



EFFECT OF FRACTIONATED SEMINAL PLASMA ON SPERM CHARACTERISTICS FOLLOWING CRYOPRESERVATION OF BOAR SEMEN*

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Abstract

This study aimed to investigate the effect of fractionated seminal plasma (SP) on boar sperm characteristics following cryopreservation. Gel filtration chromatography yielded two fractions: SP1 with more than 40 kDa (>40 kDa) and SP2 with less than 40 kDa (<40 kDa). The fractionated SP (SP1 and SP2), whole seminal plasma (wSP) and Beltsville Thawing Solution (BTS) were used for the treatment of semen before freezing-thawing. Besides the analysis of sperm motility characteristics, plasma membrane integrity (PMI), acrosome integrity, and viability (Vybrant Apoptosis Assay) were analyzed in pre-freeze and post-thaw (PT) semen. Among the analyzed pre-freeze sperm parameters, rapid movement was markedly affected by boar and treatment. Furthermore, boar and treatment were significant sources of variations in PT semen quality. Treatment with wSP caused a marked reduction in PT semen quality compared with BTS, SP1 or SP2. Wide variations in PT acrosome integrity and viability were observed in spermatozoa treated with BTS and the fractionated SP, being significantly higher in the SP1- and SP2-treated samples. However, PT semen quality did not differ between semen samples treated with SP1 and SP2. Representative electrophoretic profiles of sperm proteins from each treatment showed quantitative and qualitative differences, indicating varying effects of the cryopreservation procedure on the sperm membrane integrity. The findings of this study indicated that the cryoprotective effects of the SP components varied among boars and that different components of the fractionated SP exerted varying effects on sperm functions following cryopreservation. It could be suggested that the variable protective protein components of either fractionated SP ameliorated alterations in the sperm membranes during cryopreservation, resulting in reduced susceptibility to cryo-damage.

Key words: boar, spermatozoa, cryopreservation, seminal plasma

The limited application of cryopreserved boar semen is mainly due to the significant reduction in the sperm fertilizing ability (Knox, 2015; Yeste et al., 2017).

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Several methods, including the freezing of different ejaculate fractions, have been employed to improve the cryopreservation protocol of boar semen (Hernández et al., 2007; Saravia et al., 2008; Alkmin et al., 2014; Li et al., 2018). Analysis of the protein composition of boar seminal plasma (SP), prior to cryopreservation, has been suggested to be a useful tool to identify potential markers associated with semen freezability (Daskalova et al., 2014; Vilagran et al., 2015; Yeste, 2016).

Different factors affect the composition of boar semen (Konracki et al., 2013; Zasiadczyk et al., 2015), and could exert varying effects on the function of SP components (Perez-Patiño et al., 2016). Due to its multi-organ origin, boar SP is a complex mixture, which comprises an abundance of protein components that are implicated in various fertilization-associated events (Jonáková and Tichá, 2004; Rodríguez-Martínez et al., 2011). Even though the precise role of most of boar SP proteins in sperm function is not fully understood, there is evidence indicating the beneficial effect of SP components on sperm cryo-survival (Saravia et al., 2008; Daskalova et al., 2014; Vilagran et al., 2015; Guimarães et al., 2017; Li et al., 2018; Wasilewska et al., 2018 a, b). It has been reported that some components of boar SP could compromise post-thaw (PT) sperm functions and fertility (Eriksson et al., 2001; Okazaki et al., 2009). Specific SP components with low- and high-molecular weights are adsorbed on the surface of ejaculated spermatozoa to maintain the stability of their membranes during transport in the reproductive tract (Metz et al., 1990; Jonáková and Tichá, 2004; Rodríguez-Martínez et al., 2011). Moreover, the identification of low-abundant components of boar SP might be masked by the presence of high-abundant SP components, and chromatographic methods have been used as a potential tool to overcome this limitation (Perez-Patiño et al., 2016, 2019). It has been demonstrated that chromatographic fractionation of boar SP could yield different protein fractions that could be incorporated in the cryopreservation protocol to improve the sperm cryo-survival (Wasilewska et al., 2018 a, b). Furthermore, the intention of this study was to incorporate a 1.5-h holding time period of spermatozoa exposed to SP, prior to the cooling procedure. The aim of this study was to investigate the effect of chromatographically separated SP fractions on the characteristics of boar spermatozoa (motility parameters, membrane integrity and viability) following cryopreservation.

Material and methods

All chemicals were bought from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise stated.

Animals and semen collections

Ejaculates were collected from seven sexually mature Polish Large White (PLW) boars, using the gloved-hand technique. The ejaculates were collected from four boars stationed at the Cryopreservation Laboratory at the Faculty of Animal Bioengineering, University of Warmia and Mazury in Olsztyn (Poland), and three boars from the Artificial Insemination Station in Ciechanów (Poland) during the autumn-winter

period. Animal experiments were carried out in accordance with the guidelines set out by the Local Ethics Committee. The sperm-rich fraction (SRF) and a portion of the post-SRF, comprising a total of 100 ml, was collected in graded cylinders from each boar (Boars A to G) and used for gel filtration chromatography. For the cryopreservation procedure, a total of four or seven SRFs were collected from the boars. Prior to freezing-thawing, fresh spermatozoa exhibited more than 70% total sperm motility (TMOT) and 85% normal morphology. Sperm concentration was measured with a Bürker counting chamber (Equimed-Medical Instruments, Kraków, Poland). The study was divided into 2 experiments: Experiment 1 (Exp. 1) comprised gel filtration chromatography, while Experiment 2 (Exp. 2) included the treatment of the pre-freeze semen, semen evaluation and cryopreservation.

Experiment 1 (Exp. 1)

Gel filtration chromatography and biochemical analysis of SP

The ejaculates (100 ml) were centrifuged twice, first at $5000 \times g$ for 5 min and then at $10\,000 \times g$, 10 min) to remove the sperm pellets. The SP was examined under a microscope for the presence of spermatozoa, and centrifugation was repeated if sperm cells were detected in the SP. The whole seminal plasma (wSP) was used for gel filtration chromatography. Aliquots of the wSP were separated by gel filtration chromatography (Fast Protein Liquid Chromatography, FPLC) on Sephacryl S-200 HR HiPrep 16/60 column (Amersham-Pharmacia, Biotech) to yield two fractions: SP1 (SP1, >40 kDa) and SP2 (SP2, < 40 kDa). The column was equilibrated with phosphate-buffered solution (PBS) containing 0.02% sodium azide. A total of 10 mg proteins/SP sample was loaded into the column and elution was performed at 0.5 ml/min flow rate at room temperature (RT). Chromatographic fractions of 1 ml/tube each were collected and the protein content was determined in each fraction, prior to storage (80°C). The protein content was determined in the wSP (SP1 + SP2) and chromatographic fractions of SP1 and SP2, according to a previously described method (Lowry et al., 1951).

Electrophoretic analysis of SP proteins

One dimensional polyacrylamide gel electrophoresis, in the presence of sodium dodecyl sulfate (SDS-PAGE), was used to analyze the chromatographic fractions (SP1 and SP2), according to the method of Laemmli (1970), with some modifications (Zasiadczyk et al., 2015; Wasilewska and Fraser, 2017). Briefly, analysis was performed with the Mini-Protean II Cell electrophoresis system (BioRad, Warsaw, Poland) in 12% (wt/vol) polyacrylamide gel slabs. The SP sample (1.25 mg/ml protein) was used for each lane, and the gels were stained overnight with Coomassie brilliant blue staining solution to confirm the presence of proteins. Sigma Marker Wide Range standards (Sigma-Aldrich Inc., USA) were used to analyze the molecular masses of the protein bands.

Experiment 2 (Exp. 2)

Pre-freeze semen treatment and cryopreservation

The SRFs from seven boars (Boars A to G) were divided into four portions, in which one portion was extended in Beltsville Thawing Solution (BTS) and the oth-

er three portions were used for treatment with wSP, SP1 and SP2. Sperm samples (400×10^6 spermatozoa/ml), extended in BTS and treated with wSP, SP1 and SP2 (1.5 mg proteins/ml), were held for 1.5h at RT. After the holding time period, sperm samples held in BTS, wSP, SP1 and SP2 were extended (1:1) in BTS, and cooled for 1 h at 17°C. Parameters of semen quality were analyzed following the 1.5-h holding time period of spermatozoa in BTS, wSP, SP1 and SP2.

Semen was frozen, using lyophilized lipoprotein fractions of ostrich egg yolk, LPFo (Fraser et al., 2010; Wasilewska and Fraser, 2017). After centrifugation ($800 \times g$ for 10 min) of the cooled semen, the sperm pellets were re-suspended in a LPFo-extender containing 11% lactose (lactose-LPFo extender). The LPFo-extended semen was cooled for 2 h at 5°C, before being diluted (2:1) with another freezing extender (89.5 ml lactose-LPFo extender, 9 ml glycerol (v/v) and 1.5 ml Orvus Es Paste). Semen samples were frozen in a controlled programmable freezer (Ice Cube 14M, SY-LAB, Austria) and stored in liquid nitrogen. Samples were thawed in a water bath for 60 sec at 50°C, diluted (1:10) with BTS and incubated for 15 min at 37°C, prior to analysis of motility characteristics, membrane integrity and viability.

Evaluation of semen quality

CASA-analyzed motility characteristics

Aliquots of sperm samples (5 μ l) were placed on a pre-warmed Makler counting chamber (37°C), using the computer-assisted sperm analysis (CASA) system (HTR-IVOS 12.3, Hamilton Thorne Biosciences, MA, USA). In this study, the CASA system was used to analyze total motility (TMOT, %), progressive motility (PMOT, %) and rapid movement (%). A minimum of 5 fields per sample were assessed, with approximately 200 spermatozoa per field, using the previously established CASA parameters (Zasiadczyk et al., 2015).

Mitochondrial membrane potential (MMP)

Sperm MMP (%) was monitored with fluorescent probes, JC-1 and propidium iodide, PI (Molecular Probes, Eugene, OR, USA), according to a previously described method (Dziekońska et al., 2009; Fraser et al., 2014). Samples stained with JC-1/PI were observed at 600 \times magnification under an epifluorescence microscope (Olympus CH 30, Tokyo, Japan). A minimum of 100 cells per slide were examined and were classified as viable spermatozoa with intact mitochondria and non-viable spermatozoa damaged mitochondria. Each slide was analyzed in duplicate.

Plasma membrane integrity (PMI)

The fluorescent probes, SYBR-14 and PI (Live/Dead Sperm Viability Kit; Molecular Probes, Eugene, OR, USA), were used to assess sperm PMI, according to a previously described method (Garner and Johnson, 1995). At least 100 cells per slide were examined in each duplicate and classified as membrane-intact (%) and membrane-damaged spermatozoa (%).

Acrosome integrity

Fluorescein isothiocyanate-labeled peanut (*Arachis hypogaea*) agglutinin (FITC-PNA) staining with PI was used to assess the sperm acrosome integrity, according to

a previously described method (Soler et al., 2005), with some modifications. Aliquots of the sperm samples (3×10^6 spermatozoa/ml) in HEPES saline medium (130 mM NaCl, 4 mM KCl, 14 mM fructose, 10 mM HEPES, 1 mM CaCl_2 , 0.5 mM MgCl_2 , 0.1% bovine serum albumin) were incubated with 20 μl of FITC-PNA solution (2 mg FITC-PNA in 1 ml PBS) for 10 min at 37°C. The samples were incubated for another 5 min at 37°C following the addition of 10 μl of PI (1 mg PI in PBS) to the suspensions. The stained sperm cells (10 μl) were spread on pre-cleaned microscopic slide, mounted with 5 μl of Prolong® Diamond Antifade Mountant (Molecular Probes, Eugene, OR, USA), and covered with a coverslip. At least 100 spermatozoa in each duplicate were assessed at 600 \times magnification under a fluorescence microscope. Spermatozoa without FITC-PNA/PI staining or exhibiting uniform bright staining over the acrosome region were classified as live cells with intact acrosome (%), whereas spermatozoa exhibiting disrupted fluorescence were classified as dead cell with damaged acrosome (%).

Viability and apoptotic-like changes in spermatozoa

The Vybrant Apoptosis Assay Kit #4 (Molecular Probes Inc., Eugene, USA) was used to assess viability and the percentage of plasma membrane apoptotic-like changes in spermatozoa, according to a previously described method (Trzcińska and Bryła, 2015), with some modifications (Wasilewska and Fraser, 2017). A minimum of 100 cells per slide were examined in each aliquot, and three sub-populations were identified using this assay: viable spermatozoa (%) categorized as negative for both YO-PRO-1 and PI (YO-PRO-1⁻/PI⁻), plasma membrane apoptotic-like changes in spermatozoa (% moribund spermatozoa) were categorized as positive for YO-PRO-1⁺ but negative for PI⁻ (YO-PRO-1⁺/PI⁻), and dead spermatozoa (%), which were positive for both YO-PRO-1 and PI (YO-PRO-1⁺/PI⁺).

Electrophoretic analysis of sperm membrane proteins

Sperm membrane proteins were obtained from SRFs and PT semen using a Triton-extracted protocol (Wasilewska and Fraser, 2017). SDS-PAGE analysis was performed in sperm extracts obtained from SRFs and PT semen, according to the method of Laemmli (1970), with some modifications (Zasiadczyk et al., 2015).

Statistical analysis

The normality of the data distribution was analyzed using the one way analysis of variance (ANOVA) with the Shapiro Wilk W-test. The General Linear Model (GLM) procedure (Statistica software package, version 12.5, StatSoft Inc. Tulsa, OK, USA) was used to examine the data. In this study BTS- and wSP-treated samples were served as the control groups, whereas samples treated with SP1 and SP2 were used as the experimental groups. The effects of boar and treatment, and their interactions on the pre-freeze and PT semen were analyzed with repeated measures ANOVA, using a 7×4 factorial design. The results are expressed as the mean \pm standard error of the mean (S.E.M), and significant differences were compared using the Neuman–Keuls *post hoc* test ($P < 0.05$).

Results

Experiment 1 (Exp. 1)

Chromatographic and electrophoretic profiles

Representative chromatographic and electrophoretic profiles (SDS-PAGE) of boar SP are shown in Figure 1. Protein fractions of SP1 (> 40 kDa) and SP2 (< 40 kDa) obtained by gel filtration chromatography and SDS-PAGE analysis are shown in Figure 1 A (I, SP1 and II, SP2) and Figure 1 B (SP1 and SP2), respectively. No marked differences in either the chromatographic profiles or electrophoretic profiles were observed among boars. Protein content in the wSP averaged 36.3 ± 2.7 mg/ml (range, 24.0–45.0 mg/ml), whereas the protein content of SP from either SP1 or SP2 averaged 1.5 mg/ml (range, 1.0–2.5 mg/ml).

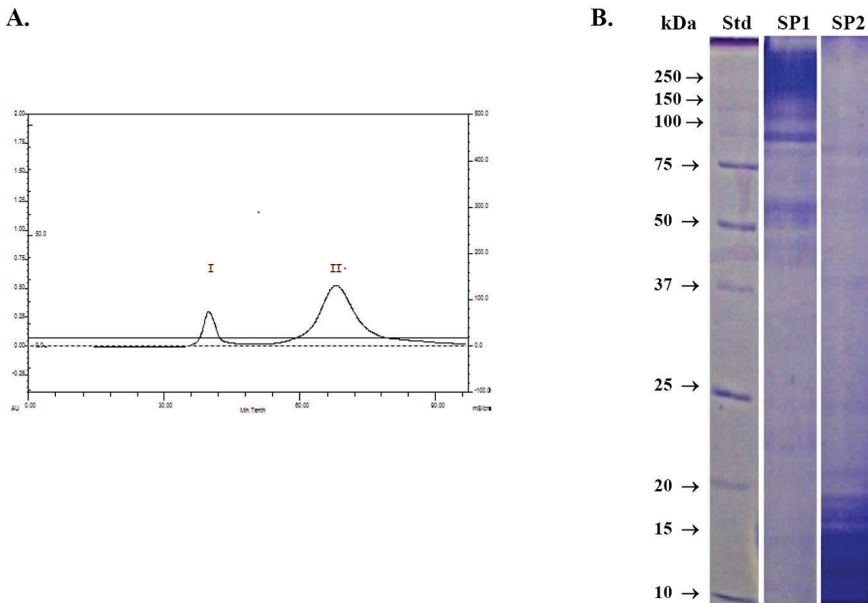


Figure 1. Chromatographic and electrophoretic profiles of boar seminal plasma (SP) (Figure 1 A and Figure 1 B, respectively). Seminal plasma 1 (I, SP1) and seminal plasma 2 (II, SP2) represent protein fractions with molecular weights greater than 40 kDa (>40kDa) and less than 40 kDa (<40kDa), respectively

Experiment 2 (Exp. 2)

Pre-freeze semen quality

No marked changes ($P > 0.05$) in the sperm quality parameters of the fresh semen were observed among the boars. The average percentage of fresh spermatozoa with TMOT was 84.5 ± 1.4 %, PMOT was 54.0 ± 2.2 %, and rapid movement was 65.8 ± 2.7 %. Likewise, the proportions of fresh spermatozoa with PMI was 86.2 ± 0.4 %, acrosome integrity was 88.6 ± 0.5 % and viability was 85.7 ± 0.7 %.

Table 1. Pre-freeze motility characteristics of boar spermatozoa held in Beltsville Thawing Solution (BTS) and seminal plasma (SP) before freezing-thawing

Sperm parameters (%)	Treatment	Boars						
		A (n=7)	B (n=7)	C (n=7)	D (n=7)	E (n=4)	F (n=4)	G (n=4)
Total motility (TMOT)	BTS	91.1±1.9	85.7±3.7	86.3±1.9	81.0±4.4	89.5±2.5	84.5±0.6	91.3±1.9
	wSP	86.1±3.1	87.6±2.5	82.7±3.7	82.1±2.8	81.3±3.3	83.5±2.1	87.5±5.2
	SP1	89.6±2.3	90.5±2.1	84.4±1.6	77.0±4.5	80.5±1.3	78.8±2.9	89.8±3.0
	SP2	87.9±0.9	87.0±2.6	88.7±1.5	81.4±2.9	83.3±0.7	82.8±2.6	87.3±3.4
Progressive motility (PMOT)	BTS	66.6±2.7 ay	67.4±5.7 a	61.4±3.8 a	56.3±4.0 ab	53.0±3.9 b	56.0±5.2 ab	68.5±2.5 a
	wSP	53.0±3.5 abx	65.4±3.6 a	52.7±5.5 ab	50.4±3.1 b	51.8±2.4 b	49.3±4.7 b	60.5±5.4 ab
	SP1	60.3±3.2 abxy	64.4±2.8 a	55.9±1.0 bc	49.7±3.7 c	50.3±4.4 bc	58.3±3.1 ab	61.8±2.6 ab
	SP2	59.9±2.3 abxy	67.1±3.1 a	56.6±2.0 b	52.6±3.8 bc	47.5±2.7 c	55.8±1.4 b	64.3±4.5 a
Rapid movement	BTS	74.6±3.9 ac	76.1±2.1 ac	69.1±3.8 aby	62.6±2.3 b	68.8±2.8 aby	67.5±2.5 aby	80.3±2.1 cy
	wSP	62.7±2.0 ad	75.3±1.9 b	51.4±3.2 cx	57.0±3.3 cd	50.5±2.3 cx	45.5±2.3 cx	67.0±2.2 ax
	SP1	67.1±2.2 ac	78.1±1.7 b	60.0±1.8 axy	58.3±2.5 a	59.5±1.2 axy	64.0±2.0 ay	72.5±2.3 bcxy
	SP2	71.4±1.5 a	69.9±1.8 ab	66.7±2.6 aby	62.3±3.2 bc	56.3±2.4 cxy	56.0±1.5 cy	73.8±4.0 axy

Values represent the means (± SEM). Within row, values with different letters (a, b, and c) are significant at $P < 0.05$. Within treatment in column values with different letters (x and y) are significant at $P < 0.05$.

wSP: whole seminal plasma, SP1: seminal plasma 1, SP2: seminal plasma 2.

Table 2. Pre-freeze membrane integrity and viability of boar spermatozoa held in Beltsville Thawing Solution (BTS) and seminal plasma (SP) before freezing-thawing

Sperm parameters (%)	Treatment	Boars						
		A (n=7)	B (n=7)	C (n=7)	D (n=7)	E (n=4)	F (n=4)	G (n=4)
Mitochondrial membrane potential (MMP)	BTS	80.9±0.9	81.4±1.3	80.2±0.7	80.3±1.0	85.4±0.5	84.8±0.8	82.8±0.9
	wSP	85.1±0.6 ab	82.0±1.1 ab	82.1±0.4 ab	77.9±3.0 b	87.0±0.6 a	86.9±0.6 a	85.0±1.0 ab
	SP1	82.3±0.8	83.0±1.4	82.0±0.8	81.1±0.9	85.8±1.0	86.9±0.5	85.1±1.3
	SP2	83.3±1.0	82.7±1.2	82.2±0.5	82.4±0.7	88.5±0.7	85.9±1.0	84.4±0.8
Plasma membrane integrity (PMI)	BTS	84.7±0.5	83.3±1.5	83.2±0.6	82.5±1.0	86.9±1.1	86.9±0.7	85.3±0.8
	wSP	81.0±0.7	82.8±1.6	80.8±0.9	78.4±1.2	85.9±0.9	83.8±0.8	82.8±1.3
	SP1	83.9±0.4 ab	88.6±0.5 a	83.6±0.9 ab	79.4±1.4 b	87.0±1.2 a	84.4±1.5 ab	86.6±1.5 ab
	SP2	83.4±0.7	82.8±1.4	83.6±0.4	81.6±1.1	87.6±0.8	85.9±1.1	83.9±1.2
Acrosome integrity	BTS	87.3±1.3 ab	91.0±1.1 a	84.8±1.9 ab	83.5±0.8 b	87.5±0.8 abxy	86.8±0.9 ab	86.1±0.6 ab
	wSP	85.3±1.5	89.2±1.3 a	84.5±2.5	80.9±1.2	84.6±2.9 ax	84.0±0.7	83.8±1.0
	SP1	87.0±1.2 ab	90.7±0.6 a	85.8±2.7 ab	83.0±2.3 b	90.4±1.8 ay	88.4±0.6 ab	86.9±1.0 ab
	SP2	87.6±1.4	90.1±0.8	86.5±2.2	84.2±0.8	86.6±1.4 axy	86.0±0.8	85.0±0.7
Viability	BTS	83.0±1.6	80.3±1.8	83.0±1.8	80.5±1.6	82.5±1.3	82.5±0.4	81.0±0.8
	wSP	83.5±1.9	82.8±0.4	81.2±2.1	79.5±1.9	86.0±0.9	83.5±0.9	83.3±0.5
	SP1	84.2±1.0 ab	86.5±1.6 a	80.0±2.9 ab	78.5±0.7 b	84.8±0.9 ab	83.0±0.6 ab	83.5±0.9 ab
	SP2	82.7±1.7	81.5±1.5	83.4±2.3	80.3±1.2	85.3±1.0	83.2±1.1	83.0±1.4

Values represent the means (± SEM). Within row values with different letters (a, b, and c) are significant at $P < 0.05$. Within treatment in column values with different letters (x, y, and z) are significant at $P < 0.05$.

wSP: whole seminal plasma, SP1: seminal plasma 1, SP2: seminal plasma 2.

Boar differences were more pronounced in sperm PMOT and rapid movement in the pre-freeze semen (Table 1). Differences among treatment effects were observed in the proportions of spermatozoa with rapid movement in most of the boars before freezing (Table 1). Also, the percentages of spermatozoa with rapid movement were lower ($P < 0.05$) in samples exposed to wSP compared with the those treated with BTS in at least four boars (Table 1).

Significant effects of boar were observed in PMI, acrosome integrity and viability in the pre-freeze spermatozoa exposed to SP1 (Table 2). It was found that SP1-treated spermatozoa from Boar D showed markedly lower ($P < 0.05$) PMI, acrosome integrity and viability compared with Boar B (Table 2). Irrespective of the treatment, the percentages of spermatozoa with apoptotic-like changes (YO-PRO-1⁺/PI⁻) averaged $6.8 \pm 0.2\%$ (range, 4.8 to 9.8%), while the proportions of dead frozen-thawed spermatozoa (YO-PRO-1⁺/PI⁺) averaged $10.7 \pm 0.3\%$ (range, 8.5 to 14.0%) in the pre-freeze semen. Variations in the percentages of spermatozoa with apoptotic-like changes were more marked in the pre-freeze semen of Boar F and Boar G, whereas markedly higher proportions of dead spermatozoa were observed in the pre-freeze semen of Boar D, regardless of the treatment.

Post-thaw (PT) semen quality

ANOVA results showed that sperm motility parameters, membrane integrity and viability of PT spermatozoa were significantly affected by boar and treatment (Table 3). However, only treatment significantly affected the proportions of spermatozoa with apoptotic-like changes and the percentages of dead cells in the PT semen (Table 3). In addition, boar \times treatment interaction markedly affected sperm MMP ($P < 0.011$) and acrosome integrity ($P < 0.002$) after freezing-thawing.

Irrespective of the treatment, consistent boar variations were observed in sperm TMOT, PMOT and rapid movement, being markedly lower ($P < 0.05$) in Boar D following freezing-thawing (Table 4). Generally, spermatozoa exposed to wSP exhibited lower ($P < 0.05$) motility characteristics in most of the boars (Table 4). Marked differences ($P < 0.05$) in TMOT were observed among samples treated with BTS, SP1 and SP2 for Boar D and Boar E, and in PMOT for Boar E following freezing-thawing (Table 4).

Table 3. ANOVA sources of variations in post-thaw quality of boar semen

Sperm parameters	Boar	Treatment
	P-value	P-value
Total motility (TMOT)	0.001	0.001
Progressive motility (PMOT)	0.001	0.001
Rapid movement	0.001	0.001
Mitochondrial membrane potential (MMP)	0.001	0.001
Plasma membrane integrity (PMI)	0.001	0.001
Acrosome integrity	0.005	0.001
Viability (YO-PRO-1 ⁻ /PI ⁻)	0.003	0.001
Plasma membrane apoptotic-like changes (YO-PRO-1 ⁺ /PI ⁻)	n.s	0.001
Dead (YO-PRO-1 ⁺ /PI ⁺)	n.s	0.001

Repeated measures ANOVA with a mixed model was used to analyze the interactions the main effects: boar and treatment (BTS, wSP, SP1 and SP2). Significant at $P < 0.05$, n.s: non-significant.

Table 4. Post-thaw (PT) motility characteristics of boar spermatozoa held in Beltsville Thawing Solution (BTS) and seminal plasma (SP) before freezing-thawing

Sperm parameters (%)	Treatment	Boars							
		A (n=7)	B (n=7)	C (n=7)	D (n=7)	E (n=4)	F (n=4)	G (n=4)	
Total motility (TMOT)	BTS	41.7±2.2 ay	38.1±3.3 aby	33.0±1.5 by	16.3±1.8 cx	19.0±0.8 cx	43.8±2.8 ay	47.3±4.0 ay	
	wSP	26.3±2.5 acx	23.4±3.4 abcx	22.3±3.4 abcx	15.7±2.2 bx	19.5±2.7 abx	26.8±3.4 acx	29.3±2.7 cx	
	SP1	47.4±4.3 ay	42.4±3.7 ay	29.4±2.3 bxy	25.3±3.6 by	27.8±2.4 by	45.3±2.3 ay	40.0±2.1 ay	
	SP2	46.1±2.9 ay	38.4±4.0 aby	34.3±3.9 by	30.4±2.1 by	34.3±1.1 by	37.5±1.7 bxy	41.3±3.2 aby	
Progressive motility (PMOT)	BTS	31.3±2.2 ay	31.7±3.3 ay	23.6±2.0 ay	11.9±2.6 bxy	14.0±1.4 bx	34.8±3.6 ay	33.0±2.5 a	
	wSP	20.4±2.9 ax	17.3±3.4 ax	14.4±3.1 abx	9.7±1.6 bx	12.0±2.0 bx	21.0±2.9 ax	22.0±2.0 a	
	SP1	34.7±4.0 ay	31.8±4.6 ay	23.0±1.9 by	16.7±2.9 by	20.0±2.9 by	36.5±3.3 ay	24.5±2.2 b	
	SP2	36.3±3.9 ay	29.5±2.9 ay	19.9±2.2 bxy	20.9±1.9 by	26.8±1.8 aby	25.5±1.7 aby	31.8±4.2 a	
Rapid movement	BTS	35.6±3.2 ay	30.2±3.9 ay	31.1±4.3 ay	15.4±4.1 bxy	14.8±1.3 bx	30.8±1.5 ay	26.5±3.8 axz	
	wSP	20.4±3.7 ax	16.0±3.9 abx	16.4±3.1 abx	9.9±1.3 bx	12.0±2.0 bx	22.4±1.6 ax	22.8±2.1 ax	
	SP1	32.3±2.2 ay	39.4±3.5 ay	22.7±2.5 bxy	17.3±2.1 by	17.8±1.7 bxy	38.8±2.2 ay	40.5±3.1 ay	
	SP2	37.9±2.5 ay	34.1±3.7 ay	20.7±2.5 bxy	20.9±1.6 by	27.5±1.7 aby	27.0±1.0 abxy	32.0±3.4 ayz	

Values represent the means (± SEM). Within row values with different letters (a, b, and c) are significant at P<0.05. Within treatment in column values with different letters (x, y, and z) are significant at P<0.05.
wSP: whole seminal plasma, SP1: seminal plasma 1, SP2: seminal plasma.

Table 5. Post-thaw (PT) membrane integrity and viability of boar spermatozoa held in Beltsville Thawing Solution (BTS) and seminal plasma (SP) before freezing-thawing

Sperm parameters (%)	Treatment	Boars							
		A (n=7)	B (n=7)	C (n=7)	D (n=7)	E (n=4)	F (n=4)	G (n=4)	
Mitochondrial membrane potential (MMP)	BTS	46.1±1.4 ay	45.0±1.6 ay	40.1±2.0 aby	35.6±1.5 by	34.9±1.7 bxz	41.8±3.0 abxy	43.6±2.5 aby	
	wSP	27.5±2.2 x	31.3±1.1 x	27.1±1.7 x	28.2±1.6 x	32.6±2.1 x	32.9±1.4 x	29.5±2.3 x	
	SP1	55.9±1.8 az	46.2±1.8 by	36.8±1.6 cy	41.2±2.1 bey	40.3±1.4 beyz	44.7±1.5 bey	44.5±0.9 bey	
	SP2	43.5±2.2 acy	42.3±2.1 axy	34.9±3.2 bxy	39.4±2.6 aby	42.8±1.2 aby	42.9±1.2 ay	52.9±2.2 cz	
Plasma membrane integrity (PMI)	BTS	44.1±1.8 ay	45.1±2.9 ay	37.5±2.2 aby	35.3±1.4 bxy	39.4±2.7 abxy	43.0±2.6 aby	44.6±1.4 ay	
	wSP	28.4±1.8 x	27.6±1.6 x	23.1±1.2 x	28.0±2.9 x	30.1±2.4 x	32.6±1.6 ax	32.8±2.1 ax	
	SP1	55.4±1.5 az	48.9±1.8 acy	36.3±2.0 by	40.7±3.2 beyz	43.3±1.9 bey	52.1±2.3 az	45.1±0.8 acy	
	SP2	45.5±2.8 ayz	43.6±3.4 aby	39.5±2.0 ay	45.2±2.7 abz	42.1±1.1 aby	49.5±1.6 byz	48.8±1.2 by	
Acrosome integrity	BTS	40.5±3.4 abx	48.1±1.8 ay	38.0±3.8 abxy	35.8±2.9 bx	36.2±1.9 bx	39.3±1.9 abx	42.5±2.3 aby	
	wSP	31.8±2.3 x	28.0±2.2 x	30.1±2.3 x	32.1±2.2 x	31.6±2.3 x	35.4±1.0 x	33.0±1.3 x	
	SP1	59.9±2.4 ay	52.3±2.9 ay	35.6±2.2 bxy	44.5±1.3 cy	43.8±1.9 cy	57.0±3.6 ay	54.8±2.7 az	
	SP2	50.4±2.9 ay	48.3±1.4 aby	42.1±1.9 by	46.8±1.2 aby	47.8±1.8 aby	50.6±2.4 ay	61.5±1.7 cz	
Viability	BTS	38.5±1.6 x	38.8±1.8 y	39.3±3.4 y	30.5±1.5 x	32.3±1.8 x	35.8±2.5 y	36.3±2.1 y	
	wSP	34.8±3.3 ax	25.5±2.4 abx	24.0±2.1 bx	24.5±1.0 bx	27.5±2.5 abx	24.6±2.8 bx	24.5±4.8 bx	
	SP1	50.0±2.9 ay	44.8±2.3 aby	29.8±2.6 cxy	40.3±2.4 by	43.0±4.2 aby	44.3±1.2 abz	40.8±4.9 by	
	SP2	46.5±1.3 ay	42.3±3.8 aby	33.8±2.4 by	42.5±4.1 ay	43.5±3.0 ay	47.3±3.4 az	43.0±2.2 y	

Values represent the means (± SEM). Within row values with different letters (a, b, and c) are significant at $P < 0.05$. Within treatment in column values with different letters (x, y, and z) are significant at $P < 0.05$.

wSP: whole seminal plasma, SP1: seminal plasma 1, SP2: seminal plasma 2.

There were wide variations in MMP, PMI, acrosome integrity and viability among treatments after thawing, being significantly lower ($P < 0.05$) in samples exposed to wSP (Table 5). Consistent boar variability in PT membrane integrity (MMP, PMI and acrosome integrity) and viability was more marked ($P < 0.05$) in spermatozoa exposed to either SP1 or SP2 (Table 5). BTS- and SP1-treated spermatozoa from Boar A showed higher ($P < 0.05$) MMP than Boar D and Boar E after freezing-thawing (Table 5). Also, SP2-treated spermatozoa from Boar G showed higher ($P < 0.05$) MMP following freezing-thawing (Table 5). Frozen-thawed spermatozoa from Boar A showed higher ($P < 0.05$) MMP in samples exposed to SP1 compared with BTS (Table 5). Compared with either the wSP or BTS, PMI was higher ($P < 0.05$) in spermatozoa exposed to SP1 from Boar A or Boar F, and in the SP2-treated spermatozoa of Boar D after freezing-thawing (Table 5). Significantly higher ($P < 0.05$) proportions of frozen-thawed spermatozoa with acrosome integrity and viability were observed in the SP1- or SP2-treated samples compared with BTS (Table 5). Wide variations in the proportions of spermatozoa with apoptotic-like changes and dead spermatozoa were observed among treatments after freezing-thawing. Furthermore, the percentages of PT spermatozoa with apoptotic-like changes were higher in semen samples treated with wSP ($15.5 \pm 1.9\%$) and lower in BTS-treated samples ($9.2 \pm 1.1\%$). Also, higher and lower proportions of dead frozen-thawed spermatozoa were observed in semen samples exposed to wSP ($59.2 \pm 2.4\%$) and SP1 ($44.7 \pm 2.3\%$), respectively.

Protein profiles of membrane proteins in spermatozoa

In this study, extracts of spermatozoa obtained from the pre-freeze and PT semen of each boar were analyzed by SDS-PAGE. SDS-PAGE densitometric analysis showed variations in the sperm profiles in the pre-freeze and PT semen and among treatments. However, the patterns of the electrophoretic profiles of the sperm membranes were found to be similar among boars. In this study we presented the representative SDS-PAGE protein profiles of spermatozoa in the pre-freeze and PT semen of only boar (Figure 2). Analysis showed that there were 11–20 protein bands in the pre-freeze semen, and 5–16 protein bands in the frozen-thawed semen. Proteins of molecular weights between 31.6 to 117.0 kDa range, comprising approximately 60% of the total band intensity, were more prominent in pre-freeze semen after treatment with either wSP or BTS. By contrast, pre-freeze semen exposed to either SP1 or SP2 showed proteins of molecular weight range between 15.7 to 56 kDa contributed approximately 60% of the total band intensity. Furthermore, the wSP-treated spermatozoa showed that 46% of the total band intensity was represented by proteins of molecular weights between 15.1 to 57.5 kDa range, while a 15.1 kDa protein (30.4%) was more predominant in the BTS-treated spermatozoa after freezing-thawing. Analysis of the PT semen showed that proteins of molecular weight range between 15.1 to 29.7 kDa and 20.9 to 30.5 kDa contributed approximately 72% of the total band intensity in the electrophoretic profiles of spermatozoa exposed to SP1 and SP2, respectively.

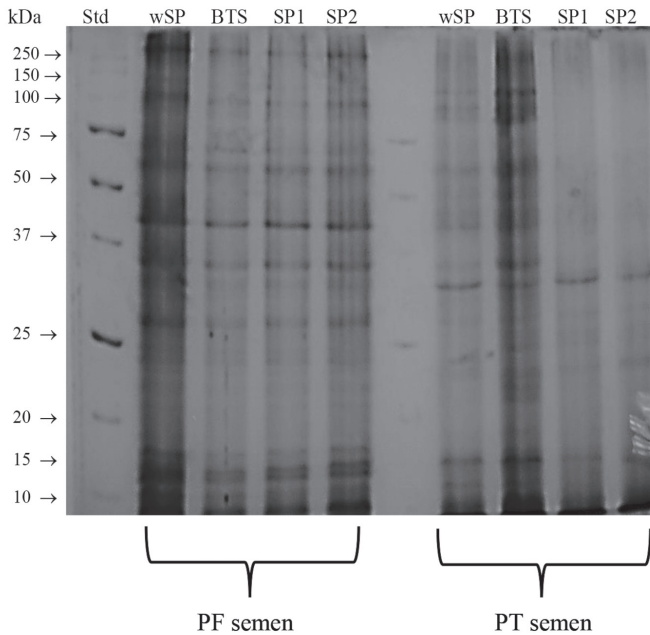


Figure 2. Sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) profiles of boar sperm membrane proteins in pre-freeze (after a 1.5-h holding time period) and post-thaw (PT) semen. Samples were standardized (1.25 mg/ml), denatured, reduced and separated in 12% polyacrylamide gel, and were stained with Coomassie brilliant blue. Std – standard protein marker; BTS – Beltsville Thawing Solution; wSP – whole seminal plasma; SP1 – seminal plasma 1; SP2 – seminal plasma 2

Discussion

In this study we incorporated a holding time period in the cryopreservation protocol, which was based on the treatment of fresh spermatozoa with SP chromatographic fractions for 1.5 h at RT. Furthermore, in this study electrophoretic analysis was used to confirm the fractionation of the chromatographically separated SP with > 40 kDa and < 40 kDa (Figure 1 B).

Consistent boar differences were observed in the pre-freeze and PT semen quality, and treatment with BTS, wSP and fractionated SP had significant effects on PT semen quality. It is noteworthy that substantial variations in PT semen quality among the boars corroborated the results of previous studies in our laboratory, indicating that boar is a significant factor affecting the quality of PT semen (Fraser et al., 2010, 2014; Wasilewska and Fraser, 2017). In the present study, treatment of the pre-freeze semen with wSP caused a rapid deterioration in PT motility characteristics, thus reaffirming the results of previous studies suggesting that the bulk of boar SP comprises components that affect sperm motility (Hernández et al., 2007; Saravia et al., 2008; Alkmin et al., 2014; Li et al., 2018). Notably, a rapid deterioration in sperm motil-

ity characteristics was concurrent with a significant loss of membrane integrity and viability in the wSP-treated spermatozoa following freezing-thawing. No consistent differences in the pre-freeze semen quality were observed among treatments following a 1.5-h holding time period. However, consistent significant differences were more prominent among treatments in the PT semen quality in all the boars, suggesting that the effects of SP proteins on sperm functions were more accentuated in the presence of components of the freezing extenders.

While understanding the functions of the SP proteins in the fertilization-related events, the underlying mechanisms that are involved in the interactions of the SP proteins with sperm membranes and egg yolk components during cryopreservation are unclear. Even though individual proteins of SP1 (>40 kDa) and SP2 (<40 kDa) that were involved in sperm cryo-tolerance were not identified in this study, it could be hypothesized that the interactions of specific SP1 and SP2 proteins with spermatozoa and egg yolk lipoproteins might result in the modifications of the sperm membrane structure, resulting in reduced susceptibility to cryo-induced damage. Remarkably, interactions of the SP proteins with spermatozoa and components of egg yolk-based or LPFo-based media exerted beneficial effects on sperm viability during semen preservation (Manjunath et al., 2007; Dziekońska et al., 2009). Moreover, differences in the SP composition among boars and ejaculates have been shown to affect PT semen quality (Hernández et al., 2007; Alkmin et al., 2014; Li et al., 2018), and some studies demonstrated that analysis of the differential expression patterns of selected SP proteins, prior to freezing, could predict semen freezability (Daskalova et al., 2014; Vilagran et al., 2015; Guimarães et al., 2017). According to Daskalova et al. (2014), the prevalence of a 80-kDa lactoferrin (LTF) in the SP of boars with good freezability ejaculates might be associated with the beneficial effect of the interaction of LTF with spermatozoa. In another study it was demonstrated that SP expression of fibronectin 1 (FN1, 272.27 kDa), which correlates with sperm motility and abnormal morphology, could be considered as a marker for the freezability of boar semen (Vilagran et al., 2015). Using 2D-electrophoresis, it was demonstrated that high expression of Fc fragment of IgG binding proteins (117.2/121.4 kDa) and a 49.8-kDa lactadherin precursor, which impair sperm motility and fertility by their binding action, was detected in boars with poor semen freezability (Guimarães et al., 2017). It seems that the aforementioned proteins markers could contribute to SP1 composition. Notably, spermadhesins (PSP-I, PSP-II, AWN, AQN-1 and AQN-3) are high-abundant low-molecular weight proteins in boar SP, which are implicated in the remodeling of the sperm membranes through their binding activity (Jonáková and Tichá, 2004; Perez-Patiño et al., 2019). Moreover, proteins in SP2, presumably spermadhesins, might play a crucial role in the protective mechanism during the cooling, freezing-thawing processes. In our laboratory, liquid chromatography tandem-mass spectrometry (LC-MS/MS) analysis has identified potential proteins of both SP1 and SP2 that are associated with sperm freezability (Wasilewska et al., 2018 b).

Interestingly, there were no consistent differences between samples treated with BTS and SP1 or SP2 with respect to motility characteristics, MMP and PMI following freezing-thawing. By contrast, marked differences were observed either between BTS and SP1 or BTS and SP2 for PT acrosome integrity and viability in most of the

boars. Although it is difficult to envisage the functional significance of such finding, it was reported that SP supplementation to the freezing or thawing extender significantly increased the percentages of viable frozen-thawed spermatozoa with intact acrosome (Okazaki et al., 2009). Accordingly, high proportions of viable spermatozoa with intact acrosome are prerequisites for successful fertilization-associated events (Daigneault et al., 2015). Despite variable protein composition, our findings indicate the lack of consistent differences in PT semen quality between SP1- and SP2-treated samples. Such observation reinforces the notion that components of either fractionated SP are involved in the mechanism associated with sperm cryo-tolerance. It is noteworthy that our results reaffirm those of previous studies suggesting that different sperm attributes react differently to the cooling and thawing-freezing processes (Holt et al., 2005; Fraser et al., 2014; Wasilewska and Fraser, 2017; Wasilewska et al., 2018 a).

The SDS-PAGE and densitometric analysis, representing the profiles of sperm membrane proteins, revealed quantitative and qualitative variations between the pre-freeze and PT semen, and among treatments. It is important to note that the SDS-PAGE analysis was performed to get more information about changes occurring in the membrane-associated proteins of frozen-thawed spermatozoa, and in the current study, the patterns of the electrophoretic profiles did not differ in most of the boars. Representative protein profiles of the frozen-thawed spermatozoa showed that samples treated with either SP1 or SP2 were characterized by mostly low-molecular-weight proteins, with the majority being <30 kDa compared with samples treated with either wSP or BTS. These differences in the protein profiles among treatments might be attributed to the cooling, and freezing-thawing processes. There has been compelling evidence indicating that cryopreservation disrupts the lipid and protein composition of the membranes of boar spermatozoa, resulting in their compromised fertility (Okazaki et al., 2009; Chen et al., 2014; Yeste, 2016; Yeste et al., 2017). However, among the potential mechanisms that could be responsible for the higher PT semen quality in spermatozoa exposed to either SP1 or SP2 might be the rearrangement of the sperm-coating layers during the cooling and freezing-thawing processes. Studies have been shown that the interactions of SP proteins with spermatozoa at ejaculation cause remodeling and rearrangement of the sperm-coating layers, which modulate the sperm functions (Jonáková and Tichá, 2004; Rodríguez-Martínez et al., 2011; Perez-Patiño et al., 2019). Despite not identifying the major SP proteins that might be implicated in the modifications of the sperm-coating layers, analysis of the expression levels of membrane-bound proteins, such as voltage-dependent anion channel 2 (VDAC2, 31.6 kDa) and a 32-kDa acrosin binding protein (ACRBP) confirmed their relevance in the assessment of semen freezability (Vilagran et al., 2013; Yeste, 2016). We did not characterize and analyze the level of expression of the sperm membrane proteins detected after different treatments of the pre-freeze semen, and are unable to specify their significant relevance during cryopreservation. Follow-up research will attempt to characterize the biological role of the sperm proteins in cryo-tolerance.

Taken together, it has been confirmed that treatment of semen with fractionated SP before freezing-thawing modulate the functions of spermatozoa (motility char-

acteristics, membrane integrity and viability), rendering them less susceptible to cryo-damage. In addition, the results of our study emphasize the beneficial effect of chromatographic fractionation of SP, and reaffirm that the protective mechanisms of SP components on sperm functions differed among boars during cryopreservation. Further research studies are prerequisite to identify individual SP protein components that could be used as potential markers associated with the freezability of boar semen.

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