Difference of seminal plasma and sperm proteins in good and poor freezability boar ejaculates

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Abstract

The present study was performed to compare the expression of sperm proteins, *i.e.* triosephosphate isomerase (TPI) and acrosin binding protein (ACRBP) and seminal plasma proteins, *i.e.* glutathione peroxidase 5 (GPX5) and fibronectin 1 (FN1), in boar semen with good, moderate and poor freezability. The study was conducted by determining the protein contents in 32 sperm samples and 38 seminal plasma samples of semen. The ejaculated semen was divided into two portions: the first portion was centrifuged to separate the pellet of sperm from the seminal plasma and the second portion was cryopreserved. After thawing, the ejaculates were classified into three groups according to their post-thawed sperm motility: good (60.2 ± 1.7%), moderate (29.3 ± 2.0%) and poor (16.6 ± 2.2%) freezabilities. The expressions of GPX5 and FN1 in seminal plasma and TPI and ACRBP in sperm were determined using Western blot analysis. It was found that, for sperm proteins, the level of TPI was negatively correlated with the post-thawed total sperm motility (r = -0.38, P = 0.029). For seminal plasma proteins, the level of FN1 in the seminal plasma was positively correlated with the post-thawed total sperm motility (r = 0.37, P = 0.021) and progressive motility (r = 0.39, P = 0.016). The expression of GPX5 was not correlated with any of the frozen–thawed sperm qualities (P > 0.05). In conclusions, boar semen containing a high level of FN1 in seminal plasma has better freezability. Frozen–thawed sperm motility was positively correlated with the level of FN1 in boar seminal plasma and negatively correlated with TPI in boar spermatozoa.

Key words: *boar; cryopreservation; freezability; protein; sperm*

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Introduction

In the current global swine industry, more than 90% of mating is performed by artificial insemination (AI) and over 99% of the semen used for AI in practice is extended fresh semen (Yeste, 2015; Knox, 2016; Žura Žaja et al., 2016). Currently, less than 1% of insemination in pigs is carried out using frozen–thawed semen (Yeste, 2016; Chanapiwat and Kaeoket, 2020). This is because AI, using extended fresh semen, results in a higher conception rate and litter size compared to frozen-thawed semen (Chanapiwat et al., 2014). However, the limitation of using of extended fresh semen in the AI industry is that the semen must be stored at 15-20 °C for no longer than 3-7 days. Although many extenders have been developed to increase the lifespan of extended fresh semen, boar semen quality can be maintained for only a short period of time (Waberski et al., 2019; Chanapiwat and Kaeoket, 2021). Thus, superior genetic boars cannot be preserved for a long time by using conventional extended fresh semen. Therefore, cryopreservation of boar semen is the only method to preserve superior genetic boar semen (Jovičić et al., 2020). Moreover, cryopreserved boar semen can be exported across countries for improving swine genetics (Suwimonteerabutr et al., 2021). Another advantage of using frozen–thawed boar semen is the limitation of the risk of disease transmission among herds. Presently, cryopreservation of boar semen has been developed for the swine industry both in pellet form and straws (Eriksson et al., 2002; Buranaamnuay et al., 2009). However, low fertility rate of sows following AI using frozen–thawed boar semen is still commonly observed (Buranaamnuay et al., 2010; Chanapiwat et al., 2014). From the commercial point of view, the main business for boar semen cryopreservation is trading genetics across countries (Waberski et al., 2019). Moreover, improving the freezing procedure for boar semen may also help to develop preserved sex-sorted semen (Johnson et al., 2005). These data indicate the importance of further development in boar semen freezing technology.

The frozen–thawed boar semen process causes a significant reduction in sperm motility, viability, acrosome integrity, DNA integrity and fertilising ability (Yeste, 2016; Rungruangsak et al., 2021; Suwimonteerabutr et al., 2021). Studies have been conducted to improve the quality of frozen–thawed boar semen including the cryopreservation process, semen extender, thawing media and searching for a freezability marker (Buranaamnuay et al., 2009; Chanapiwat et al., 2010; Yeste, 2015; Jovičić et al., 2020). The main problem with frozen–thawed boar semen quality is cryoinjury (Yeste, 2016; Jovičić et al., 2020). The swine industry continues to request a better sperm protective extender for either fresh or frozen–thawed boar semen (Waberski et al., 2019). Therefore, additional studies on the protein components in either seminal plasma or sperm cells that have protective properties on extended boar semen will eventually helpful to improve the semen cryopreservation technology (Rodriguez-Martinez, 2019). In practice, the boar semen can be classified into two groups according to its ability to withstand cryopreservation procedures, *i.e*. good and poor freezability ejaculates (Chanapiwat et al., 2010). Previous studies have demonstrated that these individual variations maybe associated with sperm proteins (Vilagran et al., 2013; Vilagran et al., 2015). Therefore, recent cryopreservation advanced research seeks a protein marker to predict ejaculate freezability (Yeste, 2015; Jovičić et al., 2020). So far, a number of proteins marker from both sperm and seminal plasma have been detected to be related

to ejaculate freezability (Rodriguez-Martinez, 2019). However, the results from different studies and different population of boars are still controversial (Thurston et al., 2002; Yeste et al., 2013; Llavanera et al., 2019; Valencia et al., 2020; Fraser et al., 2020). The difference in genetics could be responsible for these freezability differences (Thurston et al., 2002). Moreover, Yeste et al. (2013) found that poor freezability ejaculates were less resistant and had a poorer integrity of nucleoprotein structure than good freezability ejaculates. Nevertheless, during the past decade, a number of studies still reported a significant association between various sperm and/or seminal plasma proteins and the freezability of boar semen (Casas et al., 2010; Vilagran et al., 2013). In practice, the main problem is to classify good and poor ejaculates before starting cryopreservation procedures. Since conventional sperm evaluation cannot predict ejaculate freezability in boars, identifying protein markers is therefore interesting (Yeste, 2016). In previous studies, an addition of 50% of seminal plasma from good freezability boar to frozen–thawed sperm improved the quality of frozen–thawed sperm of poor freezability boars (Kaeoket et al., 2011; Fernandez-Gago et al., 2013). Vilagran et al. (2015) found that fibronectin 1 (FN1) was a positive freezability marker of boar semen. Additionally, GPX5 has also been found to be related to protection of the sperm and plasma membrane against peroxides (Noblanc et al., 2011; Vilagran et al., 2015) and was positively correlated with farrowing rates (Novak et al., 2010). Wysocki et al. (2015) found that N-acetyl-ß-hexosaminidase protein in boar seminal plasma was negatively correlated with sperm viability, motility and lipid peroxidation. Furthermore, Vilagran et al. (2013) found a positive relationship between the amount of acrosin binding protein (ACRBP) and post-thawed motility in boar sperm. On the other hand, Triosephosphateisomerase (TPI) has been described as a negative freezability marker (Vilagran et al., 2013). Nevertheless, none of these protein markers has been used in the swine industry. Moreover, additional information needs to be obtained from different population of boars and different environment before the global implementation can be done.

In the previous study, both GPX5 and TPI are markers of boar semen quality (Vilagran et al., 2016). Novak et al. (2010) found a positive correlation between GPX5 and litter size in pigs. Some sperm proteins, e.g. TPI, involved in zona pellucida binding (Petit et al., 2013), are associated with litter size in pigs (Kwon et al., 2015). In fresh semen, Vilagran et al. (2016) demonstrated that TPI levels in poor sperm quality boars were higher than in good sperm quality boars. However, a clinical study conducted under field conditions has not been done. To confirm these findings, investigation of these protein markers in different populations of boars needs to be done before clinical implementation. Moreover, investigation of these protein markers and freezability of boar semen raised under tropical climate conditions has never been done. Therefore, the aim of the present study was to investigate the difference in seminal plasma and sperm proteins in good and poor freezability boar ejaculates. The number of selected proteins in seminal plasma (*i.e.* FN1, GPX5) and sperm proteins (*i.e.* ACRBP, TPI) in relation to sperm motility patterns were also determined.

Materials and Methods

Animals

This experiment followed the guidelines of the ethical principles and guidelines for the use of animals for scientific purposes by the National Research Council of Thailand and was

approved by the Institutional Animal Care and Use Committee in accordance with the university regulations and policies governing the care and use of experimental animals (animal use protocol no. 1631032). The experiment was conducted at the Swine Research and Development Centre, Department of Livestock Development, Nakhon Ratchasima, Thailand. Semen samples were collected from 27 boars (*i.e.*, 13 Duroc, 8 Landrace and 6 Yorkshire). A total of 38 ejaculates were used for seminal plasma protein studies and 32 ejaculates were used in spermatozoa protein studies. All boars were proven sires routinely used for semen collection and were selected based on their good semen quality. To be included in the experiment, the boar ejaculates had to have ≥ 70% subjective motility and ≥ 75% normal morphology. The boars were housed in a conventional open-housing system and were allocated to individual pens. The semen was collected using a gloved-hand technique. The interval between each semen collection was 5-7 days.

Preparation of seminal plasma and sperm samples

The semen was filtered through a semen filter bag and divided equally into two portions. The first portion was prepared for determination of seminal plasma and sperm protein expression. The second portion was used for semen cryopreservation (see below). The first portion of semen was centrifuged at 800 × g at 15 °C for 10 min to separate the pellet of sperm from the seminal plasma. Following this step, the supernatant was centrifuged at 10,000 × g at 4 °C for 15 min and the supernatant was investigated under a phase contrast microscope (Olympus, Hamburg, Germany) at × 200 magnifications to verify that they did not contain sperm. Then, the seminal plasma sample was collected in a cryotube and kept in liquid nitrogen (-196 °C). Next, both sperm and seminal plasma samples were stored at -80 °C before protein extraction and analysis.

Semen cryopreservation

The second portion of the semen was cryopreserved according to our previous protocol (Buranaamnuay et al., 2009) with some modifications. Briefly, the semen was diluted (1:1, v/v) in extender I (Duragen®, Magapor, Zaragoza, Spain). The diluted semen was stored at 15 °C for 2 h. Next, the semen was centrifuged at 800 × g at 15 °C for 10 min. The sperm pellet was resuspended in extender II (lactose-egg yolk (LEY) extender: 80 mL of 11% lactose solution and 20 mL egg yolk) to achieve a 1.5 x 10^9 sperm per mL concentration. The extended semen was cooled to 4 °C for 2 h. Thereafter, the semen was resuspended and combined with extender III (LEY extender plus 10% glycerol and 1.5% Equex STM Paste; Nova Chemical Sales Inc., MA, USA) to a final sperm concentration of 1.0 x 10^9 sperm per mL and loaded into 0.5 PVC-French straws (IMV, IMV Technologies, France). For the freezing process, the straws were placed at approximately 4 cm over the liquid nitrogen level for 15 min (-120 °C) and plunged into liquid N_2 (-196 °C) for storage.

Thawing procedure and semen evaluation

For the thawing procedure, the straws were plunged into 50 °C water for 12 sec. Next, the thawed semen was diluted in Duragen® extender (Magapor, Zalagoza, Spain) 1:10 (v:v) and post-thawed sperm qualities (see below) evaluated. The post-thawed semen was classified into three groups according to the post-thawed total sperm motility, *i.e.* poor (≤ 20%), moderate (21-40%) and good (> 40%). The sperm concentration of the frozen semen was evaluated using a Bürker haemacytometre (Boeco, Hamburg,

Germany). The total motility and sperm kinematic parameters were evaluated at 37 °C using a computer assisted sperm analysis system (IVOSII, Hamilton-Thorne®, Beverly, MA, USA). The semen parameters evaluated included total sperm motility, progressive motility and kinematic characteristic parameters. The sperm kinematic characteristics included straight-line velocity (VSL, μm/ sec), curvilinear velocity (VCL, μm/ sec), average path velocity (VAP, μm/ sec), linearity (LIN, %), straightness (STR, %), wobble coefficient (WOB, %), mean lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz) (Pearodwong et al., 2019).

Sperm viability

The viability of the boar spermatozoa was determined using SYBR-14/ Ethidiumhomodimer-1 (EthD-1) (Fertilight®, Sperm Viability Kit, Molecular Probes Europe, Leiden, The Netherlands) (Pearodwong et al., 2019). Briefly, 10 μL of the semen sample was diluted using 140 μL of thawing medium. Thereafter, 50 μL of diluted semen was mixed with 2.7 μL of SYBR-14 and 10 μL of EthD-1. After incubation at 37 °C for 20 min, 200 sperm were assessed (× 1000) under a fluorescent microscope. The nuclei of sperm with an intact plasma membrane were stained green with SYBR-14, while those with damaged membranes were stained red with EthD-1. Sperm were classified into two types; live sperm, stained green and dead sperm, stained red.

Acrosome integrity

An aliquot (10 μL) of diluted semen was mixed with an equal volume of EthD-1 and incubated at 37 °C for 15 min. Thereafter, 5 μL of mixture was spread on a slide and fixed with 95% ethyl alcohol for 30 seconds. Next, the samples were smeared with 15 μL of FITC-PNA solution (FITC-PNA in phosphate buffer saline (PBS) 1:10, v/v). The slide was placed in a moist chamber at 4 °C for 30 min. The sample was rinsed gently with PBS and dried. A total of 200 sperm cells were determined under a fluorescent microscope using × 1000 magnification. The acrosome of the sperm was categorised as an intact acrosome, reacted acrosome or loose acrosome (Chanapiwat et al., 2009).

Plasma membrane integrity

The integrity of the plasma membrane was assessed using a short hypo-osmotic swelling test (sHOST). Briefly, a 100 μL semen sample was incubated with 1,000 μL of hypo-osmotic solution at 38 °C for 30 min. After incubation, 200 μL of semen and hypo-osmotic solution was loaded on 1,000 μL of hypo-osmotic solution plus 5% formaldehyde (Merck, Darmstadt, Germany). A 10 μL sample was placed on a warm slide with a cover slip. In total, 200 sperm were evaluated using bright-field microscopy at x 400 magnification. The positive result (coiled tail) was defined as sperm having active plasma membrane function (Samardžija et al., 2008; Buranaamnuay et al., 2009).

Mitochondrial activity

The mitochondrial activity was assessed by JC-1 (Molecular Probes, Molecular Probes Inc., Eugene, OR, USA). The stock solutions consisted of 0.153 mM JC-1 in DMSO, 0.02mM SYBR-14 and 2.4 mM PI. The staining solution contained 1.6 μl of 0.153 mM JC-1, 1.0 μL of 0.02 mM SYBR-14, 1.6 μL of 2.4 mM PI in 100 μL Hepes-buffered medium. A total of 12.5 μL of frozen–thawed boar semen was incubated with the staining solution (25 μL) at 37 °C for 30 min. Next, the stained sperm were examined under a fluorescent microscope at x 1,000 magnification. The sperm stained with JC-1, displaying green fluorescence, were defined as mitochondria with low to moderate membrane potential, while the sperm stained with JC-1, displaying

red-orange fluorescence, were defined as mitochondria with high membrane potential (Garner et al., 1997).

Quantification of total protein

The seminal plasma was separated from sperm by centrifugation at 800 × g at 15 °C for 10 min. The supernatant was centrifuged at 10,000 × g at 4 °C for 15 min and stored at -80 °C. The sperm pellet was washed with PBS to eliminate the remaining traces of seminal plasma and centrifuged at 800 × g at 15 °C for 10 min. Thereafter, the sperm pellet was resuspended with PBS with protease inhibitor (Gibco BRL, Life Technologies Ltd., Paisley, UK). Protein was extracted according to the protocol described by Vilagran et al. (2013) with some modifications. Briefly, after washing three times with PBS at 400 × g, the sperm pellets were resuspended in PBS at a concentration of 50 × 10^6 sperm per sample. The pellet of sperm was added to 400 µL of lysis buffer (2% sodium dodecyl sulphate, affymetrix/USB, ThermoFisher Scientific, Waltham, MA, USA), 1.0 M Triethylammonium bicarbonate buffer (Sigma-Aldrich, USA) and protease inhibitor. The samples were lysed by homogenisers (VCX750, Vibra-Cell™, USA) in ice conditions on 10 s off 5 s amp 30% for 5 min and subsequently centrifuged for 10 min at 10,000 × g at 4 °C. The supernatants were stored at -80 °C. The total proteins were quantified using the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA, USA).

Western blot analysis and quantification of protein density

Seminal plasma and spermatozoa protein (20 µg) were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Equal amounts of total protein were denatured in an electrophoresis sample buffer (0.5 M Tris-Hcl pH 6.8, glycerol, SDS, Bromophenol blue, ß-mercaptoethanol). After boiling at 95 °C for 5 min, proteins from each sample were measured in 12.5% polyacrylamide gel. Thereafter, the proteins were transferred into nitrocellulose membranes (NC) at a constant 25 volts for 30 min. The NC membrane was incubated with Odyssey blocking buffer (1×TBS) solutions (Odyssey®, Li-Cor, Lincoln, NE, USA) at room temperature for 1 h. The membranes were washed using 1× Towbin's buffer with 0.01% Tween-20 (Omnia, Huntington Beach, CA, USA) and incubated with anti-FN1 rabbit (Fab2413, Abcam, Cambridge, MA, USA) at a dilution of 1:1,000 or anti-GPX5 rabbit (SC50498, Santacruz Biotechnology, Chicago, IL, USA) at a dilution of 1:200 for seminal plasma protein. For sperm protein, the samples were incubated with anti-ACRBP rabbit (ab64809, Abcam, Cambridge, MA, USA), anti-TPI rabbit (ab96696, Abcam, Cambridge, MA, USA) or anti α-tubulin antibody 11H10 (Cell Signalling Technology, Danvers, MA, USA) at a dilution of 1:1,000 at 4 °C for 24 h. After washing with 1×Towbin buffer many times, the NC membranes were incubated with donkey anti-Rabbit IgG antibody (IRDye® 680RD, Fremont, CA, USA) at a dilution of 1:10,000 in a dark room for 60 min. After washing with 1×Towbin buffer, the NC membranes were evaluated. For protein quantification, the reactive protein band was developed by fluorescent assay. Briefly, the reactive protein band was visualised by Odyssey® (Odyssey® Fc infrared imaging system, LI-COR Biosciences, Lincoln, NE, USA). Then, the membrane was scanned by Odyssey®. Next, the band density was normalised by Coomassie staining. Briefly, the membranes were stained for 1 min at room temperature with Coomassie brilliant blue R-250 (Imperial protein stain, ThermoFisher Scientific, Waltham, MA, USA) and then the membranes were cleaned with methanol-water (1:1 v:v).

After cleaning for 20 min with a 1:5:4 (v:v:v) acetic acid-ethanol-water solution, the membranes were washed twice with distilled water. The membranes were air dried and photographs were taken by using the ChemiDoc Imaging System (Bio-Rad, CA, USA) and were analysed using Image J Software (version 1.6, National Institutes of Health, USA). The density of each band was calculated according to Vilagran et al. (2015). For sperm proteins, the band densities of interesting proteins (ACRBP and TPI) were normalised by using α-tubulin as an internal standard. Sperm protein content = band of interest (intensity) – tubulin (intensity). Each sample was normalised by dividing the density of the band corresponding to α-tubulin using Image J Software.

Statistical analyses

The statistical analyses were carried out using SAS version 9.4 (SAS Inst. Inc., Cary, NC, USA). Descriptive statistics on boar semen qualities and the number of proteins were calculated. Continuous data were presented as least square means ± SEM. Discrete data were presented as percentages. Continuous data included total sperm motility, progressive motility, subjective motility, sperm kinematic characteristic parameters (*i.e.* VCL, VSL, VAP, LIN, STR, WOB, ALH and BCF), sperm viability, acrosome integrity and functional integrity of the sperm plasma membrane. Pearson's correlation was conducted to determine the association between sperm quality parameters and the levels of protein contents of the boar sperm (ACRBP and TPI) and seminal plasma (FN1 and GPX5). Additionally, post-thawed boar semen was classified into three classes according to the post-thawed total sperm motility, *i.e.* poor (≤ 20%), moderate (21-40%) and good (> 40%). The protein content in the seminal plasma (*i.e.* FN1 and GPX5) and sperm (*i.e.* ACRBP and TPI) was compared between groups using ANOVA. Least square means were obtained from the statistical models and compared among groups using the Tukey–Kramer adjustment for multiple comparisons. For all analyses, differences of $P < 0.05$ were regarded as statistically significant.

Results

Across group, the average semen volume, sperm concentration and total sperm per ejaculates were 247 ± 74.6 mL, 283 ± 127 × 10^6 sperm/ mL and 66.5 ± 31.2 × 10^9 sperm, respectively. For fresh semen, the percentage of sperm having bent tail, coil tail, distal cytoplasmic droplet and proximal cytoplasmic droplet were 13.0%, 1.3%, 5.4% and 6.5%, respectively. For frozen-thawed semen, the percentage of sperm having bent tail, coil tail, distal cytoplasmic droplet and proximal cytoplasmic droplet were 22.4%, 2.2%, 11.3% and 13.8%, respectively. The percentage of sperm with morphologically normal was 82.1% and 74.6% for fresh and frozen-thawed semen, respectively. The average frozen-thawed sperm viability, acrosome integrity, plasma membrane integrity, mitochondrial activity, total motility, progressive motility and sperm kinematic characteristics as determined by CASA are presented in Table 1.

The expression of sperm proteins including TPI and ACRBP in good, moderate and poor freezability sperm are demonstrated in Figure 1. The expression of seminal plasma protein patterns including GPX5 and FN1 in good, moderate and poor freezability sperm are demonstrated in Figure 2.

The correlation analyses among sperm quality parameters and amount of sperm and seminal plasma proteins of the frozen–thawed boar sperm are presented in Table 1. Interestingly, the level of FN1 in the seminal plasma was positively correlated with the post-thawed total sperm motility (r = 0.37, P =

0.021) and progressive motility (r = 0.39, P = 0.016). The level of TPI in the sperm was negatively correlated with the post-thawed total sperm motility (r = -0.38, P = 0.029). Neither GPX5 nor ACRBP were correlated with any of the frozen–thawed sperm qualities (P > 0.05).

Of all the semen samples, 31.6%, 44.7% and 23.7% had good, moderate and poor freezability, respectively. Fresh and frozen–thawed boar semen characteristics in good, moderate and poor sperm freezability groups are presented in Table 2. On average, the total motility of the frozen–thawed sperm having good, moderate and poor freezabilities was 54.1 ± 1.3%, 32.1 ± 1.3% and 14.7 ± 1.3%, respectively (P < 0.001). Similarly, the sperm viability of the frozen–thawed sperm having good, moderate and poor freezabilities was 60.1 ± 1.3%, 59.5 ± 1.3% and 44.7 ± 1.3%, respectively (P < 0.001). On average, the levels of the selected sperm proteins including TPI and ACRBP were 3.79 ± 1.40 and 1.87 ± 1.01 density/mm^2, respectively. The levels of sperm proteins in good, moderate and poor sperm freezability groups are presented in Table 1. Both TPI and ACRBP in moderate freezability ejaculates was higher (P < 0.05) than good freezability ejaculates (Table 2). On average, the levels of the selected seminal plasma proteins including FN1 and GPX5 were 7.08 ± 2.88 and 14.04 ± 10.94 density/mm^2, respectively. The levels of seminal plasma proteins in good, moderate

Table 1. Pearson's correlation among sperm quality parameters and amount of sperm (n=32) and seminal plasma proteins (n=38) of frozen–thawed boar sperm.

Sperm quality parameters	Mean ± SD	Sperm		Seminal plasma	
		ACRBP	TPI	FN1	GPX5
Sperm viability (%)	58.9 ± 8.8	NS	NS	NS	NS
Acrosome integrity (%)	60.7 ± 12.6	NS	NS	NS	NS
Plasma membrane integrity (%)	53.8 ± 10.9	NS	NS	NS	NS
Mitochondrial activity (%)	63.5 ± 10.4	NS	NS	NS	NS
Total motility (%)	40.6 ± 15.8	NS	-0.38*	0.43*	NS
Progressive motility (%)	19.2 ± 10.3	NS	NS	0.39*	NS
Sperm kinematic characteristics					
VSL (μm/s)	56.0 ± 8.4	NS	NS	NS	NS
VCL (μm/s)	117.9 ± 27.5	NS	NS	NS	NS
VAP (μm/s)	69.7 ± 11.6	NS	NS	NS	NS
LIN (%)	51.6 ± 9.4	NS	NS	NS	NS
STR (%)	80.6 ± 7.0	NS	NS	NS	NS
WOB (%)	61.9 ± 7.2	NS	NS	NS	NS
ALH (μm)	4.8 ± 1.1	NS	NS	NS	NS
BCF (Hz)	34.5 ± 3.1	NS	NS	NS	NS

Significance levels indicated as * P < 0.05, ** 0.05 < P < 0.01, *** P < 0.001 and NS = P >0.05; VSL (μm/s): straight-line velocity, VCL (μm/s): velocity curved line, VAP (μm/s): velocity average path, LIN (%): linearity (VSL divided by VCL), STR (%): straightness, WOB (%): wobble coefficient, ALH (μm): mean lateral head displacement, BCF (Hz): beat cross frequency

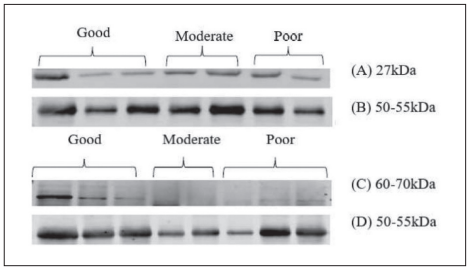

Figure 1. Sperm proteins, *i.e.* TPI (A), ACRBP (B) in good, moderate and poor freezability sperm. α–tubulin (C) and (D) were used as an internal standard in order to normalise the intensity of sperm protein bands

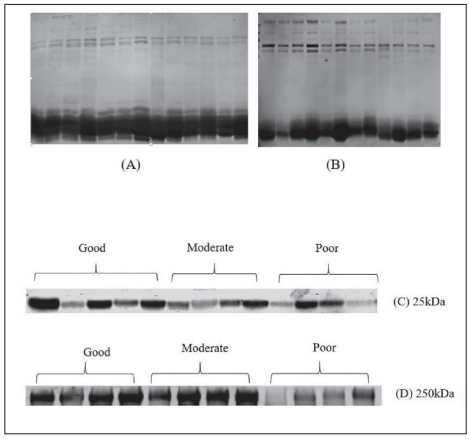

Figure 2. Seminal plasma protein patterns, *i.e.* GPX5 (C) and FN1 (D) in good, moderate and poor freezability sperm. Coomassie brilliant blue stained membranes were used to normalise the band intensity of seminal plasma proteins (A) and (B)

and poor sperm freezability groups are presented in Table 2. The levels of both FN1 and GPX5 in the seminal plasma were not different ($P > 0.05$) among groups (Table 2).

Table 2. Fresh and frozen semen characteristics and amount of sperm and seminal plasma proteins in good, moderate and poor sperm freezability groups (least square means ± SEM).

Variables	Sperm freezability		
	Good	Moderate	Poor
Fresh semen			
Semen volume (mL)	244.2 ± 12.2	238.3 ± 12.6	255.8 ± 26.6
Sperm concentration (x 10^6 sperm/mL)	328.3 ± 24.6	310.9 ± 25.5	275.0 ± 53.8
Total motility (%)	83.9 ± 1.1^a	79.2 ± 1.1^b	79.2 ± 2.3^{ab}
Progressive motility (%)	39.8 ± 2.0	35.7 ± 2.1	29.7 ± 4.4
Frozen–thawed semen			
Sperm viability (%)	60.1 ± 1.3^a	59.5 ± 1.3^a	44.7 ± 3.6^b
Total motility (%)	54.1 ± 1.3^a	32.1 ± 1.2^b	14.7 ± 2.6^c
Progressive motility (%)	26.9 ± 1.0^a	14.2 ± 1.1^b	5.7 ± 2.2^c
Sperm protein			
Number of samples	12	10	10
TPI	3.17 ± 0.39^a	4.35 ± 0.43^b	3.97 ± 0.42^{ab}
ACRBP	1.19 ± 0.21^a	1.97 ± 0.23^b	1.35 ± 0.23^{ab}
Seminal plasma protein			
Number of samples	13	16	9
GPX5	14.68 ± 3.10	14.39 ± 2.80	12.50 ± 3.74
FN1	8.00 ± 0.80	6.57 ± 0.72	6.64 ± 0.96

^{a,b,c} different superscripts within rows differ significantly ($P < 0.05$)

Discussion

Of all the cryopreserved semen samples, 31.6%, 44.7% and 23.7% had good, moderate and poor freezability, respectively. The present study demonstrated differences in some seminal plasma (GPX5, FN1) and sperm protein (TPI, ACRBP) contents in good, moderate and poor freezability ejaculates classified according to frozen-thawed boar sperm total motility. In boar seminal plasma, the FN1 content was associated with sperm quality parameters, while GPX5 was not correlated with any of the sperm quality parameters. Fibronectin 1 is a high-molecular-weight (~250 kD) glycoprotein in blood plasma, the extracellular matrix, basal lamina and on the cell surface. In humans, FN is associated with sperm maturation, sperm capacitation and fertilisation (Miranda and Tezon, 1992). Fibronectin can stimulate sperm capacitation in the oviduct through the cyclic adenosine monophosphate and protein kinase pathway (Martinez-Leon et al., 2015). In boar, FN1 is one of the most abundant proteins in seminal plasma (Druart et al., 2013). Gonzalez-Cadavid et al. (2014) found that FN1 was negatively correlated with the proportion of tail abnormalities in boar sperm. In the present study, FN1 is a potential freezability marker for boar semen cryopreservation. This finding is in agreement with a previous study (Vilagran et al., 2015). However, the mechanism underlining the effect of FN1 on the freezability of boar semen remains unknown. The functions of the boar seminal plasma proteins include the physiological modulations of sperm function during transport in the female reproductive tract, inhibiting and stabilising activity against enzyme systems and sperm chromatin, immunosuppressive and immunomodulation properties, defence mechanisms of spermatozoa and enzymes in sperm metabolism (Strzezek, 2002). Therefore, the effect of FN1 on the freezability of boar sperm may be associated with the stabilising activity and defence mechanisms of spermatozoa.

In the present study, GPX5 was not associated with the boar sperm freezability. Moreover, the level of GPX5 was not correlated with any of the frozen–thawed sperm qualities. Nevertheless, the level of GPX5 was positively correlated with FN1. The present result is in accordance with Vilagran et al. (2015) who found that the GPX5 content was no different between good and poor freezability groups. On the other hand, Barranco et al. (2016) found that the GPX5 concentration was positively associated with total sperm motility, farrowing rate and litter size. Likewise, Vilagran et al. (2016) found a negative correlation between GPX5 and boar semen qualities. However, differences in GPX5 concentration were also found among ejaculate portions, with the first 10 mL of the sperm rich fraction having a lower GPX5 concentration than the remainder (Barranco et al., 2016). These findings indicated that GPX5 is associated with some semen quality parameters and can be used as a fertility marker of the boars, but it is not associated with the boar sperm freezability.

In the present study, TPI was negatively correlated with boar sperm freezability. Triosephosphate isomerase is a glycolytic enzyme that catalyses the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3 phosphate. It is an essential enzyme for sperm capacitation and the acrosome reaction. In previous studies, the inhibition of this enzyme using ornidazole in rat spermatozoa blocked the capacitation process (Bone et al., 2001). Our results agree with Kwon et al. (2015), who revealed that the TPI level was higher in low quality sperm samples. In addition, Vilagran et al. (2013) found that

the TPI level in poor freezability semen was higher than in good freezability boar semen. Chen et al. (2014) demonstrated that an alteration of proteins, including TPI, occurred after the cryopreservation process. Vilagran et al. (2016) found that this defect was caused by handling after ejaculation, *i.e*. dilution techniques and cooling process. It could be explained that, due to the lower membrane integrity, a high level of TPI in the sperm head is related to poor sperm freezability. In our study, lower plasma membrane integrity after cryopreservation was found in poor freezability ejaculates compared to moderate and good freezability ejaculates.

Acrosin binding protein plays an important role in the maturation of acrosin (Tardif et al., 2010) and can regulate sperm capacitation and fertilisation (Arcelay et al., 2008). Expression of ACRBP occurs when sperm are capacitating and the levels of the protein in capacitated sperm and frozen–thawed sperm groups are similar. In the present study, the level of ACRBP was not different among freezability groups. Nonetheless, ACRBP was positively correlated with the percentage of total sperm motility after thawing. Vilagran et al. (2013) found that ACRBP may be involved in acrosin pathway maturation, where good freezability sperm had a high ability of acrosin maturation.

In conclusion, boar sperm proteins (*i.e*. TPI) and seminal plasma proteins (*i.e*. FN1) were significantly associated with boar sperm freezability. Boar ejaculates with a high level of FN1 had a higher freezability than those with a low level of FN1. This finding increases the possibility of improving boar sperm cryopreservation technology. Future work is required to investigate the mechanism behind the effect of these markers as well as the clinical application of these markers in boar semen cryopreservation.

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Razlika između proteina sjemene plazme i proteina sperme za dobru i lošu sposobnost smrzavanja ejakulata nerasta

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Ova studija provedena je u svrhu usporedbe ekspresije proteina sperme, *tj.* trioza-fosfat izomeraze (TPI) i akrozin-vezujućeg proteina (ACRBP) te proteina sjemene plazme, tj. glutation peroksidaze 5 (GPX5) i fibronektina 1 (FN1), u sjemenu nerasta s dobrom, umjerenom i lošom sposobnošću smrzavanja. Studija je provedena ustvrđivanjem sadržaja proteina u 32 uzorka sperme i 38 uzoraka sjemene plazme sjemena. Ejakulirano sjeme podijeljeno je u dva dijela: prvi dio je centrifugiran za odvajanje taloga sperme od sjemene plazme, a drugi je dio je krioprezerviran. Nakon odmrzavanja u skladu s pokretljivošću spermija nakon odmrzavanja ejakulati su klasificirani u tri skupine: dobra (60,2 ± 1,7%), umjerena (29,3 ± 2,0%) i loša (16,6 ± 2,2%) sposobnost smrzavanja. Ekspresije GPX5 i FN1 u sjemenoj plazmi te TPI i ACRBP u spermi ustvrđene su „Western blot" analizom. Za proteine sperme je otkriveno da je razina TPI nakon odmrzavanja negativno povezana s ukupnom pokretljivošću sperme (r = -0,38, P = 0,029). Za proteine sjemene plazme, razina FN1 u sjemenoj plazmi nakon odmrzavanja pozitivno je povezana s ukupnom pokretljivošću sperme nakon odmrzavanja (r = 0,37, P = 0,021) i progresivnom pokretljivošću (r = 0,39, P = 0,016). Ekspresija GPX5 nije povezana ni sa kakvim kvalitetama smrznute pa odmrznute sperme (P > 0,05). Zaključno, sjeme nerasta koje sadrži visoku razinu FN1 u sjemenoj plazmi ima bolju sposobnost smrzavanja. Pokretljivost sperme koja je smrznuta pa odmrznuta pozitivno je povezana s razinom FN1 u sjemenoj plazmi nerasta, a negativno s razinom TPI u spermijima nerasta.

Ključne riječi: *nerast, krioprezervacija, sposobnost smrzavanja, protein, sperma*