Effect of *Rosmarinus officinalis* (L.) Essential Oil Alone or in Association with Vitamin E and Vitamin C on Cryopreserved Bull Sperm

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Abstract. *Bull sperm cryopreservation induces significant cellular damage through reactive oxygen species generation and oxidative stress compromising motility, membrane integrity, and fertilizing capacity. Bull spermatozoa are particularly vulnerable due to limited cytoplasmic antioxidant defenses and high metabolic activity. The aim of this study was to evaluate the protective effects of vitamin C, vitamin E, their combination, and vitamin E combined with Rosmarinus officinalis essential oil on bull sperm quality following cryopreservation. Epididymal spermatozoa from ten bulls were collected and divided into five groups: control (Tris-based extender), vitamin E (0.5 mg/mL), vitamin C (0.25 mg/mL), vitamin E+C association (Vit E-C) (0.5 and 0.25 mg/mL), and vitamin E + essential oil association (Vit E-EO) (0.5 mg/mL + 1 μL/mL). Post-thaw sperm quality was assessed using computer-assisted sperm analysis for motility and kinematic parameters, hypoosmotic swelling test for membrane integrity, and thiobarbituric acid reactive substances assay for lipid peroxidation. All antioxidant treatments enhanced sperm parameters compared with control. Vitamin E + essential oil combination demonstrated the highest total (73.8 ± 5.6% vs. control 64.3 ± 3.5%) and progressive motilities (33.7 ± 7.1% vs. control 24.4 ± 3.2%). This treatment also achieved superior kinematic parameters including curvilinear velocity (47.8 ± 2.4 vs. 34.3 ± 0.7 μm/sec), straight-line velocity (23.7 ± 1.6 vs. 14.7 ± 0.5 μm/sec), and membrane integrity (23 ± 4% vs. 14 ± 2%). Vitamin E + essential oil showed the greatest reduction in lipid peroxidation (0.26 vs. 0.35 nmol MDA/10^8 spermatozoa). Antioxidant supplementation, particularly vitamin E combined with essential oil, significantly enhances bull sperm cryopreservation outcomes through synergistic protective mechanisms against oxidative damage and membrane destabilization.*

Introduction

Bull sperm cryopreservation is a cornerstone technology in cattle reproductive management, enabling the widespread dissemination of superior genetic material and facilitating long-term storage of valuable germplasm (Murphy et al., 2019). However, the freeze-thaw process inflicts significant cellular damage on spermatozoa resulting in reduced motility, compromised membrane integrity, and decreased fertilizing capacity (Sieme et al., 2016). The primary mechanism underlying this cryodamage involves the generation of reactive oxygen species (ROS) and subsequent oxidative stress, which particularly affects the polyunsaturated fatty acid-rich sperm membrane and mitochondrial function (Chatterjee and Gagnon, 2001; O'Connell et al., 2002).

Bull spermatozoa are particularly vulnerable to oxidative damage due to their limited cytoplasmic antioxidant defense systems and high metabolic activity (Aitken et al., 2012). During cryopreservation, the cellular stress response generates excessive ROS that overwhelm the natural antioxidant capacity leading to lipid peroxidation, protein oxidation, and DNA fragmentation (Lamirande and Gagnon, 1995; Bansal and Bilaspuri, 2011). Particularly, the mitochondrial compartment represents one of the most susceptible cellular organelles to cryopreservation-induced oxidative damage (Ball et al., 2001).

Conventional approaches to mitigate cryopreservation damage have focused on optimizing cryoprotectant concentrations and cooling protocols (Peña et al., 2003). However, mounting evidence suggests that supplementation of cryopreservation extenders with antioxidant compounds represents a promising strategy for improving post-thaw sperm quality (Watson, 2000; Guthrie and Welch, 2012). Various synthetic and natural antioxidants, including vitamin E (α-tocopherol), vitamin C (ascorbic acid), glutathione, and plant-derived compounds, have demonstrated protective effects against oxidative stress during sperm cryopreservation (Parks and Lynch, 1992).

Among natural antioxidants, essential oils derived from medicinal plants, have gained considerable attention due to their potent antioxidant properties and biocompatibility (Al-Essawe et al., 2018). *Rosmarinus offcinalis* (rosemary) essential oil contains a rich array

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of bioactive compounds, including rosmarinic acid, carnosic acid, and carnosol, which exhibit strong free radical scavenging activity and metal chelation properties (Shahzad et al., 2017; El-Sheshtawy et al., 2022). Previous studies have demonstrated the protective effects of plant extract on sperm quality in various species, including boar, ram, and rooster spermatozoa (Silva et al., 2012; Bucak et al., 2013). However, to the best of our knowledge, there is no previous report concerning the effect of essential oils and particularly when associated with vitamin E on cryopreserved bull sperm.

Vitamin E, as a lipophilic antioxidant, plays a crucial role in protecting cellular membranes from lipid peroxidation and has been extensively studied in sperm cryopreservation protocols (El-Sheshtawy et al., 2011). The synergistic potential of combining vitamin E with vitamin C has been suggested to provide enhanced antioxidant protection through complementary mechanisms of action (Al-Dean et al., 2024).

The interaction between different antioxidants can be complex, with some combinations showing synergistic effects while others may exhibit antagonistic interactions (Ali et al., 2023). Understanding these interactions is crucial for developing effective antioxidant cocktails that maximize protective benefits while avoiding potential negative effects on sperm function.

Despite the promising results obtained with individual antioxidants, comprehensive studies evaluating the effects of antioxidant combinations, particularly natural compounds like rosemary essential oil with conventional antioxidants, on bull sperm cryopreservation parameters are limited. Furthermore, detailed analysis of sperm motility using computer-assisted sperm analysis (CASA) provides valuable insights into the functional benefits of antioxidant supplementation beyond basic motility assessments (Mortimer, 2000).

Therefore, the objective of the present study was to evaluate the effects of vitamin C, vitamin E, their combination, and the combination of vitamin E with *Rosmarinus officinalis* essential oil on bull sperm quality parameters following cryopreservation. This comprehensive evaluation included assessment of sperm velocity parameters, total and progressive motility, membrane integrity, and oxidative stress status to provide a thorough understanding of the protective mechanisms and practical benefits of these antioxidant treatments in bovine sperm cryopreservation.

Materials and methods

Chemicals

All chemicals used in this study were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) and were of analytical grade.

Epididymal semen collection

Testes from ten bulls were obtained from a local slaughterhouse and immediately transported to the laboratory under refrigerated conditions to preserve sample integrity. Spermatozoa were collected using the retrograde flushing technique, according to the protocol established by Martinez-Pastor et al. (2006). The epididymides were carefully dissected from the testes and subsequently cleaned to remove extraneous tissue. The cauda epididymis along with the vas deferens were meticulously isolated. After ligation of the cauda epididymal blood vessels, the external surface of the cauda was thoroughly rinsed and dried to minimize contamination. Sperm recovery was achieved by applying manual pressure to the vas deferens via a syringe loaded with air and 1 mL of extender, facilitating the retrograde flow of spermatozoa. The spermatozoa were expelled through an incision in the distal region of the cauda epididymis.

Extenders preparation and sperm cryopreservation

In this study, a Tris-based extender composed of 30.8 g/L Tris, 17 g/L citric acid, and 12.5 g/L fructose, supplemented with 20% (v/v) of egg yolk, was employed for semen dilution. Each semen sample collected from the testes was divided into five distinct aliquots to evaluate the effects of various treatments:

1. Control group containing only the Tris-based extender.
2. Group supplemented with vitamin E at 0.5 mg/mL (Khellouf et al., 2025).
3. Group supplemented with vitamin C at 0.25 mg/mL (Khellouf et al., 2025).
4. Aliquot containing the combination of vitamin E and C at 0.5 mg/mL and 0.25 mg/mL, respectively (Khellouf et al., 2025).
5. Aliquot supplemented with the combination of vitamin E and *Rosmarinus officinalis* essential oil at 0.5 mg/mL and 1 µL/mL, respectively (Benberkane et al., 2019).

After 15 minutes of incubation at 22°C, all aliquots were further diluted with Tris extender + 10% glycerol + 20% egg yolk to obtain 100×10^6 spermatozoa/mL. Finally, sperm was equilibrated at 4°C for 2 hours to facilitate glycerol permeation, packaged in 0.25 mL straws, and frozen in liquid nitrogen.

The essential oils were purchased from Aromabiol Company (Algeria). The chemical composition of the extracted rosemary essential oil (REO) was analyzed using Gas Chromatography–Mass Spectrometry (GC-MS). The major bioactive constituents identified were camphor (18.88%), camphene (5.17%), 1,8-cineole (7.85%), β-thujene (13.66%), α-thujene (4.87%), chrysanthenone (12.05%), and β-cubenene (7.97%).

Motility assessment

Sperm motility was quantitatively analyzed using a Computer-Assisted Sperm Analysis (CASA) system (Sperm Class Analyzer, version 3.2.0; Microptic S.L., Barcelona, Spain) to ensure objective evaluation of kinematic parameters. Aliquots of 10 µL from frozen-thawed samples were carefully introduced into Makler Counting Chamber, featuring a chamber depth of 10 µm (Sefi-Medical Instruments Ltd., Biosigma S.r.l., Italy). To reestablish physiological conditions, the samples were equilibrated by incubating for 30 seconds at 37°C prior to analysis. Subsequently, spermatozoa movement was recorded under phase-contrast microscopy at 10× magnification. The system computed key motility indices including curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), amplitude of lateral movement of the head (ALH), beat cross frequency (BCF), and linearity percentage [LIN = (VSL / VCL) × 100] (Mortimer, 2000).

Hypoosmotic Swelling Test (HOST)

The functional integrity of the sperm plasma membrane was assessed using the hypoosmotic swelling test (HOST) according to the protocol established by Revell and Mrode (1994). In brief, 30 µL aliquots of each semen sample were incubated with 300 µL of hypoosmotic solution, prepared by dissolving 9 g of fructose and 4.9 g of sodium citrate in one liter of distilled water. The mixture was incubated for 60 minutes at room temperature to allow osmotic challenge. Following incubation, samples were examined under a phase-contrast microscope at 20× magnification. For each sample, at least 200 spermatozoa were evaluated. The spermatozoa exhibiting curled or swollen tails were considered indicative of intact and functionally competent membranes.

Thiobarbituric Acid Reactive Substances (TBARS) Assay

Lipid peroxidation was assessed through quantification of malondialdehyde (MDA), a terminal product of oxidative damage, serving as a reliable biomarker for oxidative stress. Following thawing, sperm samples (100×10^6 cells/mL) were centrifuged at 1500×g for 15 minutes, and the supernatant was discarded. The resulting sperm pellets underwent three sequential wash cycles by centrifugation at 1500×g for 10 minutes and were resuspended in 1 mL of distilled water after each wash. Following the final centrifugation, pellets were resuspended in 500 µL of distilled water and subjected to sonication in six 15-second pulses with 30-second intervals between each pulse.

MDA content levels were measured in the sonicated sperm samples through spectrophotometric analysis. Briefly, 0.5 mL of each sonicated sperm preparation was added to 1 mL of TBA-TCA-HCl reagent solution containing 15% (w/v) trichloroacetic acid, 0.375% (w/v) thiobarbituric acid, and 0.25 N hydrochloric acid. The mixture was heated at 95°C for 60 minutes to facilitate the MDA-TBA chromogenic reaction and then rapidly cooled in an ice bath. Samples were centrifuged at 18000×g for 15 minutes, and the absorbance of the supernatant was measured spectrophotometrically at 535 nm. Results were calculated and expressed as nmoles MDA per 10^8 spermatozoa using the molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} (Buege & Aust, 1978).

Statistical analysis

The entire experimental protocol was conducted in ten independent replicates to ensure reproducibility and reliability of the data. Results are presented as mean values accompanied by their standard error of the mean (mean ± SEM). Statistical analysis was performed using Statview software version 4.02 (Abacus Concepts Inc., Berkeley, CA, USA). After the normality of the data distribution was assessed, comparisons among treatment groups regarding sperm motility parameters, membrane integrity, and lipid peroxidation levels were carried out using one-way analysis of variance (ANOVA). Where significant differences were detected, post hoc pairwise comparisons were conducted using Fisher's protected least significant difference (LSD) test. Statistical significance was established at a threshold of $P < 0.05$.

Results

Total (TM) and Progressive (PM) Motility

Total motility measurements showed variable responses to the different treatments (Fig. 1). The control group exhibited TM values of 64.3 ± 3.5%. Vitamin C supplementation resulted in the highest improvement, reaching 75.8 ± 8.6% of total motility with a significant difference when compared with the control ($P < 0.04$). Individual vitamin E treatment showed values similar to the control at 66.1 ± 4.3%, while the combined vitamin treatment (Vit E-C) demonstrated a slight decrease to 64.9 ± 1.4%. The vitamin E and essential oil combination (Vit E-EO) achieved 73.8 ± 5.6% total motility.

Progressive motility displayed a different pattern compared with total motility (Fig. 1). The control group showed PM values of 24.4 ± 3.2%. Individual vitamin treatments produced moderate improvements: vitamin C achieved 32.5% ± 3.4 and vitamin E reached 29.3 ± 1.9%. The combined vitamin treatment (Vit E-C) showed values similar to the control at 27.4 ± 6.9%. However, the vitamin E and essential oil combination (Vit E-EO) demonstrated the highest progressive motility at 33.7 ± 7.1%, values statistically different compared with all the investigated treatments ($P < 0.05$).

Kinematic parameters assessment

The addition of antioxidants to cryopreservation

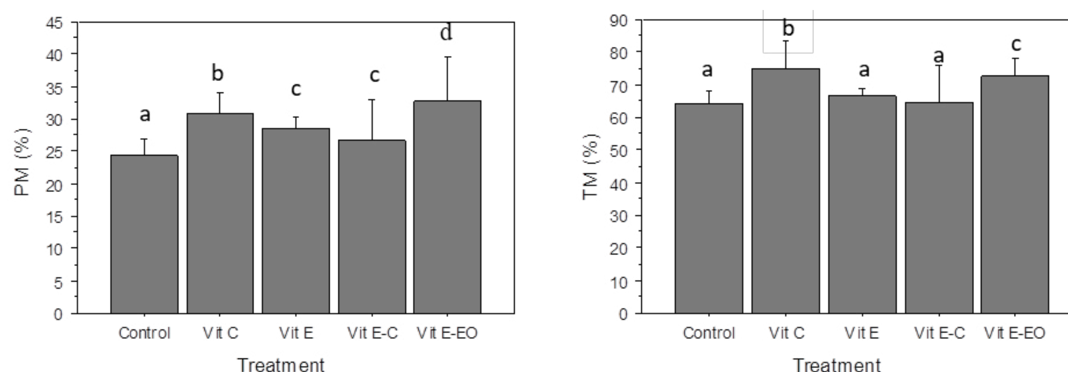

Fig. 1. Mean percentages (± S.E.M.) of total motility (TM) and progressive motility (PM) in cryopreserved bull spermatozoa across experimental groups. Semen samples were either left untreated (control) or pre-treated prior to freezing with vitamin E, vitamin C, an association of vitamin E and C, and aliquots supplemented with the association of vitamin E and Rosmarinus essential oils.

extenders significantly improved sperm motility parameters compared with the control group. Three key velocity measurements were analyzed: curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP).

VCL showed a progressive improvement across treatments (Fig. 2). The control group exhibited the lowest VCL values at approximately 34.271 ± 0.662 µm/sec. Individual vitamin supplementation resulted in modest improvements, with vitamin C achieving 37.131 ± 0.602 µm/sec and vitamin E reaching 42.066 ± 0.776 µm/sec. The combination of vitamins C and E (Vit E-C) further increased VCL to 40.961 ± 0.866 µm/sec. The most substantial improvement was observed in the vitamin E and essential oil combination (Vit E-EO), which achieved the highest VCL value with 47.835 ± 2.412 µm/sec, values significantly higher ($P < 0.03$) than the control and the other investigated treatments.

VSL measurements demonstrated a similar pattern of improvement (Fig. 2). Control samples showed VSL values of 14.654 ± 0.459 µm/sec. Vitamin C supplementation increased VSL to 16.730 ± 0.448 µm/sec, while vitamin E achieved 18.164 ± 0.520 µm/sec. The vitamin combination (Vit E-C) resulted in VSL values of 19.163 ± 0.610 µm/sec. Again, the vitamin E and essential oil combination (Vit E-EO) produced the highest VSL values (23.748 ± 1.596 µm/sec) with a significant difference, particularly when compared with Vit E-C treatment ($P < 0.002$).

VAP measurements followed the same trend as the other velocity parameters (Fig. 2). The control group recorded VAP values of 21.436 ± 0.501 µm/sec. Individual vitamin treatments showed incremental improvements: vitamin C (23.622 ± 0.481 µm/sec) and vitamin E (25.430 ± 0.561 µm/sec). The combined vitamin treatment (Vit E-C) achieved 26.072 ± 0.652 µm/sec. The vitamin E and essential oil combination (Vit E-EO) demonstrated the highest VAP values with 30.606 ± 1.689 µm/sec, indicating a significant difference ($P < 0.001$) compared with the control.

For the sperm movement pattern parameters (ALH, BCF, and LIN, Fig. 2), treatment effectiveness varied depending on the specific measured parameter. ALH and BCF showed similar progressive improvements with the hierarchy: Control < Vit C ≈ Vit E < Vit E-C < Vit E-EO. However, linearity (LIN) displayed a different pattern, where individual vitamin treatments (Vit C and Vit E) showed minimal improvements over the control, but the combined treatments (Vit E-C and Vit E-EO) demonstrated substantial enhancements. The vitamin E and essential oil combination consistently produced the most significant improvements across all three parameters ($P < 0.05$).

Hypoosmotic Swelling Test (HOST)

The hypoosmotic swelling test revealed significant variations in sperm membrane integrity across treatments (Fig. 3). The control group showed HOST values of 14 ± 2%. Individual vitamin supplementation produced contrasting effects: vitamin C treatment increased HOST to 18 ± 2%, while vitamin E showed a slight improvement to 16 ± 1%. Interestingly, the combined vitamin treatment (Vit E-C) demonstrated the lowest HOST values at approximately 13 ± 1%. However, the vitamin E and essential oil combination (Vit E-EO) achieved the highest HOST values with 23 ± 4%, which were statically different compared with the control ($P < 0.001$).

Lipid Peroxidation Assessment (TBARS Assay)

The extent of lipid peroxidation in sperm samples was evaluated through malondialdehyde (MDA) quantification using the TBARS assay. As shown in Fig. 4, all antioxidant treatments demonstrated protective effects against lipid peroxidation compared with the control group.

The control group exhibited the highest level of lipid peroxidation with MDA concentrations reaching 0.35 nmol/10^8 spermatozoa, indicating substantial oxidative damage to sperm membrane lipids. Treatment

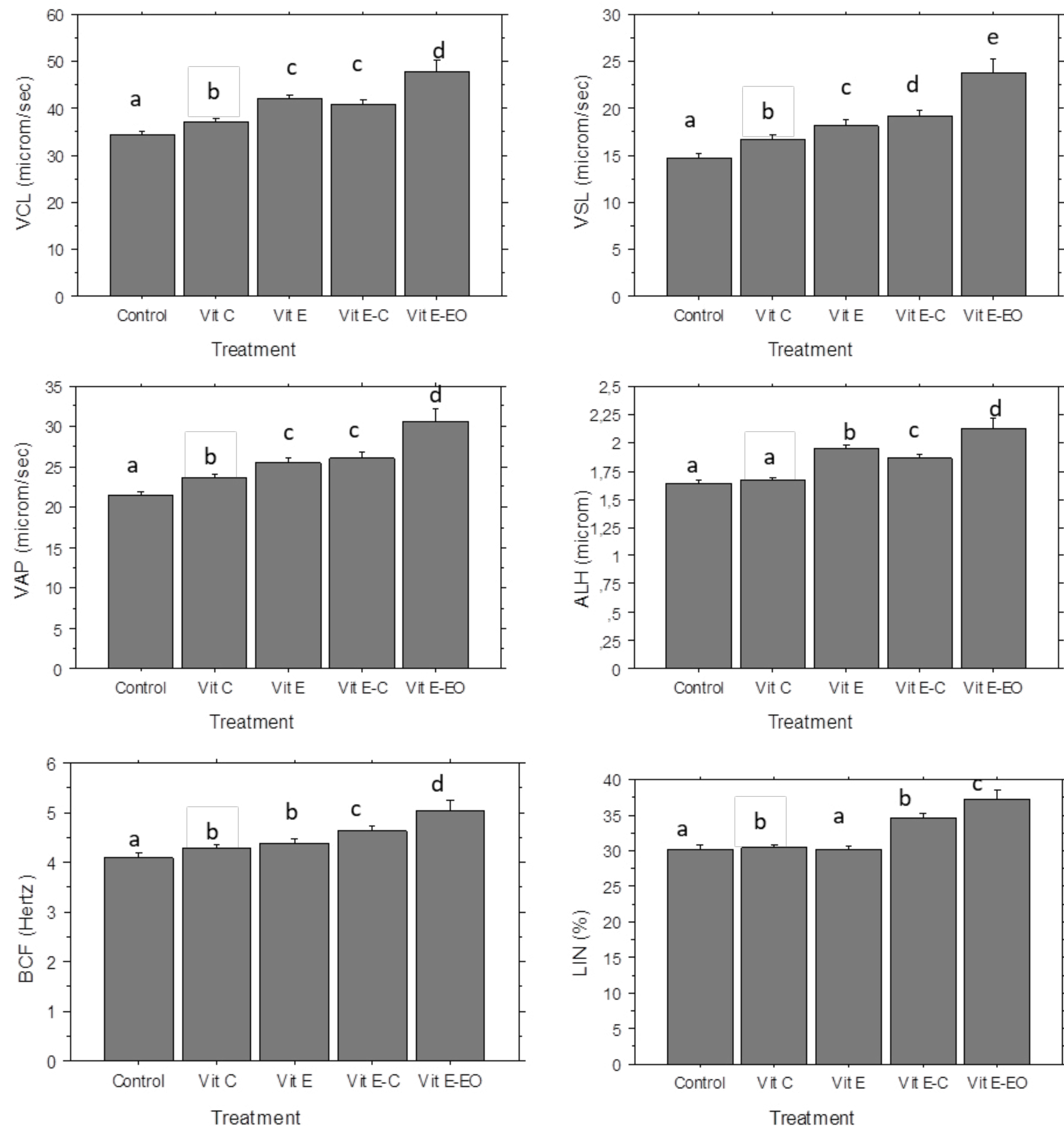

Fig. 2. Post-thaw sperm kinematic parameters in cryopreserved bull spermatozoa (mean ± S.E.M) from the control group and groups pre-treated prior to freezing with vitamin E, vitamin C, an association of vitamin E and C, and aliquots supplemented with the association of vitamin E and Rosmarinus essential oils. Parameters include curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), amplitude of lateral head displacement (ALH), and beat cross frequency (BCF). Different lowercase letters indicate statistically significant differences among groups ($P < 0.05$).

with vitamin C resulted in a notable reduction in MDA levels to 0.32 nmol/10^8 spermatozoa. Vitamin E supplementation showed enhanced protective effects, reducing MDA concentrations to 0.31 nmol/10^8 spermatozoa. The combination of vitamins E and C (Vit E-C) demonstrated superior antioxidant efficacy, achieving MDA levels of 0.27 nmol/10^8 spermatozoa.

The most pronounced protective effect was observed with the vitamin E-essential oil combination (Vit E-EO), which reduced MDA concentrations to 0.26 nmol/108 spermatozoa, representing the greatest decrease in lipid peroxidation among all treatments tested ($P < 0.05$).

Discussion

Cryopreservation remains an indispensable technique for the long-term storage of bull semen, facilitating genetic dissemination and conservation programs (Murphy et al., 2019). However, the process is known to induce substantial cellular stress, primarily through the generation of reactive oxygen species (ROS) and osmotic shock resulting to membrane destabilization (Bucak et al., 2013). These factors collectively compromise sperm motility, membrane integrity, and fertilizing capacity (Guthrie and Welch, 2012). Hence, developing extender formulations that mitigate cryodamage is a continuous objective

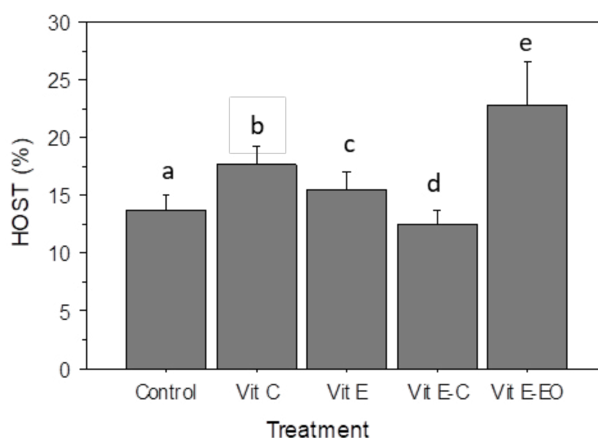

Fig. 3. Mean percentages (± S.E.M.) of hypoosmotic swelling test in cryopreserved bull spermatozoa across experimental groups. Semen samples were either left untreated (control) or pre-treated prior to freezing with vitamin E, vitamin C, an association of vitamin E and C, and aliquots supplemented with the association of vitamin E and Rosmarinus essential oils.

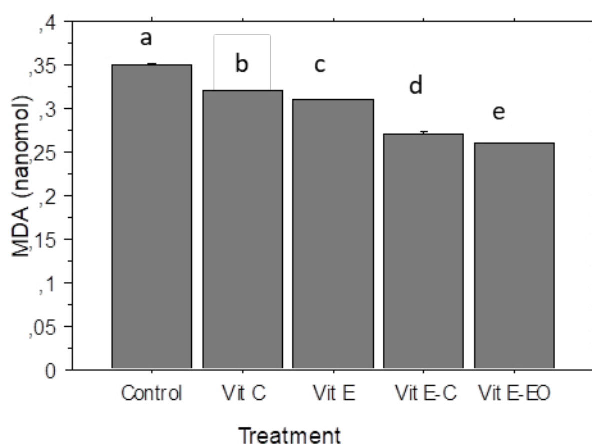

Fig. 4. Mean percentages (± S.E.M.) of MDA rate (nmol) in cryopreserved bull spermatozoa across experimental groups. Semen samples were either left untreated (control) or pre-treated prior to freezing with vitamin E, vitamin C, an association of vitamin E and C, and aliquots supplemented with the association of vitamin E and Rosmarinus essential oils.

in reproductive biotechnology (Bansal and Bilaspuri, 2011).

In this study, we evaluated the protective effects of vitamin C, vitamin E, their combination, and a novel formulation combining vitamin E with essential oils (EOs) on post-thaw sperm motility and kinematic parameters. Our findings provide compelling evidence that all antioxidant-enriched extenders conferred a degree of protection, with the most significant improvements in the group treated with vitamin E + EOs.

Vitamin C (ascorbic acid) is a potent hydrophilic antioxidant that neutralizes aqueous-phase ROS, particularly superoxide anions and hydroxyl radicals (Padayatty et al., 2003). It also has the ability to regenerate oxidized vitamin E, thereby supporting lipid-phase antioxidant defense (Padilla and Foote, 1991). However, in our results, vitamin C alone improved moderately post-thaw sperm motility and did not significantly enhance velocity parameters. This suggests that while vitamin C offers intracellular ROS protection, it may be insufficient to fully preserve membrane integrity during freezing (Silva and Gadella, 2006).

Vitamin E (alpha-tocopherol), in contrast, is a lipophilic antioxidant incorporated into the sperm plasma membrane, where it plays a direct role in inhibiting lipid peroxidation (Agarwal and Saleh, 2002). Its inclusion led to a more pronounced improvement in both progressive motility and velocity, underscoring the importance of membrane-targeted protection. This is consistent with prior studies that emphasized the centrality of membrane lipid peroxidation as a primary target of cryo-induced damage (Beconi et al., 1993).

The combined use of vitamin C and vitamin E demonstrated enhanced efficacy compared with each antioxidant alone, particularly for progressive motility and VSL (Yousef et al., 2003). This synergistic effect likely arises from the complementary actions of the two vitamins across different cellular compartments (Burton and Ingold, 1989). Vitamin C regenerates

vitamin E radicals and scavenges ROS in the cytosol, while vitamin E protects lipid domains, resulting in comprehensive antioxidant coverage.

Notably, the most remarkable improvement in both motility and velocity parameters was observed in the group supplemented with vitamin E + essential oils. This formulation outperformed even the vitamin C + vitamin E group, indicating the added value of EOs in semen cryopreservation (Askari et al., 2013). Essential oils, depending on their botanical source, are complex mixtures rich in monoterpenes, sesquiterpenes, and phenolic compounds such as thymol, carvacrol, and eugenol (Uysal and Bucak, 2007). These compounds exhibit strong antioxidant, antimicrobial, and membrane-modulating properties.

The superior performance of the EO-supplemented group may be attributed to several mechanisms. First, EOs can enhance membrane fluidity and stability, thereby reducing structural damage during freezing and thawing (Bucak et al., 2007). Second, their antioxidant constituents can directly scavenge ROS or upregulate endogenous antioxidant enzymes such as glutathione peroxidase and superoxide dismutase. Third, EOs may exert mild antimicrobial effects, reducing endotoxin-induced stress in semen samples.

The significant elevation in VSL and VAP in the vitamin E + EO group is particularly relevant, as these parameters are closely associated with the sperm's ability to reach and penetrate the oocyte (Sariözkan et al., 2009). Improved linear and average velocities suggest enhanced mitochondrial function and energy production, possibly supported by EO-induced stimulation of metabolic activity.

However, the use of essential oils requires careful consideration of concentration, as some components can be cytotoxic at high doses. In this study, the concentration was optimized to ensure efficacy without compromising cell viability. Future research should further characterize the specific EO components responsible for the observed benefits and explore their effects on fertility outcomes post-insemination.

In conclusion, this study demonstrates that antioxidant supplementation, particularly the combination of vitamin E with essential oils, enhanced significantly the resilience of bull sperm to cryodamage (Murphy et al., 2019). The findings support the development of natural, multifunctional extender additives that improve semen quality while reducing dependence on synthetic cryoprotectants. This approach aligns with current trends favoring sustainable and biocompatible solutions in animal reproduction technologies.

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