Cryobiology xxx (xxxx) xxx

Contents lists available at ScienceDirect

Cryobiology

journal homepage: http://www.elsevier.com/locate/cryo

Addition of antifreeze protein type I or III to extenders for ram sperm cryopreservation

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ARTICLE INFO

Keywords:
Cryoprotectant
Ram
Slow freezing
Spermatozoa

ABSTRACT

Antifreeze proteins (AFP) play an important role in cellular survival at sub-zero temperatures. This study assessed the effect of AFP type I or III in semen extender (TRIS-egg yolk) for ram sperm cryopreservation. Pooled semen of four rams were allocated into five treatments: Control (CONT, without AFP); AFP Type I [0.1 (AFPI-0.1) or 0.5 (AFPI-0.5) μg/mL]; or III [0.1 (AFPIII-0.1) or 0.5 (AFPIII-0.5) μg/mL], and then frozen in six replicates. Treatments affected kinetic parameters, plasma membrane integrity and morphology ($P < 0.05$). The AFPIII-0.1 presented lesser total motility. Linearity was greater in AFPI-0.1, AFPI-0.5 and AFPIII-0.5 and straightness was greater in all AFP-supplemented extenders. Plasma membrane integrity was greater in AFPI-0.1 and AFPI-0.5. All AFP groups had greater percentage of normal sperm than CONT. No differences ($P > 0.05$) were observed in hypoosmotic test, sperm acrosome status, mitochondrial activity, chromatin condensation, perivitelline membrane binding rate and lipoperoxidation. In conclusion, the use of AFP, predominantly type I, may increase sperm cell protection during cryopreservation, with no adverse effect on potential fertilization capacity or increase in reactive oxygen species, being a potential cryoprotectant to ram sperm.

1. Introduction

The use of reproductive biotechnologies in livestock production is essential for genetic improvement [30], and most of them require the use of cryopreserved semen. Despite all benefits of sperm cryopreservation, the availability of good quality frozen semen for use in artificial insemination (AI) is a challenge worldwide due to processing difficulties in sheep [19]. Therefore, the development of effective techniques to provide high quality thawed semen is essential.

In general, sperm viability decreases after freezing/thawing process, but final sperm quality is related to the initial quality of fresh semen, its ability to support the stress due temperature changes, and the processes for freezing and thawing the sample. Semen extenders are necessary to sperm protection from unfavorable conditions during freezing, increasing the rates of cell survival and increasing the sample volume [13]. However, during these processes there are membrane, mitochondrial, and molecular damages, excessive production of reactive oxygen species (ROS), osmotic stress and the formation of intracellular ice crystals, leading to a reduced motility [13,35]. These effects are especially important in ram sperm, a species in which cryopreservation has especially low effectiveness (see review [18]). Therefore, the development of new protocols [6] and potential cryoprotectant agents are needed to enhance sperm cryosurvival [35].

Antifreeze proteins (AFP) are groups of polypeptides that evolved in invertebrate and vertebrate fluids, insects and plants playing basic roles for their survival in sub-zero temperatures [29], acting as natural cryoprotectants in those species (see review [38]). These proteins induce thermal hysteresis and inhibit ice recrystallization, reduce the kinetics of ice formation, and influence the ice crystals morphology [16]. These proteins can be used for cell cryopreservation, including sperm, as their

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https://doi.org/10.1016/j.cryobiol.2020.11.001
Received 10 August 2020; Received in revised form 24 September 2020; Accepted 3 November 2020
Available online 10 November 2020
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inclusion in semen samples decrease the loss on motility, keep viability, membrane functionality and acrosome integrity in frozen-thawed semen in several farm species as ram [22], buffalo [26] and rabbit [21]. However, there are four main types of AFP (type I, type II, type III and Glycoprotein - AFGP), with different acting pathways, differences in how they bind to the ice crystal faces, among others [29]. In particular, it was demonstrated that the percentage of motile sperm after thawing is greater after adding AFP I and AFGP [22,36]. Thus, the aim of this study was to compare the effectiveness of AFP type I and III in two concentrations each added as semen extenders on the quality of frozen-thawed ram sperm.

2. Material and methods

Ethics approval

This study was approved by Ethics Committee for Use of Animals (5526080119) of Universidade Federal Fluminense and followed the guidelines of Animal Research: Reporting of *In vivo* Experiments (ARRIVE).

2.1. Reagents

All reagents used were purchased from Sigma Chemical Co (St. Louis, MO, USA), unless otherwise indicated. The AFP proteins were obtained from A/F Protein Inc (Waltham, MA, USA) and diluted according to the manufacturer instructions. The AFP I was purified from *Myoxocephalus scorpius* and AFP III from *Marcrozoarces americanus*. The eggs used to prepare TRIS egg yolk extender and to conduct the perivitelline membrane binding test were obtained in a local supermarket.

2.2. Experimental conditions and animals

Semen collection and analyses were conducted at the Unidade de Pesquisa em Caprinos e Ovinos (UniPECO), in Cachoeiras de Macacu, Rio de Janeiro, Brazil (22° 27′ S, 42° 39’ W) during august (end of the breeding season in the Southern hemisphere). Four adult Santa Inês rams (a light seasonal breed [3]) selected clinically and andrologically according to CBRA [10] were used for the study. The rams were kept under the same management conditions until the end of the study, including natural light, with access to pasture and receiving concentrate according to their nutritional requirements, with free access to water and minerals.

2.3. Experimental design and procedures

Firstly, semen was collected using electroejaculation [1] for four consecutive days, followed by two days of sexual rest to homogenize the seminal status of the four animals [15]. Afterwards, semen collection was carried out on the same four rams six times in alternate days. Semen was evaluated macroscopically (volume, odor, color, appearance) and microscopically (sperm kinetics and concentration). All ejaculates that showed patterns compatible with the species and motility ≥70% were selected for freezing [10]. Each day, semen from the four rams was pooled to minimize individual influences. The sperm concentration was determined by Neubauer chamber, and semen was diluted according to each treatment to a final concentration of 100×10^6 spermatozoa/straw to obtain the same number of straws per treatment to perform cryopreservation.

There were five treatments, including two concentrations (0.1 and 0.5 μg/mL) from two AFP types (I and III): AFP Type I with 0.1 μg/mL (AFPI-0.1); or 0.5 μg/mL (AFPI-0.5); AFP Type III with 0.1 μg/mL (AFPIII-0.1) or 0.5 μg/mL (AFPIII-0.5), and the untreated control (CONT). After sperm dilution in each treatment, the sperm kinetics, plasma membrane integrity, membrane function (hypoosmotic test), sperm acrosome and mitochondrial activity were evaluated. Immediately after thawing (0 h), the same variables and the chromatin condensation, morphology, the response to incubation in fertilization medium, sperm binding to egg perivitelline membrane and lipoperoxidation quantification of sperm cells were also done. The experimental procedures are presented in Fig. 1.

2.4. Macroscopic evaluations

The macroscopic characteristics were evaluated after collection in undiluted semen. Volume measurement was performed using graduated pipettes. Color, appearance, and odor were subjectively analyzed.

2.5. Cryopreservation and thawing

The semen extender used was TRIS egg yolk (3.63 g TRIS, 0.50 g fructose, 1.99 g citric acid, 14 mL egg yolk, 100.000 IU penicillin, 100 mg streptomycin, 5% glycerol, glass-distilled water to 100 mL; pH 7.4 and 1210 mOsm/kg). After dilution of semen in each extender treatment, semen was placed in plastic straws of 0.25 mL, identified and sealed with polyvinyl alcohol and disposed in a metal wire net frame at 37 °C for cooling in fridge for 2 h to reach temperature of 5 °C (−0.25 °C/min) and 2 h more for stabilization before liquid nitrogen vapor cooling (−15.26 °C/min). The straws were cooled in nitrogen vapor for 10 min [14], in a styrofoam box with liquid nitrogen (from +5 °C to −140 °C). Then, the straws were immediately immersed in liquid nitrogen (−196 °C) and maintained in cryogenic cylinder until thawing. The cooling rate was measured aided by a digital thermometer, in all replicates. Straws thawing was performed in water bath at 35 °C for 30 s.

2.6. Microscopic evaluations

2.6.1. Sperm kinetics

Motility parameters were analyzed by objective Computer-Assisted Semen Analysis (CASA) using SCA system (Sperm Class Analyzer Microptic, Nikon Eclipse Ci – Tokyo, Japan), with the software configured for ram sperm. Standard configuration of parameters was the spermatic head dimension detectable by system between 18 and 60 μm^2. Twenty-five images/s were captured in × 100 magnification; measurements were performed in a 24× 24 mm cover slide with a 10 μL drop of each sample. Spermatozoa were consider as immotile when the curvilinear velocity (VCL) was below 10 μm/s; slow when was between 10 and 45 μm/s; medium when it is between 45 and 75 μm/s; and rapid when it was above 75 μm/s. Sperm cells presenting straightness (STR) above 80% were featured as progressive motile sperm. The following kinetics patterns were determined: total motile sperm (%), progressive motile sperm (%), fast sperm (%), medium speed sperm (%), slow speed sperm (%), average path velocity (VAP, μm/s), curvilinear velocity (VCL, μm/s), straight line velocity (VSL, μm/s), amplitude of lateral head displacement (ALH, μm/s), beat/cross frequency (BCF, Hz), straightness (STR, %), linearity (LIN, %), and WOB (mean value of ratio between VAP and VCL, %).

2.6.2. Plasma membrane integrity

The plasma membrane integrity was determined with the eosin-nigrosin stain slightly modified according to Agarwal et al. [2]. One part of eosin at 3% stock solution was mixed to one part of nigrosin at 8% stock solution to prepare the working solution. One part of the working solution was incubated with one part of the sperm sample during 30 s at 37 °C. After that, a smear slide was prepared and evaluated in optical microscopy at 400 × magnification. At least 200 spermatozoa per slide were evaluated.

2.6.3. Hypoosmotic test

For the hypoosmotic test, aliquots of 30 μL of semen were placed in tubes with 1 mL of hypoosmotic solution of 100 mOsm/kg of MiliQ

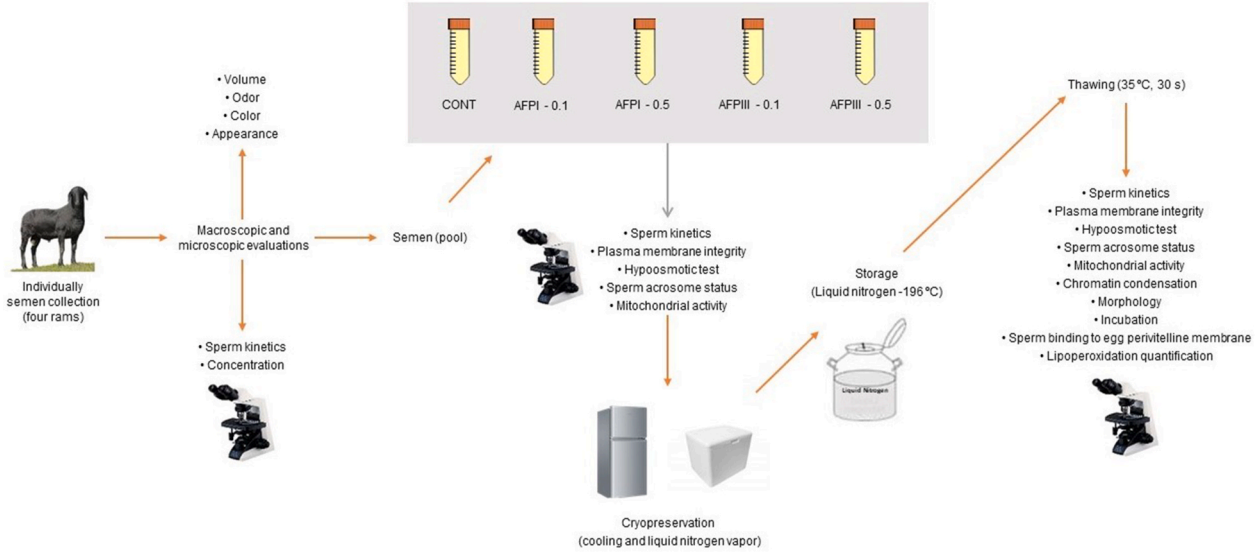

Fig. 1. Schematically representation of ram sperm collection, macroscopic and microscopic evaluations, treatment CONT-untreated; AFP Type I with 0.1 μg/mL (AFPI-0.1); or 0.5 μg/mL (AFPI-0.5); AFP Type III with 0.1 μg/mL (AFPIII-0.1) or 0.5 μg/mL (AFPIII-0.5), cryopreservation and microscopic evaluations applied before freezing and after thawing, performed in six replicates.

water. Then, these aliquots were incubated at 37 °C for 20 min and evaluated through wet preparation between slide and cover slide, by means of phase contrast microscopy in a 1000 × fold increase and immersion. At least 200 sperm cells were counted [28].

2.6.4. Sperm acrosome status

To determine the acrosome status, trypan blue/giemsa staining was applied [12,32]. One aliquot of sperm was incubated during 15 min with one part of 0.2% trypan blue at 37 °C. After that, a smear slide was prepared for each sample and kept in 10% giemsa solution for at least 1 h. Then, it was washed in running water and air dried, taken under optical microscope observation at 1000 × magnification under immersion oil. At least 200 sperm cells were evaluated. Acrosome status was classified in four types according to the presence of dye in the cellular compartment: live sperm with intact acrosome (LSIA), dead sperm with intact acrosome (DSIA), live sperm with acrosome reacted (LSAR) and dead sperm with acrosome lost (DSAL).

2.6.5. Mitochondrial activity

Mitochondrial activity was evaluated according to Rui et al. [31]. One part of the semen sample was added to one part of solution of 1 mg/mL 3,3′Diaminobenzidine (DAB) and incubated at 37 °C for 1 h in dark chamber. After that, one drop was used to prepare smear slide and was dried in air. The smears slides were fixed in 10% formalin solution for 10 min. Sperm cells were classified in four classes in optical microscopy with phase contrast at 1000 × magnification: all mitochondria active (100% of midpiece stained – DAB I); majority of mitochondria active (more than 50% of midpiece stained – DAB II); majority of mitochondria inactive (less than 50% of midpiece stained – DAB III); all mitochondria inactive (midpiece unstained – DAB IV). At least 200 sperm cells were counted.

2.6.6. Chromatin condensation

The Acidic Aniline Blue Staining was used to evaluate the chromatin condensation after cryopreservation according to Nabi et al. [20], with few modifications. For this, a smear slide was prepared with each semen sample and dried in air. Smears were fixed in 4% formalin solution for 5 min, and then washed; slides were stained in 5% of Aniline blue solution mixed with 4% acetic acid (pH 3.5) for 5 min and washed. Finally, the slides were stained in a 0.5% eosin solution for 30 s, washed in water and dried in air. For each stained smear slide, 200 sperm cells were evaluated at optical microscope in 1000 × magnification with immersion oil. Sperm cells with slightly stained nuclei were considered normal (mature chromatin) while the intense stained nuclei were considered abnormal (immature chromatin).

2.6.7. Sperm morphology

A 30 μL sample of semen was added to 1000 μL of buffered formal saline and stored at 4 °C until evaluation. Thereafter, a wet slide was prepared with one mixed drop of sample diluted in a slide with cover slide, and the spermatozoa morphology was examined under phase contrast microscopy at 1000 × magnification. At least 200 sperm cells were evaluated. Abnormal sperm were grouped into major and minor defects [10].

2.6.8. Incubation

After thawing, sperm aliquots of each treatment were incubated in FERT-TALP medium (0.33 g NaCl, 0.011 g KCl, 100 μL NaH_2PO_4, 93 μL Na lactate, 0.105 g $NaHCO_3$, 100 μL Phenol Red, 0.0135 g caffeine, 0.0147 g $CaCl_2.2H_2O$, 50 μL $MgCl_2$, 0.119 g Hepes) at 38 °C in 5% CO_2.The parameters of sperm kinetics, plasma membrane integrity, sperm acrosome status, mitochondrial activity and chromatin condensation were assessed at 1, 2 and 3 h of incubation.

2.6.9. Sperm binding to egg perivitelline membrane test

The test was conducted according to Barbato et al. [4] and Campos et al. [9]. The perivitelline membranes were obtained from fresh and non-fertile hen eggs. The perivitelline membrane was prepared by separating the egg yolk from the albumen. The intact yolks were placed on parafilm and the membrane was separated and washed with PBS. Then, the membrane was placed in a Petri dish and cut into squares of 0.5 cm^2. The membrane was covered with 1 mL of FERT-TALP and one aliquot of 20 μL of sperm sample was added. The membrane with semen sample was incubated for 1 h at 38.5 °C with 5% of CO_2 and 95% of air. After 30 min of incubation, 5 μL of orcein acetic solution was placed in the Petri dish and the samples were slowly homogenized. Then, the membrane was washed with PBS, allocated on a slide with a cover slide carefully without formation of folds or wrinkles, being sealed with nail polish. At optical microscope with 400 × magnification, five fields were counted per sample and results of spermatozoa binding were expressed as mm^2 of membrane [4,7].

2.7. Lipoperoxidation quantification

The quantification of lipoperoxidation was performed according to Sarlós et al. [33]. The method is based on the reaction between molecules of thiobarbituric acid and malondialdehyde, producing a rose color that is quantified by spectrophotometry. Reactions occur at a temperature between 90 °C and 100 °C, at acidic pH. Aliquots of 500 μL of samples from each treatment, and 1000 μL of 10% trichloroacetic acid solution (10% TCA) were centrifuged at 1800 *g* for 15 min and at 15 °C for precipitation of proteins. Aliquots of 500 μL of the supernatant were placed in tubes along with 500 μL of 1% thiobarbituric acid, dissolved in 0.05 N sodium hydroxide, freshly prepared. Tubes containing this mixture were incubated in a water boiling bath at 100 °C for 10 min and then cooled in an ice bath at 0 °C. Thiobarbituric Acid Reactive Species (TBARs) were quantified in a spectrophotometer, at a length of 532 nm and was expressed in nanograms of TBARs/mL of semen sample.

2.8. Statistical analyses

The normal distribution of the residues of all variables was determined with the Shapiro-Wilk test, and homoscedasticity with the Levene test. The variables with the residues normally distributed were analyzed with one-way analysis of variance (ANOVA) followed by Tukey test while those not normally distributed were analyzed by Kruskal Wallis followed by Dunn's test. The ANOVA with repeated measures in general linear model (GLM) was used first to check separately each effect of the concentration for each AFP type in paired samples. When sphericity was not considered, the analysis was corrected according to the Greenhouse-Geisser test followed by the Sidak test. Values of $P < 0.05$ were considered significant. The two-way ANOVA followed by Tukey test were performed for the incubation times and treatment analyses. A value of $P < 0.05$ was considered as statistically significant. All analyses were performed in IBM SPSS version 25.

3. Results

Immediately after dilution, no differences were observed in the experimental treatments in kinetic parameters, plasma membrane integrity, hypoosmotic test, acrosome status and mitochondrial activity. The results of treatments after dilution (just before cryopreservation) are presented in Table 1.

Immediately after thawing (0 h), sperm kinetics parameters, plasma membrane integrity and morphology were affected both, by AFP type and concentration (Table 1). The samples diluted with AFPIII-0.1 had lesser total motile sperm ($P = 0.009$) and lesser slow sperm ($P < 0.001$) than those from the CONT and those diluted with AFP III-0.5. The samples diluted with AFPI-0.1, AFPI0.5 and AFPIII-0.5 treatments presented greater LIN rates (AFP I: $P = 0.02$; AFP III: $P = 0.02$), whilst all AFP treatments had greater STR rates than the CONT (AFP I: $P = 0.01$; AFP III: $P = 0.02$). The percentage of sperm with intact plasma membrane after thawing was greater in AFPI-0.1 and AFPI-0.5 groups ($P = 0.003$) than in CONT.

All the AFP-diluted samples had more morphologically normal sperm (AFPI: $P = 0.03$; AFPIII: $P = 0.03$) than CONT. The extenders did not affect the percentages of sperm with major and minor defects in abnormal sperm, the hypoosmotic test, sperm acrosome status, mitochondrial activity, chromatin condensation, perivitelline membrane binding rate and lipoperoxidation. Regarding the effects of AFP concentration, only the sperm with plasma membrane integrity varied at 0.1 μg/mL ($P = 0.002$) where AFPI-0.1 was greater than AFPIII-0.1.

After 1 h of incubation, differences were only observed in LIN in favor of AFPIII treatments ($P = 0.001$) and in DSIA only in AFPIII-0.5 ($P = 0.03$). One hour later (2 h), the AFPIII-0.5 had lesser percentage of total motile sperm than CONT ($P = 0.02$), the AFPIII-0.1 had greater percentage of progressive motile sperm than CONT and AFPIII-0.5 ($P = 0.04$) and the AFPIII-0.5 had lesser BCF frequency than AFPIII-0.1 ($P =$

Table 1
Sperm parameters evaluated after dilution before freezing and immediately after (0 h) frozen-thawed ram semen subjected to extender containing different types and concentrations of antifreeze proteins (AFP) for cryopreservation (Mean + SEM).

		Before Freezing			Immediately (0 h) after frozen-thawed		
		0 μg/mL	0.1 μg/mL	0.5 μg/mL	0 μg/mL	0.1 μg/mL	0.5 μg/mL
Total Motility (%)	AFP I	96.0 ± 3.0 Aa	97.0 ± 1.6 Aa	94.2 ± 4.5 Aa	30.0 ± 2.1 Aa	27.6 ± 4.8 Aa	26.2 ± 2.0 Aa
	AFP III	96.0 ± 3.0 Aa	93.9 ± 3.0 Aa	95.3 ± 2.6 Aa	30.0 ± 2.1 Aa	13.7 ± 1.4 Ab	25.0 ± 2.3 Aa
Progressive Motility (%)	AFP I	25.2 ± 4.0 Aa	31.3 ± 6.4 Aa	28.5 ± 6.2 Aa	1.6 ± 0.3 Aa	2.5 ± 0.2 Aa	2.7 ± 0.6 Aa
	AFP III	25.2 ± 4.0 Aa	30.0 ± 4.8 Aa	25.4 ± 4.8 Aa	1.6 ± 0.3 Aa	2.1 ± 0.5 Aa	2.4 ± 0.4 Aa
Fast Sperm (%)	AFP I	64.8 ± 4.8 Aa	67.3 ± 1.8 Aa	60.3 ± 4.4 Aa	4.8 ± 1.1 Aa	5.3 ± 1.3 Aa	5.5 ± 1.9 Aa
	AFP III	64.8 ± 4.8 Aa	59.6 ± 7.1 Aa	55.3 ± 10.0 Aa	4.8 ± 1.1 Aa	1.5 ± 0.6 Aa	2.5 ± 1.1 Aa
Medium Sperm (%)	AFP I	22.2 ± 4.2 Aa	21.8 ± 4.1 Aa	24.9 ± 6.1 Aa	1.6 ± 0.2 Aa	2.1 ± 0.4 Aa	2.5 ± 0.8 Aa
	AFP III	22.2 ± 4.2 Aa	24.7 ± 7.5 Aa	24.1 ± 7.5 Aa	1.6 ± 0.2 Aa	1.6 ± 0.3 Aa	2.4 ± 0.5 Aa
Slow Sperm (%)	AFP I	10.3 ± 2.6 Aa	9.0 ± 1.9 Aa	11.9 ± 2.5 Aa	24.1 ± 1.1 Aa	15.6 ± 1.4 Ab	18.6 ± 3.1 Aa
	AFP III	10.3 ± 2.6 Aa	12.7 ± 2.2 Aa	11.7 ± 2.5 Aa	24.1 ± 1.1 Aa	8.8 ± 0.6 Ab	17.3 ± 1.3 Ac
VCL (μm/s)	AFP I	89.0 ± 8.2 Aa	95.5 ± 5.6 Aa	86.3 ± 8.3 Aa	36.2 ± 3.8 Aa	44.2 ± 3.9 Aa	41.2 ± 9.4 Aa
	AFP III	89.0 ± 8.2 Aa	87.2 ± 6.4 Aa	87.0 ± 12.6 Aa	36.2 ± 3.8 Aa	42.9 ± 6.1 Aa	37.0 ± 6.0 Aa
VSL (μm/s)	AFP I	43.5 ± 7.8 Aa	49.3 ± 9.4 Aa	45.0 ± 10.6 Aa	14.5 ± 2.1 Aa	22.3 ± 3.2 Aa	15.1 ± 5.2 Aa
	AFP III	43.5 ± 7.8 Aa	44.6 ± 7.2 Aa	40.9 ± 7.7 Aa	14.5 ± 2.1 Aa	17.0 ± 3.1 Aa	15.0 ± 2.8 Aa
VAP (μm/s)	AFP I	63.8 ± 8.4 Aa	63.5 ± 7.4 Aa	55.1 ± 7.2 Aa	21.4 ± 2.0 Aa	34.3 ± 2.3 Aa	23.7 ± 6.4 Aa
	AFP III	63.8 ± 8.4 Aa	65.2 ± 7.9 Aa	54.4 ± 8.8 Aa	21.4 ± 2.0 Aa	27.1 ± 4.1 Aa	28.6 ± 4.2 Aa
LIN (%)	AFP I	47.1 ± 5.2 Aa	50.1 ± 6.7 Aa	49.1 ± 6.8 Aa	36.8 ± 3.0 Aa	56.6 ± 3.1 Ab	56.9 ± 2.2 Ab
	AFP III	47.1 ± 5.2 Aa	49.6 ± 4.7 Aa	46.2 ± 4.8 Aa	36.8 ± 3.0 Aa	53.3 ± 2.1 Aab	64.7 ± 6.2 Ab
STR (%)	AFP I	66.0 ± 3.6 Aa	67.8 ± 4.7 Aa	67.2 ± 4.9 Aa	63.2 ± 0.8 Aa	75.4 ± 0.9 Ab	78.5 ± 2.8 Ab
	AFP III	66.0 ± 3.6 Aa	66.7 ± 3.0 Aa	64.7 ± 2.8 Aa	63.2 ± 0.8 Aa	81.9 ± 3.2 Ab	78.4 ± 4.1 Ab
WOB (%)	AFP I	70.3 ± 4.0 Aa	72.1 ± 5.0 Aa	71.4 ± 4.6 Aa	69.4 ± 7.2 Aa	68.7 ± 4.5 Aa	72.8 ± 4.2 Aa

(*continued on next page*)

Table 1 (*continued*)

		Before Freezing			Immediately (0 h) after frozen-thawed		
		0 µg/mL	0.1 µg/mL	0.5 µg/mL	0 µg/mL	0.1 µg/mL	0.5 µg/mL
	AFP III	70.3 ± 4.0 Aa	73.5 ± 3.8 Aa	70.6 ± 4.7 Aa	69.4 ± 7.2 Aa	65.8 ± 4.2 Aa	69.8 ± 6.4 Aa
ALH (µm)	AFP I	3.0 ± 0.2 Aa	3.0 ± 0.2 Aa	2.7 ± 0.2 Aa	1.9 ± 0.3 Aa	2.1 ± 0.4 Aa	1.6 ± 0.2 Aa
	AFP III	3.0 ± 0.2 Aa	2.8 ± 0.2 Aa	2.8 ± 0.3 Aa	1.9 ± 0.3 Aa	1.6 ± 0.3 Aa	2.1 ± 0.4 Aa
BCF (Hz)	AFP I	7.0 ± 0.6 Aa	7.4 ± 0.3 Aa	6.7 ± 0.4 Aa	5.2 ± 0.7 Aa	7.1 ± 1.2 Aa	6.9 ± 1.5 Aa
	AFP III	7.0 ± 0.6 Aa	7.7 ± 0.5 Aa	7.4 ± 0.4 Aa	5.2 ± 0.7 Aa	4.9 ± 0.6 Aa	7.1 ± 1.4 Aa
PM Integrity (%)	AFP I	63.9 ± 7.6 Aa	70.2 ± 5.7 Aa	71.8 ± 7.2 Aa	13.0 ± 4.4 Aa	49.1 ± 4.6 Ab	36.6 ± 7.3 Ab
	AFP III	63.9 ± 7.6 Aa	58.9 ± 3.2 Aa	58.2 ± 1.4 Aa	13.0 ± 4.4 Aa	19.8 ± 3.6 Ba	21.8 ± 4.0 Aa
Hypoosmotic (%)	AFP I	85.3 ± 1.4 Aa	86.3 ± 1.7 Aa	79.6 ± 2.1 Aa	13.6 ± 2.6 Aa	16.9 ± 4.2 Aa	22.2 ± 4.3 Aa
	AFP III	85.3 ± 1.4 Aa	81.6 ± 4.6 Aa	87.4 ± 0.3 Aa	13.6 ± 2.6 Aa	15.7 ± 3.8 Aa	11.9 ± 1.8 Aa
LSIA (%)	AFP I	28.8 ± 4.4 Aa	33.1 ± 8.8 Aa	27.8 ± 4.8 Aa	16.4 ± 6.2 Aa	13.8 ± 6.8 Aa	11.7 ± 6.1 Aa
	AFP III	28.8 ± 4.4 Aa	32.5 ± 5.6 Aa	28.1 ± 4.8 Aa	16.4 ± 6.2 Aa	7.0 ± 2.1 Aa	8.5 ± 1.4 Aa
LSAR (%)	AFP I	65.8 ± 2.4 Aa	57.8 ± 8.0 Aa	56.4 ± 6.4 Aa	8.3 ± 2.2 Aa	10.7 ± 5.0 Aa	10.6 ± 5.5 Aa
	AFP III	65.8 ± 2.4 Aa	56.8 ± 5.1 Aa	57.3 ± 8.1 Aa	8.3 ± 2.2 Aa	8.7 ± 2.8 Aa	10.0 ± 1.3 Aa
DSIA (%)	AFP I	3.8 ± 0.4 Aa	3.8 ± 0.7 Aa	4.4 ± 1.5 Aa	59.3 ± 14.1 Aa	59.0 ± 17.5 Aa	64.2 ± 14.1 Aa
	AFP III	3.8 ± 0.4 Aa	6.7 ± 1.9 Aa	2.5 ± 1.1 Aa	59.3 ± 14.1 Aa	67.8 ± 11.0 Aa	61.6 ± 10.7 Aa
DSAL (%)	AFP I	6.1 ± 1.1 Aa	6.1 ± 0.9 Aa	3.4 ± 1.0 Aa	3.9 ± 2.1 Aa	11.3 ± 5.7 Aa	7.4 ± 3.4 Aa
	AFP III	6.1 ± 1.1 Aa	4.2 ± 0.8 Aa	4.7 ± 0.7 Aa	3.9 ± 2.1 Aa	7.2 ± 5.2 Aa	8.8 ± 3.4 Aa
DAB I (%)	AFP I	95.5 ± 0.9 Aa	94.2 ± 1.2 Aa	95.2 ± 0.8 Aa	10.3 ± 2.2 Aa	12.5 ± 3.0 Aa	16.7 ± 2.5 Aa
	AFP III	95.5 ± 0.9 Aa	95.3 ± 1.1 Aa	94.7 ± 1.0 Aa	10.3 ± 2.2 Aa	12.6 ± 0.7 Aa	10.3 ± 1.7 Aa
DAB II (%)	AFP I	0.7 ± 0.3 Aa	0.9 ± 0.2 Aa	0.8 ± 0.1 Aa	9.4 ± 4.5 Aa	13.3 ± 5.3 Aa	11.8 ± 5.5 Aa
	AFP III	0.7 ± 0.3 Aa	1.0 ± 0.3 Aa	0.7 ± 0.2 Aa	9.4 ± 4.5 Aa	8.8 ± 2.3 Aa	8.0 ± 2.6 Aa
DAB III (%)	AFP I	0.8 ± 0.3 Aa	0.8 ± 0.3 Aa	1.1 ± 0.3 Aa	1.6 ± 0.3 Aa	2.0 ± 1.0 Aa	1.5 ± 0.4 Aa
	AFP III	0.8 ± 0.3 Aa	1.0 ± 0.5 Aa	0.6 ± 0.3 Aa	1.6 ± 0.3 Aa	1.5 ± 0.3 Aa	1.9 ± 0.6 Aa
DAB IV (%)	AFP I	3.0 ± 0.6 Aa	4.1 ± 1.2 Aa	2.9 ± 0.6 Aa	80.0 ± 5.7 Aa	69.6 ± 7.6 Aa	71.9 ± 6.0 Aa
	AFP III	3.0 ± 0.6 Aa	2.7 ± 0.6 Aa	4.0 ± 0.6 Aa	80.0 ± 5.7 Aa	74.3 ± 5.3 Aa	83.3 ± 3.0 Aa
Normal Chrom. (%)	AFP I	–	–	–	99.0 ± 0.3 Aa	98.9 ± 0.4 Aa	98.5 ± 0.5 Aa
	AFP III	–	–	–	99.0 ± 0.3 Aa	98.8 ± 0.3 Aa	98.8 ± 0.3 Aa
Normal Morphol. (%)	AFP I	–	–	–	65.3 ± 1.9 Aa	73.0 ± 1.0 Ab	75.7 ± 2.2 Ab
	AFP III	–	–	–	65.3 ± 1.9 Aa	73.8 ± 1.6 Ab	74.8 ± 0.5 Ab
Sperm binding (mm^2)	AFP I	–	–	–	186.7 ± 47.2 Aa	178.3 ± 31.8 Aa	175.0 ± 49.3 Aa
	AFP III	–	–	–	186.7 ± 47.2 Aa	238.3 ± 49.6 Aa	191.6 ± 44.6 Aa
TBARS (ng/mL)	AFP I	–	–	–	567.2 ± 20.0 Aa	545.8 ± 29.3 Aa	559.9 ± 18.3 Aa
	AFP III	–	–	–	567.2 ± 20.0 Aa	544.0 ± 15.6 Aa	566.6 ± 19.2 Aa

Within a column or row, values with different superscripts differ significantly for each parameter ($P < 0.05$).

[A,B] differs between AFP types (I or III), at the same concentration (0, 0.1 or 0.5 µg/mL) and at the same moment (before freezing or 0 h).

[a,b,c] differs among concentrations (0, 0.1 and 0.5 µg/mL), at the same AFP type and at the same moment (before freezing or 0 h).

Abbreviations: VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN: linearity; STR: straightness; WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat/cross frequency; PM Integrity: Plasma Membrane Integrity; LSIA: Live sperm with intact acrosome; LSAR: Live sperm with acrosome reacted; DSIA: Dead sperm with intact acrosome; DSAL: Dead sperm with acrosome lost; DAB: 3′-Diaminobenzidine (mitochondrial activity); DAB I: All mitochondria active; DAB II: Majority mitochondria active; DAB III: Majority mitochondria inactive; DAB IV: All mitochondria inactive; Normal Chrom.: Normal Chromatin condensation; Normal Morphol.: Normal Morphology; Sperm binding: Sperm binding to egg perivitelline membrane; TBARS: Thiobarbituric acid reactive substances (lipid peroxidation).

0.04). There were no differences detected after 3 h of incubation (Supplementary Table 1). Conversely, the slow sperm parameter in the AFPIII-0.1 treatment was greater at 1 h and lesser at 2 h (within the same treatment) ($P = 0.02$) (Supplementary Table 1).

4. Discussion

Overall, the results of this study demonstrate that the use of AFP I and AFP III for the dilution opens interesting perspectives for developing more effective sperm preservation techniques in sheep. The dilution *per se* had minimum effects, as immediately after dilution there were no significant differences among groups. This is important as demonstrates that despite improving sperm resistance to freezing, AFP addition to fresh semen does not have any deleterious effects. The concentrations tested were defined according to a literature screening, considering that lower concentrations of AFP improve results of cryopreservation while greater concentrations could cause cytotoxic effects when added to the

extender [16,29].

The addition of AFP to ram semen had beneficial effects increasing its cryoresistance, especially in sperm kinetics, plasma membrane integrity and morphology, maintaining the energetic metabolism without ROS increase. The AFP use as cryoprotectant in sperm was already demonstrated as a tool to reduce the loss of motility, preserve energetic metabolism and osmotic resistance [29]. During sperm cryopreservation, the ice crystals formation could irreversible damage the cells affecting plasma membrane and cell morphology [13]. The freezing procedure can alter lipid membranes, grouping proteins irreversibly, making these membranes more rigid and fragile [35]. The AFP properties probably stabilize the membrane phospholipids and unsaturated fatty acids [13,16]. It was already reported that AFP I can reduce chilling sensitivity, conferring low-temperature protection to cellular membranes by a direct interaction of this protein with the phospholipid bilayer [34]. Although combined treatments and interactions with other extender components should be tested, the use of these proteins enables to hamper a key bottleneck step in the use of ram sperm. In general, both types of AFP provided similar cryoprotectant results in ram sperm. Samples diluted with AFP III, however, had lesser percentage of motile spermatozoa than CONT samples, while AFP I enhanced the percentage of sperm with plasma membrane integrity. Thus, although not definitely, it can be suggested that AFP I is probably more effective than AFP III for ram semen cryopreservation.

The AFP improved rates of LIN and STR at 0 h and both parameters are related to sperm trajectory. Frozen-thawed sperm samples collected from *Sparus aurata* also had greater LIN after the addition of AFP I and III [5]. Considering that these variables might be predictors of *in vivo* fertility [11], these results open interesting perspectives to test the practical use of these proteins. In this sense, although, pregnancy rate in buffalos was greater with the addition *D. canadensis* antifreeze protein (DAFP), those differences did not reach significance [26]. Furthermore, test AFP in AI strategies in sheep will allows to use in a high number of animals, considering that the endpoint responses (conception rate) are statistically low sensitive and could be increased.

Although more studies are needed, it seems that 0.1 μg/mL are enough to achieve the maximum effects. It is noted that in this concentration, the percentage of slow speed sperm differed significantly from the control and the concentration of 0.5 μg/mL. In addition, in AFP I, this concentration allowed greater results in plasma membrane integrity. In buffalo, the use of 0.1 μg/mL of AFP III also enhanced membrane integrity [24], as well as the use of 1 μg/mL of AFGP [25], and 10 μg/mL of DAFP [26]. On the other hand, concentrations lower than 0.1 μg/mL of AFP as cryoprotectant in extender could not have any cryoprotective effects, such as presented in mouse spermatozoa without effects on freezing/thawing [17]. In the present study, the AFP concentrations used are similar to those previously used [8]. These values are based and corroborate previous reports showing AFP mechanism activity, where at similar concentrations these proteins perform their functions normally [27]. However, the exact dose, combination with other components, different types of AFP, as well as higher AFP concentrations that could increase the activity response in cryopreservation, reinforces the need of more detailed studies.

Although apparently AFP III did not present any advantage toward AFP I, it should be considered that this is the first report using this type of AFP (AFP III) in sheep spermatozoa. In other species, including bovine [23], buffalo [24] and cynomolgus macaque [37], AFP III had beneficial effects on sperm cryopreservation. It should be noted that no study had tested a combination of different types of AFP, including the present study, to evaluate the effects during sperm cryopreservation. This could be a future perspective, as considering that each type may act on different mechanisms [29]. It would be interesting, thus, to test if combined in a single extender the advantage of each AFP *per se* is still maintained.

In conclusion, the addition of AFP appears auspicious for cryopreserving ram sperm cells. The use of AFP, predominantly type I, increased sperm cell protection during cryopreservation, resulting in greater sperm kinetics, better plasma integrity and greater percentage of normal sperm cells. These results open interesting possibilities to use AFP as a sheep semen cryoprotectant.

Declaration of competing interest

The authors declare no conflicts of interest.

Acknowledgements

The authors would like to thank Dr. Maykon Costa (Molecular Biology Institute of Paraná, Brazil) for the kind donation of the AFP type III. The authors thank CNPq (National Council for Scientific and Technological Development) (Project 434302/2018-0) and FAPERJ (Carlos Chagas Filho Foundation for Support to Research in the State of Rio de Janeiro) for financial support. LFLC was supported by CAPES (Finance Code 001). FZB and VJFF are CNPq fellows and JMGS-F is a FAPERJ and CNPq fellow.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cryobiol.2020.11.001.

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