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Anethole improves mitochondrial activity and quality parameters in fresh and frozen-thawed ovine semen

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ABSTRACT

Anethole, an antioxidant found in plants, appears to improve the survival of spermatozoa during semen cryopreservation. This study assessed the effects of commercial trans-anethole in ram semen cryopreservation. Thirty ejaculates from six rams were diluted in media containing anethole at the following concentrations: CONT (0 μM), AN10 (10 μM), AN50 (50 μM), and AN100 (100 μM). Semen was slow-frozen, preserved in liquid nitrogen, and thawed. Anethole at 10 μM or 50 μM did not compromise any studied sperm quality parameter but increased pre-freezing functionality of membrane and mitochondrial activity. At 10 μM, anethole reduced post-thawing spermatozoa lipoperoxidation. At 50 μM, anethole sustained higher mitochondrial activity after thawing, reduced minor defects in sperm, and increased the number of sperm binding to perivitelline membrane, while keeping lipoperoxidation levels as in control. Anethole at 100 μM promoted higher pre- and post-freezing mitochondrial activity and higher number of sperm binding to perivitelline membrane, in comparison to control. Additionally, some post-thawing kinematic parameters were enhanced by anethole at 100 μM. Of note, mitochondrial activity and lipoperoxidation were higher with anethole at 100 μM in comparison to 50 μM, not differing from control. At the hypoosmotic test, the highest concentration (100 μM) tested reduced sperm osmotic resistance. The results of this study indicate that using anethole in cryopreservation media promoted mostly positive effects on the fresh and post-thawed ram semen, and the advantages vary according to its concentration.

1. Introduction

Semen cryopreservation is a leading biotechnology used for the genetic improvement of livestock but it causes unreversible damage to sperm cells, such as osmotic dysregulation, rupture of cell membranes, and non-physiological increase of reactive oxidative species (ROS) compromising sperm viability and ability to fertilize (Peris-Frau et al., 2020; Pezo et al., 2021). Overproduction of ROS intracellularly leads to lipoperoxidation, disruption of cellular membranes, damage to DNA and proteins, and impairment of mitochondrial activity and sperm motility (Aitken, 2017). Reduction in sperm viability after cryopreservation is especially problematic in the ovine species, since the cervical anatomy of the sheep challenges the intra- or transcervical process of artificial insemination, while the use of lower quantities of live sperm is currently viable only when surgical methods are applied (Souza-Fabjan et al., 2023). Thus, improving post-thawing survival of ovine sperm is mandatory for the amplification of ovine artificial insemination, which is the biotechnology that mostly propels genetic improvement in farm animals (Kershaw et al., 2005).

Cellular membranes of ram spermatozoa, as well as other farm species, have high ratios of unsaturated-to-saturated fatty acid, and this balance increases susceptibility of those cells to the disruptive effects of cold temperatures (Bailey et al., 2000). Unsaturated fatty acids are more prone to undergo lipoperoxidation when there is an overproduction of ROS, and the products of this peroxidative damage, such as malondialdehyde, react with lipids and other structures, damaging cells and leading to more cell-damaging byproducts (Bailey et al., 2000; Peris-Frau et al., 2020). Thus, because ram semen is more prone to

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lipoperoxidation, and cryopreservation reduces activity of natural antioxidant enzymes, some antioxidant compounds have been used to decrease sperm oxidative damage and enhance the overall quality of spermatozoa after thawing (Peris-Frau et al., 2020; Rostami et al., 2020). However, incorporating antioxidants to semen cryopreservation media can be costly, especially considering its use within animal production systems, and identifying least-expensive molecules that efficiently mitigate oxidative damage in sperm cells is a sought-after objective.

Numerous antioxidants have been identified in plant extracts and some of them, either contained in whole plant extract or as isolated compounds, have been tested as adjuvants for semen cryopreservation (Ros-Santaella and Pintus, 2021). Anethole [1-methoxy-4-(1-propenyl)-benzene] is one of the proposed antioxidant adjuvants. This molecule is a sweet aromatic compound found naturally in anise (*Pimpinella anisum*), star anise (*Illicium verum*), fennel (*Foeniculum vulgare*), and other plants (De Martino et al., 2009; Malo et al., 2012; Marinov and Valcheva-Kuzmanova, 2015). In the ovine species, the use of fennel plant extract in semen extender during cryopreservation promoted improvement in post-thawing semen quality (Najafi et al., 2019), but the isolated anethole has never been previously explored as an adjuvant to help cryopreservation in this species. However, the use of plant extracts can lead to difficulty in the replicability of the results, due to variations in the product composition, inherent plant geographical origin, extraction methods, parts used, and maturation stages (Diao et al., 2014; Leal et al., 2011). Anethole is considered the most efficient antioxidant present in fennel extract (Pezo et al., 2021), thus, using this molecule in isolate form might be a reliable alternative for producing consistent and controlled effects on reducing oxidative stress in the ovine sperm.

The commercially available trans-anethole is the most abundant isomer found in oily extracts of fennel and is mostly used in industry. *Trans*-Anethole increases catalase and superoxide dismutase activities, as well as reduces lipoperoxidation by inhibition of 5-lipoxygenase activity (Lee et al., 2012; Pezo et al., 2021). This molecule is available in liquid and powder and has a lower cost when compared to commonly used antioxidants, such as vitamin E and catalase (da Silva Castro et al., 2022; Qamar et al., 2023). Yet, the effects of trans-anethole in semen cryopreservation have been tested only in humans or goats, and results appear contradictory (Luo et al., 2020; Sousa et al., 2021).

Thus, it is hypothesized that trans-anethole reduces oxidative stress in the cryopreserved ovine semen and improves parameters related to seminal quality and ability to fertilize. The aim of this study was to evaluate the effects of different concentrations of anethole on cryopreservation of ram semen.

2. Material and methods

2.1. Ethics approval and location

This study was approved by the Ethics Committee for Use of Animals (3538120222) of Universidade Federal Fluminense and followed the guidelines of Animal Research: Reporting of *In Vivo* Experiments (ARRIVE). The experiment was conducted during the breeding season at UniPECO (Unidade de Pesquisa Experimental em Caprinos e Ovinos - 22° S, 42° W), in Cachoeiras de Macacu, RJ, Brazil.

2.2. Reagents

All reagents used, including liquid anethole (cat # 117870), were purchased from Sigma Chemical Co (St. Louis, MO, USA) unless otherwise indicated. The commercial anethole is available as the trans-anethole isomer but will be denominated here as simply anethole. Pasteurized yolk (Fleischmann, Brazil) was acquired in a local supermarket and used to prepare the TRIS egg yolk extender. The eggs used in the perivitelline membrane binding test were also obtained in a local supermarket.

2.3. Animals and semen collection

A total of six reproductively sound adult Santa Inês rams were selected after clinical and andrological evaluation. The rams were kept under natural light in a semi-confinement system, in which during the day, they had access to pasture and a small paddock with concentrate according to nutritional needs (NRC, 2007), *ad libitum* water and mineral mixture during the entire experiment. During the evening, the animals were kept inside the paddock. The rams underwent semen collection in three sections to renew the sperm reserve and, subsequently, had a day of sexual rest before the beginning of the experiment.

For the experiment, semen was collected daily using an artificial vagina for eight consecutive days. After each individual collection, semen was evaluated regarding macroscopic (appearance, volume, odor, and color) and microscopic (sperm kinematics and concentration) parameters. All ejaculates that had $\geq$70 % motility and were compatible with the species' normal parameters (CBRA, 2013) were selected for freezing.

2.4. Experimental design

A total of 30 ejaculates (four to six ejaculates from each ram) were used. Sperm concentration was determined using a Neubauer chamber, and each ejaculate was fractioned into four parts of the same volume, and each part was diluted in extenders containing anethole (AN) at different concentrations, according to one of the following treatments: CONT (control group, 0 μM), AN10 (10 μM), AN50 (50 μM) and AN100 (100 μM). The final concentration of sperm in cryopreservation media was 400 $\times$ 10^6 spermatozoa/mL. After sperm dilution (before freezing), the sperm kinematics, plasma membrane integrity, membrane function (hypoosmotic test), mitochondrial activity, and morphology were evaluated. Immediately after thawing (0 h), the same variables, as well as sperm capacitation status, sperm binding to egg perivitelline membrane and lipoperoxidation quantification of sperm cells, were also performed.

2.5. Semen cryopreservation and thawing

The semen extender used was TRIS egg yolk containing 5 % glycerol (3.63 g TRIS, 0.50 g fructose, 1.99 g citric acid, 15 mL egg yolk, 100.000 IU penicillin, 100 mg streptomycin, 5 mL glycerol, glass-distilled water to 100 mL, pH 7.4 and 985 mOsm/kg). A stock solution of 10 mM of AN (diluted in PBS) was prepared and used to prepare the semen extender with a final concentration of 10 μM, 50 μM or 100 μM. For the control group, extender without AN was used. After dilution in specific extender containing different concentrations of anethole, semen of different treatments was placed in plastic straws of 0.25 mL, identified, and sealed with polyvinyl alcohol. Freezing was performed using TK 3000® automatic freezing system (TK Tecnologia em Congelação Ltda, Uberaba, MG, Brazil). The curve was programmed to induce semen refrigeration at 0.25 °C/min up to 5 °C, and then the straws were kept at 5 °C for 4 h. For freezing, a - 20 °C/min speed was used until reaching the temperature of −120 °C. Straws were immersed in liquid nitrogen and subsequently organized in racks and stored in a cryogenic cylinder at −196 °C until thawing. Straws were thawed in a water bath at 37 °C for 30 s.

2.6. Sperm kinematics

Computer-assisted semen analysis (CASA) was performed using SCA® system (Sperm Class Analyzer Microptic, Nikon Eclipse Ci, Tokyo, Japan), configured for ram sperm. The spermatic head dimension detectable by the system was configured as a standard parameter between 18 and 60 μm^2. A total of 25 images per second were captured; magnifying power of microscope: ×100; measurements were performed in a 24 × 24 mm coverslip with 10 μL of sample, and at least 500 spermatozoa were evaluated per slide. Spermatozoa were identified as immotile if curvilinear velocity (VCL) was <10 μm/s. Speeds between 10

and 45 μm/s were classified as slow; between 45 and 75 μm/s were classified as medium; and above 75 μm/s were classified as rapid. Sperm cells presenting straightness (STR) above 80 % were featured as progressive. Individual ejaculates were analyzed for the following parameters: total motility (%), progressive motility (%), fast sperm (%), medium sperm (%), slow sperm (%), average path velocity (VAP), VCL, straight-line velocity (VSL), amplitude of lateral head displacement (ALH), beat/cross frequency (BCF), STR, linearity (LIN) and wobble (WOB), which is defined as the mean value of the ratio between VAP and VCL.

2.7. Hypoosmotic test

For the hypoosmotic test (HOST), 15-μL semen aliquots were placed in tubes containing 500 μL hypoosmotic solution of 100 mOsm/kg (MiliQ water). Then, these aliquots were incubated at 37 °C for 20 min and evaluated through wet preparation between slide and coverslip. The evaluation was performed using a phase-contrast microscope under 600 × magnification. Sperm with a swelled curled tail were classified as having normal membrane function, and sperm with a straight tail were classified as having abnormal membrane functionality (Ramu and Jeyendran, 2013). At least 200 sperm cells per treatment of each ejaculate were counted.

2.8. Sperm morphology

A 10 μL sample of semen was added to 120 μL 2 % paraformaldehyde solution (diluted in phosphate-buffered saline) at 37 °C and stored at 4 °C until evaluation. For morphology analysis, a wet slide was prepared with one drop of the diluted sample in a slide with a coverslip, and the spermatozoa morphology was examined under phase contrast microscopy at 1000 × magnification. At least 200 sperm cells per treatment of each ejaculate were evaluated. Abnormal sperm were grouped into major and minor defects (Chenoweth, 2005).

2.9. Epifluorescence microscopy assessment

All analyses were performed using a fluorescence microscope with a protected filter for optimized reading (Nikon Eclipse Ci- Nikon Corporation - Japan) coupled with the Pylon 7.4.0 Camera Software Suite. For each staining protocol, samples were distributed on a glass slide overlaid with a coverslip and observed under 100 x magnification for each analysis, unless otherwise indicated.

2.9.1. Plasma membrane integrity

The plasma membrane (PM) integrity was determined according to Alfradique et al. (2018). Two probes were used: acridine orange (A9231) [dilution factor: 1 (acridine orange):10.000 (distilled water), *v*/v] and 0.5 mg/mL propidium iodide (P4170). Acridine orange is PM permeable and selective to nucleic acids, labeling cells with green fluorescence. Propidium iodide is only permeable to damaged PM, and binds to nucleic acids, emitting red fluorescence. This evaluation was performed under appropriate filter sets (465–495 nm excitation and 515–555 nm emission), and cells were classified as having intact (green) or damaged (red) membranes. At least 200 sperm cells per treatment of each ejaculate were assessed.

2.9.2. Mitochondrial activity

MitoTracker Green was used for staining the sperm cells. This fluorochrome labels mitochondria of live spermatozoa only (Druart et al., 2009). A working solution with 4 mL of buffered saline and 10 μL of MitoTracker was previously prepared, reaching a final concentration of 20 μM. Semen (10 μL) was incubated with MitoTracker solution (90 μL) for 30 min at 37 °C, and then visualized at a fluorescence microscope with proper filter sets (490 nm excitation and 512 nm emission). For evaluation of fluorescence intensity of each sample, at least 10 images of different fields were obtained at 200 x magnification and were analyzed using the ImageJ (ImageJ, U.S. National Institutes of Health, imagej.nih.gov/ij/) software. For all images, values were corrected removing the background and at least 100 spermatozoa per treatment of each ejaculate were counted. Results are shown as fluorescence intensity arbitrary units.

2.9.3. Sperm capacitation status

Sperm capacitation status was assessed using chlortetracycline (CTC) staining as previously described (Alfradique et al., 2018; Cormier et al., 1997) with some modifications. A working solution was prepared with 0.75 mM CTC (pH 7.8) in a buffer containing 20 mM Tris, 130 mM NaCl, and 5 mM l-cysteine. The sperm samples were mixed with an equal volume of CTC solution (5 μL) and, after a few seconds, 2 μL of 2 % paraformaldehyde was added. Cells were observed under fluorescence using a blue-violet 2 A (BV-2 A) filter with 400–440 nm excitation and 470 nm emission. Spermatozoa were classified according to the criteria suggested by Cormier et al. (Cormier et al., 1997): uniform bright fluorescence over the whole head (non-capacitated), fluorescence-free band in the post-acrosomal region (capacitated), and dull fluorescence over the whole head with a thin and bright band of fluorescence along the equatorial region (acrosome reacted cells). At least 200 sperm cells per treatment of each ejaculate were assessed.

2.9.4. Sperm binding to egg perivitelline membrane test

This test was performed according to Barbato et al. (1998) and Campos et al. (2017). Perivitelline membranes were obtained from fresh and non-fertilized chicken eggs and prepared by separating the egg yolks from the egg whites. The intact yolks were placed on plastic paraffin film (Parafilm-M®), and the perivitelline membranes were separated and washed with PBS. Then, membranes were placed in a petri dish and cut into 0.5 cm^2 squares. Subsequently, squares were moved to one petri dish each, covered with 1 mL of FERT-TALP, and an aliquot of 20 μL of semen from each ejaculate was added. The membrane with the semen sample was incubated for 1 h at 38.5 °C with 5 % CO_2. After incubation, the membrane was gently washed three times with PBS, placed on a slide with 1 μL of Hoechst 33342 (1 mg/mL) and covered carefully with a coverslip (avoiding formation of folds or wrinkles), sealed, and protected from light. Cells were observed under proper filter sets (350 nm excitation and 461 nm emission) and five fields were counted per sample. The results of sperm binding are expressed in sperm/mm^2 of the membrane (Barbato et al., 1998; Brito et al., 2017).

2.10. Lipoperoxidation quantification

This evaluation was performed according to Sarlós et al. (2002). The method is based on quantification, by spectrophotometry, of the pink color produced by the reaction between molecules of thiobarbituric acid and malondialdehyde. The reactions occur under acidic pH, and temperature between 90 °C and 100 °C. One straw per treatment of each animal was thawed and 250 μL of thawed semen were added to 500 μL of 10 % trichloroacetic acid solution (10 % TCA) and centrifuged at 1800*g* for 15 min and at 15 °C for precipitation of proteins. Aliquots of 500 μL of the supernatant were placed in tubes along with 500 μL 1 % thiobarbituric acid, dissolved in 0.05 N sodium hydroxide, freshly prepared. Tubes containing this mixture were incubated in a water bath at 100 °C for 10 min and then cooled in an ice bath at 0 °C. Thiobarbituric Acid Reactive Species (TBARs) were quantified in a spectrophotometer, at a length of 532 nm, and were expressed in nanograms of TBARs/mL of semen.

2.11. Statistical analyses

Data analyses were performed using the software IBM SPSS Statistics (version 27). Data normality was checked using the Shapiro-Wilk test. Sperm fluorescence intensity, assessed by the mitochondrial activity

test, was detected as non-normally distributed and submitted to the non-parametric Kruskal-Wallis test. Spermatozoon was considered as the subject, and differences between treatments were attributed by the post-hoc Dunn's test. For all the remaining data, treatments were compared using a generalized linear mixed model (GLMM), including animal as the subject, treatment group (AN concentration) as the fixed effect, and ejaculate as a random effect. The pairwise comparison test of Least Significant Difference confirmed differences between groups. Parametrical data are presented as adjusted LS Means ± SEM. For all tests, significance was attributed when $P \leq 0.05$.

3. Results

Immediately after dilution with an extender containing or not anethole (pre-freezing), some differences were detected ($P < 0.05$) among experimental groups in sperm kinematics (Table 1). In the hypoosmotic test, it was detected that AN10 and AN50 had a larger ($P < 0.05$) proportion of sperm with functional membranes than CONT (Table 1). Regarding pre-freezing mitochondrial activity, all groups treated with anethole showed higher ($P < 0.05$) fluorescence intensity (*i.e.*, higher mitochondrial activity) when compared to CONT (Fig. 1).

In the frozen-thawed semen, the kinematics analyses revealed that the AN100 group had higher ($P < 0.05$) total motility, progressive motility, proportion of fast and medium-speed sperm, VCL, VSL, and VAP than one or more of the studied groups (Table 2). The kinematic parameters of AN10 or AN50 groups were not superior to any other group. Cells with higher mitochondrial activity ($P < 0.05$) were observed in AN50 and AN100 compared to CONT and AN10 (Fig. 1).

There were no differences between treatment groups in frozen-thawed sperm plasma membrane integrity (Fig. 2) and capacitation status (Fig. 3). Regarding morphology, the AN50 group had fewer sperm with minor defects compared to sperm from the CONT group (Table 3). The hypoosmotic test showed that the AN100 group had fewer ($P < 0.05$) reactive sperm cells, compared to CONT and AN50 (Fig. 2). The perivitelline membrane test revealed more ($P < 0.05$) sperm from AN100 and AN50 groups bound to the membrane, compared to CONT (Fig. 4). The lipoperoxidation test showed that AN10 had lower TBARS production ($P < 0.05$) when compared to CONT and AN100 (Fig. 5).

Table 1
Pre-freezing parameters of ram sperm diluted with extenders containing or not anethole (AN) at distinct concentrations.

	Treatment group			
Endpoints	CONT	AN10	AN50	AN100
Total motility (%)	99.9 ± 0.1	99.6 ± 0.1	99.9 ± 0.1	99.8 ± 0.1
Progressive motility (%)	14.2 ± 1.5	14.8 ± 1.5	14.4 ± 1.5	15.9 ± 1.5
Fast sperm (%)	37.3 ± 7.4^{a}	35.4 ± 7.4^{ab}	34.9 ± 7.4^{b}	35.7 ± 7.4^{ab}
Medium sperm (%)	34.4 ± 3.6	36.2 ± 3.6	37.1 ± 3.6	35.3 ± 3.6
Slow sperm (%)	28.2 ± 4.9	28.0 ± 4.9	27.8 ± 4.9	28.8 ± 4.9
VCL (μm/s)	70.4 ± 5.1^{a}	69.1 ± 5.1^{ab}	67.8 ± 5.1^{b}	68.2 ± 5.1^{ab}
VSL (μm/s)	23.4 ± 1.3	23.2 ± 1.3	22.6 ± 1.3	23.2 ± 1.3
VAP (μm/s)	40.3 ± 2.7^{a}	39.6 ± 2.7^{ab}	38.8 ± 2.7^{b}	39.4 ± 2.7^{ab}
LIN (%)	34.8 ± 1.2^{ab}	35.4 ± 1.2^{ab}	33.8 ± 1.2^{a}	36.5 ± 1.2^{b}
STR (%)	59.7 ± 1.0	60.3 ± 1.0	59.9 ± 1.0	60.4 ± 1.0
WOB (%)	58.0 ± 0.9	58.3 ± 0.9	57.8 ± 0.9	58.6 ± 0.9
ALH (μm)	5.0 ± 0.2	4.9 ± 0.2	4.9 ± 0.2	4.9 ± 0.2
BCF (Hz)	4.8 ± 0.6	4.9 ± 0.6	4.9 ± 0.6	4.9 ± 0.6
Plasma membrane integrity (%)	75.1 ± 1.4	73.7 ± 1.4	75.1 ± 1.4	75.2 ± 1.4
Functional plasma membrane (%)*	70.2 ± 1.9^{a}	72.8 ± 1.9^{b}	72.8 ± 1.9^{b}	72.7 ± 1.9^{ab}
Normal morphology (%)	93.3 ± 2.9	90.9 ± 2.8	94.0 ± 2.9	87.7 ± 2.8

Data is presented as LS Mean ± SEM. Within a line, the superscripts letters indicate difference between treatments ($P < 0.05$). *Percentage of spermatozoa reactive to the hypoosmotic test.

Abbreviations: VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN: linearity; STR: straightness; WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat/cross frequency; MMP: Mitochondrial Membrane Potential. Ram semen diluted with extenders treated or not as follows: untreated – CONT; treated with anethole: 10 μM - AN10; 50 μM – AN50; or 100 μM – AN100.

4. Discussion

The increased mitochondrial activity promoted by anethole at 10, 50, and 100 μM, detected in fresh semen, as well as after thawing on the groups treated with anethole at 50 or 100 μM, indicates a fast and lasting effect of this molecule in the sperm cells. The effect was more pronounced as the concentrations of anethole increased, indicating a dose-dependent relationship. The mechanism by which anethole increases mitochondrial activity is not clear yet but might involve actions through the activation of estradiol receptors. Fennel and anise plant extracts have been used in the human species as estrogenic agents (Albert-Puleo, 1980), and the estrogenic effects produced by anethole were confirmed by a study in yeast-bearing recombinant human estradiol receptors (ERα and ERβ) that demonstrated that trans-anethole has estrogenic activity (Howes et al., 2002). Both human (Guido et al., 2011) and ram (Gimeno-Martos et al., 2017) spermatozoa bear estradiol receptors (ERα and ERβ), and the study in humans showed that those receptors are concentrated in the mitochondria-rich midpiece. In human sperm, it was demonstrated that 17β-estradiol modified mitochondrial function, increasing it at lower concentrations or reducing it at higher concentrations (Kotwicka et al., 2016). Thus, it is reasonable to propose that activation of estradiol receptors mediated the enhanced mitochondrial activity observed after the treatment of ram semen with anethole.

Mitochondria hold oxidative phosphorylation reactions that generate energy for sperm movement (Amaral et al., 2013; Sengupta et al., 2020). Adding anethole at 100 μM to cryopreservation media elicited the highest mitochondrial activity (pre- and post-freezing) and improved total and progressive motility, as well as other sperm kinematic parameters, in comparison to one or more of the groups containing 0, 10, or 50 μM anethole. The improvement of kinematic parameters caused by anethole at 100 μM is probably not related to induction of sperm capacitation, resulting in early hyperactivation, once in this study, differences in the capacitation status were not detected between any treatment group, a finding also observed in other studies (Luo et al., 2020; Malo et al., 2012; Sousa et al., 2021). The higher post-thawing total and progressive motility of sperm induced by anethole at 100 μM, compared to the control group, corroborates with results from studies using anise extract added to the semen extender, conducted in pigs (Malo et al., 2012; Montón et al., 2015) and sheep (Najafi et al., 2019).

Besides producing ATP, mitochondria are the main generator of ROS in spermatozoa (Koppers et al., 2008), and when overproduced, ROS disrupt mitochondrial function, decreasing ATP availability and, consequently, enhance lipoperoxidation (Chianese and Pierantoni, 2021). The sperm cell membrane contains high amounts of poly-unsaturated fatty acids, such as linoleic and arachidonic acid, which have been shown to be disrupted after ram semen cryopreservation (Ofosu et al., 2023). Linoleic acid can be converted into arachidonic acid and, the latter, to leukotrienes by lipoxygenases like 5-lipoxygenase (Hamilton and Klett, 2021). Although necessary as signaling molecules, when produced in excess and for a prolonged time, leukotrienes, together with other metabolites, can promote oxidative stress, resulting

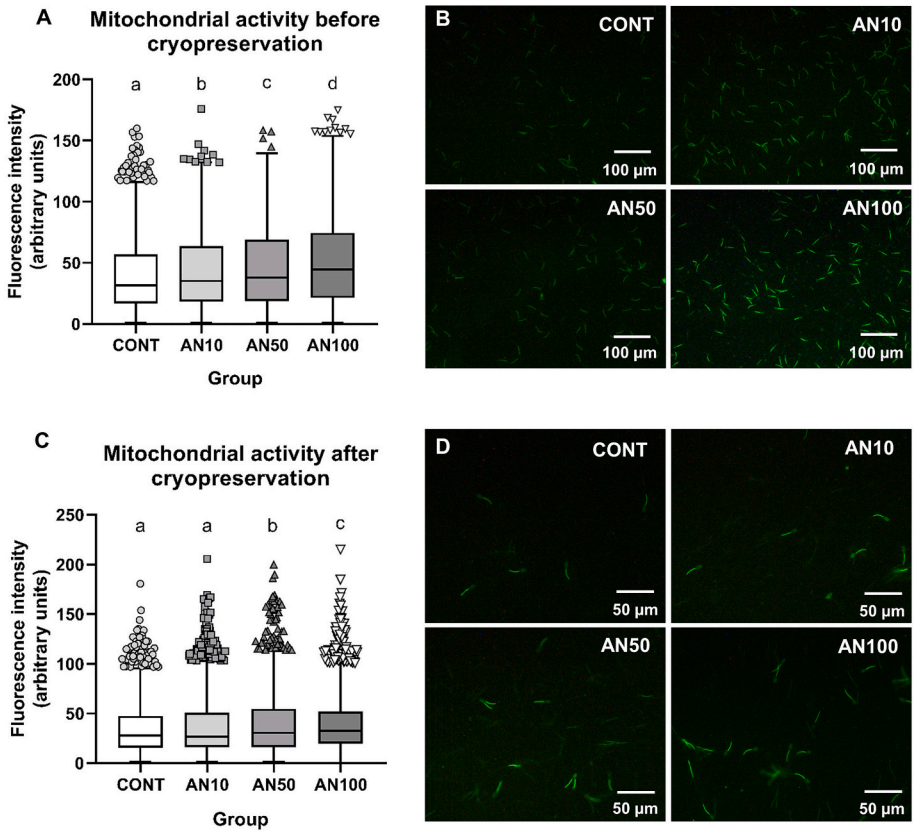

Fig. 1. Box Plots representing mitochondrial activity before (A), and after (C) cryopreservation. Representative images of each group of mitochondrial activity before (B), and after (D) cryopreservation. Cells were stained with Mitotracker Green. Ram semen diluted with extenders treated or not as follows: untreated – CONT; treated with anethole: 10 µM -AN10; 50 µM – AN50; or 100 µM – AN100. Different letters indicate differences ($P < 0.05$) between treatments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Post-thawing parameters assessed by CASA system of ram sperm cryopreserved using or not anethole (AN) on semen extender.

	Treatment group			
Endpoints	CONT	AN10	AN50	AN100
Total motility (%)	61.0 ± 2.8^{a}	62.6 ± 2.8^{a}	63.7 ± 2.8^{ab}	68.6 ± 2.8^{b}
Progressive motility (%)	9.9 ± 1.4^{a}	10.9 ± 1.4^{ab}	9.7 ± 1.4^{a}	13.0 ± 1.4^{b}
Fast sperm (%)	15.2 ± 2.4^{a}	14.5 ± 2.4^{a}	16.5 ± 2.4^{ab}	19.4 ± 2.4^{b}
Medium sperm (%)	7.4 ± 1.1^{a}	8.3 ± 1.1^{ab}	8.3 ± 1.1^{ab}	10.3 ± 1.1^{b}
Slow sperm (%)	38.4 ± 1.9	39.8 ± 1.9	38.9 ± 1.9	39.0 ± 1.9
VCL (µm/s)	46.9 ± 3.1^{ab}	45.8 ± 3.1^{a}	47.3 ± 3.1^{ab}	51.0 ± 3.1^{b}
VSL (µm/s)	21.0 ± 1.6^{ab}	20.3 ± 1.6^{a}	20.2 ± 1.6^{a}	23.1 ± 1.6^{b}
VAP (µm/s)	29.5 ± 2.1^{ab}	28.6 ± 2.1^{a}	29.2 ± 2.1^{ab}	32.5 ± 2.1^{b}
LIN (%)	44.6 ± 1.6	44.1 ± 1.6	43.3 ± 1.6	45.2 ± 1.6
STR (%)	70.3 ± 1.3	70.2 ± 1.3	68.1 ± 1.3	70.5 ± 1.3
WOB (%)	62.9 ± 1.3	62.5 ± 1.3	62.2 ± 1.3	63.6 ± 1.3
ALH (µm)	3.3 ± 0.1	3.4 ± 0.1	3.3 ± 0.1	3.5 ± 0.1
BCF (Hz)	8.8 ± 0.6	9.2 ± 0.6	8.8 ± 0.6	9.3 ± 0.6

Data is presented as LS Mean ± SEM. Within a line, the superscripts letters indicate difference between treatments ($P < 0.05$).
Abbreviations: VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN: linearity; STR: straightness; WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat/cross frequency. Ram semen diluted with extenders treated or not as follows: untreated – CONT; treated with anethole: 10 µM - AN10; 50 µM – AN50; or 100 µM – AN100.

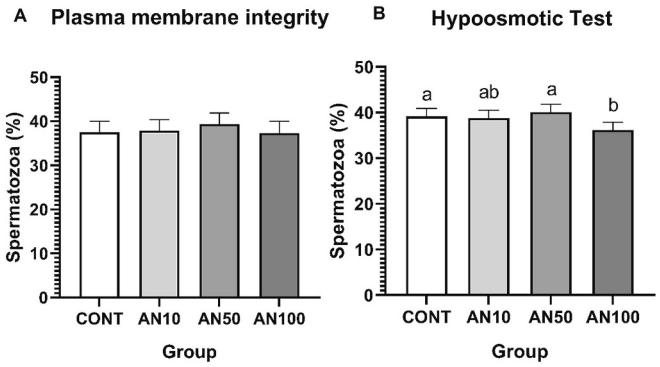

Fig. 2. Proportion of spermatozoa (A) with intact membrane in the plasma membrane integrity evaluation; (B) reactive to the hypoosmotic swelling test. Frozen-thawed ram semen with extenders treated or not as follows: untreated – CONT; treated with anethole: 10 µM - AN10; 50 µM – AN50; or 100 µM – AN100. Plasma membrane integrity, $P > 0.05$. Hypoosmotic test, different letters indicate differences between treatments ($P < 0.05$).

in DNA, mitochondrial, and membrane damages (Hamilton and Klett, 2021; Peris-Frau et al., 2020). Anethole has been shown to inhibit 5-lipoxygenase (Lee et al., 2012) efficiently, and, due to its structure, anethole is capable of sequestering hydrogen peroxide (H_2O_2) and increasing catalase and superoxide dismutase levels (Choi and Hwang, 2004; Galicka et al., 2014). According to our hypothesis, anethole has the potential to reduce the oxidative damage caused to sperm cells during cryopreservation. Herein, it was observed that anethole reduced lipoperoxidation in cryopreserved ram spermatozoa when added at the

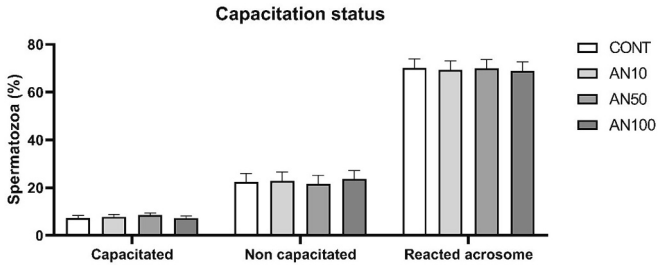

Fig. 3. Proportion of spermatozoa in each capacitation status classification. Frozen-thawed ram semen with extenders treated or not as follows: untreated – CONT; treated with anethole: 10 μM - AN10; 50 μM – AN50; or 100 μM – AN100. There was no difference in all parameters measured for all treatments ($P > 0.05$).

Table 3
Post-thawing parameters assessed by computer-assisted semen analysis (CASA) system of ram sperm cryopreserved using or not anethole (AN) on semen extender.

	Treatment group			
Morphology	CONT	AN10	AN50	AN100
Abnormal				
Major defects (%)	2.4 ± 0.3	2.6 ± 0.3	2.3 ± 0.3	2.0 ± 0.3
Minor defects (%)	4.4 ± 0.5^{b}	3.4 ± 0.5^{ab}	3.2 ± 0.5^{a}	3.8 ± 0.5^{ab}
Total (%)	6.8 ± 0.6	6.0 ± 0.6	5.5 ± 0.6	5.8 ± 0.6
Normal morphology (%)	93.2 ± 0.6	94.0 ± 0.6	94.5 ± 0.6	94.2 ± 0.6

Data is presented as LS Mean ± SEM. Within a line, the superscript letters indicate the difference between treatments ($P < 0.05$). Ram semen diluted with extenders treated or not as follows: untreated – CONT; treated with anethole: 10 μM - AN10; 50 μM – AN50; or 100 μM – AN100.

concentration of 10 μM in cryopreservation extender. Therefore, at this lower concentration, anethole seems to protect spermatozoa against oxidative damage, and its use might be advantageous during cryopreservation, especially in individuals at higher risk of oxidative stress.

At higher concentrations of anethole (50 and 100 μM), alterations in lipoperoxidation could not be observed. This might be an effect of the balance between increased metabolic activity (as seen by increased mitochondrial activity) with ROS generation *versus* protection against oxidation (Kang and Hamasaki, 2003). Another possible explanation for higher concentrations of anethole not leading to lipoperoxidation reduction is what is known as the antioxidant paradox. It has been observed that the use of antioxidants to neutralize ROS can have the opposite result, as the loss of balance between oxidants and antioxidant agents can lead to reductive stress (Sadeghi et al., 2023; Tadros and Vij, 2019). In fact, anethole, as well as some other antioxidants, have pro-oxidant activity at high concentrations (dos Santos Morais et al., 2019; Sadeghipour et al., 2005; Succu et al., 2014). Further investigation on the dose-dependent effect of anethole in semen oxidative stress is warranted.

This study also showed that the use of anethole at 100 μM may bring disadvantages to cryopreserved semen, as it increases the sensitivity of sperm cells to a hypoosmotic environment, resulting in sperm with a biochemically unstable membrane. This can be supported by previous research showing that anethole can affect the stability of biomembranes of different human tumor cell lines when different concentrations of fennel essential oil are used (Sharopov et al., 2017). Furthermore, when used in buck sperm, commercial anethole at higher concentrations (approx. 200 to 13,000 μM) induced midpiece and tail pathologies (Sousa et al., 2021). Interestingly, here we observed that lower doses (10 and 50 μM) of anethole promote more osmotic stability in fresh semen, and at 50 μM, anethole reduces minor defects in the post-thawed sperm. Thus, it appears that the stability of cell membrane and the occurrence of minor defects (majorly related to the tail) are specifically sensitive to anethole's concentration in cryopreservation media. The effect of anethole on sperm membrane might be associated with anethole's influence on the transmembrane influx of Ca^{2+}, an effect that was also

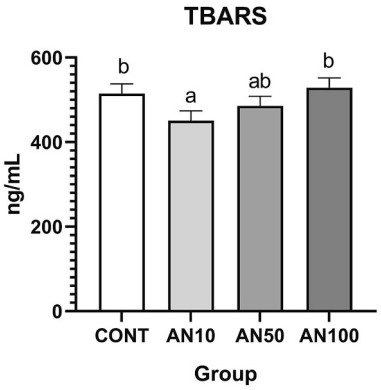

Fig. 5. Lipoperoxidation quantification of frozen-thawed ram sperm. Extenders treated or not as follows: untreated – CONT; treated with 10 μM of purified trans-anethole - AN10; 50 μM – AN50; or 100 μM – AN100. Results expressed in ng of Thiobarbituric Acid Reactive Species (TBARS) per mL. Different letters represent differences ($P < 0.05$) between treatments.

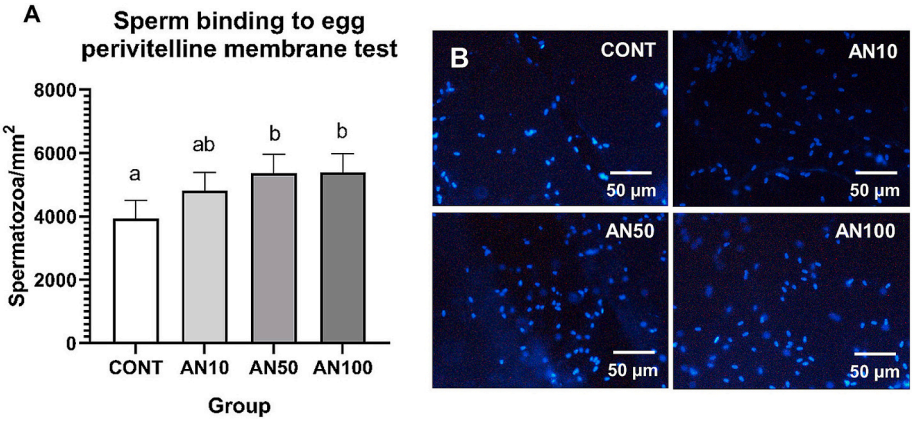

Fig. 4. (A) Number of spermatozoa bound per mm^2 of egg perivitelline membrane. (B) Representative images of each group. Cells stained with Hoechst dye. Frozen-thawed ram semen with extenders treated or not as follows: untreated – CONT; treated with anethole: 10 μM - AN10; 50 μM – AN50; or 100 μM – AN100. Different letters indicate differences between treatments ($P < 0.05$).

observed depending on its concentration: increasing it at low and reducing it at high concentrations (Soares et al., 2007). In human sperm, anethole at 10 and 100 μM reduced transmembrane Ca^{2+} influx, reducing total and progressive motility, preventing the hyperactivation and penetration capacity of these cells (Luo et al., 2020). Thus, our results indicate that anethole might produce negative or positive outcomes, depending on its concentration. In sperm cells, higher concentrations of anethole can negatively impact the functionality of membranes and resistance to osmotic stress.

Regarding the sperm binding test, more spermatozoa were bound to the egg perivitelline membrane if they were diluted in extenders containing 50 or 100 μM anethole in comparison to control, but no difference among those former two groups was observed. The egg perivitelline membrane has homologous glycoproteins to those present in the zona pellucida (ZP) of mammals, such as ZP1 and ZP3, which allows spermatozoa from other species to bind to this membrane (Bausek, 2000; Robertson et al., 2000; Waclawek et al., 1998). This test is correlated with *in vivo* fertility in roosters (Barbato et al., 1998), with the ability of collared peccary sperm to bind to swine oocytes *in vitro* (Campos et al., 2017), and with mitochondrial activity, plasma membrane integrity, and sperm motility in bulls (Losano et al., 2015) and dogs (Brito et al., 2017). Thus, the results of this test might aid in the prediction of fertilizing capacity of sperm from diverse mammalian species and this relationship might probably be valid for the ovine species. Thus, adding anethole at 50 or 100 μM in cryopreservation extender might improve ability of the ovine semen to bind and fertilize sheep oocytes. This presumption must be further elucidated.

The findings of this study indicate that adjusting anethole concentration according to a determined objective may be a strategic tool to improve specific sperm quality parameters or to avoid undesired side effects. While anethole at 10 μM reduces the oxidative stress in sperm cells, anethole at 50 or 100 μM improves some parameters of sperm quality and seems to support higher chances of fertilization after thawing. Conversely, supplementation with 100 μM of anethole leads to a higher sensitivity to osmotic stress and might compromise sperm functionality. Furthermore, the cost and commercial availability of anethole, together with its beneficial effects on post-thawed seminal parameters, support its use on a commercial scale to improve sheep-producing systems.

5. Conclusions

Altogether, our data indicate that the use of anethole in ovine semen can be advantageous, as it improves membrane stability and mitochondrial activity in fresh semen, reduces sperm lipoperoxidation, improves kinematic parameters, mitochondrial activity, and binding capacity to the perivitelline membrane in post-cryopreserved semen. The nature of the positive effect on sperm parameters depends on anethole's concentration in the semen extender, and higher concentrations can be disruptive to post-thawing osmotic stability.

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CRediT authorship contribution statement

Rachel F. Braga: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Lucas F.L. Correia:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization. **Mariana P.P. Guimarães:** Writing – review & editing, Investigation, Formal analysis. **Nathalia O. Barbosa:** Writing – review & editing, Investigation, Formal analysis. **Thais G. de Oliveira:** Writing – review & editing, Investigation, Formal analysis. **Andreza A. da Silva:** Writing – review & editing, Methodology, Formal analysis. **Glaucia M. Bragança:** Writing – review & editing, Investigation. **Felipe Z. Brandão:** Writing – review & editing, Investigation, Formal analysis. **Bruna R.C. Alves:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Data curation. **Joanna M.G. Souza-Fabjan:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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