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Short communication

Antioxidant status of chromatographic fractions of boar seminal plasma (SP) and mitochondrial functions of SP-treated spermatozoa following cryopreservation

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Abstract

In this study we investigated the antioxidant status of fractionated seminal plasma (SP1, >40 kDa; SP2, <40 kDa), obtained by gel filtration chromatography (Wasilewska-Sakowska et al. 2019a), and mitochondrial functions in SP-treated spermatozoa during pre-freeze equilibration and post-thaw storage periods. The Total Antioxidant Status (TAS), represented by enzymatic and non-enzymatic antioxidants was markedly affected by the treatments, being significantly higher in either SP1 or SP2 than in the whole seminal plasma (wSP). It was observed that ATP content was markedly higher in SP1- or SP2-treated samples following pre-freezing equilibration. Although there were no marked differences in the ATP content of frozen-thawed (FT) spermatozoa among treatments, regardless of the cryopreservation stage, mitochondrial membrane potential (MMP) of FT spermatozoa differed significantly among treatments. Furthermore, significant differences in the sperm MMP at post-thaw were not reflected in the ATP levels, suggesting that variations in the surrounding environments of FT spermatozoa might have varying effects on the sperm mitochondrial functions following cryopreservation. It seems that high TAS levels in the fractionated SP could reaffirm the protective role of antioxidants in sperm cryo-damage.

Keywords: antioxidants, ATP, boar, cryopreservation, spermatozoa

Introduction

Post-thaw sperm quality is affected by different factors, including the source of the seminal plasma (SP), and storage time after freezing-thawing (Li et al. 2018, Wasilewska-Sakowska et al. 2019b). Spermatozoa have a limited antioxidant defense system and most of the protection against reactive oxygen species (ROS) is provided by the seminal plasma (SP) (Koziorowska-Gilun et al. 2011, Gupta et al. 2021, Ünal and Uysal 2024). Evidence has been shown that semen cryopreservation induces ROS production, which compromises sperm cryo-survival (Li et al. 2018). It has been suggested that the exposure of spermatozoa to different portions of the seminal plasma (SP) could improve the cryo-survival and fertility of boar spermatozoa (Li et al. 2018, Rodríguez-Martínez et al. 2021). Boar SP comprises a wide variety of proteins and antioxidant components, which could have varying effects on the sperm functions (Rodríguez-Martínez et al. 2021). We have reported that liquid chromatography mass spectrometry analysis of fractionated SP (SP1, >40 kDa; SP2, <40 kDa) revealed that fibronectin 1 (FN1) was more abundant in SP1, whereas spermadhesins, particularly PSP-I, were highly expressed in SP2 (Fraser et al. 2021). In this study we hypothesized that the antioxidant status of the fractionated SP could also play an important role in the sperm cryo-survival. We investigated the SP antioxidant status and sperm mitochondrial functions, represented by the content of adenosine triphosphate (ATP), motility and mitochondrial membrane potential (MMP), following treatments in fractionated SP at different stages of the cryopreservation procedure.

Materials and Methods

Animals

Four sexually mature Polish large white (PLW) boars (aged 1.5 to 2 years), which were stationed at the Cryopreservation Laboratory at the Faculty of Animal Bioengineering, University of Warmia and Mazury in Olsztyn (Poland), were used in this study. The procedure regarding semen collection and processing has been described elsewhere (Wasilewska-Sakowska et al. 2019a). All animal experiments were carried out in accordance with the guidelines set out by the Local Ethics Committee.

Preparation of fractionated SP and semen processing

The fractionated fractions (SP1 and SP2) were prepared according to a previously described chromatographical method (Wasilewska-Sakowska et al. 2019a). Superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity (U/ml, respectively) was measured in the wSP and fractionated SP using Randox Superoxide Dismutase, Ransod SD125 and Randox Glutathione Peroxidase RS505 assay kits, respectively (Randox Laboratories Ltd, Crumlin, UK), according to the manufacturer's instructions. The SP Total Antioxidant Status (SP-TAS) content (μM) was determined using a TAS assay kit (NX2332, Randox Laboratories Ltd, Crumlin, UK). In Experiment 1 (Exp. 1) the sperm samples were subjected to pre-freezing equilibration in the whole SP (wSP) and fractionated SP (Wasilewska-Sakowska et al. 2019a), while in Experiment II (Exp. II) frozen-thawed (FT) sperm samples were exposed to SP at post-thaw (Wasilewska-Sakowska et al. 2019b, Fraser et al. 2021). In Exp. 1 the sperm-rich fractions were divided into four portions, and pre-freeze equilibrated in wSP, SP1, SP2 and Beltsville Thawing Solution (BTS) at room temperature (Wasilewska-Sakowska et al. 2019a). Following equilibration (1.5 h), the sperm samples were extended in BTS, before being frozen (Wasilewska-Sakowska et al. 2019a). Cooled samples were frozen, using a standard cryopreservation protocol that included lipoprotein fractions isolated from ostrich egg yolk (LPFo), as described in previously published studies (Wasilewska-Sakowska et al. 2019a, Wasilewska-Sakowska et al. 2019b, Fraser et al. 2021). In Exp. II, FT spermatozoa, suspended in BTS (prepared in Exp. I), were exposed to wSP, SP1 and SP2 after thawing (Wasilewska-Sakowska et al. 2019b, Fraser et al. 2021).

In both experiments (Exp. I and Exp. II), ATP content, motility and MMP were analyzed at different stages of the cryopreservation procedure. ATP content was measured in sperm extracts using an ATP Bioluminescence Assay Kit CLS II (Sigma Aldrich Corporation, St. Louis, MO, USA), according to the manufacturer's instructions. Sperm motility was assessed at 200 × magnification under a bright light microscope (Olympus BX 40, Tokyo, Japan), equipped with an attached heated stage (37°C). In Exp. II sperm motility was assessed following post-thaw supplementation of SP (wSP, SP1 and SP2) at 0 min (15 min post-thaw incubation), 60 min, 120 min and 180 min at 37°C. Sperm MMP was assessed using dual fluorescent staining with JC-1 with propidium iodide (Molecular Probes, Eugene, USA) (Dziekońska et al. 2009). Data are presented as the mean ± standard error of mean (SEM), and were analyzed using the Kruskal-Wallis 1-way ANOVA (IBM SPSS Statistics software package, IBM Corp. Armonk, NY, USA), or using the Tukey multiple comparison test at $p \leq 0.05$ (Statistica software package,

Table 1. Antioxidant status of whole boar seminal plasma (wSP) and fractionated SP. Values are represented by means (± SEM) of four ejaculates.

Antioxidant status	wSP	SP1	SP2
SOD activity (U/ml)	2.35 ± 0.26[a]	0.45 ± 0.04[b]	1.63 ± 0.27[a]
GPx activity (U/ml)	0.68 ± 0.05[a]	0.30 ± 0.05[b]	0.31 ± 0.06[b]
TAS (μM)	924.14 ± 65.56[a]	1232.71 ± 32.76[b]	1248.19 ± 21.60[b]

SP1 – fractionated SP with >40 kDa; SP2 – fractionated SP with <40 kDa
SOD – Superoxide dismutase, GPx – Glutathione peroxidase; TAS – Total Antioxidant Status. Values within the same row with different letters (a and b) are significant at p≤0.05 after the Bonferroni correction.

Table 2. ATP content, motility and mitochondrial membrane potential (MMP) of boar spermatozoa at different cryopreservation stages. Values are represented by means (± SEM) of 4 or 12 ejaculates.

Treatments (Exp. I)	Pre-freeze equilibration			Post-thaw analysis		
	ATP content	Motility	MMP	ATP content	Motility	MMP
	(nmol ATP/10^8 spz)	(%)	(%)	(nmol ATP/10^8 spz)	(%)	(%)
wSP	11.06 ± 0.32[ab]	79.17 ± 1.83[a]	79.58 ± 0.74[a]	2.30 ± 0.13[a]	29.58 ± 3.91[a]	31.04 ± 1.14[a]
SP1	11.56 ± 0.27[a]	81.67 ± 2.07[a]	80.66 ± 0.70[a]	2.30 ± 0.16[a]	37.08 ± 3.56[a]	43.35 ± 1.97[b]
SP2	11.75 ± 0.35[a]	83.92 ± 1.05[a]	81.68 ± 0.62[a]	2.30 ± 0.24[a]	41.67 ± 2.84[a]	39.23 ± 1.63[b]
BTS	10.30 ± 0.18[b]	81.25 ± 1.64[a]	81.40 ± 3.09[a]	2.13 ± 0.14[a]	30.83 ± 4.17[a]	42.09 ± 2.08[b]

wSP – whole seminal plasma, SP1 – fractionated SP with >40 kDa, SP2 – fractionated SP with <40 kDa, BTS – Beltsville Thawing Solution, MMP – mitochondrial membrane potential, nmol ATP/10^8 spz – nmol ATP/10^8 spermatozoa. Values within the same column with different letters (a and b) are significant at p≤0.05.

TIBCO Software Inc. Statistica, CA, USA; StatSoft Polska, Kraków, Poland).

Results and Discussion

SP-TAS, represented mainly by antioxidant enzymes and non-enzymatic antioxidants (Ünal and Uysal 2024), was markedly affected by the SP-group (p≤0.021), being significantly higher (p≤0.05) in SP1 and SP2 (Table 1). SOD and GPx are important antioxidant enzymes involved in the defense of sperm cells against ROS-induced damage (Ünal and Uysal 2024): however, the lower activity of SOD and GPX in SP1 or SP2 than in the wSP was compensated by higher TAS levels, suggesting that SP-TAS could play a significant role in the cryo-survival of SP-treated spermatozoa. It should be emphasized that SP-TAS is provided by numerous non-enzymatic antioxidants, such as phenolic compounds, hypotaurine and ascorbic acid (Gupta et al. 2021, Ünal and Uysal 2024). Recently, it has been confirmed that TAS measurements provide more valuable information about the freezability of semen than the measurements of individual antioxidants (Ünal and Uysal 2024).

There were marked variations in sperm ATP content following pre-freezing equilibration in SP and BTS (Kruskal-Wallis ANOVA, p≤0.047), being significantly higher (p≤0.05) in SP1- or SP2-treated samples (Table 2). Sperm motility and MMP following pre-freezing equilibration of spermatozoa in fractionated SP did not vary significantly (p≥0.05, respectively) among the treatments (Table 2, Exp. I). Although there were marked variations (p≤0.05) in sperm MMP, ATP content and motility of FT spermatozoa from the pre-freezing equilibration period did not significantly differ (p≥0.05) among the treatments (Table 2, Exp. I). Likewise, the percentage of FT spermatozoa with MMP differed between the wSP and fractionated SP, and was significantly lower (p≤0.05) in BTS than in the SP-treated FT spermatozoa at post-thaw (Table 3, Exp. II). No marked differences (Kruskal-Wallis ANOVA, p≥0.05) in either post-thaw ATP content or motility were observed among the SP-treated spermatozoa at post-thaw (Table 3 and Fig. 1, respectively). Prolonged post-thaw storage caused a gradual decline in the motility of FT spermatozoa, being significantly reduced (p≤0.05) at the 60 min storage period following wSP and SP2 treatments (Fig. 1).

It is noteworthy that ATP is produced in spermatozoa by mitochondrial oxidative phosphorylation (OXPHOS) and glycolysis, and its level reflects the sperm metabolic status (Dziekońska et al. 2009, Prieto et al. 2023). Moreover, ROS-induced ATP reduction in FT spermatozoa could be caused by the inhibition of the activity of enzymes that are involved in OXPHOS and glycolysis, and might result in reduced sperm motility following post-thaw storage

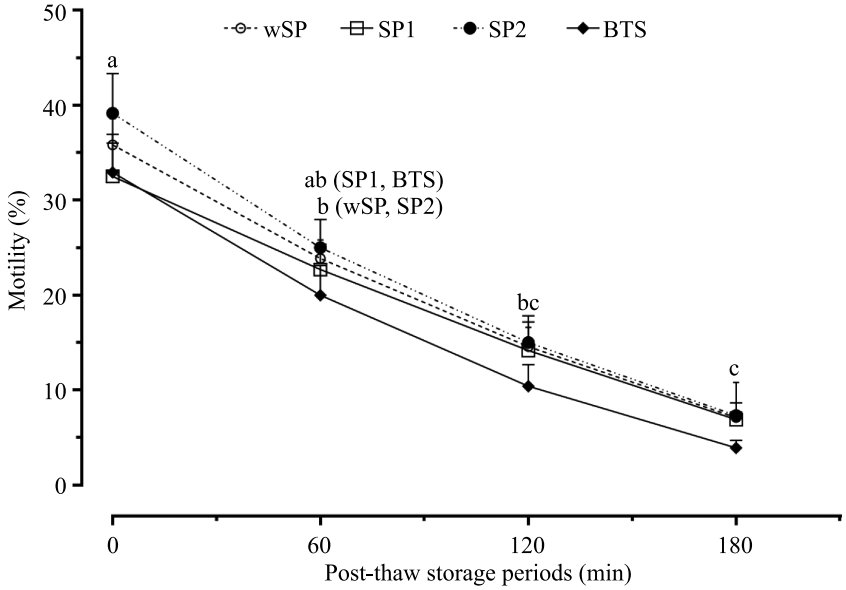

Fig. 1. Post-thaw total motility of spermatozoa following storage in whole boar seminal plasma (wSP), fractionated SP1 and SP2 (>40 kDa and <40 kDa, respectively) and Beltsville Thawing Solution (BTS). Values are expressed as the mean (± SEM) of 12 ejaculates. Values with different letters (a, b and c) among the storage period of each treatment are significant at p≤0.05.

Table 3. Post-thaw ATP content and mitochondrial membrane potential (MMP) of boar spermatozoa held in whole seminal plasma (wSP) and fractionated SP. Values are represented by means (± SEM) of 4 or 12 ejaculates.

Treatment (Exp. II)	Post-thaw supplementation with wSP, SP1 and SP2	
	ATP content	MMP
	(nmol ATP/10^8spermatozoa)	(%)
wSP	2.70 ± 0.19[a]	45.58 ± 1.66[a]
SP1	3.21 ± 0.46[a]	47.58 ± 1.61[a]
SP2	3.26 ± 0.43[a]	43.23 ± 1.63[a]
BTS	2.13 ± 0.14[a]	35.17 ± 2.10[b]

SP1 – fractionated SP with >40 kDa, SP2 – fractionated SP with <40 kDa, BTS – Beltsville Thawing Solution (BTS). Values within the same column with different letters (a and b) are significant at p≤0.05.

(de Lamirande and Gagnon 1992). It has been reported that variations in the surrounding environments of liquid-stored boar semen might affect the sperm ATP production, and the metabolic pathway (Dziekońska et al. 2009). Sperm motility, a parameter commonly used to assess semen quality, is associated with ATP production and mitochondrial functions in spermatozoa following semen preservation (Dziekońska et al. 2009, Wasilewska-Sakowska et al. 2019b). There is evidence indicating that reduced ATP production in spermatozoa is not always associated with their mitochondrial dysfunction, but might be related to the preservation conditions (Preito et al. 2023). In the present study, it seems that SP treatments might affect ATP production in FT spermatozoa, probably by relying on different substrates provided by the surrounding environment.

Notably, higher activity of SOD and GPx in the surrounding environments of FT spermatozoa could be associated with a dynamic response to oxidative stress (OS), and might promote AMP-activated protein kinase (AMPK) and reduce apoptotic-induced sperm damage (Li et al. 2023). However, the mechanism underlying the activation of AMPK is not fully understood, and in the current study we are unclear whether such a mechanism is involved in the sperm cryo-survival. In the present study significantly higher TAS levels in the fractionated SP could reaffirm the protective role of antioxidants in the sperm cryo-damage. We suggest that further studies are needed to elucidate the beneficial role of SP antioxidants in the post-thaw quality of boar semen.

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