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Addition of orange, pineapple and beet juices as extenders for cryopreservation of ram semen

Adição de sucos de laranja, abacaxi e beterraba em diluidor para criopreservação de sêmen de carneiros

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Abstract
Searching for improvements in semen cryopreservation, natural substances are commonly studied focusing to improve the sperm quality. The aim of this study were evaluated the effect of adding orange, pineapple, and beet juices in different concentrations and combinations to the ram semen cryopreservation extender. Five ejaculates from five adult rams were used. The semen pool was diluted in egg yolk-based extender and mixed with the following 15 treatments (at a final concentration of 400.10⁶ sptz/mL): orange 10% (O10) and 15% (O15); pineapple 10% (P10) and 15% (P15); beet 10% (B10) and 15% (B15); pineapple + orange 10% (PO10) and 15% (PO15); pineapple + beet 10% (PB10) and 15% (PB15); beet + orange 10% (BO10) and 15% (BO15); pineapple + beet + orange 10% (PBO10) and 15% (PBO15); and the control group (CON). Post-thaw in 0.25 mL straws semen quality analysis of cryopreserved semen was performed by CASA and flow cytometry. Analysis of variance (PROC GLM) was carried out and the averages were compared using the SNK test. Pearson's correlation test was also performed. No effect was noted in the addition of juices to the semen extender prior to cryopreservation. Post-thawed, although, statistically similar to the control group, the total motility of the B10 group reached acceptable standards of total motility. In addition, B10 group showed the highest values ($p<0.05$) of progressive motility than control group or other treatments. The addition of 10% beet juice to the ram semen extender can improve the cryopreservation of sperm motility.
Keywords: antioxidant; freezing; sperm; semen extender.

Resumo
Em busca de melhorias na criopreservação do sêmen, substâncias naturais são comumente estudadas com o objetivo de melhorar a qualidade do sêmen. O objetivo deste estudo foi avaliar o efeito da adição de sucos de laranja, abacaxi e beterraba em diferentes concentrações e combinações ao diluidor de criopreservação de sêmen ovino. Foram utilizados cinco ejaculados de cinco carneiros adultos. O pool de sêmen foi diluído em diluente à base de gema de ovo e misturado com os seguintes 15 tratamentos (na concentração final de 400x10⁶ sptz/ml): laranja 10% (O10) e 15% (O15); abacaxi 10% (P10) e 15% (P15); beterraba 10% (B10) e 15% (B15); abacaxi + laranja 10% (PO10) e 15% (PO15); abacaxi + beterraba 10% (PB10) e 15% (PB15); beterraba + laranja 10% (BO10) e 15% (BO15); abacaxi + beterraba + laranja 10% (PBO10) e 15% (PBO15); e o grupo controle (CON). Pós-descongelação em palhetas de 0,25 ml a análise da qualidade do sêmen criopreservado foi realizada pelo CASA e citometria de fluxo. A análise de variância foi realizada e as médias comparadas pelo teste SNK. O teste de correlação de Pearson também foi realizado. Nenhum efeito foi observado na adição de sucos ao diluidor de sêmen antes da criopreservação. Após o descongelamento, embora estatisticamente semelhante ao grupo controle, a motilidade total do grupo B10 atingiu padrões aceitáveis de motilidade total. Além disso, o grupo B10 apresentou os maiores valores ($p<0,05$) de motilidade progressiva que o grupo controle ou os outros tratamentos. A adição de 10% de suco de beterraba ao diluente de sêmen ovino pode melhorar a criopreservação da motilidade espermática.
Palavras-chaves: antioxidante; congelamento; sêmen; diluidor de sêmen

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1. Introduction

The use of frozen semen in artificial insemination allows optimized use of breeding animals at farms and better genetic selection of the herd. In addition, the use of this biotechnology decreases expenses for transport and acquisition of breeders. However, freezing and thawing processes are harmful to sperm cells and directly affect their fertilization efficiency. In particular, production of reactive oxygen species (ROS) due to temperature changes during cryopreservation causes damage to sperm cell membranes [1; 2; 3; 4].

Mammalian sperm cells are prone to ROS damage due to higher concentration of polyunsaturated fatty acid in their cytoplasmic membrane [5]. In addition to this structural predisposition, the lack of cytoplasmic enzymes with antioxidant function in sufficient quantity to neutralize excess ROS worsens sperm resistance and can decrease sperm motility and even cause death [5]. In physiological quantities, ROS negatively affects sperm capacitation by reducing sperm motility and causing hyperactivation, acrosomal reaction and fusion with oocytes [6].

Currently, natural antioxidant substances extracted from plants, such as phenolic compounds including flavonoids, phenolic acids and tocopherols, are used in food processing and preventive therapeutic medicine [7]. Studies have shown significant improvement of sperm preservation with the addition of natural extracts from plants in the semen diluent of several animal species [8]. Beets and various fruits are also rich in sugars used for cell respiration and can provide osmotic balance and cryoprotection to the sperm cells [9]. Fruit juices like orange, grapefruit, apple and pineapple, along with many vegetable juices, have high contents of vitamin C, carotenoids and phenolic compounds that are strongly correlated with antioxidant effects [10; 11]. According to Vinson et al. (1998) [12], beet juice had the highest concentration of phenolic compounds out of 23 juices of vegetables commonly consumed in the United States. In a study with beet pulp, Mohdaly et al. (2010) [13] found high concentrations of phenolic compounds, indicating it is a good natural source of antioxidants, suitable as a stabilizer mainly in the food industry.

The aim of the present work was to analyze the effects of adding different concentrations and combinations of juices from orange, pineapple, and beet in a ram semen extender, on the sperm kinetics and plasma, acrosomal and mitochondrial membranes after cryopreservation.

2. Materials and methods

2.1. Location and Animals

The study was carried out at the Instituto de Zootecnia (IZ/APTA/SAA) in Nova Odessa, Sao Paulo State, Brazil. The city is located at 22º42' south latitude and 47º18' west longitude. The climate is dry in the winter and hot and rainy in the summer, with average annual rainfall of around 1,270 mm. The experiment was registered with the Ethics Committee on Animal Experimentation (CEUA/IZ – no. 187).

Five adult male rams (between 2 and 4 years old) of two breeds were used, four Santa Inês and one East Friesian. The animals remained in stalls and consumed a diet based on corn silage plus nutrient concentrate daily, following the recommendations of the NRC, offered twice a day, with good quality water available during the experimental period [14].

2.2. Preparation of fruit and beet juices

The juices were prepared according to the procedures described by Adeyemo et al. (2007) [15] and Daramola et al. (2016) [16] from pineapple (*Ananas comosus* L. var. Pearl), sweet orange (*Citrus sinensis* L.) and beet (*Beta vulgaris* L). The juices were used individually and in different combinations.

The oranges, pineapples and beets were previously washed in distilled water and then peeled. The orange seeds were removed. All were separately cut into cubes with a side length of 1 cm and homogenized for five minutes to facilitate the extraction of the juice. The homogenized pieces were transferred to a nylon mesh filter and then manually squeezed and packed in graduated plastic tubes.

Then 5 mL of each juice, pure and in combinations (orange and pineapple, orange and beet, pineapple and beet; and orange, pineapple and beet), was centrifuged separately at 3000x*g* for 20 minutes. The pH of supernatant of each juice was measured, finding values of 4.54, 4.1, 6.25, 4.2, 4.8, 4.8 and 4.6 for orange, pineapple; beet, orange and pineapple; orange and beet; pineapple and beet; and orange, pineapple and beet, respectively. The pH of the juices was not neutralized before addition in the semen extender, so as to evaluate their natural potential as additives.

2.3. Semen collection and addition of juices

Five ejaculates were collected from the five rams on alternate days for 10 days using an artificial vagina at a temperature of approximately 38 ºC. In order to stimulate ejaculation, a mannequin sheep synchronized in estrus was used inside a containment trunk.

On the semen collection day, one ejaculate from each ram (n = 5) was evaluated for total motility, progressive motility and mass motility immediately after collection. Then the semen was diluted in two parts of BotuBov® diluent for each part of ejaculate (1:2) and stored in a graduated tube at 37 ºC in a water bath, until all the rams had completed the procedure.

The rams were selected in random order on each collection day (batch), and to minimize the differences

between the rams, breeds and collection timing, a pool was formed of the five ejaculates in a single graduated tube, where the concentration was corrected to 800x10^6 sptz/mL with the addition of more BotuBov® extender.

Subsequently, the pool was separated into 15 aliquots of 1 mL each and stored in test tubes previously filled with 1 mL of BotuBov ® diluent at 37 ºC, resulting in 2 mL per test tube (treatment) and a final concentration of 400x10^6 sptz/mL.

The samples were separated into 15 treatments according to the juices and concentrations used: orange at 10% (O10) and 15% (O15); pineapple at 10% (P10) and 15% (P15); beet at 10% (B10) and 15% (B15); pineapple + orange at 10% (PO10) and 15% (PO15); pineapple + beet at 10% (PB10) and 15% (PB15); beet + orange at 10% (BO10) and 15% (BO15); pineapple + beet + orange at 10% (PBO10) and 15% (PBO15); and the control treatment (CON).

Subsequently, the samples were homogenized and evaluated for total and progressive motility and sperm vigor before cryopreservation.

2.4. Semen cryopreservation

After the samples were diluted in the solution of BotuBov ® and the juices and evaluated for each of the respective treatments, they were packed in 0.25 mL straws and submitted to cryopreservation. Eight straws from each treatment were cooled from 32 ºC to 5 ºC at a cooling rate of 0.25ºC/min and stabilized for 25 minutes. Subsequently, the straws were subjected to freezing in nitrogen vapor for 20 minutes until -120 ºC and were submerged in liquid nitrogen at -196 ºC followed by storage in cylinders.

2.5. Post-cryopreservation semen analysis

Two straws from each treatment and the same batch were thawed (37 ºC/30 seconds), homogenized in a microtube and evaluated for sperm motility, plasma, acrosomal and mitochondrial membrane integrity, lipid peroxidation and oxidative stress.

To evaluate sperm motility, computer-assisted sperm analysis (CASA) was used. This analysis was performed using the sperm class analyzer software (SCA - Microptics Barcelona, Spain), with prior setup adjustment for ovine semen. A diluted semen sample was deposited in a Makler™ chamber, and the image was obtained using a camera attached to the microscope equipped with phase contrast (Nikon, Model Eclipse Ni-U 80i). The following characteristics were analyzed: total motility (TM, %), progressive motility (PM, %), path velocity (VAP, µm/s), straight-line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), lateral head displacement (ALH, µM), beat-cross frequency (BCF, Hz), straightness (STR, %) and linearity (LIN, %).

To evaluate integrity of plasma membrane and acrosome and mitochondrial membrane potential, the fluorescent probes used were: propidium iodide (PI), fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin (FITC-PSA) and 5.5',6.6'-tetrachloro-1,1,3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), respectively (adapted from Pavaneli et al., 2020) [17]. For the analysis of lipid peroxidation, we used 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a, 4a-diaza-s-indacene acid-3-undecanoic (C11-BODIPY581/591) [18] and dihydroethidium (DHE) [19]. These analyses were performed using a flow cytometer (BD Accuri™ C6). The experimental scheme of the study is shown in Figure 1.

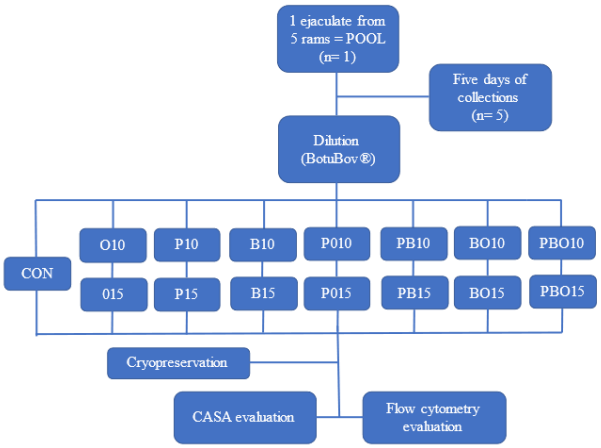

Figure 1. Experimental design of the study. Where: O10 = 10% orange; O15 = 15% orange; P10 = 10% pineapple; P15 = 15% pineapple; B10 = 10% beet; B15 = 15% beet; PO10 = 10% pineapple + orange; PO15 = 15% pineapple + orange; PB10 = 10% pineapple + beet; PB15 = 15% pineapple + beet; BO10 = 10% beet + orange; BO15 = 15% beet + orange; and PBO10 = 10% pineapple + beet + orange; PBO15 = 15% pineapple + beet + orange.

2.6. Statistical analysis

During the experiment, all data were recorded and tabulated for further statistical analysis using the Statistical Analysis System (SAS 9.4) software. Analysis of variance (PROC GLM) of the treatments and their respective batches was carried out. The averages were compared using the SNK test at 5% probability. Pearson's correlation test was also performed between the parameters observed during the experiment. Simple interactions between effects were tested, but they showed no differences ($p > 0.05$) and were excluded from the final data analysis model.

3. Results

The pre-freezing subjective kinetic parameters of the semen divided into the respective treatments were similar ($p > 0.05$) based on the observed parameters (Table I). There was no statistical difference ($p < 0.05$) between

the parameters evaluated and the different batches formulated.

Table 1. Mean and standard deviation of total (TM%) and progressive (PM%) motility and mass motility (MM, 1-5) of ram semen pool (n=5) after addition of juices (pre-cryopreservation).

Treatment	TM	PM	MM
Control	80.0 ± 0.0	66.0 ± 5.5	3.8 ± 0.4
O10%	72.0 ± 4.5	60.0 ± 0.0	4.0 ± 0.0
O15%	78.0 ± 4.5	64.0 ± 5.5	4.0 ± 0.0
P10%	76.0 ± 5.5	64.0 ± 8.9	4.0 ± 0.0
P15%	76.0 ± 5.5	66.0 ± 5.5	4.0 ± 0.0
B10%	78.0 ± 4.5	68.0 ± 4.5	4.0 ± 0.0
B15%	78.0 ± 4.5	66.0 ± 5.5	4.0 ± 0.0
PO10%	78.0 ± 4.5	68.0 ± 4.5	4.0 ± 0.0
PO15%	76.0 ± 5.5	62.0 ± 4.5	4.0 ± 0.0
PB10%	78.0 ± 4.5	66.0 ± 5.5	4.0 ± 0.0
PB15%	80.0 ± 0.0	68.0 ± 4.5	4.0 ± 0.0
BO10%	78.0 ± 4.5	64.0 ± 8.9	4.0 ± 0.0
BO15%	74.0 ± 8.9	60.0 ± 7.1	4.0 ± 0.0
PBO10%	78.0 ± 4.5	66.0 ± 5.5	4.0 ± 0.0
PBO15%	76.0 ± 5.5	62.0 ± 4.5	4.0 ± 0.0

In which: O10 = 10% orange; O15 = 15% orange; P10 = 10% pineapple; P15 = 15% pineapple; B10 = 10% beet; B15 = 15% beet; PO10 = 10% pineapple + orange; PO15 = 15% pineapple + orange; PB10 = 10% pineapple + beet; PB15 = 15% pineapple + beet; BO10 = 10% beet + orange; BO15 = 15% beet + orange and PBO10 = 10% pineapple + beet + orange; and PBO15 = 15% pineapple + beet + orange.

A statistically significant difference ($p < 0.05$) was observed between treatments for total and progressive motility in the analysis of semen cryopreservation. Treatment B - 10% showed a higher percentage of progressive motility, differing from other treatments and the control group, but it was similar to the control group, B - 15% and B + O - 10% for total motility (Table II). There was no statistical difference ($p > 0.05$) between the general average motility and semen batches.

There were no statistical differences ($p > 0.05$) in the analyses performed by CASA for curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR), amplitude of lateral head displacement (ALH) and beat-cross frequency (BCF) between treatments, as well as between general averages and semen batches.

The flow cytometry analyses showed no difference between treatments ($p > 0.05$) for lipid peroxidation measured with the C11-BODIPY581/591 probe, as well as for oxidative stress from the detection of superoxide anions measured by the DHE probe (Table III).

Table 2. Mean and standard deviation of the total (TM%) and progressive (PM%) motility of the post-thawed treatments evaluated by CASA

Treatment	TM	PM
Control	23.2 ± 6.1^{ab}	7.6 ±3.6^{b}
O10%	13.4 ± 4.8^{bc}	3.2 ± 1.2^{b}
O15%	10.3 ± 1.8^{c}	1.8 ± 0.4^{b}
P10%	10.3 ± 3.2^{c}	3.1 ± 2.3^{b}
P15%	6.8 ± 3.2^{c}	1.6 ± 1.1^{b}
B10%	31.5 ± 11.4^{a}	12.7 ±8.1^{a}
B15%	22.4 ± 9.5^{ab}	8.6 ± 5.6^{b}
PO10%	13.4 ± 5.3^{bc}	2.7 ± 1.4^{b}
PO15%	8.4 ± 2.8^{c}	1.8 ± 1.0^{b}
PB10%	13.8 ± 5.1^{bc}	3.2 ± 1.4^{b}
PB15%	12.2 ± 3.7^{bc}	4.1 ± 1.9^{b}
BO10%	22.2 ± 8.1^{ab}	6.6 ± 3.3^{b}
BO15%	13.4 ± 4.6^{bc}	3.7 ± 1.9^{b}
PBO10%	18.0 ± 6.3^{bc}	7.1 ± 3.1^{b}
PBO15%	17.7 ± 4.7^{bc}	5,9 ± 3.2^{b}

In which: O10 = 10% orange; O15 = 15% orange; P10 = 10% pineapple; P15 = 15% pineapple; B10 = 10% beet; B15 = 15% beet; PO10 = 10% pineapple + orange; PO15 = 15% pineapple + orange; PB10 = 10% pineapple + beet; PB15 = 15% pineapple + beet; BO10 = 10% beet + orange; BO15 = 15% beet + orange and PBO10 = 10% pineapple + beet + orange; and PBO15 = 15% pineapple + beet + orange.

Table 3. Mean and standard deviation of flow cytometry measuring peroxidation of plasma membrane lipids (MDHE) and assessment of oxidative stress through the detection of superoxide anions (MBP)

Treatment	MBP	MDHE
Control	1406.6 ± 505.3^{b}	2691.6 ± 752.9
O10%	2030.4 ± 448.3^{ab}	2365.0 ± 821.2
O15%	3239.2 ± 2882.6^{ab}	2240.4 ± 554.6
P10%	2663.4 ± 1429.0^{ab}	2916.4 ± 883.4
P15%	2980.1 ± 1429.5^{ab}	3390.8 ± 1027.6
B10%	3065.8 ± 469.7^{ab}	3723.0 ± 1943.3
B15%	3457.1 ± 459.2^{ab}	2786.9 ± 1401.9
PO10%	2414.9± 643.7^{ab}	3230.0 ± 752.8
PO15%	3919.3 ± 2618.6^{a}	2821.3 ±1385.6
PB10%	3000.5 ± 1139.0^{ab}	2853.8 ± 683.3
PB15%	3491.4 ± 1631.9^{ab}	2064.3 ± 1432.4
BO10%	2579.3 ± 388.3^{ab}	2443.10 ± 874.9
BO15%	2694.0 ± 481.4^{ab}	3099.1 ± 2099.4
PBO10%	3591.4 ± 877.5^{ab}	2870.8 ± 1420.9
PBO15%	3901.4 ± 1535.4^{a}	2500.4 ± 1170.7

In which: O10 = 10% orange; O15 = 15% orange; P10 = 10% pineapple; P15 = 15% pineapple; B10 = 10% beet; B15 = 15% beet; PO10 = 10% pineapple + orange; PO15 = 15% pineapple + orange; PB10 = 10% pineapple + beet; PB15 = 15% pineapple + beet; BO10 = 10% beet + orange; BO15 = 15% beet + orange and PBO10 = 10% pineapple + beet + orange; and PBO15 = 15% pineapple + beet + orange.

There was no difference between the treatments for low and high mitochondrial membrane potential, measured by the JC-1 probe (Table IV).

Table 4. Mean and standard deviation of the mitochondrial membrane with low potential (LP%) and high potential (HP%) evaluated by JC-1 probe.

Treatment	LP	HP
Control	27.31 ± 19.36	9.23 ± 1.34^{bc}
O10%	31.72 ± 29.23	9.81 ± 2.89^{bc}
O15%	24.83 ± 20.90	6.77 ± 0.93^{c}
P10%	21.62 ± 10.55	14.73 ± 6.19^{abc}
P15%	28.69 ±21.68	10.73 ± 6.80^{bc}
B10%	43.43 ± 21.68	7.38 ± 2.24^{c}
B15%	34.95 ± 21.67	6.96 ± 2.21^{c}
PO10%	31.34 ± 24.37	14.74 ± 4.56^{abc}
PO15%	27.28 ± 25.01	11.39 ± 4.29^{bc}
PB10%	19.73 ± 5.05	17.94 ± 9.32^{ab}
PB15%	21.99 ± 14.36	11.48 ± 4.57^{bc}
BO10%	33.67 ± 23.94	9.06 ± 2.29^{bc}
BO15%	33.19 ± 23.88	9.49 ± 2.95^{bc}
PBO10%	21.78 ± 7.25	21.15 ± 9.89^{a}
PBO15%	40.85 ± 23.91	13.82 ± 7.78^{abc}

In which: O10 = 10% orange; O15 = 15% orange; P10 = 10% pineapple; P15 = 15% pineapple; B10 = 10% beet; B15 = 15% beet; PO10 = 10% pineapple + orange; PO15 = 15% pineapple + orange; PB10 = 10% pineapple + beet; PB15 = 15% pineapple + beet; BO10 = 10% beet + orange; BO15 = 15% beet + orange and PBO10 = 10% pineapple + beet + orange; and PBO15 = 15% pineapple + beet + orange.

The treatment with O15 was lower than B15 for membrane integrity, and P15 was lower than all of other treatments for plasma and acrosomal membrane integrity, measured by FITC-PSA (Table V).

Table 5. Mean and standard deviation of acrosomal membrane integrity (AI%) and plasma membrane integrity (MI%) of the post-thawed extender formulations evaluated by FITC-PSA probe.

Treatment	AI	MI
Control	90.83 ± 4.80	26.82 ± 8.58^{ab}
O10%	90.96 ± 6.80	20.64 ± 7.76^{ab}
O15%	93.07 ± 3.49	12.94 ± 5.64^{b}
P10%	84.16 ± 6.69	24.21 ± 13.14^{ab}
P15%	71.38 ± 8.48^{b}	24.20 ± 13.90^{ab}
B10%	90.70 ± 5.66	33.99 ± 5.56^{ab}
B15%	91.24 ± 5.76	41.30 ± 21.30^{a}
PO10%	88.82 ± 5.60	34.24 ± 18.71^{ab}
PO15%	86.44 ± 5.57	27.34 ± 8.41^{ab}
PB10%	87.47 ± 5.82	30.81 ± 9.40^{ab}
PB15%	89.30 ± 3.65	25.46 ± 14.31^{ab}
BO10%	87.47 ± 9.73	31.01 ± 4.98^{ab}
BO15%	88.98 ± 10.28	26.68 ± 6.58^{ab}
PBO10%	90.43 ± 4.88	36.92 ± 7.40^{ab}
PBO15%	90.97 ± 5.26	28.43 ± 10.24^{ab}

In which: O10 = 10% orange; O15 = 15% orange; P10 = 10% pineapple; P15 = 15% pineapple; B10 = 10% beet; B15 = 15% beet; PO10 = 10% pineapple + orange; PO15 = 15% pineapple + orange; PB10 = 10% pineapple + beet; PB15 = 15% pineapple + beet; BO10 = 10% beet + orange; BO15 = 15% beet + orange and PBO10 = 10% pineapple + beet + orange; and PBO15 = 15% pineapple + beet + orange.

4. Discussion

The basic compounds and additives of cryoprotective media for sperm storage have been continuously revised[20]. Plant parts including fruit are known to have antioxidant, androgenic, and anti-infertility activities that can have beneficial effects in animal reproduction [8].

The analyses carried out before the freezing process showed that the addition of juices to the semen managed to maintain acceptable standards of kinetic parameters [21], without causing toxic effects to the sperm at first.

The analysis of total and progressive motility after thawing (Table II) showed a sharp drop in these values after the cryopreservation process. According to Maxwell and Salamon (1993) [22], a decline of approximately 20% in the percentage of fertile sperm is expected compared to normal semen. During the cryopreservation process, the semen is exposed to cold shock, atmospheric air, and osmotic differences, causing a higher occurrence of lipid peroxidation due to ROS action and mechanical damage to membranes by changes in osmolarity and temperature [1; 2; 4]. Thus, after thawing, sperm tend to have decreased motility and fertilization potential as a result of changes in membrane integrity and cell function caused by cryopreservation [2; 23; 24]. Bartoov et al. (1980) [25] reported that pH between 6.0 and 6.5 maintained good motility characteristics in ram semen. In the present study, beet juice was the only treatment that maintained the pH close to the results obtained by those authors, with values of 6.25, which may indicate that the pH of the other treatments negatively influenced sperm viability.

Azevedo et al. (2000) [26], studying different sperm concentrations (50, 100, and 200 million) in different straws (0.25 and 0.50 ml), found no statistical differences between progressive motility and sperm concentration. However, there was a decrease in the progressive motility when the filling was performed in 0.25 mL straws in relation to 0.50 ml ones, perhaps due to the smaller straw diameter, allowing greater damage from the thermal shock to the cells during the cryopreservation process.

Treatment B - 10% was superior to the others for progressive motility (12.7% ± 8.1), showing that the beet juice was efficient in keeping the sperm in progressive motion at levels higher than the control group after cryopreservation. The same treatment also maintained the percentage of total motility above 30%, in line with the desirable characteristics for ram regarding dose of frozen sperm according to the CBRA (2013) [21]. Sugars can promote improvements in the osmotic balance and cryoprotection of sperm cells, in addition to being used to prevent the occurrence of oxidative phosphorylation [9; 16]. Wruss et al. (2015) [27], when analyzing the juice of seven different varieties of beets, found values of 60%

betacyanins and 40% betaxanthins, which represented 70 to 100% of the total phenolic compounds. In addition, the sugar composition found was similar in all varieties, with an average of 7.7%, of which 95% was sucrose, followed by glucose and fructose. The study carried out by Fukuhara and Nishikawa (1973) [28] showed a significant effect of increasing energy to maintain motility in goat sperm when a variety of sugars was added, but there was no significant difference caused by sucrose, unlike glucose and fructose, which promoted improvements.

Daramola et al. (2016) [16], studying cucumber, pineapple and orange juices added to cryopreserved ram semen diluent, found higher values of progressive motility, especially when using concentrations of 7.5 and 10%. In the present study, pineapple and orange juices did not produce similar results, unlike beet juice, which was statistically better regarding progressive motility than the others. It is known that plant parts, such as fruits, leaves and roots, can vary in their phytochemical components [29], but as stated by Melo et al. (2008) [30], the concentration also varies according to the cultivar, variety, maturation stage, climate, and edaphic conditions, so it is unlikely that a plant of the same species will have an identical phytochemical composition as another plant. Improvements in sperm motility were observed in cryopreserved semen using fennel extract in swine semen [31]; using rosemary extract in bovine and ram semen, respectively [23; 32; 33]; with the addition of clove extract in ram semen [34]; using coconut water in swine semen [35]; using pomegranate juice in cattle semen [36; 37]; with strawberry juice in bovine and buffalo semen [38]; and with raphia sap, papaya and tomato juice and coconut water in refrigerated bovine semen [39].

Such findings show the possible benefits of the thousands of components present in plants, mainly in the case of phenolic compounds, which have high antioxidant capacity due to the ability to act as hydrogen donors and singlet oxygen suppressors, in addition to occasional action as metallic chelators [8; 40; 41; 42].

The probe C11-BODIPY581/591 consists of a fatty acid analog sensitive to oxidation, whose luminosity changes from red to green in response to the presence of several reactive species and peroxynitrite [43]. The oxidation of the DHE probe reacts specifically with the superoxide anions, emitting fluorescence, but the detection of specific products of the DHE reaction with superoxide anions can be difficult to measure due to the formation of other unspecific products of the oxidative reaction with other molecules [44]. Kasai et al. (2002) [45], using the JC-1 probe on sperm samples with different mitochondrial membrane potentials, found a significant relationship between motility and speed parameters with mitochondrial activity. Sperm with low mitochondrial membrane potential showed lower values for speed and motility parameters compared to the group with high membrane potential. In addition to the osmotic stress that occurs during the cryopreservation process, exposure of sperm to low temperatures changes the physical-chemical characteristics of the plasma membrane, changing the distribution of lipids, the communication between lipids and proteins, consequently increasing the fluidity of the plasma membrane [45]. With compromised structures, the membrane loses its semipermeable capacity and impairs all cellular functioning, favoring the formation and release of ROS [46].

5. Conclusion

The treatments did not influence the amount of superoxide anions, lipid peroxidation, mitochondrial membrane potential and integrity of plasma and acrosomal membranes of sperms. The significant increase in progressive motility with treatment B10 showed that beet juice promoted improvements in the progressive motility of ram sperm submitted to cryopreservation, without harming other sperm characteristics. It can thus be said that the beet juice added to the BotuBov® diluent promoted a beneficial effect on cryopreserved ram semen by improving sperm motility.

Conflict of interest

The authors declare no conflict of interest

Author contributions

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References

1 Holt WV. Fundamental aspects of sperm cryobiology: the importance of species and individual differences. Theriogenology. 2000; 53:47-58. Available from: https://doi.org/10.1016/S0093-691X(99)00239-3

2 Bucak MN, Atessahin A, Yuce A. Effect of anti-oxidants and oxidative stress parameters on ram semen after the freeze-thawing process. Small Ruminant Research. 2008. 75:128-134. Available from: https://doi.org/10.1016/j.smallrumres.2007.09.002

3 Castelo TS, Frota TR, Silva AR. Considerações sobre a criopreservação do sêmen de caprinos. Acta Veterinaria Brasilica. 2008. 2:67-75. Available from: https://doi.org/10.21708/

avb.2008.2.3.885

4 Câmara DR, Silva SV, Almeida FC, Nunes JF, Guerra MMP. Effects of antioxidants and duration of pre-freezing equilibration on frozen-thawed ram semen. Theriogenology. 2011. 76:341-350. Available from: https://doi.org/10.1016/j.theriogenology.2011.02.013

5 Aitken RJ, Baker MA. Oxidative stress and male reproductive biology. Reproduction, Fertility and Development., 2004. 16:581-588. Available from: https://doi.org/10.1071/RD03089

6 Savi PAP, Zavarez LB, Kipper BH, Feliciano MAR, Vicente WRR, Oliveira MEF. Uso de antioxidantes em meios diluidores para sêmen ovino: revisão de literatura. Ars Veterinaria, 2015. 31(1):12-18. Available from: http://dx.doi.org/10.15361/2175-0106.2015v31n1p12-18

7 Anwar F, Ali M, Hussain AI, Shahid M. Antioxidant and antimicrobial activities of essential oil and extracts of fennel (Foeniculum vulgare MILL.) seeds from Pakistan. Flavour Fragance Journal, 2009. 24:170-176. Available from: https://doi.org/10.1002/ffj.1929

8 Zhong R, Zhou D. Oxidative stress and role of natural plant derived antioxidants in animal reproduction. Journal of Integrative Agriculture, 2013. 12:1826-1838. Available from: https://doi.org/10.1016/S2095-3119(13)60412-8

9 Aboagla EME, Terada T. Effects of egg yolk during the freezing step of cryopreservation on the viability of goat spermatozoa. Theriogenology, 2004. 62:1160-1172. Available from: https://doi.org/10.1016/j.theriogenology.2004.01.013

10 Gardner PT, White TAC, Mcphai DB, Duthie GG. The relative contributions of vitamin C, carotenoids and phenolics to the antioxidant potential of fruit juices. Food Chemistry, 2000. 68:471-474. Available from: https://doi.org/10.1016/S0308-8146(99)00225-3

11 Cutler GJ, Nettleton JA, Ross JA. Dietary flavonoid intake and risk of cancer in postmenopausal women: The Iowa Women's Health Study. International Journal of Cancer, 2008. 123:664-671. Available from: https://doi.org/10.1002/ijc.23564

12 Vinson JA, Hao Y, Su X, Zubik L. Phenol Antioxidant Quantity and Quality in Foods: Vegetables. Journal of Agriculture and Food Chemistry., 1998. 46:3630-3634. Available from: https://doi.org/10.1021/jf980295o

13 Mohdaly AA, Sarhan MA, Smetanska I, Mahmoud A. Antioxidant properties of various solvent extracts of potato peel, sugar beterraba pulp and sesame cake. Journal of the Science of Food and Agriculture, 2010. 90:218-226. Available from: https://doi.org/10.1002/jsfa.3796

14 National Research Council, NRC. Nutrient Requirements of Small Ruminants: Sheep, Goats, Cervids, and New World Camelids. The National Academies Press, Washington DC, 2007. The Nat. Acad. Press. 2007

15 Adeyemo OK, Adeyemo OA, Oyeyemi MO, Agbede AS. Effect of semen extenders on the motility and viability of stored African Catfish (Clarias gariepinus) spermatozoa. Journal of Applied Science and Environmental Management, 2007. 11:13-16. Available from: https://doi.org/10.4314/jasem.v11i1.46827

16 Daramola JO, Adekunle EO, Onagbesan OO, Oke OE, Ladokun, AO, Abiona JA, Abioja MO, Oyewusi IK, Oyewusi JA, Isah OA, Sogunle OM., Adeleke MA. Protective effects pf fruit-juices on sperm viability of west African dwarf goat bucks during cryopreservation. Animal Reproduction, 2016. 13:7-13. Available from: http://dx.doi.org/10.4322/1984-3143-AR726

17 Pavaneli APP, Recuero S, Chaves BR, Garcia-Bonavila E, Llavanera M, Pinart E, Bonet S, Andrade AFC, Yest M. The presence of seminal plasma during liquid storage of pig spermatozoa at 17ºC modulates ability to elicit in vitro capacitation and trigger acrosomal exocytosis. International Journal of Molecular Sciences, 2020. 21:4520. Available from: https://doi.org/10.3390/ijms21124520

18 Batissaco L, Arruda RP, Alves MBR, Andrade MT, Lemes KM, Prado-Filho RR, Almeida TG, Andrade AFC, Celeghini ECC. Cholesterol-loaded cyclodextrin is efficient in preserving sperm quality of cryopreserved ram semen with low freezability. Reproductive Biolology, 2020. 20:14-24. Available from: https://doi.org/10.1016/j.repbio.2020.01.002

19 Vašíček J, Svoradová A, Baláži A, Jurčík A, Macháč M, Chrenek P. Ram semen quality can be assessed by flow cytometry several hours after post-fixation. Zygote, 2021. 29:130-137. Available from: https://doi.org/10.1017/S0967199420000581

20 Manjunath P. New insights into the understanding of the mechanism of sperm protection by extender components. Animal Reproduction, 2012. 9:809-815. Available from: https://www.animalreproduction.org/article/5b5a6053f7783717068b46cc/pdf/animreprod-9-4-809.pdf

21 Colégio Brasileiro de Reprodução Animal – CBRA, 2013. Manual para exame andrológico e avaliação de sêmen animal, 3, 104.

22 Maxwell WMC, Salamon S. Liquid storage of ram semen: a review. Reproduction, Fertility and Development, 1993. 5:613-638. Available from: https://doi.org/10.1071/RD9930613

23 Motlagh MK, Sharafi M, Zhandi M, Mohammadi-Sangcheshmeh, A, Shakeri M, Soleimani M, Zeinoaldini S. Antioxidant effect of rosemary (Rosmarinus officinalis L) extract in soybean lecithin-based semen extender following freeze-thawing process of ram sperm. Cryobiology, 2014. 69:217-222. Available from: https://doi.org/10.1016/j.cryobiol.2014.07.007

24 Aitken RJ. Pathophysiology of human spermatozoa. Current Opinion in Obstetrics & Ginecology, 1994. 6:128-135. Available from: https://europepmc.org/article/MED/8193251

25 Bartoov B, Bar-Sagie D, Mayevsky A. The effect of pH on ram sperm collective motility driven by mitochondrial respiration. International Journal of Andrology, 1980. 3:602-612. Available from: https://doi.org/10.1111/j.1365-2605.1980.tb00148.x

26 Azevedo HC, Machado R, Simplício AA, Soares AT. Características do sêmen caprino congelado: influência do tipo de palheta e concentração espermática. Rev. Científica Rural, 2000. 5:148-157. Available from: http://www.alice.cnptia.embrapa.br/alice/handle/doc/45681

27 Wruss J, Waldenberger G, Huermer S, Uygun P, Lanzerstorfer P, Muller U, Hoglinger O, Webhuber J. Compositional characteristics of commercial beterrabaroot products and beterrabaroot juice prepared from seven beterrabaroot varieties grown in Upper Austria. Journal of Food Composition and Analysis, 2015. 42:46-55. Available from: https://doi.org/10.1016/j.jfca.2015.03.005

28 Fukuhara R, Nishikawa Y. Effects of various substrates on respiration, glycolysis and motility of goat spermatozoa. Japanese. Journal of Zootechnical Science, 1973. 44:271-274. Available from: https://www.jstage.jst.go.jp/article/chikusan1924/44/5/44_5_271/_pdf

29 Simón BF, Pérez-Ilzarbe J, Hernández T, Gómez-Cordovés C, Estrella I. Importance of phenolic compounds for the characterization of fruit juices. Journal of Agriculture and Food Chemistry, 1992. 40:1531-1535. Available from: https://doi.org/10.1021/jf00021a012

30 Melo EA, Maciel MIS, Galvão de Lima VLA, Nascimento RJ. Capacidade antioxidante das frutas. Revista Brasileira de Ciencias Farmaceuticas, 2008. 44:193-200. Available from: https://doi.org/10.1590/S1516-93322008000200005

31 Malo C, Gil R, Cano N, Gonzales V, Luño V. Fennel (Foeniculum vulgare) provides antioxidante protection for boar semen cryopreservation. Andrologia, 2012. 44:710-715. Available from: https://doi.org/10.1111/j.1439-0272.2011.01254.x

32 Daghigh-Kia H, Olfati-Karaji R, Hoseinkhani A, Ashrafi I. Effect of rosemary (Rosmarinus officinalis) extracts and glutathione antioxidants on bull semen quality after cryopreservation. Spananish Journal of Agriculture Research, 2014. 12(1):98-105. Available from: https://doi.org/10.5424/sjar/2014121-4486

33 Gil L, Mascaró F, Mur P, Gale I, Silva A, González N, Malo C, Cano R. Freezing ram semen: The effect of combination of soya and rosemary essences as freezing extender on post-thaw sperm motility. 10th Int Cong Spanish Association of Animal Reproduction (AERA), Cáceres (Spain), June 2-5. p: 91. 2010.

34 Baghshahi H, Riasi A, Mmahdavi AH, Shirazi A. Antioxidant effects of clove bud (Syzygium aromaticum) extract used with different extenders on ram spermatozoa during cryopreservation. Cryobiology, 2014. 69:482-487. Available from: https://doi.org/10.1016/j.cryobiol.2014.10.009

35 Silva MA, Peixoto GCX, Lima GL, Bezerra JAB, Campos LB, Paiva ALC, Paula VV, Silva AR. Cryopreservation of collared peccaries (*Tayassu tajacu*) semen using powdered coconut water (ACO-116c) based extender plus various concentrations of egg yolk and glycerol. Theriogenology, 2012. 78:605–611. Available from: https://doi.org/10.1016/j.theriogenology.2012.03.006

36 Perumal P, Rajkhowa C. Effect of addition of pomegranate (Punica granatum) juice on the liquid storage (5ºC) of mithun (Bos frontalis) semen. Indian Journal of Animal Research, 2014. 49:470-473. Available from: https://doi.org/10.5958/0976-0555.2015.00091.6

37 El-Sheshtawy RI, El-Nattat WS, Ali A. Effect of clarified tris egg yolk extender supplemented with strawberry juice (*Fragaria* spp.) on some characteristics of chilled and frozen cattle semen. International Journal of ChemTech. Research, 2016. 9(6):203-207. Available from: https://www.sphinxsai.com/2016/ch_vol9_no6/1/(203-207)V9N6CT.pdf

38 El-Sheshtawy RI, El-Sisy GA, El-Nattat WS. Effect of pomegranate juice in Tris-based extender on cattle semen quality after chilling and cryopreservation. Asian Pacific Journal of Reproduction, 2016. 5:335-339. Available from: https://doi.org/10.1016/j.apjr.2016.06.001

39 Bayemi PH, Banla NR, Leinyuy I, Niba AT, Nsongka VM, Mario G, Shamshuddin M. Use of fruits and raffia palm sap (Raffia hookeri) in chilled bull semen extenders in Cameroon. Agricultural and Biological Science Journal, 2015. 1:142-149. Available from: http://files.aiscience.org/journal/article/html/70040066.html

40 Krishnaiah D, Sarbatly R, Nithyanandam R. A review of the antioxidant potential of medicinal plant species. Food and Bioproducts Process., 2011. 89:217-233. Available from: https://doi.org/10.1016/j.fbp.2010.04.008

41 Djeridane A, Yousfi M, Nadjemi B, Boutassouma D, Stocker P, Vidal N. Antioxidant activity of some Algerian medicinal plants extracts containing phenolic compounds. Food Chemistry, 2006. 97:654-660. Available from: https://doi.org/10.1016/j.foodchem.2005.04.028

42 Ferguson LR. Role of plant polyphenols in genomic stability. Mutation Research, 2001. 475:89-111. Available from: https://doi.org/10.1016/S0027-5107(01)00073-2

43 Drummen GPC, Van Liebergen CM, Den Kamp JAF, Post JA. C11-BODIPY$^{581/591}$, a oxidation-sensitive fluorescent lipid peroxidation probe: (micro) spectroscopic characterization and validation of methodology. Free Radical Biology and Medicine, 2002. 33(4):473-490. Available from: https://doi.org/10.1016/S0891-5849(02)00848-1

44 Nazarewicz RR, Bikineyeva A, Dikalov SI. Rapid and specific measurements of superoxide using fluorescence spectroscopy. SLAS Discovery, 2013. 18:498-503. Available from: https://doi.org/10.1177/1087057112468765

45 Kasai T, Ogawa K, Mizuno K, Nagai S, Uchida Y, Ohta S, Fujie M, Suzuki K, Hirata S, Hoshi K. Relationship between sperm mitochondrial membrane potential, sperm motility, and fertility potential. Asian Journal of Andrology, 2002. 4:97-103. Available from: http://www.asiaandro.com/archive/1008-682x/4/97.htm?mpclwjsbcesrixsb

46 Grotter LG, Cattaneo L, MarinI PE, Kjelland ME, Ferré B. Recent advances in bovine sperm cryopreservation techniques with a focus on sperm post-thaw quality optimization. Reproduction Domestic Animal, 2019. 54:655-665. Available from: https://doi.org/10.1111/rda.13409