USE OF ETHANOLIC EXTRACT OF PEAR ORANGE PEEL IN THE CRYOPRESERVATION OF BOVINE SPERMATOZOA

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ABSTRACT

Brazil is the country with the largest commercial cattle herd, and with that, there is a need to increasingly improve the efficiency of the processes involved in this sector. For this, biotechniques applied to reproduction, such as cryopreservation, are used. In order to avoid the damage caused by this technique, seminal diluters are used. However, the standard dilutors routinely used have ingredients of animal origin, such as egg yolk and skim milk. These are difficult to standardize and present a risk of contamination. There is therefore a great interest in the development of plant-based extenders. Orange is a fruit rich in secondary metabolites, such as carbohydrates and phenolic compounds, these are related to its antioxidant activity. In Brazil, the pear orange (Citrus sinensis (L.) Osbeck) is the most important citrus variety and its use by the juice industry generates tons of agricultural waste, mainly from its peel. This work seeks to use pear orange peel, a residue with possible bioactive potentials, for the production of crude extract. These can be beneficial within the formulation of a seminal diluter due to their antioxidant and antimicrobial characteristics. Thus, this study aimed to evaluate the use of ethanolic extract from the husk in the production of a new extender for the conservation of bovine spermatozoa. The ethanolic extract of orange peel was obtained and characterized for its phytochemical composition, antioxidant potential and antimicrobial activity. After obtaining, the ethanolic extract was included in sperm freeze thinners, with or without the presence of egg yolk and addition of 10 and 20% of this extract. The results showed that the ethanolic extract of orange peel has alkaloids, steroids, tannins and flavonoids, in addition to a high concentration of reducing sugars and antioxidant potential, however, the sperm evaluations indicated that there was no difference between the standard diluter added to the extract and the standard extender alone. In view of these results, it is concluded that the ethanolic extract of orange peel should be better evaluated for its cryoprotective potential in bovine sperm cells.

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INTRODUCTION

According to the Confederation of Agriculture and Livestock of Brazil, the second place in the ranking of the gross value of production of Brazilian agriculture is occupied by beef cattle, with R$ 183.31 billion, in 2023 (CNA, 2024). To achieve these expressive numbers, biotechniques applied to reproduction are commonly used, such as artificial insemination, embryo transfer and cryopreservation of oocytes, sperm and embryos (CARVALHO; DICK; CARMO, 2023).

Seminal cryopreservation is an important technique within livestock, as it allows the storage of samples from animals with high genetic and commercial value, enabling their use for a long period of time (ZUIDEMA; KERNS; SUTOVSKY, 2021). In addition, it also contributes to the conservation of endangered species (BOLTON *et al.*, 2022), reduces risks and costs with the acquisition and transport of breeding stock and minimizes the possibility of introducing transmissible infections via semen in a region and/or country and the transmission and spread of sexually transmitted infections among herds (QUELHAS *et al.*, 2023).

Even with the best preservation techniques, post-thaw survival rates are still low, with an approximate viability reduction of around 50%, considering the initial population of spermatozoa submitted to freezing. In order to minimize the damage caused by the cryopreservation process, a seminal extender is added to the cells, which must have adequate pH and osmolarity values and also provide protection to the morphological structure of the spermatozoa, keeping it viable even in the face of injuries caused by the cryopreservation process (BUSTANI; BAIEE, 2023).

However, the standard extenders used in these techniques currently use components of animal origin, such as egg yolk and skim milk. These are difficult to standardize and present a risk of microbial contamination. Therefore, it is of great interest to research additives that contribute to the reduction of cryoinjuries and the growth of microorganisms (SOUZA *et al.*, 2023).

Orange (Citrus sinensis), fruit of the tree of the Rutaceae family, is rich in secondary metabolites, such as carbohydrates, phenolic compounds, flavonoids, among others (BERNARDI *et al.*, 2010). In Brazil, the pear orange [Citrus sinensis (L.) Osbeck] is the most important citrus variety, being widely used by the industry, as well as by the domestic and foreign markets of fresh fruit.

As a consequence of the industrial activity of food processing, such as juice extraction, a large volume of waste is generated, whose destination can be problematic, as it occupies large spaces, and when poorly managed can represent environmental risks,

such as water and soil pollution, in addition to risks to the health of the population (FARHAT *et al.*, 2011). According to Law No. 12,305 of August 2010, of the National Waste and Solids Policy (BRASIL, 2020), all use alternatives must have been exhausted for a waste to be considered waste.

Therefore, it is increasingly important to develop and apply new technologies that lead to the use and transformation of this waste into co-products. When outlining formulations of extenders for sperm cryopreservation with defined composition and protective activity, the search for residues that could contain cryoprotective properties was considered. In this context, the alternative of using orange-pear peel emerges, representing the residues from the manufacture of juices, as raw material for the production of co-products, such as crude ethanolic extract and essential oil (FERRONATO; ROSSI, 2018).

Based on the above, when considering the economic impact and environmental importance of the use of orange residues, in view of the antioxidant and antimicrobial potential from orange peel, the objective of this work was to evaluate the effect of extenders added to the ethanolic extract of pear orange peel on the conservation of bovine epididymal spermatozoa.

METHODOLOGY

This study was developed in three stages, the first refers to obtaining and characterizing the ethanolic extract of orange peel; the second, the post-dilution tests of the extract in the diluter commonly used for cryopreservation and the third, the tests with the freezing of epididymal spermatozoa from cattle

OBTAINING AND CHARACTERIZING THE ETHANOLIC EXTRACT OF ORANGE PEEL

Obtaining and Preparing Orange Peel

Pear-type orange peels [*Citrus sinensis* (L.) Osbeck] acquired in João Pessoa-PB (7°06'35.5"S 34°49'55.3"W) were used. The peels were washed under running water, dried, weighed and placed in an oven (60 °C) for three days. Subsequently, the dry peels were removed and previously crushed in a mixer, with subsequent crushing in a blender, until the formation of a fine powder, which was weighed, stored and wrapped in aluminum foil, kept at room temperature until its use in the next step.

Elaboration of ethanolic extract from orange peel

127 grams of the dry peel powder obtained in the previous stage were used. The powder was placed in an Erlenmeyer and 1.2 liters of 95% ethanol (v/v) were added, following the approximate ratio of 1:10, referring to the peel and solvent, respectively.

This mixture was filtered every three days. At each filtration, the liquid fraction was separated in another Erlenmeyer and the solid material was maintained, along with it was added another 1.2 liters of ethanol. This process was repeated three times and all liquid fraction obtained was taken to the rotevaporator for drying and obtaining the ethanolic extract.

Phytochemical screening

The phytochemical screening was performed to qualitatively identify the presence/absence of five groups of chemical compounds in the ethanolic extract, namely: alkaloids, steroids, tannins, flavonoids and saponins. For all groups analyzed, the test was performed using 20 mg of the crude extract in glass test tubes. The methodology was used according to the description of Melo *et al.* (2024).

Quantification of reducing sugars by the DNS method

The DNS method is based on the reduction of 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid at the same time that the aldehyde group of the sugar is oxidized to the carboxylic group, with the development of a reddish coloration, read in the spectrophotometer at a wavelength of 540 nm (MILLER, 1959).

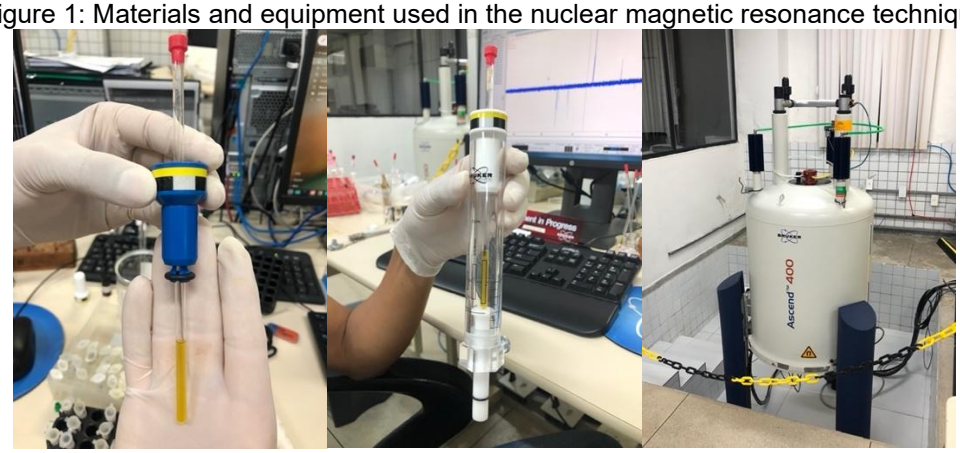
Different dilutions made with the crude extract previously obtained and distilled water were used. In glass test tubes, 0.5 mL of the sample and 0.5 mL of the DNS reagent were added, this mixture was stirred in vortex and incubated in a thermostabilized bath at 90 °C for 5 minutes. After this period, the sample was cooled in ice water to room temperature. Then, 3.0 mL of distilled water was added (Figure 15) and the absorbance at 540 nm was verified in a UV-visible double-beam spectrophotometer model IL-0082-Y-BI. The white of the analysis was prepared with 0.5 mL of distilled water instead of the sample, following the same procedure.

The absorbance values of the samples at the different dilutions were compared with a standard curve of reducing sugars (glucose and fructose) previously prepared following the same method, allowing the conversion of the absorbance read into glucose concentration in grams per liter or milligrams per milliliter (SANTOS *et al.*, 2017).

Nuclear magnetic resonance (NMR) of hydrogen 1H and carbon 13C

The NMR spectra of 1H hydrogen and 13C carbon in the APT technique were recorded in a nuclear magnetic resonance spectrometer (Bruker, Ascend model), operating at 400 MHz for 1H and 100 MHz for 13C. For the analysis, the sample was prepared by dissolving 20 mg of ethanolic extract in methanol (Cambridge Isotope Laboratories). The sample was placed in the tube suitable for the NMR equipment with an internal diameter of 5 mm, and then it was inserted into the equipment to obtain the spectra (Figure 1).

Figure 1: Materials and equipment used in the nuclear magnetic resonance technique



Source: Authorship (2022).

Determination of total phenolic compounds (CFT) content

The Folin-Ciocalteu method was used, which takes its name from the main reagent used in the technique. This reagent is yellow in color, but when in contact with reducing agents (phenolic compounds) at alkaline pH, there is a change in color to blue. This change allows an estimate of the concentration of these compounds to be obtained. For this, a calibration curve of the standard was used, with gallic acid, in different concentrations. The samples and calibration curve were read on a spectrophotometer at a wavelength of 765 nm. The results were expressed in milligrams of gallic acid equivalent per gram of sample (mg EAG/g sample).

Before starting the test, the reagents used in the analyses (Appendix II) were prepared and the 1.0 mg/mL sample solution of the ethanol extract in methanol was prepared.

For the test, an aliquot of 120 µL of the sample solution and 500 µL of the 10% Folin-Ciocalteu reagent was added to a microtube, this reaction was maintained for 8 minutes. After this period, 400 µL of the 7.5% sodium carbonate solution was added, this reaction was performed and maintained under the shelter of light and at room temperature for two hours. At the end, 200 µL of the samples were transferred to a 96-well plate, which was

read on a spectrophotometer at a wavelength of 765nm. The white was prepared with 120 µL of the sample solution of the ethanolic extract plus 900 µL of distilled water.

Determination of antioxidant potential by the DPPH method

To perform the test, plates from 96 flat-bottomed wells were used, in which each well had 100 µL of the samples (including the positive control) added. After that, under a light shelter, 100 µL of DPPH solution (Sigma-Aldrich) at 0.3 mM was added to each well. For the preparation of the blank, 100 µL of the sample and 100 µL of methanol were used, and for the negative control, 100 µL of the DPPH solution at 0.3 mM and 100 µL of methanol were used (BRAND-WILLIAMS *et al.*, 1995).

The plate was kept in the dark for 30 minutes without shaking. After this time, the absorbance was measured in a multidetector microplate reader (Biotek, Synergy HT model), at a wavelength of 517 nm. From the values obtained from the equipment, the value of the percentage of free radical scavenging (% SRL) or also known as percentage of inhibition (% I) was calculated for each sample (Equation 1).

Equation 1: Free Radical Scavenging Percentage Equation

$$\%SRL\ ou\ \%I = \left[\frac{(Absorbância\ do\ controle - Absorbância\ da\ amostra)}{Absorbância\ do\ controle}\right] x100$$

The values of the concentration necessary to reach the IC 50 were also calculated, referring to the concentration necessary to reduce the initial concentration of the DPPH radical in solution by 50%. This value was found from the plot on a graph, in which the X axis was the sample concentration, and the Y axis was the mean percentage of inhibition (% I) (DUARTE-ALMEIDA *et al.*, 2006).

For the ethanolic extract, tests were carried out with stock solutions of 1, 2 and 3 mg/mL in methanol. Finally, for the extenders, the same ones were used as standard solutions already ready in their final dilution, which was used for the sperm evaluations.

POST-DILUTION TESTS OF ETHANOLIC EXTRACT OF ORANGE PEEL IN SEMINAL EXTENDER

Experimental design of tests with ethanolic extract of orange peel

For the tests with the ethanolic extract (n=6), it was diluted in water in a ratio of 1:100. This aqueous solution was used to formulate the tested dilutors. Therefore, the experimental groups used for the ethanolic extract tests were:

- Control Group: Tris-gema;
- Dilute Ethanolic Extract Group 1 (EE1): Tris-yolk + 10% of the aqueous solution of the extract;
- Dilute Ethanolic Extract Group 2 (EE2): Tris-yolk + 20% of the aqueous solution of the extract;
- Dilute Ethanolic Extract Group 3 (EE3): Tris + 10 % of the aqueous solution of the extract;
- Dilute Ethanolic Extract Group 4 (EE4): Tris + 20% of the aqueous solution of the extract;

Microbiological tests

The microbiological tests aimed to evaluate the antimicrobial potential of the orange-pear peel extract included in the cryopreservation diluter, Tris-yolk (3.605 g of Tris-hydroxymethylaminomethane; 2.024 g of citric acid; 1.488 g of fructose; 100 mL of bidistilled water; 20% of egg yolk; 5% of glycerol) or in the buffer solution of Tris (3.605 g of Tris-hydroxymethyl aminomethane; 2.024 g of citric acid; 1.488 g of fructose; 100 mL of bidistilled water; 5% glycerol).

To perform the microbiological tests, the following groups were used: Tris-yolk + 10% ethanolic extract, Tris-yolk + 20% ethanolic extract, Tris + 10% ethanolic extract and Tris + 20% ethanolic extract. The method of microdilution in 96-well flat-bottomed plates was used. A plate was reserved for each bacterium used, namely: the standard strains Staphylococcus aureus UFPEDA02, Klebsiella pneumoniae ATCC-13883, and the isolate Escherichia coli AV12. The three species used in this work are part of the WHO list of priority bacteria (Asokan *et al.*, 2019). The AV12 isolate has a multidrug-resistant characteristic, with resistance to amoxilin, cephalexin and cephalothin, and sensitivity to ciprofloxacin, gentamicin, imipenem, meropenem, norfloxacin and sulfonamide.

The microorganisms were isolated from a recent three-day culture and were suspended in saline solution (0.9% NaCl) until the standardized turbidity of 1.0 on McFarland's scale was obtained.

Determination of the minimum inhibitory concentration (MIC)

Initially, a serial dilution of each diluter was performed to obtain the following concentrations: 20%; 10%; 5%; 2,5%; 1.25% and 0.25% of the ethanolic extract. Each concentration was performed in triplicate.

In each well of the 96-well plate, an aliquot of 100 µL of sterilized Mueller-Hinton juice was added. Soon after, 100 µL of the test diluter was added to the first well and with the highest concentration of the serial dilution, at a concentration of 40%, so that the concentration in this well reached 20%. A volume of 100 µL was then removed from the well, being taken to the next one, following the concentrations already mentioned above. At the end, a 10 µL aliquot of the bacterial cell suspension to be analyzed was added to each well. The plates were wrapped by plastic film and were kept for 48 h in an oven at a temperature of approximately 37 °C.

The value of the minimum inhibitory concentration was determined as the lowest concentration which presented absence of turbidity, which indicates absence of bacterial growth (GENHARTDT *et al.*, 1994).

Determination of the minimum bactericidal concentration (MBC)

To determine the minimum bactericidal concentration, redox reazurin dye was used. This molecule has a blue color, and when it enters the cell that is viable and with normal metabolic activity, this compound undergoes reduction reactions resulting in resorufin, a pink molecule that has fluorescence (GENHARTDT *et al.*, 1994). The test consisted of applying a 10 µL aliquot of resazurin in each well of the plates used in the previous test. After an incubation time of two hours, at room temperature and on a benchtop, the plates were analyzed for their resulting staining.

SPERM FREEZING TESTS WITH THE ADDED EXTENDERS OF ETHANOLIC EXTRACT OF ORANGE PEEL

Obtaining epididymal sperm from bovine animals

Testicle complexes/epididymis of mixed-breed cattle were obtained from a slaughterhouse located in the city of Santa Rita/PB (7°07'10.7"S 34°59'33.7"W) and transported in a cooler to the Laboratory of Biotechnology in Animal Reproduction of the Federal University of Paraíba (Campus I). In the laboratory, the epididymis and vas deferens were separated from the testicle, tied with a string so that there was no extravasation of its internal contents, cut and sanitized with saline solution (0.9% NaCl) at room temperature (FARIAS *et al.*, 2019).

The spermatozoa were isolated by the flotation technique (Figure 2). Initially, the tail of the epididymis was sliced, avoiding the cutting of apparent blood vessels, and then this sliced region was immersed in 2.0 mL of sterile saline solution (0.9% NaCl) at 37 °C contained in slightly inclined Petri dishes.

Figure 2: Flotation technique to obtain bovine epididymal sperm

Source: Authorship (2022).

The spermatozoa obtained in the suspension were evaluated for motility (0-100%) and vigor (0-5). Samples with motility greater than 50% were homogenized in a 15 mL falcon tube for the formation of the pool (spermatozoa diluted in saline solution) in order to avoid alterations or variability between individuals in the experiment (ALMEIDA *et al.*, 2017).

1.0 mL of the pool obtained was distributed in eppendorf tubes, which were taken to the centrifuge (Solab Refrigerated Benchtop Centrifuge, model SL-706) for 10 minutes at 3000 rpm (revolutions per minute), with acceleration and deceleration of 120 seconds and at room temperature. Then, by pipetting, the largest possible volume of the supernatant saline solution was removed, keeping the pellet from the sperm cells. In all replications, the cells were resuspended in a volume of 2.0 mL of the diluter/group to be tested.

Pre-freezing sperm analyses

Evaluation of sperm motility

Sperm motility was performed by subjective evaluation expressed as a percentage, ranging from 0 to 100%, with the average of two evaluators. This analysis was performed using an optical microscope with a 40x objective. An aliquot of 10 µL of the sample was placed between the slide and the coverslip, which was evaluated.

Plasma membrane integrity test

The plasma membrane integrity test was performed using double staining with eosin and nigrosin dyes (CBRA, 2013). Eosin is a supravital dye that is unable to penetrate cells with their plasma membrane intact. On the other hand, in dead or injured cells, where there is no longer an integrity of the plasma membrane, eosin can penetrate the cell, and these have been stained pink. Nigrosin is responsible for giving the darkest contrast of the bottom of the slide, allowing a better visualization of the unstained spermatozoa (SWANSON;

BEARDEN, 1951). The unstained cells indicated the live cells, where there was no entry of the dye, and the pink cells represented cells with non-intact plasma membrane

25 µL of each experimental group, 25 µL of eosin-nigrosine dye solution and 50 µL of saline solution (0.9% NaCl) were added to a microtube. After dilution of these components, the stretching was performed from 10 µL of each sample, this stretching was also done on two glass slides. Next, 200 cells were counted with the aid of a brightfield microscope with a 40x objective. The value of stained and unstained cells was recorded for each experimental group both at 0 h, before freezing, and after thawing.

Plasma membrane functionality test

The hyposmotic test (HOST) was used to evaluate the functional integrity of the plasma membrane. This is based on the properties of maintaining osmotic balance between the intra- and extracellular environment (CBRA, 2013). In this technique, the sperm is incubated in a hyposmotic solution, in which water influxes until osmotic equilibrium is reached. Consequently to the entry and expansion of the membrane, there is the curling of the tail in a physiological process. If the membrane is not intact, either due to damage or death of the sperm, this reaction will not occur, resulting in a stretched tail (JEYENDRAN *et al.*, 1984). We have that, for the experiment, sperm cells with functional plasma membranes were considered those that had their tails curled and cells with non-functional plasma membranes were those that had a stretched tail.

For the test, 10 µL of each experimental group was diluted in a microtube together with 100 µL of the hyposmotic solution (50 mOsm/Kg H2O) (Appendix II). This solution was incubated in a water bath at 37 °C for 30 minutes. After incubation, 50 µL of a formaldehyde saline solution was added in order to stop the osmotic reaction. The evaluation was made by placing 10 µL of each solution on a slide, covered by a coverslip, and this was analyzed under an optical microscope with a 40x objective. 100 cells were counted and the number of cells with curled tails and stretched tails was noted for each experimental group.

Freezing and thawing of bovine epididymal sperm

For freezing (Figure 3), the samples were initially filled in 0.25 mL straws and sealed with polyvinyl alcohol. These straws were then placed in plastic bags and were submerged in a beaker with water at room temperature. This was taken to the refrigerator, kept at a temperature of 5 °C, for the refrigeration curve and subsequent maintenance at 5 °C for four hours, for equilibrium time (ALMEIDA *et al.*, 2017). After this period, they were subjected to a 15-minute liquid nitrogen vapor freezing curve (4 cm distance between the

straws and the liquid nitrogen slide). Subsequently, the straws were immersed in liquid nitrogen and transferred to the cylinder, until the moment of evaluation.

Figure 3: Steps of freezing samples

Source: Authorship (2022).

Post-thaw sperm analyses

The samples were thawed at 37 °C in a water bath for 30 seconds and fractionated in microdilution tubes at 10 µL. The samples were diluted in saline solution (0.9% NaCl) in a 1:3 ratio and fluorescent probes were added (except for the samples submitted to kinetic evaluation), which will be described below. All probes were purchased from Sigma-Aldrich (St. Louis, MO, USA). The fluorophore stock solutions were prepared as follows: propidium iodide (25 mg/mL), JC-1 (5 mg/mL), and FITC conjugated with peanut agglutinin (FITC-PNA, 1 mg/mL) in phosphate buffer solution The working solutions were JC-1 (153 µM) in dimethyl sulfoxide (DMSO), FITC-PNA (0.04 mg/mL) and PI (0.5 mg/mL) in PBS and carboxyfluorescein diacetate (DIC; 0.46 mg/mL in DMSO). The evaluations were made soon after thawing and repeated again after 1 h in a dry bath at 37 °C (thermoresistance test).

Assessment of kinetic parameters

Sperm kinetics were analyzed using a computerized sperm analysis system (Sperm Class Analyzer - SCATM software, Microptics, v. 5.1, S.L., Barcelona, Spain). An aliquot (5 µL) of each sample was deposited on a previously heated slide (37 °C), covered with coverslip and analyzed by phase contrast microscope (100x magnification; NikonTM H5505, Eclipse 50i, Tokyo, Japan) and images were captured using a video camera (Basler Vision TechnologyTM A312FC, Ahrensburg, Germany) (Figure 16). For each sample, five non-consecutive fields were analyzed, randomly selected, with a record of at least 2,000 spermatozoa.

The following variables were evaluated: total motility (MT; %), progressive motility (MP; %), curvilinear velocity (VCL; µm/s), straight-line velocity (VSL; µm/s), mean travel velocity (VAP; µm/s), linearity (LIN; %), straightness (STR, %), oscillation index (WOB, %), lateral amplitude of the sperm head (ALH, µm) and flagellar crossbeat (BCF, Hz). The values were measured with the following configurations: temperature of 37 °C; 100x magnification; number of images, 25; images per second, 25; head area, 4 to 75 µm²; VAP: slow 10 µ/s < medium 45 µ/s < fast 90 µm/s; progressivity, 75% STR, 50% LIN(VERSTEGEN; IGUER-OUADA; ONCLIN, 2002).

Plasma membrane integrity (iMP)

Plasma membrane integrity (iMP) was evaluated according to Araújo Silva *et al.* (2019), by the double staining method of propide iodide (IP) and carboxyfluorescein diacetate (DIC) fluorophores, detected by the inclusion of PI in the cell nucleus. The previously diluted sample was stained with 5.0 µL of DIC and 5.0 µL of PI and incubated for a period of seven minutes at 37 °C. Spermatozoa were evaluated using DBP 485/520 nm and DBP 580–630 nm excitation filters. Sperm stained green were found to be intact and those stained red were considered to have a damaged membrane.

Mitochondrial Membrane Potential (PMM)

For this test, 5 µL of JC-1 (5', 6.6' iodide – tetrachlore - 1, 1, 3, 3' – tetraethylbenzimidazolylcarbocyanine; 153 µM) was added to each sample and incubated for 7 minutes, then evaluated under the fluorescence microscope. Green fluorescence (530 nm) indicates the formation of aggregated J monomers and orange (590 nm) indicates the formation of dimers as the mitochondrial membrane becomes more polarized. Thus, cells stained in orange were classified as having high mitochondrial membrane potential, while

those stained in green were classified as having low membrane potential (ALMEIDA *et al.*, 2017).

Acrosomal Membrane Integrity (iAC)

The evaluation of acrosome integrity (iAC) was done by Fluorescein Isothiocyanate conjugated to peanut agglutinin (FITC-PNA). A 10 µL aliquot of the sample was used to make an air-dried smear. The slides were stained with aliquots of 20 µL of FITC-PNA and incubated in a humid chamber at 4 °C for 20 minutes in the absence of light. Then, the slides were immersed in PBS (Saline Phosphate Buffer) twice and air-dried. Immediately prior to the evaluation, 5.0 µL of UCD solution (4.5 mL glycerol, 0.5 mL PBS, 5.0 mg p-phenylenediamine, and 5.0 mg sodium azide) were placed on the slide and then covered with a coverslip. Spermatozoa were evaluated using an excitation filter BP 450–490 nm and LP emission 515 nm (ARAÚJO-SILVA *et al.*, 2019).

STATISTICAL ANALYSIS

The data were expressed as the mean and standard deviation of the replicates for each experiment. For the analysis of the data obtained, the T test and analysis of variance (ANOVA) were used for two samples and multiple samples, respectively. Values of $p < 0.05$ were considered statistically significant. The tests were carried out in the Past4 version 4.11 program.

RESULTS AND DISCUSSION

OBTAINING ETHANOLIC EXTRACT FROM ORANGE PEEL

A brown extract was obtained, with high viscosity and smell similar to that of orange peel essential oil.

CHARACTERIZATION OF THE ETHANOLIC EXTRACT OF ORANGE PEEL

Phytochemical screening

The results of the phytochemical screening indicated the absence of saponins and the presence of alkaloids, steroids, tannins and flavonoids. In the evaluation of saponin, there was no foam formation in the system. For alkaloids, steroids and tannins, there was precipitate formation, indicating the presence of these compounds in their respective tests. And for flavonoids, the result was positive due to the presentation of the color change of the system, showing a pink color after performing the test.

Similar data regarding the presence of these compounds in orange peel were described by Pereira *et al.* (2020), Silveira (2019) and Oliveira (2017). The presence of steroids may indicate a possible protection to the plasma membrane, since they have a composition similar to cholesterol, which promotes this protection to spermatozoa submitted to cryopreservation. Flavonoids may have antioxidant activity, which is important in the thawing of seminal samples, as it promotes balance after the high formation of oxidants after cryopreservation. Thus, the ethanolic extract of orange peel has functional characteristics for sperm cryopreservation.

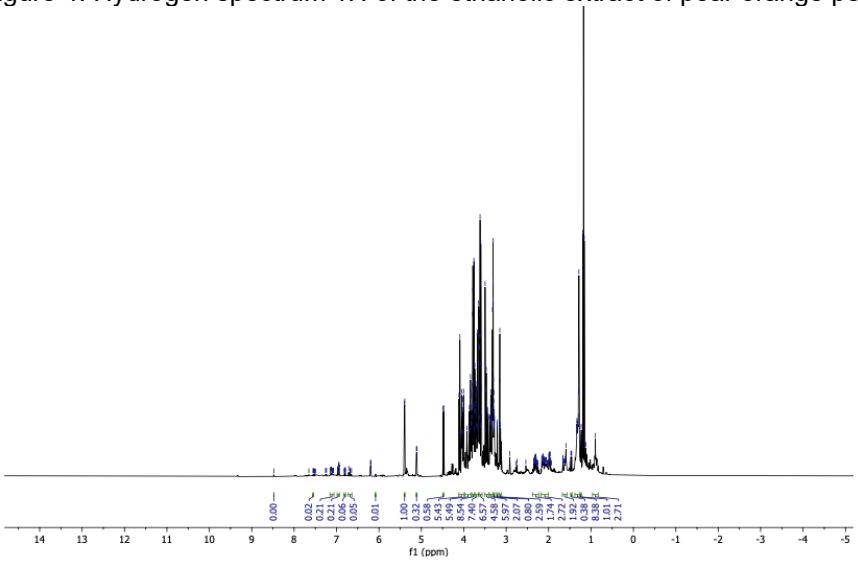
Quantification of reducing sugars by the DNS method

The concentration of reducing sugars found in the orange peel extract was 305±20 mg/mL. The soluble sugars in orange peel vary between 15 and 40% of the total weight, they act as an important substrate for biological processes (Ahmed *et al.*, 2016). Among them, the main sugars found are glucose, fructose and sucrose, but it is possible to find xylose and glycopolysaccharides in smaller quantities (Rivas *et al.*, 2008; Torrado *et al.*, 2011). The data obtained here indicate that the extract produced and used in this work has a high concentration of reducing sugars, this result is in line with other studies that have also performed this technique in different extracts of orange peel (Locatelli, Finkler, Finkler, 2019; Ayala *et al.*, 2021). Under the conditions of this experiment, the presence of sugars in the extract would act in two moments; sucrose, in the refrigeration/freezing phase, which can increase the osmolarity of the medium and promote cellular dehydration, important for the exit of water from the intracellular medium to the extracellular medium, preventing the formation of ice crystals within the sperm and, like glucose and fructose, providing substrate for post-thaw sperm motility, since mitochondria use this energy to convert into ATP and boost flagellar beating.

Nuclear magnetic resonance (NMR) of hydrogen 1H and carbon 13C

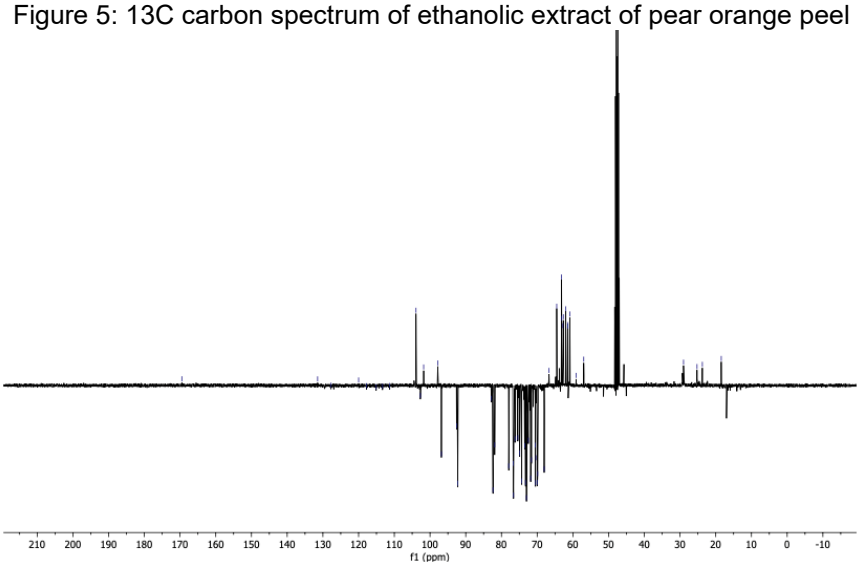
The 1H hydrogen spectrum of the ethanolic extract is indicated in Figure 4 and the 13C carbon spectrum is indicated in Figure 5.

Figure 4: Hydrogen spectrum 1H of the ethanolic extract of pear orange peel



Source: Authorship (2022).

Figure 5: 13C carbon spectrum of ethanolic extract of pear orange peel



Source: Authorship (2022).

Regarding the 1H spectrum, it was possible to perceive the presence of three distinct intervals, which indicate the presence of certain classes of chemical compounds. The first range is between 1.9 and 2.4 ppm (mg/kg), and shows signs of aliphatic hydrogens characteristic of terpene substances. The second range is between 3 and 4 ppm and presents a signal envelope of sugar or glycoside characteristics. The third range is between 6 and 8 ppm, and shows characteristic signs of aromatic substances.

From the 1980s onwards, the presence of carbohydrates began to be established using chemical displacement data, which can be evaluated by the NMR technique. H NMR signals around 4.5 to 5 ppm may indicate the presence of anomeric hydrogens from sugars, associated with the presence of multipets in the range of 3 and 4 ppm. As for the NMR spectra of C, the presence of carbohydrates can be given by the presence of signals

between 90 and 115 ppm, corresponding to anomeric carbons, and signals around 70 to 80 ppm, relative to hydroxylated carbons. In addition to these, the presence of glycosides can be highlighted by the existence of a signal around 61 ppm (BORGES, 2008; GIL; GERALDES, 1987). The general aspect of the spectrum obtained in this work is very similar to the spectrum equivalent to the class of carbohydrates observed by Lima (2013), and can be considered as the group of major compounds present in this extract.

According to Pavia *et al.* (2016), hydrogens bonded to aromatic rings can be easily identified within the 1H chemical displacement range between 6.5 and 8 ppm. In this range, few other types of hydrogen will present absorption, thus characterizing a positive correlation when there is the presence of signals in this range, equivalent to the aromatic functional group in organic compounds. In addition, the presence of aromatic compounds, more specifically of the flavonoid class, can be reinforced by the presence of signals with chemical displacements close to 175 and 180 ppm in carbon spectra (Lima, 2013). The presence of this class of compounds in orange peel extracts was also found by Mencherini *et al.* (2012).

A similar result regarding spectrum disposition can be found in Pei *et al.* (2022), which identified and described in detail the presence of 102 orange components, from its peel to its juice, in different varieties of the fruit, based on the evaluation of nuclear magnetic resonance spectra.

Based on the results obtained by the NMR technique and the DNS technique, it can be considered that the ethanolic extract of the evaluated orange peel has a high concentration of carbohydrates, including reducing sugars.

Determination of total phenolic compounds (CFT) content

The spectrophotometric reading of the 1.0 mg/mL solution of the ethanolic extract resulted in an absorbance of 0.191. Thus, from the standard curve of gallic acid, this absorbance value indicated the presence of 340.88 mgEAG/100g of ethanolic extract. Regarding the ethanolic extract solution at a 1:100 dilution, which was used in the formulation of the extenders tested, the absorbance value obtained was 1.335 and, from the same standard curve, it corresponded to 2003.00 mgEAG/100g of crude extract.

The result obtained in this study (equivalent to 1.0 mg/mL) showed a higher CFT value compared to the commercial citrus extracts (142.19±8.35 mgGAE/g of extract) evaluated by Fiorentini *et al.* (2022). Chemically, orange peel extract is characterized by the presence of phenolic compounds with great antioxidant power. In general, there is this positive correlation between the total phenolic content and the antioxidant property of fruits

and vegetables (KAUR; KAPOOR, 2002). However, other authors have not evidenced this correlation (ISMAIL; MARJAN; FOONG, 2004). Thus, it is necessary that in order to explain the antioxidant capacity of an extract, one must take into account factors other than its total phenolic content. The characterization of the structure of the active compound and its biological function should be observed (HEINONEN, LEHTONEN, HOPIA, 1998; MELO *et al.* 2018).

Determination of antioxidant potential by the DPPH method

Based on the standard Trolox curve (Appendix A), Tables 1 and 2 present the Trolox equivalent values for each concentration of the extract tested by the DPPH method and the T+10%EB and T+20%EB diluters. The data obtained for the TG+10% and TG+20% extenders are not described because it was not possible to perform this analysis, due to the presence of egg yolk that made it impossible to measure absorbance. The composition of the egg yolk, rich in proteins, when in contact with the DPPH solution diluted in methanol, triggered a protein denaturation, making it impossible to spectrophotometric evaluation of the samples.

Table 1: Antioxidant potential by the DPPH method for ethanolic extract of orange peel

Crude extract concentration	**TEAC (µg/mL)**
1.0 mg/mL	400,3
2.0 mg/mL	597,1
3.0 mg/mL	768,6

Source: Authorship (2022).

Table 2: Antioxidant potential by the DPPH method for the extenders used in sperm evaluations

Diluters	**TEAC (µg/mL)**
Tris	0,0
Tris+10%EB	409,0
Tris+20%EB	771,1

Source: Authorship (2022).

The result obtained at the concentration of 1.0 mg/mL already demonstrates that the ethanolic extract of orange peel used in this work has antioxidant activity, which is equivalent to 400 µg/mL of Trolox. As for the extenders that were possible to evaluate, we have that the Tris solution alone did not present antioxidant potential, as expected. On the other hand, the addition of the ethanolic extract solution, at concentrations of 10 and 20%, offered an antioxidant activity. Thus, it is concluded that the antioxidant activity obtained in

the Tris+10%EB and Tris+20%EB extenders is due to the action of the crude extract and not to the Tris solution.

The antioxidant potential of fruits is the result of the synergistic action of several compounds, including polyphenols or phenolic compounds. However, the effectiveness of this antioxidant action depends on the chemical structure and concentration of these active ingredients in the extract (Kaur; Kapoor, 2002). The pear orange is described as a fruit with strong antioxidant properties, with a percentage of DPPH radical sequestration of more than 70% (Melo *et al.*, 2018). These data are in agreement with the results obtained here, since the orange peel extract used has a high concentration of phenolic compounds in its composition.

Determination of hydrogen potential

The pH value obtained for the ethanolic extract was initially 5. Table 3 shows the results of the pH measurement of the standard diluter based on Tris and egg yolk (Tris-yolk) and of the extenders formulated with the crude extract (EB), respectively.

Table 3: pH values of extenders with the crude extract of pear orange peel

Groups	pH
Tris-gem	7,0
Tris-gema + 10% EB (EB1)	7,0
Tris-gema + 20% EB (EB2)	7,0
Tris + 10% EB (EB3)	6,5
Tris + 20% EB (EB4)	6,5

Source: Authorship (2022).

The diluters added to the extract had higher pH values, being 7 for the groups added to the Tris-yolk, and 6.5 for the groups added to the Tris.

Sperm freezing should occur in the presence of seminal extenders, in order to minimize the damage caused by thermal shock, maintain the pH and adequate osmolarity for these cells (SALAMON; MAXWELL, 2000).

A buffer system must be one of the constituents of these dilutors, allowing the hydrogen ions produced by sperm metabolism to be neutralized, and thus, causing the pH of the solution to be maintained close to neutrality (6.8 to 7.1), optimal pH for spermatozoa (BORGES, 2008).

Since the extenders added to the orange peel extract present acceptable pH values for cryopreservation, maintaining the average pH of the standard extender commonly used, it can be stated that they present a promising alternative for this process.

Microbiological tests

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The values obtained for the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) for the extenders with the ethanolic extract of orange peel are shown in Tables 4 and 5, respectively.

Table 4: Minimum inhibitory concentration of extenders plus ethanolic extract of orange peel

	Staphylococcus aureus **UFPEDA02**	***Escherichia coli*** **AV12**	***Klebsiella pneumoniae*** **ATCC-13883**
TG+10%EB	0,75%	-	5,00%
TG+20%EB	1,25%	-	5,00%
T+10%EB	-	5,00%	10,00%
T+20%EB	0,75%	20,00%	20,00%

Source: Authorship (2022).

Table 5: Minimum bactericidal concentration of extenders plus ethanolic extract of orange peel

	Staphylococcus aureus **UFPEDA02**	***Escherichia coli*** **AV12**	***Klebsiella pneumoniae*** **ATCC-13883**
TG+10%EB	0,75%	-	0,75%
TG+20%EB	0,75%	-	1,25%
T+10%EB	5,00%	20,00%	10,00%
T+20%EB	20,00%	20,00%	20,00%

Source: Authorship (2022).

The TG+10%EB and TG+20%EB groups, at the concentrations used in the study (10 and 20%, respectively) inhibited growth and killed the colonies of Staphylococcus aureus UFPEDA02 and Klebsiella pneumoniae ATCC-13883, but not of Escherichia coli AV12. This result may be related to the multidrug-resistant characteristics of the latter species, which is more difficult to inhibit. Despite this, due to their inhibitory and bactericidal properties of the other two bacteria, these groups can be considered to have antimicrobial activity. This potential is interesting for these groups, since the crude extract added to the diluter makes it possible to use the egg yolk, an ingredient that naturally presents high risks of contamination, in a safer way.

As for the T+10% and T+20% groups, these also inhibited bacterial growth and made the cells unviable, this time for the three bacteria tested. However, the concentration values required for this were higher than previously observed. It is then clear that the crude extract of orange peel has antimicrobial activity for the microorganisms tested.

The chemical composition of orange (Citrus sinensis) residues indicates the presence of carbohydrates, flavonoids, glycosides, coumarins, volatile compounds, organic acids and oils (OIKEH; ORIAKHI; OMOREGIE, 2013) Among these groups, phenolic compounds, essential oils, tannins, alkaloids, flavonoids and saponins are the molecules

considered responsible for the antimicrobial effect in this plant (RAHMAN *et al.*, 2011) The synergistic action of these compounds is described in terms of inhibition of the growth of pathogens (NWANKWO; ONWUAKOR; ANINWEZE, 2014).

The results of these analyses are similar to those obtained by Shetty *et al.* (2016), El-Desoukey *et al.* (2018), Baba *et al.* (2018) and Oikeh *et al.* (2020), who also investigated the antimicrobial activity of orange peel extract on pathogenic bacteria and different fungi. These authors suggest that the antimicrobial activity of this extract is the result of the synergistic action of the phenolic compounds, flavonoids and tannins present in the sample.

Pre-freeze sperm tests

Evaluation of sperm motility

The mean values of sperm motility in the post-dilution and post-thawing are indicated in Table 6.

Table 6: Total motility (%) of post-dilution and post-thaw bovine epididymal sperm with or without the addition of orange peel extract

	Tris-gem	**TG+10%EB**	**TG+20%EB**	**T+10%EB**	**T+20%EB**
Post-dilution (%)	75,0±5,2aA	79,1±3,7aA	80,8±3,4aA	19.1±13.1 bA	12.5±6.3b A
Post-thaw (%)	48.3±9.9aB	50.8±9.3aB	51.6±9.9aB	0,6±0,5bB	0,0±0,0bB

Source: Authorship (2022). Legend: Lowercase letters indicate difference ($p<0.05$) between the groups of the same condition (0 h or post-thaw) and uppercase letters indicate difference between the same group in the two conditions (0 h and post-thaw).

The addition of 10 and 20% of crude extract of orange peel obtained similar results to the control group (Tris-yolk) regarding sperm motility, both at 0h and post-thaw. There was a loss of motility in all groups after thawing, this process is already expected as a result of the cryoinjuries caused by the cryopreservation process (Silva; Guerra, 2011). The groups that contained only the Tris solution added to the crude extract, both at concentrations of 10 and 20%, did not show protection to bovine epididymal sperm cells, since they presented values well below those of the control group (at time 0h) and almost total loss of motility after thawing.

Plasma membrane integrity test

The mean values of spermatozoa with non-intact plasma membrane in the post-dilution and post-thawing are shown in Table 7.

Table 7: Plasma membrane integrity (%) of post-dilution and post-thaw bovine epididymal sperm with or without the addition of orange peel extract

	Tris-gem	TG+10%EB	TG+20%EB	T+10%EB	T+20%EB
Post-dilution (%)	55,8±10,8a A	57,0±8,8aA	56,8±10,4aA	85.0±23.8b A	110.0±22.3b A
Post-thaw (%)	59,1±6,3aA	55,8±4,8aA	69,8±5,3bB	187,5±6,6c B	197,6±1,3dB

Source: Authorship (2022). Legend: Lowercase letters indicate difference ($p<0.05$) between the groups of the same condition (0 h or post-thaw) and uppercase letters indicate difference between the same group in the two conditions (0 h and post-thaw).

There was no difference ($P>0.05$) between the Tris-yolk and TG+10%EB groups, both for the 0 h and post-thawing time, as well as between them. There was an increase in the number of cells with non-intact PM in the TG+20%EB group after thawing, this increase is compared to the control group (Tris-gema) in the same condition and to the group itself at 0 h. As for the groups T+10%EB and T+20%EB, they presented higher values than the other groups, both at 0 h and after thawing.

This increase in the number of cells with non-intact PM indicates that the extract alone, and at these concentrations tested, does not have sufficient membrane protective action for bovine epididymal spermatozoa that will undergo the freezing process.

Plasma membrane functionality test

The mean values of spermatozoa with functional plasma membrane (curled tail) in post-dilution and post-thawing are indicated in Table 8.

Table 8: Plasma membrane functionality (%) of post-dilution and post-thaw bovine epididymal sperm with or without the addition of orange peel extract

	Tris-gem	TG+10%EB	TG+20%EB	T+10%EB	T+20%EB
Post-dilution (%)	80,5±4,2aA	83,5±5,5aA	85,6±4,8aA	69.3±7.1b A	68.6±11.8 bA
Post-thaw (%)	46.6±3.6aB	39.6±10.6aB	34.1±6.9aB	8,6±3,9bB	4,6±2,0bB

Source: Authorship (2022). Legend: Lowercase letters indicate difference ($p<0.05$) between the groups of the same condition (0 h or post-thaw) and uppercase letters indicate difference between the same group in the two conditions (0 h and post-thaw).

At the same time, there was no difference ($P>0.05$) between the control group and the Tris-yolk groups added to the crude extract, both for 10 and 20%. However, as well as the parameters of motility and membrane integrity, there was a statistical difference between the groups with only the crude extract and without the addition of egg yolk. Regarding plasma membrane functionality, for these two groups, there was a decrease in the number of cells with functional plasma membrane when compared to the control, both for the time 0 h and after thawing.

This result is complementary to the previous ones, and corroborates the understanding that the crude extract alone is not able to protect the plasma membrane of these cells. And since the damage to this structure is directly related to cell death, there is a notable decrease in sperm motility in these groups (MORAES; MEYERS, 2018).

Post-thaw sperm analyses

Assessment of kinetic parameters

From the data obtained, the values of total (Table 9) and progressive motility (Table 10) are described here.

Table 9: Total motility (%) of bovine epididymal sperm after thawing and after 1 h at 37°C, with or without the addition of orange peel extract

	Tris-gem	TG+10%EB	TG+20%EB	T+10%EB	T+20%EB
Post-thaw (%)	55,2±20,0aA	66,7±15,5aA	57,5±9,0aA	8,3±2,6b	7,0±3,4b
1 h post-thaw (%)	40.5±15.7aB	48.6±17.1aB	43,0±8,0aA	0,0±0,0	0,0±0,0

Source: Authorship (2022). Legend: Lowercase letters indicate a difference ($p<0.05$) between groups of the same condition (post-thawing or after 1 h) and uppercase letters indicate a difference between the same group in both conditions (post-thawing and after 1 h).

Table 10: Progressive motility (%) of bovine epididymal sperm after thawing and after 1 h at 37°C, with or without addition of orange peel extract

	Tris-gem	TG+10%EB	TG+20%EB	T+10%EB	T+20%EB
Post-thaw (%)	23,8±11,7aA	27,2±13,6aA	22,03±6,4aA	0,1±0,3b	0,1±0,2b
1 h post-thaw (%)	14.2±6.5aB	17.4±6.2aB	13.5±7.6aB	0,0±0,0	0,0±0,0

Source: Authorship (2022). Legend: Lowercase letters indicate a difference ($p<0.05$) between groups of the same condition (post-thawing or after 1 h) and uppercase letters indicate a difference between the same group in both conditions (post-thawing and after 1 h).

It is noted that there was no difference ($P>0.05$) between the control groups (Tris-yolk) and the TG+10%EB and TG+20%EB, both post-thawing and in the analysis after 1 h at 37 °C. On the other hand, there was a difference ($P<0.05$) between the same group in terms of time, and for the groups mentioned, there was a decrease in progressive and total motility in the evaluation after 1 h. Only the TG+20%EB group did not obtain statistical difference for total motility between the post-thaw analysis and after 1 h.

The T+10%EB and T+20%EB groups had both total and progressive motility lower than the control group, reaching values close to zero in terms of progressive motility. This evaluation, unlike the sperm motility previously assessed, was performed using a computerized and more robust method (ARRUDA *et al.* 2011). However, a correlation can be observed between the two results obtained, in which both show a decrease in sperm motility in the post-freezing condition.

Plasma membrane integrity (iMP)

The mean values of spermatozoa with non-intact plasma membrane (stained in red) after thawing and after 1 h are shown in Table 11.

Table 11: Plasma membrane integrity of post-thaw and post-1 h epididymal sperm at 37 °C

	Tris-gem	TG+10%EB	TG+20%EB	T+10%EB	T+20%EB
Post-descong.	47,1±7,6aA	39,3±9,6aA	34.1±16.5bA	3,3±2,7c	1,0±2,4c
After 1h	42,7±11,4aA	41,0±14,4aA	33,8±11,3aA	-	-

Source: Authorship (2022). Legend: Post-descong. – Average of cells stained in post-thaw red; After 1h – Average of cells stained red after 1h; Data presented regarding the number of non-intact cells in 200 cells counted; Lowercase letters indicate a difference ($p<0.05$) between the groups of the same condition (post-thawing or after 1h) and uppercase letters indicate a difference between the same group in both conditions (post-thawing and after 1h).

There was no difference ($P>0.05$) between the control group (Tris-yolk) and the TG+10%EB group, both after thawing and in the evaluation after 1 hour. For the TG+20%EB group, in the post-thawing group, there was an increase, when compared to the control group, in the number of cells with non-intact plasma membrane. This statistical difference did not appear in the data obtained in the time after 1h. For T+10%EB and T+20%EB, there was an increase ($P<0.05$) in the number of cells stained red, indicating loss of plasma membrane integrity in these groups. This result presents an interpretation similar to the plasma membrane integrity test previously evaluated.

Mitochondrial Membrane Potential (PMM)

The mean values of spermatozoa with high mitochondrial membrane potential (stained orange) after thawing and after 1 hour are shown in Table 12.

Table 12: Mitochondrial membrane potential (MPP) of post-thaw and post-thaw epididymal sperm 1 h at 37 °C

	Tris-gem	TG+10%EB	TG+20%EB	T+10%EB	T+20%EB
Post-descong.	64,0±28,9aA	65,5±17,7aA	68,9±22,5aA	4,8±3,4b	1,3±1,2b
After 1h	64,5±28,4aA	66,8±18,3aA	64,6±26,9aA	-	-

Source: Authorship (2022). Caption: Post descong. – Average of cells stained in post-thaw orange; After 1h – Average of cells stained orange after 1h; Lowercase letters indicate a difference ($p<0.05$) between the groups of the same condition (post-thawing or after 1h) and uppercase letters indicate a difference between the same group in both conditions (post-thawing and after 1h).

For PPM, there was no difference ($P>0.05$) between the control groups (Tris-yolk), TG+10%EB and TG+20%EB, both for post-thawing evaluation and for the time after 1h. There was also no difference between these two halves for the same group. Only the T+10%EB and T+20%EB groups showed statistical differences from the others, characterizing a decrease in the number of cells with high membrane potential.

Acrosomal Membrane Integrity (iAC)

The mean values of spermatozoa with intact acrosomal membrane (stained bright green) after thawing and after 1 h are shown in Table 13.

Table 13: Acrosomal membrane integrity (iAC) of post-thaw and post-thaw epididymal sperm 1 h at 37 °C

	Tris-gem	TG+10%EB	TG+20%EB	T+10%EB	T+20%EB
Post-descong.	69,5±17,3aA	73,4±14,8aA	74,5±16,8aA	49,1±19,0b	39,1±19,9b
After 1h	68,2±19,8aA	70,9±12,8aA	71,9±17,4aA	-	-

Source: Authorship (2022). Caption: Post descong. – Average of cells with intact acrosome after thawing; After 1h – Average of cells with intact acrosome after 1h; Lowercase letters indicate a difference ($p<0.05$) between the groups of the same condition (post-thawing or after 1h) and uppercase letters indicate a difference between the same group in both conditions (post-thawing and after 1h).

As in the previous test, there was no difference ($P>0.05$) between the control groups (Tris-yolk), TG+10%EB and TG+20%EB, both immediately after thawing, as well as in the evaluation after 1h. There was also no difference ($P>0.05$) for these groups between the values in the two times. There was a statistical difference for the T+10%EB and T+20%EB groups, with a decrease in the number of spermatozoa with intact acrosomal membrane.

From the data obtained in the post-thaw kinetic evaluations, it is concluded that the addition of pear orange peel extract, both at 10 and 20% concentrations, in general, did not present statistical difference to the control group (Tris-yolk).

In addition, it was possible to perceive that the groups with only the crude extract (10 and 20%), and that there was no presence of the yolk, showed that they did not protect bovine epididymal spermatozoa regarding the integrity and functionality of their plasma membrane, integrity of the acrosomal membrane and mitochondrial membrane potential.

The lack of protection for these structures (plasma membrane, acrosome and mitochondria) is related to the loss of motility (total and progressive) of these cells, especially when subjected to the freezing technique (SILVA; GUERRA, 2011).

The addition of antioxidant substances in the seminal extender is one of the factors that influence the improvement of sperm evaluation parameters, especially regarding sperm motility (total and progressive) (BOZZI, 2017; DANELUZ, 2016; TONIOLLI, 2012). The increase in these parameters indicates an increase in the quality of semen after thawing (KALTHUR *et al.*, 2011).

FINAL CONSIDERATIONS

The ethanolic extract of orange-pear peel has a high concentration of carbohydrates, including reducing sugars, a high concentration of total phenolic compounds, as well as antioxidant activity. Despite the antioxidant potential, the extenders formulated with 10 and 20% of the extract added to Tris-yolk did not show improvement compared to their absence

regarding the parameters of post-thaw sperm evaluation. Extenders formulated only with Tris and the addition of crude extract (10 and 20%) are not efficient in the conservation of epididymal spermatozoa submitted to cryopreservation. Although the extenders tested in this study do not present cryoprotective activity, it is necessary to improve the methodologies used in the preparation of the extenders, in addition to new tests with different concentrations of the ethanolic extract, to evaluate its cryoprotective potential more deeply.

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