IRIS AperTO

UNIVERSITÀ DEGLI STUDI DI TORINO

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Is the protective effect of egg yolk against osmotic and cryogenic damage on dog spermatozoa dose-dependent?

This is a pre print version of the following article:

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/1726631 since 2022-08-03T10:02:55Z

Published version:

DOI:10.1016/j.anireprosci.2019.106259

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

25 April 2024

Is the protective effect of egg yolk against osmotic and cryogenic damage on canine spermatozoa dose-dependent?

Alessia Gloria[a], Daniele Zambelli[b], Augusto Carluccio[a], Marco Cunto[b], Patrizia Ponzio[c], Alberto Contri[d]*

[a]*Faculty of Veterinary Medicine, University of Teramo, Loc. Piano d'Accio, 64100 Teramo, Italy*

[b]*Department of Veterinary Medical Sciences, Alma Mater Studiorum - University of Bologna, via Tolara di Sopra 50, 40064 Ozzano dell'Emilia, Bologna, Italy*

[c]*Department of Veterinary Sciences, University of Turin, largo Braccini 2, 10095 Grugliasco, Turin, Italy*

[d]*Faculty of Biosciences and Technologies for Agriculture Food and Environment, University of Teramo, via Balzarini 1, 64100 Teramo, Italy*

*Corresponding author: Alberto Contri, Faculty of Biosciences and Technologies for Agriculture Food and Environment, University of Teramo, via Balzarini 1, 64100 Teramo, Italy. Phone/fax: +39 0861 266995.

E-mail address: acontri@unite.it

ABSTRACT

Egg yolk (EY) is conventionally used to reduce sperm cryodamage, but a dose-dependent effect has not been previously tested. To improve the knowledge about the protective effect of EY during cryopreservation of canine semen, a specific study has been designed to evaluate the dose-dependent protection of the EY against osmotic and cryogenic damage in sperm collected from 21 healthy dogs. In the first experiment, extended sperm at increasing EY concentrations (0%, 5%, 10%, and 20%) were diluted with hypo- or hyperosmotic solutions (final osmolality of 75 mOsm/kg, 150 mOsm/kg, 300 mOsm/kg, 500 mOsm/kg, 1000 mOsm/kg). Sperm kinetics, membrane integrity (MI), mitochondrial activity, and normal morphology showed that osmotic stress impacts especially on the kinetic ability of spermatozoa but without direct effects on mitochondrial activity. In both hypo- and hyperosmotic conditions, EY was found to exert a protective effect regardless of its concentration. In the second experiment, semen samples were diluted in extenders at increasing EY concentrations (0%, 5%, 10%, and 20%) and cryopreserved. Sperm kinetics, membrane and acrosome integrity and mitochondrial membrane potential showed better results when EY concentration was 5% and 10%, and lower when it was 20%. These results suggest, for the first time, that EY reduces osmotic and cryogenic damage when used at 5% or 10%, and that these concentrations protect canine spermatozoa more effectively than the conventional used (20%).

Keywords: Canine; Egg yolk; Cryopreservation; Sperm kinetics

1. Introduction

Semen cryopreservation allows long-term storage of viable and function spermatozoa (Leroy et al., 2011). This technology shows several advantages along the decades since semen can be stored for an undefined time, moved at great distances, or can also be used when the female is in oestrous, avoiding the contextual presence of the male (Thomassen and Farstad, 2009).

On the other hand, cryopreservation exerts a relevant detrimental effect on mammalian sperm viability and fertilizing ability. A reduction in progressive motility (Jones and Stewart, 1979), alterations of membrane permeability and stability (Holt and North, 1986; Watson, 2000), and an increase in the radical species of oxygen (ROS) generation (Alvarez and Storey, 1992; Chatterjee and Gagnon, 2001) have been reported in cryopreserved mammalian spermatozoa. To reduce the impact of extremely low temperatures on spermatozoa, extenders with specific composition were developed. Among the different components of the freezing extender, egg yolk (EY) appears to be an unavoidable ingredient. Previous studies showed that the use of EY in the freezing medium reduces cellular damage (De Leeuw et al., 1993; Pace and Graham, 1974; Phillips and Lardy, 1940). Although the protective role of the EY was demonstrated, the underline mechanism of action appeared evanescent. Most studies suggested that the protective ability of EY is related to the content of low density lipoproteins (LDL) (Bencharif et al., 2010; Moussa et al., 2002; Pace and Graham, 1974). It was proposed that these components adhere to and interact with the sperm membrane (Bergeron et al., 2004; Foulkes, 1977; Graham and Foote, 1987; Manjunath et al., 2002). On the other hand, some studies evidenced that respiration of spermatozoa can be inhibited by some EY components, and this could affect sperm motility (Pace and Graham, 1974; Wall and Foote, 1999).

Traditionally, while EY is added at the dog freezing extender at 20% (Anderson, 1972; Peña et al., 1998; Silva et al., 2002), few studies have addressed whether the cryoprotective effect of this compound is dose-dependent. In other species, such as the stallion, EY is effectively used for semen cryopreservation at 2%, without a reduction of semen quality and fertility (Pillet et al., 2008).

The damage induced by cryopreservation on spermatozoa is multimodal since several papers evidenced a combination of cold shock (Amann and Pickett, 1987), peroxidation (Slaweta et al., 1988), and osmotic stress (Watson, 2000). As the temperature reduces subzero, water forms ice crystals first in the extracellular compartment, with an increase of the solute concentration in the free uncrystallised water outside the cell, inducing hyperosmotic stress (Sieme et al., 2016). On the contrary, during thawing, the ice crystals melt in the free water that enters the plasma membrane, thus sperm experience hypo-osmotic stress.

Although Foulkes (Foulkes, 1977) suggested that the protective effect of the egg yolk during cryopreservation could contribute to the colloid pressure maintenance of the external medium, few studies focused the attention on the role of EY as a protective compound against the osmotic stress. Despite the crucial role and wide use of egg yolk for sperm cryopreservation in dogs, surprisingly few trials have been conducted to clarify the dose-dependent protective effect of this component. Furthermore, a relevant part of the damage during cryopreservation could be attributed to the osmotic stress, but few studies focused on the protective role of the egg yolk against osmotic stress in canine spermatozoa. Thus, to increase the knowledge of the biology and manipulation of reproduction in dogs, this study was designed to verify, for the first time, the protective effect of egg yolk at different concentrations (0%, 5%, 10%, and 20%) on canine spermatozoa in different anisosmotic conditions. Moreover, the aim of the present study was the evaluation of the cryoprotective effect of egg yolk, added at the same concentrations (0%, 5%, 10%, and 20%) to the freezing medium, for canine sperm cryopreservation.

2. Materials and Methods

2.1. Animals and semen collection

The study involved 21 healthy dogs of known fertility aged between 2 and 6 years. The breed represented were: Newfoundland (n = 6), Pitbull (n = 5), American Staffordshire (n = 5), and Labrador retriever (n = 5). All dogs were presented for routine reproductive examination at the Hospital of the

University of Veterinary Medicine of Teramo, Italy. For all the dogs included in the study, the consent was obtained by the owner for the use of part of the semen sample of their dogs. Animals were managed in agreement with the Italian legislation concerning animal care (DL *n*.116, 27/01/1992).

Semen collection was performed by the digital manipulation from the same person to avoid any effects on sperm quality. Only the sperm-rich fraction was evaluated and used for experiments. From each animal two ejaculates were collected, for a total of 42 samples. Only ejaculates with motility > 70% and concentration > 200 x 10^6 sperm/mL were included in the trials.

2.2. Experimental designs

2.2.1. Experiment 1. Protective effect of egg yolk on canine sperm during osmotic stress

In this study a hyperosmotic solution based on TRIS formula (hyper-TRIS) was prepared using 119.8 g/L TRIS, 67.32 g/L citric acid and 12.375 g/L glucose (pH 6.9; mOsm/kg 1218). This solution was diluted with bi-distilled sterile water to achieve isosmotic TRIS (iTRIS, pH, 6.8, Osm 302 mOsm/kg). Semen samples were split in four aliquots and diluted at 240 x 10^6 sperm/mL in iTRIS, then each sample was diluted 1:1 with 40% EY (final concentration 20% EY - EY20), 20% EY (final concentration 10% - EY10), 10% EY (final concentration 5% EY - EY5), and 0% EY (EY0 – as control). The 40% EY, 20% EY, and 10% EY extenders showed an osmolality of 1864 mOsm/kg, 1839 mOsm/kg, and 1821 mOsm/kg, respectively. Each treatment (EY20, EY10, EY5, and EY0) was in turn divided into five aliquots, in duplicate. To evaluate the protective effect of different EY concentrations, each treatment was then diluted with a combination of hyper-TRIS and bi-distilled water to a final osmolality of 75 mOsm/kg, 150 mOsm/kg, 300 mOsm/kg, 500 mOsm/kg, 1000 mOsm/kg. The final sperm concentration was 40 x 10^6 sperm/mL. Part of the first series, immediately after dilution, was used for objective motility evaluation by CASA system as described below. The evaluation was performed at T0, after 20 min of dilution (T20) and after 45 min of dilution (T45), following the procedure described below for the kinetc evaluation. The remaining sample was used for morphology assessment by phase contrast microscopy, as described below.

The second series was split into two aliquots and used for flow cytometry evaluation of MI (first aliquot) and mitochondrial potential (second aliquot), as described below. The evaluation was performed after 20 min (T20) and after 45 min (T45) after staining, described as below.

2.2.2. Experiment 2. Protective effect of egg yolk during cryopreservation

Semen samples were diluted 1:1 (v:v) with iTRIS (pH, 6.7, Osm 304 mOsm/kg), centrifuged for 10 min at 700 X g and resuspended in iTRIS at the concentration of 300 x 10^6 sperm/mL. The semen was then diluted 1:1 (v:v) with iTRIS supplemented with 8% glycerol (final concentration 4%) (Peña et al., 1998) and 40% EY (final concentration 20% EY - EY20), 20% EY (final concentration 10% - EY10), 10% EY (final concentration 5% EY - EY5), and 0% EY (EY0 – as control). The samples were then cooled and equilibrated at 4°C for 2h in a passive refrigerator, packaged in 0.25 straws (IMV Technologies, L'Aigle, France) and sealed mechanically. Straws were suspended 4 cm above the liquid nitrogen surface for 10 min, plugged into liquid nitrogen (Anderson, 1972), and stored for at least 5 days. Samples were evaluated for sperm objective motility, MI, acrosome integrity, and mitochondrial potential at the end of equilibration (EQ) and after thawing (FT). To this, straws were plunged into the water bath at 37°C for 30 seconds (Bencharif et al., 2008), the sample was then transferred to a 2-ml plastic tube and additionally incubated at 37°C to reach 5 minutes of total incubation.

2.3. Semen evaluation

2.3.1. Semen concetration

Fresh semen was evaluated within 10 min after collection. Sperm concentration was determined using a Bürker counting chamber (Merck, Leuven, Belgium) after dilution 1:1000 with a formol-saline solution.

2.3.2. Sperm kinetic

The kinetic evaluation was performed using the computer-assisted sperm analyzer (CASA) system IVOS 12.3 (Hamilton Thorne Biosciences, Beverly, MA, USA) for objective evaluation of motility following the guidelines for CASA use (Iguer-ouada and Verstegen, 2001; Rijsselaere et al., 2003). The correct identification of spermatozoa was obtained using the playback function and adjusting the detection gates. Samples from the experiment 1 were analysed without further dilution, while frozen/thwed samples were diluted with relevant extender at 40 x 10^6 sperm/mL. An aliquot of each sample was rewarmed at 37°C for 5 min and a 5-µL drop was loaded onto a Makler chamber (Sefi Medical Instruments, Haifa, Israel). Motility parameters were collected and recorded by the analysis of 12 nonconsecutive fields. The anti-collision algorithm was activated. Motility parameters considered included total motility (TM; %), progressive motility (PM; %), average path velocity (VAP; µm/s), straight line velocity (VSL; µm/s), curvilinear velocity (VCL; µm/s), amplitude of lateral head displacement (ALH; µm), beat cross frequency (BCF; Hz), straightness (STR, as VSL/VAP; %), and linearity (LIN, as VSL/VCL; %). Spermatozoa with VAP ≥80 µm/s and STR ≥75% were considered to be progressive cells.

2.3.3. Sperm membrane and acrosome integrity

In Experiment 1, MI in the different osmotic conditions was evaluated by the use of Propidium Iodide (PI) exclusion test, as previously validated (Ball and Vo, 2001) with some modifications. Semen was diluted at 10 x 10^6 sperm/mL with the relevant extender, and aliquots (500 µL) were incubated with PI at the final concentration of 12 µM for 5 min at 22°C and then were analysed using the flow cytometer EPICS XL (Beckman Coulter, San Jose, CA, USA). Acquisitions were conducted using the System II software (Beckman Coulter, USA). The sperm population was selected on the basis of the forward-scatter and side-scatter, and a gate was created on these parameters. Samples were excited with a 20-mW argon ion 488-nm laser, and PI fluorescence was obtained using the FL3 sensor through a 660/20 nm long pass filter. Forward and side-scatter values were recorded on a linear scale and fluorescence values on a logarithmic scale. Flow cytometric analysis was performed at a

flow rate of 6 to 24 μL/min, and acquisitions were stopped at 30,000 events. Events with red fluorescence were considered sperm with membrane damage, those without fluorescence were considered spermatozoa with MI.

In Experiment 2, sperm MI and acrosome integrity were evaluated simultaneously by flow cytometry, as previously described (Gloria et al., 2018). Briefly, samples at 10 x 10^6 sperm/mL (1 mL) were stained with 2.4 μM of PI and 5 μg/mL of FITC-conjugated agglutinin derived from Pisum sativum (FITC-PSA). After 10 min of dark incubation at 22°C, each sample was analysed using a flow cytometer (EPICS XL). FITC-PSA fluorescence was collected through FL1 sensor using a 530/28 nm band-pass, while PI fluorescence was obtained using the FL3 sensor through a 660/20 nm long pass filter. Compensation was used between the fluorescent channels. The sperm population was selected on the basis of the forward-scatter and side-scatter, and a gate was created on these parameters. Forward and side-scatter values were recorded on a linear scale and fluorescence values on a logarithmic scale. Flow cytometric analysis was performed at a flow rate of 6 to 24 μL/min, and acquisitions were stopped at 30,000 events. Combination of these two fluorochromes resulted in four different subpopulations: sperm with MI and acrosome integrity showing no fluorescence (PI-/PSA-); sperm with MI and an acrosome reaction (PI-/PSA+); sperm with a damaged membrane and acrosome integrity (PI+/PSA-); and sperm with a damaged membrane and a reacted acrosome (PI+/PSA+).

2.3.4. Mitochondrial membrane potential assay

Mitochondrial membrane potential (MMP) of spermatozoa was evaluated using the fluorescent stain 5,5,6,6-tetrachloro-1,1,3,3-tetraethyl-benzimida zolylcarbocyanine chloride (JC-1) as reported by Gloria et al. (Gloria et al., 2018). The sperm suspension was adjusted to a concentration of 5×10^6 sperm/mL and incubated for 45 min at 37°C in the dark with JC-1 (final stain concentration 8 μM). At the end of the incubation period, cells were washed in the same medium without stain and evaluated using the flow cytometer EPICS XL (Beckman Coulter) equipped with the System II

197 software (Beckman Coulter) as previously reported (Garner and Thomas, 1999). The sperm
198 population was selected on the basis of the forward-scatter and side-scatter, and a gate was created
199 on these parameters. The green fluorescent emissions of the monomeric form of JC-1 (mitochondria
200 with low potential - LMMP) were collected by a 530 ± 15 - nm filter (FL 1), and the orange emission
201 of the polymeric form of JC-1 (mitochondria with high membrane potential - HMMP) by a 585 ± 21
202 – nm filter (FL 2). The flow cytometric analysis was performed at a flow rate of 8 to 30 µL/min, and
203 the acquisitions were stopped at 30,000 events. No compensation was used between the fluorescent
204 channels.
205
206 *2.3.5. Sperm morphology*
207 Sperm morphology was evaluated using a phase contrast microscope (BX-51 - Olympus Italia,
208 Milan, Italy) at 1000 X magnification. Spermatozoa at different osmolalities were immobilized by
209 the addition of 3% glutaraldehyde (Hancock, 1957), a drop (6 µl) was dispensed on a slide a covered
210 by a 22 x 22 mm coverslip. Spermatozoa were then classified as normal sperm, sperm with abnormal
211 head, sperm with abnormal midpiece, and sperm with abnormal tail, that were in turn subdivided in
212 sperm with complete coiling (more than the 50% of the tail length was involved in the
213 twisting/coiling), sperm with partial coiling (the twisting/coiling involved the distal part of the tail),
214 and sperm with other tail abnormalities. Sperm abnormalities were computed on at least 400
215 spermatozoa.
216
217 *2.4. Statistical analysis*
218 Data are presented as mean ± standard error of the mean (SEM). The data were evaluated with
219 Shapiro-Wilk test (normal distribution) and Levene test (homogeneity of variances). When data were
220 not normally distributed, a log transformation was performed before data analysis.
221 In Experiment 1, the effect of the concentration of EY on the different sperm parameters
222 (kinetic parameters, MI, mitochondrial membrane potential, morphological subclasses) was tested

using a general linear model (GLM) based on Univariate ANOVA. The dog was included as a random factor. Post-hoc evaluation was performed using the Scheffé's test.

In Experiment 2, the cryoprotective effects of the different concentrations of EY, in terms of kinetic parameters, membrane and acrosome integrity, and mitochondrial membrane potential were tested using a GLM based on Univariate ANOVA, with Scheffé's test for the post-hoc evaluation. The dog was included as a random factor.

In Experiment 1, correlations between total and progressive motility, MI, and mitochondrial membrane potential were performed using Pearson's correlation coefficient.

For both the experiments, differences were considered significant when $P < 0.05$. Statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Experiment 1

In this study, the protective effect of EY against anisosmotic stress was tested in canine spermatozoa. Data showed that EY exerted a protective effect, irrespective of the concentration used.

In samples diluted in iTRIS without EY at T0, the kinetic parameters were similar to those recorded using EY, with the exception of ALH, which was significantly lower in samples with EY, irrespective the concentration, compared with those without EY ($P < 0.05$) (Table 1). However, in these samples, kinetic parameters dropped in both hypo-osmotic and hyperosmotic conditions. At 75 mOsm/kg and at 1000 mOsm/kg no spermatozoa moved, while at 150 mOsm/kg and at 500 mOsm/kg the proportion of sperm with total motility was below 10% in all the cases. In samples diluted with 5% EY, kinetic parameters recorded at 150 mOsm/kg were significantly lower than those observed at 300 mOsm/kg ($P < 0.01$). Values at 500 mOsm/kg were similar to those recorded in the control for total motility (Table 1). However, mild hyperosmotic condition seemed to reduce kinetic parameters, since spermatozoa that move showed lower velocities (VAP, VSL, and VCL) ($P < 0.05$) and progressiveness (STR, LIN) ($P < 0.05$), with an increase in the amplitude of the movement (ALH) (*P*

249 < 0.05), resulting in a reduced proportion of sperm with progressive motility ($P < 0.05$). Kinetic
250 parameters were related to the presence of EY, but were not affected by the concentration of this
251 component, since the values recorded with 5%, 10%, and 20% of EY were similar (Table 1). The
252 time seemed to exert a negligible effect on sperm kinetic. Values recorded after 20 min (data not
253 shown) and 45 min (Table 2) were similar to those recorded soon after dilution, with a relevant impact
254 of anisosmotic conditions on samples without EY and on the sample in hypo-tonic media.

255 Membrane integrity and mitochondrial membrane potential were evaluated at 20 and 45 min
256 after dilution, due to the time required for the incubation of the stain. At T20, MI in EY 0% samples
257 was higher at 300 mOsm/kg, while it was significantly lower at 75 mOsm/kg (Table 3). Similarly, as
258 osmolality increased, MI decreased ($P < 0.05$) (Table 3). Differently, in the presence of EY, MI was
259 similar at all osmolarities, regardless of EY concentration (Table 3). A similar trend was found for
260 HMMP in samples without EY compared with those with 5%, 10% and 20% EY (Table 3). In general,
261 LMMP showed lower values in samples with a high percentage of sperm with HMMP, and higher
262 values in samples with a low percentage of sperm with HMMP. Samples diluted in the extender
263 without EY at 1000 mOsm/kg made an exception since the percentage of sperm with HMMP and
264 with LMMP was in both the cases low (Table 3). Values for MI, HMMP, and LMMP at 45 min of
265 incubation showed similar values compared with those recorded at the correspondent EY
266 concentration and osmolality. Acrosome integrity showed similar values during the experiment and
267 appeared less affected by both EY concentration and osmolality (data not shown).

268 In samples with 0% EY, significant correlations were found between sperm TM, and MI (r =
269 0.896, $P < 0.01$), TM and HMMP (r = 0.824, $P < 0.01$), and MI and HMMP (r = 0.792, $P < 0.01$) at
270 300 mOsm/kg, while in anisosmotic conditions MI was correlated with HMMP (r = 0.786, $P < 0.01$),
271 but not with TM (r = 0.318, $P > 0.05$). In samples containing 5%, 10%, and 20% EY, significant
272 correlations were found at 300 mOsm/kg between TM and MI (r = 0.916, $P < 0.01$; r = 0.934, $P <$
273 0.01; r = 0.928, $P < 0.01$, respectively), TM and HMMP (r = 0.874, $P < 0.01$; r = 0.836, $P < 0.01$; r
274 = 0.792, $P < 0.01$, respectively), and MI and HMMP (r = 0.816, $P < 0.01$; r = 0.842, $P < 0.01$; r =

0.758, $P < 0.01$). Similarly, in all the samples with EY significant correlations were found at 500 mOsm/kg TM and MI (r = 0.826, $P < 0.01$ for 5% EY; r = 0.842, $P < 0.01$ for 10% EY; r = 0.816, $P < 0.01$ for 20%EY), TM and HMMP (r = 0.682, $P < 0.01$ for 5% EY; r = 0.648, $P < 0.01$ for 10% EY; r = 0.586, $P < 0.05$ for 20% EY), and MI and HMMP (r = 0.798, $P < 0.01$ for 5% EY; r = 0.816, $P < 0.01$ for 10% EY; r = 0.786, $P < 0.01$ for 20% EY). On the other hand, at 75 mOsm/kg, 150 mOsm/kg, and 1000 mOsm/kg MI was significantly correlated with HMMP (r = 0.638, P < 0.05; r = 0.682, P < 0.05; r = 0.568, P < 0.05, respectively in 5% EY samples; r = 0.672, $P < 0.05$; r = 0.684, $P < 0.05$; r = 0.548, $P < 0.05$, respectively in 10% EY samples; and r = 0.626, $P < 0.05$; r = 0.584, $P < 0.05$; r = 0.526, $P < 0.05$, respectively in 20% EY samples) but not with TM.

Sperm abnormal head and midpiece were not affected by the osmolality of the medium, or by EY concentration (Table 4, $P > 0.05$). A significant effect was found for the dog in the model ($P < 0.05$).

As expected, hyperosmotic conditions marginally affected sperm tail morphology. Soon after dilution (T0), morphological subclasses recorded at 500 mOsm/kg and 1000 mOsm/kg in samples were similar to those recorded at 300 mOsm/kg ($P > 0.05$). Moreover, spermatozoa in the hypoosmotic condition showed a typical twisting/coiling of the tail. The percentage of spermatozoa with a tail response was similar at both 75 and 150 mOsm/kg, however, the percentage of sperm with complete tail coiling was significantly higher at 75 mOsm/kg compared with 150 mOsm/kg ($P < 0.05$) that was, in turn, higher than the iso-osmotic samples ($P < 0.01$). On the other hand, the percentage of spermatozoa with partial coiling higher at 150 mOsm/kg compared with 75 mOsm/kg ($P < 0.05$) (Figure 1). The presence of EY seemed to exert a partial protective effect against the hypo-osmotic stress since the percentage of complete coiling was significantly lower in samples extended with 5%, 10%, and 20% EY compared with samples without EY at both 75 mOsm/kg and 150 mOsm/kg ($P < 0.05$). However, no differences ($P > 0.05$) in the proportion of the morphological tail subclasses were found in samples diluted with EY5, EY10, and EY20 (data not shown).

The effect of the time on the sperm morphological subclasses was not found, since the subclasses were similar soon after the dilution (T0), at T20, and T45 (data not shown).

3.2. Experiment 2

In Experiment 2, the dose-dependent cryoprotective nature of EY was tested. In the samples, soon after dilution, EY seemed to exert an effect on the velocity of canine sperm, since progressive motility, VAP, VSL, VCL, STR, and LIN were all higher in samples diluted with 5% EY and 10% EY compared with samples without EY ($P < 0.05$). On the other hand, samples diluted with 20% EY showed values similar to those reported for 0% EY, and lower ($P < 0.05$) compared with 5% EY and 10% EY (Table 5). Total motility, ALH, and BCF seemed slightly affected by EY concentration, since values were similar for all treatments ($P > 0.05$). Membrane and acrosome integrity, such as sperm with HMMP, were similar in samples without EY and 5%, 10%, and 20% EY (Table 5).

During equilibration, the values recorded for semen samples diluted with different EY concentration showed similar values compared to those recorded at the corresponding samples soon after dilution ($P > 0.05$). Cryopreservation reduced all sperm parameters but the impact on spermatozoa seemed to be related to EY concentration. As shown in Table 6, samples in extender without EY reduced significantly the kinetic parameters, MI, and HMMP compared with samples in extender with EY 5% and EY 10% ($P < 0.05$). Unexpectedly, in samples diluted with EY 20%, total and progressive motilities were lower compared with the values of EY 5% and 10% ($P < 0.05$), even if the values were significantly higher compared with EY 0% ($P < 0.05$). The other kinetic parameters recorded in 20% EY samples seemed similar to those of EY 5% and EY 10%. Although PI+/PSA- and PI+/PSA+ subpopulations were not significantly different between samples at EY 20% compared with EY 5% and EY 10% ($P > 0.05$) (Table 6) the total amount of sperm with membrane damage (PI+/PSA- plus PI+/PSA+) was significantly higher in samples at EY 20% compared with EY 5% and EY 10% ($P < 0.05$).

4. Discussion

The data reported in the present study suggested that EY acts preserving spermatozoa against osmotic stress. Spermatozoa are able to adapt to the solute concentration in the medium through the passage of water across the plasma membrane and the modification of the cytoskeleton (Correa et al., 2007). In a simple salt solution, spermatozoa respond to anisosmotic conditions as linear osmometers, since a correlation was found between bovine sperm volume and medium osmolality (Guthrie et al., 2002). Several studies reported the osmotic tolerance limit in spermatozoa of different domestic animals, including bull (Guthrie et al., 2002; Liu and Foote, 1998), horse (Ball and Vo, 2001; Glazar et al., 2009), boar (Gilmore et al., 1998), ram (Curry and Watson, 1994). Although specific studies on the osmotic tolerance limit in canine spermatozoa lack in literature, canine spermatozoa were likely to have similar behaviour. The definition of the osmotic tolerance limit was not the primary aim of this study; however, our data in medium without EY corroborated this hypothesis.

On the other hand, all the previous studies on the osmotic stress response of spermatozoa did not verify the response of sperm in a complex medium supplemented with colloidal components, that could modulate the cellular adaptation. In dogs, the addition of EY seemed to protect cellular structures involved in the regulation of sperm motility at 500 mOsm/kg, even if a general reduction in the progressiveness was present. Similarly, the protective role of EY was more evident on sperm MI. In samples diluted with EY 5%, EY 10%, and EY 20%, percentages of sperm MI at 150 mOsm/kg, and at 500 and 1000 mOsm/kg were similar compared to the isosmotic condition. Although the protective role of EY in anisosmotic conditions seemed to be clear, our data suggested that this action was neither dose-dependent, nor time-dependent, as demonstrated by the similar values recorded for all the seminal parameters with EY 5%, EY 10%, and EY 20% at all incubation times.

The wide discrepancy between sperm motility and MI, as underlined by the lack of correlations between these parameters, in anisosmotic conditions suggests that the sperm function could be lost before the integrity of the plasmalemma. Our data confirmed previous studies in the

352 bull, in which the proportion of total motile spermatozoa at 100 and 150 mOsm/kg was lower
353 compared to the MI found in the same conditions, but both parameters were lower than those
354 measured in near-isosmotic conditions. In hyperosmotic conditions, sperm motility was significantly
355 low, whereas sperm MI was similar to the values recorded from 300 mOsm/kg to 936 mOsm/kg (Liu
356 and Foote, 1998). A similar trend was also reported in other studies on human and ram spermatozoa
357 (Curry and Watson, 1994; Gao et al., 1995).

358 However, differently to what previously hypothesized (Liu and Foote, 1998), the mechanism
359 of the kinetic loss was not related to the mitochondrial dysfunction since the values seemed to follow
360 MI. This suggested that the reduced kinetic ability of spermatozoa during osmotic stress could be
361 marginally due to the dysfunction of the metabolic activity of mitochondria. Thus, the suppressive
362 effect of the hyperosmotic conditions on kinetic sperm requires a different mechanism, likely at the
363 cytoskeletal level. Correa et al. (Correa et al., 2007) reported a reorganization of the cytoskeletal actin
364 in hyperosmotic condition, responsible for the decreased motility recorded in that conditions.
365 However, specific studies should be designed to verify this second hypothesis.

366 In a previous study, it was suggested that the response of sperm to the anisosmotic condition
367 could not only be related to plasma MI but also to the membrane permeability to ions and to the
368 cytoskeletal integrity (Petrunkina et al., 2004). Our data seemed to corroborate these data since the
369 different degrees of curing/twisting in sperm incubated in hypo-osmotic conditions recorded without
370 EY could suggest a different response of spermatozoa at the structural level. The ability of canine
371 spermatozoa to modify their tail morphology in hypo-osmotic condition was reported by Kumi-Diaka
372 (1993). Unfortunately, in that study the degree of sperm response to the hypo-osmotic stress was not
373 reported; thus, it was not possible to compare the data directly. However, in contrast with findings by
374 Kumi-Diaka (1993), the number of sperm that curled was similar soon after dilution, at 20 min and
375 after 45 min of incubation. Differently to those findings, in our study, the osmotic adaptation onset
376 closely to the induction of osmotic stress and remained at the same level during incubation. The
377 timing of sperm response to the osmotic stress recorded in our study was similar to that previously

reported (Pinto and Kozink, 2008), in which no difference in the percentage of sperm with curled tail was found at 1 or 60 min of incubation.

Data reported for the protective effect of EY against osmotic stress could suggest a role during cryopreservation since sperm survival after freezing-thawing procedures seemed to be related to the sperm ability to undergo cell volume regulation (Petrunkina et al., 2004).

As expected, the addition of EY to the medium resulted in a protective effect during cryopreservation, as reported in most studies in the cryopreservation of dog semen (Silva et al., 2002). Although the usual concentration of EY used in the canine semen extender was 20% (Anderson, 1972; Peña et al., 1998; Silva et al., 2002), our study demonstrated that the concentration of EY seemed to be less relevant. In samples without EY, we found lower percentages of motile and membrane intact spermatozoa, similarly to the data reported in a previous study (Silva et al., 2002). However, an improvement in sperm characteristics was found starting from EY 5%.

Unexpectedly, the total amount of motile and progressive sperm was slightly lower in samples diluted in extender with EY 20% compared with 5% and 10% EY. This finding suggests that higher EY concentration could be detrimental for canine frozen spermatozoa, as previously proposed in other species (Amirat et al., 2004; Moussa et al., 2002). To the authors' knowledge, no study compared different EY concentrations on the same sample during canine semen cryopreservation, thus the superior protective effect of the EY at 20% was never clearly demonstrated.

The data reported in our study demonstrated that canine spermatozoa could be successfully frozen with lower concentrations of EY. This finding was in agreement with other species, in which EY concentration in the extender is 2% (Pillet et al., 2008). The optimal concentration of low density lipoproteins (LDL), which are the active fraction of EY, could be species-specific. In the bull, Moussa et al. (Moussa et al., 2002) found similar post-thaw sperm kinetics by the use of EY 20% and 2.5% LDL, while the values were significantly higher when 5% to 10% LDL was added to the extender. With a high concentration of LDL (15% and 20%), post-thaw sperm motility reduced. In the dog, Bencharif et al. (2008) found higher values in the cryopreserved spermatozoa with the use of LDL

compared with conventional EY concentration, but among the different LDL concentrations, higher values were found using 6% LDL. It is possible that the purification of LDL performed in that study could affect the results, explaining the different values we reported. However, both studies showed that the use of EY at high concentration (20%) could reduce canine sperm quality after thawing, and the use of lower concentrations (5%, 10%) or a purified LDL preparation, resulted in better post-thaw canine semen characteristics.

The data reported in this study suggest that at least part of the cryoprotective effect of EY in canine cryopreserved spermatozoa is related to the protection against osmotic stress. However, the lack of correlation between total and progressive motility, highly reduced, and MI, less affected by the anisosmotic environment, in both hypo- and hyperosmotic conditions, indicates that the damage was directed to the cytoskeleton or to a metabolic pathway not located in the mitochondria. The osmoprotective effect of the EY was not dose-dependent since similar values were found at 5%, 10%, and 20% EY. During sperm cryopreservation, EY at 5% and 10% seemed to be more effective compared with EY at 20% during the canine semen cryopreservation.

5. Conclusions

The data reported in this study showed that EY exert a protective role during the osmotic stress in canine spermatozoa. The protective effect seem not to be dose-dependent, since no differences were found in sperm characteristics after dilution with extender at 5%, 10%, or 20% EY. Furthermore, spermatozoa exposed to hyperosmotic conditions lost the kinetic ability more extensively than their membrane integrity, suggesting a primary cytoskeletal damage. Finally, EY did not show a dose-dependent protection effect during cryopreservation in canine spermatozoa. Characteristics of fozen-thawed sperm using 5% and 10% EY were significantly higher compared with 0% EY, but slightly significantly higher than 20% EY, suggesting that canine spermatozoa could be effectively frozen at lower EY concentration.

Funding sources

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

Alvarez, J.G., Storey, B.T., 1992. Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. J. Androl. 13, 232–241. https://doi.org/10.1002/j.1939-4640.1992.tb00306.x

Amann, R.P., Pickett, B.W., 1987. Principles of cryopreservation and a review of cryopreservation of stallion spermatozoa. J. Equine Vet. Sci. 7, 145–173. https://doi.org/10.1016/S0737-0806(87)80025-4

Amirat, L., Tainturier, D., Jeanneau, L., Thorin, C., Gérard, O., Courtens, J.L., Anton, M., 2004. Bull semen in vitro fertility after cryopreservation using egg yolk LDL: A comparison with Optidyl®, a commercial egg yolk extender. Theriogenology 61, 895–907. https://doi.org/10.1016/S0093-691X(03)00259-0

Anderson, K., 1972. Fertility of frozen dog semen. Acta Vet. Scand. 13, 128–130.

Ball, B.A., Vo, A., 2001. Osmotic tolerance of equine spermatozoa and the effects of soluble cryoprotectants on equine sperm motility, viability, and mitochondrial membrane potential. J. Androl. 22, 1061–1069. https://doi.org/10.1002/j.1939-4640.2001.tb03446.x

Bencharif, D., Amirat, L., Anton, M., Schmitt, E., Desherces, S., Delhomme, G., Langlois, M.L., Barrière, P., Larrat, M., Tainturier, D., 2008. The advantages of LDL (Low Density Lipoproteins) in the cryopreservation of canine semen. Theriogenology 70, 1478–1488. https://doi.org/10.1016/j.theriogenology.2008.06.095

Bencharif, D., Amirat, L., Pascal, O., Anton, M., Schmitt, E., Desherces, S., Delhomme, G., Langlois, M.L., Barrière, P., Larrat, M., Tainturier, D., 2010. The advantages of combining low-density lipoproteins with glutamine for cryopreservation of canine semen. Reprod.

456 Domest. Anim. 45, 189–200. https://doi.org/10.1111/j.1439-0531.2008.01198.x
457 Bergeron, A., Crête, M.-H., Brindle, Y., Manjunath, P., 2004. Low-density lipoprotein fraction
458 from hen's egg yolk decreases the binding of the major proteins of bovine seminal plasma to
459 sperm and prevents lipid efflux from the sperm membrane. Biol. Reprod. 70, 708–717.
460 https://doi.org/10.1095/biolreprod.103.022996
461 Chatterjee, S., Gagnon, C., 2001. Production of reactive oxygen species by spermatozoa undergoing
462 cooling, freezing, and thawing. Mol. Reprod. Dev. 59, 451–458.
463 https://doi.org/10.1002/mrd.1052
464 Correa, L.M., Thomas, A., Meyers, S.A., 2007. The macaque sperm actin cytoskeleton reorganizes
465 in response to osmotic stress and contributes to morphological defects and decreased motility.
466 Biol. Reprod. 77, 942–953. https://doi.org/10.1095/biolreprod.107.060533
467 Curry, M.R., Watson, P.F., 1994. Osmotic effects on ram and human sperm membranes in relation
468 to thawing injury. Cryobiology 31, 39–46. https://doi.org/10.1006/cryo.1994.1005
469 De Leeuw, F.E., De Leeuw, A.M., Den Daas, J.H.G., Colenbrander, B., Verkleij, A.J., 1993. Effects
470 of various cryoprotective agents and membrane-stabilizing compounds on bull sperm
471 membrane integrity after cooling and freezing. Cryobiology 30, 32–44.
472 https://doi.org/10.1006/cryo.1993.1005
473 Foulkes, J.A., 1977. The separation of lipoproteins from egg yolk and their effect on the motility
474 and integrity of bovine spermatozoa. Reproduction 49, 277–284.
475 https://doi.org/10.1530/jrf.0.0490277
476 Gao, D.Y., Liu, J., Liu, C., McGann, L.E., Watson, P.F., Kleinhans, F.W., Mazur, P., Critser, E.S.,
477 Critser, J.K., 1995. Prevention of osmotic injury to human spermatozoa during addition and
478 removal of glycerol. Hum. Reprod. 10, 1109–1122.
479 https://doi.org/10.1093/oxfordjournals.humrep.a136103
480 Garner, D.L., Thomas, C.A., 1999. Organelle-specific probe JC-1 identifies membrane potential
481 differences in the mitochondrial function of bovine sperm. Mol. Reprod. Dev. 53, 222–229.

https://doi.org/10.1002/(SICI)1098-2795(199906)53:2<222::AID-MRD11>3.0.CO;2-L

Gilmore, J.A., Liu, J., Peter, A.T., Critser, J.K., 1998. Determination of plasma membrane characteristics of boar spermatozoa and their relevance to cryopreservation. Biol. Reprod. 58, 28–36. https://doi.org/10.1095/biolreprod58.1.28

Glazar, A.I., Mullen, S.F., Liu, J., Benson, J.D., Critser, J.K., Squires, E.L., Graham, J.K., 2009. Osmotic tolerance limits and membrane permeability characteristics of stallion spermatozoa treated with cholesterol. Cryobiology 59, 201–206. https://doi.org/10.1016/j.cryobiol.2009.07.009

Gloria, A., Wegher, L., Carluccio, A., Valorz, C., Robbe, D., Contri, A., 2018. Factors affecting staining to discriminate between bull sperm with greater and lesser mitochondrial membrane potential. Anim. Reprod. Sci. 189, 51–59. https://doi.org/10.1016/j.anireprosci.2017.12.007

Graham, J.K., Foote, R.H., 1987. Effect of several lipids, fatty acyl chain length, and degree of unsaturation on the motility of bull spermatozoa after cold shock and freezing. Cryobiology 24, 42–52. https://doi.org/10.1016/0011-2240(87)90005-8

Guthrie, H.D., Liu, J., Critser, J.K., 2002. Osmotic tolerance limits and effects of cryoprotectants on motility of bovine spermatozoa. Biol. Reprod. 67, 1811–1816. https://doi.org/10.1095/biolreprod67.6.1811

Hancock, J.L., 1957. The morphology of boar spermatozoa. J. R. Microsc. Soc. (Great Britain) 76, 84–97.

Holt, W. V., North, R.D., 1986. Thermotropic phase transitions in the plasma membrane of ram spermatozoa. Reproduction 78, 447–457. https://doi.org/10.1530/jrf.0.0780447

Iguer-ouada, M., Verstegen, J.P., 2001. Evaluation of the "Hamilton Thorn computer-based automated system" for dog semen analysis. Theriogenology 55, 733–749. https://doi.org/10.1016/S0093-691X(01)00440-X

Jones, R.C., Stewart, D.L., 1979. The effects of cooling to 5 degrees C and freezing and thawing on the ultrastructure of bull spermatozoa. J. Reprod. Fertil. 56, 233–238.

508 Kumi-Diaka, J., 1993. Subjecting canine semen to the hypo-osmotic test. Theriogenology 39, 1279–
509 1289. https://doi.org/10.1016/0093-691X(93)90230-3
510 Leroy, G., Danchin-Burge, C., Verrier, E., 2011. Impact of the use of cryobank samples in a
511 selected cattle breed: A simulation study. Genet. Sel. Evol. 43, 36.
512 https://doi.org/10.1186/1297-9686-43-36
513 Liu, Z., Foote, R.H., 1998. Osmotic effects on volume and motility of bull sperm exposed to
514 membrane permeable and nonpermeable agents. Cryobiology 37, 207–218.
515 https://doi.org/10.1006/cryo.1998.2116
516 Manjunath, P., Nauc, V., Bergeron, A., Ménard, M., 2002. Major proteins of bovine seminal plasma
517 bind to the low-density lipoprotein fraction of hen's egg yolk. Biol. Reprod. 67, 1250–1258.
518 https://doi.org/10.1095/biolreprod67.4.1250
519 Moussa, M., Martinet, V., Trimeche, A., Tainturier, D., Anton, M., 2002. Low density lipoproteins
520 extracted from hen egg yolk by an easy method: cryoprotective effect on frozen–thawed bull
521 semen. Theriogenology 57, 1695–1706. https://doi.org/10.1016/S0093-691X(02)00682-9
522 Pace, M.M., Graham, E.F., 1974. Components in egg yolk which protect bovine spermatozoa
523 during freezing. J. Anim. Sci. 39, 1144–1149. https://doi.org/10.2527/jas1974.3961144x
524 Peña, A., Barrio, F., Quintela, L., Herradón, P., 1998. Effect of different glycerol treatments on
525 frozen-thawed dog sperm longevity and acrosomal integrity. Theriogenology 50, 163–174.
526 https://doi.org/10.1016/S0093-691X(98)00122-8
527 Petrunkina, A.M., Gröpper, B., Günzel-Apel, A.-R., Töpfer-Petersen, E., 2004. Functional
528 significance of the cell volume for detecting sperm membrane changes and predicting
529 freezability in dog semen. Reproduction 128, 829–842. https://doi.org/10.1530/rep.1.00296
530 Phillips, P.H., Lardy, H.A., 1940. A yolk-buffer pabulum for the preservation of bull semen. J.
531 Dairy Sci. 23, 399–404. https://doi.org/10.3168/jds.S0022-0302(40)95541-2
532 Pillet, E., Batellier, F., Duchamp, G., Furstoss, V., LE Vern, Y., Kerboeuf, D., Vidament, M.,
533 Magistrini, M., 2008. Freezing stallion semen in INRA96®-based extender improves fertility

534 rates in comparison with INRA82. Dairy Sci. Technol. 88, 257–265.
535 https://doi.org/10.1051/dst:2008002
536 Pinto, C.R.F., Kozink, D.M., 2008. Simplified hypoosmotic swelling testing (HOST) of fresh and
537 frozen-thawed canine spermatozoa. Anim. Reprod. Sci. 104, 450–455.
538 https://doi.org/10.1016/j.anireprosci.2007.07.005
539 Rijsselaere, T., Van Soom, A., Maes, D., Kruif, A. de, 2003. Effect of technical settings on canine
540 semen motility parameters measured by the Hamilton-Thorne analyzer. Theriogenology 60,
541 1553–1568. https://doi.org/10.1016/S0093-691X(03)00171-7
542 Sieme, H., Oldenhof, H., Wolkers, W.F., 2016. Mode of action of cryoprotectants for sperm
543 preservation. Anim. Reprod. Sci. 169, 2–5. https://doi.org/10.1016/j.anireprosci.2016.02.004
544 Silva, A.R., de Cássia Soares Cardoso, R., Uchoa, D.C., Machado da Silva, L.D., 2002. Effect of
545 Tris-buffer, egg yolk and glycerol on canine semen freezing. Vet. J. 164, 244–246.
546 https://doi.org/10.1053/tvjl.2002.0704
547 Slaweta, R., Waşowicz, W., Laskowska, T., 1988. Selenium content, glutathione peroxidase
548 activity, and lipid peroxide level in fresh bull semen and its relationship to motility of
549 spermatozoa after freezing-thawing. J. Vet. Med. Ser. A 35, 455–460.
550 https://doi.org/10.1111/j.1439-0442.1988.tb00058.x
551 Thomassen, R., Farstad, W., 2009. Artificial insemination in canids: A useful tool in breeding and
552 conservation. Theriogenology 71, 190–199.
553 https://doi.org/10.1016/j.theriogenology.2008.09.007
554 Wall, R.J., Foote, R.H., 1999. Fertility of bull sperm frozen and stored in clarified egg yolk-tris-
555 glycerol extender. J. Dairy Sci. 82, 817–821. https://doi.org/10.3168/jds.S0022-
556 0302(99)75301-4
557 Watson, P., 2000. The causes of reduced fertility with cryopreserved semen. Anim. Reprod. Sci.
558 60–61, 481–492. https://doi.org/10.1016/S0378-4320(00)00099-3
559
560

561 **Figure legend**

562 Fig. 1. Bar charts of the tail defect proportions at the different osmolarities (75 mOsm/kg, 150
563 mOsm/kg, 300 mOsm/kg, 500 mOsm/kg, and 1000 mOsm/kg) in canine spermatozoa diluted with
564 0% egg yolk (EY 0%) and with 10% egg yolk (EY 10%). Bars with different letters differ significantly
565 ($P < 0.05$).