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Assessment of high hydrostatic pressure for the cryopreservation procedure of boar semen with low initial sperm motility

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Cryopreservation of biological material is critical for programmes of animal conservation and provides insurance for calamities, such as the loss of breeds due to animal diseases. The creation of biological material cryocollections depends on an effective cryopreservation procedure. The treatment of spermatozoa using high hydrostatic pressure (HHP) before the freezing procedure is a technological solution increasing cryopreservation efficiency. Our previous study demonstrated that HHP treatment (Applied Cell Technology, Hungary) with 35 MPa at 21°C for 1.5 h prior to boar semen cryopreservation improves the quality of post-thaw spermatozoa. This study aimed to evaluate the effect of fresh boar semen HHP treatment on the post-thaw sperm parameters. Only ejaculates with sperm progressive motility (PM%) below 70% (7 boars, 4-6 ejaculates/boar) were used in the experiment. All ejaculates (control samples without HHP treatment and samples treated with 35 MPa at 21°C for 1.5 h) were cryopreserved using a patented method (no. PL 228192). The results showed that post-thaw sperm motility (TM%) of the HHP-treated sperm was significantly higher ($P<0.05$; $P<0.01$) than that of the control sperm (43.0 vs. 37.5%; 58.9 vs. 54.9%, 40.1 vs. 35.5%; and 57.7 vs. 51.0% in boars nos. I, II, IV, and V, respectively). There were no significant differences ($P>0.01$) in the percentage of sperm displaying DNA fragmentation after cryopreservation between the treated and untreated samples. The analysis of ejaculates from three boars revealed a significantly higher ($P<0.05$) percentage of viable sperm ($YO\text{-}PRO\text{-}1^{-}/PI^{-}$) in the HHP-treated samples than in the control samples (37.2 vs. 32.0%; 40.5 vs. 33.8% and 54.7 vs. 46.6%).

Our study demonstrates that the HHP treatment applied in the freezing procedure of boar semen with initial sperm motility below 70% protects spermatozoa against cryodamage. However, the increase in semen tolerance to the cryopreservation procedure is an individual predisposition of specific boars.

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The cryopreservation of boar semen is useful for long-term preservation of genetic resources, improving genetic progress and enhancing transportation of genetic material across countries [Kaeoket 2012]. Moreover, freezing of biological material offers the possibility of postponing the reproductive period and using boar semen in the future, which may contribute to reduced inbreeding. Cryopreservation of biological material also enables the reconstruction of breeding herds that are currently being liquidated for epidemiological reasons. A biosecure reserve of cryopreserved semen could minimize the effects of a sudden outbreak of a contagious illness or natural disaster [Bailey *et al*. 2008].

Boar semen differs from the semen of other domestic animals in several aspects, since boar semen is produced in a large volume and is mainly liquid due to high sensitivity to cold shock [Kajabova *et al*. 2020]. Boar spermatozoa are highly susceptible to oxidative damage due to the high level of polyunsaturated fatty acids in the plasma membrane and the low scavenging activity in the cytoplasm [Waterhouse *et al*. 2004]. The formation of reactive oxygen species (ROS) during the cryopreservation procedure is a major concern, because ROS-induced oxidative damage can impair post-thaw sperm quality [Buranaamnuay *et al*. 2011]. The cryoinjuries that occur during semen cryopreservation can be minimalized by the addition of cryoprotectants, supplementation of freezing and thawing extenders with antioxidants, as well as optimalization of the freezing protocols and cooling rate [Silva *et al*. 2015, Jovičić *et al*. 2020]. Our research in this area led to the development and patenting of the composition of semen cryopreservation extenders [Trzcińska and Bryła 2018] that ensure good post-thaw sperm quality with high fertilizing capacity [Trzcińska *et al*. 2015].

When selecting ejaculates for cryopreservation the assessment of fresh sperm motility (above 70%) is the most important indicator of quality and a crucial predictor of semen freezability [Rath *et al*. 2009, Knox 2015, Yeste *et al*. 2017]. However, this general criterion for semen selection for cryopreservation based on the assessment of sperm motility is insufficient and prevents effective freezing of many ejaculates [Trzcińska and Bryła 2021]. Therefore, to create a cryocollection of such biological material an effective cryopreservation procedure with initial sperm motility below 70% should be optimized. One of the possibilities for increasing sperm cryotolerance is the application of high hydrostatic pressure (HHP) in the cryopreservation protocol. The use of HHP to preserve the quality of food products can be traced to the 1890s, when Hilte [1899] found that this approach results in extending the shelf life and improving milk quality and sterilization of fruits. Pressure treatment reduces the microbial load in foodstuffs with minimal adverse effects on the product compared with the results obtained with other preservation methods. In contrast to these observations, the use of sublethal stress treatment (cold shock and hydrostatic pressure) significantly increased the proliferation of Listeria monocytogenes. The biological effects of the first sublethal treatment protected the bacteria from the detrimental effects of the second sublethal treatment [Wemekamp-Kamphuis *et al*. 2002]. Based on its superior

effect on food quality the hydrostatic pressure, which was selected as the stressor, improved cryotolerance of mouse embryos, *in vitro*-produced bovine embryos, and semen from bulls and boars [Pribenszky *et al*. 2005a, Pribenszky *et al*. 2005b, Kuo *et al*. 2008, Du *et al*. 2008]. The application of sublethal stress to fresh boar semen before cryopreservation reportedly increases both total and progressive sperm motility after thawing.

The application of sublethal stress to fresh boar semen before cryopreservation reportedly increases total and progressive sperm motility after thawing. The optimal pressure treatment was in the range of 20-40 MPa (applied for 90-120 min) [Pribenszky *et al*. 2005c, Huang *et al*. 2009]. Moreover, after insemination with freezing-thawing sperm similar pregnancy rates and higher litter sizes were observed with the treated semen [Kuo *et al*. 2008].

In the current study the quality of fresh semen with initial sperm motility below 70% and frozen-thawed spermatozoa was verified using a computer-assisted sperm analysis to assess sperm motility and DNA fragmentation, while fluorescence microscopy was used to detect changes in sperm membrane permeability and acrosome integrity.

Material and methods

Animals

Thirty-five ejaculates of seven Polish Landrace and Polish Large White boars (4-6 ejaculates/boar) aged 2 to 3 years were used in this study. The boars were maintained at the Boar AI Station in Wet-Rol in Górka Stagniowska. All boars were housed in buildings with stable conditions of controlled temperature and humidity and were fed an adjusted commercial diet.

Ethical approval

All procedures that involved animals were approved by the Local Ethics Committee, Krakow, Poland (decision no. 126/2018).

Concept of the study

The concept of the study with the application of high hydrostatic pressure (HHP) before boar semen cryopreservation and assessment of the sperm quality before and after freezing is presented in Figure 1.

Semen collection

The sperm-rich fraction was collected by hand manipulation into water-jacketed vessels and diluted (1:1) in the *Biosolwens Plus* extender (Biochefa, Sosnowiec, Poland). Only the sperm-rich fraction of the ejaculates with progressive motility below 70% was used for further processing. After dilution the semen was divided into the control and treatment samples.

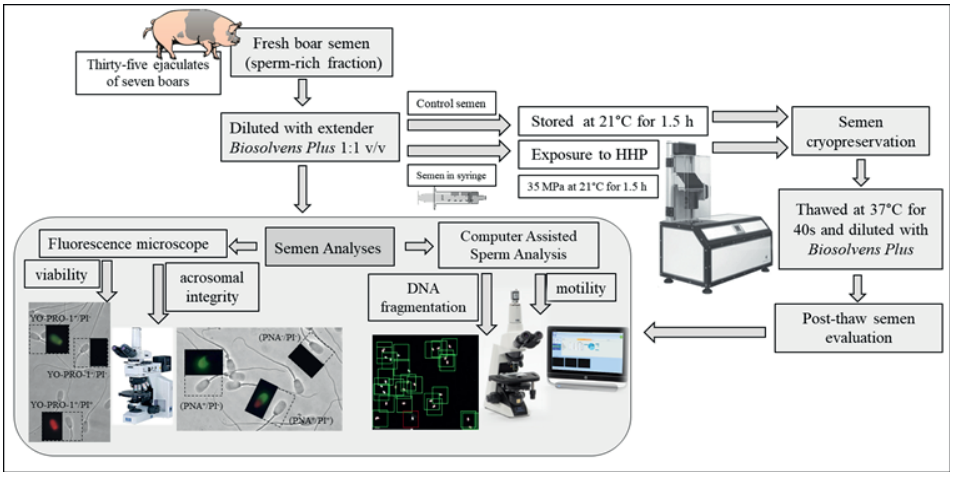

Fig. 1. Concept of the study with the application of high hydrostatic pressure (HHP) before boar semen cryopreservation and assessment of sperm quality before and after freezing.

Exposure to hydrostatic pressure

The HHP treatments were executed by a computer-controlled pressurizing device (Applied Cell Technology, Budapest, Hungary). Diluted sperm was loaded into 5-mL plastic Luer-lock syringes (B. Braun Melsungen, Melsungen, Germany) without air bubbles and the syringes were tightly sealed with plastic Luer-lock caps. The syringes were placed into a pressure chamber containing distilled water as the pressure medium and treated with 35 MPa at 21°C for 1.5 h. The control semen without HHP treatment was stored in syringes at 21°C for 1.5 h.

Semen cryopreservation

Spermatozoa were cryopreserved using a patented method [Trzcińska and Bryła 2018]. After pressurization both the control and treatment samples were centrifuged at 800 g for 25 min. The supernatant was discarded and the sperm pellet was resuspended in LEY extender (80 mL of 11% lactose solution and 20 mL of egg yolk) at a concentration of $1.5x10^9$ spermatozoa/mL. Then the samples were cooled to 5°C for 120 min. Subsequently, the semen was mixed with one part of LEYG extender (89.5% LEY extender with 9% glycerol and 1.5% Equex-STM paste in sodium dodecyl sulfate, Nova Chemical Sales, Scituate Inc., MA, USA) to obtain final concentrations of $1.0x10^9$ spermatozoa/mL, 3% glycerol and 1.0 mM butylated hydroxytoluene (BHT). The diluted and cooled semen was loaded into 0.5-mL straws (Minitüb). The straws were sealed with polyvinyl chloride powder and then placed in contact with nitrogen vapour (-120°C) for 15 min in a topped polystyrene box. Afterwards the straws were plunged into liquid nitrogen (-196°C) for storage.

Evaluation of semen quality

The fresh and post-thaw semen quality was evaluated by assessing sperm motility, DNA fragmentation, sperm viability and acrosome integrity.

Assessment of sperm motility and DNA fragmentation using Computer-assisted sperm analysis (CASA)

Frozen semen was thawed in a water bath at 37°C for 40 s. Post-thaw sperm motility was measured based on a computer-assisted sperm analysis using a Sperm Class Analyser (S.C.A. V5.1, Microptic, Barcelona, Spain) equipped with Plan 10/0.30 Ph1 DL objective lenses (Nikon). Before the examination a drop of semen from the thawed package was reextended (1:20) in Biosolwens Plus extender to obtain a concentration of 50 to 60×10^6 spermatozoa/mL and the frozen semen was incubated at 38°C for 20 min before 2 µl of semen was placed in a Leja Standard Count 8 Chamber Slide 20 micron (Leja Products B.V., GN Nieuw-Vennep, The Netherlands) on a heated stage (38°C). Each sample was measured twice, three fields were evaluated and at least 1000 cells were counted in each analysis. The motility patterns, including total sperm motility (TM%) and progressive sperm motility (PM%), were measured.

Sperm DNA fragmentation was assessed using a commercial variant of the sperm chromatin dispersion test (Sperm Sus-halomax®; Halotech DNA, Madrit, Spain) following the manufacturer's instructions. Biosolvens Plus diluted sperm samples contained a total of 15-20×10^6 spermatozoa/ml. To each sample 25 µL of diluted spermatozoa were added to a vial with low-melting agarose and mixed. A drop of the cell suspension was spread onto the treated face of the provided slides, which were covered with a glass coverslip. After 5 min at 4°C the coverslips were removed and the slides were placed horizontally in 10 mL of the lysing solution (provided in the kit) for 5 min at room temperature. The slides were washed with distilled water for 5 min and dehydrated in an increasing series of ethanol baths (70% and 100%) for 2 min at each concentration. Then, the dry slides were stained with 2 µL (1:1; vol/vol) of 10× SYBR® Green (Sigma-Aldrich, Saint Louis, USA) in Vectashield Mounting Medium H-1000 (Vector Laboratories, Burlingame, CA, USA) placed into the well of the slide for fluorescent staining of sperm chromatin.The slides were observed under a fluorescence microscope (Nikon Eclipse E200, Japan) equipped with 20/0.40 DH1 DL objective lenses (Nikon). Two hundred sperm cells in each slide were automatically counted by the Sperm Class Analyser® CASA System (Microptic S.L. Barcelona, Spain). The percentages of sperm with intact DNA (without a halo of chromatin dispersion) and fragmented DNA (DFI) (with a large and spotty halo of chromatin dispersion) were calculated in each semen sample.

Fluorescence microscope analysis of sperm viability and acrosomal integrity

Sperm viability and acrosome integrity of fresh semen and frozen-thawed semen were evaluated under a Nikon Eclipse E600 compound microscope (Nikon Corp., Tokyo, Japan) equipped with PlanFluo 40×/0.75 DIC M objective lenses (Nikon).

Fluorescence staining was measured by the microscopic observation of at least 200 cells in one field per sample per slide by one observer.

The Vybrant Apoptosis Assay Kit #4 (Molecular Probes Inc., Eugene, USA) was used to detect changes in plasma membrane permeability to YO-PRO-1 [10]. In total, 2×10^6 thawed sperm cells were diluted in 1 mL of PBS (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and 1 µL of YO-PRO-1 (100 µmol/L) was added. The tubes were gently mixed and incubated for 20 min at room temperature, afterwards 2 µmol/L propidium iodide (PI) were added to each tube. After the incubation period at least 200 spermatozoa per sample were evaluated using appropriate filters for YO-PRO-1 (Ex:491nm/Em:507nm) and PI (|Ex:538 nm/Em:619nm).

The results are presented as percentages of viable spermatozoa (YO-PRO-1^-/PI^-), viable spermatozoa with apoptotic-like changes (YO-PRO-1^+/PI^-) and nonviable spermatozoa (YO-PRO-1^+/PI^+) [Trzcińska and Bryła 2015].

The acrosome status was assessed by fluorescein isothiocyanate conjugated with peanut agglutinin (FITC-PNA) and PI staining as previously described by Trzcińska and Bryła [2015]. Briefly, 100 µL of thawed spermatozoa (30×10^6 cells/mL in HEPES buffer) were diluted with 10 µL of FITC-PNA solution (1 µg/mL in double-distilled water) and 5 µL of PI solution. The samples were incubated at room temperature in the dark for 5 min before the fluorescence analysis. The spermatozoa were classified as viable with an intact acrosome (PNA^-/PI^-), viable with a reacted acrosome (PNA^+/PI^-), or nonviable (PNA^+/PI^+).

Cryosurvival rate of ejaculates

The cryosurvival rate was measured as TM% after freeze-thawing/TM% of fresh semen×100.

Statistical analysis

Traits that did not exhibit a normal distribution were subjected to a logarithmic transformation and subsequently analysed by one-way and two-way ANOVA for fresh and frozen-thawed semen, respectively. When a significant effect was detected by ANOVA, the significance of the difference between means was determined followed by Duncan's test. The computations were performed using the Statistica 6.0 program (StatSoft, Tulsa, OK, USA).

Results and discussion

Ejaculates from Polish Landrace and Polish Large White boars with sperm motility below 70% were used in the present study. The results of the analysis of the fresh semen quality using the CASA system are summarized in Table 1. Significant differences ($P<0.01$) were observed in progressive and total sperm motility among individual boars. The highest PM% and TM% were recorded for boar no. II (62.2 and 67.2%). There were no significant differences ($P>0.01$) in the percentage of

Table 1. Computer-assisted sperm analysis of motility and DNA fragmentation of fresh semen

Boar no. (no. of ejaculates)	Motility		DNA fragmentation
	progressive motility PM (%)	total motility TM (%)	DFI (%)
I (n=6)	56.5^{BCD} (2.9)	64.0^{AB} (2.4)	1.4^{A} (0.1)
II (n=5)	62.2^{A} (3.0)	67.2^{A} (2.0)	1.5^{A} (0,1)
III (n=4)	60.7^{AB} (1.4)	63.5^{B}(2.5)	1.6^{A} (0.2)
IV (n=5)	57.1^{BC}(2.6)	64.1^{AB} (1.0)	1.5^{A} (0.2)
V (n=4)	59.4^{AB}(1.1)	61.5^{B} (1.2)	1.7^{A} (0.2)
VI (n=5)	53.7^{CD}(2.5)	60.4^{B} (2.6)	1.6^{A} (0.3)
VII (n=6)	52.2^{D} (2.0)	61.8^{B}(0.9)	1.7^{A} (0.1)

^{ABCD} Within columns means bearing different superscripts differ significantly at P<0.01.

Table 2. Quality parameters of fresh semen analysed by fluorescence microscopy

Boar no. (no. of ejaculates)	Viability (%)			Acrosome integrity (%)		
	viable sperm ($YO\text{-}PRO\text{-}1^{-}/PI^{-}$)	viable sperm with apoptotic-like changes ($YO\text{-}PRO\text{-}1^{+}/PI^{-}$)	nonviable sperm ($YO\text{-}PRO\text{-}1^{+}/PI^{+}$)	viable sperm with acrosome integrity (PNA^{-}/PI^{-})	viable sperm with reacted acrosome (PNA^{+}/PI^{-})	nonviable sperm (PNA^{+}/PI^{+})
I (n=6)	51.3^{C} (2.0)	24.2^{A} (4.3)	24.6^{B} (4.4)	50.3^{D} (2.5)	22.6^{A} (4.5)	26.0^{C} (4.6)
II (n=5)	63.0^{A} (2.9)	3.9^{B} (1.2)	33.1^{A} (4.2)	$60.9^{A,B}$ (2.5)	5.7^{B} (1.3)	35.2^{AB} (4.2)
III (n=4)	62.1^{A} (2.5)	4.0^{B} (1.5)	33.9^{A} (2.4)	62.0^{A} (2.3)	5.5^{B} (2.7)	34.0^{B} (2.3)
IV (n=5)	53.4^{BC} (4.6)	23.7^{A} (3.4)	22.9^{B} (5.4)	55.5^{ABCD} (5.0)	24.2^{A} (3.8)	20.8^{C} (3.7)
V (n=4)	57.6^{AB} (3.0)	3.4^{B} (1.1)	39.0^{A} (2.1)	58.5^{ABC} (3.3)	4.7^{B} (1.3)	38.1^{AB} (3.3)
VI (n=5)	52.0^{BC} (3.7)	4.6^{B} (0.5)	43.4^{A} (3.9)	51.8^{CD} (4.2)	3.9^{B} (1.0)	43.6^{A} (4.0)
VII (n=6)	51.6^{BC} (3.4)	23.4^{A} (3.1)	25.0^{B} (5.2)	54.8^{BCD} (4.1)	26.7^{A} (1.9)	21.7^{C} (2.9)

^{ABCD}Within columns means bearing different superscripts differ significantly at P<0.01.

spermatozoa displaying DNA fragmentation among the boars, with this value ranging from 1.4 to 1.7%. The fresh semen qualities, i.e. sperm viability and acrosome integrity, are presented in Table 2. For boars I, IV and VII similar proportions of viable sperm with apoptotic-like changes (from 23.4 to 24.2%) and reacted acrosomes (from 22.6 to 26.7%) were identified. Meanwhile, for boars II, III, V and VI the percentage of these sperm subpopulations ranged from 3.4 to 4.6% and from 3.9 to 5.7%. Moreover, the results indicated that boars I, IV and VII significantly differed (P<0.01) from boars II, III, V and VI in the percentage of spermatozoa $YO\text{-}PRO\text{-}1^{+}/PI^{-}$ and PNA^{+}/PI^{-}.

All samples of fresh semen were treated with high hydrostatic pressure at 35 MPa for 90 min before the cryopreservation procedure [Bryła and Trzcińska 2018]. The effects of the HHP treatment on post-thaw sperm quality are presented in Table 3 and Table 4. The proportion of total motile spermatozoa after HHP treatment (43.0, 40.1 and 57.7%) was significantly higher (P<0.01) than that in the untreated samples (37.5, 35.5 and 51.0% in boars nos. I, IV and V, respectively) (Tab. 3). Simultaneously, in boar no. II the same difference was observed at P<0.05. Roca *et al*. [2006] also observed such differences in sperm motility after freeze-thawing, when boars were divided into the following three groups: poor sperm freezers (less than 40%), moderate sperm

Table 3. Means and standard deviations (in parenthesis) of post-thaw quality sperm parameters analysed by CASA

Boar no. (no. of ejaculates)	Motility						DNA fragmentation			Cryosurvival (%)		
	progressive motility PM (%)			total motility TM (%)			DFI (%)					
	control	HHP	P-value	control	HHP	P-value	control	HHP	P-value	control	HHP	P-value
I (n=6)	31.3^{C} (4.8)	36.8^{C} (2.9)	0.040	37.5^{B} (1.6)	43.0^{D} (2.0)	<0.001	1.6^{A} (0.2)	1.6^{A} (0.3)	0.815	58.6^{B} (1.0)	67.2^{C} (2.5)	<0.001
II (n=5)	52.5^{A} (2.6)	56.0^{A} (1.9)	0.041	54.9^{A} (2.1)	58.9^{A} (2.9)	0.039	1.5^{A} (0.2)	1.6^{A} (0.1)	0.877	81.8^{A} (4.6)	$87.6^{A,B}$ (4.4)	0.076
III (n=4)	$50.2^{A,B}$ (3.5)	$52.0^{A,B}$ (3.5)	0.512	53.8^{A} (0.8)	$54.2^{B,C}$ (1.3)	0.637	1.6^{A} (0.1)	1.7^{A} (0.1)	0.239	84.7^{A} (3.8)	85.4^{B} (2.1)	0.763
IV (n=5)	32.2^{C} (1.6)	36.7^{C} (2.1)	0.005	35.5^{B} (2.0)	40.1^{E} (0.8)	0.002	1.5^{A} (0.1)	1.7^{A} (0.3)	0.243	55.3^{B} (2.8)	$62.5^{C,D}$ (0.6)	<0.001
V (n=4)	$45.3^{A,B}$ (3.8)	54.4^{A} (2.7)	0.009	51.0^{A} (3.2)	$57.7^{A,B}$ (1.4)	0.009	1.6^{A} (0.3)	1.7^{A} (0.1)	0.865	83.0^{A} (6.7)	93.8^{A} (3.7)	0.035
VI (n=5)	43.1^{B} (1.1)	46.0^{B} (4.2)	0.186	51.6^{A} (2.9)	52.5^{C} (2.1)	0.599	1.5^{A} (0.2)	1.6^{A} (0.2)	0.909	85.6^{A} (4.7)	$87.0^{A,B}$ (2.9)	0.584
VII (n=6)	31.9^{C} (3.4)	33.5^{C} (3.8)	0.456	36.6^{B} (3.0)	37.5^{E} (1.2)	0.509	1.9^{A} (0.4)	1.7^{A} (0.3)	0.516	57.4^{B} (1.1)	60.2^{D} (3.9)	0.135

^{ABCD}Within columns means bearing different superscripts differ significantly at P<0.01.

freezers (40-60%) and good sperm freezers (more than 60%). In our study all the analysed boars could be classified based on post-thaw total sperm motility only into two following groups: poor sperm freezers (boars I, IV and VII) and moderate sperm freezers (boars II, III, V, and VI). Significantly higher motility parameters were observed for boars I and IV after the application of high hydrostatic pressure. The obtained results allowed us to classify these samples as moderate sperm freezers. Moreover, the significant increase in sperm motility resulted in a cryosurvival rate greater than

60%. A significant increase in the cryosurvival rate compared with the control was observed in boars nos. I (67.2 vs. 58.6%), IV (62.5 vs. 55.3%) and V (93.8vs. 83.0%) at P<0.01 and P<0.05 (Tab. 3).

In the present study in all analysed boars the level of post-thaw DNA fragmentation was low in both treated and untreated samples. There were also no significant differences in the percentage of sperm with DNA fragmentation among the boars (Table 3). In boar spermatozoa the factors responsible for DNA fragmentation of post-thaw sperm are oxidative stress and the activation of apoptotic-like changes [Fraser and Strzeżek 2007]. All ejaculates (treated and untreated) were cryopreserved in an extender supplemented with an antioxidant, which causes high sperm antioxidant activity, resulting in an effective reduction in oxidative damage [Trzcińska *et al*. 2015] As it was shown by the present study, it effectively protects sperm against the induction of DNA fragmentation caused by the cryopreservation process.

The assessed viability and acrosome integrity of frozen-thawed spermatozoa are summarized in Table 4. A significantly lower percentage of viable sperm with apoptotic-like changes was observed in boars I (27.2%) and IV (28.0%) in the treated samples compared with the control samples (33.9% and 35.8%). Additionally, the percentage of viable sperm

Table 4. Means and standard deviations (in parenthesis) of post-thaw quality sperm parameters analysed by fluorescence microscopy

Boar no. (no. of ejaculates)	Viability (%)								Acrosome integrity (%)							
	viable sperm (YO-PRO-1$^-$/PI$^-$)			viable sperm with apoptotic-like changes (YO-PRO-1$^+$/PI$^-$)			nonviable sperm (YO-PRO-1$^+$/PI$^+$)			viable with acrosome integrity (PNA$^-$/PI$^-$)			viable sperm with reacted acrosome (PNA$^+$/PI$^-$)			nonviable sperm (PNA$^+$/PI$^+$)		
	control	HHP	P-value	control	HHP	P-value	control	HHP	P-value	control	HHP	P-value	control	HHP	P-value	control	HHP	P-value
I (n=6)	32.0^{D} (4.3)	37.2^{BC} (3.2)	0.045	33.9^{A} (3.6)	27.2^{A} (3.0)	0.006	34.1^{BC} (7.7)	35.7^{B} (5.6)	0.636	33.2^{C} (5.1)	39.0^{C} (1.3)	0.038	31.5^{A} (1.6)	27.4^{A} (3.5)	0.024	35.2^{BC} (4.4)	33.6^{B} (6.2)	0.588
II (n=5)	53.0^{A} (2.8)	54.8^{A} (3.6)	0.409	7.7^{B} (2.8)	6.0^{B} (2.2)	0.319	39.3^{ABC} (5.6)	39.2^{AB} (5.5)	0.979	55.1^{A} (3.6)	57.0^{A} (3.5)	0.407	7.9^{B} (2.4)	7.6^{B} (2.6)	0.840	37.0^{BC} (5.9)	35.4^{B} (5.8)	0.696
III (n=4)	51.3^{A} (2.0)	52.1^{A} (2.4)	0.659	6.9^{B} (1.0)	7.0^{B} (1.5)	0.912	41.8^{ABC} (2.1)	40.9^{AB} (3.2)	0.653	54.6^{A} (2.0)	53.6^{A} (3.7)	0.634	8.2^{B} (4.1)	7.2^{B} (2.9)	0.751	37.1^{BC} (4.4)	39.1^{A,B} (4.3)	0.541
IV (n=5)	33.8^{CD} (4.0)	40.5^{B,C} (3.8)	0.027	35.8^{A} (6.8)	28.0^{A} (3.3)	0.049	30.4^{C} (9.4)	31.5^{B} (0.7)	0.658	33.0^{C} (4.9)	37.1^{CD} (1.7)	0.121	31.5^{A} (3.1)	27.7^{A} (2.0)	0.048	35.5^{BC} (7.6)	35.2^{B} (3.5)	0.979
V (n=4)	46.6^{AB} (4.6)	54.7^{A} (3.2)	0.029	6.7^{B} (1.7)	6.1^{B} (1.6)	0.625	46.6^{AB} (4.7)	39.1^{A,B} (3.5)	0.043	47.4^{AB} (1.3)	52.5^{A} (2.9)	0.017	9.2^{B} (0.9)	8.1^{B} (0.7)	0.049	43.4^{AB} (1.8)	39.4^{AB} (3.4)	0.089
VI (n=5)	39.9^{BC} (6.0)	43.8^{B} (6.8)	0.375	6.8^{A} (1.3)	6.4^{B} (1.6)	0.666	53.3^{A} (7.0)	49.8^{A} (7.9)	0.469	43.4^{B} (3.1)	45.0^{B} (3.1)	0.440	7.7^{B} (1.4)	7.1^{B} (1.5)	0.518	48.9^{A} (3.7)	47.9^{A} (3.4)	0.669
VII (n=6)	34.6^{CD} (4.0)	35.9^{C} (4.1)	0.592	35.1^{A} (6.2)	33.2^{A} (5.9)	0.592	30.3^{C} (8.3)	30.9^{B} (8.3)	0.903	33.5^{C} (1.8)	55.4^{D} (1.6)	0.396	33.2^{A} (0.9)	31.7^{A} (0.9)	0.051	33.2^{C} (2.4)	33.9^{B} (1.7)	0.547

^{ABCD}Within columns means bearing different superscripts differ significantly at P<0.01.

with reacted acrosomes in the frozen-thawed sperm after HHP treatment in boars I (27.4%), IV (27.7%) and V (8.1%) was significantly lower than that in the control sperm (31.5%, 31.5% and 9.2%). Simultaneously, in these boars a significantly higher ($P<0.05$) percentage of viable sperm (YO-PRO-1^-/PI^-) was noticed in the treated samples (Table 4). Moreover, the statistical analysis indicated that the treated and untreated semen from boars I, IV and VII significantly differed ($P<0.01$) from those from boars II, III, V and VI in the percentage of YO-PRO-1^+/PI^- and PNA^+/PI^-. Our results demonstrate that applying sublethal stress treatment to gametes significantly reduced the percentage of viable sperm with apoptotic-like changes and the percentage of viable sperm with reacted acrosomes in some boars. Simultaneously, a significant increase in the post-thaw percentage of viable sperm was also observed after HHP treatment.

We aimed to verify whether triggering survival mechanisms of poor-quality gametes by subjecting them to sublethal stress levels could improve their resistance to cryopreservation. The hypothesis that controlled stress before storage may protect sperm has been investigated. Pribenszky and Vajta [2011] showed that stressing boar semen with hydrostatic pressure compared to conventional processing resulted in higher progressive motility.

Our results demonstrate that the application of HHP treatment before the cryopreservation procedure provides promising results for poor-quality boar ejaculates. This study showed that it is possible to positively impact the semen quality using high hydrostatic pressure in a cryopreservation procedure, but the results depend on the individual predisposition of the boars. Cryopreservation of biological material is critical for programmes of animal conservation because it prevents loss of genetic diversity, an essential factor for protecting populations during unforeseen situations.

Conclusion

The results obtained in this study demonstrate that applying HHP treatment to boar semen before cryopreservation may improve post-thaw sperm quality parameters. However, the positive impact of HHP depends on the individual susceptibility of boars. This finding is very important for semen banking, particularly for cryopreservation of biological material with low initial sperm motility.

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