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Comparative evaluation between the extenders TES-TRIS and ACP-112® and the association of Sálva Marajó oil (*Lippia origanoides*) in the quality of cryopreserved buffalo sperm

Avaliação comparativa entre os diluidores TES-TRIS e ACP-112® e a associação com o óleo da Sálva do Marajó (*Lippia origanoides*) na qualidade do sêmen bubalino criopreservado

Adriana Novaes dos Reis[1*]; Moysés dos Santos Miranda[2]; Lílian Kátia Ximenes Silva[3]; Aluizio Otavio Almeida da Silva[1]; José Silva de Sousa[4]; Eziquiel de Morais[5]; Alexandre Rossetto Garcia[6]; Sheyla Farhayldes Souza Domingues[7]; Joyce Kelly do Rosário da Silva[2]; Haroldo Francisco Lobato Ribeiro[8]

Abstract

For artificial insemination, it is essential to use frozen semen, however the freezing process causes deleterious changes to the structure and integrity of sperm membranes that compromise the function of sperm. To avoid this cellular damage, extenders and suitable substrates must be used to recover the highest possible number of viable cells post-thaw. To this end, in the first experiment, we evaluated three different extenders: TES-TRIS, which is widely used for buffaloes; and an extender composed of powdered coconut water-based (ACP-112®) with or without milk (ACP-112®-milk) for buffalo semen freezing. In the second experiment, we evaluated the effect of *Lippia origanoides* oil extract on protecting buffalo sperm against cryoinjury arising from freezing semen. Semen was collected from ten buffalo bulls (10 ejaculates/bull) and diluted in TES-TRIS (control), ACP-112® or ACP-112®-Milk in the first experiment. In the second experiment, the samples were diluted in the diluent with the best results for sperm quality obtained in experiment I, and 2.5 μg mL^{-1}, 5 μg mL^{-1} or 10 μg mL^{-1} of the plant extract was added to treatments; and a control group containing only the diluent was also included. The fresh semen was analyzed for conventional features such as motility, concentration, morphology and viability. After thawing, the samples were evaluated again for motility, vigor and supra-vital staining, and then, were performed the of thermal-resistance test, hypoosmotic test and evaluated sperm membrane integrity with the fluorescent probes PI, FITC-PSA and JC-1 using flow cytometry. The data were submitted to ANOVA, and the results were compared by Tukey's test at a significance of 5%. In the first experiment, the extender TES-TRIS showed better results for the various characteristics evaluated compared to ACP-112® and ACP-112®-Milk ($P < 0.05$), demonstrating greater protection of the buffalo sperm structures during cryopreservation. In the second experiment, the treatments with different concentrations of *Lippia origanoides* essential oil extract showed no differences among the assessed variables regarding the protection of sperm structures during cryopreservation ($P > 0.05$).

[1] Profs. Drs., Pesquisadores, Universidade Federal do Pará, UFPA/CEBRAN, Castanhal, PA, Brasil. E-mail: areis@ufpa.br; otavio@ufpa.br
[2] Profs. Drs., Pesquisadores, UFPA/ICB, Belém, PA, Brasil. E-mail: moyses_m@yahoo.com; joycekellys@yahoo.com.br
[3] Profª Drª, Pesquisadora, Universidade da Amazônia, UNAMA, Santarém, PA, Brasil. E-mail: silva_lilian@yahoo.com.br
[4] Prof. M.e, Pesquisador, UFPA/CEBRAN, Castanhal, PA, Brasil. E-mail: jss@ufpa.br
[5] Prof. M.e, Pesquisador, Instituto Federal do Pará, IFPA, Castanhal, PA, Brasil. E-mail: eziquielmorais@yahoo.com.br
[6] Pesquisador, Dr., Embrapa Pecuária Sudeste, CPPSE, São Carlos, SP, Brasil. E-mail: alexandre.garcia@embrapa.br
[7] Profª Drª, Pesquisadora, UFPA/BIOMEDAN, Castanhal, PA, Brasil. E-mail: shfarha@ufpa.br
[8] Prof. Dr., Pesquisador, Universidade Federal Rural da Amazônia, UFRA, Belém, PA, Brasil. E-mail: haroldo.ribeiro@ufra.edu.br
[*] Author for correspondence

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Based on these data, we demonstrated the beneficial effects of TES-TRIS for post-thaw buffalo sperm quality; however, no protective effect was observed for buffalo sperm cryopreserved with the different tested concentrations of *Lippia origanoides* extract oil.
Key words: Powdered Coconut Water. *Lippia.* Cryopreservation. Spermatozoa. Buffalo.

Resumo

Para a implantação da inseminação artificial é indispensável à utilização de sêmen congelado, que pode provocar mudanças deletérias na estrutura e na integridade das membranas espermáticas, comprometendo sua função. Para evitar estes danos celulares, há a necessidade de se utilizar meios diluidores e substratos adequados que recuperem o maior número possível de células viáveis pós-descongelação. Para isso, foram avaliados, no experimento I, três diferentes diluidores, o diluidor TES-TRIS, bastante utilizado para bubalinos, e um diluidor a base de água de coco em pó (ACP-112□), associado ou não ao leite (ACP-112□-Leite), na congelação do sêmen de bubalinos; e no experimento II, foi avaliado o efeito do óleo extraído da *Lippia origanoides* na proteção dos espermatozóides contra as crioinjúrias decorrentes da congelação do sêmen bubalino. Foram utilizados 10 touros bubalinos para as colheitas de sêmen (10 ejaculados/touro), sendo os ejaculados diluídos em TES-TRIS (controle), ACP-112□ e ACP-112□-Leite no experimento I; e no experimento II, os ejaculados foram diluídos no melhor diluidor obtido no experimento I, acrescido de 2.5 µg mL^{-1}, 5 µg mL^{-1} e 10 µg mL^{-1} da planta e o grupo controle, constituído somente do diluidor. O sêmen recém colhido foi analisado quanto as características convencionais, tais como, motilidade, concentração, morfologia e viabilidade. Após a descongelação das amostras foram avaliados novamente, motilidade e viabilidade espermática, e posteriormente, foram realizados os testes de termo-resistência, hiposmótico e de avaliação das membranas dos espermatozóides, através das sondas fluorescentes PI, FITC-PSA e JC-1, utilizando a citometria de fluxo. Os dados obtidos foram submetidos à ANOVA e ao Teste de Tukey a 5%. No experimento I, o diluente TES-TRIS apresentou melhores resultados para as várias características avaliadas quando comparado com o ACP-112® e ACP-112®-Leite ($P < 0.05$), demonstrando maior proteção deste diluidor às estruturas espermáticas durante a criopreservação do sêmen de bubalinos. No experimento II, as diferentes concentrações do óleo extraído da *Lippia origanoides* não demonstraram nenhuma diferença ($P > 0.05$) entre as variáveis avaliadas, quanto à proteção das estruturas espermáticas durante a criopreservação. Com base nestes dados, pôde-se demonstrar os efeitos benéficos do diluidor TES-TRIS na qualidade pós-descongelação dos espermatozóides bubalinos; entretanto, nenhum efeito protetor foi observado sobre os espermatozóides bubalinos criopreservados em associação com o óleo extraído da *Lippia origanoides*, nas diferentes concentrações utilizadas.
Palavras-chave: Água de coco em pó. *Lippia*. Criopreservação. Espermatozóides. Búfalo.

Introduction

Bhattacharya and Srivastava (1955) initiated research on buffalo semen cryopreservation for artificial insemination in India, and since then, several studies have been carried out; however, only by the middle of 1979 was there some progress in this area of study (VALE et al., 1984; OBA et al., 1993; BALRAM et al., 1997). Several diluents, such as TES-TRIS (SILVA et al., 2002), TRIS-Yolk (ANSARI et al., 2011; SHAHVERDI et al., 2014) Ringer's lactate (SILVA et al., 2002; MIYASAKI et al., 2014), Lactose-TRIS (MIYASAKI et al., 2014), Bioxcell® (AKHTER et al., 2010), Botu-Bov® (ZORZETTO et al., 2017), and Skim Milk (MIYASAKI et al., 2014), have been tested in buffalo, but currently, there is no consensus concerning the best diluent for use in this species to confer high post-thaw semen viability at an accessible cost (AKHTER et al., 2010; ANSARI et al., 2011; SHAHVERDI et al., 2014).

TES (N-Tris (hydroxymethyl)methyl-2-aminoethane sulfonate) is a diluent that has been used for more than 20 years that is specific for buffalo, which contains constituents that allow homogeneous post-cryopreservation results (VALE et al., 1984; RASUL et al., 2000; SILVA et al.,

2002; MOOSE et al., 2007). Today, TES is still widely used and even serves as a control diluent in experiments evaluating the use of other diluents for freezing buffalo semen (MOOSE et al., 2007; GONÇALVES et al., 2011; BRITO, 2014). This diluent is composed of two isoelectric buffers (TES and TRIS) that are associated with glycerol and, in contrast to other diluents, it also has two important high molecular weight compounds, egg yolk and milk, which according to Brito (2014), act as complementary cryoprotectants during cooling, preventing cold shock and improving post-thaw motility rates; these compounds were therefore used as control group in this experiment. According to Prado (2009), egg yolk has protective action that is mediated through low-density lipoproteins, which adhere to the cell membrane during cryopreservation and preserve the sperm membrane; according to Foote et al. (2002), milk provides benefits mainly through the supply of casein, which is a protein that acts as a natural antioxidant and assists in the protection of spermatozoa during cryopreservation.

As an alternative to traditional diluents, there are also some diluents consist of products of plant origin, as the coconut water (*Cocus nucifera*) either in natural form (SALGUEIRO et al., 2002) or powder (ACP®) (ACP Biotecnologia®, Fortaleza, Ceará, Brazil), as an efficient alternative that is simple and cost effective for the cryopreservation of semen from some species. According to Nunes (1997), this protective effect is due to the presence of a phytohormone that acts as a sperm cell protector, now known as 3-indole acetic acid (IAA) (NUNES, SALGUEIRO, 1999; TONIOLLI et al., 1999). ACP® was initially tested in goats (SALGUEIRO et al., 2002) and was shown to be efficient, which led to research on the use of this diluent for the cryopreservation of semen from other species, such as swines (TONIOLLI et al., 2010), equines (SAMPAIO NETO et al., 2002), canines (CARDOSO et al., 2005), ovines (SALGUEIRO et al., 2007) and caprines (OLIVEIRA et al., 2011).

As new diluents have been evaluated for the cryopreservation of mammalian semen, antioxidant compounds such as synthetic antioxidants (Pentoxifylline, Trolox®), vitamins (Vitamin C and E, Selenium) and antioxidants obtained from extracted plant essential oils have been evaluated as additives to diluents to balance the production of toxic substances with the viability of semen (MAIA; BICUDO, 2009; GONÇALVES et al., 2011; DAMASCENO et al., 2011; CARDOSO, 2013; TEIXEIRA et al., 2014).

Studies of the effectiveness of these plant oils in spermatozoa conservation have recently been reported. Zhao et al. (2009) observed that *Rhodiola sacra* extract improved the characteristics of cryopreserved boar spermatozoa by strong sequestering activity against free radicals. In a similar experiment, Malo et al. (2010) showed that Rosemary (*Rosmarinus officinalis*) improved the viability of swine semen and produced better rates of cleavage and blastocysts after the use of semen for in vitro fertilization. Daghigh-Kia et al. (2014) also found that the addition of Rosemary to the diluent medium increased the intracellular defense and cellular membrane protection systems against the production of ROS after the cryopreservation of bovine semen, which improved motility, membrane integrity and spermatozoa viability.

Although the Amazon has a high biodiversity of available plants with approximately 30 thousand existing species, less than 1% have been investigated from a chemical or pharmacological viewpoint (FERRO, 2008). One group of plants that have already been studied biochemically are those of the genus *Lippia*, which has approximately 200 species of herbs, shrubs and small trees, and approximately 120 species are found in Brazil, including *L. alba, L. gracilis, L. origanoides, L. organoides, L. sidoides*, and *L. triplinervis* (OLIVEIRA et al., 2007; DAMASCENO et al., 2011; SOARES; TAVARES-DIAS, 2013; OLIVEIRA et al., 2014). Extracted oil from *L. origanoides* has been evaluated in somatic cells; it showed antioxidant properties that retard oxidative degradation reactions, reducing the

oxidation rate and decreasing cell death (OLIVEIRA et al., 2007; DAMASCENO et al., 2011; TEIXEIRA et al., 2014).

ACP® for buffaloes (ACP-112®), which was developed by ACP Biotechnology® (Fortaleza, Ceará, Brazil), until now has not yet been tested on buffalo, nor have the antioxidant effects of *L. origanoides* essential oil been evaluated for their role in spermatozoa protection during the cryopreservation process; thus, the present work aimed to evaluate the efficiency of ACP-112® either with or without milk in the cryoprotection of buffalo semen, as well as evaluated the effect of the addition of natural antioxidants from *L. origanoides* essential oil on buffalo semen quality after cryopreservation.

Materials and Methods

Semen collection and initial evaluation

Ten Murrah buffalo bulls (3-5 years) were used in the experiment. The semen was collected by artificial vagina (42°C) with an interval of 15 days between the collections and with 10 ejaculates collected per animal. A total of 100 samples were obtained.

Immediately after semen collection, routine analyses were performed to select ejaculates that were suitable for cryopreservation according to the sperm parameters established by the Brazilian College of Animal Reproduction (2013); under these guidelines, the ejaculates should have at least 70% progressive motility, 70% normal morphology and 70% sperm viability, as determined by microscopic evaluation (Nikon Eclipse E600, Tokyo, Japan), and a sperm concentration between 300 and 1200×10^6 mL^{-1} spermatozoa (Photometer SDM5, Tiefenbach, Alemanha).

Cryopreservation

In this work, two experiments were carried out. In experiment I, three diluents for buffalo semen cryopreservation were tested (ACP-112®, ACP-112®-Milk, TES-TRIS) as described in Table 1. In experiment II, *L. origanoides* essential oil extract was added to the diluent with the best result from experiment I. Three different oil concentrations were used (2.5 μg mL^{-1}, 5 μg mL^{-1} and 10 μg mL^{-1}) in addition to a control group without the addition of essential oil. The reported chemical composition of the sample provided by the Laboratory of Natural Products Engineering – ICB / UFPa indicated Thymol (88.2%) as the major component.

Table 1. Composition of different diluents used in experiment I.

	Diluents		
	ACP	**ACP-Milk**	**TES-TRIS (Control)**
Base buffer	ACP-112®	ACP-112®	4.9% TES 1.06% TRIS
Skim milk **(w v^{-1})**	-	11%	11%
Egg yolk **(v v^{-1})**	20%	20%	20%
Glycerol **(v v^{-1})**	7%	7%	7%
Fructose **(w v^{-1})**	-	-	0.21%
Antibiotics	- -	- -	1000 UI mL^{-1} Penicillin 1 g mL^{-1} Streptomycin

TES (Sigma-Aldrich, St. Louis, USA), TRIS (Sigma-Aldrich, St. Louis, USA), Fructose (Merck, Darmstadt, Germany), Penicillin (Sigma-Aldrich, St. Louis, USA) and Streptomycin (Sigma-Aldrich, St. Louis, USA), Skim Milk (Molico, Nestlé Brazil Ltda, Brazil), Glycerol (Merck, Darmstadt, Germany), ACP-112® (Coconut powder – Buffalo Diluent, ACP Biotechnology® , Fortaleza, Brazil) (SALGUEIRO et al., 2002).

In all experiments, equal aliquots of the semen were diluted in the different experimental groups so that the final sperm concentration was 10×10^6 mL^{-1} spermatozoa. After mixing with the diluents, the semen was loaded into 0,25 mL French straws (IMV, France) and cooled over an equilibrium period for 3 hours at 5°C, subsequently submitted to liquid nitrogen vapor for 20 minutes, and then plunged into liquid nitrogen (-196°C) for storage until the thawing of samples for analysis.

Immediate post-thaw analysis

The semen straws were thawed in water at 37-40°C for 30 seconds, and immediately after thawing, an aliquot of the diluted semen was used to assess the motility (10 µl), the number of spermatozoa with swollen tails (50 µl), and the viability of the spermatic membranes by staining with 1% Eosin (w v^{-1}). The sperm motility was evaluated subjectively by percentage values by the same evaluator, as well as, the number of swollen tails and the viability of the membrane were assessed for 100 spermatozoa.

Hypoosmotic test (HOST)

Sperm plasma membrane integrity was evaluated by the hypoosmotic swelling test (HOST) as described by Tartaglione and Ritta (2004). For this purpose, a 50 µl aliquot of fresh semen was diluted in 500 µl of pre-warmed hypoosmotic solution (150 mOsm kg^{-1}) composed of 7.35 g L^{-1} sodium citrate (Merck, Darmstadt, Germany) and 13.51 g L^{-1} fructose (Merck, Darmstadt, Germany). This mixture was incubated in a water bath at 37°C for 15 minutes; subsequently, a semen sample aliquot was mixed with a buffered saline formaldehyde solution for later analysis under a phase contrast microscope. One hundred cells were evaluated per slide, and the percentage of spermatozoa with swollen tails was determined to indicate the integrity and functionality of the plasma membrane.

Thermal-resistance test (TRT)

To perform this test, the Brazilian College of Animal Reproduction – CBRA (2013) methodology was recommended, in which the semen samples are incubated in a water bath at 46°C for 30 minutes to evaluate spermatozoa resistance to thermal stress after incubation. At the end of the test, the semen was analyzed for sperm motile progression, and the samples were considered high quality if they retained at least 20% motility.

Evaluation of plasmatic and acrosomal membrane integrity and mitochondrial membrane potential

After thawing, approximately 250 µL of semen was deposited on an 800 µL Percoll column (90 and 45%) (GE Healthcare, Sao Paulo, Brazil) and centrifuged for 7 minutes at 700×g. The pellet was subsequently washed in 800 µl TALP medium with HEPES (Tyrode's Albumin Lactate Pyruvate; 114 mM NaCl, 3.2 mM KCl, 2.0 mM $CaCl_2$, 0.4 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 25 mM $NaHCO_3$, 0.1 mM Sodium Pyruvate, 10% FBS, 2.4 mg mL^{-1} HEPES Acid, and 50 µg mL^{-1} Gentamycin) (BALL et al., 1983), which was similar to the previous procedure. From the final pellet, 10 µL of semen was added to microtubes already containing 150 µL of TALP-HEPES medium according to the different experimental groups so that the final concentration of the samples was 5×10^6 mL^{-1} spermatozoa. The samples were then stained with 3 µl of propidium iodide fluorescent probe (PI, 0.5 mg mL^{-1}, Sigma-Aldrich, St. Louis, MO, USA) and 30 µl *Pisum sativum* agglutinin conjugated to fluorescein isothiocyanate (FITC-PSA, 100 µg mL^{-1}, Sigma-Aldrich, St. Louis, MO, USA) associated, or with 3 µl of the probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1; 154 µM; Sigma-Aldrich, St. Louis, MO, USA), and subsequently incubated in a water bath at 37°C for 10 minutes protected from light (CELEGHINI et al., 2007) modified. After this period, the samples were analyzed by a BD FACSCanto II flow cytometer

(Franklin Lakes, Becton Dickinson Biosciences, NJ, USA).

For each semen sample, 10.000 spermatozoa were evaluated by cytometry using BD FACSDiva™ 6.1 software (Becton Dickinson Biosciences, USA) to assess the percentages of the cells that reacted for each experimental group. A semen sample was submitted to cold thermal shock and was used as a positive control, and an aliquot of sperm that were not stained with fluorescent probes was used as a negative control (SANTANA et al., 2016).

PI is a marker of plasma membrane integrity that has DNA affinity and produces a red stain in cell nuclei with damaged membranes (ARRUDA, 2000). The agglutinin of *Pisum sativum* (PSA), which binds to the glycoconjugates of the acrosomal matrix when conjugated to fluorescein isothiocyanate (FITC), successfully marks injured acrosomes in green (ARRUDA, 2000). The JC-1 identifies the populations of mitochondria with different membrane potentials through color codes. The color changes from green to orange or red with increased membrane potential (COSSARIZZA et al., 1993).

For the association of PI and FITC-PSA, the cells were classified as damaged plasma membrane and intact acrosome (DPMIA), damaged plasma membrane and acrosome (DPMDA), intact plasma membrane and acrosome (IPMIA), and intact plasma membrane and damaged acrosome (IPMDA). The spermatozoa sub-populations stained with JC-1 were differentiated into cells with high, medium and low mitochondrial membrane potential ($\Delta\Psi m$).

This study was carried out following the technical standards of biosafety and ethics under the animal ethics committee approval (Animal Ethics Commission – CEUA, Federal University of Pará – UFPA, Case No. 3419040816).

Statistical analysis

The data were submitted to analysis of variance (ANOVA) by R CORE TEAM software (2014), and the differences between the means were compared by Tukey's test at 5% probability. Correlations were assessed using Pearson's test with a significance level of $P<0.05$.

Results

Experiment I

Post-thaw motility, motility and viability of spermatozoa post-TRT, and HOST-positive tails decreased significantly ($P<0.05$) in the treatment groups compared to the control group (TES-TRIS), except for the viability of the plasma membrane of spermatozoa immediately after thawing, where higher indices were observed in the ACP and TES-TRIS groups than in the ACP-M group ($P<0.05$) (Table 2).

A higher percentage of spermatozoa with intact membranes (IPMIA – Q3) was observed in the TES-TRIS group when compared to ACP and ACP-M groups ($P<0.05$), and lesion indices in both membranes (DPMDA – Q2) were higher in the ACP group ($P<0.05$) than in the ACP-M and TES-TRIS groups. Regarding the lesion index for only the acrosomes (IPMDA – Q4) or plasma membranes (DPMIA – Q1), there were no differences ($P>0.05$) between the ACP, ACP-M and TES-TRIS groups (Table 3, Figure 1).

Table 2. Effect of ACP®, ACP®-M and TES-TRIS diluents on motile and post-thawing and post-TRT sperm viability and on the positive tails of the Hypoosmotic Test (HOST) (mean ± SD) after cryopreservation of buffalo semen.

Parameters (%)	n	ACP	ACP-M	TES-TRIS
Motility	50	33.75±7.74[a]	31.50±6.62[a]	42.25±6.98[b]
Coloration Eosin	50	45.30±8.03[ab]	41.18±8.32[b]	46.18±7.62[a]
Motility TRT	50	25.25±6.79[a]	25.00±6.41[a]	32.00±5.16[b]
Coloration Eosin TRT	50	32.18±8.25[a]	31.58±6.80[a]	38.92±5.15[b]
HOST	50	36.80±6.89[a]	35.68±5.52[a]	49.93±7.90[b]

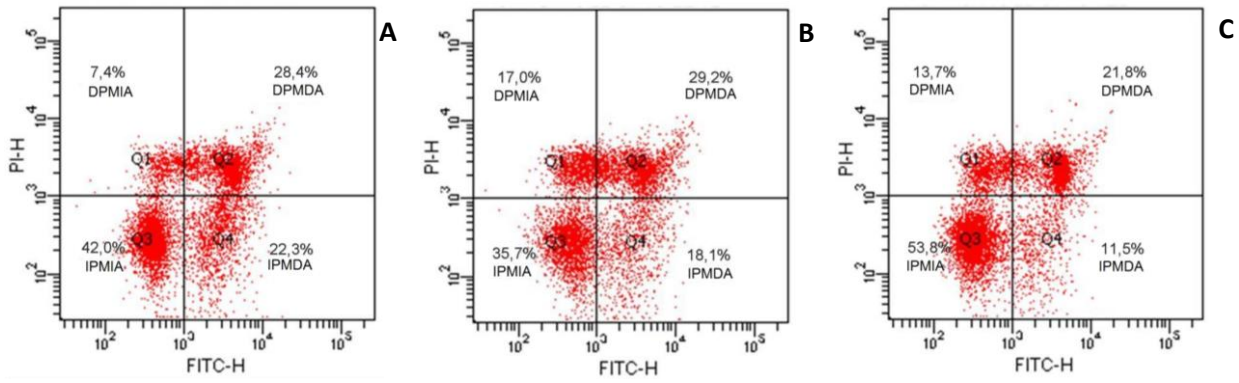
Values with different letters in the same row indicate statistical differences (P<0.05).

Table 3. Effect of ACP®, ACP®-M and TES-TRIS diluents on the integrity of the plasma and acrosomal membranes and the mitochondrial potential of post-thaw buffalo spermatozoa (mean ± SD) stained with PI + FITC-PSA and JC- 1 and evaluated by flow cytometry.

Parameters	n	ACP	ACP-M	TES-TRIS
Membranes Integrity (%)				
DPMIA (Q1)	50	10.41±4.85[a]	10.87±5.18[a]	9.95±4.35[a]
DPMDA (Q2)	50	29.25±5.75[a]	22.92±3.19[b]	20.86±3.63[b]
IPMIA (Q3)	50	34.71±8.55[a]	35.76±8.35[a]	47.37±8.91[b]
IPMDA (Q4)	50	25.63±11.61[a]	30.55±10.04[a]	21.80±8.84[a]
Mitochondrial Potential (%)				
High ΔΨm (Q1)	50	23.19±9.42[a]	24.22±8.60[a]	32.23±10.98[b]
Medium ΔΨm (Q2)	50	34.11±7.58[a]	34.09±9.23[a]	34.95±6.15[a]
Low ΔΨm (Q4)	50	38.99±11.69[a]	39.88±10.00[a]	32.87±10.40[b]

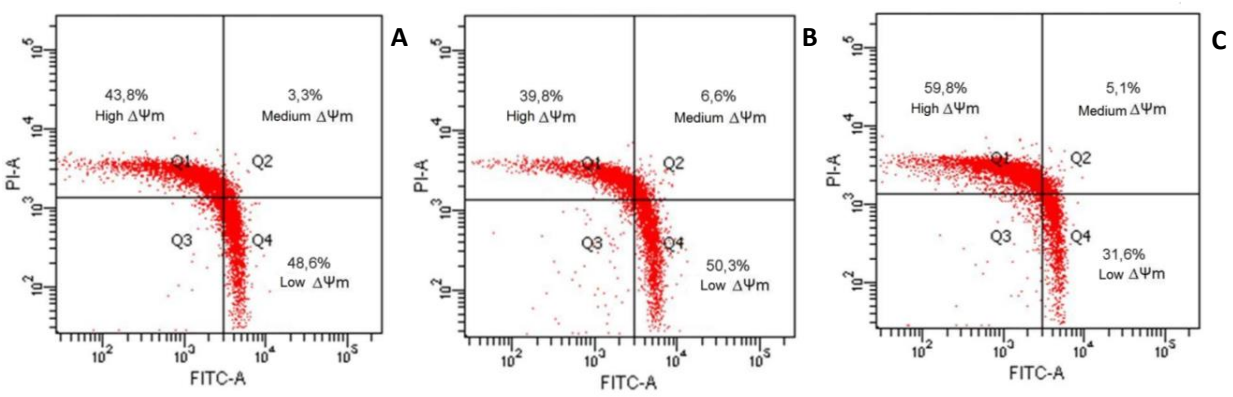
Values with different letters in the same row indicate statistical differences (P<0.05).

Figure 1. Dot plot sample obtained by flow cytometry in the analysis of the integrity of the sperm membranes of cryopreserved buffalo spermatozoa in the different experimental groups stained with PI-associated FITC-PSA. **A** – ACP; **B** – ACP-Milk and **C** – TES-TRIS.



Analysis of the data regarding the mitochondrial membrane potential of buffalo spermatozoa cryopreserved in ACP, ACP-M and TES-TRIS showed that there was a higher percentage of spermatozoa with high ΔΨm (Q1) in the TES-TRIS group than in the ACP and ACP-M groups; however, no differences in spermatozoa counts with the mean mitochondrial membrane potential (Q2) were observed between the ACP, ACP-M and TES-TRIS groups. The percentage of spermatozoa with low mitochondrial membrane potential (Q4) was lower in the TES-TRIS group than in the ACP and ACP-M groups (Table 3, Figure 2).

Figure 2. Dot plot sample obtained by flow cytometry in the analysis of the mitochondrial membrane potential of cryopreserved buffalo spermatozoa in the different experimental groups stained with JC-1. **A** - ACP; **B** – ACP-Milk and **C** – TES-TRIS.



Experiment II

For experiment II, the semen samples were diluted in TES-TRIS, which was the diluent that presented the best results for post-thaw semen quality and that was used as a control group for experiment II (without the addition of *Lippia*).

The percentages (mean ± SD) of the motility and viability of the spermatozoa plasma membrane post-thaw and post-TRT and the number of positive tails from the HOST did not differ between the experimental groups (P>0.05) (2.5 µg mL^{-1}, 5 µg mL^{-1}, 10 µg mL^{-1}, Control) (Table 4).

Table 4. Effect of different concentrations of *Lippia origanoides* on motile and post-thaw and post-thaw sperm viability and positive tails on the Hypoosmotic Test (HOST) (mean ± SD) after cryopreservation of buffalo semen.

Parameters (%)	n	2.5 µg mL^{-1}	5 µg mL^{-1}	10 µg mL^{-1}	Control
Motility	50	35.00±6.72	37.81±6.59	37.50±8.80	37.81±7.51
Coloration Eosin	50	43.81±8.50	49.19±7.18	47.97±9.32	45.28±9.42
Motility TRT	50	24.69±6.71	25.94±4.99	24.69±6.71	26.25±4.92
Coloration Eosin TRT	50	32.22±6.60	33.19±8.24	33.59±7.07	35.66±4.21
HOST	50	42.78±8.07	42.09±8.79	38.69±8.92	42.88±8.14

Values in the table did not show significant difference (P>0.05).

The flow cytometric data using the FITC-PSA, PI and JC-1 probes showed no differences (P>0.05) in plasma and acrosomal membrane integrity between the treatment groups and the control group after thawing the semen samples. The highest indices were found for the IPMIA (Q3) group with intact plasma membranes and acrosomes, and the lowest indices were found in the groups with lesions only

in the plasma membrane (DPMIA – Q1) and lesions in both membranes (DPMDA – Q2). However, relatively high indices of acrosome injury (IPMDA – Q4) were shown for the post-thaw experimental groups (Table 5; Figure 3). No differences in the percentages of spermatozoa were observed with high ΔΨm (Q1), medium ΔΨm (Q2) and low ΔΨm (Q4) (P>0.05) based on the analyses performed with the JC-1 probe between the treatment groups and the control group post-thaw (Table 5, Figure 4).

Table 5. Effect of different concentrations of *Lippia origanoides* on the integrity of the plasma and acrosomal membranes and on the mitochondrial potential of post-thaw buffalo spermatozoa (mean ± SD) (%) stained with PI + FITC-PSA and JC-1 and evaluated by flow cytometry.

Parameters	n	2.5 µg mL^{-1}	5 µg mL^{-1}	10 µg mL^{-1}	Control
Membranes Integrity (%)					
DPMIA (Q1)	50	8.86±6.57	7.59±6.41	6.44±5.28	7.02±4.97
DPMDA (Q2)	50	14.92±7.16	13.22±6.29	13.17±6.52	13.02±7.91
IPMIA (Q3)	50	53.49±9.49	54.29±8.70	55.97±5.58	54.09±7.01
IPMDA (Q4)	50	22.81±8.57	25.02±8.17	24.57±4.79	26.09±5.14
Mitochondrial Potential (%)					
High ΔΨm (Q1)	50	32.17±5.41	31.47±5.60	30.89±6.03	31.87±6.71
Medium ΔΨm (Q2)	50	25.53±7.25	27.33±7.40	26.98±6.48	27.59±7.84
Low ΔΨm (Q4)	50	42.28±8.65	41.18±8.50	42.12±8.36	40.54±10.33

Values in the table did not show significant difference (P>0.05).

Figure 3. Dot plot sample obtained by flow cytometry in the analysis of the integrity of sperm membranes from cryopreserved buffalo spermatozoa in the different experimental groups stained with PI-associated FITC-PSA.
A – 2.5 µg mL^{-1}; **B** – 5 µg mL^{-1}; **C** – 10 µg mL^{-1} and **D** – Control (TES-TRIS).

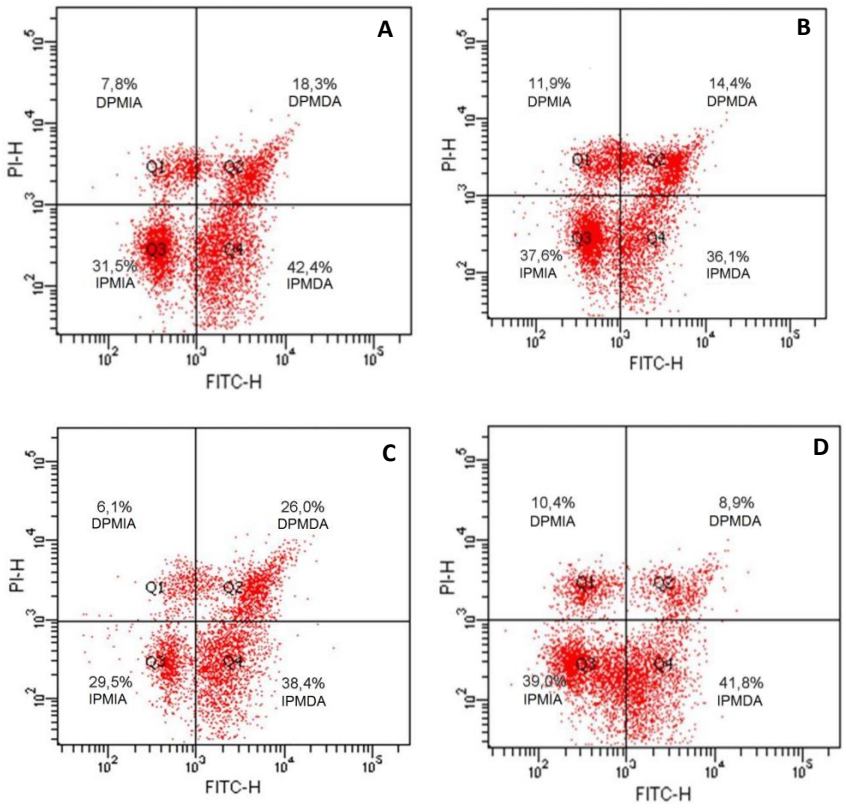
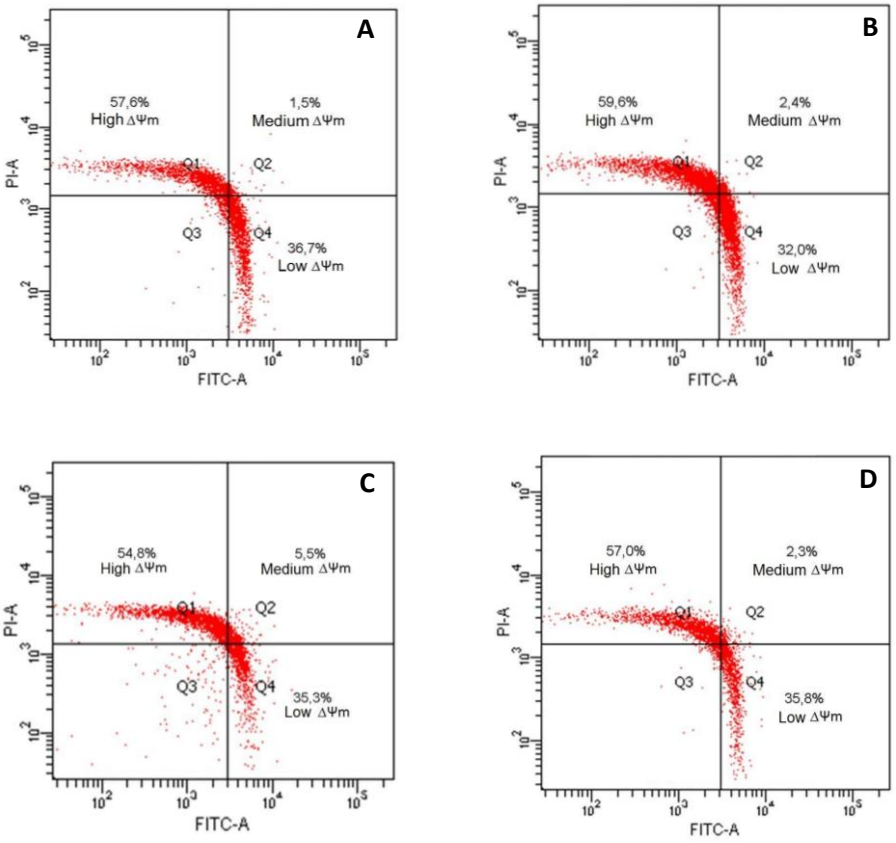


Figure 4. Dot plot sample obtained by flow cytometry in the analysis of the mitochondrial membrane potential of cryopreserved buffalo spermatozoa in the different experimental groups stained with JC-1.
A - 2.5 µg mL^{-1}; **B** – 5 µg mL^{-1}; **C** – 10 µg mL^{-1} and **D** – Control (TES-TRIS).



Discussion

ACP® has been used as an efficient alternative for semen cryopreservation in some species, such as swines (TONIOLLI et al., 2010), equines (SAMPAIO NETO et al., 2002), canines (CARDOSO et al., 2005), ovines (SALGUEIRO et al., 2007), and caprines (OLIVEIRA et al., 2011); however, this efficiency is still controversial in terms of its effectiveness among and within species (TONIOLLI et al., 2010, CAVALCANTE, 2012; CAVALCANTE et al., 2014) mainly when ACP® is compared to other diluents, as was observed in the present study. Our study showed that semen diluted in ACP-112® presented lower indices than TES-TRIS in terms of progressive motility, plasma membrane integrity (HOST), and the degree of intact plasma and acrosomal membranes (IPMIA) and showed low rates of spermatozoa with high mitochondrial potential. These results corroborate with the findings of Zorzetto et al. (2017), who evaluated the ACP-111® in buffalo and reported lower total and progressive motility results assessed by CASA and lower IPMIA percentages after flow cytometry than the Botu-Bov® diluent.

This inferiority of ACP® in terms of post-thaw sperm viability may indicate that some of the components of this diluent do not interact adequately with sperm structures, resulting in less protection and lower post-thaw viability; this was demonstrated by Cavalcante (2012), who observed that the constitution of sheep seminal plasma interfered differently with the diluent used in spermatozoa freezing and was more efficient in TRIS than in ACP-102c®, suggesting a possible

interaction of the semen constituents with different constituents of the diluent used for spermatozoa cryopreservation.

Some authors have shown that when milk is part of the diluent composition, good post-thaw viability of buffalo semen is obtained. Swelum et al. (2011) reported good rates of motility, viability, and plasma membrane integrity (HOST) when skim milk was added to the TRIS diluent, and Miyasaki et al. (2014) reported that when associated with Ringer's Lactate, milk improved the motility of post-thawed spermatozoa compared to skim milk tested alone. These results indicate that milk may have different effects on sperm depending on the interactions with the diluent components used. In our study, skim milk with ACP® did not effectively contribute to the improvement of the quality of cryopreserved buffalo semen since the assay results for motility, plasma membrane integrity (HOST), IPMIA and spermatozoa with high mitochondrial potential did not differ from those of ACP® without milk.

The best post-thaw indices of the present experiment were obtained in the TES-TRIS group, which presented higher rates of progressive motility (42.25%), plasma membrane integrity (HOST) (49.93%) and IPMIA (47.37%) after cytometry, as well as higher rates of spermatozoa with high mitochondrial potential (32.23%). Most likely, these higher rates occurred due to the presence of two amines cited as the most efficient for the recovery of semen characteristics after thawing compared to other conventional buffers (VALE et al., 1991; RASUL et al., 2000; MOOSE et al., 2007), having the advantages of not crossing the cellular barrier, stabilizing the plasma membrane, and preventing possible inhibition of metabolism; these amines also have binding properties for heavy metals, which are considered the true inhibitors of sperm motility (BALRAM et al., 1997; MOOSE et al., 2007).

When compared to the results obtained by other authors in studies of diluents for freezing buffalo semen, our results obtained with TES-TRIS validate the efficiency and current use of this diluent. For example, Akhter et al. (2010), in an experiment testing the TRIS and Bioxcell® diluents, reported motile values (45.3% and 45.0%) similar to those obtained in our experiment (42.25%) and to values obtained by Mahmoud et al. (2013) and Shahverdi et al. (2014) using Bioxcell® (42.51% and 40.10%, respectively) and by Brito (2014) using Botu-Bov® (40.97%). However, higher indices were obtained by Zorzetto et al. (2017) (47.56%) and Albuquerque et al. (2017) (59.97%) using the Botu-Bov® diluent in buffalo, and lower indices were obtained by Shahverdi et al. (2014) (32.40%) for TRIS and by Brito (2014) for TRIS (32.81%) and TES-TRIS (37.13%) diluents in buffalo. The HOST data from the present experiment (49.90%) were similar to those obtained by Mahmoud et al. (2013) using Bioxcell® (52.70%) and by Brito (2014) using TRIS, Botu-Bov® and TES-TRIS (50.17%, 47.33% and 49.17%, respectively); however, the values in the current study were lower than those obtained by Akhter et al. (2010) (60.4% and 59.2%, respectively) and Shahverdi et al. (2014) (62.7% and 68.70%, respectively) using TRIS and Bioxcell® diluents.

With respect to the sperm parameter compartments such as the analysis of plasma and acrosomal membrane integrity (IPMIA) by cytometry, the values obtained in our experiment with the TES-TRIS diluent were higher (47.37%) than those reported by Zorzetto et al. (2017) in a study evaluating TRIS and Botu-Bov® (25.90% and 29.72%, respectively) by flow cytometry; by Brito (2014) using TRIS, Botu-Bov® and TES-TRIS (29.83%, 39.17% and 27.39%, respectively) evaluated by epifluorescence; and by Albuquerque et al. (2017) evaluating the Botu-Bov® diluent (33.74%) by flow cytometry. These results indicate that perhaps the constituents of the TES-TRIS diluent evaluated in our experiment were more efficient in interacting with and protecting the integrity of sperm membranes, which is an essential function to increase the fertility indices of cryopreserved semen. For this reason, perhaps the

TES-TRIS diluent has been used since the 1980s and is still used efficiently by numerous researchers for freezing buffalo semen (OBA et al., 1993; SILVA et al., 2002; MOOSE et al., 2007; PESSOA et al., 2011; BRITO, 2014; GONÇALVES et al., 2014; REIS, 2015).

The cryopreservation of semen with different diluents has been well studied, and some of these studies have reported the use of different synthetic or natural antioxidants that may help protect the spermatozoa after the freezing and thawing process (GONÇALVES et al., 2011; CÂMARA et al., 2011; DAMASCENO et al., 2011; SILVA et al., 2011; CARDOSO, 2013; TEIXEIRA et al., 2014). Recently, the use of plant extracts has been reported to improve spermatozoa quality after freezing for some species as well as the rates of sperm penetration and embryo cleavage (ZHAO et al., 2009; MALO et al., 2011, 2012; DAGHIGH-KIA et al., 2014; MOTLAGH et al., 2014; SHAHBAZZADEH et al., 2014).

Zhao et al. (2009) observed that *Rhodiola sacra* extract (RSEA) had strong free radical scavenging activity and improved the viability of cryopreserved boar spermatozoa as the concentration of RSEA in the semen diluent (0, 2, 4, 6, 8 and 10 mg L^{-1}) was increased, resulting in better indices for sperm motility, acrosome integrity, mitochondrial activity and plasma membrane integrity. These results contrast those of the present experiment, in which no differences in the characteristics of the thawed semen, such as progressive motility, plasma membrane integrity (HOST), intact plasma and acrosomal membranes (IPMIA) and mitochondrial potential, were observed with increased *L. origanoides* concentrations in the diluent (0, 2.5, 5 and 10 μg mL^{-1}). These differences are probably due to the plant concentrations being approximately 1000 times lower in the current experiment compared to the study by Zhao, which produced no positive or negative (toxicity) effects on cryopreserved buffalo spermatozoa.

Malo et al. (2010) carried out a similar experiment using *Rosmarinus officinalis* (Alecrim) and also showed an improvement in semen viability and higher rates of cleavage and blastocysts in the group treated with the herb extract (1 mg mL^{-1}) that in the control group. Daghigh-Kia et al. (2014), using the same plant, found that extract addition to the diluent medium (5 and 10 g L^{-1}) increased intracellular and cellular membrane defense systems against ROS production after bovine semen cryopreservation, resulting in improved motility, membrane integrity and viability.

Lippia oil has high antioxidant potential due to the high concentrations of carvacrol and thymol, which are derived from phenolic compounds capable of donating hydrogen atoms (TEIXEIRA et al., 2014). This feature was shown in a study by Pereira (2015) that tested the effects of the addition of *Lippia origanoides* extract (0, 2.5, 5 and 10 μg mL^{-1}; the same concentrations used in this experiment) in buffalo oocyte IVM, which showed improvement in the embryo cell numbers produced by supplementation, providing promising results for the future incorporation of this substance in systems of in vitro production of buffalo embryos.

Conclusions

Based on the data obtained in this experiment, it can be concluded that the TES-TRIS diluent showed greater protection of post-thaw buffalo spermatozoa compared to ACP-112® with or without skim milk and that TES-TRIS should be used as the standard diluent for the cryopreservation of buffalo semen, and the *Lippia origanoides* essential oil did not show protective antioxidant effects on sperm integrity during cryopreservation at the different concentrations used.

However, there is a need to improve the protocols for the freezing of buffalo semen that optimize the use of ACP-112®, which may be used as a potential alternative diluent given its simplicity, practicality and low cost. Further studies are needed to evaluate

other plant concentrations and the effect of this species on buffalo spermatozoa structures during cryopreservation.

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