2 and F3 showed marked differences in the electrophoretic profiles of proteins with molecular weights ranging from 6.5 to 200 kDa (Fig. 3A-C). Generally, SDS-PAGE analysis showed proteins of molecular weights in the range from 50 to 55 kDa were more prevalent in the chromatographic fractions 13 to 16 of F1 (Fig. 3A), whereas the F2 consisted predominately a 60-kDa protein (Fig. 3B) in fractions 14 to 16. Furthermore, a 200 kDa-protein band was more prominent in fractions 8 to 12 of either F2 (Fig. 3B) or F3 (Fig. 3C). Additionally, high intensity protein bands, representing LPFo-F1, LPFo-F2 or LPFo-F3, with molecular weights of 6.5 kDa, 36 kDa, and in the range from 48-50 kDa, were more marked in the selected chromatographic fractions (Fig. 3D-F, Table 1).

Experiment 2

The quality of the pre-freeze semen of the SRFs did not differ ($p>0.05$) among the boars. Boar, treatment, storage time and boar × storage time interaction were significant sources of variations in PT sperm motility (Table 2). There was a gradual reduction in PT sperm motility in each boar, irrespective of the treatment (Fig. 4A-D). It was found that wSP-treated spermatozoa of Boar 3 exhibited higher ($p<0.05$) percentages of PT motility than Boar 1 at 60 and 180 min after thawing (Fig. 4A). Significantly higher ($p<0.05$) PT motility at 0 min after thawing was observed in F1- and F2-treated spermatozoa of Boars 1 to 3 compared with other boars (Fig. 4B and Fig. 4C, respectively). Marked differences among the boars were more noticeable in F3 at 180 min after thawing (Fig. 4D).

ANOVA results revealed that boar and treatment had marked ($p<0.001$) effects on the analyzed PT sperm parameters (PMI, MMP, NAR acrosome integrity and DNA fragmentation). Sperm PMI, analyzed by SYBR-14/PI assay and the HOS test, was markedly lower ($p<0.05$) in the wSP-treated samples after freezing-thawing (Fig. 5A-B). Significantly higher ($p<0.05$) PMI was observed in frozen-thawed spermatozoa of Boars 1 to 2, particularly in the samples exposed to F1 or F2 (Fig. 5A-B).

Sperm MMP and NAR acrosome integrity in frozen-thawed spermatozoa were lower ($p<0.05$) in the samples treated with wSP (Fig. 6A-B). Cryo-induced DNA damage was less marked in spermatozoa exposed to either wSP or F3 in most of the boars compared with SP-treated samples (Fig. 6C).

Discussion

Chromatographic analyses of the SP of the ejaculated fractions showed that F1 and F2 comprised similar protein composition compared to F3. Due to the lack of clarity regarding the analysis of the chromatographic profiles of LPFo-SP, we considered that comparative

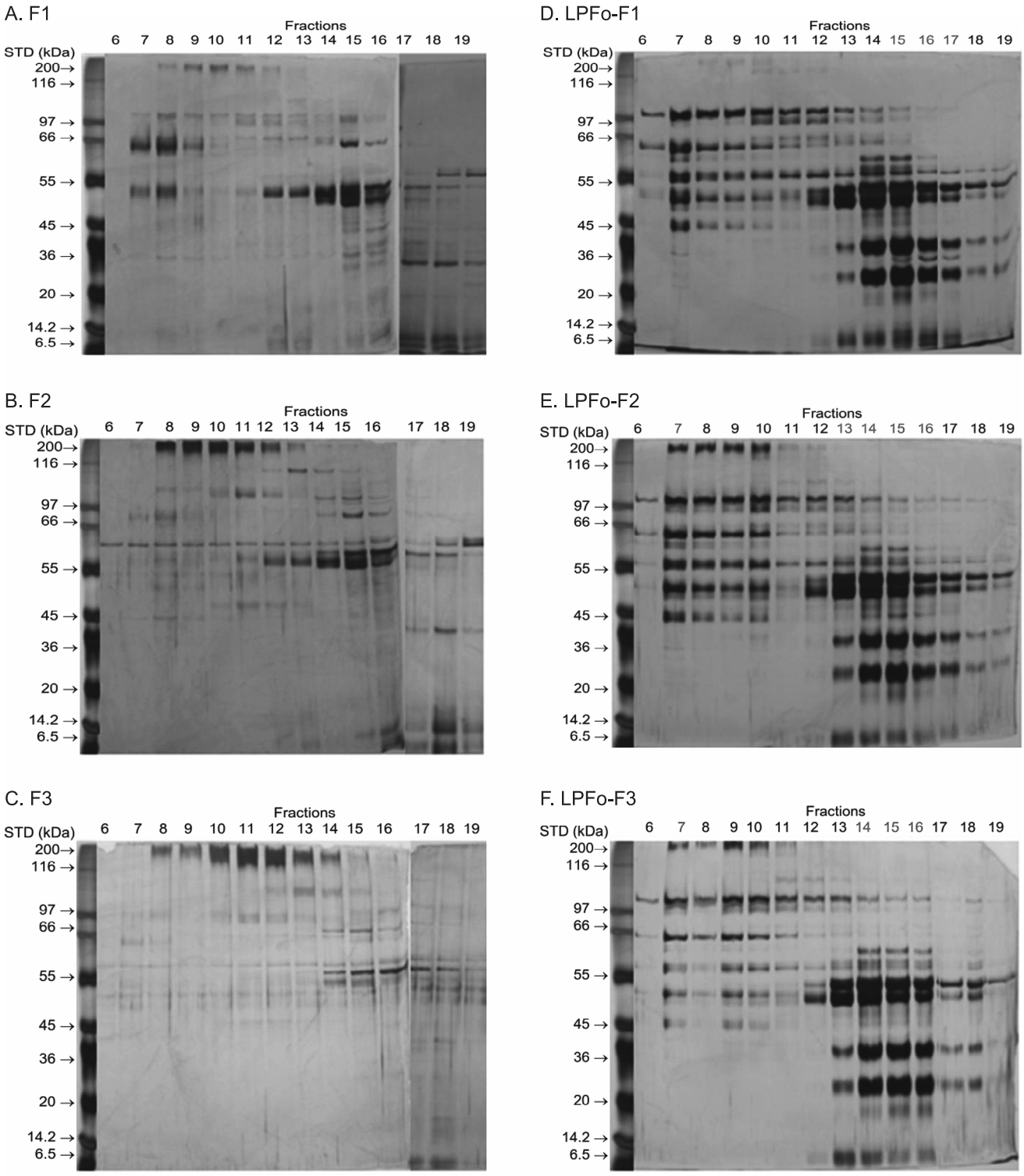

Fig. 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) profiles of chromatographic fractions (F) of seminal plasma (SP) of fractionated ejaculates (A, B and C) and LPFo-F (D, E and F). Protein complexes formed after the interactions of LPFo with SP are indicated by the appearance of bands with high intensity, as shown in the lanes marked in red. Lanes 6 to 19 represent the chromatographic fraction. LPFo–ostrich egg yolk lipoprotein fractions. STD–standard.

visualization of the SDS-PAGE profiles would be a better approach to investigate the association between LPFo and SP. It should be emphasized that the mechanism through which LPFo exerts its cryo--protective effects on sperm function during the freezing--thawing procedure is not fully understood. Similar assumption was reported for LDL, when used as the supplement to the freezing extender (Manjunath et al. 2002, Plante et al. 2015). However, some hypotheses were suggested to explain the mechanism of the

Table 2. ANOVA sources of variations in post-thaw motility of boar spermatozoa.

Sources	Sperm motility
	p-value
Boar	<0.001
Treatment	<0.001
Storage time	<0.001
Boar × Treatment	>0.987
Boar × Storage time	<0.003
Treatment × Storage time	>0.797

Repeated measures ANOVA with a factorial design, 5 × 4 × 4, with main effects of boar, treatment (wSP, F1, F2 and F3) and storage time 0, 60, 120 and 180 min) and their interactions.

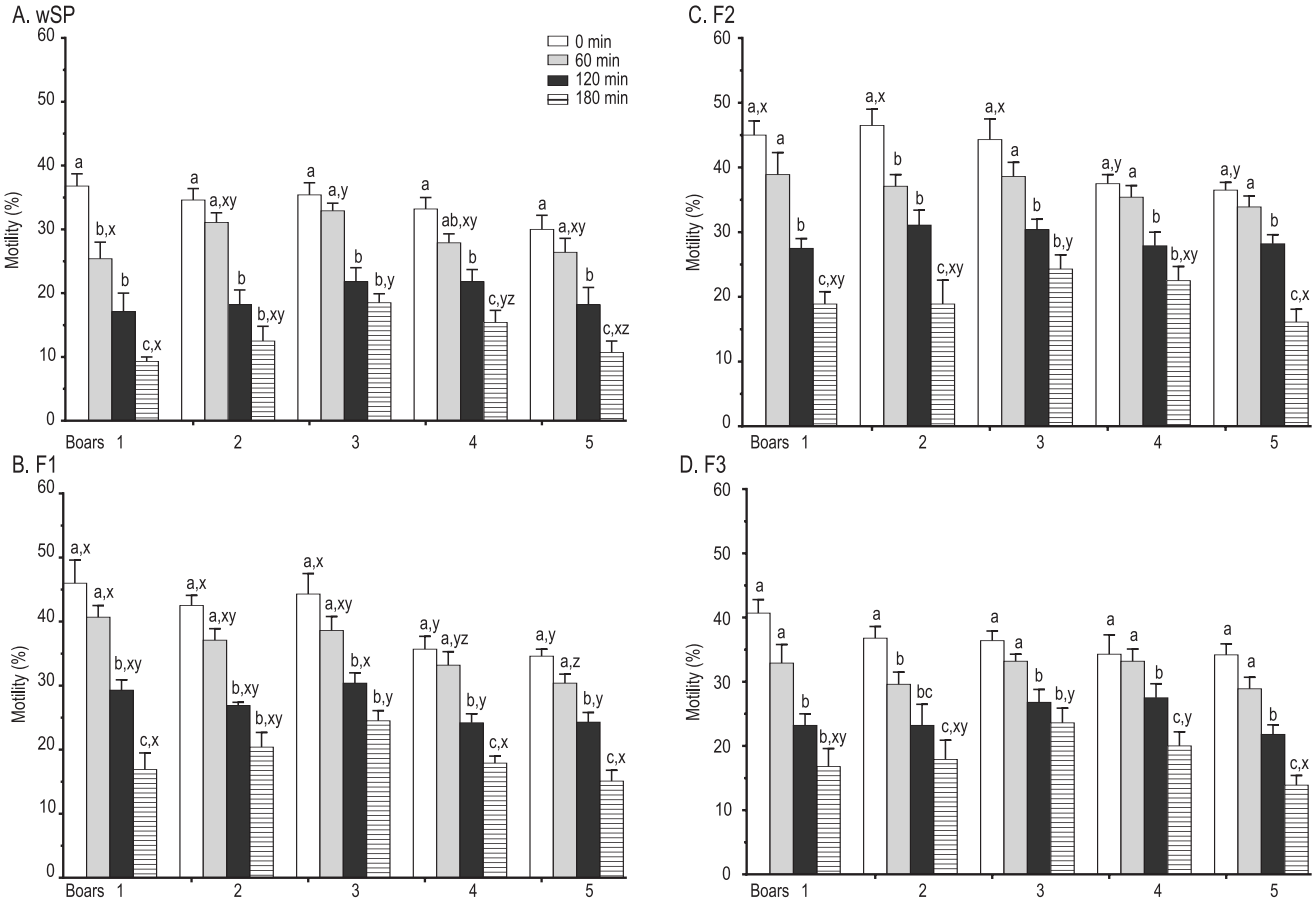

Fig. 4. Post-thaw motility of spermatozoa following treatment with whole seminal plasma (wSP) and different chromatographic fractions (F1, F2 and F3) from individual boars at different storage time periods. Values are expressed as the mean (± SEM) of seven ejaculates from each of the boars. Within boars values with different letters (a, b and c) are significant at $p<0.05$. Within incubation time values with different letters (x, y and z) are significant at $p<0.05$.

cryo-protective action of LDL. Firstly, it was reported that LDL interacted with spermatozoa and formed a protective coating at the sperm surface, or might replace membrane phospholipids that were lost during the freezing-thawing procedure (Manjunath et al. 2002, Bergeron et al. 2004, Plante et al. 2015). Even though a similar mechanism of LPFo binding was confirmed in boar spermatozoa (Fraser et al. 2010), it is still not clear about the role of cholesterol, phospholipids and protein substances in this sperm-coating mechanism after the disruption of LPFo in the presence of SDS, a component of OEP used in the freezing extender for boar semen (Fraser and Strzeżek 2007). In our experimental conditions, it is likely that such hypothesis might partly explain the cryo-protective action of LPFo on sperm function. Secondly, it was hypothesized that the interaction of LDL with Binder of Sperm (BSP) proteins, a family of closely related major proteins from bull SP, reduced the negative effect of the BSP proteins on the sperm membrane integrity

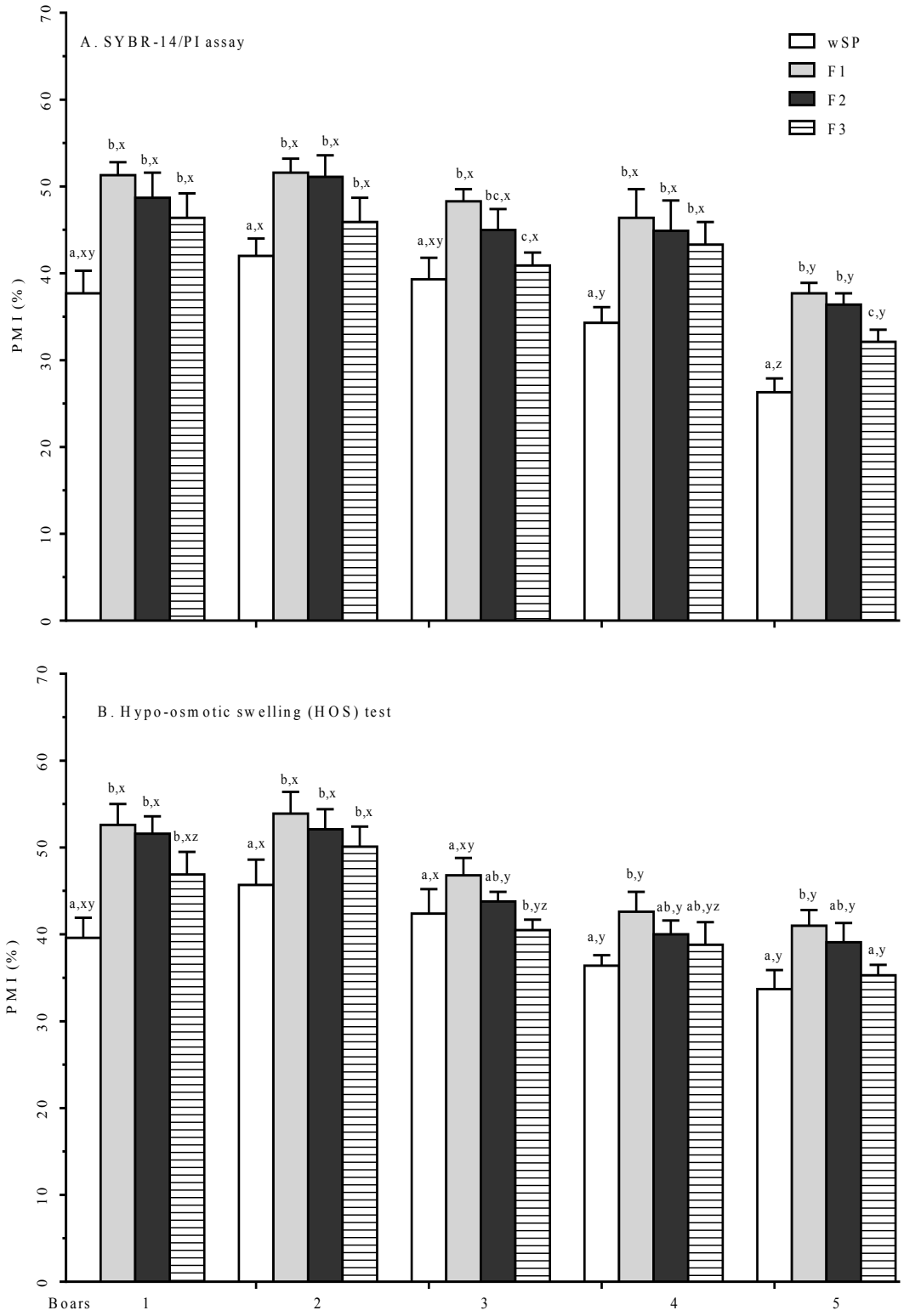

Fig. 5. Plasma membrane integrity of frozen-thawed spermatozoa following treatment with whole seminal plasma (wSP) and different chromatographic fractions (F1, F2 and F3). Values are expressed as the mean (± SEM) of seven ejaculates from each of the boars. Within boars values with different letters (a, b and c) are significant at $p<0.05$. Within ejaculate fractions values with different letters (x, y and z) are significant at $p<0.05$.

during semen preservation (Plante et al. 2015). In our study it is plausible that the sequestration of SP proteins by LPFo might occur during the cooling period of the semen, however, it is likely that fractionation of the SP, which allowed the selection of specific chromatographic fractions, might minimize the negative impact of SP components on sperm function. Thirdly, we have demonstrated that the interactions of LPFo

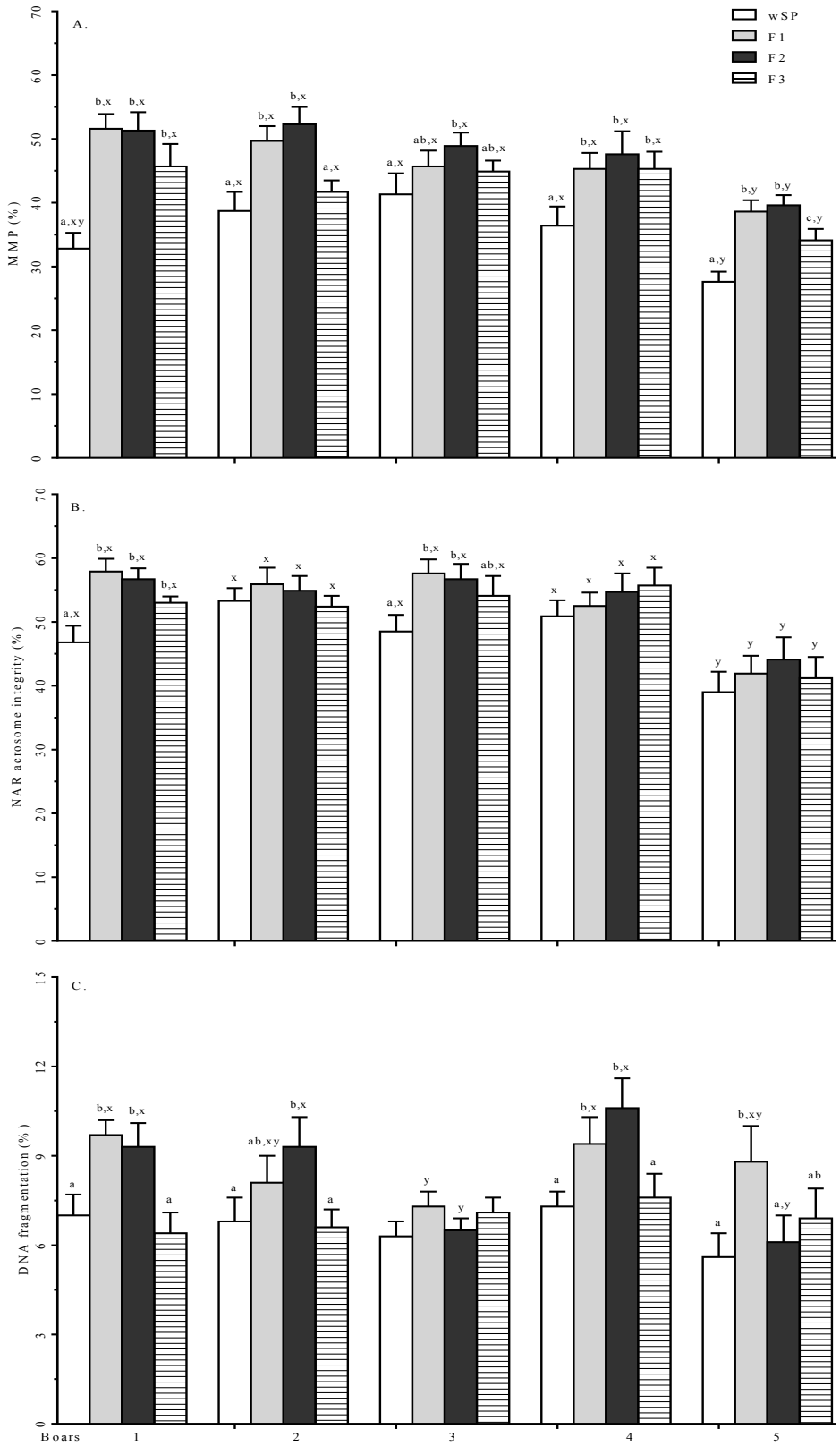

Fig. 6. Mitochondrial membrane potential (MMP), normal apical ridge (NAR) acrosome integrity and DNA fragmentation of frozen-thawed spermatozoa following treatment with whole seminal plasma (wSP) and different chromatographic fractions (F1, F2 and F3) from individual boars. Values are expressed as the mean (± SEM) of seven ejaculates from each of the boars. Within boars values with different letters (a, b and c) are significant at $p<0.05$. Within ejaculate fraction values with different letters (x, y and z) are significant at $p<0.05$

with SP proteins resulted in the formation of LPFo-SP complexes, which confer protection to the sperm membranes during the freezing-thawing procedure. In the present study such protective action in PT sperm function was more prominent in samples exposed to SP originating from either F1 or F2. It seems likely that the interactions of the LPFo-SP protein complexes with spermatozoa induce modifications in their membranes, resulting in the accessibility of more binding sites on the sperm membranes. Similar findings were reported in previous studies following the cryopreservation of bull and boar semen (Manjunath et al. 2002, Bergeron et al. 2004, Daskalova et al. 2014, Plante et al. 2015). We suggest that cryo-induced modifications in the sperm membranes might play a key role in the protective action of LPFo-SP protein complexes on sperm function during the freezing-thawing procedure. It should be reaffirmed that the interaction between BSP proteins and HEY, LDL or milk components has been suggested to be the basis for sperm protection during semen preservation (Manjunath et al. 2002, Plante et al. 2015)

In this study, we confirm that the interactions of LPFo with SP proteins of the chromatographic fractions exert beneficial effects on PT semen quality, particularly for samples treated with the SP derived from either F1 or F2. Furthermore, the analyzed sperm quality parameters (motility, PMI and DNA integrity) were markedly affected during the freezing-thawing procedure, and the extent of the cryo-damage differed among the boars. Unlike PT motility, PMI and DNA fragmentation, there was a lack of consistent marked variations in sperm NAR acrosome integrity among the treatment groups after freezing-thawing. A possible explanation for this phenomenon could be due to the Giemsa staining protocol, which does not seem to be a robust staining assay for the assessment of the acrosome integrity of frozen-thawed spermatozoa (Wasilewska and Fraser 2017).

The results of the current study indicated that variations in PT sperm motility and MMP were mainly associated with the proportions of frozen-thawed spermatozoa with PMI in each treatment group. Generally, spermatozoa exposed to the F3 or wSP exhibited markedly reduced PT semen quality compared with those treated with either F1 or F2. Such findings suggest that the presence of components in F3 or wSP exerted unfavorable effect on the interactions of LPFo with SP proteins, resulting in reduced PT semen quality. Irrespective of the SP source, this phenomenon was observed mainly in the SP harvested from Boar 4 and Boar 5, reaffirming the findings of previous studies indicating that there are marked variations in the SP components between boars (Hernández et al. 2007, Yeste 2016). Besides sperm DNA fragmentation, we have demonstrated that treatment of spermatozoa with wSP did not significantly improve PT semen quality (Fraser and Strzeżek 2007). A similar finding was observed in the current study when the spermatozoa were treated with F3 from each boar. Although we did not identify the proteins comprising the LPFo-SP complexes, the results of our study confirm that SP harvested from the chromatographic fractions of both F1 and F2 provide better interactions with LPFo, contributing to improved PT semen quality. Several reports confirmed that the beneficial effects of boar SP on sperm function during cooling or freezing-thawing were attributable mainly to the presence of low-molecular components, such as the non–heparin-binding spermadhesins, PSP-I and PSP-II (Hernández et al. 2007, Saravia et al. 2008, Rodríguez-Martínez et al. 2011), or specific 26-kDa and 55-kDa fertility associated proteins (Daskalova et al. 2014). In the present study it is unclear whether these SP components might mediate the interactions between LPFo and the SP, however, high-intensity protein bands, with molecular weights in the range of 6.5 kD to 50 kDa, were more prominent in the LPFo-F1 and LPFo-F2 complexes in three boars (Boar 1, Boar 2 and Boar 3), whereas these complexes consisted predominately of protein bands with molecular weights greater than 30 kDa in the other two boars (Boar 4 and Boar 5). In the current study we suggest that the association of LPFo with SP proteins of the chromatographic fractions is a crucial factor affecting the cryo-survival of boar spermatozoa.

Taken together, we confirmed that comparative electrophoretic analyses allowed the identification of LPFo-SP protein complexes whose beneficial effects on PT semen quality were dependent on the SP source. It could be suggested that the treatment of spermatozoa with SP harvested from either F1 or F2, prior to freezing, contributed to modifications in the sperm membranes that were accompanied by reduced cryo-susceptibility. We suggest that further investigations are required to better understand the functional significance of the associations of ostrich egg yolk lipoproteins with SP proteins in the cryo-tolerance of boar spermatozoa.

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