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Original article

Effect of post-thaw supplementation of fractionated seminal plasma on survival of boar spermatozoa

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Abstract

In our recent study we demonstrated that the holding of fresh semen in fractionated seminal plasma (SP1, >40 kDa; SP2, <40 kDa), obtained by gel filtration chromatography, significantly improved the sperm quality characteristics following cryopreservation (Wasilewska-Sakowska et al. 2019). In this study we investigated the effect of post-thaw (PT) supplementation of fractionated SP (SP1 and SP2) on the survival of spermatozoa from boars with good and poor semen freezability, GSF and PSF, respectively. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis showed distinct differences in the protein profiles of SP1 and SP2 from boars with GSF or PSF regarding the number of protein spots. Sperm motility characteristics and the motion patterns, assessed using the computer-assisted sperm analysis (CASA) system, were markedly higher in PT semen supplemented with SP1 and SP2 from boars with GSF. Post-thaw supplementation of either SP1 or SP2 from boars with GSF significantly improved mitochondrial function, plasma membrane and acrosome integrity, and viability during storage. The findings of this study have confirmed that the presence of protective protein components in varying abundance in either fractionated SP from boars with good freezability ejaculates significantly improved the sperm survival following PT storage.

Key words: boar, semen, fractionated seminal plasma, cryopreservation

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Introduction

Cryopreservation causes irreversible alterations in the sperm membranes, resulting in reduced fertilization potential of frozen-thawed semen (Yeste 2016). Sperm cryo-survival is influenced by many factors, such as the sperm source and individual differences (Alkmin et al. 2014, Fraser et al. 2014). There is a general consensus that the identification of seminal plasma (SP) markers associated with semen freezability would improve the freezing protocol of boar semen (Vilagran et al. 2015, Yeste, 2016). Even though progress in proteomic technology of boar SP has provided information about the role of boar SP components in fertilization-related events (Rodríguez-Martínez et al. 2011, Perez-Patiño et al. 2016), there is still limited research on the functional relevance of SP proteins in sperm cryo-tolerance.

Boar SP comprises numerous proteins and antioxidant components, and differences in the SP composition could have a significant impact on sperm functions (Strzeżek et al. 2002, 2005, Rodríguez-Martínez et al. 2011, Perez-Patiño et al. 2016). It is well accepted that specific SP components bind to spermatozoa to protect their membrane integrity during semen preservation (Manjunath et al. 2007, Dziekońska et al. 2009). Cumulative evidence has indicated the varying effects of SP components on boar sperm function following cryopreservation (Garcia et al. 2010, Alkmin et al. 2014). It has been demonstrated that freezing of semen in SP from boars with good freezability ejaculates improved the sperm cryo-survival (Hernández et al. 2007), and that the supplementation of SP to the thawing extender significantly improved post-thaw (PT) semen quality (Garcia et al. 2010).

In one of our recent studies, we showed that the fractionation of boar SP by affinity chromatography is an important procedure that could be used to obtain two SP fractions (SP1, >40 kDa; SP2, <40 kD) that have varying effects on sperm-cryo-tolerance (Wasilewska-Sakowska et al. 2019). Moreover, the incorporation of a 1.5 h holding period for semen in fractionated SP has been shown to significantly improve the sperm quality characteristics following freezing-thawing (Wasilewska-Sakowska et al. 2019). Given such a finding, we performed this study to investigate the effect of PT supplementation of fractionated SP on the quality characteristics of spermatozoa from boars with good and poor semen freezability, GSF and PSF, respectively. Also, the electrophoretic profiles of fractionated SP from boars with different freezability were analyzed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis.

Materials and Methods

Animals and ejaculate collections

Seven sexually mature Polish large white (PLW) boars (aged 1.5 to 2 years) were used in this study. 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), using the gloved hand technique. A total of 100 ml ejaculates, comprising the sperm-rich fraction (SRF) and a portion of the post-SRF, were collected from each boar for SP analysis. For the cryopreservation procedure, a total of four or seven SRFs were collected from the seven boars. All ejaculates and SRFs were collected in pre-warmed graded cylinders during the autumn-winter period. The procedure regarding semen collection and analysis has been described elsewhere (Wasilewska-Sakowska et al. 2019). All animal experiments were carried out in accordance with the guidelines set out by the Local Ethics Committee.

Preparation of fractionated SP

The procedure used for the preparation of SP samples (100 ml) from each boar for gel filtration chromatography has been described in a recent study (Wasilewska-Sakowska et al. 2019). The two fractions, SP1 (>40 kDa) and SP2 (<40 kDa), obtained by gel filtration chromatography, were used for two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis, and for PT treatment of frozen-thawed semen. The chromatographic fractions of SP1 or SP2 were pooled for each boar. The protein content was measured in SP1and SP2, and in the whole seminal plasma (wSP, non-fractionated SP) in the pooled samples from each boar (Lowry et al. 1951).

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analysis

Following the PT sperm quality assessment procedure (Wasilewska-Sakowska et al. 2019), the SP1 or SP2 from four boars (Boar A, Boar B, Boar F and Boar G) with good semen freezability (GSF) was pooled and used for 2D-PAGE analysis. A similar procedure was carried out for SP1 and SP2 from the other three boars (Boar C, Boar D and Boar E), which showed poor semen freezability (PSF). The electrophoresis procedure was prepared from protein samples of SP1 and SP2 from boars with GSF or PSF. Fifty micrograms of proteins of SP1 and SP2 from boars of either freezability group were diluted in a buffer comprising 9.5M

urea, 2% Triton X-100, 0.065M dithiothreitol (DTT), 2% ampholytes (pH 3-10) and 0.1% bromophenol blue. The SP samples were subjected to 2D-PAGE analysis according to a previously described method (O'Farrell et al. 1977, Mogielnicka-Brzozowska et al. 2017). Precision Plus Protein Standards (Bio-Rad, Rockville, MD, USA) were used as the molecular weight standards. PDQuest software was used to analyze the 2D-PAGE gels in terms of the number of protein spots, detection of optical density, determination of the molecular weights (MWs) and isoelectric points (pIs).

Semen cryopreservation

The SRF samples were extended in Beltsville Thawing Solution (BTS) for 1.5 h at room temperature, before being cooled for 1 h at 17°C (Wasilewska-Sakowska et al. 2019). Following cooling, the extended samples were processed according to a previously described cryopreservation protocol (Fraser et al. 2010).

Post-thaw semen treatment and analysis

Following thawing in a water bath for 60 sec at 50°C, the samples (500 × 106 spermatozoa/ml) from each boar were divided into three equal portions, and each portion was diluted with BTS (control), BTS+SP1 (1:1) and BTS+SP2 (1:1). For each boar PT semen was diluted with homologous fractionated SP, supplemented with BTS, and incubated for 0 min (15 min PT incubation), 60 min, 120 min and 180 min at 37°C, prior to analysis of motility characteristics and motion patterns. Assessment of PT membrane integrity and viability was performed at 0 and 120 min after thawing. The final sperm concentration after dilution was 50 × 106 spermatozoa/ml. Sperm quality was analyzed in fresh, pre-freeze semen and PT semen in each boar.

Sperm quality assessment

Motility parameters

A computer-assisted sperm analysis (CASA) system (HTR-IVOS 12.3, Hamilton Thorne Biosciences, MA, USA) was used to assess sperm motility characteristics and the motion patterns (Zasiadczyk et al. 2015). The sperm parameters analyzed by the CASA system included total motility (TMOT, %), progressive motility (PMOT, %), rapid movement (%), velocity average pathway (VAP, µm/s), velocity straight line (VSL, µm/s) and velocity curvilinear (VCL, µm/s).

Mitochondrial membrane potential (MMP)

The percentages of spermatozoa with high MMP were assessed by fluorescent staining with JC-1 with propidium iodide (PI) (Molecular Probes, Eugene, USA), according to a previously described method (Dziekońska et al. 2009, Fraser et al. 2014).

Plasma membrane integrity (PMI)

The sperm plasma membrane integrity (PMI) was assessed by florescent staining with SYBR-14 and PI, using a Live/Dead Sperm Viability Kit (Molecular Probes, Eugene, OR, USA), according to a previously described method (Garner and Johnson 1995).

Acrosome integrity

The sperm acrosome integrity was evaluated using fluorescein isothiocyanate-labeled peanut (*Arachis hypogaea*) agglutinin (FITC-PNA) staining with PI, according to a previously described method (Soler et al. 2005, Wasilewska-Sakowska et al. 2019). At least 100 spermatozoa were analyzed in each duplicate slide at 600× 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 cells with damaged acrosome (%).

Viability and apoptotic-like changes in spermatozoa

The percentages of viable and plasma membrane apoptotic-like changes in spermatozoa were assessed using the Vybrant Apoptosis Assay Kit #4 (Molecular Probes Inc., Eugene, USA, according to a previously described method (Trzcińska and Bryła 2015, Wasilewska and Fraser 2017).

Statistical analysis

The normality of the data distribution was analyzed using the one way analysis of variance (ANOVA, Shapiro Wilk test). In our recent study (Wasilewska-Sakowska et al. 2019), assessment of the post-thaw (PT) semen quality showed that four boars (Boar A, Boar B, Boar F and Boar G) exhibited GSF, while the other three boars (Boar C, Boar D and Boar E) showed PSF. A total of 22 SRFs collected from four boars with GSF and 18 SRFs from three boars with PSF were examined by repeated measures ANOVA using the General Linear Model (GLM) procedure from the Statistica software package, version 12.5 (StatSoft Incorporation, Tulsa OK., USA). A 3 × 4 × 2 factorial design was performed to determine if treatment (BTS, SP1 and SP2), storage time (0 min, 60 min, 120 min and 180 min), freezability group (GSF and PSF) or their interactions affected PT sperm motility characteristics

Table 1. Sperm quality characteristics in fresh pre-freeze semen. Values are expressed as the means (± SEM) of 22 and 18 ejaculates from boars with good and poor semen freezability, GSF and PSF, respectively.

Parameters	GSF	PSF
TMOT (%)	88.2 ± 1.3	81.3 ± 1.9
PMOT (%)	61.2 ± 2.1	53.9 ± 4.0
Rapid movement (%)	69.8 ± 3.6	62.1 ± 4.6
VAP (μm/s)	82.5 ± 3.1	86.0 ± 4.9
VSL (μm/s)	58.6 ± 1.9	61.6 ± 2.8
VCL (μm/s)	146.9 ± 6.2	152.8 ± 10.2
MMP (%)	86.4 ± 0.4	85.6 ± 0.5
PMI (%)	87.6 ± 0.6	87.1 ± 0.4
Acrosome integrity (%)	89.2 ± 0.5	87.8 ± 0.8
YO-PRO-1$^-$/PI$^-$ (%)	86.5 ± 1.0	84.3 ± 0.9
YO-PRO-1$^+$/PI$^-$ (%)	5.1 ± 0.4	6.6 ± 0.5
YO-PRO-1$^+$/PI$^+$ (%)	8.4 ± 0.7	9.1 ± 0.6

TMOT – total motility; PMOT – progressive motility; VAP – velocity average path;
VSL – velocity straight line; VCL – velocity curvilinear; MMP – mitochondrial membrane potential; PMI – plasma membrane integrity; YO-PRO-1$^-$/PI$^-$ – viability; YO-PRO-1$^+$/PI$^-$ – plasma membrane apoptotic-like changes; YO-PRO-1$^+$/PI$^-$ – dead spermatozoa

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

Sperm parameters	Treatment		Freezability		Storage time	
	F-value	p-value	*F*-value	p-value	*F*-value	p-value
TMOT	44.614	0.001	202.774	0.001	182.056	0.001
PMOT	43.108	0.001	200.935	0.001	172.149	0.001
Rapid movement	22.794	0.001	195.164	0.001	172.614	0.001
VAP	5.013	0.007	16.490	0.001	46.691	0.001
VSL	3.182	0.045	12.380	0.001	26.720	0.001
VCL	8.223	0.001	16.157	0.001	39.068	0.001
MMP	20.946	0.001	15.006	0.001	384.322	0.001
PMI	22.419	0.001	10.976	0.001	202.465	0.001
Acrosome integrity	7.246	0.001	29.690	0.001	294.516	0.001
YO-PRO-1$^-$/PI$^-$	23.603	0.001	100.497	0.001	338.987	0.001
YO-PRO-1$^+$/PI$^-$	0.845	n.s	0.305	n.s	23.714	0.001
YO-PRO-1$^+$/PI$^+$	13.326	0.001	58.934	0.001	305.972	0.001

Significant at $p<0.05$; n.s – non significant; TMOT – total motility; PMOT – progressive motility; VAP – velocity average path; VSL – velocity straight line; VCL – velocity curvilinear; MMP – mitochondrial membrane potential; PMI – plasma membrane integrity; YO-PRO-1$^-$/PI$^-$ – viability; YO-PRO-1$^+$/PI$^-$ – plasma membrane apoptotic-like changes; YO-PRO-1$^+$/PI$^-$ – dead spermatozoa

and motion patterns. The effects of treatment, storage time (0 min and 120 min), freezability group and their interactions with PT membrane integrity and viability were analyzed under a 3 × 2 × 2 factorial design. All results are expressed as the mean ± standard error of the mean (SEM), and values were considered to differ significantly at $p<0.05$. Significant effects were compared using the Neuman-Keuls multiple comparison test ($p<0.05$). Significant differences within treatment between the GSF and PSF groups were compared using a paired Student's t-test ($p<0.05$).

Results

The parameters of the pre-freeze semen did not differ ($p>0.05$) between the freezability groups (Table 1).

2D-PAGE analysis

Protein content in the wSP averaged 36.4 mg/ml (24 – 40 mg/ml), whereas it averaged 1.1 mg/ml (range, 0.5 to 2.5 mg/ml) in both SP1 and SP2. A representative 2D-PAGE profile of fractionated SP from boars with

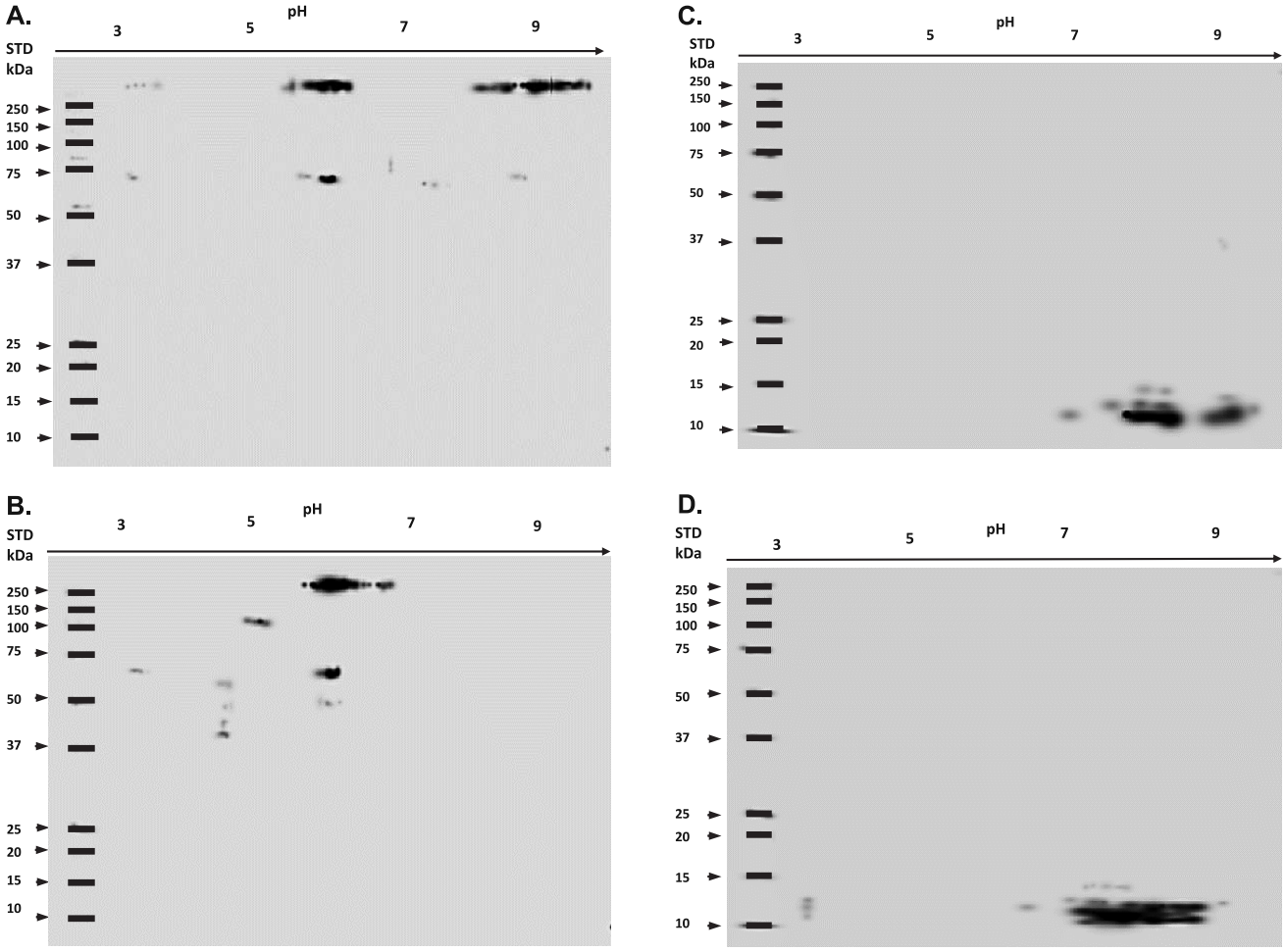

Fig. 1. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of proteins of fractionated seminal plasma (SP) from boars with good and poor semen freezability, GSF and PSF, respectively. A – SP1 from GSF boars, B – SP1 from PSF boars, C – SP2 from GSF boars, D – SP2 from PSF boars, STD – Precision Plus Protein Standards

GSF and PSF is shown in Fig. 1. The image analysis software detected approximately 32 protein spots with 60 to 280 kDa MWs and pIs of 3.5 to 9.7 pH range in SP1 from boars with GSF (Fig. 1A). However, only about 24 proteins, with MWs ranging from 41 to 280 kDa and pIs of 3.5 to 9.7 pH range, were detected in SP1 from boars with PSF (Fig. 1B). In addition, approximately 30 protein spots, with 11 to 37 kDa MWs (pIs of 7.1 to 9.6 pH range), were detected in SP2 from boars with GSF (Fig. 1C), whereas about 39 protein spots, with 11 to 14 MWs (pIs of 3.5 to 9.2 range), were detected in SP2 from boars with PSF (Fig. 1D).

Post-thaw semen assessment

Analysis of variance (ANOVA) showed that the PT sperm quality characteristics were significantly affected by treatment, freezability and storage time (Table 2). Furthermore, treatment × freezability and freezability × storage time interactions significantly affected most of the CASA-analyzed sperm parameters after freezing-thawing.

Figure 2 shows PT motility characteristics and motion patterns of spermatozoa treated with BTS extender and SP1 and SP2 at different storage time periods. Higher ($p<0.05$) TMOT (Fig. 2A), PMOT (Fig. 2B) and rapid movement (Fig. 2C) were observed in PT semen exposed to SP1 or SP2 compared with BTS-treated samples, regardless of the freezability group. Prolonged storage time caused a marked decline in TMOT, PMOT and rapid movement, particularly in PT semen treated with BTS. In addition, TMOT, PMOT and rapid movement were higher ($p<0.05$) in PT spermatozoa from boars with GSF than in the PSF boars, irrespective of the storage time (Fig. 2A-C). Significantly higher ($p<0.05$) VAP (Fig. 2D), VAP (Fig. 2E) and VCL (Fig. 2F) were observed in frozen-thawed spermatozoa from boars with GSF at 0 min after storage (15 min PT incubation), irrespective of treatment. Furthermore, PT semen from boars with GSF exhibited markedly higher ($p<0.05$) values for VAP and VSL at 0 min after storage in SP1 and SP2 (Fig. 2D and Fig. 2E, respectively). For each treatment, VCL was higher ($p<0.05$) in PT spermatozoa from

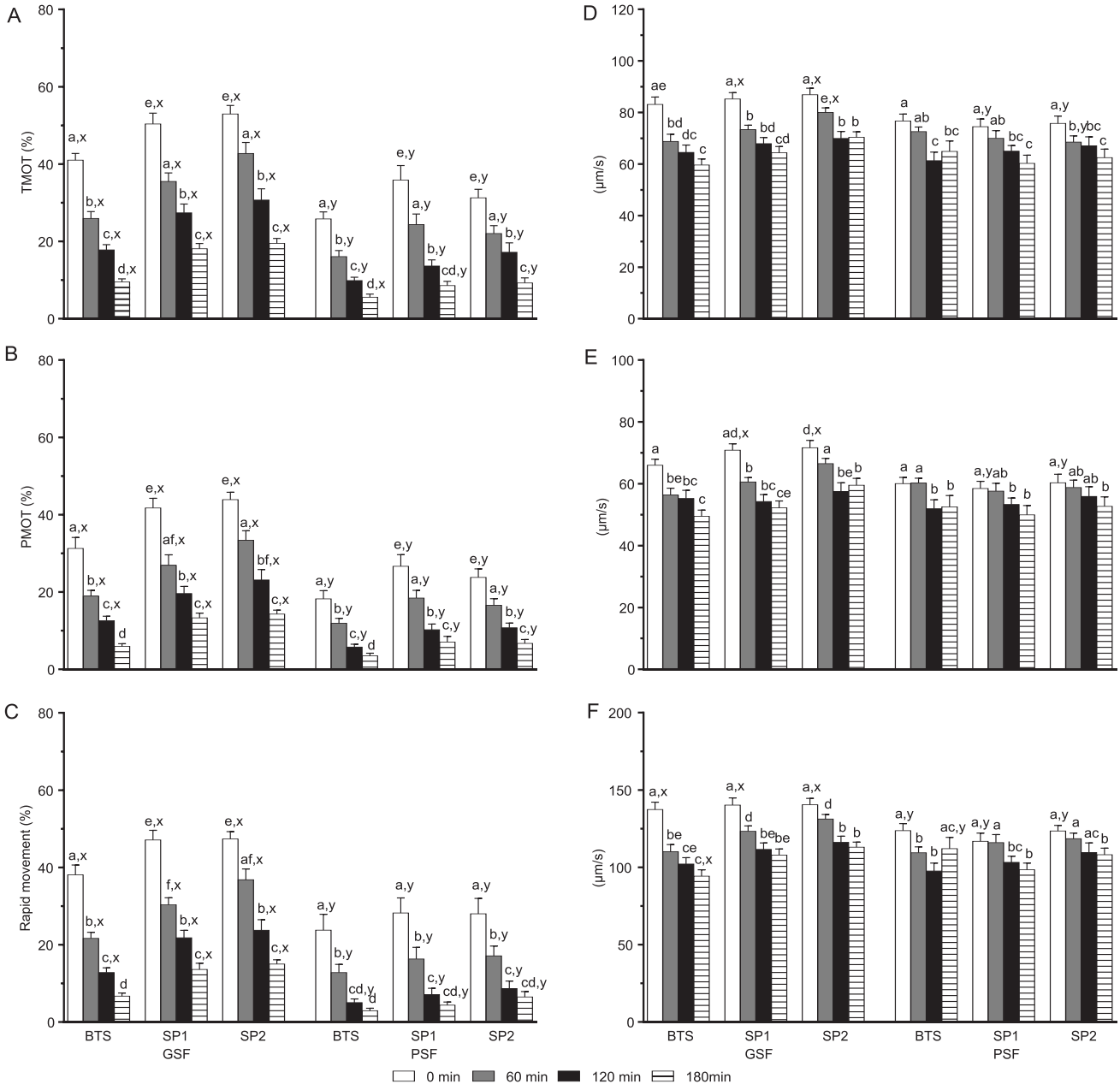

Fig. 2. Post-thaw motility characteristics and motion patterns of boar spermatozoa held in Beltsville Thawing Solution (BTS) and fractionated seminal plasma (SP) at different storage time periods. Values are expressed as the means (± SEM) of 22 and 18 ejaculates from boars with good and poor semen freezability, GSF and PSF, respectively. Values with different letters (a-f) within freezability group are significant at $p<0.05$. GSF vs PSF within storage time period (x and y) is significant at $p<0.05$. A – total motility (TMOT); B – progressive motility (PMOT); C – rapid movement; D – velocity average pathway (VAP); E – velocity straight line (VSL); F – velocity curvilinear (VCL)

boars with GSF at 0 min after storage (Fig. 2F).

Frozen-thawed spermatozoa exposed to SP1 or SP2 from boars with GSF exhibited higher ($p<0.05$) MMP (Fig. 3A), PMI (Fig. 3B) and acrosome integrity (Fig. 3C) than those treated with BTS at 0 min after thawing. There were wide variations in MMP, PMI, acrosome integrity and viability at 120 min after storage, being markedly higher ($p<0.05$) in PT spermatozoa from boars with GSF than those from the PSF boars (Fig. 3A-C). Post-thaw viability (YO-PRO-1$^-$/PI$^-$) was higher ($p<0.05$) in sperm samples exposed to either fractionated SP from boars with GSF, irrespective of the storage time (Fig. 3D). The proportions of dead sperm cells (YO-PRO-1$^+$/PI$^+$) observed during PT storage were markedly lower ($p<0.05$) in SP1 or SP2 from boars with GSF (Fig. 3E). Irrespective of the treatment, the percentages of PT spermatozoa with apoptotic-like changes (YO-PRO-1$^+$/PI$^-$) were lower at 120 min after storage, and ranged from 7.5 to 9.8% and 7.3 to 7.5% for boars with GSF and PSF, respectively.

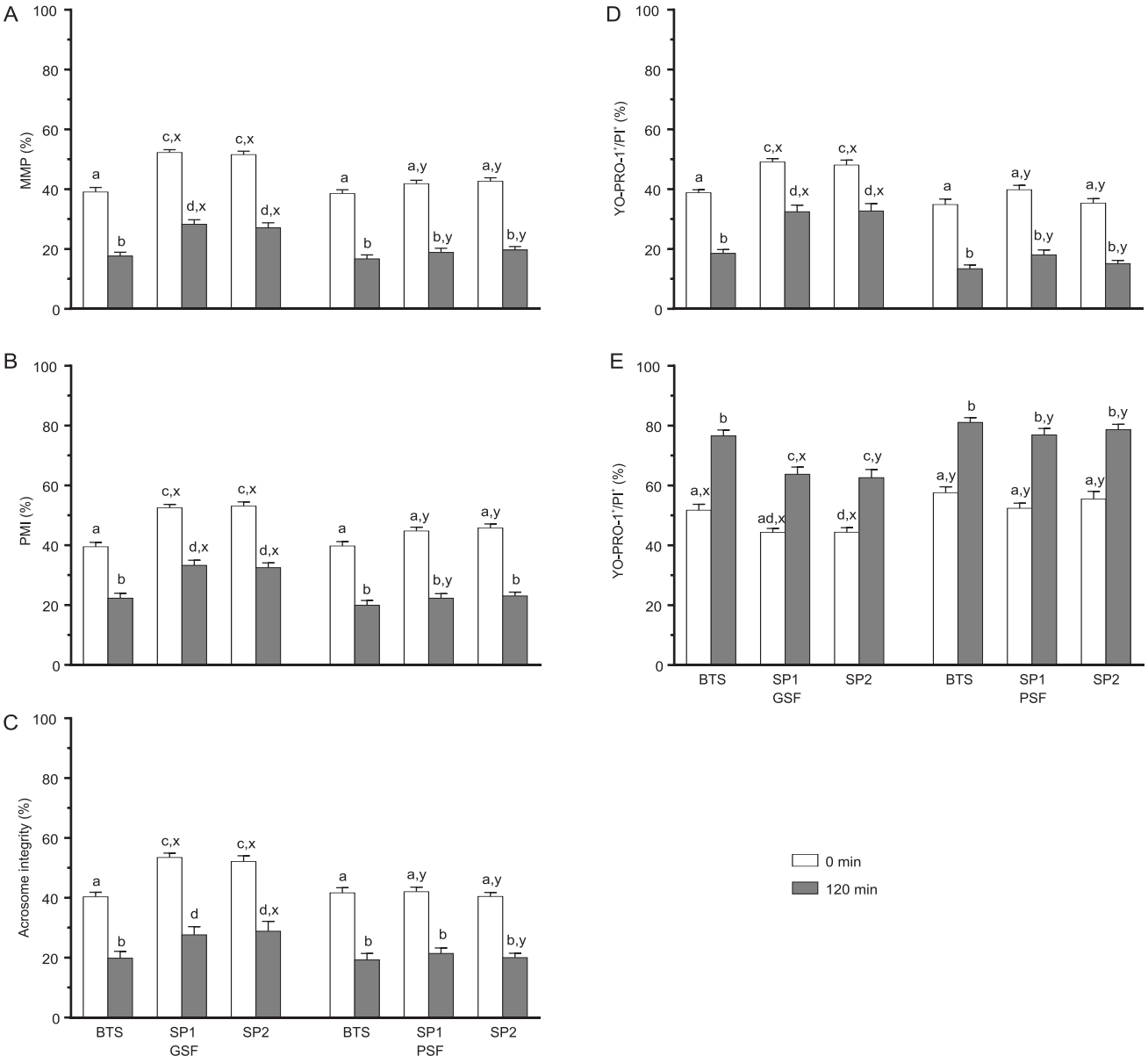

Fig. 3. Post-thaw membrane integrity, viability and (B) plasma membrane apoptotic-like changes in boar spermatozoa held in Beltsville Thawing Solution (BTS) and fractionated seminal plasma (SP) at different storage time periods. Values are expressed as the means (± SEM) of 22 and 18 ejaculates from boars with good and poor semen freezability, GSF and PSF, respectively. Values with different letters (a-d) within freezability group are significant at $p<0.05$. GSF vs PSF within storage time period (x and y) is significant at $p<0.05$. A – mitochondrial membrane potential (MMP); B – plasma membrane integrity (PMI); C– acrosome integrity; D – viability ($YO\text{-}PRO\text{-}1^{-}/PI^{-}$); E – dead sperm cells ($YO\text{-}PRO\text{-}1^{+}/PI^{-}$).

Discussion

In our recent study we showed that fractionated SP exerts varying effects on sperm cryo-survival and that there were marked differences among the individual boars (Wasilewska-Sakowska et al. 2019). In our study the effect of PT supplementation of fractionated SP on the survival of frozen-thawed spermatozoa was assessed in four boars with GSF and three boars with PSF. A preliminary study based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis shows varying abundance of different proteins in the composition of SP1 and SP2 from boars of either freezability group (Wasilewska et al. 2018).

Although SP1 is compositionally different from SP2 from boars with GSF (Wasilewska et al. 2018), there were no marked differences in PT survival of spermatozoa between the fractionated SP, irrespective of the storage time. At post-thaw there were marked variations between the GSF and PSF boars, with respect to the motility characteristics and motion patterns. It should be emphasized that motility is an important sperm quality assessment parameter that is significantly affected by cryopreservation (Fraser et al. 2010).

The findings of the current study showed that PT storage of semen from boars with GSF in either SP1 and SP2 was accompanied by greater TMOT, PMOT and rapid movement compared to boars with PSF. Furthermore, differences between the freezability groups were marked in the motion patterns during PT storage, being significantly higher in BTS and the fractionated SP from boars with GSF. Our results reaffirm the beneficial effects of the SP components on sperm functions during PT storage of semen from boars with GSF. Notably, boar-dependent variations in the amounts of SP protein components have been shown to affect sperm cryo-survival (Hernández et al. 2007).

In the current study PT membrane integrity and viability of frozen-thawed spermatozoa were significantly higher in both SP1 and SP2 from boars with GSF compared with the PSF boars following storage. According to Fernández-Gago e al. (2013), PT supplementation of 50% to the thawing medium had negative effects on sperm functionality, whereas in a previous study it was reported that there was an improvement in PT sperm function and fertility when 50% SP was added to the semen (Garcia et al. 2010). We suggest that the inconsistency in the SP-based studies on PT semen quality might be due to the final concentrations of SP and its composition, or source of the SP. It has been demonstrated that the loss of membrane integrity and reduced viability of boar spermatozoa following PT storage have been attributed to increased oxidative stress, resulting in an excessive production of reactive oxygen species (ROS) (Fernández-Gago et al. 2013, Alkmin et al. 2014). Moreover, the presence of high proportions of dead spermatozoa in frozen-thawed semen has been shown to compromise the sperm fertilizing ability (Roca et al. 2013). In the current study, it appears that the increased proportions of dead spermatozoon in SP1 or SP2 from boars with PSF were concomitant with higher ROS production, which probably exacerbated the PT sperm damage. It is noteworthy that boar SP comprises numerous antioxidant components (Strzeżek et al. 2002, 2005), and certain antioxidants have been shown to exert a beneficial effect on the sperm cryo-tolerance (Li et al. 2018). It is plausible that such cryo-protective components were more prominent in SP1 and SP2 from boars with GSF, resulting in improved PT sperm longevity.

In the current study we did not characterize the protein composition comprising the fractionated SP; however, 2D-PAGE analysis showed significant differences in the electrophoretic profiles of SP1 and SP2 from either freezability group. Using label-free quantitative LC-MS/MS analysis, several categories of proteins, with high and low abundance that might be associated with semen freezability, have been identified in the fractionated SP (Wasilewska et al. 2018). In our preliminary study LC-MS/MS-based analysis has detected high-abundance proteins in SP1, such as fibronectin 1 (FN1) and kinesin family member 15 (kinesin-like protein KIF 15), and low-molecular weight spermadhesins (PSP-I, PSP-II, AWN, AQN-1 and AQN-3) and cathepsin B **(**CTSB**)** in SP2 from boars with GSF (Wasilewska et al. 2018). It should be stressed that there is still insufficient information about the functional relevance of most of the SP proteins in the cryopreservation of boar semen. However, studies have demonstrated that high expression of FN1 correlates with greater PT sperm motility (Vilagran et al. 2015, Yeste 2016), whereas KIF15 is a microtubule-associated protein that is implicated in the modulation of ATPase activity (Tomar et al. 2011). It should be emphasized that the role of spermadhesins in sperm function has been widely studied (Strzeżek et al. 2005, Rodríguez-Martínez et al. 2011, Perez-Patiño et al. 2016), and their association with improved sperm viability and fertility following semen preservation has been reported by several authors (Hernández et al. 2007, Garcia et al. 2010). Moreover, CTSB is a protease protein that is involved in metabolism (Inayat et al. 2012); however, the significant relevance of this protein in PT sperm longevity is unclear. It is widely known that spermatozoa interact with SP proteins and extender components to form a protective coating-layer, which stabilizes the sperm membranes during semen preservation (Manjunath et al. 2007, Dziekońska et al. 2009). In this regard, it seems likely that the beneficial effect of several membrane-coating proteins of SP1 or SP2 from boars with GSF could explain the higher PT sperm survival.

The findings of our study show that PT supplementation of fractionated SP from boars with GSF ameliorates sperm survival during storage. We suggest that the improved survival of spermatozoa from boars with GSF is attributed mainly to the cryo-protective effect of several protein components of SP1 and SP2 on the PT sperm functionality. The findings of this study have reaffirmed that the PT supplementation of fractionation SP from boars with good freezability ejaculates is a useful approach to improving the survival of frozen-thawed spermatozoa. However, identification of high and low abundance proteins in the fractionated SP that are associated with sperm freezability would facilitate the improvement in the freezing protocol of boar semen.

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