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Original article

Effect of commercial long-term extenders on metabolic activity and membrane integrity of boar spermatozoa stored at 17°C

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Abstract

This study was aimed to analyze the metabolic activity and membrane integrity of boar spermatozoa following storage in long-term semen extenders. Boar semen was diluted with Androhep® EnduraGuard™ (AeG), DILU-Cell (DC), SafeCell Plus™ (SCP) and Vitasem LD (VLD) extenders and stored for 10 days at 17°C. Parameters of the analyzed sperm metabolic activity included total motility (TMOT), progressive motility (PMOT), high mitochondrial membrane potential (MMP) and ATP content, whereas those of the membrane integrity included plasma membrane integrity (PMI) and normal apical ridge (NAR) acrosome. Extender type was a significant ($P < 0.05$) source of variation in all the analyzed sperm parameters, except for ATP content. Furthermore, the storage time had a significant effect ($P < 0.05$) on the sperm metabolic activity and membrane integrity during semen storage. In all extenders the metabolic activity and membrane integrity of the stored spermatozoa decreased continuously over time. Among the four analyzed extenders, AeG and SCP showed the best performance in terms of TMOT and PMI on Days 5, 7 and 10 of storage. Marked differences in the proportions of spermatozoa with high MMP were observed between the extenders, particularly on Day 10 of storage. There were not any marked differences in sperm ATP content between the extenders, regardless of the storage time. Furthermore, the percentage of spermatozoa with NAR acrosomes decreased during prolonged storage, being markedly lower in DC-diluted semen compared with semen diluted with either AeG or SCP extender. The results of this study indicated that components of the long-term extenders have different effects on the sperm functionality and prolonged semen longevity by delaying the processes associated with sperm ageing during liquid storage.

Key words: boar, spermatozoa, extender, storage time, metabolic activity

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Introduction

In pig production artificial insemination (AI) is widely performed with extended boar semen stored at 15-17°C (Kuster and Althouse 1999, Gerrits et al. 2005, Fantinati et al. 2009). Laboratory assays are commonly used to assess the quality of liquid-stored or frozen-thawed semen prior to AI. Moreover, multiple attributes of spermatozoa are assessed simultaneously to provide adequate information on the sperm fertilizing ability (Rodríguez-Martínez 2003, Waberski et al. 2011).

Sperm motility, assessed subjectively or objectively with the computer-assisted semen analysis (CASA), is one of the most important characteristics commonly used to assess the quality of liquid-stored semen (Johnson et al. 2000, Huo et al. 2002, Vyt et al. 2004, De Ambrogi et al. 2006, Gączarzewicz et al. 2010). Even though motility is related to the sperm metabolic performance, it does not give enough information about the fertilizing ability of spermatozoa following liquid semen storage (Kuster and Althouse 1999, Kommisrud et al. 2002, Fraser et al. 2003, Dziekońska et al. 2009). Besides sperm motility assessments, the sperm mitochondrial function can be monitored with a cationic fluorescent probe, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1), which accumulates in the mitochondria, depending on the mitochondrial membrane potential (MMP), and the measurements of adenosine triphosphate (ATP) content (Thomas et al. 1998, Dziekońska et al. 2009, Gogol et al. 2009). Both MMP and ATP production are indispensable for flagellar beat and motility of spermatozoa (Guthrie et al. 2008, Storey 2008, Waberski et al. 2011), and have been used to assess the sperm metabolic activity in liquid-stored boar semen (Huo et al. 2002, Fraser et al. 2003, Trzcińska et al. 2008, Dziekońska et al. 2009, Dziekońska and Strzeżek 2011). Furthermore, the plasma membrane integrity (PMI) and the acrosome integrity are essential for cell viability, and their maintenance during prolonged liquid semen storage is crucial for the events leading to fusion with the oocyte (Flesch and Gadella 2000).

There has been increasing interest in the modification of the composition of long-term extenders compared with short-term extenders (Gadea 2003, Fantinati et al. 2009). Moreover, a long-term boar semen extender is generally preferred to ensure semen longevity and thereby reduces the need for frequent semen delivery (Anil et al. 2004, Vyt et al. 2004); however, prolonged semen storage reduces sperm viability and subsequently compromises the sperm fertilizing ability (Johnson et al. 2000, Waberski et al. 2011). Hence, the choice of long-term extenders is very important to maintain optimal storage conditions of extended boar semen (Vyt et al. 2004). This study investigated the effect of long-term semen extenders on the metabolic activity and membrane integrity of boar spermatozoa stored at 17°C. Assessments of the sperm metabolic activity included total motility (TMOT), progressive motility (PMOT), high MMP and measurements of ATP content, whereas sperm membrane integrity included the PMI and normal apical ridge (NAR) acrosome.

Materials and Methods

Ejaculate collections

Ejaculates were collected from three Polish Large White boars (aged 1.5 to 2.5 years) over a 2-month period, beginning from November through December. The boars were kept under standard hygienic conditions and fed a commercial porcine ration. The gloved-hand technique was used to collect ejaculates from the boars, giving a total number of 15 ejaculates, 5 ejaculates from each boar. We used only semen samples with more than 70% sperm TMOT and less than 15% morphologically abnormal spermatozoa. Sperm concentration was calculated with a hemocytometer. Permission to conduct this study was granted by the Local Ethics Committee.

Liquid semen processing

The ejaculates were split into four parts and diluted with the following extenders: Androhep® EnduraGuard™, AeG (Minitüb, Tiefenbach, Germany), DILU-Cell, DC (Alfred Hein-K&WB, Germany), SafeCell Plus™, SCP (IMV Technologies, France) and Vitasem LD, VLD (Megapor SL, Spain). All extenders were prepared according to the manufactures' instructions. The diluted semen samples (30×10^6 spermatozoa/ml) were dispersed into 100-ml plastic bottles and equilibrated for 2 h at room temperature (Day 0) prior to storage at 17°C (Thermobox, Minitub Gmbh, Tiefenbach, Germany). Sperm metabolic activity and membrane integrity were analyzed in the diluted semen after 2 h (Day 0), 72 h (Day 3), 120 h (Day 5), 198 h (Day 7) and 240 h (Day 10) of storage.

Assessment of sperm metabolic activity

Motility

Sperm TMOT and PMOT (%) were evaluated using the CASA system (VideoTesT Sperm 2.1, Russia). Aliquots of sperm samples were placed in a Makler

Chamber and examined at 37ºC under a phase-contrast microscopy system coupled to a video camera adapted to the VideoTest Sperm system. Sperm motility parameters were analyzed in accordance with the recommendations given by the World Health Organization (1999).

Mitochondrial membrane potential (MMP)

Sperm MMP (%) was assessed in semen samples using the dual fluorescent probes, JC-1 (Molecular Probes, Eugene, USA) with propidium iodide (PI), according to a previously described method (Thomas et al. 1998), with some modifications (Dziekońska et al. 2009). Sperm cells displaying orange-red fluorescence at the mid-piece region were considered viable spermatozoa with high MMP (active mitochondria), while those exhibiting green fluorescence were considered as nonviable spermatozoa with low MMP. A minimum of 100 spermatozoa were counted per slide, using epifluorescence microscopy (Olympus CH 30 RF-200, Tokyo, Japan) at 600× magnification. The epifluorescence microscopy was equipped with a blue excitation filter (DMB) for JC-1 and a green excitation filter (DMG) for PI.

ATP content

Sperm ATP content was measured in semen samples using a Bioluminescence Assay Kit CLSII protocol (Roche Molecular Biochemical Company). A Junior bioluminometer (Berthold Technologies, GmbH & Co. KG, Germany) was used to measure ATP content, which was calculated from a standard ATP curve and expressed as nmol/10^8 spermatozoa.

Sperm membrane integrity assessments

Plasma membrane integrity (PMI)

Sperm PMI (%) was assessed with the dual fluorescent probes, SYBR-14 and PI (Live/Dead Sperm Viability Kit, Molecular Probes, Eugenie, OR. USA), according to a previously described method (Garner and Johnson 1995). Sperm cells exhibiting green fluorescence were classified as viable spermatozoa with intact membrane, while those exhibiting red fluorescence were classified as membrane-damaged spermatozoa. A minimum of 100 spermatozoa were counted per slide, using epifluorescence microscopy (Olympus CH 30 RF-200, Tokyo, Japan) with blue excitation filter (DMB) for SYBR-14, at 600\ magnification.

Normal apical ridge (NAR) acrosome

The percentage of spermatozoa with 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 under a bright field microscope at 1000 × magnification and were classified as spermatozoa with NAR acrosome or damaged apical ridge acrosome.

Statistical analysis

All results are expressed as the mean ± standard error of the mean (SEM). The data were analyzed by statistical analysis of variance (ANOVA) followed by the Duncan multiple comparison test ($P < 0.05$). A two-way ANOVA was used to assess the effect of extender type (AeG, DC, SCP and VLD), storage time (Days 0, 3, 5, 7 and 10) and their interaction on the sperm metabolic activity and membrane integrity. Statistical analyses were performed using the Statistica software package (StatSoft Incorporation, Tulsa, OK, USA).

Results

There were no significant differences in fresh semen quality between the three boars used in this study. In the fresh semen, the average percentage (mean ± SEM) of sperm TMOT was 81.8 ± 1.8 (range, 68.2 to 90.5), whereas that for sperm PMOT was 41.1 ± 4.2 (range, 21.4 to 70.6). The percentage of spermatozoa with high MMP ranged from 88.0 to 92.0, with a mean of 89.7 ± 0.3, whereas ATP content (nmol/10^8 spermatozoa) ranged from 6.7 to 14.3, with a mean of 12.2 ± 1.6. Sperm PMI averaged 90.2 ± 0.4% (range, 89.0% to 95.0%), whereas with NAR acrosome the mean was 90.2 ± 0.4% (range, 87.0% to 96.0%).

Sperm metabolic activity

The ANOVA results showed that extender type (AeG, DC, SCP and VLD) significantly affected ($P < 0.001$) sperm TMOT, PMOT and high MMP during liquid semen storage. Also, the storage time (Days 0, 3, 5, 7 and 10) had a marked effect on sperm TMOT ($P < 0.001$), PMOT ($P < 0.027$), high MMP ($P < 0.001$) and ATP content ($P < 0.001$). Only sperm TMOT ($P < 0.003$) and PMOT ($P < 0.004$) were significantly affected by the extender type × storage time interaction.

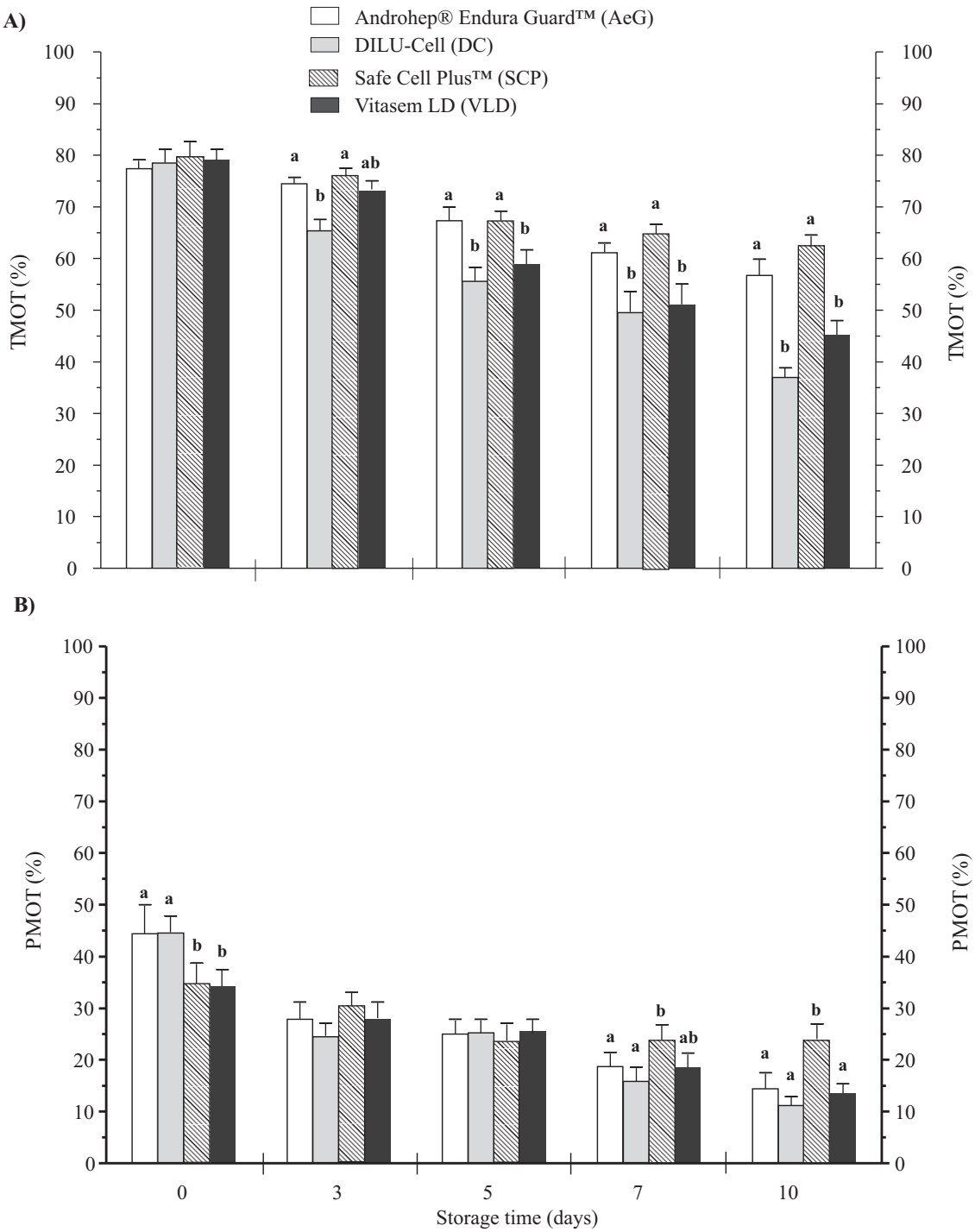

Fig. 1. Total motility, TMOT (A) and progressive motility, PMOT (B) of boar spermatozoa following liquid semen storage in different long-term extenders at 17°C. Values represent the means (± SEM) of 15 ejaculates. Values with different letters (a-b), with the same storage time, are significantly different ($P < 0.05$).

There were not any marked significant differences ($P > 0.05$) in sperm TMOT between the extenders on Day 0 of storage (Fig. 1A). Sperm TMOT decreased continuously over time, being significantly higher ($P < 0.05$) in semen diluted with AeG and SCP, particularly on Days 5, 7 and 10 of storage (Fig. 1A). It was found that sperm PMOT was higher ($P < 0.05$) in semen diluted with AeG and DC extenders on Day 0 of storage, however, SCP-diluted semen exhibited significantly higher ($P < 0.05$) percentage of spermatozoa with PMOT on Days 7 and 10 of storage (Fig. 1B). Higher proportions of spermatozoa with high MMP were observed in AeG-diluted semen on Day 5 of storage compared with semen diluted with either

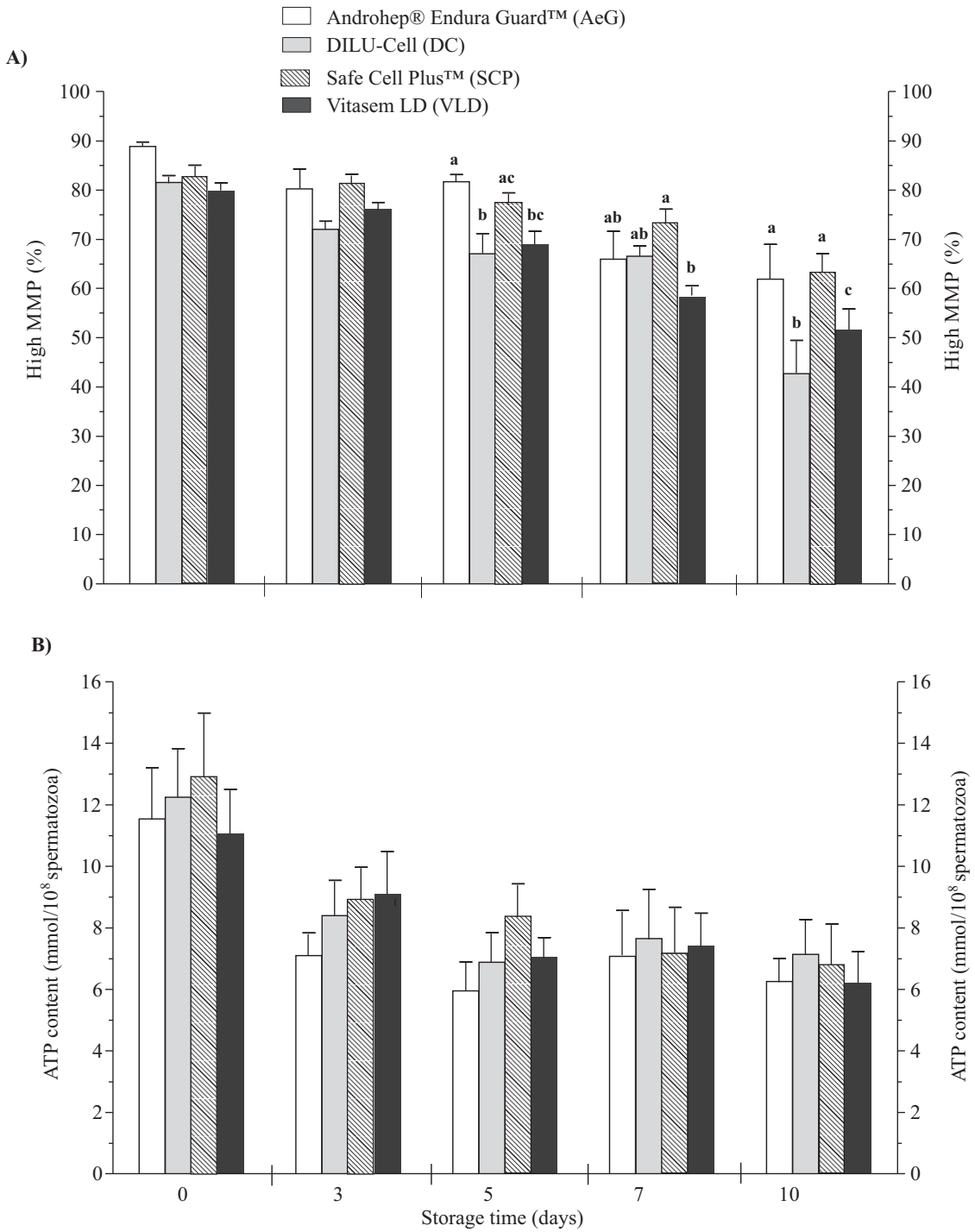

Fig. 2. High mitochondrial membrane potential, MMP (A) and ATP content (B) of boar spermatozoa following liquid semen storage in different long-term extenders at 17°C. Values represent the means (± SEM) of 15 ejaculates. Values with different letters (a-c), with the same storage time, are significantly different ($P < 0.05$).

DC or VLD extender (Fig. 2A). Furthermore, differences in sperm MMP between the extenders were more pronounced on Day 10 of storage (Fig. 2A). Sperm ATP content decreased continuously over time, but there were not any marked differences ($P > 0.05$) between the extenders within the storage period throughout this study (Fig. 2B).

Sperm membrane integrity

It was found that extender type affected sperm PMI ($P < 0.001$) and NAR acrosome ($P < 0.065$) during semen storage. Similarly, the storage time was a significant source ($P < 0.001$) of variation for the analyzed sperm membrane parameters. There were

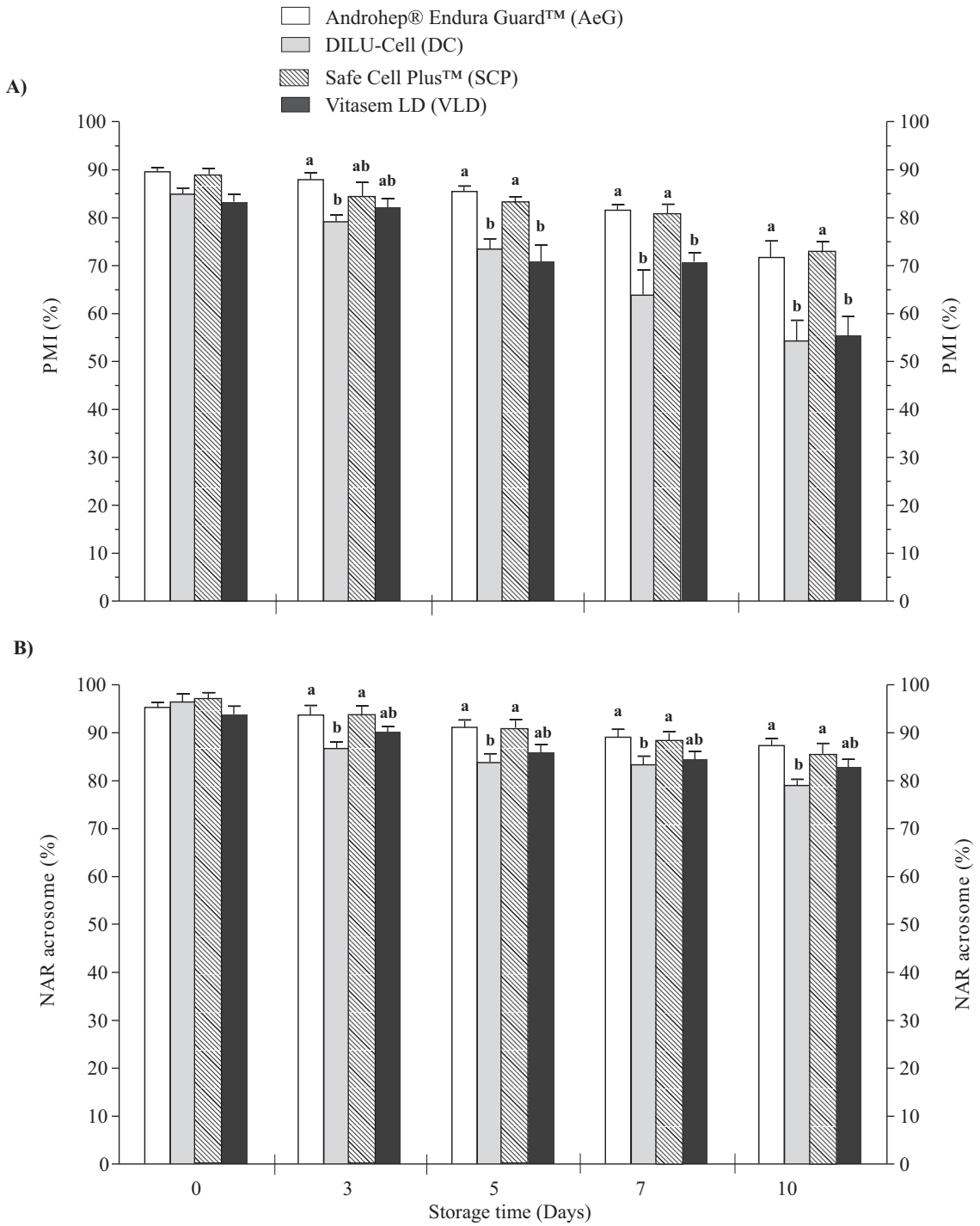

Fig. 3. Plasma membrane integrity, PMI (A) and normal apical ridge (NAR) acrosome (B) of boar spermatozoa following liquid semen storage in different long-term extenders at 17°C. Values represent the means (± SEM) of 15 ejaculates. Values with different the letters (a-b), with the same storage time, are significantly different ($P < 0.05$).

marked decline in sperm PMI in each extender over time (Fig. 3A). The percentage of spermatozoa with intact membrane in AeG-diluted semen was significantly higher ($P < 0.05$) on Day 3 of storage compared with DC-diluted semen (Fig. 3A). Also, semen diluted with either AeG extender or SCP extender exhibited higher ($P < 0.05$) sperm PMI during prolonged storage (Fig. 3A). The proportions of spermatozoa with NAR acrosomes markedly decreased during storage, irrespective of the extender type (Fig. 3B). Besides Day 0 of storage, the percentage of spermatozoa with NAR acrosomes was significantly lower ($P < 0.05$) in DC-diluted semen compared with either AeG or SCP extender over time (Fig. 3B).

Discussion

The findings of this study showed that the sperm metabolic performance and membrane integrity were compromised during storage for 10 days at 17ºC, regardless of the extender type. It was observed that sperm TMOT was better preserved in AeG and SCP extenders, however, the latter preserved sperm PMOT more efficiently than the former during prolonged storage. Sperm motility, an indicator of the sperm metabolic performance, is an important parameter that is routinely used to assess the quality of liquid-stored boar semen (Kuster and Althouse 1999, Kommisrud et al. 2002, Fraser et al. 2003, Vyt et al. 2004, Dziekońska et al. 2009). In the current study sperm motility decreased in all extenders over time, and it was approximately 60% in AeG-diluted semen on Day 10 of storage. Several studies have shown that the degree of decrease in the percentage of motile boar spermatozoa is not similar in different long-term extenders during prolonged storage (Kommisrud et al. 2002, De Ambrogi et al. 2006, Fantinati et al. 2009, Gączarzewicz et al. 2010, Kaeoket et al. 2010). Contrary to our results, Fantinati et al. (2009) reported that sperm motility in AeG-diluted semen did not significantly drop below 60% until Day 15 of storage, whereas sperm motility in SCP-diluted semen was approximately 60% on Day 9 of storage (Gączarzewicz et al. 2010). In another study, a significant decrease in sperm motility was seen in semen diluted with X-Cell on Day 4 of storage, being slightly less than 60% (De Ambrogi et al. 2006). The findings of this study and those of other studies reaffirmed that components of the long-term extenders and the storage conditions might exert varying effects on the sperm motility apparatus.

Sperm MMP and ATP production decreased in all extenders, irrespective of the storage time. Moreover, motility has been demonstrated to be highly correlated with mitochondrial function of boar spermatozoa following liquid semen storage (Fraser et al. 2003, Trzcińska et al. 2008, Dziekońska et al. 2009, Dziekońska and Strzeżek 2011). In the present study differences in the proportions of spermatozoa with high MMP between the extenders were more marked during prolonged storage, particularly in the AeG and SCP extenders. It must be emphasized that the maintenance of high MMP facilitates the production of ATP, which is required to sustain sperm movements (Guthrie et al. 2008, Gogol et al. 2009). However, in the current study the magnitude of the loss in sperm motility and MMP during storage in the diluted semen was not associated with the decline in ATP production, regardless of the extender type. Such findings might suggest that the measurements of sperm ATP content were less sensitive to detect differences in the sperm metabolic performance between long-term extenders used for liquid storage of boar semen. It has been reported that ATP is derived from mitochondrial oxidative phosphorylation and glycolysis, but the former is more efficient in supplying sperm cells with adequate amounts of ATP during liquid semen storage (Medrano et al. 2005, Dziekońska et al. 2009). The findings of this study suggest that prolonged liquid storage contributed to a dysfunction in mitochondrial oxidative phosphorylation in the spermatozoa, resulting in a decline in sperm MMP and ATP production, which compromises sperm motility. According to Storey (2008), spermatozoa can utilize different substrates and therefore activate different metabolic pathways, depending on of the available substrates. Such phenomenon has been reported in boar spermatozoa during liquid semen storage (Medrano et al. 2005), and could be responsible for the wide variations in sperm ATP content observed during prolonged storage in the different extenders.

In the current study, both AeG and SCP extenders preserved higher proportions of spermatozoa with intact-membrane and NAR acrosomes during storage compared with the other extenders, suggesting that differences in the extender composition delayed the storage-dependent ageing changes in spermatozoa. High sperm PMI and acrosome integrity have been reported in other studies during prolonged storage of boar semen in different long-term extenders (Kommisrud et al. 2002, De Ambrogi et al. 2006, Fantinati et al. 2009, Gączarzewicz et al. 2010, Kaeoket et al. 2010). There is increasing evidence indicating that the deterioration in sperm viability following liquid storage might be associated with excessive production of reactive oxygen species (ROS), reduced storage temperature, or differences in extender components (Waberski et al. 1989, Johnson et al. 2000, Gadea 2003, Kumaresan et al. 2008, Gogol et al. 2009, Kaeoket et al. 2010). Even though the exact composition of the long-term extenders used in this study is not fully known, it can be suggested that the extender components of AeG and SCP had a more beneficial effect on the semen longevity. It is well noteworthy that the addition of different substances to boar semen extenders, such as bovine serum albumin (BSA), L-cysteine and EDTA, helps to enhance semen longevity during prolonged storage, probably by delaying the processes associated with sperm ageing (Waberski et al. 1989, Johnson et al. 2000, Kaeoket et al. 2010). Several authors have emphasized the importance of the buffering capacity of AeG and SCP extenders for the maintenance of boar sperm viability during prolonged storage (Fantinati et al. 2009, Gączarzewicz et al. 2010). In this study, it is possible that the buffering

capacity of AeG and SCP could also contribute to greater sperm metabolic performance and membrane integrity during semen storage. Also, individual differences between the boars could affect the quality of the liquid-stored semen (Dziekońska and Strzeżek 2011). However, in the current study there were not any marked differences between the three boars, with respect to the analyzed sperm metabolic activity and membrane integrity, during prolonged storage at 17°C.

Taken together, the finding of this study strongly suggest that the type of extender selected for long-term storage of boar spermatozoa is important in order to increase the semen longevity with minimum deterioration in the sperm function. According to the storage conditions used in this study, AeG and SCP extenders possess greater ability to preserve the metabolic activity and membrane integrity of boar spermatozoa during long-term storage of liquid semen. However, more studies are needed to assess the sperm function in conjunction with *in vitro* assays following prolonged liquid semen storage.

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