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# THE EFFECT OF DIFFERENT CONCENTRATIONS OF CAFFEINE, PENTOXIFYLLINE AND 2'-DEOXYADENOSINE ON THE BIOLOGICAL PROPERTIES OF FROZEN-THAWED CANINE SEMEN*

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**Abstract**
**Artificial insemination (AI) and semen cryopreservation are the most accessible and commonly used techniques for breeding domestic animals. Among many parameters, such as plasma membrane integrity and acrosome structure, one of the key factors that determine the quality of frozen-thawed samples for artificial insemination is sperm motility. Sperm motility is one of the key parameters that determine the quality of frozen-thawed samples for AI. The total number of progressively motile spermatozoa in thawed canine semen is correlated with fertility. A variety of substances were used to compare sperm motility with the control. The aim of this study was to determine the effect of semen extender supplementation with motility stimulants, pentoxifylline (PTX), caffeine (CAF) and 2'-deoxyadenosine (DX), after different post-thaw incubation times (30, 60, 120 min) on the motility, selected kinematic parameters, plasma membrane integrity and mitochondrial membrane potential of cryopreserved canine spermatozoa. During attempts to improve the quality of cryopreserved semen, the applied substances exerted beneficial effects at a concentration of 10 mM. We demonstrated that both phosphodiesterase inhibitors, caffeine and pentoxifylline, as well as 2'-deoxyadenosine increased the motility and selected kinematic parameters of thawed canine spermatozoa.**

**Key words: canine, cryopreservation, motility stimulants, kinematic motility**

Artificial insemination (AI) and semen cryopreservation are the most accessible and commonly used techniques for breeding domestic animals. However, fewer assisted reproduction techniques have been developed for dogs than farm animals (Jewgenow et al., 2017; Lucio et al., 2017). Despite the above, there is a growing interest in frozen dog semen. The freezing-thawing procedure supports the preservation and propagation of genetic material. Cryopreservation also enables long-dis-

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tance transport of semen, reduces animal transport costs and increases the genetic diversity of species (Comizzoli et al., 2009; Belala et al., 2016; Lucio et al., 2017). Canine semen has low resistance to freezing due to the alternations in phospholipids/cholesterol ratio triggered by freezing process, which makes cells vulnerable with the spermatozoa plasma membrane destabilization and consequent reduced sperm fertility (Inanc et al., 2018). Cryopreservation can contribute to a decrease in sperm motility, loss of membrane integrity and acrosome structure, as well as damage to sperm DNA (Kim et al., 2010; Karger et al., 2017). The above risks necessitate new solutions to enhance the post-thaw parameters of canine spermatozoa, optimize oocyte fertilization and increase contraception rates.

Sperm motility is one of the key parameters that determine the quality of frozen-thawed samples for AI (Van den Berghe et al., 2018). The total number of progressively motile spermatozoa in thawed canine semen is correlated with fertility (Mason et al., 2017).

Sperm motility can decrease as a result of changes in plasma membrane permeability in the tail region of spermatozoa, and the formation of ice crystals in mitochondria and axonemes during cryopreservation (Rasul et al., 2001; Yu et al., 2002; Prapaiwan et al., 2016). In the past, light microscopy was routinely used to evaluate the main semen parameters. Today, spermatozoa are accurately and rapidly assessed with the use of computer-assisted sperm analysis systems (CASA). These systems are capable of detecting even minor changes in sperm motility, and they are highly useful tools for improving the quality of cryopreserved canine semen (Rijsselaere et al., 2012). In addition, it is possible to visualize the trajectory of movement of individual spermatozoa, enabling a detailed description of parameters related to motility and type of movement (Antończyk et al., 2010). Furthermore, evaluation of kinematic parameters of sperm after cryopreservation process could be insufficient to determine the structural and biochemical changes in gametes. The maintenance of the sperm fertilization potential depends also on plasma membrane integrity and mitochondrial membrane potential of spermatozoa (Fraser et al., 2002; Dziekońska et al., 2009).

Various substances have been used to improve the quality of cryopreserved semen. The most popular motility stimulants include methylxanthines such as caffeine (CAF) and pentoxifylline (PTX) which are competitive and non-selective inhibitors of cyclic adenosine monophosphate (cAMP) phosphodiesterase that increase intracellular cAMP concentration and enhance tyrosine phosphorylation in the sperm tail region (Yunes et al., 2005; Esteves et al., 2007; Brie et al., 2016). It is known that PTX inhibits tumour necrosis factor-alpha which is responsible for DNA fragmentation and programmed cell death. It also reduces superoxide anions and lipid peroxidation associated with sperm membrane damage (Peeker et al., 1997; Mundle et al., 1999; Zhang et al., 2005).

2'-deoxyadenosine (DX), an adenosine analogue with a modified ribose ring, delivers similar effects. 2'-deoxyadenosine stimulates motility via a different molecular mechanism which probably involves the activation of A2-receptor-mediated adenylate cyclase which increases the intracellular concentration of cAMP (Milani et al., 2010).

Motility enhancers exerted positive effects in fresh and frozen-thawed semen of several animal species, including humans (Carrel and Aston, 2013), bulls (Zhang et al., 2014), stallions (Stephens et al., 2013), rams (Maxwell et al., 1995), dogs (Milani et al., 2010) and turkeys (Slanina et al., 2018).

The aim of this study was to determine the effect of semen extender supplementation with motility stimulants, caffeine (CAF), pentoxifylline (PTX) and 2'-deoxyadenosine (DX), after different post-thaw incubation times (30, 60, 120 min) on the motility, selected kinematic parameters, plasma membrane integrity and mitochondrial membrane potential of cryopreserved canine spermatozoa.

## Material and methods

### Chemicals
The chemicals were purchased from: Sigma-Aldrich Chemical Company (Saint Louis, USA) – caffeine, pentoxifylline, 2'-deoxyadenosine, Tris; POCh (Gliwice, Poland) – citrate; Chempur (Piekary Śląskie, Poland) – fructose; Minitüb (Tiefenbach, Germany) – Orvus Es Paste (Equex STM).

### Semen collection
Semen was obtained from four mixed-breed dogs. 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*. 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. Sperm-rich fractions of ejaculates were collected once weekly over a period of 10 weeks. The dogs were housed in individual pens and fed commercial canine food. Water was available *ad libitum*. Permission to conduct this study was granted by the Local Ethics Committee for Animal Experiments (LKE.065.01.2017).

A preliminary assessment of the ejaculates involved an evaluation of sperm motility and determination of sperm cell concentrations by the cytometric method in a hemocytometer. Semen samples with total motility higher than 80% and sperm counts higher than $200 \times 10^6$ cells per ml of semen were qualified for further analyses.

### Freezing-thawing procedure
The procedure of cryopreservation was conducted according to Nizanski et al. (2001) with some modifications (Strzeżek et al., 2015). Briefly, after individual evaluation, semen samples were pooled and centrifuged ($700 \times g$, 5 min). Seminal plasma was removed, semen sediments were diluted with standard Tris-citrate-fructose (TCF) extender (Rota et al., 1995) to a concentration of $2 \times 10^8$ spermatozoa/ml and left to stand for 30 minutes at room temperature (20°C). The samples were then cooled to 5°C for 60 minutes. After chilling, a second dilution (1:1) was performed

with an extender containing 5.0 ml of TCF, 4.0 ml of chicken egg yolk, 0.8 ml of glycerol and 0.2 ml of Orvus Es Paste to obtain a concentration of $1 \times 10^8$ spermatozoa/ml. The final content of chicken egg yolk, glycerol and Orvus Es Paste in diluted semen samples was determined at 20%, 4% and 1%, respectively. Equilibration time at 5°C was 15 minutes. Semen was loaded into 0.25 ml plastic straws (Minitüb, Germany) and frozen in a closed styrofoam box by placing filled straws on a rack suspended 4 cm above the surface of liquid nitrogen. Freezing time was 10 minutes. Frozen straws and tubes were placed in a container with liquid nitrogen. Straws were thawed in a water bath at 70°C for 5 s (Nöthling et al., 2005).

### Application of motility stimulants

Thawed semen was aliquoted and mixed with: 1) TCF extender plus caffeine at final concentrations of 5 mM, 10 mM, 20 mM; 2) TCF extender plus pentoxifylline at final concentrations of 5 mM, 10 mM, 20 mM; 3) TCF extender plus 2'deoxyadenosine at final concentrations of 5 mM, 10 mM, 20 mM.

Sperm viability (motility, plasma membrane integrity and mitochondrial function) was examined at different time intervals of 0, 60 and 120 min for different variants of the extender relative to the control. The incubation temperature was 22°C (Kordan et al., 2010).

### Motility

Sperm motility characteristics were evaluated using the Hamilton-Thorne Sperm Analyser IVOS version 12.3 (Hamilton-Thorne Biosciences, USA). Software settings recommended by the manufacturer for canine sperm analyses were used: frame acquired – 30, frame rate – 60 Hz, minimum cell contrast – 75, minimum cell size – 6 pixels, straightness threshold – 75%, path velocity threshold – 100 μm/s, low velocity average pathway VAP (velocity straight line) cut-off – 9.9 μm/s, low VSL (velocity straight line) cut-off – 20 μm/s, static size gates – 0.80–4.93, static intensity gates – 0.49–1.68, static elongation gates – 22–84. Total motility (%), progressive motility (%), velocity average pathway (VAP, μm/s), velocity straight line (VSL, μm/s), curvilinear velocity (VCL, μm/s), amplitude lateral head (ALH, μm), beat cross frequency (BCF), linearity (LIN, %) and straightness (STR, %) were determined by the IVOS analyser.

### Plasma membrane integrity

Sperm plasma membrane integrity was assessed by dual fluorescent staining with SYBR-14 and PI (Live/Dead Sperm Viability Kit; Molecular Probes, Eugene, USA), as described by Garner and Johnson (1995), with minor modifications. Briefly, aliquots of sperm samples ($20 \times 10^6$ spermatozoa/ml) were incubated with SYBR-14 (1 mM SYBR-14 in DMSO) and PI solutions (2.4 μM PI in Tyrode's salt solution) for 10 min at 37°C. After incubation, stained sperm cells were placed on microscopic slides and examined under a fluorescence microscope (Olympus CH 30 RF-200, Tokyo, Japan) at 600 × magnification. Sperm cells displaying only bright green fluorescence were considered as viable spermatozoa with an undamaged plasma membrane. A minimum of 200 cells per slide was examined in random fields of each aliquot.

### Mitochondrial function assessed by fluorescent microscopy

Sperm mitochondrial function was assessed by 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 (Garner and Thomas, 1999) with some modifications (Dziekońska et al., 2009). Aliquots of sperm samples ($20 \times 10^6$ spermatozoa/ ml) were incubated with JC-1 solution (1 mg of JC-1/ml dimethyl sulfoxide, DMSO) for 15 min at 37°C. After incubation, sperm samples were stained with PI (10 μl of PI solution in 0.5 mg/ml phosphate buffered solution) for 10 min at 37°C, washed ($600 \times g$, 5 min at room temperature), and sperm pellets were resuspended 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 ($22 \times 22$ mm) and examined under a fluorescence microscope (Olympus CH 30 RF-200, Tokyo, Japan). Viable spermatozoa with functional mitochondria emitted orange-red fluorescence. Two slides were evaluated per sample, and 200 spermatozoa were counted per slide.

### Statistical analysis

Data were expressed as means ± SEM and analysed by one-way analysis of variance (ANOVA). The mean values were compared between the control group and treatment groups using Duncan's multiple range test in the Statistica software package (StatSoft Inc., Tulsa, OK, USA). Differences were considered significant at $P \leq 0.05$.

## Results

### Total motility

The influence of motility stimulants on total sperm motility in samples incubated over different times is shown in Figure 1A. Total motility was significantly higher in thawed semen samples supplemented with motility stimulants at a concentration of 10 mM, regardless of stimulant type and incubation time, relative to control. Caffeine and 2'-deoxyadenosine applied at a concentration of 5 mM improved total motility after 0 and 60 minutes of incubation.

### Progressive motility

Progressive motility was significantly higher in all semen samples treated with CAF, PTX and 2 DX at concentrations of 5 and 10 mM, regardless of incubation time (Figure 1B).

### Kinematic parameters of sperm motility

When applied at a concentration of 10 mM, all of the tested stimulants significantly influenced VAP after 0, 60 and 120 minutes of incubation. A similar result was noted in VSL values, excluding semen samples treated with PTX. VCL was

most influenced by CAF at a concentration of 10 mM, however, CAF did not exert a significant influence on VCL values immediately after supplementation. DX and PTX did not influence the analysed parameter, but in non-incubated samples, VCL was significantly higher under exposure to pentoxifylline at a concentration of 10 mM. ALH increased significantly after treatment with 10 mM of CAF and 10 mM of PTX, regardless of incubation time. BCF remained constant through incubation with no significant differences (Figure 2). The highest percentage of spermatozoa characterized by linear movement was observed in semen samples treated with 10 mM of CAF after 60 minutes of incubation. The highest STR values were noted after the application of CF, DX and PTX at a concentration of 10 mM immediately after supplementation, and after 120 minutes of incubation with 10 mM of CAF and 10 mM of DX.
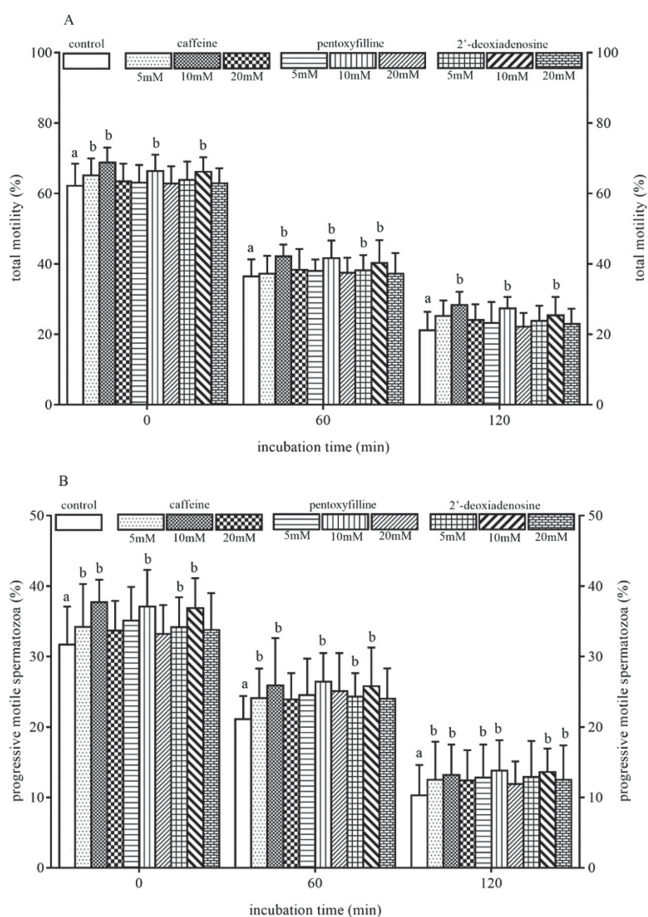


Figure 1. Percentage of total (A) and progressive motility (B) of frozen-thawed canine spermatozoa supplemented with different concentrations of caffeine, pentoxifylline and 2'-deoxyadenosine. The results are expressed as the mean values ± SEM of 20 ejaculates from 4 dogs. Values (a, b) with different letters are significant at P≤0.05 for every incubation time
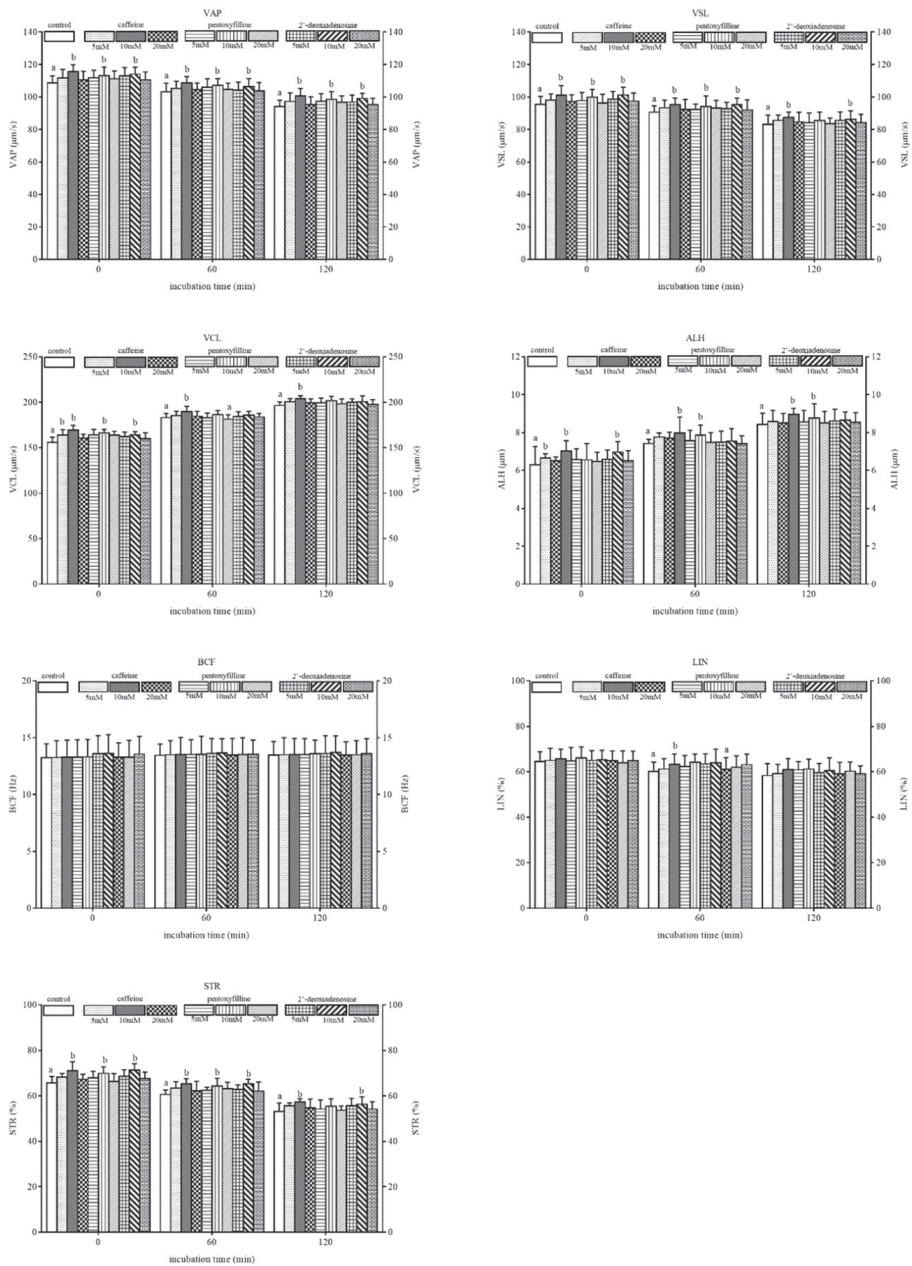
Figure 2. The mean values ± SEM of VAP, VSL, VCL, ALH, BCF, LIN and STR in thawed semen supplemented with different concentrations of motility stimulants during 120 minutes of incubation, determined by the CASA method. The results are expressed as mean values ± SEM. Values (a, b) with different letters are significant at P≤0.05 for every incubation time
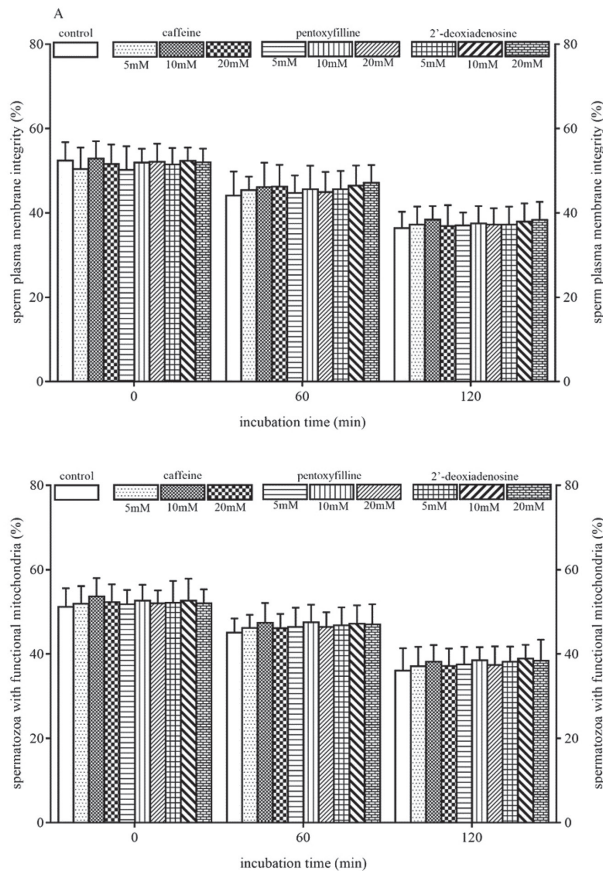
Figure 3. The percentage of canine spermatozoa with integral plasma membrane determined in the SYBR-14/PI assay (A) and the percentage of canine spermatozoa with functional mitochondria determined in the JC-1/PI assay (B) after the addition of different concentrations of caffeine, pentoxifylline and 2'-deoxyadenosine. The results are expressed as mean values ± SEM

### Plasma membrane integrity and mitochondrial membrane potential

The influence of different concentrations of motility stimulants after 0, 60 and 120 minutes of incubation on plasma membrane integrity and mitochondrial activity is shown in Figure 3A and Figure 3B, respectively. Significant differences in the percentage of spermatozoa with intact plasma membrane and higher mitochondrial membrane potential were not observed relative to control.

### Discussion

Despite the availability of sophisticated assisted reproductive techniques, frozen-thawed semen is characterized by lower motility and viability than freshly ejaculated

sperm. Cryopreservation induces crystallization and osmotic changes which can cause cellular damage (Stănescu and Bîrţoiu, 2011; Setyawan et al., 2015). An intact plasma membrane is essential for the maintenance of sperm cell integrity and the events associated with oocyte fertilization. However, most freezing-induced injuries in spermatozoa affect the plasma membrane (Bailey et al., 2000; Lucio et al., 2017).

A variety of extenders and cryopreservation protocols have been developed over the years, but the whelping rates of frozen dog semen are still unsatisfactory in comparison with fresh semen (Uchoa et al., 2012; Rodenas et al., 2014; Johnson et al., 2014). In dogs, the success of AI with cryopreserved semen is considerably limited due to problems associated with insemination timing (Alhaider and Watson, 2009). The selection of the appropriate extender, the freezing process and the thawing protocol are critical for the success of AI and the quality of cryopreserved canine sperm (Silva et al., 2006; Karger et al., 2017).

The purpose of our study was to verify whether post-thaw motility parameters, plasma membrane integrity and mitochondrial membrane potential can be improved by adding motility stimulants at different concentrations to the thawing medium. The results indicate that both phosphodiesterase inhibitors, caffeine and pentoxifylline, as well as 2'-deoxyadenosine increased the motility and selected kinematic parameters (VAP, VSL, VCL, ALH, BCF, LIN, STR) of thawed canine spermatozoa relative to control. Nevertheless, we did not observe any significant influence of used substances on plasma membrane integrity and mitochondrial membrane potential. We found that the tested substances exerted beneficial effects at a concentration of 10 mM. Similar results were reported by López and Alvariño (2000) in rabbits. The authors found that sperm motility increased at a higher concentration of caffeine (10 mM), but was not affected at lower caffeine concentrations (2.5 or 5 mM). In other studies, high caffeine concentrations exerted opposite effects on sperm motility. Stephens et al. (2013) demonstrated a non-significant increase in motility parameters after the addition of CAF at concentrations of 1, 2 and 3.5 mmol/L to thawed equine semen. The addition of 5 mM of caffeine also significantly enhanced the motility of bovine semen (Barakat et al., 2015). Caffeine also improved the qualitative parameters of spermatozoa in boars (Yamaguchi et al., 2013) and laboratory mice (Nabavi et al., 2013). These findings suggest that caffeine's effect on sperm motility parameters could be species-specific.

In the present study, PTX exerted a similar effect on sperm motility parameters. Semen samples supplemented with 10 mM of pentoxifylline were characterized by significantly higher motility parameters relative to control. Similar findings were reported by Koutsarova et al. (1997). The authors found that the addition of pentoxifylline at a concentration of 0.0036 mol/L significantly increased the percentage of progressively motile spermatozoa in fresh semen, and that the addition of PTX at a concentration of 0.0072 mol/L to semen samples at thawing improved the quality of frozen-thawed sperm.

The addition of PTX to semen samples before cryopreservation significantly decreased the velocity, linearity and motility of spermatozoa, which could result from the depletion of the available energy substrate (Yovich et al., 1990). However, various authors demonstrated that the addition of PTX to thawed semen significantly

increased sperm parameters. In a study by Gradil and Ball (2010), the addition of pentoxifylline (3.5 or 7.0 mM) to cryopreserved equine semen immediately after thawing significantly increased total and progressive motility relative to control. Furthermore, Stephens et al. (2013) observed that use of pentoxifylline increased sperm VCL, VAP and VSL in equine semen. Similar results were noted in previous studies where PTX treatment increased the percentage of motile spermatozoa in frozen-thawed human, feline and boar semen (Hammitt et al., 1989; Stachecki et al., 1995). In many previous studies, pentoxifylline was used at a concentration of 3.6 mM of (Tesarik et al., 1992; Kay et al., 1993; Lewis et al., 1993) but the beneficial effects of this motility stimulant were observed at both lower and higher concentrations (Kay et al., 1993; Lewis et al., 1993; Centola et al., 1995; Sharma et al., 1996). Esteves et al. (2007) demonstrated that the addition of pentoxifylline to low-quality human sperm before freezing did not prevent acrosomal loss during the cryopreservation process and did not improve post-thaw motility or viability. Despite the above, pentoxifylline improved the ability of thawed spermatozoa to undergo the acrosome reaction in response to calcium ionophore.

Bani-Hani et al. (2011) suggested that the stimulatory effect of PTX on semen motility could be modulated by higher energy production. When added at a concentration of 5 mM, PTX increased the activity of seminal creatine kinase, an enzyme which catalyses the formation of ATP by the creatine-creatine phosphate shuttle (Banihani and Abu-Alhayjaa, 2016).

There is a general scarcity of published studies investigating the influence of 2'- deoxyadenosine on frozen-thawed spermatozoa. This motility stimulant can increase the proportion of motile spermatozoa by approximately 21–39% and induce changes in motility characteristics, linear velocity and the frequency of sperm head rotation in human semen. The percentage of penetrated oocytes was determined at 80.8% in the hamster zona-free ovum test (Mbizvo et al., 1993; Lanzafame et al., 1994) demonstrated that DX exerted a prolonged effect on hyperactivated motility. Similarly to our findings, Milani et al. (2010) reported that the addition of 5 mM and 7.5 mM of DX had a positive effect on the motility of thawed canine semen. Imoedemhe et al. (1992) observed a significant improvement in sperm motility and the number of recoverable human spermatozoa relative to unexposed control samples. In the present study, sperm membrane integrity was nearly equally enhanced by the addition of pentoxifylline and 2-deoxyadenosine to sperm prepared with the two-layer Percoll gradient method.

There are limited reports concerning the influence of pentoxifylline and 2-deoxyadenosine on sperm membrane integrity. A positive correlation between the percentage of sperm tail swelling patterns and motile spermatozoa was found in a study investigating membrane functional integrity in mouse spermatozoa supplemented with pentoxifylline (Ponce et al., 1999). Laokirkkiat et al. (2007) demonstrated that the addition of pentoxifylline or 2-deoxyadenosine to human semen prepared with the two-layer Percoll gradient method led to nearly equal enhancement of sperm membrane integrity.

In theory, spermatozoa are the most suitable candidates for cryopreservation on account of their general properties. However, freezing-thawing can severely impair

the function of male gametes. Cryoinjury can be caused by various factors, including membrane disturbances and changes in osmotic pressure which lead to changes in cell volume (Pena, 2007). The motility of frozen-thawed spermatozoa can be compromised by active transport and changes in plasma membrane permeability in the tail region of sperm cells (Yu et al., 2002).

In conclusion, the motility enhancers used in our study (CAF, PTX, DX) improved post-thaw motility, selected kinematic parameters of motility (VAP, VSL, VCL, ALH, BCF, LIN, STR) and the overall quality of frozen-thawed canine semen. These effects were concentration-dependent, in particular in CAF and PTX. All of the tested motility enhancers produced the most satisfactory results when administered at a concentration of 10 mM. However, the applied treatments did not exert a significant influence on plasma membrane integrity or mitochondrial membrane potential in cryopreserved dog semen.

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