Zygote

cambridge.org/zyg

Effect of the addition of antifreeze protein type I on the quality of post-thawed domestic cat epididymal sperm

L.P. Alcaráz, P.V.S. Pereira, T.A. Oliveira, L.F.L. Correia, E.M. Vasconcelos, F.Z. Brandão and J.M.G. Souza-Fabjan

Faculdade de Veterinária, Universidade Federal Fluminense, Av. Vital Brasil Filho, 64, CEP 24230-340, Niterói, RJ, Brazil

Research Article

Cite this article: Alcaráz LP *et al.* (2023). Effect of the addition of antifreeze protein type I on the quality of post-thawed domestic cat epididymal sperm. *Zygote*. page 1 of 6. doi: 10.1017/S0967199422000521

Received: 18 January 2022
Revised: 12 August 2022
Accepted: 22 September 2022

Keywords:
AFP; Cryoprotectant; Feline; Slow freezing; Sperm

Author for correspondence:
L.P. Alcaráz. Faculdade de Veterinária, Universidade Federal Fluminense, Av. Vital Brasil Filho, 64, CEP 24230-340, Niterói, RJ, Brazil. E-mail: leticiaaalcaraz@gmail.com; J.M.G. Souza-Fabjan. Faculdade de Veterinária, Universidade Federal Fluminense, Av. Vital Brasil Filho, 64, CEP 24230-340, Niterói, RJ, Brazil. E-mail: joannavet@gmail.com

Summary

Cryopreservation of domestic cat semen is mainly performed as a model for the establishment of endangered wild feline protocols. The supplementation of antifreeze protein type I (AFP I) to cryopreservation medium has shown improvement in frozen–thawed sperm quality in other species, but its effect on cat semen has not yet been tested. This study aimed to assess the addition of AFP I to cryopreservation medium in domestic cats. Sperm was obtained from the cauda epididymis of orchiectomized cats; sperm was then pooled in Tris buffer and allocated into three treatments, according to AFP I final concentration: 0 (control), 0.1, and 0.5 µg/ml. Nine replicates were cryopreserved in a two-step protocol and subsequently thawed at 37°C for 30 s. There was no difference ($P > 0.05$) among the control, 0.1 and 0.5 µg/ml groups for parameters such as motility, vitality, functional membrane integrity, mature chromatin, normal morphology, and sperm binding to egg perivitelline membrane. In the 0.5 µg/ml group only, percentages of live sperm with intact acrosome and of sperm with most inactive mitochondria (DAB III) showed a significant reduction, along with a tendency ($P = 0.053$) to an increase in the percentage of sperm with most active mitochondria (DAB II). In conclusion, the supplementation of 0.1 and 0.5 µg/ml of AFP I did not promote consistent beneficial effects on the overall sperm cryotolerance in domestic cats.

Introduction

There are currently 40 species of wild cats described and 18 of them are listed as vulnerable or threatened with extinction (IUCN, online). Therefore, a great effort is required for the conservation of these animals and assisted reproductive technologies are strong allies for this purpose. However, the establishment of protocols in wild cats is very difficult, as their biological material is scarce. Therefore, due to genetic similarities and availability, the domestic cat is often used as a research model (Klaus *et al.*, 2016).

Cryopreservation of semen is an essential tool for long-term storage, allowing genetic preservation, transporting, and application in artificial insemination and *in vitro* fertilization, thereby contributing to the conservation of threatened species. However, cat semen cryopreservation protocols are not well established, resulting in below-expected post-thaw outcomes regarding sperm quality (Buranaamnuay, 2017). This process potentially induces spermatozoa damage (Luvoni, 2006), resulting in a reduction in viability, motility, and structural integrity (Watson, 2000; Isachenko *et al.*, 2003). So, it is important to test different substances that may have a role as a cryoprotectant to increase the overall cryopreservation efficiency.

Antifreeze proteins (AFPs) are a class of polypeptides first described in Arctic fish as an adaptive mechanism at sub-zero temperatures and then in arthropods, plants, algae, bacteria, and fungi (De Vries and Wohlschlag, 1969; Kim *et al.*, 2017). Four fish-derived types of AFP (I, II, III, and IV) and AFGP (glycoprotein) have been described, these differ according to their composition and tertiary structure (Cheung *et al.*, 2017). These proteins play a role in inhibiting the normal growth and morphology of ice crystals; lowering the freezing point in a non-colligative manner, at a process called thermal hysteresis; inhibiting the recrystallization; and protecting the plasma membrane (Kim *et al.*, 2017). Therefore, the addition of AFP I to the cryopreservation medium could benefit post-thaw semen quality as it presents positive results for sperm cryoresistance, resulting in higher sperm kinetics, higher normal sperm rates and better plasma integrity, in species such as cattle (Prathalingam *et al.*, 2006) and ram (Payne *et al.*, 1994; Upreti *et al.*, 1996; Correia *et al.*, 2021). To the authors' best knowledge, there have been no data published about the use of any AFP for cat semen cryopreservation. Therefore, this study aimed to assess the use of different concentrations of AFP I added into the extender for sperm cryopreservation in domestic cats.

© The Author(s), 2023. Published by Cambridge University Press.

Materials and methods

Animals

This study did not require the Ethics Committee of Animal Use approval of the University, as the domestic male cat ($n = 86$) testes were collected at local veterinary clinics, as byproducts from owner-requested routine orchiectomies.

Experimental design

Cat spermatozoa were collected from the cauda epididymis; sperm were incubated in Tris buffer, and pooled. The pooled fresh sperm were evaluated and allocated into three experimental groups according to the final concentration of AFP I after its addition in both Extenders I and II: control (0 μg/ml), 0.1 μg/ml (10 μg), and 0.5 μg/ml (50 μg). The AFP I concentrations used were based on the study of Correia *et al.* (2021). Subsequently, the pooled sperm samples were frozen at a final concentration of 25×10^6 spermatozoa/ml (Buranaamnuay, 2015). Pre-freezing evaluation consisted of motility, functional membrane integrity, mitochondrial activity, vitality, chromatin condensation, and viability, and acrosome status. For thawing, the straws were placed at 37°C for 30 s and promptly evaluated for the analyses cited above, plus sperm kinetics, morphology, and binding to egg perivitelline membrane assessment.

Sperm harvesting

The removed testicles from each cat were sent to the laboratory in sterile 0.9% (w/v) NaCl solution at 4°C within 2 h after surgery. Epididymis and proximal *vasa deferentia* were isolated and dissected free from visible blood vessels and connective tissues. Afterward, the epididymis was transversely cut and placed for 10 min in 400 μl of Tris buffer at 37°C. After that, the tissue fragments were removed, and the fresh samples were pooled and evaluated for sperm motility, vigour, and concentration. All sperm samples used in this study exhibited values of ≥50% subjective motility and 3 for vigour. Nine replicates were conducted, ranging between seven and 13 animals each.

Preparation of medium

Tris buffer [3.025 g Tris, 1.4% citric acid, 0.8% glucose, 0.1% antibiotic and antimycotic solution (streptomycin, penicillin, and amphotericin B), in MilliQ water], Extender I (the same composition as Tris buffer, with added 3% glycerol, 20% egg yolk, and AFP I according to the experimental group) and II [same as Extender I, but containing 7% glycerol and 0.6% sodium dodecyl sulfate (SDS)] were prepared according to Chatdarong *et al.* (2010) and Mizutani *et al.* (2010) (Table 1).

Cryopreservation process

Sperm samples were pooled, allocated into one of the three groups according to AFP I concentrations (Figure 1), centrifuged at 700 *g* for 6 min (Thammapradit and Ponglowhapan, 2018), and their supernatant was removed. The pellets were diluted in Extender I and kept at 4°C for 1 h. Thereafter, the respective Extender II was added and an aliquot from each experimental group was removed for pre-freezing analyses. The 0.25 ml straws were filled, and kept at 4°C for 1 h to equilibrate. Then, the straws were suspended horizontally 7 cm above the liquid nitrogen level for 10 min and immediately immersed (Toyonaga *et al.*, 2010).

Sperm assessments

Sperm concentration, subjective motility and vigour

Sperm concentration was determined by Neubauer chamber. Subjective (percentage of motile spermatozoa) and vigour (scale from 0 to 5, with 0 being immotile sperm and 5 being fast, steady forward progression) were evaluated using a phase-contrast microscope at ×400 magnification. These parameters were only assessed in fresh samples.

Sperm kinetics

Sperm motility patterns were only assessed in frozen–thawed samples using computer-assisted sperm analysis (CASA) and the SCA® system (Sperm Class Analyzer Microptic, Nikon Eclipse Ci – Tokyo, Japan) connected to a phase-contrast microscope. Software settings were adjusted for cat spermatozoa. Standard configuration of parameters was the spermatic head dimension detectable by the system between 5 and 60 μm^2. In total, 25 images per second were captured; measurements were performed in a 24 × 24 mm cover slide with a 10-μl volume, at ×100 magnification. The following parameters were analyzed: Total Motility (%), Progressive Motility (%), Non-progressive Motility (%), Fast Sperm (%), Medium Sperm (%), Slow Sperm (%), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), average path velocity (VAP, μm/s), linearity (LIN, %), straightness (STR, %), WOB (mean value of the ratio between VAP and VCL, %), amplitude of lateral head displacement (ALH, μm), beat/cross frequency (BCF, Hz). Spermatozoa were classified according to their curvilinear velocity (VCL). It was considered fast when it was above 75 μm/s; medium when it was between 45 and 75 μm/s; slow when it was between 10 and 45 μm/s; and immobile when the value was less than 10 μm/s. Sperm cells showing straightness (STR) above 80% were characterized as progressive motile sperm.

Functional membrane integrity

For functional integrity, the hypo-osmotic swelling test was performed (Ramu and Jeyendran, 2013). Briefly, sperm was mixed with a hypo-osmotic solution of 100 mOsm/kg of MilliQ water (1:9) and incubated at 37°C for 20 min. At least 200 sperm cells were counted, through wet preparation in phase-contrast microscopy (×1000 magnification). The results were based on counting spermatozoa that folded the tail, meaning that their membrane was still intact when exposed to the hypo-osmotic environment.

Sperm viability and acrosome status

Trypan blue/Giemsa staining was applied as reported by Kovács and Foote (1992), with minor modifications. Briefly, the sperm were incubated for 15 min with trypan blue 0.2% at 37°C (1:1). Then, a smear slide was prepared, air dried and Giemsa stain (10%) was applied for at least 1 h. Ultimately, the slides were rinsed with running water, air dried, and evaluated under an optical microscope at ×1000 magnification. At least 200 sperm cells were evaluated in each smear. Sperm were classified as live sperm with intact acrosome (LSIA): purple; dead sperm with intact acrosome (DSIA): violet; live sperm with acrosome reacted (LSAR): light pink; and dead sperm with acrosome lost (DSAL): post acrosome region violet and light pink acrosome.

Chromatin condensation

The acidic aniline blue staining was applied to assess the chromatin condensation according to Dadoune *et al.* (1988), with

https://doi.org/10.1017/S0967199422000521 Published online by Cambridge University Press

Table 1. Composition of sperm buffer and extenders prepared and used in this study

	Tris buffer	Extender I			Extender II		
Tris (g)	3.025	3.025			3.025		
Citric acid (g)	1.4	1.4			1.4		
Glucose (g)	0.8	0.8			0.8		
Glycerol (ml)	–	3.0			7.0		
Egg yolk (ml)	–	20			20		
Antibiotic–antimycotic (ml)	0.1	0.1			0.1		
SDS (g)	–	–			0.6		
AFP I (μg)	–	–	10	50	–	10	50
MilliQ water added to (ml)	100	100			100		

Abbreviations: SDS: sodium dodecyl sulfate; Tris: tris(hydroxymethyl)aminomethane.

modifications. The sperm smear was fixed in 4% formalin solution for 5 min. Then, it was washed and stained in 5% aniline blue solution mixed with 4% acetic acid (pH 3.5) for 5 min and once more washed. Finally, the slide was stained in a 0.5% eosin solution for 30 s, washed in water, and dried in air. In total, 200 sperm cells were evaluated under an optical microscope at ×1000 magnification and immersion. Sperm were differentiated into either intense stained (immature chromatin) or slightly stained nuclei (mature chromatin).

Sperm vitality and morphology

Eosin–nigrosin dye (3% and 8%, respectively) was mixed with the sperm sample (1:1), smeared on a slide, and immediately evaluated for sperm vitality (Cedenho and Miyamoto, 1990). Furthermore, the fixed sperm smears allowed the assessment of morphological abnormalities of the sperm tails, cytoplasmic droplets, detached heads, and abnormal mid-pieces (Cocchia *et al.*, 2010). In the absence of any abnormality, sperm were classified as presenting normal morphology. For both analyses, 200 sperm cells were counted and evaluated under an optical microscope at ×1000 magnification.

Mitochondrial activity

Mitochondrial activity was assessed according to Rui *et al.* (2017). Briefly, the sperm was diluted (1:1) in a 1 mg/ml solution of 3,3′-diaminobenzidine (DAB) and incubated at 37°C for 1 h in the dark. After this period, smears were fixed in 10% formaldehyde for 10 min. At least 200 sperm cells were counted under a phase-contrast optical microscope (×1000 magnification). The sperm were categorized as: all mitochondria active (100% of the midpiece stained: DAB I); most mitochondria active (more than 50% of the midpiece stained: DAB II); most mitochondria inactive (less than 50% of the midpiece stained: DAB III); and all mitochondria inactive (absence of staining in the midpiece: DAB IV).

Sperm binding to egg perivitelline membrane test

Fresh and non-fertile chicken egg yolks were separated from the albumen for perivitelline membrane acquisition. Then, they were washed with phosphate-buffered saline (PBS) and the membranes were cut into squares (0.5 cm^2). Finally, the membranes were incubated for 1 h in 1 ml of FERT-TALP and 20 μl of sperm sample at 38.5°C and 5% of CO_2. For evaluation, the membrane was washed three times in PBS, placed on a slide, and 5 μl of Hoechst 33342 stain (1 mg/ml) were added. Five fields per sample were visualized and counted under an epifluorescence microscope (Nikon Eclipse Ci, Nikon Corporation, Japan), at ×600 magnification, and the results were expressed as spermatozoa binding per mm^2 of the membrane (Barbato *et al.*, 1998).

Statistical analysis

All variables were subjected to normality (Shapiro–Wilk and Kolmogorov–Smirnov tests) and homoscedasticity tests. Parametric data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test, whereas non-parametric data were evaluated using Kruskal–Wallis test, followed by Dunn's test. Data are presented as mean ± standard error of the mean (SEM). *P*-values < 0.05 were considered significant and those at $0.05 \leq P \leq 0.1$ as a tendency. All analyses were performed using IBM SPSS 25 software version (IBM Corporation, Armonk, NY, USA).

Results

Fresh pooled spermatozoa data regarding subjective motility, concentration and total spermatozoa were: 69.4 ± 2.3%, 49.3 ± 5.7 × 10^6 spermatozoa/ml, and 124.7 ± 11.5 × 10^6 spermatozoa, respectively.

Data regarding pre-freezing evaluation are presented in Table 2. The subjective motility, vitality, functional membrane integrity, mature chromatin, and acrosome status showed no difference ($P > 0.05$) among groups. Furthermore, the mitochondrial activity assay indicated a tendency ($P = 0.069$) for the DAB I category, with the control group presenting a greater ($P < 0.05$) value compared with both AFP I-supplemented groups. DAB II, III, and IV categories did not exhibit any differences ($P > 0.05$).

In post-thawing assessments (Table 2), differences were detected in the acrosome status and mitochondrial activity. The LSIA percentages in the control and 0.1 μg/ml groups were greater ($P < 0.05$) when compared with 0.5 μg/ml. For mitochondrial activity, the DAB II category presented a tendency ($P = 0.053$) favouring the 0.5 μg/ml group, when compared with the control, whereas 0.1 μg/ml supplementation was similar to both treated groups. Concerning DAB III, the 0.5 μg/ml AFP I addition resulted in a lower percentage compared with the control group. Supplementation of 0.1 μg/ml showed no difference ($P > 0.05$) compared with the other groups. All other parameters were similar ($P > 0.05$) among the groups. Similarly, the presence of AFP did not affect ($P > 0.05$) any sperm kinetic parameter assessed by CASA and the results are shown in Table 3.

Discussion

The results presented in this study were not as expected in our initial hypothesis that the supplementation of AFP I in cryopreservation extender would have a beneficial effect on the quality of frozen–thawed spermatozoa. In fact, the addition of AFP I did not result in any major effect; although it negatively affected the acrosome status (LSIA) and positively affected the mitochondrial activity in the 0.5 μg/ml group.

It is important to note that most studies using AFP I in semen cryopreservation extenders have resulted in a beneficial effect. In ram, an improvement in sperm kinetics, motility, membrane integrity, and normal morphology was reported using 0.1–10 μg/ml (Payne *et al.*, 1994; Upreti *et al.*, 1996; Correia *et al.*, 2021), whereas

https://doi.org/10.1017/S0967199422000521 Published online by Cambridge University Press

Table 2. Pre-freezing and post-thawed sperm parameters related to the supplementation of 0, 0.1 or 0.5 µg/ml of antifreeze protein (AFP) I in cryopreservation medium of domestic cats. Values are expressed as mean ± standard error of the mean (SEM)

	Pre-freezing			Post-thawed (0 h)		
	Control	AFP I 0.1 µg/ml	AFP I 0.5 µg/ml	Control	AFP I 0.1 µg/ml	AFP I 0.5 µg/ml
Total motility (%)*	56.7 ± 3.8	60.0 ± 1.8	60.8 ± 4.7	13.8 ± 2.1	14.3 ± 2.3	11.7 ± 2.6
Sperm vitality (%)	59.2 ± 4.3	57.0 ± 5.4	57.6 ± 7.1	16.2 ± 3.8	13.9 ± 2.9	13.2 ± 2.6
Hypo-osmotic (%)**	83.6 ± 2.7	85.4 ± 3.8	82.5 ± 2.3	9.1 ± 1.6	8.2 ± 1.1	9.4 ± 1.7
DAB I (%)	24.3 ± 1.7	18.4 ± 1.2	18.1 ± 2.5	5.8 ± 1.1	6.4 ± 1.0	7.8 ± 1.5
DAB II (%)	65.5 ± 3.2	72.8 ± 1.9	68.9 ± 3.1	54.6 ± 4.0	63.0 ± 2.9	65.6 ± 1.4
DAB III (%)	8.3 ± 1.9	7.8 ± 1.7	11.4 ± 2.6	29.6 ± 2.8^{a}	22.1 ± 2.7^{a,b}	20.3 ± 2.1^{b}
DAB IV (%)	1.8 ± 0.7	1.0 ± 0.3	1.5 ± 0.5	12.7 ± 2.3	9.2 ± 1.1	7.2 ± 0.8
Mature chromatin (%)	70.3 ± 3.5	65.6 ± 2.5	66.7 ± 4.4	66.9 ± 2.0	67.4 ± 0.8	65.3 ± 3.1
LSIA (%)	44.4 ± 10.6	53.8 ± 10.1	51.3 ± 15.1	47.8 ± 6.4^{a}	44.3 ± 11.3^{a}	19.8 ± 5.6^{b}
LSAR (%)	40.8 ± 12.9	31.8 ± 10.4	30.3 ± 14.3	21.4 ± 4.4	16.6 ± 4.5	26.6 ± 9.2
DSIA (%)	12.5 ± 7.6	12.7 ± 9.3	16.2 ± 11.9	32.3 ± 8.8	45.6 ± 12.6	49.9 ± 10.6
DSAL (%)	2.3 ± 0.9	1.8 ± 1.1	2.3 ± 1.2	6.8 ± 2.3	6.3 ± 2.3	9.1 ± 2.4
Normal morphology (%)	–	–	–	53.3 ± 2.1	56.9 ± 1.9	55.5 ± 2.8
Sperm binding (per mm^2)	–	–	–	461.3 ± 33.5	415.7 ± 51.9	421.3 ± 59.0

^{a,b}Within a row, values differ significantly at the same moment (either before freezing or 0 h) ($P < 0.05$).
Abbreviations: DAB: 3′-diaminobenzidine (mitochondrial activity); DAB I: all mitochondria active; DAB II: majority of mitochondria active; DAB III: majority of mitochondria inactive; DAB IV: all mitochondria inactive; DSAL: dead sperm with acrosome lost; DSIA: dead sperm with intact acrosome; LSAR: live sperm with acrosome reacted; LSIA: live sperm with intact acrosome; sperm binding: sperm binding to egg perivitelline membrane.
*Subjective and objective assessments were performed to estimate pre-freezing and post-thawed total motility, respectively.
**Results based on the counting of spermatozoa that folded the tail (intact membrane).

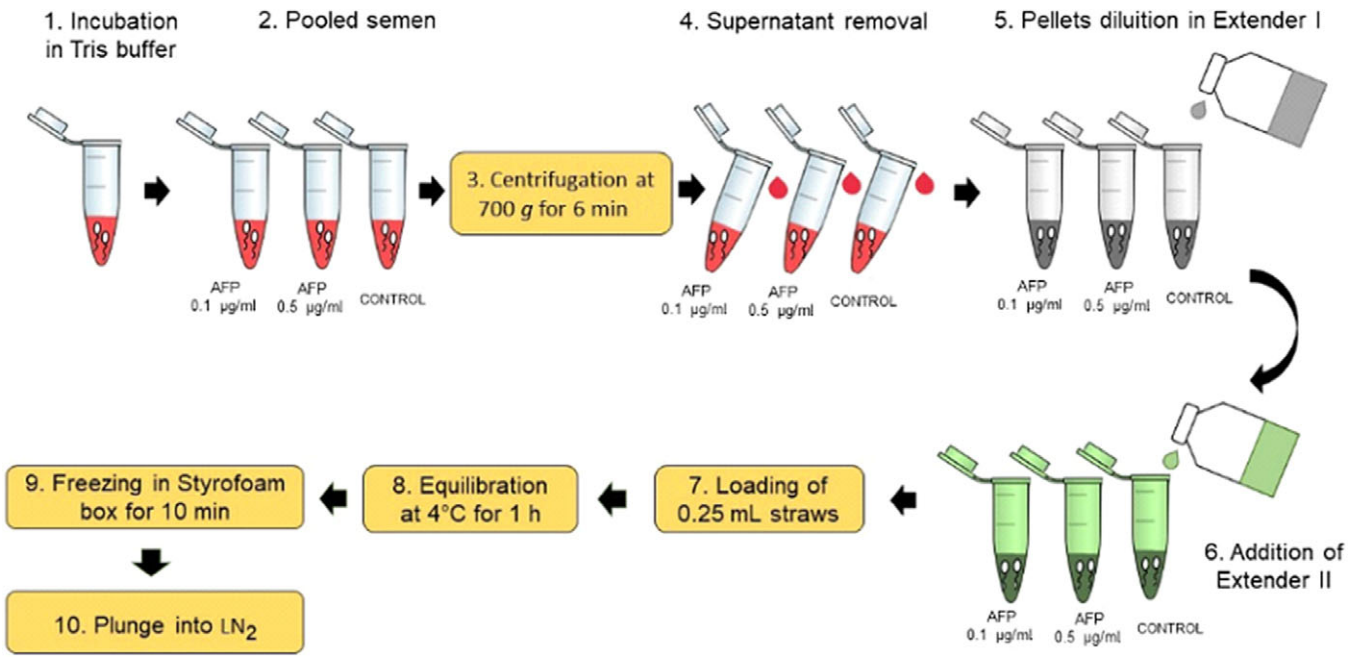

Figure 1. Illustrative diagram of the semen cryopreservation process: 1. Incubation in Tris buffer. 2. Formation of pooled semen. 3. Centrifugation at 700 *g* for 6 min. 4. Supernatant removal. 5. Pellets dilution in Extender I. 6. Addition of Extender II. 7. Loading of 0.25 ml. 8. Equilibration at 4°C for 1 h. 9. Freezing in Styrofoam box for 10 min. 10. Plunge into LN_2.

in cattle, the membrane integrity parameter was superior following supplementation with 0.1–10 µg/ml of AFP I (Prathalingam *et al.*, 2006). However, this was not true for all species studied to date. In mice, supplementation of 0.01–100 µg/ml did not improve sperm quality, in contrast, the results showed that AFP I decreased motility (Koshimoto and Mazur, 2002). We can hypothesize that similar effects regarding physical stress during cryopreservation are expected to occur in species presenting similar sizes of sperm

https://doi.org/10.1017/S0967199422000521 Published online by Cambridge University Press

Table 3. Post-thawed sperm kinetics parameters performed by computer-assisted sperm analysis regarding the supplementation of 0, 0.1 or 0.5 µg/ml of antifreeze protein (AFP) I in cryopreservation medium of domestic cats. Values are expressed as mean ± standard error of the mean (SEM)

	Control	AFP I 0.1 µg/ml	AFP I 0.5 µg/ml
Total motility (%)	2.2 ± 0.4	2.0 ± 0.5	2.2 ± 0.5
Progressive motility (%)	0.7 ± 0.2	0.7 ± 0.3	0.7 ± 0.2
Non-progressive motility (%)	1.6 ± 0.3	1.3 ± 0.2	1.6 ± 0.4
Fast sperm (%)	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
Medium sperm (%)	1.0 ± 0.2	1.0 ± 0.3	1.0 ± 0.3
Slow sperm (%)	0.9 ± 0.2	0.8 ± 0.1	1.0 ± 0.2
VCL (µm/s)	17.9 ± 0.8	17.8 ± 1.2	19.1 ± 1.7
VSL (µm/s)	7.1 ± 0.8	8.0 ± 1.1	9.0 ± 1.4
VAP (µm/s)	10.2 ± 0.7	10.8 ± 1.0	11.8 ± 1.3
LIN (%)	39.1 ± 3.3	45.4 ± 5.9	46.8 ± 6.1
STR (%)	68.5 ± 3.7	73.0 ± 4.3	74.1 ± 3.7
WOB (%)	56.4 ± 2.2	60.9 ± 4.5	61.7 ± 4.5
ALH (µm)	1.9 ± 0.3	1.7 ± 0.3	2.5 ± 0.3
BCF (Hz)	1.6 ± 0.3	1.3 ± 0.3	1.5 ± 0.4

Abbreviations: ALH: amplitude of lateral head displacement; BCF: beat/cross frequency; LIN: linearity; STR: straightness; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight-line velocity; WOB: mean value of the ratio between VAP and VCL.

heads. Similarly, this parameter might also affect binding between AFP I and the cell membrane and, consequently, the protective effects of the molecule during cryopreservation. For example, sperm from mice (6.30 and 3.29 µm) and cats (5.56 and 3.10 µm) have greater proximity in size (length and width, respectively) when compared with cattle (7.87 and 4.05 µm), and ram (7.13 and 3.79 µm). It has been proposed that the area and shape of the sperm head can cause variations in water movement, exchanges of heat, ions, and cryoprotectants (Curry, 2000; Esteso *et al.*, 2013). Therefore, these differences in physical properties dependent on size and shape could be one of the factors responsible for resistance to freezability.

Another possibility is related to the composition of Extender II. It is important to note that all media used for this article were based on previous studies on domestic cats. Indeed, SDS is generally added to the cryopreservation medium (Buranaamnuay, 2017) as its outcomes have shown improvement in the quality and cryotolerance of the spermatozoa (Axnér *et al.*, 2004; Mizutani *et al.*, 2010; Zambelli *et al.*, 2010). For these reasons, SDS was supplemented to our cryopreservation extender to a final concentration of 3 mg/ml, as suggested by Mizutani *et al.* (2010). However, SDS could have a detrimental effect on the structure of AFP I, by causing disturbances in the protein's secondary structure (Kar *et al.*, 2016), which could possibly explain the absence of consistent positive effects of the AFP I molecule in this study.

It can be speculated that the concentration range tested in the current study was not adequate to reach the beneficial effects of AFP. Another important factor is the origin of the sperm. Epididymal sperm seem to exhibit lower cryotolerance compared with that of ejaculated sperm (Luvoni and Morselli, 2017), which could be attributed, for example, to the absence of contact with seminal plasma (SP) proteins. As these proteins have important effects on sperm cryopreservation due to their role in membrane stabilization (Bernardini *et al.*, 2011), their absence could explain why, even when adding some theoretically useful substances such as AFP, the results are still contrasting. For instance, in ram (Colás *et al.*, 2009), cattle (Patel *et al.*, 2016), and Iberian red der (Martínez-Pastor *et al.*, 2006), the supplementation of semen with SP was beneficial for cryopreservation. Therefore, further studies are needed that aim to detect the most appropriate concentration of AFP I and/or to assess different parameters, such as the role of SP when using epididymal sperm.

In conclusion, the supplementation of 0.1 and 0.5 µg/ml of AFP I did not exhibit beneficial effects on the overall cryopreservation quality of epididymis spermatozoa from domestic cats. However, it remains unclear whether an adjustment to the cryopreservation protocol, such as a change in AFP I concentration, could lead to a more promising outcome.

Data availability. The data that support the study findings are available from the corresponding author upon reasonable request.

Acknowledgements. The authors thank the following organizations for financial support: Fluminense Federal University (FOPESQ), the Brazilian National Council for Scientific and Technological Development (CNPQ; project #434302/2018–0), and the Carlos Chagas Filho Foundation for Research Support in the State of Rio de Janeiro (FAPERJ). The authors also thank the Instituto Municipal de Medicina Veterinária Jorge Vaitsman for the surgical procedures and donation of traits for this study. The authors are grateful to Rachel Ferreira Braga and Viviane Lopes Brair for their technical assistance. LPA, PVSP, TAO, and LFLC are CAPES fellows (Finance code 001) and EMV was supported by CNPq. FZB and JMGS-F are both FAPERJ and CNPq fellows.

Author contributions. LPA, LFLC, and JMGS-F designed the study. LPA, PVSP, and TAO conducted the experiments. LPA, LFLC, EMV, and JMGS-F analyzed the data. LPA wrote the first manuscript, and PVSP, TAO, LFLC, EMV, FZB, and JMGS-F critically revised and approved the final version of the manuscript.

Conflicts of interest. The authors declare no conflict of interest.

Ethical standards. The authors declare that all procedures were performed according to national and institutional guides on the care and use of animals.

References

Axnér, E., Hermansson, U. and Linde-Forsberg, C. (2004). The effect of Equex STM paste and sperm morphology on post-thaw survival of cat epididymal spermatozoa. *Animal Reproduction Science*, **84**(1–2), 179–191. doi: 10.1016/j.anireprosci.2003.11.003

Barbato, G. F., Cramer, P. G. and Hammerstedt, R. H. (1998). A practical *in vitro* sperm-egg binding assay that detects subfertile males. *Biology of Reproduction*, **58**(3), 686–699. doi: 10.1095/biolreprod58.3.686

Bernardini, A., Hozbor, F., Sanchez, E., Fornés, M. W., Alberio, R. H. and Cesari, A. (2011). Conserved ram seminal plasma proteins bind to the sperm membrane and repair cryopreservation damage. *Theriogenology*, **76**(3), 436–447. doi: 10.1016/j.theriogenology.2011.02.020

Buranaamnuay, K. (2015). Determination of appropriate cryopreservation protocols for epididymal cat spermatozoa. *Reproduction in Domestic Animals*, **50**(3), 378–385. doi: 10.1111/rda.12496

Buranaamnuay, K. (2017). Protocols for sperm cryopreservation in the domestic cat: A review. *Animal Reproduction Science*, **183**, 56–65. doi: 10.1016/j.anireprosci.2017.06.002

Cedenho, A. P. and Miyamoto, I. T. (1990). Comparison of supravital staining techniques for spermatozoa: eosin and eosin-nigrosin. *Jornal Brasileiro de Urologia*, **16**(4), 234–236.

Chatdarong, K., Thuwanut, P., Manee-in, S., Lohachit, C. and Axnér, E. (2010). Effects of thawing temperature and post-thaw dilution on the quality of cat spermatozoa. *Reproduction in Domestic Animals*, **45**(2), 221–227. doi: 10.1111/j.1439-0531.2008.01218.x

Cheung, R. C. F., Ng, T. B. and Wong, J. H. (2017). Antifreeze proteins from diverse organisms and their applications: An overview. *Current Protein and Peptide Science*, **18**(3), 262–283. doi: 10.2174/1389203717666161013095027

Cocchia, N., Ciani, F., El-Rass, R., Russo, M., Borzacchiello, G., Esposito, V., Montagnaro, S., Avallone, L., Tortora, G. and Lorizio, R. (2010). Cryopreservation of feline epididymal spermatozoa from dead and alive animals and its use in assisted reproduction. *Zygote*, **18**(1), 1–8. doi: 10.1017/S0967199409990256

Colás, C., Junquera, C., Pérez-Pé, R., Cebrián-Pérez, J. A. and Muiño-Blanco, T. (2009). Ultrastructural study of the ability of seminal plasma proteins to protect ram spermatozoa against cold-shock. *Microscopy Research and Technique*, **72**(8), 566–572. doi: 10.1002/jemt.20710

Correia, L. F. L., Espírito-Santo, C. G., Braga, R. F., Carvalho-de-Paula, C. J., da Silva, A. A., Brandão, F. Z., Freitas, V. J. F., Ungerfeld, R. and Souza-Fabjan, J. M. G. (2021). Addition of antifreeze protein type I or III to extenders for ram sperm cryopreservation. *Cryobiology*, **98**, 194–200. doi: 10.1016/j.cryobiol.2020.11.001

Curry, M. R. (2000). Cryopreservation of semen from domestic livestock. *Reviews of Reproduction*, **5**(1), 46–52. doi: 10.1530/ror.0.0050046

Dadoune, J. P., Mayaux, M. J. and Guihard-Moscato, M. L. (1988). Correlation between defects in chromatin condensation of human spermatozoa stained by aniline blue and semen characteristics. *Andrologia*, **20**(3), 211–217. doi: 10.1111/j.1439–0272.1988.tb01058.x

DeVries, A. L. and Wohlschlag, D. E. (1969). Freezing resistance in some Antarctic fishes. *Science*, **163**(3871), 1073–1075. doi: 10.1126/science.163.3871.1073

Esteso, M. C., Soler, A. J., Fernández-Santos, M. R., Quintero-Moreno, A. A. and Garde, J. J. (2013). Functional significance of the sperm head morphometric size and shape for determining freezability in Iberian red deer (*Cervus elaphus hispanicus*) epididymal sperm samples. *Journal of Andrology*, **27**(5), 662–670. doi: 10.2164/jandrol.106.000489

Isachenko, E., Isachenko, V., Katkov, I. I., Dessole, S. and Nawroth, F. (2003). Vitrification of mammalian spermatozoa in the absence of cryoprotectants: From past practical difficulties to present success. *Reproductive Biomedicine Online*, **6**(2), 191–200. doi: 10.1016/s1472-6483(10)61710-5

IUCN (International Union for Conservation of Nature and Natural Resources). IUCN Red List of Threatened Species. Available online: https://www.iucnredlist.org [Accessed 2020]

Kar, R. K., Mroue, K. H., Kumar, D., Tejo, B. A. and Bhunia, A. (2016). Structure and dynamics of antifreeze protein–model membrane interactions: A combined spectroscopic and molecular dynamics study. *Journal of Physical Chemistry. B*, **120**(5), 902–914. doi: 10.1021/acs.jpcb.5b11164

Kim, H. J., Lee, J. H., Hur, Y. B., Lee, C. W., Park, S. H. and Koo, B. W. (2017). Marine antifreeze proteins: Structure, function, and application to cryopreservation as a potential cryoprotectant. *Marine Drugs*, **15**(2), 27. doi: 10.3390/md15020027

Klaus, C., Eder, S., Franz, C. and Müller, K. (2016). Successful cryopreservation of domestic cat (*Felis catus*) epididymal sperm after slow equilibration to 15 or 10°C. *Reproduction in Domestic Animals*, **51**(2), 195–203. doi: 10.1111/rda.12666

Koshimoto, C. and Mazur, P. (2002). Effects of warming rate, temperature, and antifreeze proteins on the survival of mouse spermatozoa frozen at an optimal rate. *Cryobiology*, **45**(1), 49–59. doi: 10.1016/s0011-2240(02)00105-0

Kovács, A. and Foote, R. H. (1992). Viability and acrosome staining of bull, boar and rabbit spermatozoa. *Biotechnic and Histochemistry*, **67**(3), 119–124. doi: 10.3109/10520299209110020

Luvoni, G. C. (2006). Gamete cryopreservation in the domestic cat. *Theriogenology*, **66**(1), 101–111. doi: 10.1016/j.theriogenology.2006.03.012

Luvoni, G. C. and Morselli, M. G. (2017). Canine epididymal spermatozoa: A hidden treasure with great potential. *Reproduction in Domestic Animals*, **52**(Suppl. 2), 197–201. doi: 10.1111/rda.12820

Martínez-Pastor, F., Anel, L., Guerra, C., Alvarez, M., Soler, A. J., Garde, J. J., Chamorro, C. and de Paz, P. (2006). Seminal plasma improves cryopreservation of Iberian red deer epididymal sperm. *Theriogenology*, **66**(8), 1847–1856. doi: 10.1016/j.theriogenology.2006.04.036

Mizutani, T., Sumigama, S., Nagakubo, K., Shimizu, N., Oba, H., Hori, T. and Tsutsui, T. (2010). Usefulness of addition of orvus ES paste and sodium lauryl sulfate to frozen feline semen. *Journal of Veterinary Medical Science*, **72**(1), 23–27. doi: 10.1292/jvms.08-0332

Patel, M., Gandotra, V. K., Cheema, R. S., Bansal, A. K. and Kumar, A. (2016). Seminal plasma heparin binding proteins improve semen quality by reducing oxidative stress during cryopreservation of cattle bull semen. *Asian-Australasian Journal of Animal Sciences*, **29**(9), 1247–1255. doi: 10.5713/ajas.15.0586

Payne, S. R., Oliver, J. E. and Upreti, G. C. (1994). Effect of antifreeze proteins on the motility of ram spermatozoa. *Cryobiology*, **31**(2), 180–184. doi: 10.1006/cryo.1994.1021

Prathalingam, N. S., Holt, W. V., Revell, S. G., Mirczuk, S., Fleck, R. A. and Watson, P. F. (2006). Impact of antifreeze proteins and antifreeze glycoproteins on bovine sperm during freeze–thaw. *Theriogenology*, **66**(8), 1894–1900. doi: 10.1016/j.theriogenology.2006.04.041

Ramu, S. and Jeyendran, R. S. (2013). The hypo-osmotic swelling test for evaluation of sperm membrane integrity. *Methods in Molecular Biology*, **927**, (21–25). doi: 10.1007/978-1-62703-038-0_3

Rui, B. R., Angrimani, D. S. R., Losano, J. D. A., Bicudo, L. C., Nichi, M. and Pereira, R. J. G. (2017). Validation of simple and cost-effective stains to assess acrosomal status, DNA damage and mitochondrial activity in rooster spermatozoa. *Animal Reproduction Science*, **187**, 133–140. doi: 10.1016/j.anireprosci.2017.10.017

Thammapradit, K. and Ponglowhapan, S. (2018). Evaluation of two cryopreservation protocols on cauda epididymal spermatozoa characteristics in domestic cats. *Thai Journal of Veterinary Medicine*, **48**(1), 1–7.

Toyonaga, M., Sato, Y., Morita, M., Watanabe, M., Oba, H., Mizutani, T., Hori, T. and Tsutsui, T. (2010). The qualities of cryopreserved epididymal sperm collected from feline epididymides stored at low temperature. *Journal of Veterinary Medical Science*, **72**(6), 777–780. doi: 10.1292/jvms.09-0260

Upreti, G. C., Payne, S. R., Duganzich, D. M., Oliver, J. E. and Smith, J. F. (1996). Enzyme leakage during cryopreservation of ram spermatozoa. *Animal Reproduction Science*, **41**(1), 27–36. doi: 10.1016/0378-4320(95)01442-X

Watson, P. F. (2000). The causes of reduced fertility with cryopreserved semen. *Animal Reproduction Science*, **60–61**, 481–492. doi: 10.1016/s0378-4320(00)00099-3

Zambelli, D., Iacono, E., Raccagni, R. and Merlo, B. (2010). Quality and fertilizing ability of electroejaculated cat spermatozoa frozen with or without Equex STM paste. *Theriogenology*, **73**(7), 886–892. doi: 10.1016/j.theriogenology.2009.11.012

https://doi.org/10.1017/S0967199422000521 Published online by Cambridge University Press