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Original article

Apoptotic-like changes of boar spermatozoa in freezing media supplemented with different antioxidants

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Abstract

This study evaluated the effect of supplementing the freezing extender with exogenous antioxidants on apoptotic-like changes in post-thaw boar spermatozoa. A total of 36 ejaculates were resuspended in standard lactose-egg yolk-glycerol extender supplemented with antioxidant to final concentrations of 0 (as control), 2.5mM GSH (group I), 5.0 mM GSH (group II), 150 IU/mL SOD (group III), 300 IU/mL SOD (group IV), 200 IU/mL CAT (group V), 400 IU/mL CAT (group VI), 150 IU/mL SOD+200 IU/mL CAT (group VII), 300 IU/mL SOD+400 IU/mL CAT (group VIII). Sperm motility and apoptotic-like changes were determined before and after freeze-thawing. The various markers of apoptotic-like changes were measured: plasma membrane permeability by YO-PRO-1/PI assay, phosphatidylserine (PS) translocation across the plasma membrane using fluorescein-labeled Annexin-V, mitochondrial transmembrane potential detected by JC-1, and DNA fragmentation evaluated by TUNEL assay. The highest percentage of progressive motile sperm was noticed in group II (PM% 64.2±15.4) compared with control (PM% 36.8±5.5). The supplementation of 400 IU/mL CAT (group VI) revealed significant ($P<0.01$) reduction of apoptotic-like changes (YO-PRO-1^+/PI^-: 13.1±7.5%, AnV^+/PI^-: 9.9±4.1%) in frozen-thawed spermatozoa compared with extender supplemented with 200 IU/mL CAT (group V). Irrespective of the concentration used, SOD and CAT in combination (group VII and group VIII) significantly ($P<0.01$) improved post-thaw sperm survival compared with the control. Evaluation by TUNEL assay revealed that cryopreservation and thawing did not induce DNA fragmentation in boar spermatozoa.

Key words: apoptotic-like changes, antioxidants, cryopreservation, spermatozoa, boar

Introduction

The processes of cooling, freezing, and thawing produce physical and chemical stress on the sperm membrane and reduce sperm viability. Moreover, a cryopreservation protocol produced cold shock which decreased sperm survival and freezing ability, leading to the death of the sperm. The cold shock of spermatozoa is associated with oxidative stress induced by reactive oxygen species (ROS) generation

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(Gadea et al. 2005). Sperm oxidative damage is the result of an imbalance between ROS generation and scavenging activities. The scavenging potential of the ejaculate is normally maintained by adequate levels of antioxidants present in the seminal plasma (SP) (Kowalowka et al. 2008, Koziorowska-Gilun et al. 2011). Studies have demonstrated that seminal plasma contains a number of enzymatic antioxidants such as superoxide dismutase (SOD; Alvarez and Storey 1992), glutathione peroxidase/glutathione reductase (GPX/GRD), catalase (CAT) and contains a variety of non-enzymatic antioxidants such as ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), and reduced glutathione (GSH) (Silva and Gadella 2006). Koziorowska-Gilun et al. (2011) reported differences in the activity of antioxidants in the boar seminal plasma, showing low and high activity of CAT and SOD regardless of the level of antioxidant activity during the cryopreservation process of boar semen seminal plasma discarded by centrifugation and the beginning of semen preparation, which results in a lack of antioxidant property of seminal plasma in protecting sperm from cryoinjury. A way to protect the spermatozoa from oxidative damage could be the addition of antioxidants to the freezing extenders. Previous studies have shown that supplementation of antioxidants in extenders for both chilled and frozen-thawed semen, such as butylated hydroxytoluen (BHT), SOD, CAT, and GSH has improved boar semen quality (Roca et al. 2005, Yeste et al. 2014, Trzcińska et al. 2015).

The main damage to sperm due to cryo-injury occurs at the level of the plasma membrane and appears to be more closely related to fertility than sperm motility (Rodriguez-Martinez 2003). Following detection by fluorescence techniques, about 50% of boar sperm are commonly classified as „dead" after the cryopreservation procedure. The additional detection of sub-lethal changes in plasma membrane function seems to be of high diagnostic relevance.

Recently, apoptotic-like changes during cryopreservation of buffalo (Kadirvel et al. 2012), equine (Ortega-Ferrusola et al. 2008) and boar sperm (Trzcińska et al. 2015) have been reported. The presence of a population of spermatozoa with apoptotic-like changes could explain the reduced life span of the surviving population after freezing and thawing. During apoptosis the cytoplasmic membrane becomes slightly permeable. YO-PRO-1 stain selectively passes through the plasma membranes of apoptotic cells and labels them with moderate green fluorescence (Bryła et al. 2010). When the cell membrane is disturbed the phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane (Pena et al. 2003). Sperm cryopreservation may also affect DNA fragmentation. DNA fragmentation is considered the most frequent cause of paternal DNA anomaly transmitted to progeny. Damaged sperm DNA may be incorporated into the genome of the embryo, and participate or lead to errors in DNA replication, transcription or translation during embryo development, ultimately contributing to diseases in future generations (Katari et al. 2009).

The aim of the present study was to evaluate the effect of exogenous antioxidants: GSH, SOD and /or CAT supplementation to the freezing extender on post-thaw sperm parameters. Frozen-thawed sperm quality was assessed based on motility and the multi-parametric study of apoptotic-like changes. Plasma membrane integrity (YO-PRO-1/PI assay); phosphatidylserine externalization (Annexin-V/PI); mitochondrial membrane potential (JC-1); and DNA fragmentation (TUNEL assay) were evaluated.

Material and Methods

Procedures involving animals were approved by the Local Ethics Board for Animal Experiments in Kraków (Poland). All reagents were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

Animals

Six crossbreds of Polish Landrace and Large White boars aged 2 to 4 years and selected according to normal semen quality and proven fertility were used in this study. The semen (six ejaculates from each male) was collected once weekly during a 2-month period, from October through November. Boars were kept at the Boar AI Station in Klecza Dolna. All boars were housed in buildings with stable conditions of controlled temperature and humidity and were fed an adjusted commercial diet.

Semen collection and handling

The sperm-rich fraction was collected by hand manipulation into water-jacketed vessels. The volume, concentration, and motility of the sperm were estimated immediately after collection. The average of boar sperm-rich fraction volume was 79.8±5.3 mL and the concentration was approximately 1.426 x 10^9 spermatozoa/mL. Only ejaculates with greater than 70% progressively motile sperm and 80% morphologically normal spermatozoa were used for cryopreservation. The semen was diluted (1:1 v/v) with *Biosolwens Plus* (BP) extender (Biochefa, Sosnowiec, Poland) and were transported to the laboratory within 1 hr at 15°C.

Freezing and thawing procedure

Semen samples were processed using the freezing procedure described by Trzcińska et al. (2013). The freezing procedure started with the centrifugation of semen aliquots at 800 x g for 25 minutes. Pellets were then recovered and diluted at 1.5 x 10^9spermatozoa/mL in freezing extender containing lactose and egg yolk (LEY). Spermatozoa were cooled to 5°C for 120 minutes and subsequently diluted in LEYG extender (89.5% LEY extender with 9% glycerol and 1.5% Equex-STM paste; Nova Chemical Sales, Scituate Inc, MA, USA). Each aliquot was resuspended in LEY and LEYG extender supplemented with antioxidant to final concentrations of 0 (as control), 2.5 mM GSH (group I), 5.0 mM GSH (group II), 150 IU/mL SOD (group III), 300 IU/mL SOD (group IV), 200 IU/mL CAT (group V), 400 IU/mL CAT (group VI), 150 IU/mL SOD+200 IU/mL CAT (group VII), 300 IU/mL SOD+400 IU/mL CAT (group VIII). The final concentration of semen was 1.0 x 10^9 spermatozoa/mL and 3% glycerol. The processed semen was loaded into 0.5 mL straws (Minitub), and sealed with polyvinyl chloride powder before being placed in contact with nitrogen vapor for 15 minutes in a polystyrene box. The straws were plunged into liquid nitrogen (-196°C). The straws were then stored in liquid nitrogen until thawing.

Thawing was carried out by immersing the straws in a circulating water bath at 37°C for 40 seconds. Immediately after thawing, the semen was diluted in BP extender at 37°C.

Semen evaluation

The assessment of sperm motility and apoptotic-like changes was made in fresh and frozen-thawed boar spermatozoa.

Assessment of sperm motility

Sperm motility parameters were analyzed using a computer-assisted sperm analysis (CASA) system (SM-CMA; MTM, Montreaux, Switzerland). Before examination, a drop of the semen from the thawed package was reextended in BP extender to give a concentration of 50 to 60 x 10^6 spermatozoa/mL. Thus, extended FT semen was incubated (38°C) for 20 minutes before being placed in a Leja counting chamber (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 per analysis were counted. Sperm motility parameters were recorded according to the percentage of total motile spermatozoa (TM%) (average path velocity ≥ 20 µm/s) and the percentage of motile spermatozoa showing rapid and progressive movement (PM%) (straight line velocity ≥ 40 µm/s).

Assessment of apoptotic-like changes in spermatozoa

Fluorescent staining to assess sperm viability using the YO-PRO-1/PI assay

The Vybrant Apoptosis Assay Kit#4 (Molecular Probes Inc., Eugene, USA) was used to detect changes in plasma membrane permeability to YO-PRO-1 that are related to apoptosis. A total of $2x10^6$ sperm cells were diluted in 1 ml PBS (phosphate buffered saline, 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 and 2 µmol/l PI were added to each tube. After the incubation period, the sperm cell suspensions were analyzed under a fluorescence microscope at 400x magnification. At least 200 spermatozoa per sample were evaluated using the appropriate filters for red (PI) and green (YO-PRO-1) fluorescence detection. The fluorescent staining was measured by microscopic observation in at least 200 cells/sample per slide in one field by one observer. The results were presented as the percentage of viable spermatozoa ($YO\text{-}PRO\text{-}1^-/PI^-$), viable spermatozoa with apoptotic-like changes ($YO\text{-}PRO\text{-}1^+/PI^-$), and nonviable spermatozoa ($YO\text{-}PRO\text{-}1^+/PI^+$).

Fluorescent examinations of mitochondrial function using JC-1 probe

For evaluation of the mitochondrial membrane potential, spermatozoa were labeled with JC-1 -5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide (Molecular Probes Inc., Eugene, USA). The semen was centrifuged for 15 min at 300 *g* at room temperature and the pellet of sperm was then washed with PBS (without calcium and magnesium, Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Finally, the sample was centrifuged and washed twice, and the sperm pellet was then resuspended in 1 mL PBS. The sample was stained with 10 µg/mL JC-1 (final concentration; stock solution 1 mg/mL in DMSO) at 37°C for 15 min (Trzcińska et al. 2011). After incubation, sperm smears were evaluated with a fluorescence microscope. At least

200 spermatozoa per sample were evaluated using appropriate filters. In viable sperm, JC-1 is taken up by the mitochondria, where it forms aggregates which exhibit red/orange fluorescence. In dysfunctional sperm (possibly apoptotic/necrotic), JC-1 cannot aggregate in the mitochondria due to alterations in membrane potential. The stain remains as a monomer in the cytoplasm where it fluoresces green. The increase in $\Delta\Psi m$ causes a fluorescence emission shift from green to red-orange due to the reversible formation of JC-1 aggregates in polarized mitochondria.

Detection of membrane phosphatidylserine (PS) exposure

Another early apoptotic change, the translocation of PS onto the outer leaflet of the cell membrane, was determined using an Annexin-V-Fluos staining kit (Roche, Mannheim, Germany). In brief, semen samples containing $1x10^6$ sperm were washed twice (300 g for 10 min) with PBS medium (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The washed sperm were resuspended in Annexin-V labeling solution containing Hepes buffer, 4 µL Annexin V-FITC protein and 2 µL propidium iodide (PI). After incubation at room temperature for 15 min, the sperm were analyzed immediately under a fluorescence microscope at 400x magnification. At least 200 spermatozoa per sample were evaluated using the appropriate filters. Using an Annexin-V-Fluos staining kit we observed four subpopulations of sperm under the fluorescence microscope: viable (Annexin-V^-/PI^-), in which no fluorescence was observed; early apoptotic (Annexin-V^+/PI^-) showing only green fluorescence; late apoptotic or early necrotic (Annexin-V^+/PI^+), which fluoresced green and red; and necrotic (Annexin-V^-/PI^+) showing only red fluorescence.

Detection of DNA fragmentation

TUNEL analysis was performed according to the manufacturer's instructions in the In Situ Cell Death Detection Kit (Roche Diagnostics, Germany) with minor modifications. In brief, 3.10^6 cells were washed with phosphate-buffered saline 1 x PBS, pH 7.4 and centrifuged at 800 x g. Then samples were fixed with 200 µl of 1% paraformaldehyde for 10 min at room temperature in the dark. Afterwards, sperm cells were washed with 1xPBS and permeabilized using 0.1% Triton X-100 for 10 min. After washing with PBS, sperm DNA was labeled by incubating spermatozoa with 50 µl of the TUNEL reaction mixture (Tdt enzyme and FITC-labeled nucleotides) in a humidified atmosphere for 60 min at 37°C in the dark. After reaction the samples were washed twice in 1xPBS, transferred through a drop of Vecta-Shield (Vector Laboratories, Burlingame, CA, USA) and mounted on a glass slide. Labeled nuclei were examined under a Nikon Eclipse E600 microscope fitted with epifluorescent illumination. The negative control was prepared by omitting the fluorescein and the positive control by adding 1 µL of DNAse. A minimum of 200 cells per slide was examined in random fields of each aliquot under a fluorescence microscope using appropriate filters. The results were presented as the percentage of spermatozoa with DNA fragmentation.

Statistical analysis

Results were expressed as the means (±SD) and data were analyzed using the Statistica 6.0 program (StatSoft, Tulsa, OK, USA). One-way ANOVA was used to check if there were differences between the boars before and after freezing. No differences between the animals were found. To determine the effect of different additives (control – without treatment, 2.5 mM GSH 5.0 mM GSH 150 IU/mL SOD 300 IU/mL SOD, 200 IU/ml CAT, 400 IU/ml CAT, 150 IU/mL SOD+200 IU/ml CAT, 300 IU/mL SOD+400 IU/ml CAT) in freezing extender on semen quality, ANOVA, followed by Duncan's multiple range test was used.

Results

Motility

Motility parameters of frozen-thawed boar spermatozoa are provided in Table 1.

The results obtained indicate that the addition of 150 SOD, 300 SOD and 200 CAT did not improve motility parameters compared to the control extender ($P>0.01$). The addition of 5 mM GSH to semen extender significantly ($P<0.01$) increased the post-thaw rates of progressive sperm motility (PM% 64.2±15.4), compared with the extender supplemented with 2.5 mM GSH (PM% 38.1±8.5). The addition of SOD (150 and 300 IU/mL) to the freezing extender did not positively affect the proportion of total and progressive motility. The addition of CAT (200 and 400 IU/mL) to the freezing extender revealed that only supplementation with 400 IU/mL CAT significantly improved ($P<0.01$) the post-thaw percentage of sperm motility (TM% 69.2±9.3, PM% 59.3±17.1). The combined SOD plus CAT in the freezing extender signifi-

Table 1. Percentages of total and progressive motile spermatozoa in fresh and frozen-thawed boar semen.

Population of sperm (%)	Fresh	Frozen-thawed spermatozoa							
		Control	Group I (2.5m GSH)	Group II (5.0mM GSH)	Group III (150 IU SOD)	Group IV (300IU SOD)	Group V (200 IU CAT)	Group VI (400IUCAT)	Group VII (150 SOD +200 CAT)	Group VIII (300 SOD +400 CAT)
Total motility (TM%)	90.1±12.4	51.7±7.4^{C}	58.2±9.51^{B}	71.5±12.6^{A}	49.7±6.3^{C}	49.1±9.4^{C}	49.3±8.7^{C}	69.2±9.3^{A}	69.6±12.4^{A}	66.7±9.2^{A}
Progressive motility (PM%)	82.3±11.1	36.8±5.5^{C}	38.1±8.5^{C}	64.2±15.4^{A}	37.4±5.7^{C}	39.4±6.2^{C}	36.7±9.8^{C}	59.3±17.1^{B}	63.6±15.1^{A,B}	61.5±8.3^{A,B}

A,B,C Values in rows with different letters are statistically different (P<0.01)

Table 2. Results (mean±SD) of the assessment of apoptotic-like changes in fresh and frozen-thawed boar spermatozoa.

Population of sperm (%)	Fresh	Frozen-thawed spermatozoa							
		Control	Group I (2.5m GSH)	Group II (5.0mM GSH)	Group III (150 IU SOD)	Group IV (300IU SOD)	Group V (200 IU CAT)	Group VI (400IUCAT)	Group VII (150 SOD +200 CAT)	Group VIII (300 SOD +400 CAT)
Viable (YO-PRO-1^{-}/PI^{-})	81.6±9.7	36.4±4.7^{B}	39.5±11.4^{B}	65.8±11.6^{A}	35.4±13.3^{B}	39.2±13.7^{B}	42.2±12.4^{B}	58.2±12.5^{A}	63.6±13.1^{A}	61.2±12.4^{A}
Viable with apoptotic-like changes (YO-PRO-1^{+}/PI^{-})	4.3±2.1	25.3±3.2^{A}	19.1±4.3^{B}	10.3±4.3^{C}	21.1±10.6^{B}	20.5±7.8^{B}	18.5±9.5^{B}	13.1±7.5^{C}	10.4±3.8^{C}	11.5±5.1^{C}
Nonviable (YO-PRO-1^{+}/PI^{+})	14.1±5.2	38.3±4.3^{B,C}	41.4±6.3^{A,B}	23.9±4.2^{E}	43.5±9.4^{A}	40.3±6.3^{A,B,C}	37.3±4.2^{C}	28.7±3.7^{D}	26.0±4.8^{D,E}	27.3±4.9^{D,E}
Viable sperm (AnV^{-}/PI^{-})	79.6±3.1	35.2±3.9^{C,D}	35.4±9.7^{C,D}	62.7±9.8^{A}	32.3±10.3^{D}	34.2±6.4^{C,D}	39.1±8.1^{C}	56.8±7.3^{B}	61.0±9.5^{A,B}	60.9±11.2^{A,B}
Viable with early apoptotic-like changes (AnV^{+}/PI^{-})	3.7±0.5	22.6±1.2^{A}	18.6±4.2^{A,B}	9.7±4.9^{C}	19.5±9.2^{A,B}	19.2±7.2^{A,B}	16.9±8.4^{B}	9.9±4.1^{C}	9.4±2.6^{C}	8.9±2.3^{C}
Late apoptotic-like changes/ Early necrotic (AnV^{+}/PI^{+})	12.8±1.3	31.5±5.1^{B}	36.5±7.3^{A}	19.4±9.4^{C}	39.1±7.9^{A}	37.8±8.3^{A}	36.8±9.3^{A}	20.4±7.6^{C}	19.6±9.3^{C}	18.8±6.5^{C}
Nonviable (AnV^{-}/PI^{+})	3.9±0.8	10.7±2.3^{A,B}	9.5±5.4^{B,C}	8.2±5.1^{B,C}	9.1±5.8^{B,C}	8.8±3.4^{B,C}	7.2±4.6^{B,C}	12.9±3.9^{A}	10.0±3.1^{A,B}	11.4±3.8^{A,B}
High $\Delta\Psi m$	87.7±9.3	40.2±8.5^{B}	42.4±11.4^{B}	61.6±12.6^{A}	40.6±9.6^{B}	42.7±9.5^{B}	41.5±11.5^{B}	60.4±13.6^{A}	62.5±8.7^{A}	60.6±10.4^{A}
DNA fragmentation	2.1±0.8	2.3±1.1	2.4±0.5	2.3±0.7	2.1±0.3	2.2±0.5	2.1±0.6	2.2±0.4	2.3±0.6	2.4±0.4

A,B,C,D,E Values in rows with different letters are statistically different (P<0.01)
Abbreviations: GSH, reduced glutathione; SOD, superoxide dismutase; CAT, catalase; $\Delta\Psi m$, mitochondrial transmembrane potential

cantly (P<0.01) improved the motility parameters compared to the control group.

Apoptotic-like changes in spermatozoa

The results of the assessment of the apoptotic-like changes in frozen-thawed spermatozoa are presented in Table 2.

An increase in percentage of spermatozoa with apoptotic-like changes (YO-PRO-1$^+$/PI$^-$ and AnV$^+$/PI$^-$) was observed after cryopreservation in all experimental extenders. In the control group the highest percentage of spermatozoa with apoptotic-like changes measured by YO-PRO-1/PI (25.3±3.2) and AnV/PI assay (22.6±1.2) was noticed. Moreover, in extender without supplementation of antioxidants the lowest percentage of sperm with high $\Delta\Psi m$ (40.2±8.5) was observed.

When comparing the supplementation of the different GSH concentrations to the freezing extender, it was found that the 5 mM GSH concentration significantly improved the post-thaw percentage of viable spermatozoa (YO-PRO-1$^-$/PI$^-$ and AnV$^-$/PI$^-$) and the percentage of spermatozoa with high $\Delta\Psi m$. Also, the lowest percentage of spermatozoa with apoptotic-like changes (YO-PRO-1$^+$/PI$^-$: 10.3±4.3%, AnV$^+$/PI$^-$: 9.7±4.9%) was observed in sperm cryopreserved with the addition of 5mM GSH.

In freezing extender with addition of SOD (150 or 300 IU/mL) a significantly lower percentage of spermatozoa with apoptotic-like changes (YO-PRO-1$^+$/PI$^-$: 21.1±10.6 *vs* 20.5±7.8) compared with control (YO-PRO-1$^+$/PI$^-$: 19.1±4.3) was noticed.

The supplementation of CAT (200 or 400 IU/mL) to the freezing extender revealed that the addition of CAT significantly (P<0.01) reduced the appearance of apoptotic-like changes (YO-PRO-1$^+$/PI$^-$, AnV$^+$/PI$^-$) in frozen-thawed spermatozoa compared with the control group. At the same time, comparison of the different CAT concentrations showed that the 400 IU/mL supplement better protects the sperm from cryogenic damage.

The combination of SOD plus CAT in the freezing extender provided the best post-thaw sperm survival parameters compared with SOD and CAT alone. The highest percentage of spermatozoa with high $\Delta\Psi m$ (62.5±8.7) from among the analyzed groups was noticed in group VII (150 SOD plus 200 IU/mL CAT).

Freezing and thawing increased the mean percentage of nonviable spermatozoa (AnV$^-$/PI$^+$) and no significant differences in this subpopulation of sperm in all experimental groups compared with control was reported.

The results revealed no differences for DNA fragmentation between fresh and frozen-thawed semen. The TUNEL-positive sperm nuclei were 2.1±0.8 in fresh semen and ranged from 2.1±0.3 to 2.4±0.5 in frozen-thawed semen, which did not differ significantly.

Discussion

Boar semen differs in many aspects from the semen of other domestic animals. The semen is produced in a large volume and is highly sensitive to cold shock; the viability of the sperm is dramatically reduced during the cryopreservation process. During cryopreservation boar spermatozoa are especially vulnerable to oxidative damage. Oxidative stress has long been reported for diminished membrane integrity, impaired sperm function and decreased sperm motility. This process could be blocked, at least in part, by the addition of exogenous antioxidants to the freezing extender.

Glutathione is a tripeptide ubiquitously distributed in living cells and it plays an important role as an intracellular defense mechanism against oxidative stress. During the cryopreservation process a significant reduction in the glutathione content of the semen was observed (Gadea et al. 2004). This can be prevented by using glutathione-supplemented extender for cryopreservation. On the basis of our results, we can conclude that 2.5 mM glutathione is not sufficient to protect the sperm from oxidative damage. In experiments carried out by Gadea et al. (2005) and Yeste et al. (2014), this positive effect was observed with the addition of 1 mM and 2 mM of GSH to the freezing extender, while Gadea et al. (2004) reported that the addition of 5 mM GSH to the freezing extender did not result in any improvement in standard semen parameters. In our study, the addition of 5 mM GSH to the freezing extender resulted in: an improvement in motion parameters of thawed spermatozoa, a higher number of viable sperm and sperm with high $\Delta\Psi$m, and a decrease in percentage of sperm with apoptotic-like changes (YO-PRO-1$^+$/PI$^-$ and AnV$^+$/PI$^-$). In our study, 5 mM of exogenous GSH added to the freezing extender had a positive effect on post-thaw sperm quality. On the other hand, Gadea et al. (2005) demonstrated that the protective effect on sperm function was more pronounced with 1 mM of GSH than with 5 mM of GSH. Moreover, a previous study of Gadea et al. (2004) revealed that the addition of GSH to freezing extender did not results in any significant improvement in standard semen parameters, but GSH addition to the thawing extender resulted in a significant increase in the fer-

tilizing ability of post-thaw sperm. The explanation for the different effect of GSH on sperm function is associated with different levels of endogenous GSH. Spermatozoa GSH content before and after freezing may show variation between boars (Gadea et al. 2004). Therefore, when planning further studies, it is appropriate to focus also on evaluating the amount of endogenous GSH. This may explain differences in the response of sperm to exogenous GSH levels.

In the present study we evaluated the potential benefits of the ROS-scavenging enzymes superoxide dismutase and catalase for boar sperm freezing. Enzymatic antioxidants such as SOD and CAT protect spermatozoa from superoxide anion and H_2O_2. The superoxide dismutase in the spermatozoa catalyzes the O_2^- radicals to form O_2 and H_2O_2. Our results suggest that H_2O_2 rather than O_2^- is more harmful to the spermatozoa. The negative effect of H_2O_2 was significantly minimized after the addition of 400 IU CAT to the freezing extender. As is well known, catalase converts H_2O_2 to H_2O and O_2, eliminating potential ROS toxicity (Aitken 1995). In agreement with Roca et al. (2005), addition of SOD and CAT in combinations of 150 plus 200 IU/mL or 300 plus 400 IU/mL had a beneficial effect on sperm during cryopreservation.

Regardless of the variant of the extender used for freezing, cryopreservation and thawing did not induce DNA fragmentation in boar spermatozoa. A similar result was found for cryopreserved bull and human spermatozoa (Duru et al. 2001, Martin et al. 2004). Few studies have focused on the effects of cryopreservation on apoptotic manifestations in spermatozoa (Duru et al. 2001, Anzar et al. 2002). They have shown that cryopreservation is associated with induction of membrane PS translocation, but this membrane modification is not correlated with DNA fragmentation or with free radical production. In the case of boar semen, it was found that the freezing and thawing procedure facilitated destabilization in the chromatin structure, resulting in an unstable DNA that was highly susceptible to fragmentation irrespective of the sperm source, extender type and packaging (Fraser and Strzeżek 2007, Fraser et al. 2011). The differences in results may be due to male-to-male individual response to oxidative stress during the cryopreservation process. In addition, differences in the results may be caused by the use of different assessment methods for identification of DNA fragmentation.

Generally, the results showed that cryopreservation has a negative effect on spermatozoa $\Delta\Psi m$ and results in mitochondrial dysfunction. This mitochondrial dysfunction can be responsible for decreased sperm motility observed after thawing. The low percentage of viable spermatozoa with apoptotic-like changes measured by YO-PRO-1/PI and AnV/PI assay was observed in fresh semen, before the cryopreservation process. However, after cryopreservation an increased permeability of plasma membranes to YO-PRO-1 (YO-PRO-1^+/PI^-) and in translocation of phosphatidyloserine in viable spermatozoa (AnV^+/PI^-) was noted. Simultaneously, cryopreservation induced an increase in percentage of the late apoptotic-like changes (AnV^+/PI^+) in spermatozoa in all freezing extenders. This observation is in agreement with that of Pena et al. (2003), who evaluated the changes in translocation of phosphatidyloserine in post-thaw boar spermatozoa. The detection of the apoptotic-like changes in spermatozoa is a very important parameter for evaluating the fertilizing ability of frozen-thawed spermatozoa. As previously reported by our laboratory (Trzcińska et al. 2015) the low value of post-thaw spermatozoa with apoptotic-like changes results in higher insemination success.

In conclusion, this study of apoptotic-like changes in frozen-thawed boar spermatozoa revealed that the addition of 5 mM GSH and SOD in combination with CAT (150+200 or 300+ 400 IU/mL) as well as 400 IU/mL catalase caused the highest reduction in apoptotic-like changes in frozen-thawed boar spermatozoa compared with the standard lactose-egg yolk-glycerol extender. Moreover, analysis of apoptotic-like changes could be a valuable tool for assessing the quality of frozen-thawed boar semen.

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