

DOI 10.1515/pjvs-2015-0095

Original article

Effects of storage in different semen extenders on the pre-freezing and post-thawing quality of boar spermatozoa

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Abstract

The aim of this study was to investigate the effects of storage of semen in different commercial extenders on the pre-freezing and post-thawing quality of boar spermatozoa. Semen was diluted in BTS, Androhep (AH) and Gedil (GD), stored for 24 h at 17°C, and then frozen in accordance with the cryopreservation protocol. Analyses of the quality of spermatozoa included: motility, normal apical ridge (NAR) acrosome, plasma membrane integrity (PMI), mitochondrial membrane potential (MMP), measurements of ATP content and activity of superoxidase dismutase (SOD) and glutathione peroxidase (GPx). Prior to the freezing process, no significant effect of the extender on the sperm quality parameters was noted. After thawing the spermatozoa it was demonstrated that the type of extender used influenced PMI, MMP, ATP content and activity of GPx. In the AH extender the percentage of spermatozoa with PMI and ATP content in spermatozoa was significantly higher ($P<0.05$) as compared to the BTS or GD extenders. In addition, semen stored in the AH was characterised by a statistically higher ($P<0.05$) percentage of spermatozoa with MMP and increased activity of GPx as compared with the BTS. The results obtained indicate that for the cryopreservation process, boar spermatozoa stored for 24 hours in liquid state can be used. However, the type of extender used prior to freezing may have a significant effect on the post-thawing quality of the spermatozoa. The AH extender better secured the quality of thawed boar spermatozoa as compared with the BTS or GD.

Key words: boar, spermatozoa, cryopreservation, extender

Introduction

Artificial insemination (AI) is commonly used in the reproduction of swine all over the world. Approximately 90% of sows are primarily inseminated using semen stored in a liquid state, which is associated with

much better results as regards their fertility as compared to the use of cryopreserved semen (Johnson et al. 2000, Didion et al. 2013).

The use of cryopreserved semen is insignificant, as only 1% of all insemination procedures are performed using such semen (Didion et al. 2013). However, a sig-

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nificant increase in interest in this method has been observed, as well as progress in the development of this preservation technology. This is due to the fact that cryopreservation of semen allows both the storage of genetic material for a long time, and the introduction of desired traits to the currently run breeding lines (Johnson et al. 2000, Gerrits et al. 2005, Bailey et al. 2008). In addition, it contributes to the prevention of possible biological hazards in the production of high quality pork, and allows the reproduction of parents' genetic traits eliminated during an epidemic (Bailey et al. 2008, Didion et al. 2013). Therefore, in this aspect, cryopreservation of semen is of great significance in the long-term storage of genetic material. However, the use of this method of semen storage is subject to numerous restrictions. Boar spermatozoa are very sensitive to cold-shocked temperature resulting in reduced survivability during preservation (Fraser et al. 2007, Gączarzewicz et al. 2015). Cryopreservation is an expensive process requiring appropriate conditions, time, sterility, a suitable extender, and equipment allowing gradual, controlled cooling of the semen, followed by storage in liquid nitrogen.

For the cryopreservation of semen, fresh ejaculate is used immediately or soon after collection. However, materials derived from individuals characterized by outstanding traits are often located far from the laboratory. Therefore, it is recommended that the materials should be appropriately secured through, e.g. dilution with a suitable extender used for the storage of semen, and transported under appropriate conditions to the location where they will be subjected to the cryopreservation process. The quality of boar semen stored in a liquid state is influenced by the type of extender and storage time (Johnson et al. 2000). Currently, many different diluents are used for the storage of semen, which help maintain the viability of the spermatozoa for several days (Gadea, 2003). However, taking into account the criteria which must be met by semen for artificial insemination, its ability to fertilize quickly falls. Research has shown that in short and medium-term extenders (up to 5 days), as early as on the second day of storage, spermatozoa motility and the percentage of normal sperm morphology decrease and DNA disintegration increases, which makes them unsuitable for insemination (Wrona et al. 2009, Klimont et al. 2015). Long-term extenders (over 6 days) compared to short-term ones are enriched with substances which better protect the quality of the stored spermatozoa and thus their capacity for fertilization, which may significantly affect their improved suitability for technological treatment (Johnson et al. 2000, Gadea 2003).

The aim of this paper was to investigate the effects of the extender used on selected parameters of quality

of boar spermatozoa prior to freezing, and then following thawing. The analysis of the quality of spermatozoa included the motility and motion parameters, plasma membrane integrity, acrosome integrity, mitochondrial function (mitochondrial membrane potential), ATP content and the activity of selected antioxidant enzymes.

Materials and Methods

Animals and semen collection

Ejaculates were collected from five sexually mature Polish Landrace boars (average age 2 years) stationed at the Artificial Insemination (AI) Centre in Szczecinek (Poland). A total of 11 ejaculates were collected from the boars from September to December, using the gloved hand technique. During collection, the semen was passed through sterile gauze to remove the gelatinous fraction. Boars were provided with water and fed under standard housing protocols. Animal experiments were carried out in accordance with the guidelines set out by the Local Ethics Committee.

Semen processing and preservation

The semen samples were subjected to macro- and microscopic analyses. Only semen samples that exhibited more than 70% of total sperm motility (assessed using a computer-assisted sperm analysis (CASA) system) and less than 15% abnormal spermatozoa were used in this study. Sperm concentration was determined using a Burkner counting chamber (Equimed-Medical Instruments, Kraków, Poland).

Whole ejaculate was diluted in a 1:1 proportion in three commercial extenders: BTS (Minitüb, Tiefenbach, Germany), Androhep (AH, Minitüb, Tiefenbach, Germany), and Gedil (GD, Genes Diffusion, France) at the AI Centre. The extended semen was transported to the cryopreservation laboratory of the Department of Animal Biochemistry and Biotechnology (Kortowo-Olsztyn, Poland) at 17°C in a Thermobox (Minitüb GmbH, Tiefenbach, Germany), where it was stored for 24 h in the same conditions, semen samples were then frozen according to a cryopreservation protocol by Strzeżek et al. (1985) with some modifications (Fraser and Strzeżek 2007). The samples of extended semen were centrifuged for 10 minutes at 800 x g at room temperature, and sperm pellets were re-suspended in extender 1 containing 11% lactose and lipoprotein fraction isolated from ostrich egg yolk (LPFo). After mixing, the semen samples were cooled at 5°C over a 2-h period. The

cooled semen samples were diluted (2:1) with a second extender, extender 2, consisting of 89.5 ml of extender 1, 9 ml of glycerol, and 1.5 ml of Orvus Es Paste (OEP). The final concentrations of LPFo, glycerol and OEP were 5%, 3%, and 0.5%, respectively. The cooled semen samples were packaged into 10-ml sterilized aluminum tubes (Polfa, S.A., Bolesławiec, Poland) and loaded onto a programmable computer freezing machine (Ice Cube 1411, SY-LAB, Austria), using an appropriate cooling rate. Frozen semen samples were stored in liquid nitrogen containers (VHC 35, Taylor-Wharton Germany GmbH, Mildstedter Landstr. 1, Mildsted, Germany) at the cryolaboratory of the department for a two-week period. Analyses of sperm quality were performed before freezing (24 h after dilution of fresh semen in extender) and following thawing. For the post-thawing analysis, frozen semen samples were thawed in a water bath at 50°C for 60 s prior to assessment of sperm quality.

Analysis of sperm quality

Motility evaluations

Motility evaluations were analysed using a CASA system (Hamilton-Thorne research, HTR, IVOS version 12.3; Beverley, MA, USA). Aliquots of semen samples (5 µl) were placed on a pre-warmed Makler counting chamber and examined at 37°C. A minimum of five fields per sample were assessed. The CASA system analysed total motile spermatozoa (TMOT, %), progressive motile spermatozoa (PMOT, %), velocity average path (VAP, µm/s); low velocity straight line (VSL, µm/s), curvilinear velocity (VCL, µm/s), mean amplitude of lateral head displacement (ALH, µm), beat cross frequency (BCF, Hz), straightness (STR, ratio of VSL/VAP x 100%), and linearity (LIN, ratio of VSL/VCL x 100%). The CASA sperm motion parameters were measured according to settings of the program system required for boar.

Normal apical ridge (NAR) acrosome

The percentage of spermatozoa with normal apical ridge (NAR) acrosomes was assessed using the Giemsa staining method described by Watson (1975), with some modifications (Fraser et al. 2007). A minimum of 100 sperm cells per slide were examined at 1000 × magnification under a bright light microscope (Olympus CH 30), and were considered as spermatozoa with NAR (acrosome-intact) or damaged apical ridge acrosome.

Plasma membrane integrity (PMI)

The percentage of spermatozoa with intact membrane (PMI) was assessed using dual fluorescent staining, SYBR-14 and PI (Live/Dead Sperm Viability Kit, Molecular Probes, Eugene, OR, USA), as described by Garner and Johnson (1995). Approximately 100 sperm cells per slide were examined at 600 x magnification under an epifluorescence microscope (Olympus CH 30, RF-200, Tokyo, Japan). Spermatozoa exhibiting green fluorescence were considered as viable cells with an intact membrane.

Mitochondrial function

The percentage of spermatozoa with functional mitochondria (high and medium mitochondrial membrane potential, MMP) was assessed in semen samples, using dual fluorescent staining, JC-1 (Molecular Probes, Eugene, USA) and PI, according to a previously described method (Thomas et al. 1998), with some modifications (Dziekońska et al. 2009). Aliquots (10 µl) of the stained sperm cells were examined at 600 x magnification under an epifluorescence microscope (Olympus CH 30 RF-200, Tokyo, Japan) equipped with a blue excitation filter (DMB) for JC-1 and a green excitation filter (DMG) for PI. Sperm cells displaying only orange-red fluorescence in the mid-piece region were considered as viable spermatozoa with functional mitochondria. Two slides were assessed per sample, and approximately 100 spermatozoa were counted per slide.

ATP content

Sperm ATP content was measured in semen samples using a Bioluminescence Assay

Kit CLS II protocol (Roche Diagnostics GmbH, Germany). A Junior bioluminometer (Berthold Technologies, GmbH & Co. KG, Germany) was used to measure bioluminescence in semen samples. ATP content was calculated from a standard ATP curve and expressed as nmol/10⁸ spermatozoa.

Activity of intracellular antioxidant enzymes

The activities of SOD and GPx were measured using commercial kits, according to the manufacturers' instructions, in sperm extracts prepared as follows. Semen samples with a concentration of 20 x 10⁸ spermatozoa/ml, were rinsed with 1 ml of 0.85% NaCl solution, centrifuged for 5 minutes at 900 x g with the

Table 1. Influence of type of extender on quality parameters of pre-freezing boar spermatozoa.

Parameters	Androhep (AH)	BTS	Gedil (GD)
MOT ($\mu\%$)	87.54 \pm 1.37	86.09 \pm 1.65	87.00 \pm 1.89
PMOT (%)	57.36 \pm 2.68	59.54 \pm 3.93	58.18 \pm 2.82
VAP ($\mu\text{m/s}$)	80.72 \pm 4.17	85.57 \pm 5.45	76.85 \pm 2.98
VSL ($\mu\text{m/s}$)	60.62 \pm 3.06	63.93 \pm 4.22	57.82 \pm 2.22
VCL ($\mu\text{m/s}$)	137.32 \pm 7.43	146.29 \pm 9.16	134.05 \pm 4.99
ALH ($\mu\text{m/s}$)	6.81 \pm 0.27	6.78 \pm 0.20	7.07 \pm 0.23
BCF (Hz)	25.24 \pm 1.06	24.21 \pm 0.94	26.75 \pm 0.94
STR (%)	74.54 \pm 1.35	74.45 \pm 1.81	74.27 \pm 1.13
LIN (%)	46.09 \pm 1.53	45.91 \pm 2.16	44.91 \pm 1.25
NAR acrosome (%)	91.73 \pm 0.67	90.45 \pm 1.32	91.00 \pm 1.19
PMI (%)	82.49 \pm 1.43	81.62 \pm 1.51	79.25 \pm 1.31
MMP (%)	64.35 \pm 1.25	64.31 \pm 1.46	60.10 \pm 1.17
ATP (nmol/10 ⁸ sperm)	8.09 \pm 0.97	8.33 \pm 0.61	10.46 \pm 1.21
SOD (U/10 ⁸ sperm)	3.97 \pm 0.03	3.93 \pm 0.04	3.97 \pm 0.04
GPx (mU/10 ⁸ sperm)	0.15 \pm 0.01	0.15 \pm 0.02	0.15 \pm 0.01

MOT – total motile spermatozoa, PMOT – progressive motile spermatozoa, VAP – velocity average path, VSL – velocity straight line, VCL – curvilinear velocity, ALH – mean amplitude of lateral head displacement, BCF – beat cross frequency, STR – straightness (ratio of VSL/VAP x 100%), LIN – linearity (ratio of VSL/VCL x 100%), NAR – normal apical ridge acrosome, PMI – plasma membrane integrity, MMP – mitochondrial membrane potential, ATP – adenosine triphosphate content, SOD – superoxidase dismutase, GPx – glutathione peroxidase. Values represent the mean (\pm SEM) of 11 ejaculates from 5 boar.

Table 2. Influence of type of extender on quality parameters of post-thawing boar spermatozoa.

Parameters	Androhep (AH)	BTS	Gedil (GD)
MOT (%)	20.55 \pm 4.84	30.18 \pm 5.02	21.55 \pm 3.64
PMOT (%)	15.00 \pm 3.88	22.00 \pm 4.043	15.18 \pm 2.72
VAP ($\mu\text{m/s}$)	66.53 \pm 2.32	66.53 \pm 3.14	64.32 \pm 1.79
VSL ($\mu\text{m/s}$)	56.60 \pm 2.27	56.76 \pm 3.07	55.85 \pm 1.68
VCL ($\mu\text{m/s}$)	103.24 \pm 4.45	107.77 \pm 4.87	100.71 \pm 3.81
ALH ($\mu\text{m/s}$)	4.50 \pm 0.31	4.72 \pm 0.24	4.45 \pm 0.23
BCF (Hz)	23.56 \pm 1.86	27.12 \pm 0.96	25.49 \pm 1.49
STR (%)	84.72 \pm 1.66	84.54 \pm 1.33	86.18 \pm 1.16
LIN (%)	59.18 \pm 2.86	56.09 \pm 2.25	58.18 \pm 2.77
NAR acrosome (%)	54.36 \pm 2.56	55.54 \pm 2.75	56.55 \pm 2.67
PMI (%)	60.51 \pm 2.24 ^a	51.92 \pm 2.50 ^b	51.65 \pm 1.80 ^b
MMP (%)	42.05 \pm 2.41 ^a	33.79 \pm 3.46 ^b	36.54 \pm 3.47 ^{ab}
ATP (nmol/10 ⁸ sperm)	6.89 \pm 0.96 ^a	2.93 \pm 0.26 ^b	4.03 \pm 0.65 ^b
SOD (U/10 ⁸ sperm)	2.44 \pm 0.28	2.43 \pm 0.24	2.48 \pm 0.25
GPx (mU/10 ⁸ sperm)	0.14 \pm 0.03 ^a	0.08 \pm 0.01 ^b	0.09 \pm 0.01 ^b

MOT – total motile spermatozoa, PMOT – progressive motile spermatozoa, VAP – velocity average path, VSL – velocity straight line, VCL – curvilinear velocity, ALH – mean amplitude of lateral head displacement, BCF – beat cross frequency, STR – straightness (ratio of VSL/VAP x 100%), LIN – linearity (ratio of VSL/VCL x 100%), NAR – normal apical ridge acrosome, PMI – plasma membrane integrity, MMP – mitochondrial membrane potential, ATP – adenosine triphosphate content, SOD – superoxidase dismutase, GPx – glutathione peroxidase. Values represent the mean (\pm SEM) of 11 ejaculates from 5 boars. Values with different letters (a, b) in the same row are different at $p < 0.05$.

supernatant liquid being discarded, and suspended in 1 ml of 0.85% NaCl solution. The prepared samples were homogenized using Ultra Turrax T8 homogenizer (IKA-Werke, Staufen, Germany) in an ice bath for 5 minutes, and centrifuged at 10,000 x g for 10 minutes. The obtained supernatant liquid was separated from the sediment, and stored at a temperature of -20°C for further analyses.

SOD assay

The activity of SOD was measured using the Randox RANSOD assay (Randox Laboratories, Crumlin, Great Britain). This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. One unit (U) of SOD activity was defined as the amount of the enzyme that caused 50% inhibition in the rate of reduction of INT at 37°C (pH 7.0). The activity of SOD was measured spectrophotometrically at 505 nm, and expressed as U/10⁸ spermatozoa.

GPx assay

The activity of GPx was determined using the Ransel Glutathione Peroxidase kit (Randox Laboratories, UK). In this assay GPx catalyses the oxidation of glutathione (GSH) with cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH, oxidized glutathione (GSSG) is converted to GSH with a concomitant oxidation of NADPH to NADP⁺. The decrease in absorbance was measured at 340 nm at 37°C (pH 7.2), and the GPx activity was expressed as mU/10⁸ spermatozoa.

The activities of SOD and GPx were measured using commercial kits, according to the manufacturers' instructions. All assays were performed in duplicates.

Statistical analysis

The results are expressed as the mean ± standard error of the mean (SEM). Differences between means were determined by ANOVA, followed by the Duncan Multiple Comparison test. A significant difference was noted at P<0.05. In addition, following the thawing of semen, the effects of the extender on the analysed parameters of sperm quality were investigated using single-factor variance analysis ANOVA (Fisher's test, F). The results were processed in the

Statistica application (v. 10; StatSoft Incorporation, Tulsa, OK, USA).

Results

The characteristics of the quality of boar spermatozoa prior to freezing are presented in Table 1. Irrespective of the extender used, the study indicated that the percentage of motile spermatozoa amounted to over 86%, while the acrosome integrity was not lower than 90%. No statistically significant differences (p<0.05) were found between the extenders in the analysis of the parameters of sperm quality: TMOT, PMOT, VAP, VSL, VCL, ALH, BCF, STR, LIN, NAR, PMI, MMP, ATP content, SOD and GPx activity.

Following thawing, a significant reduction in the values of all analysed parameters was demonstrated, irrespective of the extender used (Table 2). It was observed, however, that the type of extender used had a significant effect on the quality of thawed spermatozoa. In the AH extender, the highest values of PMI, MMP, and the ATP content and GPx activity in spermatozoa were demonstrated. Statistically significant differences (p<0.05) were found between AH extender and the BTS and GD extenders in the analysis for PMI, the ATP content and GPx activity. In addition, statistically significant differences (p<0.05) between the AH and BTS extenders were also demonstrated in MMP. In the analysis of sperm motility (TMOT, PMOT) and motion parameters (VAP, VSL, VCL, ALH, ALH, BCF, STR, LIN), no statistically significant differences (p>0.05) between the extenders were found. However, the highest values of TMOT, PMOT, VAP, VSL and VCL were observed in the BTS extender. In turn, when using ANOVA single-factor variance analysis, significant effects of the extender used on PMI (F = 5.80; p<0.008), MMP (F = 3.36; P<0.049) and ATP content (F = 8.675; p<0.001) in the thawed spermatozoa were demonstrated.

Discussion

The use of spermatozoa stored for several hours prior to freezing is not commonly practiced, and not fully understood. Hence, in this study, boar semen diluted in different commercial extenders (Androhep, BTS and GEDIL) and stored for 24 hours at a temperature of 17°C was used for cryopreservation, and the quality of spermatozoa prior to freezing and following thawing was then investigated. Prior to freezing, no statistically significant differences were

found in the quality of spermatozoa stored in different extenders, which probably resulted from the quality of fresh semen intended for the study (data not presented), and the short storage period (24 h). This is because the spermatozoa used in the experiment were collected from selected boars from the AI Center, which were characterized by the good quality of the ejaculates, and the extenders used secured the biological properties of the spermatozoa.

Following the thawing of the sperm, a reduction in sperm motility, motion parameters, integrity of acrosomal and plasma membranes, as well as impaired function of the sperm mitochondria and a reduction in the antioxidant efficiency were observed, irrespective of the extender used. Numerous studies have demonstrated that liquid storage or cryopreservation of boar semen induces marked changes in the structural and functional integrity of the sperm membranes (Roca et al. 2006, Gączarzewicz et al. 2010, 2015). During the cryopreservation procedure, damage to the sperm mitochondria occurs, which in turn leads to the disruption of the oxidative phosphorylation process, and a reduction in post-thaw sperm motility (Johnson et al. 2000, Fraser et al. 2007, Peña et al. 2011, Guthrie and Welch 2006, 2012). Hence, in our study, irrespective of the extender used, a reduction in mitochondrial functionality was also observed, which was reflected in a reduction in mitochondrial membrane potential, ATP content and the motility of the thawed spermatozoa. Motility is a parameter commonly used for the assessment of sperm vitality in an ejaculate both prior to, and after the cryopreservation process (Johnson et al. 2000). In our study, the percentage of spermatozoa exhibiting motion amounted to approx. 20-30%, which may indicate significant damage to the spermatozoa following thawing. The obtained results were slightly lower than those obtained earlier for fresh sperm subjected to the cryopreservation process (approx. 26-40%), but the earlier results concerned other individuals (Fraser and Strzeżek 2007, Fraser et al. 2007). In the case of other parameters used to assess the quality of semen (NAR acrosome, PMI, mitochondrial activity and ATP content), the results were also similar to the ones previously received for post-thawing fresh boar semen (Fraser and Strzeżek 2007, Fraser et al. 2007). This can only confirm that the use of extender to store semen for one day allows the preservation of such spermatozoa quality as fresh semen has, and this may suggest that it can be used for the cryopreservation process.

One of the main sources of damage to boar spermatozoa occurring during the cryopreservation procedure, particularly during thawing, is oxidative stress (Peña et al. 2011, Guthrie and Welch 2012), which is

associated with the excessive generation of reactive oxygen species (ROS). In addition, ROS causes an adverse effect on the physiological functions of sperm cells through the induction of peroxidation in the plasma membranes of the spermatozoa (Agarwal et al. 2008, Saryozkan et al. 2013). Boar spermatozoa contain many polyunsaturated fatty acids, which makes them particularly susceptible to the lipid peroxidation processes. Lipid peroxidation is responsible for disturbances in the integrity of plasma membranes, and leads to the inhibition of motility (Cerolini et al. 2000).

The elements of the sperm antioxidant system include antioxidant enzymes and low-molecular antioxidants. As compared with other animal species, ejaculated boar spermatozoa are characterized by a poor enzymatic antioxidant system (Strzeżek 2002). The main enzyme in boar spermatozoa and seminal plasma is SOD (Kowalowka et al. 2008, Kozirowska-Gilun et al. 2011). In addition, low GPx and glutathione reductase activity was demonstrated in boar spermatozoa (Jelezarsky et al. 2008). No presence of CAT was found in cauda epididymal spermatozoa (Kozirowska-Gilun et al. 2011). Given the limited possibilities for the antioxidant protection of boar spermatozoa, it is believed that the antioxidants of the seminal plasma play a main role in protecting them against ROS (Strzeżek 2002). As a result of diluting the sperm with extenders, followed by the cryopreservation procedure, a significant inhibition of the spermatozoa's antioxidant occurs. Our study demonstrated a significant reduction in SOD and GPx activity in the thawed spermatozoa as compared with the spermatozoa prior to freezing, irrespective of the extender used. Previous studies also demonstrated that the cryopreservation process is responsible for the decrease in the level of antioxidants such as glutathione or SOD in the post-thaw spermatozoa of both the human and bull (Alvarez and Storey 1992, Bilodeau et al. 2000). During the cryopreservation process, an increase in lipid peroxidation occurs as a result of a reduction in SOD activity in cryopreserved human spermatozoa (Alvarez and Storey 1992).

Our study demonstrated that the type of extender used for the storage of semen prior to freezing had a significant effect on the quality of spermatozoa after thawing. The type of extender used affected plasma membrane integrity, mitochondrial function, ATP content, and GPx activity. A probable cause of the differences between the extenders was their different compositions. As regards the analysed extenders, i.e. Androhep (AH), BTS and GEDIL (GD), the AH extender proved to be the most suitable for the storage of boar sperm. As compared with the short-term BTS, the AH is enriched with Hepes (N-2-hydroxyethyl-

piperazine-N²-ethanesulfonic acid) and bovine serum albumin (BSA) (Gadea 2003). Unfortunately, little is known about the composition of the long-term GD extender. It can only be assumed that its composition is different from that of the AH, and possibly more similar to that of the BTS, since the quality of the spermatozoa stored in it was not statistically significantly different from that of the spermatozoa stored in the BTS.

Where the pH value decreases below 7.2, a restriction in spermatozoa metabolism and motility occurs (Johnson et al. 2000). The AH extender contains Hepes, which can affect the buffering capacity of the extended semen. This phenomenon might affect the sperm metabolic activity as indicated in a recent study (Gączarzewicz et al. 2015). Many properties are attributed to the BSA contained in the Androhep, such as the ability to stimulate sperm motility in various animal species (Harrison et al. 1982, Sariözkan et al. 2013). Blank et al. (1976) proposed an explanation for this phenomenon, according to which BSA could have contributed to the flowing of Ca²⁺ ions, important to the maintenance of sperm motility, into the cytoplasm. Previous studies indicated that BSA exhibits protective properties towards the membranes, which involve its specific interaction with the membrane phospholipids, thus reducing the extent of damage to the stored spermatozoa (El-Kon 2011). Alvarez and Storey (1995) reported that BSA is a very potent inhibitor of lipid peroxidation in the spermatozoa, and this type of damage reduces sperm motility. It was proven that BSA added to the Modena extender exhibits antioxidant properties, reduces oxidative stress, and enhances the quality of boar semen stored at a temperature of 17°C (Zhang et al. 2015). The content of antioxidants in the stored semen plays an important role in the survivability of the cryopreserved spermatozoa (Bilodeau et al. 2000, Funahashi and Sano 2005). This could suggest that the differences between the extenders in terms of the quality of the spermatozoa following thawing were a result of the presence of BSA contained in the AH. Hence, in the extender concerned, as compared with the BTS and GD, a better integrity of plasma membranes was demonstrated, which in turn had a positive effect on the functioning of mitochondria and the activity of one of the antioxidant enzymes, GPx. GPx protects the spermatozoa cell membrane against oxidative damage (Jelezarsky et al. 2008). In our study the SOD activity did not change depending on the extender, which may suggest that it is more resistant to cryogenic damage than GPx. Moreover, its low activity in the spermatozoa following thawing, as compared with that of the spermatozoa prior to freezing, may indicate a low level of ROS generation, which was previously suggested by Guthrie and Welch (2012).

Our study demonstrated the highest post-thaw motility in the spermatozoa stored in the BTS and not, as could have been expected, in the Androhep in which TMOT was at the 20% level, which is not very high and not satisfactory for the cryopreserving process of boar semen. It would be better if sperm motility was greater than 30%, as in BTS. The situation is not clear since other analysed parameters of spermatozoa quality (PMI, MMP, ATP content, GPx activity) were highest in Androhep. The obtained results remain partially different from those previously obtained (Dubé et al. 2004, Dziekońska et al. 2013), which demonstrated that the AH, as compared with the BTS, helped maintain better motility and the percentage of spermatozoa with progressive motion, as well as the integrity of membranes in the stored semen. However, the present study lacks statistically significant differences between the extenders in the analysis of sperm motility, in spite of the considerable difference between the values, which could indicate the need for more replications. Therefore, this study may be regarded as preliminary to further research which would allow a better understanding and explanation of the changes and their mechanisms in boar spermatozoa stored in extenders prior to freezing and following thawing.

Based on the obtained results, it can be concluded that boar semen stored in commercial extenders for 24 hours at a temperature of 17°C may be used for the cryopreservation process. The type of extender used for the storage of semen prior to freezing may affect the biological properties of the spermatozoa following thawing (PMI, MMP, ATP content and GPx activity). The Androhep extender secured the quality of spermatozoa better than the BTS and GEDIL, which could have resulted from the presence of BSA in its composition. This study may contribute to considerable progress in the cryopreservation process, and requires continuation.

Acknowledgements

This study was supported by the University of Warmia and Mazury in Olsztyn (No. 11.610.003-300).

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