Specific phosphodiesterase type-10 inhibitor, papaverine, added after the cooling period improves canine sperm quality

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Abstract

The use of chilled semen has gained increasing interest in canine reproductive services. The addition of phosphodiesterase (PDE) inhibitors that increase the intracellular cyclic adenosine monophosphate levels may improve sperm motility. The purpose of this study was to examine the quality of sperm under the effect of the specific PDE-10 inhibitor (papaverine) added after storage for 1, 2, and 3 days at 5°C. The ejaculates were obtained from 5 healthy Beagle dogs by digital manipulation. After collection, ejaculates were pooled, extended and cooled at 5°C during 3 days. Sperm parameters were tested 30 min after the addition of different papaverine (PA) concentrations: 0, 5, 10 and 20μM. Sperm motility (CASA), viability (PI/FITC-PNA) and capacitation status (chlortetracycline assay) were evaluated. The results showed that the addition of PA has no effect on sperm samples at day 0. However, concentrations of 5 and 10μM increased ($P<0.05$) sperm motility kinetics and viability significantly compared to the control at day 1, day 2 and day 3 of cooling. The addition of 20μM PA decreased ($P<0.05$) sperm quality parameters significantly and increased the percentage of capacitated/acrosome-reacted spermatozoa. In conclusion, the addition of 5 and 10μM PA concentrations after cooled storage improved canine sperm quality.

Keywords

Canine sperm; Phosphodiesterase type-10 inhibitor; Papaverine; Sperm quality

Introduction

In recent years, artificial insemination with chilled semen has become the most utilised reproduction biotechnology in canine species. Semen conservation at 5ºC has several advantages related to easy handling and shipping, low cost because it does not require special equipment and minor legal requirements for import and export compared to frozen semen (Shahiduzzaman and Linde-Forsberg 2007). In addition, pregnancy rates and litter size are higher compared to cryopreserved sperm (Linde-Forsberg 1995).

The goal of chilled semen is to increase the lifespan of sperm, decrease the basal metabolism and preserving the quality parameters and fertility during short periods of time (Iguer-Ouada

and Verstegen 2001; Hori et al. 2013). However, semen longevity is limited due to the several changes produces it in the sperm, called collectively "cold shock". Motility decrease, damages in plasma and acrosomal membrane, loss of intracellular components and increase in plasma membrane permeability are observed (Watson 1990).

One strategy to prevent the deleterious effects of cooling storage is the use of extenders. The primary function is to provide essential nutrients to sperm, to prevent bacterial growth, maintaining stable medium, and to protect the integrity and functionality of the plasma membrane (Johnston et al. 2001). However, sometimes these media are insufficient to maintain seminal quality. Several researches have described the utilisation of different motility enhancers that did not affect longevity and increase sperm functionality (Linde- Forsberg 1995; Milani et al. 2009; Mirshokraei et al. 2011).

The sperm activators most widely used on human and animal reproduction are phosphodiesterase (PDE) inhibitors. These enzymes are metallohydrases that hydrolyse cyclic nucleotides (cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP)) in their corresponding 5'-nucleoside monophosphate in the presence of divalent cations. They can be specific for cAMP or cGMP or both substrates. The PDE superfamily contains 11 families (PDE1 to PDE11) identified according to their affinity to the substrate, biochemical properties, regulation and inhibitor sensitivities (Beavo 1995; Conti and Beavo 2007). Three of the PDE families are capable of hydrolysing exclusively cAMP (PDE4, PDE7 and PDE8), 3 are specific for cGMP (PDE5, PDE6, and PDE9) and the remaining 5 degrade both cyclic nucleotides (PDE1, PDE2, PDE3, PDE10 and PDE11).

The importance of the action of PDE on sperm functionality is related to their ability to hydrolyse cAMP. It participates in the acquisition of motility, sperm capacitation and acrosomic reaction, essential events for fertilization process (Visconti et al. 1999). Numerous PDE inhibitor substances have been used as activators of sperm motility in different species (Maxwell et al. 1995; Milani et al. 2009; Gil et al. 2010; Guasti et al. 2017). The most utilised are the family of methylxanthines, such as caffeine and pentoxifylline. The incorporation of caffeine and pentoxifylline in cryopreservation extender increased the kinetic parameters on dog

85 sperm (Milani et al. 2009). However, pentoxifylline supplementation in doses greater than 10
86 mM produces a capacitating effect and increases the levels of reacted and non-viable
87 spermatozoa (Mirshokraei et al. 2011). These effects have been described in numerous species
88 after incubation of sperm samples with different types of methylxanthines (Maxwell et al. 1995;
89 Gil et al. 2010; Guasti et al. 2017).

90 One of the PDE inhibitory substances less studied on seminal quality is papaverine (PA). It
91 is a specific PDE10 inhibitor, which increases tyrosine phosphorylation protein levels in sperm,
92 associated with sperm capacitation and hypermotility and regulating cAMP and cGMP
93 concentrations (Matthiesen and Nielsen 2011). In addition, PDE10 together PDE3 are the main
94 enzymes that catalyse cytosolic changes of cAMP in the sperm, so its regulation could
95 efficiently modify sperm functionality, such as motility (Matthiesen and Nielsen 2011). In
96 human and bovine species, PA produced an increase in cAMP levels but not sperm quality
97 (Torres-Flores et al. 2008; Bergeron et al. 2017). However, in a recent study, Terriou et al.
98 (2015) observed that supplementation with PA increased motility in human sperm. In the cases
99 of canine and mice species, the presence of PDE10 has been determined in spermatocytes
100 (Wayman et al. 2005) and in reproductive tract tissues (Coskran et al. 2006), indicating a
101 possible role in the physiological processes in both species.

102 The goal of this study was to determine the quality of sperm under the effect of the specific
103 PDE-10 inhibitor (papaverine) added after storage for 3 days at 5 ° C**.**

104 **Materials and methods**

105 ***Reagents and media***

106 All chemicals were obtained from Sigma-Aldrich Quimica, S.A. (Madrid, Spain), unless
107 otherwise indicated. The basic extender used to dilute canine sperm was tris-citrate-fructose
108 (TCF) medium containing 259 mM Trizma base, 80 mM citric acid and 69 mM fructose at 6.8
109 pH and an osmolality of 300-330 mOsm/kg, supplemented with 20% (vol/vol) egg yolk.
110 Papaverine was dissolved in bidistilled water.

111 *2.2 Animals and semen collection*

112 The study was performed following approval by the Veterinary Ethical Committee of
113 University of Zaragoza (PD04/18NE). The care and use of animals were performed
114 according to the Spanish Policy for Animal Protection RD53/2013, which meets the
115 European Union Directive 2010/63/EU on the protection of animals used for
116 experimental and other scientific purposes. The dogs were individually housed and fed on a
117 balanced diet, and water was provided *ad libitum.* Thirty ejaculates were obtained from 5
118 clinically healthy beagle dogs (mean age: 4.5 years) by digital manipulation. The 3 different
119 fractions were collected separately into pre-warmed sterile glasses, and only the second sperm-
120 rich fraction was utilized. Sperm concentration was determined with a photometer (SpermaCue,
121 Minitub GmbH, Tiefenbach, Germany). Only sperm samples with concentration ≥200 × 10^6
122 spermatozoa/ml, total motility ≥ 80% and normal morphology ≥75% were included in the
123 study.
124 *Sperm processing*
125 Ejaculates were diluted in TCF extender at room temperature and centrifuged at 700 g for 3
126 min. The sperm pellet was resuspended in TCF extender supplemented with egg yolk to yield a
127 concentration of 100 x 10^6/ml. Then, samples were cooled gradually in a glass beaker
128 containing water and placed in the refrigerator at 5ºC. The cooled extended sperm were
129 incubated with different concentrations of PA (C: control group without PA, P5: 5 μM PA,
130 P10:10 μM PA and P20: 20 μM PA) during 30 min at 37°C before sperm analysis at day 0, day
131 1, day 2 and day 3. The concentrations of PA selected are according to previous studies (Terriou
132 et al., 2015; Bergeron et al., 2017). Each experiment was replicated 6 times, and a total of 30
133 sperm rich fractions per experiment were processed.
134 ***Semen evaluation***
135 ***Motion parameters*** were determined using a computer- assisted sperm analysis (CASA)
136 system (ISAS®; PROISER; Valencia; Spain). The samples were analysed at a concentration of
137 20 × 10^6 sperm/ml.The parameters evaluated were total motile spermatozoa (TM %), motile

progressive spermatozoa (PM %), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity of the curvilinear trajectory (LIN, ratio of VSL/VCL, %) and amplitude of lateral head displacement (ALH, μm). A 5-μl aliquot of each sperm sample was placed in a pre-warmed Makler counting chamber. The setting parameters were: 25 frames/s in which spermatozoa had to be present in at least 15 frames in order to be counted. The sperm total motility variable used in the statistical analysis was the overall percentage of motile spermatozoa (VCL > 20 μm/sec). Images were obtained at 200x magnification in a contrast phase microscope.

Sperm viability was defined as spermatozoa with plasma and acrosome membrane intact. It was assessed by fluorescein isothiocyanate conjugated with peanut agglutinin (FITC-PNA) and propidium iodide (PI) staining. An aliquot of 500 μL sperm suspension from each treatment group was added with 10 μL of FITC-PNA solution (200 μg/mL stock solution) and 4 μL PI solution (500 μg/mL stock solution), incubated at 38ºC for 5 min, and finally fixed with paraformaldehyde (4%, v/v) in saline solution. At least 200 spermatozoa were examined determining the percentages of viable spermatozoa (intact plasma and acrosome membranes; PNA−/PI−).

Chlortetracycline (CTC) staining was used to assess dog sperm capacitation, following the procedure described by Guerin et al. (1999). The CTC-staining solution was prepared by mixing 20 mM Tris, 130 mM NaCl, 5 mM cysteine and 750 mM CTC. For staining, 20 μl of sperm suspension was mixed with 20 μl CTC staining solution prior to the addition of 10 μl fix solution (2% paraformaldehyde in Phosphate- Buffered Saline, PBS). A drop was placed on a slide and mounted with a cover-slip and DABCO anti-fading medium. Two hundred spermatozoa were counted at 1,000× magnification. Sperm were classified according to CTC staining patterns: F (uniform fluorescent head-uncapacitated and acrosome intact), B (fluorescence-free band on the postacrosomal region-capacitated), and AR (nonfluorescent head or a thin fluorescent band on the equatorial segment- acrosome reacted).

Statistical analysis

165 The statistical analysis was performed using SPSS version 22.0 for Windows. Differences
166 between sperm quality parameters between groups were submitted to an analysis of variance
167 using the general linear model procedure (GLM). Data were expressed as mean value ± SEM.
168 Differences were considered statistically significant at $P < 0.05$.

169 **Results**

170 The effect of PA supplementation on sperm motility parameters added after chilling storage
171 are summarised in Figure 1 and Table 1. There was a significant decrease ($P<0.05$) in the values
172 of all the variables studied throughout storage time, except ALH. At day 0, the results of MT,
173 MP and the different sperm kinetics parameters were not modified after the incorporation of the
174 different PA concentrations in the sperm samples. However, significant differences ($P<0.05$)
175 were observed in each one of the parameters studied after PA addition during the storage time
176 (up to 3 days). At day 1, MT and MP, as well as the VCL, VAP and ALH parameters, showed
177 significantly lower data in control samples than in the samples incubated with PA
178 concentrations of 5 or 10 µM. At days 2 and 3, the higher concentration of PA (20 µM)
179 decreased TM and PM percentages significantly, and concentrations between 5 and 10 µM
180 significantly improved ($P<0.05$) the parameters evaluated in relation to control sperm samples.
181 In addition, P5 and P10 sperm samples significantly increased ($P <0.05$) the values of VCL,
182 VCL, VAP and ALH compared to control and P20 sperm samples after the addition of PA.

183 In relation to viability, the addition of different concentrations of PA did not produce any
184 effect during day 0 of evaluation, although after 24h of storage, the addition of 20 µM PA
185 decreased the percentage of viable spermatozoa (Figure 2). At days 2 and 3, P5 and P10 sperm
186 samples showed significantly higher percentages of sperm with the intact plasma membrane
187 than control and P20 samples ($P <0.05$).

188 Figure 3 shows the capacitation status of spermatozoa at days 0, 1, 2 and 3 after incubation
189 with PA during 30 min at 37ºC. At day 0, there was a significant reduction of F pattern
190 spermatozoa and a significant increase of B pattern and AR pattern spermatozoa at
191 concentration of 20 µM PA compared to control ($P < 0.05$). Concentrations of 5 and 10 µM of
192 PA did not show differences in any patterns evaluated in relation to control; however, the

number of F and B patterned spermatozoa was significantly higher than those of 20 µM PA samples ($P< 0.05$). At days 1, 2 and 3, we determined similar changes on sperm patterns. PA20 samples showed significantly lower values of F pattern spermatozoa in relation to the others experimental groups, whereas B and AR pattern spermatozoa were significantly higher ($P< 0.05$). P5 and P10 samples of F pattern and B patterns spermatozoa increased significantly compared to the control ($P< 0.05$); however, no differences were found in AR pattern.

Discussion

PDE inhibitors have been utilised to stimulate sperm activity and improve semen functionality after storage and artificial insemination in different animal species. Nevertheless, to the best of our knowledge, this is the first report on the effect of PA (specific PDE10 inhibitor) on the quality canine semen added after the cooling period.

Regardless of the extender used in canine semen, there is a linear decrease in sperm quality during refrigeration storage after the first 48h of storage (Iguer-Ouada and Verstegen 2001). Sperm motility enhancers are utilised to improve seminal functionality and fertility after several types of damage produced during the conservation process that may affect sperm energy metabolism (Tardif et al. 2014; Terriou et al. 2015 Bergeron et al. 2017). In our study, the addition of 5 and 10 µM PA increased the kinetic parameters of chilled canine sperm. There are different sperm activators, although the most commonly used are PDE inhibitors (Tardif et al. 2014; Bergeron et al. 2017), such as PA, a selective PDE10 inhibitor. Incubation of low-motility human sperm with 93 µM concentrations of PA produced an increase in total motility of samples, improving the final quality of the ejaculates (Terriou et al. 2015). A study conducted by Bergeron et al. (2017), showed that 400 nM of PA produced high activity of cAMP-PDE in bovine sperm after 3 h of incubation, although this concentration did not change sperm capacitation status due to the low concentration of PA utilised. Similar results were obtained byTorres-Flores et al. (2008) on human sperm. PA improves sperm motility depending on the concentration applied.

In dogs, pentoxifylline (another PDE inhibitor), has shown a beneficial effect on sperm motility (Koutsarova et al. 1997). It was observed that the addition of pentoxifylline at

concentrations of 0.0036 mol/µl significantly increased the percentages of progressive motility in fresh semen, while we have observed this change with 5 and 10 µM of PA on chilled semen. However, according to another study with frozen canine semen (Milani et al. 2009), concentrations of 5 mM of pentoxifylline (after 120 min at 37°C) produced a significant increase in total and progressive motility, as well as VSL, while concentrations of 2.5 mM decreased VAP and VSL values. In this same study, it was observed that 7.5 mM of caffeine decreased in both progressive motility and VCL parameter, demonstrating a negative effect of caffeine depending on time and concentration. We determined similar effects with concentrations of 20 µM PA after incubation during 30 min at 37ºC. The values of VCL and ALH were the parameters most influenced by the addition of caffeine and pentoxifylline in canine sperm, as we observed in our study.

Metallohydrolases hydrolyse cAMP, cGMP or both in the presence of divalent cations. PA produces an increase in cAMP levels on sperm that enhances sperm motility and stimulates capacitation status (Visconti et al. 1999). Capacitation is characterised by complex biochemicaland biophysical changes that produce structural andmorphological modifications in the sperm that allow fertilisation of the egg (Suarez and Osman 1987). In our study, PA concentrations of 20 µM significantly increased the levels of reacted, non-viable and capacitated sperm. By contrast, in the presence of 5 and 10 µM of PA, although the percentage of acrosome-reacted spermatozoa did not change significantly, the percentage of capacitated sperm cells assessed by CTC assay significantly increased. In the case of the dog, the presence of PDE10 has been determined in the postacrosomal region and midpiece of the sperm (Coskran et al. 2006; Bergeron et al. 2017). In these areas, there are high tyrosine protein phosphorylation levels related to increasedsperm motility and capacitation status (Jin and Yang 2016), as occurred in our study. However, studies in bull sperm have determined that 400 nM of PA did not change the percentages of viable and capacitated spermatozoa after 3h of incubation (Bergeron et al. 2017). In relation to pentoxifylline, the incorporation of 10 and 100 mM on canine semen produced an increase in the reacted sperm; although low concentration did not produce any modifications compared to the control (Mirshokraei et al. 2011). In other species,

such as ram, boar and stallion sperm, similar effects have been described (Maxwell et al. 1995; Gil et al. 2010; Guasti et al. 2017). The use of other PDE inhibitors, such as caffeine or sildenafil, at concentrations greater than 5 mM and 2.5 mM, respectively, increased the percentages of reacted sperm by decreasing motility in buck, ram and boar (Pereira et al. 2000; Yeste et al. 2008; Ioki et al. 2016).

The incubation time seems to be a determining factor in the effect of PA on dog spermatozoa. Terriou et al. (2015) observed an increase on sperm motility after 20-30 min of incubation of PA with human sperm. However, on bull semen, changes related to capacitation status were observed after 4 h of incubation with PA (Tardif et al. 2014). Different authors have studied the effect of several PDE inhibitors on sperm (caffeine, pentoxifylline, kallikrein) and determined significant changes after 30 and 90 minutes of incubation (Yeste et al. 2008; Mirshokraei et al. 2011 ; Barakat et al. 2015). We observed an improvement in sperm quality parameters after 30 min of incubation at 37ºC with concentrations ranged 5-10 μM of PA. These effects are produced after 3 days of sperm refrigeration at 5ºC; therefore, PA may increase the longevity of canine semen.

Semen fertility generally decreases after short cold storage time compared to fresh semen (Hori et al. 2013). The reason for this deterioration is the reduction of motility, damage in plasma membrane and acrosomal and loss of intracellular components of spermatozoa (Watson 1990). The addition of motility enhancing substances in seminal doses may increase the motility values of spermatozoa and subsequently improve the fertilisation rates. The experimental and clinical use of pentoxifylline and caffeine is contradictory in the literature. Some studies have suggested deleterious effects on blastocyst development (Tournaye et al. 1993; Scott and Smith 1995). However, Yamaguchi et al. (2013) showed that concentrations of 10 mM of caffeine added to frozen-thawed boar sperm increased fertility rates but not litter size and the incorporation of pentoxifylline on cryopreserved bovine and porcine semen improved *in vitro* fertilisation rates (Numabe et al. 2000; Gil et al. 2010). Further studies are necessary to test embryo toxicity of PA.

Conclusions

In conclusion, our finding has shown that supplementing with 5 and 10 μM of PA canine chilled semen can significantly improve sperm quality parameters and their longevity *in vitro*. Therefore, PA may be a promising and safe alternative agent to enhance canine sperm quality.

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Disclosure statement

The authors have no conflict of interest to declare.

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Figure legend

Figure 1. Effect of PA on total motility (A) and progressive motility (B) of chilled canine spermatozoa during 3 days of cold storage (Mean ± SEM).

a,b,c: Different letters in the same day indicate significant differences $P<0.05$.

Figure 2. Effect of PA on canine sperm viability during 3 days of cold storage (Mean ± SEM).

a,b,c: Different letters in the same day indicate significant differences $P<0.05$.

Figure 3. Effect of different concentrations of PA on CTC patterns of spermatozoa at day 0 (A), day 1 (B), day 2 (C) and day 3 (D) of cold storage (Mean ± SEM). The CTC-binding patterns were uncapacitated (F pattern), capacitated (B pattern) and acrosome-reacted (AR) pattern.

a,b,c: Different letters in the same day indicate significant differences $P<0.05$.