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Original article

Effect of platelet activating factor (PAF) supplementation in semen extender on viability and ATP content of cryopreserved canine spermatozoa

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Abstract

The aim of this study was to investigate the effect of platelet activating factor (PAF) on the quality characteristics of cryopreserved canine spermatozoa. Cryopreserved semen of 5 mixed-breed dogs was treated with different concentrations of exogenous PAF (1×10^{-3}M, 1×10^{-4}M, 1×10^{-5}M and 1×10^{-6}M) and examined at different time intervals (0, 30, 60 and 120 min). Cryopreserved semen treated without PAF was used as the control. Sperm quality was evaluated for motility (computer-assisted semen analysis, CASA), mitochondrial function (JC-1/PI assay) and plasma membrane integrity (SYBR-14/PI assay and Hoechst 33258). Also, ATP content of spermatozoa was determined using a bioluminescence assay. Treatment of cryopreserved semen with 1×10^{-3} M PAF at 120 min of incubation resulted in significantly higher total sperm motility compared with the control. It was observed that PAF-improved total sperm motility was concurrent with enhanced sperm motility patterns after treatment of cryopreserved semen. Treatment of cryopreserved semen with PAF did not improve either sperm mitochondrial function or plasma membrane integrity, as monitored by different fluorescent membrane markers. Furthermore, ATP content of cryopreserved spermatozoa was significantly higher when PAF was used at a concentration of 1×10^{-3} M compared with the control and other PAF treatments, regardless of the incubation time. The findings of this study indicated that treatment with 1×10^{-3} M PAF at 120 min of incubation rendered better quality of cryopreserved canine semen, which was associated with improved sperm motility parameters and ATP content. It can be suggested that exogenous PAF addition is beneficial as a supplement for canine semen extender used for cryopreservation.

Key words: canine, spermatozoa, PAF, viability, ATP content, cryopreservation

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Introduction

In recent years, there has been an increased interest in assisted reproductive techniques. Artificial insemination (AI) with chilled or frozen-thawed semen is a useful tool that allows improvement in canine breeding management (Tsutsui et al. 2003). Cryopreservation of canine semen is gaining interest because it allows the transfer of a desired genetic pool among valuable dog studs, both within and between countries (Peña et al. 2006). However, cryopreservation compromises sperm function, resulting in a reduced life-span of post-thaw spermatozoa and subsequently lower conception rates (Linde-Forsberg and Forsberg 1989, Rota et al. 1997). All these factors must be taken into consideration in order to optimize the design of new thawing protocols that could improve post-thaw survival of canine spermatozoa.

Platelet activating factor, PAF (1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphorylcholine) has been implicated in numerous reproductive processes, which are associated with sperm-egg interactions (Kordan et al. 2003). The action of PAF on preserved spermatozoa has been studied in different mammalian species. Recent research studies in our laboratory have shown that exogenous PAF improves motility parameters of boar spermatozoa during liquid semen storage (Kordan and Strzeżek 2002, 2006) or cryopreservation of semen (Kordan et al. 2009). Moreover, treatment with exogenous PAF increased motility of post-thaw human spermatozoa (Hellstrom et al. 1991). The molecular mechanism of PAF action on sperm motility apparatus is associated with an increase in phosphatidylinositol 4,5-biphosphate (PIP2) breakdown and the subsequent formation of inositol 1,4,5 triphosphate (IP3) and diacylglycerol (DAG), which in turn cause an increase in calcium mobilization, a prerequisite for hyperactivation of sperm movement (Ricker et al. 1989). To our knowledge, there is a lack of information regarding the effect of exogenous PAF on post-thaw survival of canine spermatozoa. The aim of this study was to investigate the effect of PAF supplementation in semen extender on the quality characteristics of cryopreserved canine spermatozoa at different time intervals. Assessments of sperm quality characteristics included viability, such as motility, mitochondrial function and plasma membrane integrity, and ATP content.

Materials and Methods

Animals and ejaculate collections

Five dogs of mixed-breed, aged from 4 to 9 years (body weight of 15 to 20 kg) were used in this study. The animals were housed at the laboratory of the Department of Animal Biochemistry and Biotechnology. They were kept individually, fed a commercial dry food (Purina Dog Chow®) with mineral-vitamin supplementation (Can-Vit®) and had access to water *ad libitum*. Permission to conduct this study was granted by the Local Ethics Committee for Animal Experiments

Ejaculates were collected once weekly by digital manipulations (Linde-Forsberg 1991) and only the second fraction (the sperm-rich fraction) was used in the experiments. A total number of 16 sperm-rich fractions (3 sperm-rich fractions each from 4 dogs and 4 sperm-rich fractions from 1 dog) were collected within a period of 3-4 weeks. Only semen samples showing a minimum of 70% sperm motility were used. Sperm concentration was determined cytometrically, using a Bürker chamber (Bielański 1979).

Extenders

All reagents were purchased from the Polish Chemical Reagents, Joint-Stock Company, Gliwice, Poland (POCH S.A), unless otherwise stated. A stock solution containing 3.025 g Tris (hydroxymethyl, aminomethane), 1.70 g citric acid, 1.25 g fructose in 100 cm^3 distilled water was prepared for equilibration of the semen (Extender 1). A second extender (Extender 2) containing the same composition as that of Extender 1, but with lipoprotein fraction isolated from ostrich egg yolk (LPFo), glycerol and Orvus Es Paste (OEP, Nova Chemical Sales, Inc., Scituate MA, USA) was prepared for chilling and freezing of the semen. The final concentration of LPFo, glycerol and OEP was 10%, 4% and 1%, respectively. A brief description of the isolation procedure of LPFo has been described elsewhere (Strzeżek et al. 2005). The LPFo was lyophilized and stored, until required.

Semen cryopreservation

Semen was cryopreserved according to the method of Rota et al. (1997), with some modifications. In brief, after collection, the semen was centrifuged (700 × g, 6 min room temperature) and the sperm pellets were re-suspended in Extender 1 at a concentration of 200 × 10^6 spermatozoa/cm^3. The diluted semen was allowed to equilibrate for 1 h at room temperature and then cooled at 5°C over a period of 30 min. After chilling of the semen, a second dilution was carried out with Extender 2 (1:1) to result in a final concentration of 100 × 10^6 spermatozoa/cm^3. Finally, the diluted semen was loaded into 0.25 cm^3 plastic straws (Minitüb Gmbh, Tiefenbach, Germany)

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at 5°C and frozen in a controlled programmable freezer (CryoCell 1205; SY-LAB, Austria), using an appropriate freezing protocol (cooled from +5°C to -20°C at 4.46°C/min and then further cooled from -20°C to -80°C at 5°C/min). The frozen straws were stored in liquid nitrogen (-196°C), until required for laboratory analysis.

Thawing was performed for 6 sec at 70°C in a water bath. Post-thaw semen samples were diluted with Extender 1 to give a concentration of 20×10^6 spermatozoa/cm^3 and kept at 22°C prior to PAF treatment.

Treatment of cryopreserved spermatozoa with PAF

Cryopreserved spermatozoa (20×10^6 spermatozoa/cm^3) were treated with different concentrations of PAF: 1×10^{-3} M, 1×10^{-4} M, 1×10^{-5} M and 1×10^{-6}M at 22°C. Cryopreserved semen diluted with Extender 1, without PAF supplementation, was used as the control. Sperm viability (motility, plasma membrane integrity and mitochondrial function) and ATP content were examined at different time intervals: 0, 30, 60 and 120 min for each PAF treatment together with the control (without PAF).

Sperm viability assessments

Motility

Sperm motility was evaluated using the computer assisted semen analysis (CASA) system (VideoTesT Sperm 2.1). Aliquots of sperm samples were placed on 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 analyzed in accordance with the recommendations given by the World Health Organization (1999). The parameters analyzed included total sperm motility (%), linear motile spermatozoa (%), circular motile spermatozoa (%) and locally motile spermatozoa (%).

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 previously described methods (Thomas et al. 1998, Garner and Thomas 1999), with some modifications (Dziekońska et al. 2009). Aliquots of sperm samples (20×10^6 spermatozoa/cm^3) were incubated with JC-1 solution (1 mg 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 × g, 5 min at room temperature) and the sperm pellets were re-suspended in a HEPES buffered solution (10 mM HEPES, 0.85% NaCl, 0.1% bovine serum albumin, pH 7.4). Stained sperm samples were placed on microscopic slides, covered with coverslips (22 × 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 100 spermatozoa were counted per slide.

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 (20×10^6 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 × magnification under a fluorescence microscope (Olympus CH 30 RF-200, Tokyo, Japan). Sperm cells displaying only bright green fluorescence were considered viable spermatozoa with an undamaged plasma membrane. A minimum of 100 cells per slide was examined in random fields of each aliquot.

The sperm plasma membrane integrity was also assessed using a membrane-impermeable DNA binding stain, Hoechst 33258 (Calbiochem-Behring Corporation, La Jolla, CA, USA), as described by Woelders (1988). Aliquots of sperm samples (20×10^6 spermatozoa/cm^3) were stained with H 33258 solution (1 mg/10 cm^3 sodium citrate buffer, pH 7.0) in the dark for 5 min at room temperature. The stained sperm samples were observed under a fluorescence microscope (Olympus CH 30 RF-200) and classified as spermatozoa with an undamaged or damaged plasma membrane. A minimum of 100 cells per slide was examined in random fields of each aliquot.

Sperm ATP content

Sperm suspensions (20×10^6 spermatozoa/cm^3) were added to a boiling extraction medium containing 100 mM Tris and 4 mM ethylenediamine tetra acetic

acid, EDTA (pH 7.75). The mixture was boiled in a water bath for 5 min (100°C), cooled and subsequently centrifuged (15 000 × g, 15 min). ATP content was measured in the supernatants using a Bioluminescence Assay Kit CLSII (Roche Diagnostics, GmbH, Basel, Switzerland), according to the manufacturer's protocol. Luminescence was read with a Junior Bioluminometer (Berthold Technologies, GmbH & Co. KG, Germany). The ATP content of spermatozoa was calculated from an ATP standard curve and expressed as nmol ATP/10^8 spermatozoa.

Statistical analysis

Values were expressed as the mean ± standard deviation (SD) of 16 ejaculates collected from the 5 dogs. The data were analyzed by ANOVA followed by Duncan multiple comparison test, using the Statistica software package (StatSoft Incorporation, Tulsa OK., USA). Differences between means were considered significant at $p \leq 0.05$.

Results

There were no significant differences ($p \geq 0.05$) in pre-freezing sperm quality characteristics among the dogs. In fresh semen, the overall percentage (mean ± S.D) of total sperm motility was 87.5.0 ± 4.1 and sperm concentration was 740.0 ± 290.0 × 10^6 spermatozoa/cm^3.

The effect of different concentrations of PAF on total motility of cryopreserved canine spermatozoa is shown in Fig. 1. There was a slightly higher proportion of cryopreserved motile spermatozoa following treatment with 1 × 10^{-3} M PAF at 30 and 60 min of incubation compared with the control. It was observed that PAF used at a concentration of 1 × 10^{-3} M exerted a more beneficial effect ($p \leq 0.05$) on total motility of cryopreserved spermatozoa at incubation for 120 min compared with the control and other PAF treatments (1 × 10^{-4} to 10^{-6} M). Total motility of cryopreserved spermatozoa was approximately 2-fold greater than the control after treatment with 1 × 10^{-3} M PAF at 120 min of incubation.

The effect of different concentrations of PAF on sperm motility patterns, represented by linear and locally motile spermatozoa, following treatment of cryopreserved canine semen is shown in Fig. 2. The percentage of spermatozoa showing linear movement following treatment of cryopreserved semen with PAF was significantly higher ($p \leq 0.05$) at 120 min of incubation compared with the control, regardless of PAF concentrations (Fig. 2A). There were no marked differences ($p \geq 0.05$) among other PAF treatments at 120 min of incubation, with respect to linear motility of cryopreserved spermatozoa. No significant differences ($p \geq 0.05$) in the percentage of spermatozoa showing circular movements were observed following treatment of cryopreserved semen with different concentrations of PAF, independent to the incubation time (data not shown). However, there was a significant effect ($p \leq 0.05$) of PAF treatment of cryopreserved semen on the percentage of locally motile spermatozoa at 120 min of incubation (Fig. 2B). The percentage of locally motile spermatozoa was approximately 2-fold greater than the control after treatment of cryopreserved semen with 1 × 10^{-3} M PAF at 120 min of incubation and there were no significant differences when PAF was used at a very low concentration (1 × 10^{-6} M).

Mitochondrial function and the plasma membrane integrity of cryopreserved spermatozoa deteriorated over time, as indicated by fluorescence staining with JC-1/PI assay (Fig. 3A), SYBR-14/PI assay (Fig. 3B) and Hoechst 258 (Fig. 3C). Treatment of cryopreserved semen with different concentrations of PAF did not improve the sperm mitochondria functional (Fig. 3A) or the sperm plasma membrane integrity (Fig. 3B and 3C). In addition, no significant differences ($p \geq 0.05$) in mitochondrial function or plasma membrane integrity of cryopreserved spermatozoa were observed among PAF treatments at the same time.

The effect of different concentrations of PAF on ATP content of cryopreserved canine spermatozoa is shown in Fig. 4. Treatment of cryopreserved semen with 1 × 10^{-3} M PAF showed consistently higher ($p \leq 0.05$) ATP content of spermatozoa compared with the control or other PAF treatments (1 × 10^{-4} M to 1 × 10^{-6} M). It was observed that treatment of cryopreserved semen with 1 × 10^{-3} M PAF was the best concentration in which there was a consistent enhancement of sperm ATP production, irrespective of the incubation time. No marked differences in sperm ATP content among other PAF treatments were observed, regardless of the incubation time.

Discussion

Even though there has been an unexpected surge in canine semen technologies, there are still problems facing the use of cryopreserved semen. This study was conducted mainly to find out whether exogenous PAF supplementation in the thawing extender could improve post-thaw survival of canine spermatozoa.

Motility is the criterion mostly used for routine semen evaluation, both before and after cryopreservation (Ford 2006, Pena et al. 2006). Spermatozoa need to be motile and gain hyperactivated movement in order to fertilize the oocyte (Ricker et al. 1989).

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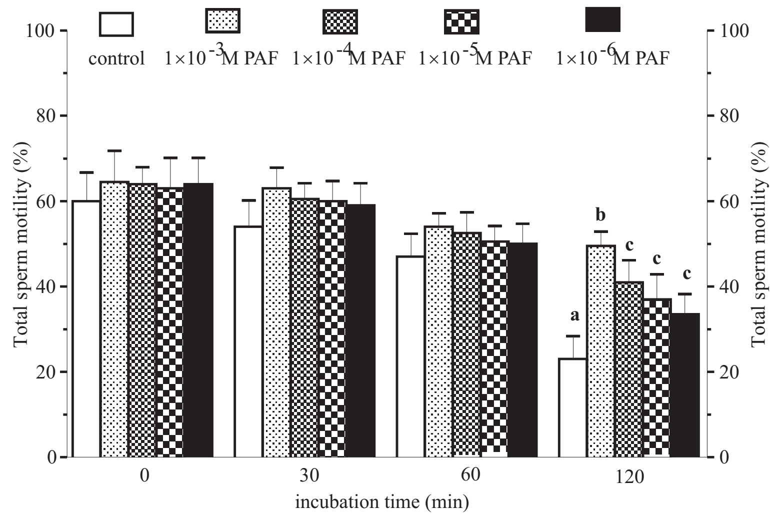

Fig. 1. Percentage of total motility of canine spermatozoa following treatment of cryopreserved semen with different concentrations of platelet-activating factor (PAF). Sperm motility was assessed by the computer-assisted semen analysis (CASA) system. Values represent the means ± SD of 16 ejaculates from 5 dogs. Within incubation time, values (a, b, c) with different letters are significant at $p \leq 0.05$.

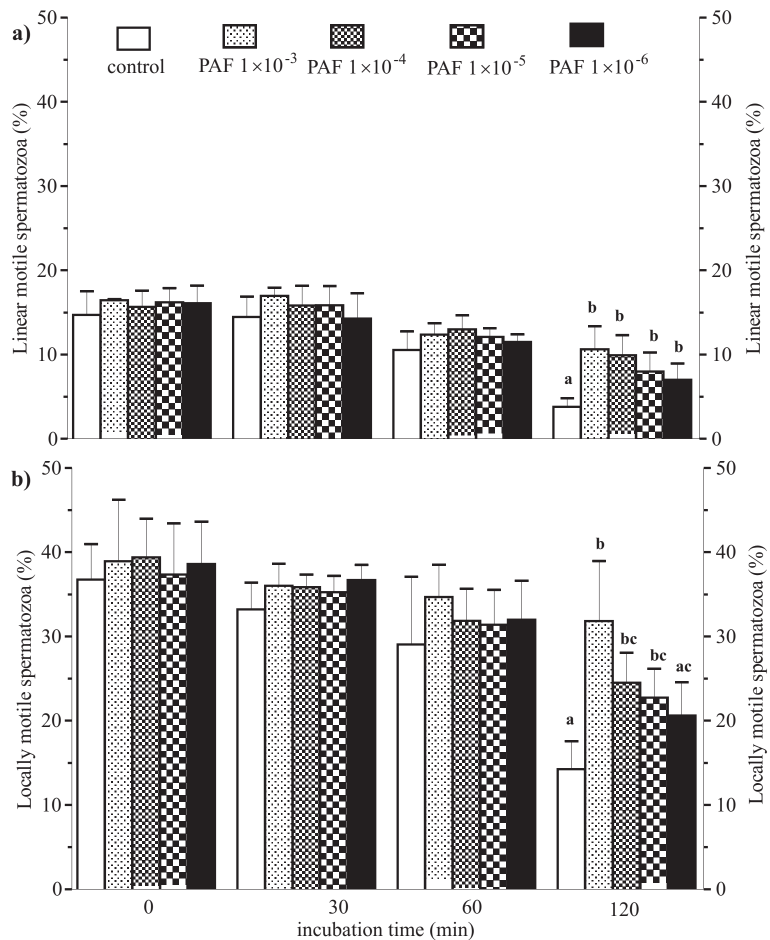

Fig. 2. Percentage of linear (A) and locally motile (B) canine spermatozoa following treatment of cryopreserved semen with different concentrations of platelet-activating factor (PAF). Sperm motility patterns were analyzed by the computer-assisted semen analysis (CASA) system. Values represent the means ± SD of 16 ejaculates from 5 dogs. Within incubation time, values (a, b, c) with different letters are significant at $p \leq 0.05$.

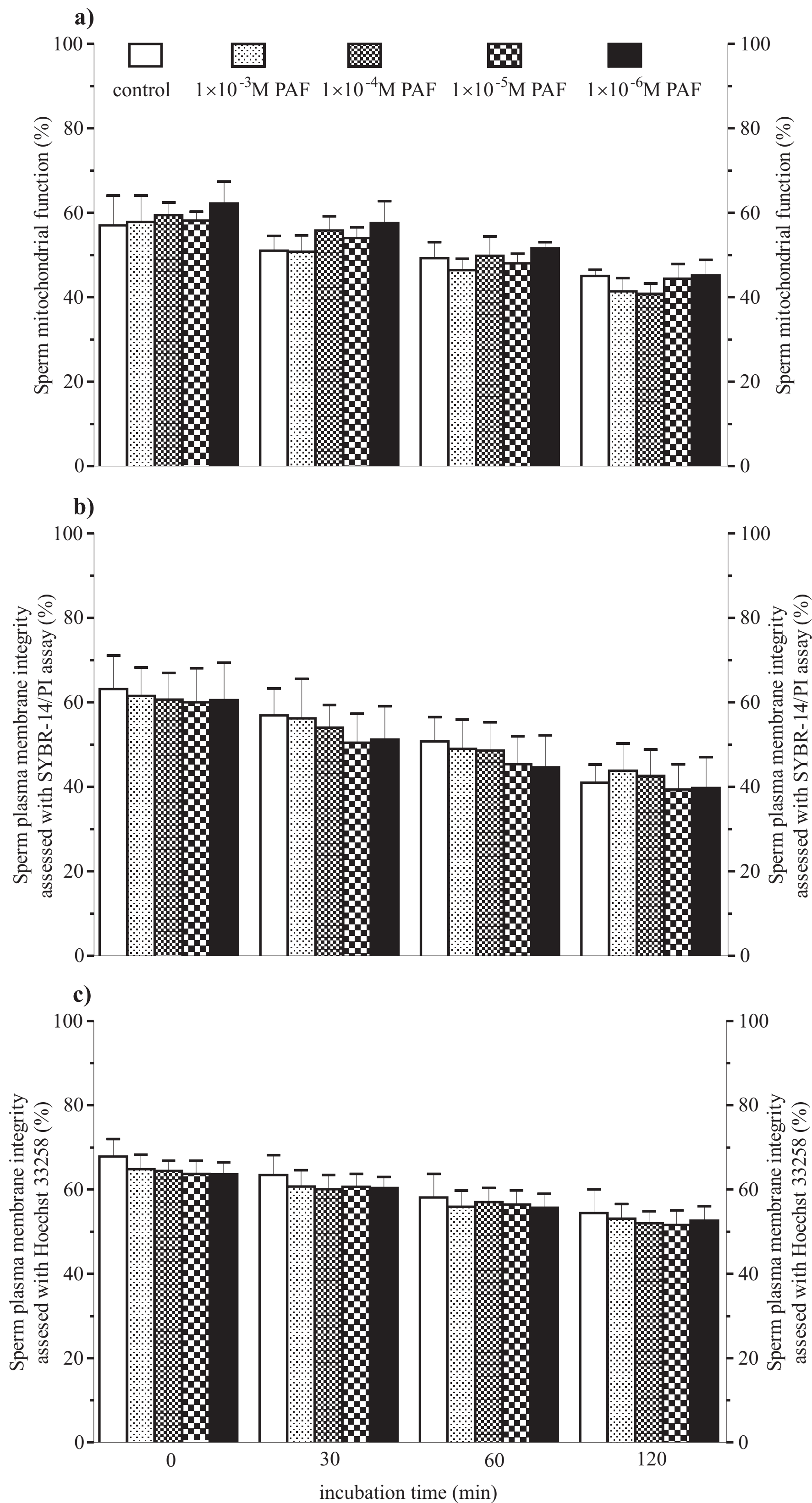

Fig. 3. Percentage of mitochondrial function assessed with JC-1/PI assay (A) and plasma membrane integrity assessed with SYBR-14/PI assay (B) and Hoechst 33258 (C) of canine spermatozoa following treatment of cryopreserved semen with different concentrations of platelet-activating factor (PAF). Values represent the means ± SD of 16 ejaculates from 5 dogs.

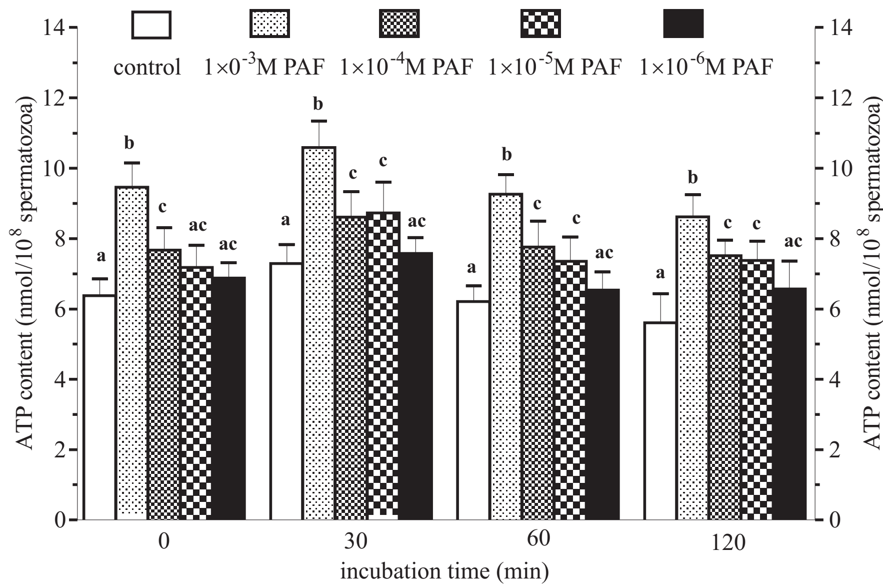

Fig. 4. ATP content of canine spermatozoa following treatment of cryopreserved semen with different concentrations of platelet-activating factor (PAF). Values represent the means ± SD of 16 ejaculates from 5 dogs. Within incubation time, values (a, b, c) with different letters are significant at $p \leq 0.05$.

Recent studies have shown that PAF induces changes in sperm motility parameters that are related to capacitation (Odeh et al. 2003, Kordan et al. 2009). In the present study, cryopreserved spermatozoa exposed to 1×10^{-3} M PAF at 120 min of incubation resulted in a significantly higher percentage of motile spermatozoa. Furthermore, PAF-improved motility following treatment of cryopreserved semen was associated with enhanced sperm motility patterns, such as linear and locally motile spermatozoa. It was demonstrated that the incorporation of 1×10^{-7} M PAF into boar semen extender during liquid storage or exposure of cryopreserved spermatozoa to PAF concentrations ranging from 1×10^{-5}M to 1×10^{-6} M stimulated sperm motility (Kordan and Strzeżek 2002, Kordan et al. 2009). Moreover, it has been reported that there was a 3-fold improvement in motility of fresh human spermatozoa after exposure to 1×10^{-8} M PAF (Krausz et al. 1994). Treatment of fresh stallion semen with PAF concentrations ranging from 1×10^{-9} M to 1×10^{-12} M resulted in a significant increase in sperm motility (Odeh et al. 2003), whereas exposure of fresh bovine semen to PAF did not stimulate sperm motility (Parks and Hough 1990). A previous study showed that PAF content in boar spermatozoa was positively correlated with progressive sperm motility and related to the fertilizing ability of spermatozoa (Roudebush and Diehl 2000). In contrast, PAF content in ram spermatozoa was not correlated either with motility or fertility status (O'Meara et al. 2008). These findings and those of the present study indicate that PAF action on sperm motility apparatus is dose-dependent and varies among species, probably due to differences in the sperm plasma membrane composition, such as proteins, phospholipids or cholesterol.

It is interesting to note that PAF-improved ATP content of cryopreserved spermatozoa at each incubation time was not consistently accompanied by a similar improvement in sperm motility. Furthermore, cryopreserved semen treated with PAF did not show significant differences in JC-1/PI staining, suggesting a lack of direct effect on the sperm mitochondrial activity. In addition, the results of the present study demonstrated that even though treatment of cryopreserved semen with 1×10^{-3} M PAF at 120 min of incubation caused a marked improvement in sperm motility, similar changes were not observed in the sperm mitochondrial function, as monitored by the JC-1/PI assay. It has been confirmed that ATP is synthesized by glycolysis in the cytoplasm of the principal sperm flagellum or through oxidative phosphorylation in the mitochondria of spermatozoa (Kamp et al. 2003, Ford 2006). Even though considerable advances have been made in understanding the role of glycolysis and oxidative phosphorylation in ATP production of spermatozoa, the results shown in the literature are still inconclusive. According to Ford (2006), sperm motility is dependent on intracellular ATP content. Moreover, impairment of the mitochondrial function of spermatozoa has been associated with reduced sperm motility (Thomas et al. 1998, Fraser et al. 2002). In the present study it is possible that the predominance of post-thaw sperm subpopulations with PAF-improved metabolic activity seems to rely on glycolytic ATP production. It seems that the improvement in post-thaw motility and ATP content of PAF-treated spermatozoa, without enhancement in

their mitochondria activity, might be due to dysfunction of ATP synthesis by the mitochondria rather than the impairment of the glycolytic pathway. More recently, it has been demonstrated that glycolysis is the major energy source and probably more important than oxidative phosphorylation in supporting sperm motility, hyperactivation and protein tyrosine phosphorylation (Hung et al. 2008).

Deterioration in post-thaw sperm membrane integrity is a factor that seriously compromises the quality of cryopreserved canine semen (Pena et al. 2006). In our study, the use of different fluorescence membrane markers, SYBR-14/PI assay and Hoechst 33258, showed that exogenous PAF did not render better effects on the plasma membrane integrity of cryopreserved spermatozoa. A recent study has shown that PAF used at a concentration of 1×10^{-7} M PAF caused destabilization in the plasma membrane integrity of boar spermatozoa during liquid semen storage (Kordan and Strzeżek 2006). Moreover, cryopreserved boar spermatozoa exposed to PAF concentrations ranging from 1×10^{-7} M to 1×10^{-8} M exhibited a slight increase in membrane permeability (Kordan and Strzeżek 2009). It has been suggested that PAF exerts its biological effect through specific membrane sites, which are predominantly concentrated in the midpiece and equatorial regions of spermatozoa, as indicated by immunofluorescence microscopy (Roudebush and Diehl 2000, Odeh et al. 2003). The findings of the current study indicated that exogenous PAF could exert different modes of action on the structures of sperm subpopulation by binding to specific membrane receptors following treatment of cryopreserved canine semen. However, the exact role of exogenous PAF in these mechanisms still remains to be elucidated.

Taken together, the findings of this study confirmed that sperm motility parameters, in conjunction with sperm ATP content, were best maintained when the cryopreserved semen was treated with 1×10^{-3} M PAF at 120 min of incubation. Such findings indicate that exogenous PAF addition rendered a better quality of cryopreserved canine semen, suggesting that it can be beneficial as a supplement for semen extender.

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