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Storage temperature of boar semen and its relationship to changes in sperm plasma membrane integrity, mitochondrial membrane potential, and oxidoreductive capability

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Abstract: The aim of this study was to analyze changes in sperm plasma membrane integrity, mitochondrial activity, and extracellular environment during storage of boar semen at 5 °C, 16 °C, and 25 °C for 10 days. Progressive sperm motility, plasma membrane integrity (SYBR-14/PI-test and HOS test), aspartate aminotransferase (AAT), and mitochondrial activity (JC-1-test and NADH-dependent NBT assay), as well as pH and osmolality were assessed in nine ejaculates of Polish Landrace boars. The plasma membrane integrity of the semen stored at 5 °C was similar to that of the semen stored at 16 °C and 25 °C; however, an increase in AAT activity of the semen stored at 5 °C revealed sperm membrane disorders as early as day 2. Significant differences in the progressive motility of the semen stored at 5 °C and 16 °C were observed at each of the evaluation times. This reduced motility was consistent with decreased sperm mitochondrial transmembrane potential and oxidoreductive capability. We inferred that the cooling of boar semen to 5 °C increases the permeability of the sperm plasma membrane, which may not be revealed by SYBR-14/PI staining or HOS testing. The loss of boar sperm motility that occurs during hypothermic liquid preservation may be connected with alterations in the plasma membrane stability and disorders of mitochondrial transmembrane potential and oxidoreductive capability.

Key words: Boar spermatozoa, liquid storage, storage temperature, plasma membrane integrity, mitochondrial activity

1. Introduction

The successful processing of boar semen prior to its use in artificial insemination (AI) depends on an understanding of the factors that influence the capacity of spermatozoa to survive during preservation of any type and the interactions of these factors. It is also well known that the survival of boar sperm is much higher when the semen is stored in liquid form rather than frozen (Johnson et al., 2000). For this reason, boar spermatozoa are commonly stored in liquid form at 15–18 °C for routine use in AI (Johnson et al., 2000; Purdy et al., 2010; Schmid et al., 2013; Schulze et al., 2013). In addition, swine AI involves the use of semen collected within the previous 3–7 days almost exclusively, although there is evidence to indicate that optimal fertility requires AI within 48 h after collection and extension in short- and long-term extenders (Johnson et al., 2000; Großfeld et al., 2008; Garcia et al., 2010). The components of the preservation solution and the temperature at which the semen is collected and stored following dilution are the main factors that influence the storage tolerance of spermatozoa preserved in a liquid state (Johnson et al., 2000; Martín-Hidalgo et al., 2013a; Schulze et al., 2013).

Boar spermatozoa are susceptible to rapid cooling from 35 °C to 15 °C and are especially sensitive to cold shock when cooled below 15 °C (Bailey and Buhr, 1995; Canvin and Buhr, 1989; Althouse et al., 1998; Johnson et al., 2000; Purdy et al., 2010; Kim et al., 2011; Schmid et al., 2013; Schulze et al., 2013). This is associated with a thermotropic phase transition in sperm membrane lipids and contributes to an irreversible impairing of the cellular functions of the spermatozoa (Drobnis et al., 1993; Johnson et al., 2000). The range of sperm damage during cooling depends on the chilling rate and threshold temperature (Schmid et al., 2013), but resistance of boar sperm to cold-induced damage may be enhanced by an appropriately long and slow cooling to 5 °C (Casas and Althouse, 2013). The sperm damage that occurs when lowering the temperature below the cold-shock temperature has been attributed to the specific plasma membrane composition of boar spermatozoa. These have a relatively high content of polyunsaturated fatty acids and a low concentration of cholesterol and, in consequence, show a lower sterol-to-phospholipid ratio which renders boar sperm plasma membranes more labile to chilling than spermatozoa from

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other mammalian species (Bailey et al., 2008; Martín-Hidalgo et al., 2011; López Rodrigeuz et al., 2012; Schulze et al., 2013).

The storage temperature, depending closely on storage length, is capable of destroying the integrity and function of a whole series of boar sperm cellular structures. It is commonly accepted that chilling injury impairs the functional and molecular state of the membrane and is accompanied by an alteration of the membrane proteins and membrane fluidity and an increase in membrane permeability, with a subsequent leakage of ions and reduction of enzyme activity (Drobnis et al., 1993; Johnson et al., 2000; López Rodrigeuz et al., 2012; Casas and Althouse, 2013; Schmid et al., 2013). The plasma membrane defects and the loss of important intracellular ingredients (such as calcium ions, adenine nucleotides, antioxidants, and enzymes) induce metabolic disruption that may affect sperm motility and vitality. Cooling-induced reductions in sperm function may also be due to oxidative damage from the harmful formation of reactive oxygen species (ROS) and membrane lipid peroxidation (Brouwers et al., 2005; Aitken, 2006; Großfeld et al., 2008; Awda et al., 2009). In addition to the plasma membrane, mitochondria are the sperm structures most sensitive to the stress induced by cooling and freezing (Flores et al., 2009; Peña et al., 2009). These organelles play a crucial role in diverse cellular functions, such as energy production, the modulation of redox status, osmotic regulation, and Ca^{2+} homeostasis (Bailey and Buhr, 1995; Kim et al., 2008; Storey, 2008; Costello et al., 2009; Piomboni et al., 2012; Ramió-Lluch et al., 2012; Vadzyuk and Kosterin, 2015), as well as cellular death (Flores et al., 2009; Peña et al., 2009; Amaral et al., 2013). Moreover, sperm mitochondria may also contribute significantly to oxidative stress (Koppers et al., 2008; Flores et al., 2009; Kim et al., 2011), which can lead to a decrease in boar-sperm motility (Bailey et al., 2008; Guthrie et al., 2008; Guthrie and Welch, 2012), independent of mitochondrial oxidative phosphorylation (Peña et al., 2009; Ramió-Lluch et al., 2011; Guthrie and Welch, 2012; Amaral et al., 2013).

Given these data, an understanding of boar sperm metabolism and, particularly, the dynamic mechanisms of energy-level regulation in cells, seems important for optimizing liquid semen hypothermic storage and developing appropriate semen cryopreservation methods. The main aim of this work was, thus, to investigate the effects of temperature (5 °C, 16 °C, and 25 °C) and length on boar semen storage for 10 days on specific sperm cell structures, as determined by plasma membrane integrity, mitochondrial activity, and extracellular environment alterations.

2. Materials and methods

2.1. Experimental animals, semen collection, and dilution

Nine Polish Landrace boars (1 ejaculate per boar) 2 years of age and with proven fertility from the local AI center were used for this study. The animals were healthy and were kept in individual pens under uniform management practices. According to farm standards, the boars were fed a complete diet to meet all of their nutritional needs and had access to water ad libitum.

Sperm-rich ejaculate fractions were collected by gloved hand technique into disposable filtering bags enclosed in insulated plastic thermos cups preheated to 37 °C. Immediately after collection, the initial semen characteristics (sperm concentration, motility, viability, and acrosome integrity) were microscopically evaluated using routine laboratory procedures. The ejaculate sperm samples (with more than 70% motile spermatozoa) were then isothermically diluted in Vitasem LD long-term commercial extender (Magapor S.L., Spain) so that each AI dose (closed plastic bags, 100 mL) contained 2.7×10^9 spermatozoa.

2.2. Conditions of semen storage and time points of semen evaluation

In the present study, the following cooling protocols were used: AI doses were separated after storage at room temperature for 2 h; one-third of all semen samples were transferred to an incubator and stored at 25 °C, while the remainder of the semen doses were cooled to 16 °C in a cooling chamber. After a 2-h holding time at 16 °C, half the semen samples were transferred to a 5 °C cooling chamber. AI doses were then stored for 10 days, with twice daily gentle agitation.

After dilution, the AI doses were kept at room temperature for 2 h. At this time point (day 0: the day of collection), the semen samples were first analyzed as described below. Further analyses were performed on every second day of the storage period (days 2, 4, 6, 8, 10) on semen samples stored at 5 °C, 16 °C, and 25 °C. At each time point for semen quality evaluation, newly opened AI doses were used. Before proceeding to assessment, the semen samples were prewarmed to 37 °C.

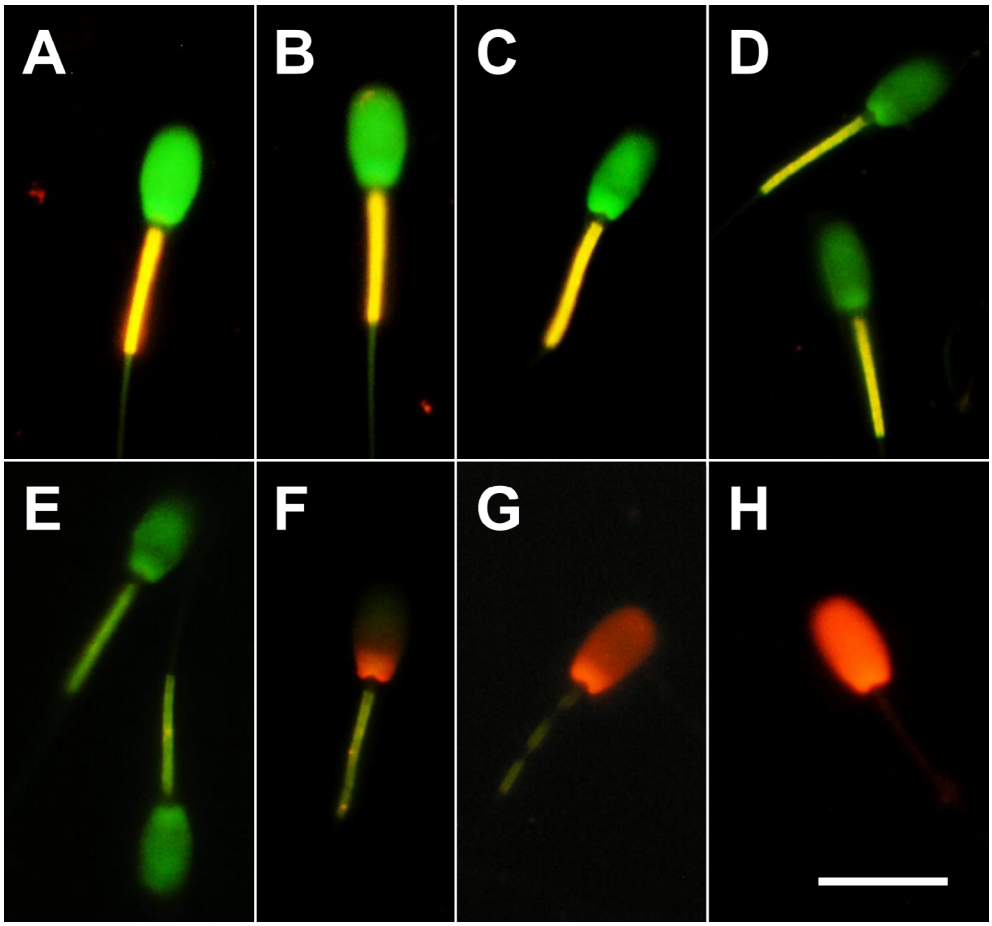
2.3. Assessment of sperm parameters

2.3.1. Motility

Sperm motility was assessed subjectively by placing a drop (5 µL) of semen on a prewarmed slide, covering it with a glass cover slide (22 × 22 mm), and estimating the percentage of sperm possessing progressive motility at ×400 magnification using a phase-contrast microscope (BX 41; Olympus Optical Co., Tokyo, Japan) and a heating stage (37 °C).

2.3.2. Fluorescent staining

A combination of SYBR-14 and propidium iodide (PI) triple fluorescent stains, PI (LIVE/DEAD sperm viability kit; Molecular Probes, Eugene, OR, USA), and JC-1 (5,5',6,6'-tetrachloro-1,1'3,3'tetraethylbenzimidazolyl carbocyanine iodide; Molecular Probes), was used for simultaneous evaluation of sperm plasma membrane integrity at the level of the sperm head and mitochondrial activity. SYBR-14 and PI are DNA-specific dyes that stain living cells green (SYBR-14) and dead cells red (PI). JC-1 has the unique ability to differentially label mitochondria with high and low mitochondrial transmembrane potential (ΔΨm). In mitochondria with high ΔΨm (active polarized organelles with ΔΨm above 80–100 mV), JC-1 forms multimeric aggregates that emit in the high orange wavelength of 590 nm when excited at 488 nm. At the same excitement wavelength (488 nm), however, JC-1 forms monomers within mitochondria with low ΔΨm (depolarized organelles with ΔΨm below 80–100 mV), emitting in the green wavelength (520–530 nm). For analysis, 2.5 µL of 20 µmol/L SYBR-14 in dimethyl sulfoxide (DMSO) and 5 µL of 153 µmol/L JC-1 in DMSO were added to 500 µL of prewarmed (37 °C) sperm sample and incubated at 37 °C for 20 min under lightproof conditions. Then 2.5 µL of 2.4 mmol/L PI in water was added, and the samples were further incubated at 37 °C for 10 min. With this staining, three primary sperm subpopulations were distinguished: (i) sperm cells that fluoresced green over the head (SYBR-14 positive and PI negative) and emitted intense or diminished yellow-orange fluorescence over the midpiece (representing mitochondria with high ΔΨm or mitochondria with decreasing ΔΨm, respectively; Figures 1A–1D); (ii) sperm cells that fluoresced green over the head (SYBR-14 positive and PI negative) while emitting green fluorescence over the midpiece (mostly representing mitochondria with low ΔΨm; Figure 1E);



Figures 1A–H. SYBR-14–, PI-, and JC-1–stained boar spermatozoa. Live sperm cells (SYBR-14 positive and PI negative) with intact plasma and mitochondrial membrane integrity exhibit green fluorescence over the head and intense (A–C, cells with high ΔΨm) or diminished (D, cells with decreasing ΔΨm) yellow-orange fluorescence over the midpiece. Sperm cells with intact plasma membrane integrity and low ΔΨm show green fluorescence over the head and midpiece (E). Dead spermatozoa with damaged plasma membrane reveal partial (F) or more significant (G–H) red fluorescence over the head and display less green (G) or no green (H) fluorescence over the midpiece. Scale bar: 10 µm.

(iii) spermatozoa that fluoresced red or red and green over the head (PI and SYBR-14 positive) but did not emit any fluorescence over the midpiece (Figures 1F–1H). A total of two hundred sperm cells were evaluated per sample under an epifluorescence microscope (BX 41; Olympus Optical Co., Tokyo, Japan) at ×400 magnification. A filter set (U-N51004 F/R V2; Ex/Em 475–492 and 545–565/503–533 and 582–622 nm) for simultaneous viewing of SYBR-14 (Ex/Em 490/516 nm), PI (Ex/Em 535/617 nm), and JC-1 monomers and aggregates (at the time of detection; Ex/Em 485/530 nm and 535/590 nm, respectively) was used.

2.3.3. Cytochemical analysis

The oxidoreductive capability of mitochondria was assessed by screening with cytochemical NADH-dependent nitro blue tetrazolium (NBT; Sigma-Aldrich) assay (related to NADH-dependent diaphorase test) using reduced form of nicotinamide adenine dinucleotide (NADH; Sigma-Aldrich) as a donor and NBT as an artificial acceptor of electrons. The cytochemical reaction was performed in the sperm suspension under dark conditions at 37 °C with 5% CO_2 for 60 min (incubator MCO-17AI; Sanyo, Japan), according to Piasecka et al. (2001) and Gączarzewicz et al. (2003). The intensity of the cytochemical reaction was evaluated by a computer image analyzing system (Quantimet 600S; Cambridge, UK) measuring the mean optical density (MOD) and the integrated optical density (IOD) of the reaction product formazans (reduced NBT) deposited within the sperm midpiece. The densitometric measurements were taken as arithmetic means of a hundred nonoverlapping and noncrossing sperm cells.

2.3.4. Hypo-osmotic swelling test

The integrity of the sperm membrane tail was evaluated using the hypo-osmotic swelling (HOS) test. A hypotonic solution containing fructose and sodium citrate was prepared with an osmolality of 150 mOsm/kg. Then 0.1 mL of prewarmed (37 °C) semen was mixed with 1 mL of the hypotonic solution and incubated at 37 °C for 30 min (Gączarzewicz et al., 2010). Smears were made from the incubated semen sample; a minimum of one hundred cells were assessed under phase-contrast microscope at ×1000 magnification for the tail coiling/swelling indicative of the hypo-osmotic reaction.

2.3.5. Aspartate aminotransferase activity

The measurement of aspartate aminotransferase (AAT; EC 2.6.1.1) activity in the extracellular environment was used to determine the changes in plasma membrane integrity in response to external stress factors (Ciereszko et al., 1994). Enzyme activity was assessed with the kinetic method at 37 °C in the extracellular fluid (obtained by centrifugation for 15 min at 10,000 × *g*) of stored semen using reagents manufactured by Alpha Diagnostics (Poland), according to the manufacturer's protocol. In the method used, AAT catalyzes the transamination of aspartic acid and 2-oxopentanedioic acid, forming glutamic acid and oxaloacetic acid. The oxaloacetic acid is reduced to malic acid by malate dehydrogenase, while NADH is simultaneously converted to nicotinamide adenine dinucleotide (NAD^+). The decrease in absorbance due to the consumption of NADH is measured at 340 nm (Epoll 20; Poll, Warsaw, Poland) and is proportional to the enzyme activity in the sample. The results were expressed as AAT activity per 10^9 spermatozoa (mU/10^9 spermatozoa). Sperm concentration was determined using a Bürker hemocytometer.

2.3.6. pH and osmolality

The pH of the extracellular fluid of the semen was measured using a pH meter (CP-315M; Elmetron, Poland. Electrode type ERH-13-6; Hydromet, Poland). Osmolality (mOsm/kg) was determined using a two-point calibrated (0 mOsm/kg and 400 mOsm/kg) freezing point depression osmometer (OS 3000; Marcel, Poland).

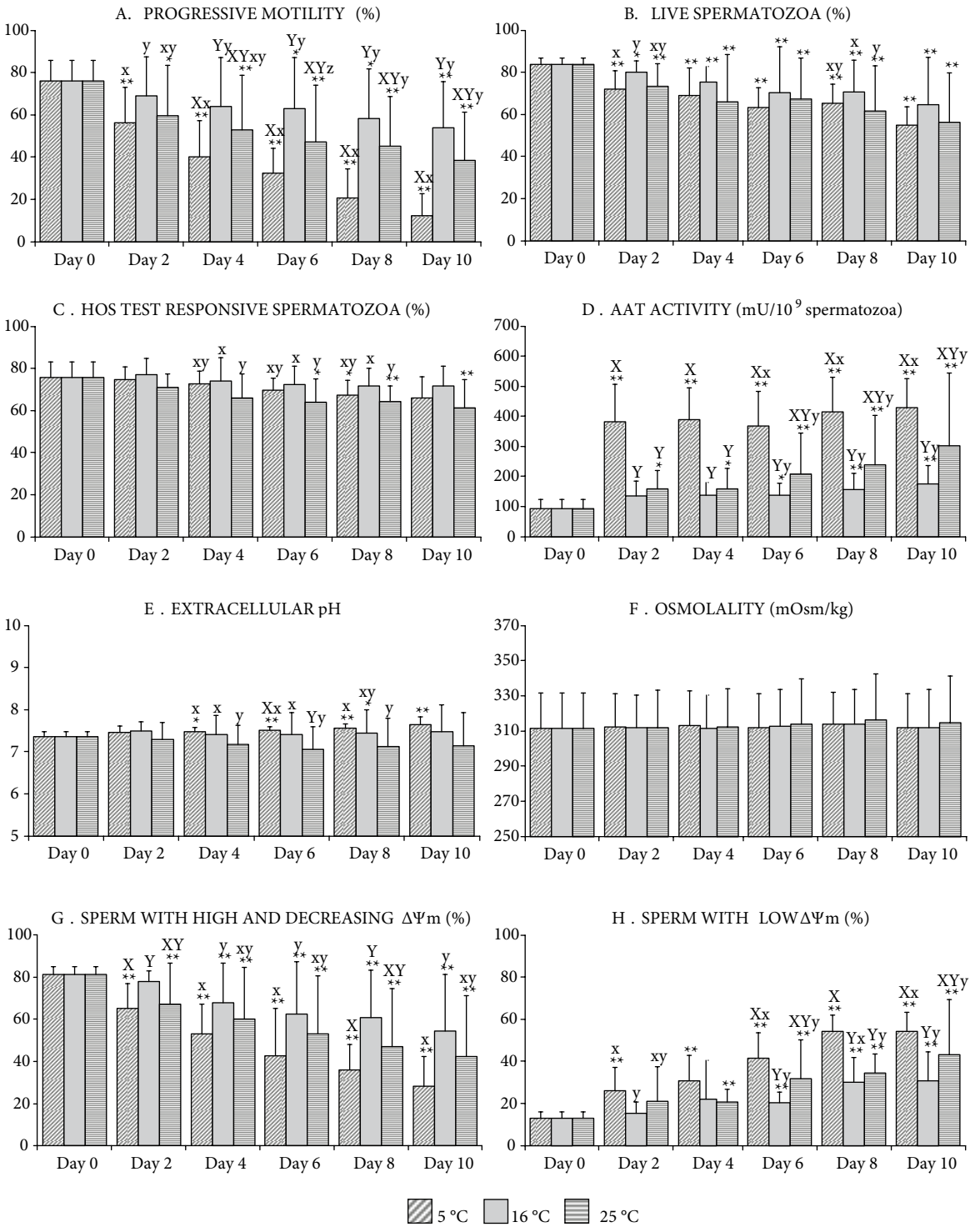
2.4. Statistical analysis

Statistical analyses were performed using the Statistica software package (version 10.0; StatSoft Incorporation, Tulsa OK, USA). The data were examined using the Shapiro–Wilk *W*-test for the possibility that they significantly deviated from a normal distribution. The Friedman ANOVA test (Friedman's nonparametric repeated measurements comparison) was applied to determine the influence of storage temperature and preservation time. Comparisons between storage temperatures and storage time points (to determine which values differed significantly from day 0) were performed using Wilcoxon's rank sum test. Differences were considered significant if the probability of their occurring by chance was less than 5% ($P < 0.05$). All results are expressed as means ± SD (standard deviation). Correlations between the measured sperm quality parameters were calculated by means of Spearman's rank correlation coefficient with $P < 0.05$ as the significance level.

3. Results

3.1. Motility and membrane status assessed by fluorescent staining and HOS test

The motility and membrane integrity parameters of boar spermatozoa stored at various temperatures are shown in Figures 2A–2C. There was an influence of both storage temperature and storage time on the movement of spermatozoa. Progressive motility was reduced with decreasing storage temperature. Significant differences ($P < 0.05$) were observed between semen storage at 5 °C and at 16 °C at each of the times evaluated. The initial motility (76.11 ± 9.61%) after ten days of storage at 5 °C, 16 °C, and 25 °C was significantly ($P < 0.01$) reduced (Figure 2A).



Figures 2A–H. Changes in the sperm-quality parameters in boar semen diluted with Vitasem LD extender (day 0) and subsequently stored for 10 days (days 2–10) at 5 °C, 16 °C, and 25 °C. Different letters indicate significant differences between the semen stored at various temperatures. Lowercase (xyz): $P < 0.05$; uppercase (XY): $P < 0.01$. Asterisks indicate significant differences between the day of collection (day 0) and each day of semen storage (days 2–10) for the applied temperature (*$P < 0.05$ and **$P < 0.01$). ΔΨm: mitochondrial transmembrane potential; HOS test: hypo-osmotic swelling test; AAT: aspartate aminotransferase. Bars represent means ± SD. Wilcoxon's rank-sum test was used for multiple comparisons.

Sperm viability was assessed by SYBR-14/PI staining and HOS test. The spermatozoa with intact membranes (SYBR-14 positive/PI negative or HOS-test positive) stored at 16 °C were present in higher numbers than at the other temperature groups at all time-points. The percentage of PI-unstained spermatozoa decreased ($P < 0.05$) from 83.84 ± 2.76% for samples on day 0 to 54.75 ± 8.79%, 64.43 ± 22.60% and 56.30 ± 23.44% for samples stored 10 days at 5 °C, 16 °C, and 25 °C, respectively. In semen stored at 16 °C, a higher percentage ($P < 0.05$) of spermatozoa survived than among sperm cells stored at 5 °C on day 2 and at 25 °C on day 8 (Figure 2B). In turn, the incidence of HOS-test–responsive spermatozoa on the day of collection was 75.56 ± 7.54%. The percentage of coiled/swollen-tailed spermatozoa significantly decreased only in the 25 °C group after the sixth day of semen storage, but on day 10 was still 61.22 ± 13.28%. Statistically significant differences ($P < 0.05$) were observed between the percentages of HOS-test–responsive cells stored at 25 °C and at 16 °C on days 4–8 (Figure 2C).

3.2. Changes in extracellular fluid determined by AAT activity, pH, and osmolality

The changes in AAT activity, pH, and osmolality are shown in Figures 2D–2F. The average AAT activity measured on the day of collection amounted to 93.4 ± 30.0 mU/10^9 spermatozoa. The storage of semen at 5 °C, 16 °C, and 25 °C caused an increase in AAT activity; storage at 5 °C resulted in the highest enzyme activity (382.4 ± 124.5 mU/10^9 spermatozoa; $P < 0.01$) as early as the second day of preservation. From then to day 10, this increased slightly to 429.5 ± 95.2 mU/10^9 spermatozoa. At all time-points tested, the values of the AAT activity for semen stored at 16 °C and 25 °C were significantly lower than those of semen stored at 5 °C. There were 176.2 ± 60.6 and 301.8 ± 242.3 mU/10^9 spermatozoa on day 10 at 16 °C and 25 °C, respectively. A significant increase was observed in AAT activity for semen samples stored at 25 °C on day 2 and at 16 °C on day 6 (Figure 2D).

The initial pH of 7.36 ± 0.12 increased significantly ($P < 0.05$) on day 4 and very significantly ($P < 0.01$) after the sixth day of semen storage at 5 °C (Figure 2E), reaching 7.64 ± 0.19 on the last day tested. Storage for 10 days did not result in pH changes in the 16 °C or 25 °C groups. However, the pH level of semen stored at 25 °C was lower than the pH level at 5 °C on days 4–8 and 16 °C on days 4 and 6. In turn, for all temperatures tested, storage to day 10 did not result in significant changes in osmolality, as compared to day 0 (Figure 2F). The osmolality of the samples on the day of collection was 311.6 ± 19.8 mOsm/kg; for the samples stored 10 days at 5 °C, 16 °C, and 25 °C, the osmolality was 311.7 ± 19.3, 311.8 ± 21.8, and 314.4 ± 26.8 mOsm/kg, respectively. Over the duration of storage, there were no differences ($P > 0.05$) among semen stored at 5 °C, 16 °C, or 25 °C.

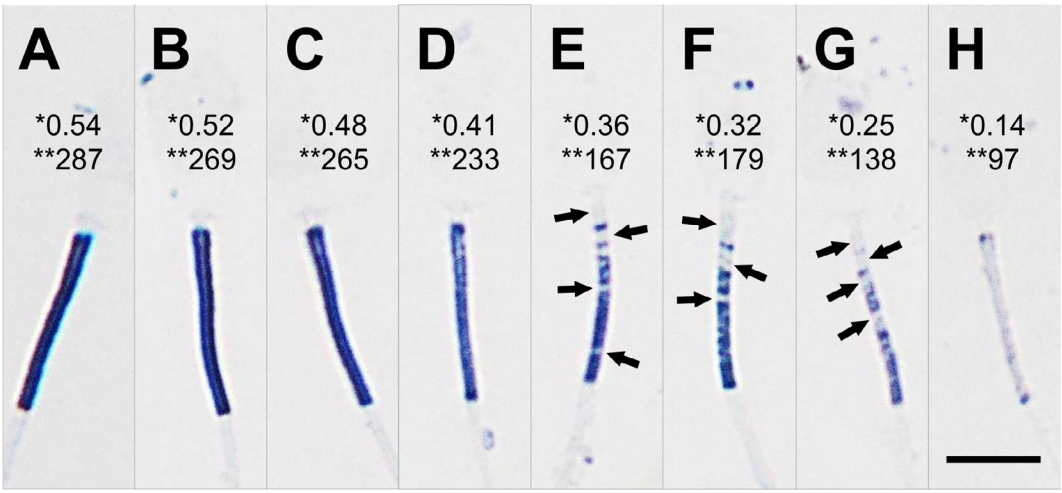
3.3. Mitochondrial activity determined by JC-1

Figures 2G and 2H show the results of JC-1 staining of the sperm. The storage temperature and storage time had a significant impact on mitochondrial membrane potential. The percentage of sperm cells with high and decreasing ΔΨm declined from 81.24 ± 3.68% on the day of collection to 28.19 ± 14.25%, 54.37 ± 26.78%, and 42.38 ± 28.77% on day 10 in semen stored at 5 °C, 16 °C, and 25 °C, respectively. This subpopulation of spermatozoa significantly ($P < 0.01$) decreased upon storage for 2 days at 5 °C and 25 °C, and considerable decreases ($P < 0.01$) were observed after 4 days in the samples stored at 16 °C. The proportion of spermatozoa with high and decreasing ΔΨm was higher ($P < 0.05$) over the duration of storage at 16 °C than for semen stored at 5 °C, with no differences found between the 16 °C and 25 °C groups or the 5 °C and 25 °C groups (Figure 2G). The observed reduction in the percentage of sperm cells with active mitochondria was manifested by changes in the proportion of spermatozoa with low ΔΨm for all storage temperatures. Storage at 5 °C, 16 °C, and 25 °C for 10 days resulted in an increase in the proportion of spermatozoa with low ΔΨm (41%, 18%, and 30%, respectively) (Figure 2H).

3.4. Mitochondrial oxidoreductive capability

The analysis of the NADH-dependent NBT assay revealed the presence of blue formazan deposits filling the active mitochondria in the sperm midpieces. Spermatozoa in which abundant formazan deposits (a product of the cytochemical reaction) filled the entire length of the midpiece (compact pattern of formazans; Figures 3A–3C) predominated, particularly in the early storage period. The sperm cells with less intense staining of the midpiece, as well as those showing an absence of formazan deposits in parts of the sperm midpiece (diffuse-focal formazan pattern; Figures 3D–3H), were also found. The incidence of these spermatozoa was higher when storage time was longer. Qualitative analysis of formazan deposits was performed by computer image analysis in which the signal intensities were expressed as the MOD and the IOD of the reaction product (Figure 4).

The average initial values (day 0) of the MOD and the IOD were 0.48 ± 0.09 and 240.0 ± 39.4, respectively (Figure 4). There was an influence of both storage temperature and storage time on the MOD and IOD. The MOD decreased ($P < 0.01$) over the duration of storage to 0.18 ± 0.07 at 5 °C, 0.24 ± 0.04 at 16 °C, and 0.22 ± 0.07 at 25 °C; the IOD decreased ($P < 0.01$) to 110.8 ± 22.4, 130.5 ± 11.4, and 121.0 ± 23.1, respectively. Compared with the day of semen collection, the MOD values significantly decreased ($P < 0.01$) after the fourth day of sperm storage at 5 °C and 25 °C and on days 4 and 10 when the semen samples were stored at 16 °C. In turn, the IOD values had decreased ($P < 0.01$) on day 2 for the spermatozoa stored at 5 °C and



Figures 3A–H. Representative micrographs of cytochemical NADH-dependent NBT test in boar spermatozoa diluted in Vitasem LD extender and stored for 10 days at 5 °C, 16 °C, and 25 °C. The figure shows the sperm midpieces from high (A) to low (H) mitochondrial oxidoreductive activity. A–C: compact pattern of formazan deposits showing intense reaction throughout the length of the midpieces with active mitochondria. D–H: diffuse-focal formazan pattern showing a decrease in the staining intensity, reflecting weakened (D) or extinguishing (H) cytochemical reaction throughout the length of midpiece; arrows (E–G) indicate the absence of formazan deposits (bright areas) in inactive mitochondria in various parts of the midpieces located among active mitochondria (blue areas). *MOD: mean optical density; **IOD: integrated optical density. Scale bar: 5 µm.

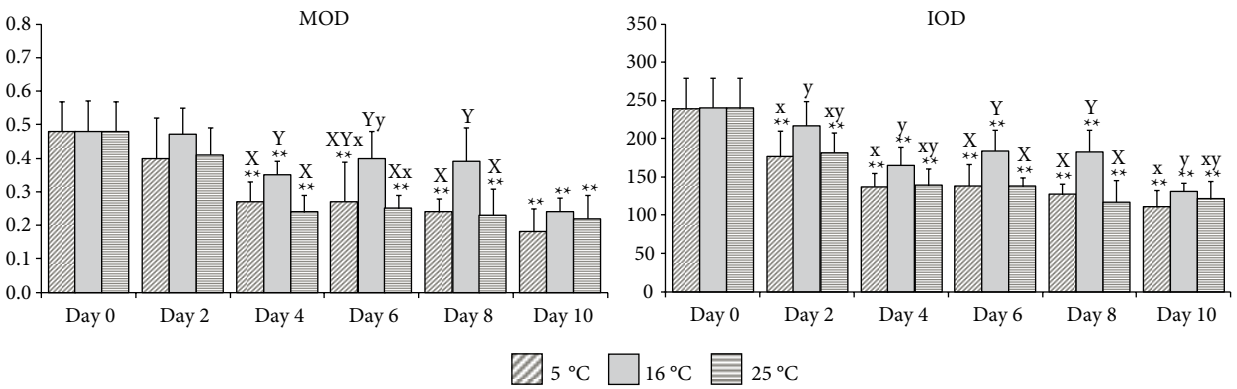


Figure 4. Changes in mean optical density (MOD) and integrated optical density (IOD) of formazans deposited in the midpiece of boar spermatozoa diluted with Vitasem LD extender (day 0) and subsequently stored for 10 days (days 2–10) at 5 °C, 16 °C, and 25 °C. Different letters indicate significant differences between semen stored at various temperatures. Lowercase (xy): $P < 0.05$; uppercase (XY): $P < 0.01$. Asterisks indicate significant differences between the day of collection (day 0) and each day of semen storage (days 2–10) for applied temperatures ($P < 0.01$). Bars represent means ± SD. Wilcoxon's rank-sum test was used for multiple comparisons.

25 °C and after the fourth day for all temperatures tested. The values of both densitometric parameters for the semen stored at 16 °C were higher than those obtained for the remaining tested temperatures. The MOD at 16 °C was significantly higher on days 4–8. The IOD values at 16 °C were significantly higher over the duration of storage than in semen stored at 5 °C, while a significant difference was found at 25 °C only on days 6 and 8 (Figure 4).

3.5. Correlation coefficient

These results indicate that the correlation coefficients are moderate (or high) and show significant correlations between most of the semen quality parameters compared (Table). PI-negative and HOS-positive sperm cells positively correlate ($P < 0.001$) with sperm motility (R_s = 0.78 and 0.50, respectively), high and decreasing $\Delta\Psi m$ (R_s = 0.90 and 0.49, respectively), and oxidoreductive

Table. Spearman rank correlation coefficient (R_s) between the sperm variables analyzed (n = 144).

		1.	2.	3.	4.	5.	6.	7.	8.	9.
1.	Progressive motility (%)									
2.	Live spermatozoa (%)	0.78**								
3.	Sperm with high and decreasing ΔΨm (%)	0.88**	0.90**							
4.	Sperm with low ΔΨm (%)	–0.77**	–0.78**	–0.87**						
5.	HOS test responsive spermatozoa (%)	0.50**	0.49**	0.49**	–0.43**					
6.	AAT activity (mU/10^9 spermatozoa)	–0.72**	–0.73**	–0.73**	0.65**	–0.46**				
7.	Extracellular pH	n.s.	n.s.	n.s.	0.18*	0.20*	0.17*			
8.	Osmolality (mOsm/kg)	–0.41**	–0.19*	–0.35**	0.21**	n.s.	n.s.	n.s.		
9.	MOD	0.52**	0.57**	0.58**	–0.52**	0.38**	–0.54**	n.s.	n.s.	
10.	IOD	0.61**	0.63**	0.67**	–0.60**	0.38**	–0.58**	n.s.	n.s.	0.92**

ΔΨm: mitochondrial transmembrane potential; HOS test: hypo-osmotic swelling test; AAT: aspartate aminotransferase; MOD, IOD: mean or integrated optical density deposited in the midpieces; *P < 0.05; **P < 0.01; n.s.: no significant relationship among variables.

mitochondrial capability (range of R_s: 0.38–0.63). Furthermore, AAT activity negatively correlates (P < 0.001) with sperm motility, PI-negative and HOS-responsive spermatozoa, and mitochondrial activity (range of R_s: –0.46–0.73). As shown in the Table, the parameters for the activity of sperm mitochondria correlated with motility and also with each other.

4. Discussion

The findings of this study show that boar sperm motility, mitochondrial activity, and plasma membrane integrity are impaired by storage for 10 days, depending on the investigated storage temperature. In general, fewer dysfunctional and morphological sperm disorders were observed in the semen stored at 16 °C, in particular, compared to the samples preserved at 5 °C. Relationships were also found between the examined parameters determining the status and functionality of the sperm cellular structures. These results confirm the previously reported negative effects of time on boar sperm during long-term preservation at temperatures above 15 °C (Medrano et al., 2005; Pérez-Llano et al., 2006; Guthrie et al., 2008; Fantinati et al., 2009; Gączarzewicz et al., 2010; Martín-Hidalgo et al., 2011; Dziekońska et al., 2013; Schulze et al., 2013) as well as the susceptibility of boar sperm to cold shock when cooled below 15 °C (Drobnis et al., 1993; Bailey and Buhr, 1995; Huo et al., 2002; Bailey et al., 2008; Kim et al., 2011) and then stored (Althouse et al., 1998; Paulenz et al., 2000; Zou and Yang, 2000; Petrunkina et al., 2005; Funahashi and Sano, 2005; Casas and Althouse, 2013; Namula et al., 2013; Schmid et al., 2013).

4.1. The influence of temperature and time of semen storage on the sperm motility

As expected, the storage temperature used in our study was the main factor to influence the storage tolerance of spermatozoa, controlled by evaluating motility. The progressive motility decreased at all treatment temperatures, but the semen stored at 5 °C showed the largest reduction rate over storage time. Sperm motility reflects cell membrane intactness and the lack of impediment to the metabolism and function of the sperm; thus, motility is one of the most important indicators used in AI centers. The minimum percentage of motile spermatozoa for AI dose has been established at 60%; under this threshold, lower farrowing rates can be experienced (Johnson et al., 2000; Vyt et al., 2004; Fantinati et al., 2009). In the present study, semen stored at 16 °C failed to meet the 60% threshold of spermatozoa with progressive motility after the sixth day, while in the semen preserved at 5 °C and 25 °C this happened on the second day. The particular extenders used in porcine AI may have different effects on motility and other features of sperm quality (Johnson et al., 2000; Fantinati et al., 2009; Martín-Hidalgo et al., 2013a; Dziekońska et al., 2013). A drop in motility below 60% could, therefore, occur at different points in time (Martin-Hidalgo et al., 2011; Dziekońska et al., 2013) and under various semen storage temperatures (Paulenz et al., 2000).

4.2. The influence of temperature and time of semen storage on the sperm plasma membrane integrity

The differences in motility noted for temperature and time of storage were not accompanied by differences in the parameters characterizing the plasma membrane assessed

by SYBR-14/PI-staining and the HOS test. Despite a gradual decrease over time, the proportion of sperm with intact plasma membranes remained at a relatively high level until the end of storage. On the tenth day, the percentage of PI-negative (live) and HOS-test–responsive spermatozoa was higher than the proportion of sperm motility (Figures 2A–2C). These results suggest that the plasma membrane of boar spermatozoa can maintain its integrity and functionality even under cold-shock temperatures. This is consistent with the observations of some authors who showed that hypothermic storage may have no drastic effect on the lipid composition or organization of the boar sperm cellular membranes (Schmid et al., 2013), although our study also indicates the multifarious nature of the cell membrane damage (morphological and/or biochemical) that occurs during cooling and storage of boar spermatozoa. As noted earlier, both SYBR-14/PI staining and HOS testing point to a relatively high level of integrity and function of the sperm membrane for 10 days at all temperatures. However, the measurement of AAT activity showed that the cell membranes of sperm stored at 5 °C suffer damage within the first 2 days of storage (as a result of chilling injuries) (Figure 2D). At this temperature, enzyme activity remains high until the end of the storage period; in the semen stored at 16 °C and 25 °C, an increase in enzyme activity occurs gradually (as a result of increasing aging changes). The massive leak of AAT triggered by cold shock could result from destabilization of the plasma membrane in a substantial proportion of spermatozoa, while its structural stability and integrity are maintained at the sperm head level (the membrane being resistant to PI) and/or through the maintenance of the structural continuity (stability/integrity) and membrane channel efficiency, which controls the influx and efflux of water (e.g., aquaporins) across the sperm tail membrane (in response to the hypotonic environment of the HOS test). The specificity of the cell membrane tests seem important both for detecting the intrinsic nature of that damage and determining the morphological structures of the spermatozoa in which these defects occur (e.g., head or tail of the spermatozoon). Furthermore, it is conceivable that the cell membrane in different areas of the sperm structures may respond differently to experimental conditions and differences in the analytical procedures of the membrane tests employed here. In boar spermatozoa, different domains of the sperm membrane may present variable responses to decreasing temperatures, but a general increase in fluidity occurs at temperatures of 5 °C (Casas and Althouse, 2013). Canvin and Buhr (1989) reported that pig-sperm head membranes are less fluid than the tail membranes and when undergoing cooling and reheating show differential temperature-sensitivity changes in fluidity.

Boar sperm membranes are the primary site of cold-induced damage (Bailey et al., 2008; Kim et al., 2011) and highly susceptible to temperature and storage effects (Waberski et al., 2011; Casas and Althouse, 2013). The membrane destabilization associated with cooling may stimulate capacitation-like calcium-dependent processes (Bailey and Buhr, 1995; Huo et al., 2002; Bailey et al., 2008; Costello et al., 2009; Waberski et al., 2011; Schmid et al., 2013; Gadella and Luna, 2014). Moreover, the disruption of cellular calcium homeostasis may be associated with the excessive formation of ROS and lipid peroxidation in sperm cells (Awda et al., 2009; Peña et al., 2009) affecting ion transport and enzyme activity (Großfeld et al., 2008; Kim et al., 2008; Awda et al., 2009; Kumaresan et al., 2009). Brouwers et al. (2005) indicate that the midpiece and tail of boar spermatozoa are more susceptible than the sperm head to lipid peroxidation, and that living sperm cells are intensely peroxidized after freeze-thawing. Based on our results, it is likely that cold-shock temperatures in the initial period of storage result in the greatest amount of membrane damage and, thus, leakage of cellular components essential to sperm function. Due to the presence of AAT in sperm cytoplasm, the increase in its extracellular activity is mainly related to damage of the tail plasma membrane (Ciereszko et al., 1994) where the movement apparatus of the spermatozoa is located. Moreover, the release of AAT in response to biophysical stress can be associated with an increase in sperm motility inhibitor factor (SMIF) activity and changes in sperm antigenic structure (Strzeżek, 2002). This may have contributed to the results of our study in which the sperm motility at 5 °C was the most impaired.

4.3. The influence of temperature and semen storage time on mitochondrial activity

Preservation of boar spermatozoa for prolonged periods requires a decrease in metabolic activity, which, in AI procedures, is carried out by lowering the temperature to 15–17 °C and diluting in an appropriate medium (Johnson et al., 2000; Rodriguez-Gil, 2006). Storage at 5 °C would theoretically allow a greater slowdown in sperm metabolism and, thus, preserve functionality for longer than storage at 15–17 °C. However, in AI practice boar semen is not stored at 5 °C due to the high susceptibility of sperm cells to damage caused by cooling to this temperature (Casas and Althouse, 2013). The impairment of mitochondria function by cooling to low (5 °C) and moderate (15–17 °C) temperatures and the subsequent storage has been described (Huo et al., 2002; Dziekońska et al., 2009; Fantinati et al., 2009; Kumaresan et al., 2009; Dziekońska and Strzeżek, 2011; Trzcińska et al., 2011). Our study also indicates the functional response of sperm mitochondria to temperature and period of storage. The proportion of spermatozoa with high and decreasing $\Delta\Psi m$ diminished at all temperatures over storage time;

however, preservation at 5 °C resulted in a higher decline in percentage of sperm with active mitochondria than storage at 16 °C. Concurrently, the weakness of mitochondrial capacity during storage at all temperatures was reflected by an increase in the proportion of spermatozoa showing low ΔΨm. Hence, the changes in ΔΨm observed during semen storage are due to alterations in the metabolic processes dependent on or regulated by sperm mitochondria. The changes in ΔΨm observed in the present study are in agreement with the results of other authors, who reported a decrease in the proportion of sperm cells with high ΔΨm (JC-1 staining) during storage of semen in Vitasem LD at 17 °C (Martín-Hidalgo et al., 2011; Dziekońska et al., 2013).

The present study has also shown that prolonged liquid storage of boar semen has a harmful and temperature-dependent effect on the oxidoreductive capability of sperm mitochondria, as evaluated by NADH-dependent NBT assay. Generally, better maintenance of sperm mitochondrial oxidoreductive capability was observed after storage at 16 °C than at other temperatures. NADH-dependent NBT assay reveals the mitochondrial ability to oxidize NADH. As a result of these events, the mitochondria form deposits of formazan in accordance with their oxidoreductive capabilities; thus, the amount of reduced NBT salts within the mitochondria corresponds to the intensity of cytochemical reaction (Piasecka et al., 2001; Gączarzewicz et al., 2003). We observed that the incidence of spermatozoa with diffuse-focal formazan patterns (Figure 3) was strengthened with prolonged storage time at all studied temperatures, but the increase in these patterns at 5 °C and 25 °C was higher. These observations were confirmed by densitometric measurements related to the oxidoreductive system of mitochondria (Figure 4). The measurement of MOD and IOD using computer image analysis allows qualitative assessment of the staining; high or low densitometric values reflect a normal or diminished ability to oxidize NADH, respectively, and is common to all mitochondria in one sperm midpiece. Previously it was reported that various factors may affect the mitochondrial oxidoreductive activity of human (Fraczek et al., 2012), rat (Piasecka et al., 2001), mouse (Golas et al., 2010), and boar (Gączarzewicz et al., 2003; Lydka et al., 2012) spermatozoa. The present study has established that storage temperature of boar semen may also affect the oxidoreductive system of sperm mitochondria. Furthermore, the results of the NADH-dependent NBT test correspond to those obtained by JC-1 staining, and the relationship between both techniques was demonstrated.

We observed that the differences in mitochondrial activity (especially as evaluated by JC-1 staining) between temperature and time of semen storage were consistent with the differences registered in motility. Because the metabolic activity of boar sperm is dependent on the storage temperature (Dziekońska et al., 2009; Hurtado de Llera et al., 2012; Martín-Hidalgo et al., 2013b), it seems likely that various causes could have led to the observed decrease in ΔΨm and the oxidoreductive capability of sperm mitochondria. These changes in semen stored at 5 °C are probably connected with the infringement of plasma membrane impermeability (see above), mainly resulting from the response of spermatozoa to chilling. In turn, exposure to 25 °C provoked premature aging in the stored sperm caused by insufficient inhibition of metabolic processes. This may explain the slight negative impact of stress associated with the loss of mitochondrial function caused by long-term storage at 16 °C.

4.4. The influence of temperature and time of semen storage on pH and osmolality

In present study, pH tended towards significant increase or slight decrease over time in semen stored at 5 °C or 25 °C, respectively. These changes contributed to significant differences in the pH observed at these temperatures. Similar to the current study, Paulenz et al. (2000) found a significant decrease in the pH of semen stored at 25 °C and 20 °C, a nonsignificant increase when stored at 15 °C, and a significant increase at 10 °C. It should be emphasized that the buffering capacity of various extenders can affect the quality of semen during long-term storage at the optimal holding temperature of approximately 16 °C (Johnson et al., 2000; Vyt et al., 2004; Fantinati et al., 2009). The extenders that have and maintain the lower pH are better at preserving sperm quality (Vyt et al., 2004; Fantinati et al., 2009). The storage of boar sperm with the increasing pH of extenders resulted in a decrease in motility, probably through the pH sensitivity enzyme systems responsible for premature activation of motility (Vyt et al., 2004; Fantinati et al., 2009). Additionally, this event may be enhanced by contact with high amounts of the bicarbonate buffering systems present in most extenders of porcine semen (Vyt et al., 2007). In the present study, the drop in sperm motility was also accompanied by elevated mean values of pH in semen stored at 5 °C. At this temperature, the metabolic processes of sperm cells should, theoretically, be most inhibited (Johnson et al., 2000; Paulenz et al., 2000); however, the buffering capacity of the extender may have also been limited, thus contributing to a greater loss of spermatozoa quality. In turn, the pH tended to decrease in semen preserved at 25 °C, which may indicate greater metabolic activity, because the tendency of the extracellular environment to acidify reflects the production of lactic acid by stored spermatozoa (Paulenz et al., 2000). On the other hand, the changes in mean pH value observed at all temperatures were small and within the normal physiological range (6.8–7.9) of boar semen (Paulenz et al., 2000). As with the control pH, the

present study has shown that the use of an extender can provide adequate regulation of osmotic pressure. Neither temperature-dependent nor time-dependent changes in osmolality were found, and the extender caused slightly hypertonic conditions that boar spermatozoa tolerate well (Johnson et al., 2000).

4.5. Concluding remarks

Our findings indicate that the quality of liquid stored boar semen is closely related to storage temperature and length. Extending the storage time at any of the tested temperatures may be connected with the loss of sperm mitochondrial function and plasma membrane integrity. During long-term liquid preservation of boar semen, the disruption of mitochondrial transmembrane potential and oxidoreductive capability are likely important contributors to the reduction in sperm motility and functional integrity. Cooling semen to 5 °C escalates the permeability of the boar sperm plasma membrane. It seems that the changes in membrane integrity may not always be revealed by SYBR-14/PI staining or the HOS test.

References

Aitken RJ (2006). Sperm function tests and fertility. Int J Androl 29: 69–75.

Althouse GC, Wilson ME, Kuster C, Parsley M (1998). Characterization of lower temperature storage limitations of fresh-extended porcine semen. Theriogenology 50: 535–543.

Amaral A, Lourenço B, Marques M, Ramalho-Santos J (2013). Mitochondria functionality and sperm quality. Reproduction 146: R163–R174.

Awda BJ, Mackenzie-Bell M, Buhr MM (2009). Reactive oxygen species and boar sperm function. Biol Reprod 81: 553–561.

Bailey JL, Buhr MM (1995). Regulation of internal Ca^{2+} by chilled bull and boar spermatozoa. Cryobiology 32: 259–269.

Bailey JL, Lessard C, Jacques J, Breque C, Dobrinski I, Zeng W, Galantino-Homer HL (2008). Cryopreservation of boar semen and its future importance to the industry. Theriogenology 70: 1251–1259.

Brouwers JF, Silva PFN, Gadella BM (2005). New assays for detection and localization of endogenous lipid peroxidation products in living boar sperm after BTS dilution or after freeze-thawing. Theriogenology 63: 458–469.

Canvin AT, Buhr MM (1989). Effect of temperature on the fluidity of boar sperm membranes. J Reprod Fertil 85: 553–540.

Casas I, Althouse GC (2013). The protective effect of a 17 °C holding time on boar sperm plasma membrane fluidity after exposure to 5 °C. Cryobiology 66: 69–75.

Ciereszko A, Glogowski J, Demianowicz W, Strzeżek J (1994). Stimulation of aspartate aminotransferase from farm animal semen by pyridoxal 5'-phosphate. Anim Reprod Sci 34: 327–341.

Costello S, Michelangeli F, Nash K, Lefievre L, Morris J, Machado-Oliveira G, Barratt C, Kirkman-Brown J, Publicover S (2009). Ca^{2+}-stores in sperm: their identities and functions. Reproduction 138: 425–437.

Drobnis EZ, Crowe LM, Berger T, Anchordoguy TJ, Overstreet JW, Crowe JH (1993). Cold shock damage is due to lipid phase transitions in cell membranes: a demonstration using sperm as a model. J Exp Zool 265: 432–437.

Dziekońska A, Fraser L, Majewska A, Lecewicz M, Zasiadczyk Ł, Kordan W (2013). Effect of commercial long-term extenders on metabolic activity and membrane integrity of boar spermatozoa stored at 17 °C. Pol J Vet Sci 16: 517–525.

Dziekońska A, Fraser L, Strzeżek J (2009). Effect of different storage temperatures on the metabolic activity of spermatozoa following liquid storage of boar semen. J Anim Feed Sci 18: 638–649.

Dziekońska A, Strzeżek J (2011). Boar variability affects sperm metabolism activity in liquid stored semen at 5 °C. Pol J Vet Sci 14: 21–27.

Fantinati P, Zannoni A, Bernardini C, Forni M, Tattini A, Seren E, Bacci ML (2009). Evaluation of swine fertilisation medium (SFM) efficiency in preserving spermatozoa quality during long-term storage in comparison to four commercial swine extenders. Animal 3: 269–274.

Flores E, Fernández-Novell JM, Peña A, Rodríguez-Gil JE (2009). The degree of resistance to freezing-thawing is related to specific changes in the structures of motile sperm subpopulations and mitochondrial activity in boar spermatozoa. Theriogenology 72: 784–797.

Fraczek M, Piasecka M, Gaczarzewicz D, Szumala-Kakol A, Kazienko A, Lenart S, Laszczynska M, Kurpisz M (2012). Membrane stability and mitochondrial activity of human ejaculated spermatozoa during in vitro experimental infection with *Escherichia coli*, *Staphylococcus haemolyticus* and *Bacteroides ureolyticus*. Andrologia 44: 315–329.

Funahashi H, Sano T (2005). Select antioxidants improve the function of extended boar semen stored at 10 °C. Theriogenology 63: 1605–1616.

Gączarzewicz D, Piasecka M, Udała J, Błaszczyk B, Laszczyńska M, Kram A (2003). Oxidoreductive capability of boar sperm mitochondria in fresh semen and during their preservation in BTS extender. Reprod Biol 3: 161–172.

Gączarzewicz D, Piasecka M, Udała J, Błaszczyk B, Stankiewicz T, Laszczyńska M (2010). Plasma membrane changes during the liquid storage of boar spermatozoa: a comparison of methods. Acta Vet Hung 58: 105–116.

Gadella BM, Luna C (2014). Cell biology and functional dynamics of the mammalian sperm surface. Theriogenology 81: 74–84.

Garcia JC, Dominguez JC, Pena FJ, Alegre B, Gonzalez R, Castro MJ, Habing GG, Kirkwood RN (2010). Thawing boar semen in the presence of seminal plasma: effects on sperm quality and fertility. Anim Reprod Sci 119: 160–165.

Golas A, Malek P, Piasecka M, Styrna J (2010). Sperm mitochondria diaphorase activity: a gene mapping study of recombinant inbred strains of mice. Int J Dev Biol 54: 667–673.

Großfeld R, Sieg B, Struckmann C, Frenzel A, Maxwell WMC, Rath D (2008). New aspects of boar semen freezing strategies. Theriogenology 70: 1225–1233.

Guthrie HD, Welch GR (2012). Effects of reactive oxygen species on sperm function. Theriogenology 78: 1700–1708.

Guthrie HD, Welch GR, Long JA (2008). Mitochondrial function and reactive oxygen species action in relation to boar motility. Theriogenology 70: 1209–1215.

Huo LJ, Yue KZ, Yang ZM (2002). Characterization of viability, mitochondrial activity, acrosomal integrity and capacitation status in boar sperm during in vitro storage at different ambient temperatures. Reprod Fert Develop 14: 509–514.

Hurtado de Llera A, Martin-Hidalgo D, Gil MC, Garcia-Marin LJ, Bragado MJ (2012). AMP-activated kinase AMPK is expressed in boar spermatozoa and regulates motility. PLoS ONE 7: e38840.

Johnson LA, Weitze KF, Fiser P, Maxwell WMC (2000). Storage of boar semen. Anim Reprod Sci 62: 143–172.

Kim JC, Li Y, Lee S, Yi YJ, Park CS, Woo SH (2008). Effects of cryopreservation on Ca^{2+} signals induced by membrane depolarization, caffeine, thapsigargin and progesterone in boar spermatozoa. Mol Cells 26: 558–565.

Kim S, Lee YJ, Kim YJ (2011). Changes in sperm membrane and ROS following cryopreservation of liquid boar semen stored at 15 °C. Anim Reprod Sci 124: 118–124.

Koppers AJ, De Iuliis GN, Finnie JM, McLaughlin EA, Aitken RJ (2008). Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. J Clin Endocr Metab 93: 3199–3207.

Kumaresan A, Kadirvel G, Bujarbaruah KM, Bardoloi RK, Das A, Kumar S, Naskar S (2009). Preservation of boar semen at 18 °C induces lipid peroxidation and apoptosis like changes in spermatozoa. Anim Reprod Sci 110: 162–171.

López Rodrigeuz A, Rijsselaere T, Vyt P, Van Soom A, Maes D (2012). Effect of dilution temperature on boar semen quality. Reprod Domest Anim 66: e63–e66.

Lydka M, Piasecka M, Gaczarzewicz D, Koziorowski M, Bilinska B (2012). Administration of flutamide alters sperm ultrastructure, sperm plasma membrane integrity and its stability, and sperm mitochondrial oxidative capability in the boar: in vivo and in vitro approach. Reprod Domest Anim 47: 635–643.

Martín-Hidalgo D, Barón FJ, Bragado MJ, Carmona P, Robina A, García-Marín LJ, Gil MC (2011). The effect of melatonin on the quality of extended boar semen after long-term storage at 17 °C. Theriogenology 75: 1550–1560.

Martín-Hidalgo D, Barón FJ, Robina A, Bragado MJ, Hurtado de Llera A, García-Marín LJ, Gil MC (2013a). Inter- and intra-breed comparative study of sperm motility and viability in Iberian and Duroc boar semen during long-term storage in MR-A and XCell extenders. Anim Reprod Sci 139: 109–114.

Martín-Hidalgo D, Hurtado de Llera A, Yeste M, Gil MC, Bragado MJ, García-Marín LJ (2013b). Adenosine monophosphate-activated kinase, AMPK, is involved in the maintenance of the quality of extended boar semen during long-term storage. Theriogenology 80: 285–294.

Medrano A, Peña A, Rigau T, Rodríguez-Gil JE (2005). Variations in the proportion of glycolytic/non-glycolytic energy substrates modulate sperm membrane integrity and function in diluted boar samples stored at 15–17 °C. Reprod Domest Anim 40: 448–453.

Namula Z, Sato Y, Kodama R, Morinaga K, Luu VV, Taniguchi M, Nakai M, Tanihara F, Kikuchi K, Nagai T, Otoi T (2013). Motility and fertility of boar semen after liquid preservation at 5 °C for more than 2 weeks. Anim Sci J 84: 600–606.

Paulenz H, Kommisrud E, Hofmo PO (2000). Effect of long-term storage at different temperatures on the quality of liquid boar semen. Reprod Domest Anim 35: 83–87.

Peña FJ, Rodríguez Martínez H, Tapia JA, Ortega Ferrusola C, González Fernández L, Macías García B (2009). Mitochondria in mammalian sperm physiology and pathology: a review. Reprod Domest Anim 44: 345–349.

Pérez-Llano B, Enciso M, García-Casado P, Sala R, Gosálvez J (2006). Sperm DNA fragmentation in boars is delayed or abolished by using sperm extenders. Theriogenology 66: 2137–2143.

Petrunkina AM, Volker G, Weitze KF, Beyerbach M, Töpfer-Petersen E, Waberski D (2005). Detection of cooling-induced membrane changes in the response of boar sperm to capacitating conditions. Theriogenology 63: 2278–2299.

Piasecka M, Wenda-Różewicka L, Ogoński T (2001). Computerized analysis of cytochemical reactions for dehydrogenases and oxygraphic studies as methods to evaluate the function of the mitochondrial sheath in rat spermatozoa. Andrologia 33: 1–12.

Piomboni P, Focarelli R, Stendardi A, Ferramosca A, Zara V (2012). The role of mitochondria in energy production for human sperm motility. Int J Androl 35: 109–124.

Purdy PH, Tharp N, Stewart T, Spiller SF, Blackburn HD (2010). Implications of the pH and temperature of diluted, cooled boar semen on fresh and frozen-thawed sperm motility characteristics. Theriogenology 74: 1304–1310.

Ramió-Lluch L, Fernández-Novell JM, Peña A, Bucci D, Rigau T, Rodríguez-Gil JE (2012). "In vitro" capacitation and subsequent acrosome reaction are related to changes in the expression and location of midpiece actin and mitofusin-2 in boar spermatozoa. Theriogenology 77: 979–988.

Ramió-Lluch L, Fernández-Novell JM, Peña A, Colás C, Cebrián-Pérez JA, Muiño-Blanco T, Ramírez A, Concha II, Rigau T, Rodríguez-Gil JE (2011). 'In vitro' capacitation and acrosome reaction are concomitant with specific changes in mitochondrial activity in boar sperm: evidence for a nucleated mitochondrial activation and for the existence of a capacitation-sensitive subpopulational structure. Reprod Domest Anim 46: 664–673.

Rodriguez-Gil JE (2006). Mammalian sperm energy resources management and survival during conservation in refrigeration. Reprod Domest Anim 41: 11–20.

Schmid S, Henning H, Oldenhof H, Wolkers WF, Petrunkina AM, Waberski D (2013). The specific response to capacitating stimuli is a sensitive indicator of chilling injury in hypothermically stored boar spermatozoa. Andrology 1: 376–386.

Schulze M, Henning H, Rüdiger K, Wallner U, Waberski D (2013). Temperature management during semen processing: Impact on boar sperm quality under laboratory and field conditions. Theriogenology 80: 990–998.

Storey BT (2008). Mammalian sperm metabolism: oxygen and sugar, friend and foe. Int J Dev Biol 52: 427–437.

Strzeżek J (2002). Secretory activity of boar seminal vesicle glands. Reprod Biol 2: 243–266.

Trzcińska M, Bryła M, Smorąg Z (2011). Apoptotic-like changes in the spermatozoa of fresh and stored boar semen and the quality of embryos produced in vivo. Anim Reprod Sci 124: 90–97.

Vadzyuk O, Kosterin S (2015). Diazoxide attenuates ROS generation and exerts cytoprotection under conditions of ROS overproduction in rat uterus cells. Turk J Biol 39: doi: 10.3906/biy-1410-75.

Vyt P, Maes D, Dejonckheere E, Castryck F, Van Soom A (2004). Comparative study on five different commercial extenders for boar semen. Reprod Domest Anim 39: 8–12.

Vyt P, Maes D, Sys SU, Rijsselaere T, Van Soom A (2007). Air contact influences the pH of extended porcine semen. Reprod Domest Anim 42: 218–220.

Waberski D, Henning H, Petrunkina AM (2011). Assessment of storage effects in liquid preserved boar semen. Reprod Domest Anim 46: 45–48.

Zou CX, Yang ZM (2000). Evaluation on sperm quality of freshly ejaculated boar semen during in vitro storage under different temperatures. Theriogenology 53: 1477–1488.