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Effect of adding anethole to the cryopreservation medium (powdered coconut water) on morphology, kinetics, and oxidative stress of buck sperm

Efeito da adição de anethole ao meio de criopreserção (água de coco em pó) na morfologia, cinética e estresse oxidativo do sêmen de bode

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Highlights

Evaluation of membrane and acrosome with flow cytometry was unaffected by anethole.
Morphological damage did not affect sperm kinetics.
Higher concentrations of anethole resulted in increased damage to the middle piece.
The antioxidant did not improve sperm kinetics.

Abstract

The quality of post-thawing goat sperm is critical to the success of artificial insemination protocols and may be influenced by extenders, cryoprotectants, and antioxidant substances. Therefore, the objective of this study was to evaluate the effects of the antioxidant anethole on goat sperm diluted in preservation medium based on powdered coconut water (ACP-101c) and frozen. For that, each ejaculate was submitted to the following treatments: ACP-101c (control); control plus supplementation with 30, 300, or 2000 µg/mL anethole. The samples were thawed and evaluated for morphology, kinetics, membrane integrity, and reactive oxygen species (ROS). The addition of anethole increased morphological abnormalities ($P < 0.05$), however, it did not affect sperm kinetics. Flow cytometry analysis showed that sperm cells cryopreserved

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with 300 µg/mL anethole had lower acrosome integrity than those cryopreserved in other treatments. Evaluation of oxidative stress revealed that cells stored in the presence of 2000 µg/mL anethole had small amounts of ROS when compared to those preserved in the control medium alone or supplemented with 300 µg/mL anethole (P < 0.05). After cryopreservation of sperm with 2000 µg/mL anethole, the highest percentage of viable sperm without ROS was observed (P < 0.05). In conclusion, despite reducing ROS levels, the supplementation of anethole in ACP-101c did not affect sperm kinetics or membrane integrity post-thawing, however, it did cause morphological damage to sperm.
Key words: Cryopreservation. Oxidative stress. Reproduction. Semen.

Resumo

A qualidade do espermatozoide caprino pós-descongelação é crítica para o sucesso dos protocolos de inseminação artificial e pode ser influenciada por extensores, crioprotetores e substâncias antioxidantes. Portanto, o objetivo deste estudo foi avaliar os efeitos do antioxidante anetole sobre espermatozoides caprinos diluídos em meio de conservação à base de água de coco em pó (ACP-101c) e congelados. Para tanto, cada ejaculado foi submetido aos seguintes tratamentos: ACP-101c (controle); controle mais suplementação com 30, 300 ou 2000 µg / mL de anetole. As amostras foram descongeladas e avaliadas quanto à morfologia, cinética, integridade de membranas e espécies reativas de oxigênio. A adição de anetole aumentou as anormalidades morfológicas (P < 0,05), no entanto, não afetou a cinética dos espermatozoides. A análise da citometria de fluxo mostrou que as células de esperma criopreservadas com 300 µg / mL anethole tinham integridade acrosma menor do que aquelas criopreservadas em outros tratamentos. A avaliação do estresse oxidativo revelou que as células armazenadas na presença de 2000 µg / mL anethole apresentaram pequenas quantidades de ROS quando comparadas às preservadas em meio de controle isoladamente ou suplementadas com 300 µg / mL anethole (P < 0,05). Após a criopreservação de espermatozoides com 2000 µg / mL anethole, observou-se a maior porcentagem de espermatozoides viáveis sem ROS (P < 0,05). A população com espermatozoides viáveis sem ROS foi maior quando utilizado 2.000 µg / mL (P < 0,05). Em conclusão, apesar de reduzir os níveis de ROS, a suplementação de anetole em ACP-101c não afetou a cinética espermática e a integridade da membrana pós-descongelação, entretanto, causou danos morfológicos nos espermatozoides.
Palavras-chave: Criopreservação. Estresse oxidativo. Reprodução. Sêmen.

Introduction

Several studies have been conducted aiming at improvements in post-thawing sperm quality for artificial insemination, either by testing extenders (Oliveira et al., 2011), cryoprotectants (Bispo et al., 2011; Chelucci et al., 2015), or the addition of antioxidant substances (Elsayed, El-Shamy, Abdelrazek, & El-Badry, 2019; Memonet al., 2013; Naijian, Kohram, Shaneh, & Sharafi, 2013).

Temperature variation in the seminal cryopreservation process can cause sperm damage, inducing osmotic stress and ice crystal formation, damage to the plasma membrane and acrosome, and and reactive oxygen species (ROS) rate deregulation (Igbokwe et al., 2019; Souza et al., 2019). However, when unbalanced, they can induce membrane, metabolic, or chromatin damage (Elsayed et al., 2019).

Chelating metals and enzymes such as reduced glutathione (GSH), glutathione peroxidase (GSH-Px), catalase (CAT), and superoxide dismutase (SOD), present in spermatozoa and seminal fluid, act by minimizing the effects of oxidative stress (Bucak, Sariözkan, Tuncer, Uluas, & Akçadag, 2009; Silva et al., 2011). However, their actions are often insufficient to maintain sperm quality during the freeze-thaw process (Elsayed et al., 2019). Accordingly, several substances with antioxidant action have been studied to assist in the regulation of ROS concentration in post-thawing sperm (Elsayed et al., 2019; Memon et al., 2013; Silva et al., 2011).

In buck sperm, the enzyme phospholipase hydrolyzes the egg yolk lecithin widely present in the diluent, converting it to lysolecithin and fatty acids. In turn, these act directly on the sperm membrane releasing ROS (Nunes, 1982). In this case, phospholipase A, which is a natural antioxidant responsible for the removal of peroxyl agents, may act in a reverse way, increasing damage through the degradation of unsaturated fatty acids in the sperm membrane by lysolecithin (Aitken, Wingate, De Iuliis, & McLaughlin, 2007; Nunes, 1982).

Anethole (1-methoxy-4-[(1E)-prop-1-en-1-yl]benzene) is a natural anisole derivative that is abundantly present in aromatic essential oils with the *Croton zehntneri* plant (Polzin, Stanfill, Brown, Ashley, & Watson, 2007). Studies with anethole have shown no genotoxicity or teratogenesis in somatic cell culture (Yea et al., 2006) and a positive effect on the elimination of ROS *in vivo* (Freire, Morais, Catunda, & Pinheiro, 2005), and *in vitro* (Sá et al., 2017).

There are no reports on the use of anethole in the cryopreservation of sperm cells. Therefore, the present work aimed to evaluate the antioxidant effects of anethole in different concentrations in a medium based on powdered coconut water (ACP-101c) on the morphology, kinetics, and oxidative stress of cryopreserved goat sperm.

Materials and Methods

Ethics approval

The present study was carried out at the Integrated Biotechnology Center of the State University of Ceará (UECE) (latitude, 3° 43′ 6″ S and longitude, 38° 32′ 36″ W). The present study was approved by the Ethical Committee on Animal Use at the UECE, under registration number 6305558/2014.

Animals and sperm collection

Five bucks (Capra hircus) aged between 3-5 years old were used. They were individually housed, fed with concentrate with approximately 18% crude protein, crushed Tifton hay (Cynodon sp.), goat mineral salt, and ad libitum water. Semen collections were performed every four days using the artificial vagina method. In total, nine collections were performed per animal, resulting in 45 samples.

Extenders and cryopreservation

After the analyses, the semen was diluted in a powdered coconut water-based medium (ACP-101c; 300 mOsm/L; pH 6.8; ACP Biotechnology, Fortaleza-CE, Brazil) plus 2.5% egg yolk, 40 mg of antibiotic (gentamicin), and 7% of glycerol (cryoprotectant), until reaching a concentration of 400 x 106 sptz/mL, as

described in Câmara et al. (2019), which was the control treatment. The other treatments consisted of the addition of 10 µL mL^{-1} in three different anethole concentrations (Sigma-Aldrich, St Louis, USA) to the extender: 30, 300, or 2000 µg mL^{-1} (Sá et al., 2017).

Each ejaculate was divided into four aliquots, which were submitted to the treatments. Semen dilution was standardized to a final sperm concentration of approximately 600 x 10^6 cells mL^{-1}. After dilution, the semen was packaged in 0.25 mL (150 x 10^6 cells) straws and sealed with polyvinyl alcohol. Semen was cryopreserved using the programmable freezer TK 3000® (TK Technology in Freezing Ltd., Uberaba, Brazil), adopting a specific refrigeration and freezing curve for buck sperm (−0.25 °C $min.^{-1}$ up to 4 °C, and −10 °C $min.^{-1}$ from 4 °C to −120 °C). At −120 ºC, the straws were dipped in liquid nitrogen (−196 ºC), stored for at least one month in a cryogenic cylinder. All extender preparation and dilution processes were performed in a 37 °C water bath in a partially dark room due to anethole photosensitivity.

Sperm morphology

Sperm morphological evaluation was performed as bromophenol blue-stained smears. To this end, 5 µL of the sperm sample was added over 5 µL of the dye on a glass slide preheated to 37 ºC. The prepared slide was dried at room temperature and subsequently observed under an optical microscope (1000x). Two hundred cells per slide were quantified between normal and abnormal spermatozoa. Abnormalities were evaluated as head, middle piece, and tail changes. The morphological evaluation was performed in triplicate.

Sperm kinetics

A computerized semen analysis system was used, with the Sperm Class Analyzer® (SCA) software configured for goat sperm (head size between 10 and 70 µm, slow between 10 and 45 µm s^{-1}, medium between 45 and 75 µm s^{-1}, fast > 75 µm s^{-1}, progressive > 80%, circular > 50%, and connectivity 12). The evaluations were carried out individually. For each thawed straw, a re-dilution was performed to obtain a final concentration of 25 x 106 cells mL^{-1}. The sperm parameters provided by the system were: total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, µm s^{-1}), straight line velocity (VSL, µm s^{-1}), average path velocity (VAP, µm s^{-1}), linearity (LIN, %), straightness (STR, %), wobble (WOB, %), amplitude of lateral head (ALH, µm), and beat cross frequency (BCF, Hz). Each evaluation was performed in triplicate.

Flow cytometry

Analyses were performed on a BD FACSVerse™ flow cytometer (BD Biosciences, USA) equipped with three excitation lasers (4.2.2). For cytometer calibration, an aliquot of fresh, unlabeled semen was used to identify the spermatozoa population (Figure 1A), and another labeled with propidium iodide (Figure 1B), FITC, and DCHF-DA probes, individually and in combination. To adjust the sperm concentration for incubation and analysis, each sample was diluted to approximately 5 x 10^6 cells in PBS with 5% glycerol. Sequentially, the samples were re-diluted in PBS to a final concentration of 2.5 x 10^6 cells mL^{-1} and evaluated by flow cytometer (Kumaresan, Johannisson, Al-Essawe, & Morre, 2017). After evaluation, data were analyzed using FlowJo software (FlowJo, LLC, Ashland, Ore.) to quantify different sperm populations.

Plasma membrane and acrosome integrity analysis

Plasma membrane and sperm acrosome integrity were verified by the combination of propidium iodide probes - PI (Sigma-Aldrich, St Louis, USA) and *Pisum sativum* agglutinin-conjugated fluorescein isothiocyanate - FITC-PSA (Sigma-Aldrich, St Louis, USA). For the evaluation, 150 µL sperm aliquots were added of 3 µL PI and 6 µL FITC into black opaque 1.5 mL tubes incubated at 37 °C for 10 min. At the end of the analysis, four populations were obtained: PI+/FITC−; PI+/FITC+; PI−/FITC+; PI−/FITC− (Figure 1C).

Reactive oxygen species detection

The presence of intracellular ROS was assessed using 2',7'-dichlorofluorescein diacetate - DCHF-DA (Sigma-Aldrich, St Louis, USA) which was oxidized to green fluorescence upon penetration into the cell (Okano et al., 2019). For the evaluations, 500 µL sperm aliquots were placed in opaque black 1.5 mL tubes, added with 0.5 µL DCHF-DA, and incubated at 37 °C for 30 min. Then, 3 µL PI was added and incubated for 10 min. In the end, four populations were obtained: PI^+/$DCHF\text{-}DA^-$; PI^+/$DCHF\text{-}DA^+$; PI^-/$DCHF\text{-}DA^+$; PI^-/$DCHF\text{-}DA^-$ (Figure 1D).

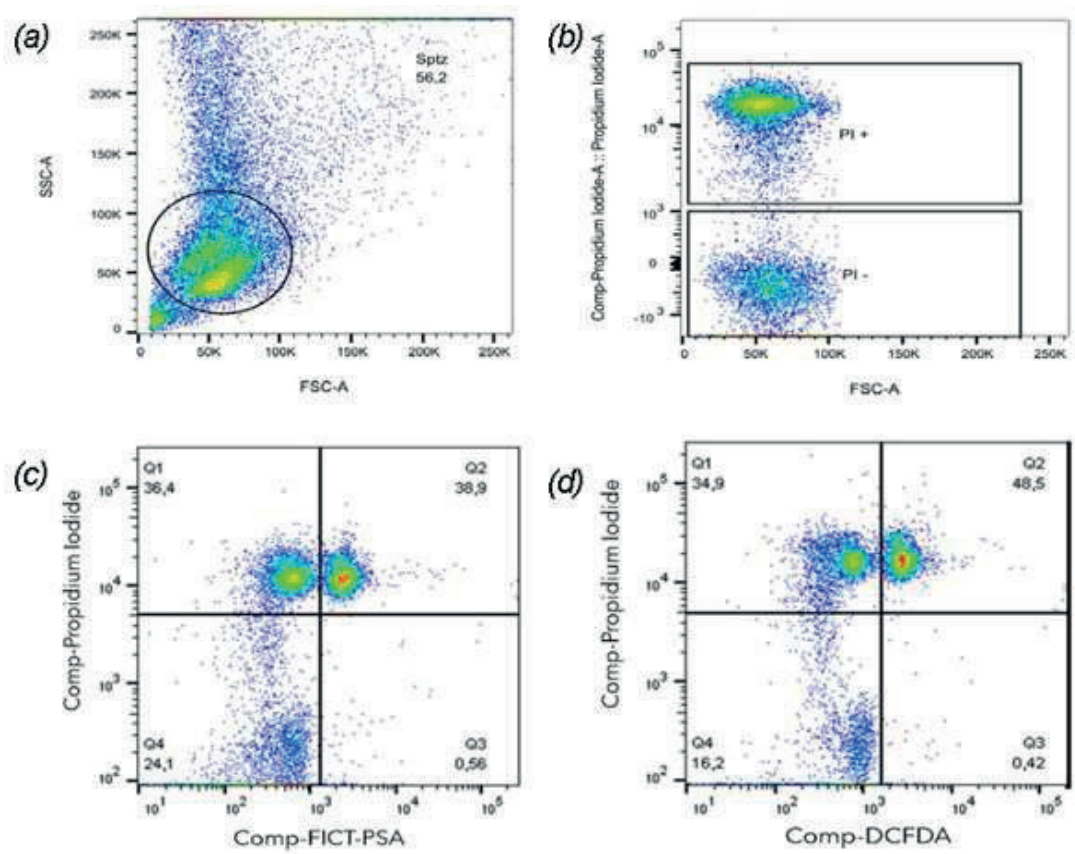

Figure 1. Representative image of spermatozoa populations by flow cytometry. (A) selection of sperm population by granulometry. (B) sperm subpopulations with intact or damaged plasmatic membrane (PI). (C) evaluation of sperm viability and acrosomal membrane integrity (PI/FITC). (D) sperm viability and hydrogen peroxide presence in the spermatozoa (PI/DCFDA).

Statistical analysis

Data were analyzed using Sigma Plot 11 software, version 23.0 (Systat Software Inc., San Jose, CA, USA). To reduce the variability of the results, the Z-score was performed to identify outliers. The Levene test was used to verify the homogeneity of variances. For verification of normality, the Shapiro-Wilk test was adopted. For comparison between treatments, ANOVA was performed, followed by Fisher's test (DMS). Data were presented as mean (± SEM), and statistical significance was set at $P < 0.05$.

Results and Discussion

Morphological damage

The powdered coconut water-based medium (ACP-101c; control) was effective in protecting the sperm morphology during the freeze-thaw process, maintaining a percentage of normal cells above 75% (Table 1). The addition of anethole at any concentration (30, 300, and 2000 µg mL^{-1}) reduced the percentage of normal spermatozoa ($P < 0.05$). The results indicate that the addition of anethole to the seminal extender, at all concentrations, significantly increased sperm tail damage ($P < 0.05$). However, only the addition of 30 µg mL^{-1} to ACP-101c did not cause significant damage in the middle piece compared to the control ($P > 0.05$). When comparing treatments with anethole, the addition of 30 µg mL showed less damage to the intermediate part when compared to treatment with 2000 µg mL ($P < 0.05$).

Table 1
Mean and error of the mean of sperm morphology of different anethole concentrations added to ACP-101c diluent

Parameters	N	ACP-101c (control)	Anethole		
			30 µg mL^{-1}	300 µg mL^{-1}	2000 µg mL^{-1}
Normal spermatozoa	45	$77.8 + 1.05^{a}$	$70.7 + 1.16^{b}$	$67.4 + 1.15^{b}$	$67.4 + 1.32^{b}$
Head defect	45	$1.31 + 0.13^{a}$	$1.64 + 0.13^{a}$	$1.84 + 0.14^{a}$	$1.98 + 0.17^{a}$
Middle piece defect	45	$10.1 + 0.68^{a}$	$13.0 + 0.78^{ab}$	$15.7 + 0.87^{bc}$	$16.1 + 0.90^{c}$
Tail defect	45	$9.61 + 0.48^{a}$	$13.4 + 0.64^{b}$	$13.9 + 0.59^{b}$	$13.2 + 0.61^{b}$

*Different superscript lowercase letters on the same line ($P < 0.05$). Data presented as mean + standard error. ACP-101c = extender medium based on powdered coconut water.

Evaluation of sperm kinetics

The total and progressive motility; the curvilinear, straight line and average path velocities; the linearity, straightness, wobble, amplitude of lateral head, and beat cross frequency were verified using the CASA system (Table 2). The addition of anethole to ACP-101c did not jeopardize the kinetics of post-thawed spermatozoa ($P > 0.05$).

Table 2
Mean and error of the mean of kinetic parameters of spermatozoa of different anethole concentrations added to ACP-101c diluent

Parameters	N	ACP-101c (control)	Anethole		
			30 µg mL $^{-1}$	300 µg mL $^{-1}$	2000 µg mL $^{-1}$
TM (%)	45	33.4 + 2.09	30.7 + 2.58	31.9 + 2.31	33.0 + 2.42
PM (%)	45	17.7 + 1.48	15.5 + 1.64	15.7 + 1.60	15.9 + 1.62
VCL (µm s^{-1})	45	67.5 + 1.98	65.3 + 1.88	65.8 + 2.17	64.4 + 2.04
VSL (µm s^{-1})	45	50.4 + 2.11	47.6 + 1.74	48.8 + 2.17	47.0 + 1.88
VAP (µm s^{-1})	45	50.9 + 2.13	56.7 + 1.88	50.7 + 2.19	55.6 + 2.01
LIN (%)	45	73.8 + 1.09	71.6 + 0.93	72.4 + 1.02	72.2 + 1.02
STR (%)	45	84.5 + 0.64	83.2 + 0.62	83.1 + 0.64	83.8 + 0.60
WOB (%)	45	86.4 + 0.76	85.8 + 0.66	86.5 + 0.62	85.9 + 0.70
ALH (µm)	45	2.07 + 0.39	2.06 + 0.43	2.05 + 0.33	2.06 + 0.49
BCF (Hz)	45	8.10 + 0.14	7.85 + 0.14	7.98 + 0.88	8.09 + 0.11

*P > 0.05. Data presented as mean + standard error. ACP-101c = extender medium based on powdered coconut water; TM = total motility; PM = progressive motility; VCL = curvilinear velocity; VSL = straight line velocity; VAP = average path velocity; LIN = linearity; STR = straightness; WOB = wobble; ALH = amplitude of lateral head; BCF = beat cross frequency.

Sperm membrane and acrosome integrity

The addition of anethole to ACP-101c did not improve sperm membrane integrity at any of the concentrations studied (P > 0.05) (Table 3). The same occurred when the membrane integrity and acrosome parameters were evaluated together (P > 0.05). However, the percentage of spermatozoa that presented intact acrosome in the treatment with the addition of 2000 µg mL^{-1} of anethole to ACP-101c (53.2 ± 3.1%) was higher than the 300 µg mL^{-1} (43.1 ± 3, 7%; P < 0.05), and similar to the control and treatment with the addition of 30 µg mL^{-1} of anethole to ACP-101c (P > 0.05).

Table 3
Mean and error of the mean of spermatic membrane and acrosomal integrity parameters and low levels of reactive oxygen species of different anethole concentrations added to ACP-101c diluent

Parameters	ACP-101c (control)	Anethole		
		30 µg mL $^{-1}$	300 µg mL $^{-1}$	2000 µg mL $^{-1}$
Intact membrane (%)	24.4 ± 1.7	25.1 ± 2.1	25.0 ± 1.7	28.1 ± 1.6
Intact acrosome (%)	48.1 ± 3.0^{ab}	47.0 ± 3.3^{ab}	43.1 ± 3.7^{b}	53.2 ± 3.1^{a}
Intact membrane and acrosome (%)	24.3 ± 2.5	28.7 ± 1.9	24.4 ± 3.7	25.8 ± 3.0
ROS low concentration (%)	30.9 ± 10.2^{bc}	60.4±12.6^{ab}	29.2 ± 8.9^{c}	64.9 ± 11.8^{a}
Intact membrane/ROS low concentration (%)	7.3 ± 2.8^{b}	16.3 ± 3.9^{a}	7.82 ± 3.5^{b}	16.80 ± 3.8^{a}

*Different superscript lowercase letters on the same line (P < 0.05). ACP-101c = extender medium based on powdered coconut water. ROS = reactive oxygen species.

ROS concentration

Table 3 shows the percentage of sperm with or without ROS. When DCHF-DA negative sperm populations were compared, for low or no ROS concentrations, it was found that the 2000 µL treatment (64.9%) was superior ($P < 0.05$) to the control (30.9%) and the 300 µL (29.2%) treatment, but did not differ ($P > 0.05$) from the 30 µL (60.4%) treatment. However, the control did not differ from the 30 µL and 300 µL treatments ($P > 0.05$). Regarding sperm populations with intact membranes and low ROS concentrations, the 2000 µL (16.8%) and 30 µL (16.3%) treatments were superior ($P < 0.05$) to the control (7.30 %) and 300 µL (7.82%) treatments.

The addition of anethole to the cryopreservation diluent did not affect the percentage of mobile and progressive sperm and did not influence sperm motility. Comparatively, the eugenol addition to bovine seminal extended, another short-chain phenolic compound, demonstrated efficiency in sperm motility parameters at concentrations of 10 and 50 µM (Castelo Branco et al., 2020). It is noteworthy that the results obtained in this study regarding anethole were compared to the control (diluent ACP-101c) and showed good kinetic results for goat spermatozoa, as described by Câmara et al. (2019). In addition, the results of the present study show that anethole is beneficial to the maintenance integrity of the acrosome membrane at concentrations of 30 and 2,000 µL. Other results presented demonstrate that anethole (0.1 µM, 1 µM, 10 µM, and 100 µM) did not show deleterious effects on sperm viability or integrity of the human sperm acrosome (Luo et al., 2020).

Regarding morphology, the proportions found in this study show that anethole increased the percentage of sperm abnormalities, especially tail changes. In the qualitative evaluation of morphology, the tail reflex or strongly folded tail were the most common alterations found in samples treated with anethole. The reflex was observed in the transition from the intermediate piece to the flagellum. This phenomenon can be explained by the flagellum structures' disposition, where small damage to the mitochondrial network can lead to an asymmetry in energy conduction causing morphological changes in the region of the middle piece and flagellum (Gu, Zhao, Wang, & Sun, 2019). Another mechanism indicates that anethole, in high concentrations (10 and 100 µM), reduced basal tyrosine phosphorylation and increased progesterone, [Ca2+] ion, and CatSper current, a predominant cationic sperm channel for the influx of Ca2+. This mechanism occurred mainly in the middle piece due to the presence of mitochondria in the region (Luo et al., 2020).

When analyzing the results of oxidative stress, it was observed that concentrations of 30 and 2000 µg/mL of anethole in the seminal extender reduced the rates of ROS production. This can be explained by the inhibition of ROS generation and its consequent degradation into hydroxyl radical (OH−), since anethole has an affinity for the incorporation of hydroxyl groups in the double bond of the molecules' side chain, increasing the antioxidant capacity of the diluent (Freire et al., 2005). The treatment with 300 µg/mL showed inferior results, it is not possible to detail the mechanism of this result with only the tests performed in this experiment, however, this concentration proved to be prejudicial, as

suggested by Majzoub and Agarwal (2018). In these cases, the treatment with 30 µg/mL had an action dose, in which there was a reduction in intracellular OH^- by inducing the intracellular activity of SOD and CAT (Choi & Hwang, 2004).

This result indicate the anethole it seems to be a primary antioxidant, with a higher efficiency action on free radicals than on H_2O_2, which is not a primary oxidative agent (Chainy, Manna, Chaturvedi, & Aggarwal, 2000). In cell incubation with 1 mM anethole, the intracellular efflux of glutathione (GSH) and glutathione-S-transferase (GST) demonstrated an efficient intracellular antioxidant action in reducing the levels of primary radicals such as hydroxyl (Dringen, Hamprecht, & Drukarch, 1998; Drukarch, Schepens, Stoof, & Langeveld, 1997). The same was verified in this study when the DCHF-DA probe was used in the 2000 µg/mL treatment. By reducing the concentration of ROS, anethole demonstrated a potent antioxidant action as pointed out by Oktay, Gülcin and Küfrevioglu (2003), when they used fennel extract (*Foeniculum vulgare*), of which the main component is anethole.

Conclusion

In general, despite reducing ROS levels, the supplementation of anethole in ACP-101c did not affect sperm kinetics and membrane integrity post-thawing, however, it caused morphological damage in sperm. Thus, it is not recommended for buck sperm.

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