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Original article

Effects of the platelet-activating factor (PAF) on selected quality parameters of cryopreserved bull semen (AI) with reduced sperm motility

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Abstract

The aim of the study was to determine the effects of platelet-activating factor (PAF) on selected quality parameters of cryopreserved bull semen with reduced sperm motility used for artificial insemination. The aim of experiment 1 was to identify the optimal concentration of the phospholipid able to preserve sperm viability. Cryopreserved semen was treated with different PAF concentrations: $1x10^{-5}$M, $1x10^{-6}$M, $1x10^{-7}$M, $1x10^{-8}$M and $1x10^{-9}$M. The experiment demonstrated that PAF at concentration $1x10^{-9}$M increased most the sperm viability parameters (motility parameters, plasma membrane integrity and mitochondrial function) after 120 min of incubation of thawed semen at 37°C. Cryopreserved bull semen with reduced sperm motility (below 70%) was supplemented with PAF in a concentration of $1x10^{-9}$M. A statistically significant increase in sperm motility, percentage of linear motile spermatozoa and VSL value was observed after 120 min incubation of sperm with $1x10^{-9}$M PAF. Sperm supplementation with PAF also had positive effects on plasma membrane integrity and percentage of spermatozoa with preserved mitochondrial transmembrane potential, but the differences were not statistically significant. The results indicated positive effects of PAF supplementation at a concentration of $1x10^{-9}$M on the selected sperm quality parameters in cryopreserved bull semen with reduced motility.

Key words: bull, spermatozoa, PAF, viability, cryopreservation.

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Introduction

Insemination with cryopreserved semen is the most popular practice used in the controlled reproduction of cattle. The main factor determining the success of the insemination procedure is a semen quality (Walsh et al. 2011). Sperm motility is the basic parameter of semen quality analysed at insemination centres. Many researchers have suggested that the percentage of motile spermatozoa is an indicator of plasma membrane integrity and metabolic activity of semen (Johnson et al. 2000, Estienne et al. 2007, Kaeoket et al. 2010). For effective insemination, the cryopreserved semen has to be of a high biological quality.

The platelet-activating factor (1-0-alkyl-2-acetyl-sn-glycerol-3-phosphoryl-choline) (PAF) is a specific signalling phospholipid that has pleiotropic biological properties in addition to platelet activation (Braquet et al. 1987, Roudebush and Diehl 2001). This naturally occurring, acetylated membrane glycerophospholipid plays an important role in the reproductive process, both in males and females (Kordan et al. 2003).

The synthesis of PAF from membrane phospholipids in various types of activated cells, mainly basophiles, neutrophils, monocytes, macrophages and endothelial cells, is catalysed by phospholipase A2 (Roudebush et al. 2002). PAF plays an important role in mammalian reproduction, affecting, for example, the ovulation and fertilization process. In addition, it is involved in the pre-implantation development of the embryo and its subsequent implantation (Harper 1989, Roudebush et al. 2002).

PAF is present in human spermatozoa (Minhas et al. 1991), and in other primates, including squirrel monkey (Roudebush and Mathur 1998), macaque (Diaz et al. 1999) and rhesus monkey (Roudebush et al. 2002), and in domestic animals such as rabbit, rooster, boar, bull and stallion (Kumar et al. 1988, Hough and Parks 1994, Roudebush and Diehl 2001). Many studies carried out in various animal species and in humans have demonstrated the positive effect of supplementation with exogenous PAF on sperm motility, plasma membrane integrity, capacitation and the acrosome reaction of semen preserved either in liquid form or cryopreserved (Aravindakshan and Sharma 1996, Roudebush et al. 2002, Odeh et al. 2003, Kordan and Strzeżek 2006, Kordan et al. 2009, Kheradmand et al. 2009, Kordan et al. 2010, Esmaeilpour et al. 2014).

The aim of the study was to determine the effects of PAF on selected quality parameters of cryopreserved bull semen with reduced sperm motility.

Materials and Methods

Experiment 1

The experimental material was cryopreserved semen obtained from 10 bulls reared at the Animal Breeding and Insemination Centre in Bydgoszcz, used for insemination practice. Cryopreserved spermatozoa ($20x10^6$ spermatozoa/cm^3) were treated with different concentrations of PAF: $1x10^{-5}$M, $1x10^{-6}$M, $1x10^{-7}$M, $1x10^{-8}$M and $1x10^{-9}$M at 37°C. Cryopreserved semen without PAF supplementation was used as the control. Sperm viability (motility parameters, plasma membrane integrity and mitochondrial function) was examined at different time intervals: 0, 30, 60, 90 and 120 min for each PAF treatment, together with the control (without PAF).

Experiment 2

The experimental material was cryopreserved semen with reduced sperm motility obtained from 10 bulls reared at the Animal Breeding and Insemination Centre in Bydgoszcz, used for insemination practice. PAF was used at a concentration of 10^{-9}M. The assessments of the quality parameters of the cryopreserved bull semen and sample incubation times were identical to those in the experiment 1.

Sperm viability assessments

Motility

Sperm motility was evaluated using the computer assisted sperm analysis (CASA) system (VideoTesT Sperm 2.1, St. Petersburg, Russia). Aliquots of sperm samples were placed in a Makler Chamber and examined at 37°C under a phase-contrast microscopy system coupled to a video camera adapted to the VideoTesT Sperm system. All motility parameters were analysed in accordance with the recommendations given by the World Health Organization (Elia et al. 2010). The parameters analysed included total sperm motility (%), linear motile spermatozoa (%), non-linear motile spermatozoa (%) and VSL (Straight Line Velocity).

Plasma membrane integrity

Sperm plasma membrane integrity was assessed using dual fluorescent staining, SYBR-14 and PI (Live/Dead Sperm Viability Kit; Molecular Probes),

as described by Garner and Johnson (1995), with slight modifications. Briefly, aliquots of sperm samples ($20x10^6$ spermatozoa/cm^3) were incubated with SYBR-14 (1mM SYBR-14 in DMSO) and PI solutions (2.4 µM PI in Tyrode's salt solution) for 10 minutes at 37°C. Following incubation, stained sperm cells were placed on microscopic slides and examined at 600 x magnification under a fluorescence microscope (Olympus CH 30 RF-200, Tokyo, Japan). Only sperm cells displaying only bright green fluorescence were considered viable spermatozoa with an undamaged plasma membrane. A minimum of 200 cells per slide were examined in random fields of each aliquot.

Mitochondrial function assessed by fluorescent microscopy

The sperm mitochondrial function was assessed using dual staining with fluorescent probes, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide, JC-1 (Molecular Probes, Eugene, USA) with propidium iodide (PI, Sigma Chemical Co., St. Louis, MO, USA), according to a previously described method (Thomas et al. 1998), with some modifications (Dziekońska et al. 2009). Aliquots of sperm samples ($20x10^6$ spermatozoa/cm^3) were incubated with JC-1 solution (1mg JC-1/cm^3 dimethylsulfoxide, DMSO) for 15 min at 37°C. Following incubation, sperm samples were stained with PI (10 µl of PI solution in 0.5 mg/cm^3 phosphate buffered solution) for 10 minutes at 37°C, washed (600 x g, 5 min at room temperature) and the sperm pellets were re-suspended in a HEPES buffered solution (10mM HEPES, 0.85% NaCl, 0.1% bovine serum albumin, pH 7.4). Stained sperm samples were placed on microscopic slides, covered with coverslips (22 x 22 mm) and examined under a fluorescence microscope (Olympus CH 30 RF-200). Viable spermatozoa with functional mitochondria emitted orange-red fluorescence. Two slides were evaluated per sample and 200 spermatozoa were counted per slide.

Statistical analysis

Values were expressed as the mean + standard deviation (SD). The data were analysed by ANOVA, followed by the Duncan multiple comparison test (Experiment I), and by the t-Student test (Experiment 2), using the Statistica software package (StatSoft Incorporation, Tulsa OK., USA). Differences between means were considered significant at $p \leq 0.05$.

Results

Experiment 1

Changes in the percentage of motile spermatozoa are presented in Fig. 1A. A higher percentage of motile spermatozoa was found from the first minutes of incubation of samples with different concentrations of exogenous PAF when compared to the control. This trend continued throughout the entire incubation period. Statistically significant differences at $p \leq 0.05$ were found starting from minute 30 of the experiment. The highest percentage of motile sperm was found for PAF at a concentration of $1x10^{-9}$M.

Regardless of PAF concentration and incubation time, the supplementation of samples with the phospholipid had a positive effect on all sperm kinematic parameters, i.e. VCL, VAP, ALH, BCF (data not shown) and VSL (Fig. 1B). In minute 90 of the experiment the supplementation with PAF at concentrations $1x10^{-8}$M and $1x10^{-9}$M caused a statistically significant increase in the linear motility of spermatozoa as compared to the control. In minute 120 statistically significant differences were found between the control and samples treated with PAF at concentrations of $1x10^{-6}$M and $1x10^{-9}$M.

The effect of different PAF concentrations on the percentage of cryopreserved linear motile spermatozoa from bull is presented in Fig. 2A. A higher percentage of linear motile spermatozoa was found after 30 min of incubation of thawed semen treated with different concentrations of exogeneous PAF. Statistically significant differences were recorded in minute 90 of incubation.

Fig. 2B presents changes in the percentage of nonlinear motile spermatozoa. Regardless of the incubation time, all samples of semen treated with PAF contained a higher percentage of nonlinear motile spermatozoa. As in the case of linear motility, statistically significant differences were observed in the final period of the experiment.

Changes in the percentage of sperm with plasma membrane integrity are presented in Fig. 3A. Throughout the experiment, regardless of the PAF concentration, semen incubated with the phospholipid contained a higher percentage of spermatozoa with plasma membrane integrity. However, no statistically significant differences were found for this parameter. A similar trend was observed for the percentage of spermatozoa with a high mitochondrial transmembrane potential (Fig. 3B). No statistically significant differences were found for this parameter, either.

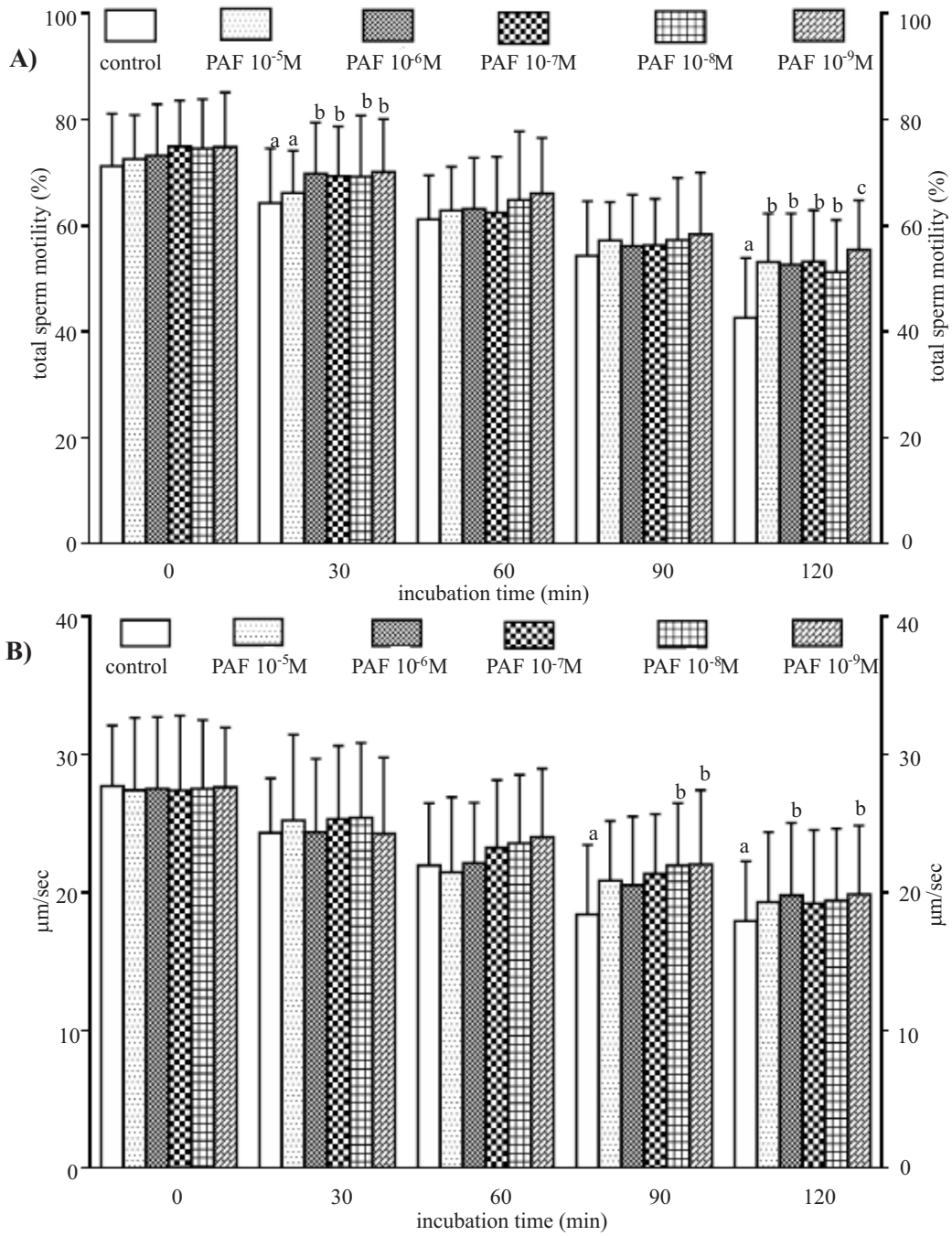

Fig. 1. Percentage of total motility of bull spermatozoa (A) and values of VSL (Straight Line Velocity) (B) following treatment of cryopreserved semen with different concentrations of platelet-activating factor (PAF). Sperm motility was assessed by the computer-assisted semen analysis (CASA) system. Values represent the means ± SD from 10 bulls. Within incubation time, values (a,b,c) with different letters are significant at $p \leq 0.05$.

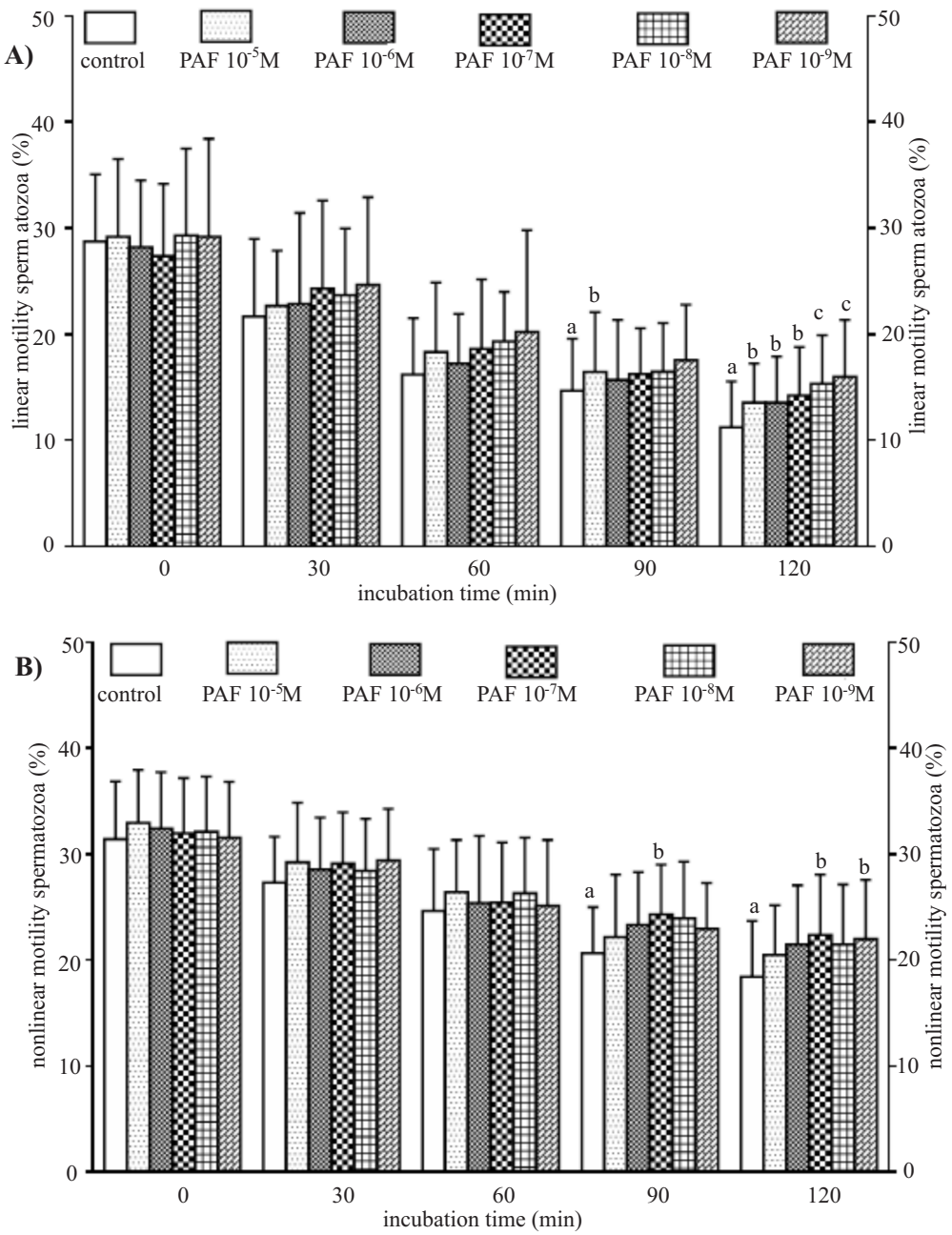

Fig. 2. Percentage of linear (A) and nonlinear (B) bull spermatozoa following treatment of cryopreserved semen with different concentrations of platelet-activating factor (PAF). Sperm motility patterns were analysed by the computer-assisted semen analysis (CASA) system. Values represent the means ± SD from 10 bulls. Within incubation time, values (a,b,c) with different letters are significant at $p \leq 0.05$.

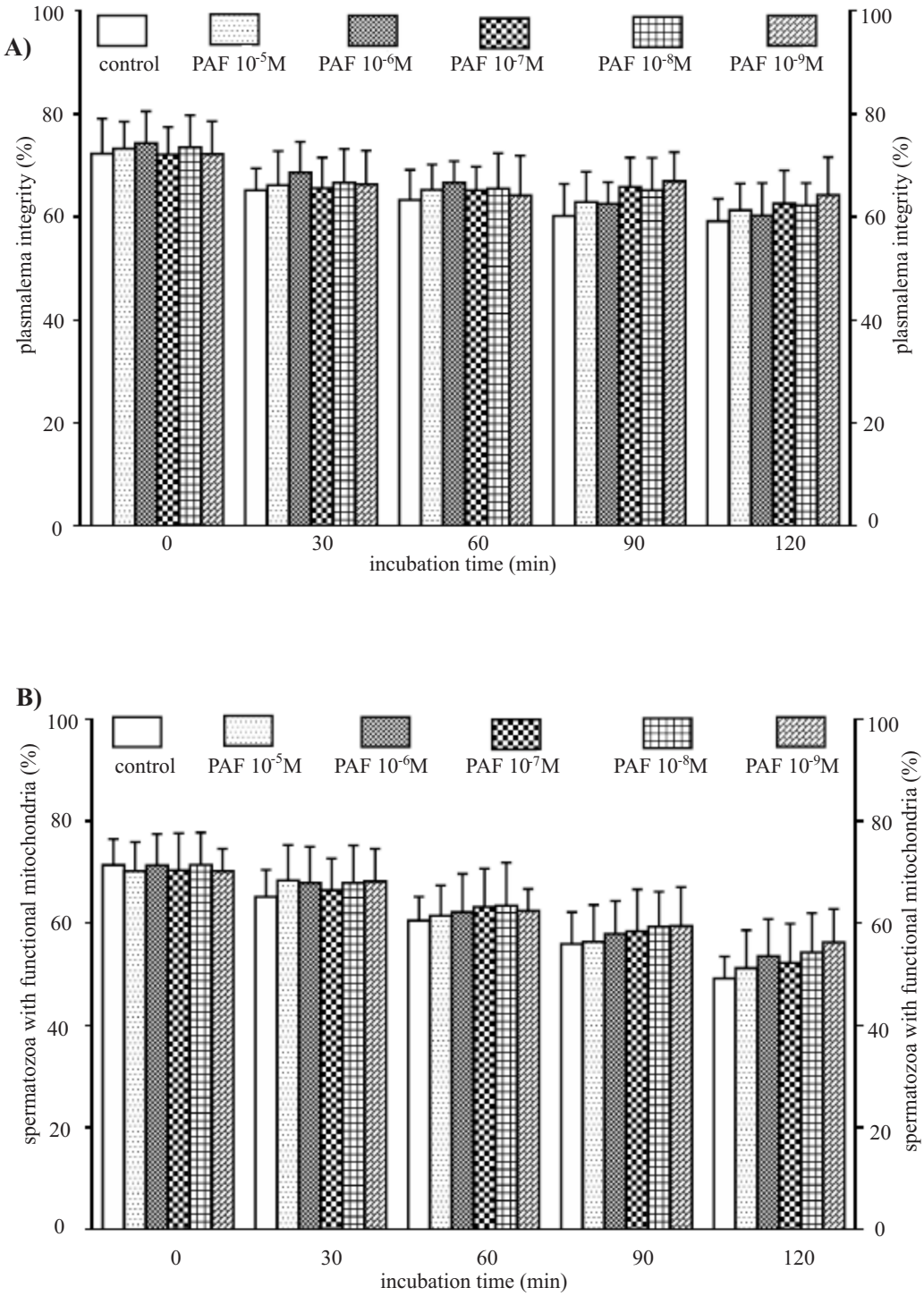

Fig. 3. Percentage of plasma membrane integrity assessed with the SYBR-14/PI assay (A), mitochondrial function assessed with the JC-1/PI assay (B) of bull spermatozoa following treatment of cryopreserved semen with different concentrations of platelet-activating factor (PAF). Values represent the means ± SD from 10 bulls.

Experiment 2

Results from experiment 1 demonstrated that $1x10^{-9}$M PAF was the optimal concentration, and it had the most beneficial effect on the analysed quality parameters of the cryopreserved bull semen. After selecting the optimal PAF concentration, its effect on the cryopreserved bull semen with reduced motility was investigated. Experiment II was carried out on semen samples in which the percentage of motile sperm was below 70%.

Results from the analysis of sperm motility are

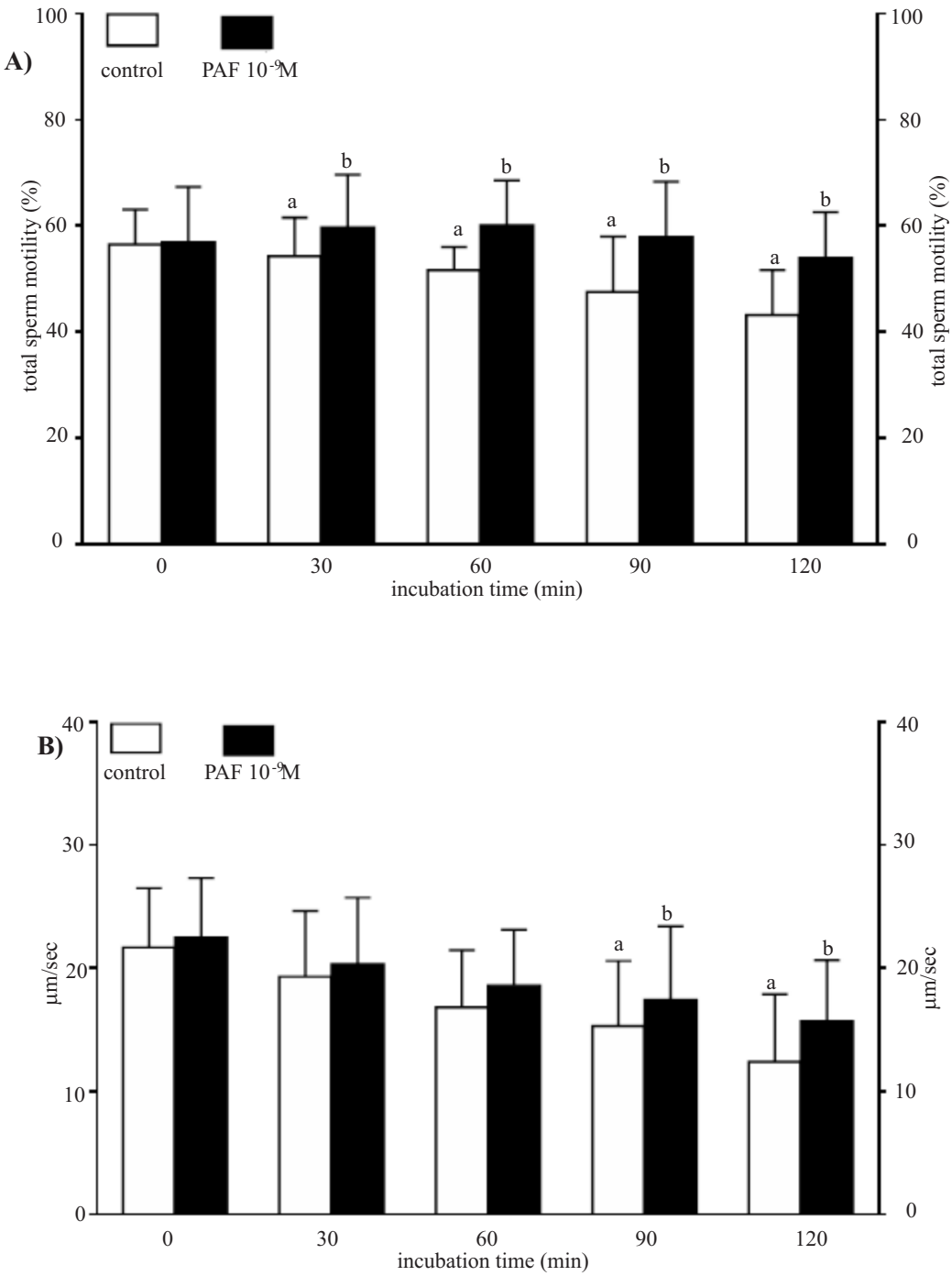

Fig. 4. Percentage of total motility of cryopreserved bull spermatozoa (A) and values of VSL (Straight Line Velocity) (B) for semen samples with reduced motility treated with 10^{-9}M exogeneous PAF. Sperm motility was assessed by the computer-assisted semen analysis (CASA) system. Values represent the means ± SD from 10 bulls. Within incubation time, values (a,b) with different letters are significant at p≤0.05.

presented in Fig. 4A. The mean sperm motility in the control sample was 56.5%. From minute 30 statistically significant differences (p≤0.05) in the percentage of motile sperm (59.69%) were found between the control and samples treated with $1x10^{-9}$M PAF.

Treatment of samples with PAF had positive effects on all kinematic parameters of sperm, i.e. VCL, VAP, ALH, BCF (data not shown) and VSL. It was found that in minute 90 of the experiment the supplementation with PAF at optimal concentration caused a statistically significant increase in the VSL values of spermatozoa (Fig. 4B).

The effect of the optimal PAF concentration on the percentage of cryopreserved linear motile bull

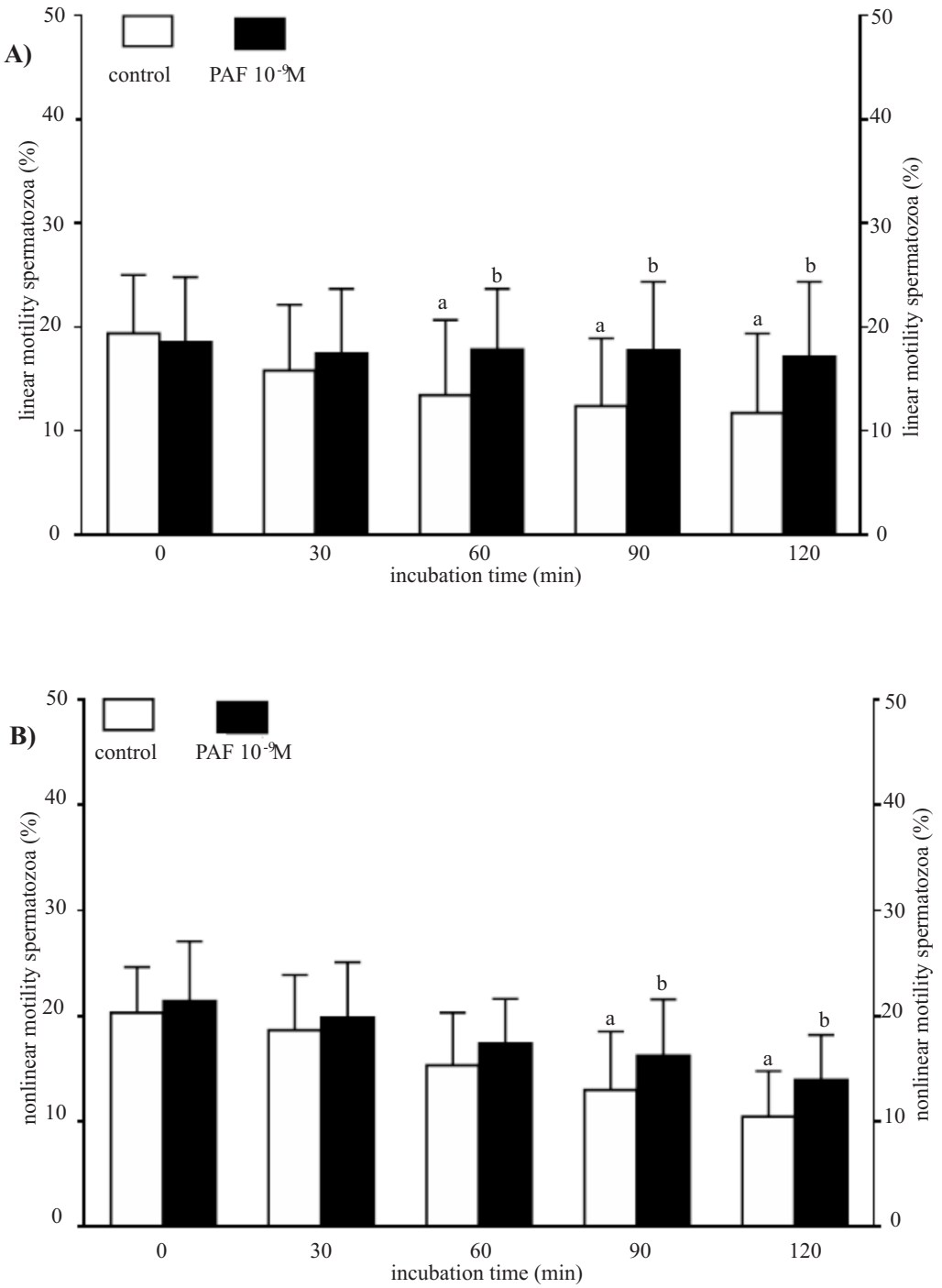

Fig. 5. Percentage of linear (A) and nonlinear (B) of cryopreserved bull spermatozoa in semen samples with reduced motility treated with 10-9M exogenous PAF. Sperm motility patterns were analysed by the computer-assisted semen analysis (CASA) system. Values represent the means ± SD from 10 bulls. Within incubation time, values (a,b) with different letters are significant at $p \leq 0.05$.

spermatozoa is presented in Fig. 5A. An increase in the percentage of linear motile spermatozoa was observed throughout the entire incubation period. Statistically significant differences in this parameter were observed as early as in minute 30 of semen incubation. A similar trend was observed for nonlinear motile spermatozoa (Fig. 5B). An increase in the percentage of nonlinear motile spermatozoa was observed from the first minutes of sample incubation with PAF. In the final period of incubation a statistically significant effect ($p \leq 0.05$) of supplementation with PAF on the percentage of nonlinear motile spermatozoa was found.

Changes in the percentage of sperm with the inte-

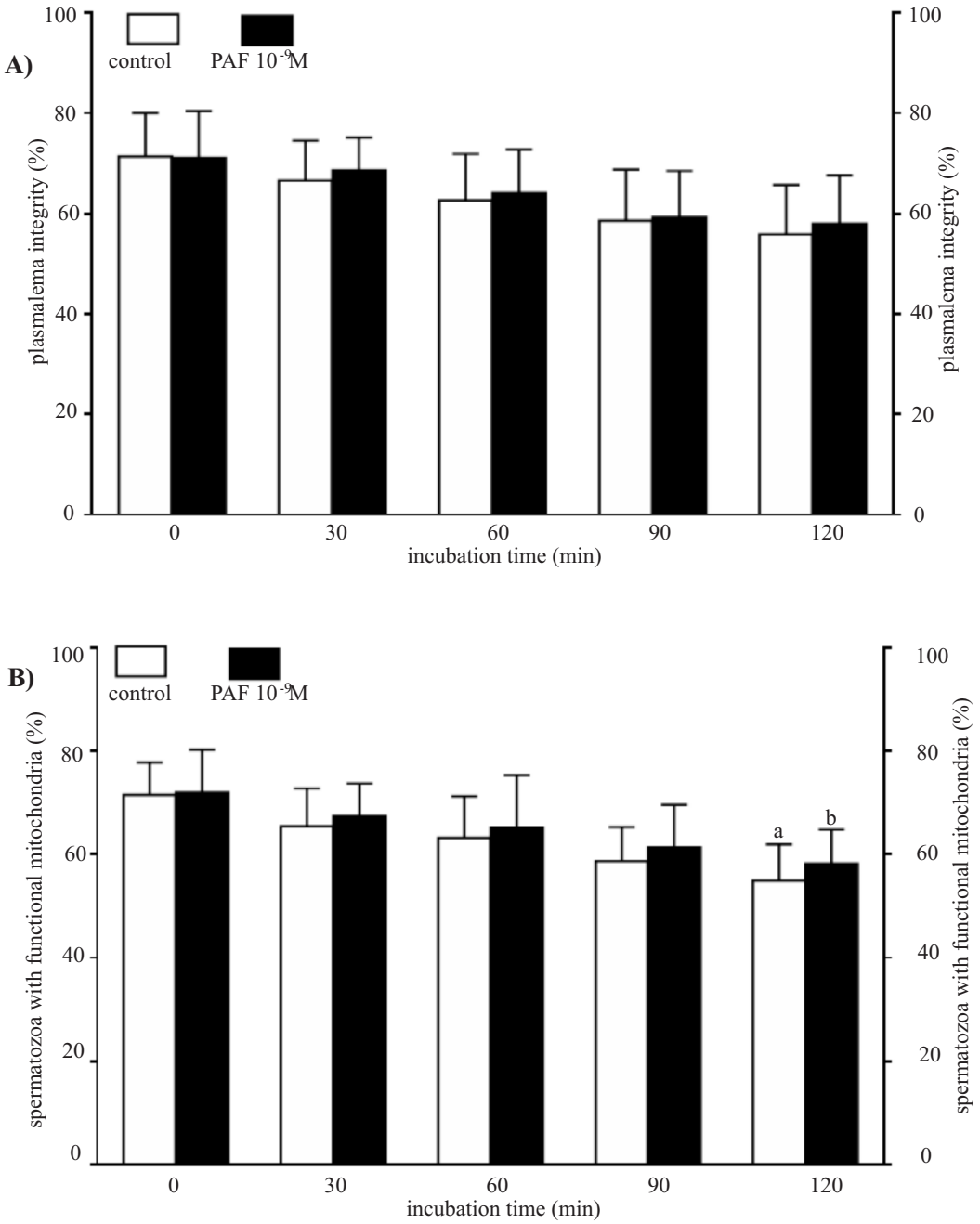

Fig. 6. Percentage of plasma membrane integrity assessed with the SYBR-14/PI assay (A), mitochondrial function assessed with the JC-1/PI assay (B) of cryopreserved bull semen samples with reduced motility treated with 10^{-9}M exogenous PAF. Values represent the means ± SD from 10 bulls. Within incubation time, values (a,b) with different letters are significant at p≤0.05.

gral plasma membrane are presented in Fig. 6A. Throughout the experiment, treatment of samples with the optimal concentration of PAF caused an increase in the percentage of sperm with the integral plasma membrane. However, the differences were not statistically significant. Moreover, throughout the entire experiment semen incubated with phospholipid contained a higher percentage of sperm with functional mitochondria (Fig. 6B). Statistically significant differences in this parameter at p≤0.05 were observed after 120 min of incubation.

Discussion

The aim of the first experiment was to select the optimal concentration of exogenous PAF that would have a positive effect on the basic parameters of cryopreserved bull semen after thawing. The study revealed that sperm incubated with $1x10^{-9}$M PAF contained the highest percentage of motile spermatozoa at all time intervals of the experiment. Moreover, an increase in sperm kinematic parameters, i.e. VCL, VAP, ALH, BCF (data not shown) and VSL, as well

as in the percentage of linear and nonlinear motile spermatozoa was observed. These results are in line with observations by Ricker et al. (1989), who demonstrated that treatment of human semen for 5 min with synthetic PAF at concentrations from 10^{-7} to 10^{-13}M resulted in a significant increase in motility, whereas treatment with 10^{-5}M PAF resulted in immediate cell death. A threefold improvement in the motility of human spermatozoa was reported after exposure to $10x10^{-9}$M exogenous PAF for 4 h (Krausz et al. 1994). Improved motility of stallion sperm exposed to PAF at concentrations from 10^{-10} to 10^{-13}M was reported, with the best motility maintained after 120 min of incubation (Odeh et al. 2003).

On the other hand, Kheradmand et al. (2009) reported that a 30-minute incubation of sperm with $10x10^{-9}$M PAF initiates the acrosome reaction in the ram without a drastic decrease in sperm motility. The effect of exogenous PAF on the biological parameters of human and animal semen can be explained by the function of this endogenous phospholipid. Studies by Reinhardt et al. (1999) and Roudebush et al. (2000) demonstrated the presence of PAF receptors on the surface of the plasma membranes of human sperm. The highest number of receptors was found at the connecting piece and midpiece of the spermatozoon, which is extremely important because of the functions performed by these structures. The midpiece of the spermatozoon, a structure rich in mitochondria, is involved in maintaining sperm motility, while the connecting piece contains proximally located centrioles, participating in the pre-implantation development of the embryo (Santhanthan et al. 1996).

Binding PAF to its receptor protein on the surface of the sperm plasma membrane activates the phospholipase C system, causing the release of the second messengers in the cell – phosphatidylinositol derivatives – responsible for the increase in the intracellular levels of Ca^{2+} (Lapetina et al. 1982). All these events increase actin membrane network depolymerisation and phospholipase activation, both of which can increase cellular movement and sperm motility (Roudebush et al. 2000). Studies carried out by Roudebush et al. (2004) demonstrated that damaged human spermatozoa have a modified distribution of PAF receptors on their surface. Abnormal distribution of PAF receptors is associated with disturbed expression of the gene responsible for the synthesis of PAF (Roudebush et al. 2000).

The aim of the second experiment was to assess the effects of the optimal concentration of exogenous PAF on the samples of cryopreserved and thawed bull semen, containing less than 70% of motile sperm, which in practice do not meet the criteria required by insemination centres. Sperm motility analysis demonstrated a statistically significant increase in the percentage of motile sperm in samples incubated with the optimal concentration of exogenous PAF throughout the entire experiment. Similar results were obtained when analysing the percentage of cryopreserved linear and nonlinear motile spermatozoa. Of all the analysed kinematic parameters of sperm with reduced motility, the addition of the phospholipid caused a statistically significant increase in VSL. Antończyk et al. (2010) indicated a strong correlation between the kinematic parameters of sperm, such as VSL, VCL and ALH and fertility. Similar findings on sperm motility and its basic kinematic parameters were made by Grassi et al. (2010) in their study on human sperm with reduced motility intended for intra-uterine insemination. The researchers reported that a 60 min exposure of sperm to 0.5 μM PAF caused a significant increase in the motility parameters. Studies by Briton-Jones et al. (2001) demonstrated that incubation of cryopreserved oligospermic human semen with exogenous PAF increased the percentage of motile spermatozoa. In addition, the exposure of semen samples from patients diagnosed with acute asthenozoospermia to PAF significantly improved motility, but also facilitated the identification of viable sperm necessary for the intracytoplasmic sperm injection (ICSI).

The plasma membrane is responsible for intracellular homeostasis necessary to preserve the *in vitro* fertilizing capacity of previously preserved sperm (de Andrade et al. 2007). Both the first and the second part of the experiment revealed the positive effects of PAF on the sperm plasma membrane and mitochondrial activity. Similar results were obtained in our previous studies investigating the effect of PAF supplementation on the plasma membranes of cryopreserved boar and canine spermatozoa (Kordan et al. 2009, Kordan et al. 2010). Sperm with a stable plasma membrane have a high transmembrane potential (Trzcińska et al. 2008), which is necessary to generate energy in the form of ATP to maintain sperm motility and other processes (de Andrade et al. 2007). Considering the specific distribution of PAF receptors in the structure of the sperm, it can be assumed that its biological function is also related to the protection of the plasma membrane and sperm motility due to the effect on the mitochondrial transmembrane potential.

In conclusion, the treatment of cryopreserved bull semen characterised by reduced motility (below 70%) with 10^{-9} M exogenous PAF has a positive effect on semen quality parameters. This is manifested by increased sperm motility, and also improved kinematic parameters and increased stability of the plasma membrane, which is important for insemination practice.

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