Research Article

Influence of *Mentha spicata* Essential Oil on the Quality of Chilled Canine Spermatozoa

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Abstract | Oxidative stress is one of the main factors causing sperm quality deterioration during preservation in canine sperm. This study was conducted to evaluate the protective effect of *Mentha spicata* essential oil when added to sperm dilutions, with the aim of enhancing the preservation and maintenance of canine sperm functional quality under chilling conditions. Several important parameters including progressive motility, viability, plasma membrane integrity and lipid peroxidation levels were analyzed to comprehensively evaluate the quality and oxidative stability of sperm. The experimental results showed that the effect of *Mentha spicata* essential oil was clearly concentration-dependent. At concentrations below 15 µg/mL, the essential oil showed positive effects, helping to reduce oxidative damage and improve overall sperm function during the chilling stages. In contrast, concentrations exceeding 20 µg/mL were associated with reduced sperm motility and viability, suggesting that cytotoxicity and redox imbalance predominated over antioxidant protection at higher doses. Notably, the supplementation of *Mentha spicata* essential oil at a concentration of 15 µg/mL was determined to be optimal, significantly reducing lipid peroxidation and maintaining sperm motility, membrane integrity and viability throughout the storage period. In conclusion, *Mentha spicata* essential oil is a promising natural antioxidant in canine semen preservation. The supplementation of essential oil at an optimal concentration of 15 µg/mL effectively protected sperm from oxidative stress, improved sperm quality after storage, and provided a potential natural solution to enhance the efficiency of sperm chilling procedures in canine reproductive technology.

Keywords | Canine sperm, *Mentha spicata,* Essential oil, Antioxidant, Chilled, Sperm quality

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INTRODUCTION

During sperm preservation, canine sperm are susceptible to oxidative stress, which can lead to biochemical changes and functional impairment. In the natural seminal environment, sperm are protected by antioxidant enzymes present in seminal plasma, such as superoxide dismutase, glutathione peroxidase, phospholipid hydroperoxide glutathione peroxidase (PHGPx), and catalase (Neagu *et al.*, 2011). These enzymes play an important role in neutralizing reactive oxygen species (ROS) and maintaining redox balance (Ighodaro and Akinloye, 2017). However, during the processing of semen for chilling or cryopreservation, seminal plasma is often removed to avoid cryogenic injury and prevent contamination (Hori *et al.*, 2017). This removal of seminal plasma reduces the natural antioxidant defenses, making sperm more vulnerable to oxidative stress during preservation.

To overcome this effect, researchers have added antioxidants

to sperm extenders to improve sperm preservation. Antioxidant supplementation can eliminate free radicals, reduce oxidative stress, and improve sperm quality. Many synthetic and natural antioxidants have been tested for this purpose. However, antioxidant effectiveness is variable and often inconsistent, depending largely on their chemical properties, concentration, and interactions with sperm physiology (Michael *et al*., 2009; Monteiro *et al*., 2009; Thiangtum *et al*., 2012; Lucio *et al*., 2016; Andersen *et al*., 2018).

Recently, there has been increasing scientific interest in the use of natural antioxidants derived from plants; these are considered safer, more biocompatible, and environmentally friendly. Several studies have demonstrated the positive effects of plant-derived bioactive compounds in improving sperm quality during storage, including improved motility, membrane integrity, and fertilization ability (Calabria *et al*., 2023; Vui *et al*., 2024; Partyka *et al*., 2024; Vui and Tan, 2025; Vui *et al*., 2025).

Mentha spicata is a fragrant herb widely used worldwide in the food, cosmetic, and pharmaceutical industries. In addition to its culinary uses, *Mentha spicata* is also valued for its essential oil, which contains a variety of bioactive plant compounds such as carvone, limonene, β-bourbonene, cis-dihydrocarveol, trans-caryophyllene, 1,8-cineole, and terpinen-4-ol all of which are known for their potent antioxidant and antimicrobial properties (Shahbazi *et al*., 2015; Mahboubi, 2021). These compounds can effectively neutralize free radicals, prevent lipid peroxidation, and protect cell membranes from oxidative damage.

Based on these properties, *Mentha spicata* essential oil is considered a potential candidate for addition to semen dilutions to improve the preservation of canine sperm. With the increasing demand for dog breeding and the popularity of sperm conservation techniques, the identification of optimal extenders and effective antioxidant supplements has become extremely important to maintain sperm fertility. Therefore, the aim of this study was to evaluate the protective potential of *Mentha spicata* essential oil when added to sperm dilutions during preservation. Through comprehensive analysis of sperm quality factors such as motility, viability, plasma membrane integrity and lipid peroxidation levels, the study aimed to elucidate the protective mechanism and antioxidant effects of *Mentha spicata* essential oil, and to determine the optimal concentration to maintain sperm quality during preservation.

MATERIALS AND METHODS

Essential oils extraction

Fresh *Mentha spicata* plants were collected in October 2024 at an herbal garden in Vinh Long province, Vietnam. The plants were grown under natural conditions without the use of chemical fertilizers, pesticides or growth stimulants, to ensure the purity of the plant material. The botanical identification and designation were carried out by taxonomists from the Department of Plant Science, Tra Vinh University, Vietnam.

After harvesting, the leaves were washed with distilled water to remove dirt and impurities on the surface, then left to dry naturally at room temperature for 24 hours. Next, the leaves were cut into pieces of approximately 3 cm in length and dried in an oven at 40°C until reaching a constant mass, to prevent evaporation or decomposition of volatile compounds.

To extract the essential oil, 150 g of dried leaves were placed in a 1000 mL round-bottom flask containing 500 mL of distilled water, which was then connected to a Clevenger-type steam distillation apparatus. The distillation was carried out at 100°C for 5 h, during which the steam carrying the volatile compounds was condensed and collected. The essential oil layer was separated from the aqueous phase, followed by drying with anhydrous sodium sulfate to completely remove the remaining water. The purified essential oil was transferred to airtight amber glass vials to prevent photo-oxidation and stored at 4°C until chemical analysis.

The qualitative and quantitative composition of *Mentha spicata* essential oil was analyzed by gas chromatography-mass spectrometry (GC–MS). This analysis was conducted to identify and characterize the bioactive compounds based on their retention times and mass spectra, compared with reference standards and spectral libraries (NIST and Wiley) to ensure accuracy in identifying the chemical constituents of the essential oils.

Evaluation of the antioxidant properties of essential oils

The antioxidant potential of *Mentha spicata* essential oil was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method described by Blois (1958), with some minor modifications to optimize it for microplate analysis. The principle of this method is based on the reduction of the deep purple DPPH radical to a pale-yellow diphenyl-picrylhydrazine compound upon contact with hydrogen-donating antioxidants. The stock DPPH solution was freshly prepared in methanol and stored in the dark at room temperature until use to avoid photodegradation. *Mentha spicata* essential oil was dissolved in methanol to obtain different concentrations, while vitamin E (α-tocopherol) was used as an antioxidant reference standard and prepared under the same conditions.

During the experiment, 100 µL of essential oil solution or standard solution was added to each well of the 96-well plate, followed by 100 µL of DPPH solution. The mixture was shaken gently to mix well, then incubated in the dark at room temperature for 30 minutes to limit photodegradation of free radicals. After incubation, the decrease in optical absorption was measured at 517 nm using a spectrophotometer (Thermo Fisher Scientific, USA). Methanol was used as the blank, while DPPH solution without antioxidants was used as the negative control. The IC_{50} was determined using linear regression of percentage inhibition against log concentration.

Animals

Five clinically healthy, sexually mature male Rottweiler dogs, aged 2–5 years and weighing 35–45 kg, were used in this study. All dogs were housed under uniform management conditions at a kennel in Vinh Long province, Vietnam, and confirmed to be reproductively sound through clinical and semen evaluations. They were fed a commercial dry diet twice daily and provided with free access to clean drinking water. The dogs were well-trained for semen collection using manual stimulation and samples were obtained once weekly. All experimental procedures were conducted in accordance with institutional ethical guidelines and approved by the Institutional Animal Care and Use Committee of Tra Vinh University, Vietnam.

Semen collection and initial evaluation of canine semen quality

Semen samples were collected once a week from each dog using digital manipulation as described by Linde-Forsberg (1991). The collection was performed in a quiet environment to minimize stress and allow the dog to ejaculate completely. After collection, the semen sample was divided into three parts: pre-sperm, sperm-rich and prostatic. Only the sperm-rich semen was used for further analysis.

The semen was collected in sterile graduated tubes, pre-warmed at 37°C, and then transferred to the laboratory within 10 minutes of collection to ensure sperm motility and survival. Upon arrival at the laboratory, each semen sample underwent an initial macroscopic and microscopic evaluation. Macroscopic parameters including volume, color and pH were carefully recorded.

Sperm motility was assessed under a light microscope at 400× magnification using glass slides and coverslips warmed at 37°C. Results were expressed as the percentage of progressively motile spermatozoa, averaged from at least five random fields. Sperm concentration was determined using a Neubauer counting chamber after dilution with sodium bicarbonate–formalin solution, and results were expressed as $\times 10^6$ spermatozoa/mL.

Evaluation of sperm morphology and viability was performed by Eosin–Nigrosin staining according to the instructions of Tamuli and Watson (1994). Only semen samples that met strict quality standards were selected for testing, including: progressive motility ≥ 70%, viability ≥ 90%, sperm concentration ≥ 200 × 10^6 sperm/mL, and abnormal sperm ≤ 5%. Samples that did not meet the above criteria were excluded from the study to ensure the uniformity, reliability, and accuracy of the experimental results.

Semen extender formulation and experimental framework

The semen dilution solution was prepared based on Tris–citric acid–fructose medium supplemented with 20% chicken egg yolk as the base. The detailed composition of the dilution medium is presented in Table 1, and all chemicals used were of analytical grade provided by Sigma-Aldrich (USA). To evaluate the effect of *Mentha spicata* essential oil, dilution medium samples were prepared with essential oil concentrations of 0, 5, 10, 15, 20 and 25 µg/mL, respectively. The essential oil was pre-dissolved in dimethyl sulfoxide (DMSO) solvent, and then the final DMSO concentration was adjusted to 0.8% in all samples to ensure uniformity between treatments. The entire solution preparation process was carried out under sterile conditions, using sterile distilled water.

Table 1: Composition of chilled canine sperm extenders.

Components	T0	T1	T2	T3	T4	T5
Tris (mg)	3025	3025	3025	3025	3025	3025
Citric acid (mg)	1700	1700	1700	1700	1700	1700
Fructose (mg)	1250	1250	1250	1250	1250	1250
Egg yolk (mL)	20	20	20	20	20	20
Essential oil (µg)	0	500	1000	1500	2000	2500
Streptomycin (mg)	100	100	100	100	100	100
Penicillin (mg)	60	60	60	60	60	60
DMSO (mL)	0.8	0.8	0.8	0.8	0.8	0.8
Distilled water (mL)	Up to 100	Up to 100	Up to 100	Up to 100	Up to 100	Up to 100

T0: Control; T1, T2, T3, T4, and T5: 5, 10, 15, 20, and 25 µg/mL of *Mentha spicata* essential oil, respectively. All components are expressed as amounts added per 100 mL of final extender volume, and DMSO (dimethyl sulfoxide) was maintained at a constant level across all treatments.

Semen from five healthy dogs was pooled, mixed, and then divided into six sterile test tubes. Seminal plasma was removed by centrifugation at 720×g for 5 min according to the instructions of Rijsselaere *et al.* (2002). After centrifugation, the sperm pellets were resuspended in the

respective dilutions, to achieve a final concentration of 100×10^6 sperm/mL.

The diluted semen samples were gradually cooled at a rate of 0.3°C per minute, according to the manual cooling method of Bouchard *et al.* (1990), until reaching a final temperature of 5°C. The samples were then stored at 5°C for 12 days. During the storage period, semen quality parameters were periodically assessed to monitor the effect of *Mentha spicata* essential oil on the ability to preserve semen in cold conditions.

The experiment was arranged in a completely randomized design with six treatments (five essential oil concentrations and one control), each treatment repeated four times. Sperm quality parameters were evaluated repeatedly during 12 days of storage to determine the effectiveness of *Mentha spicata* essential oil in maintaining preserved sperm quality. The experimental process is depicted in a summarized form in Figure 1.

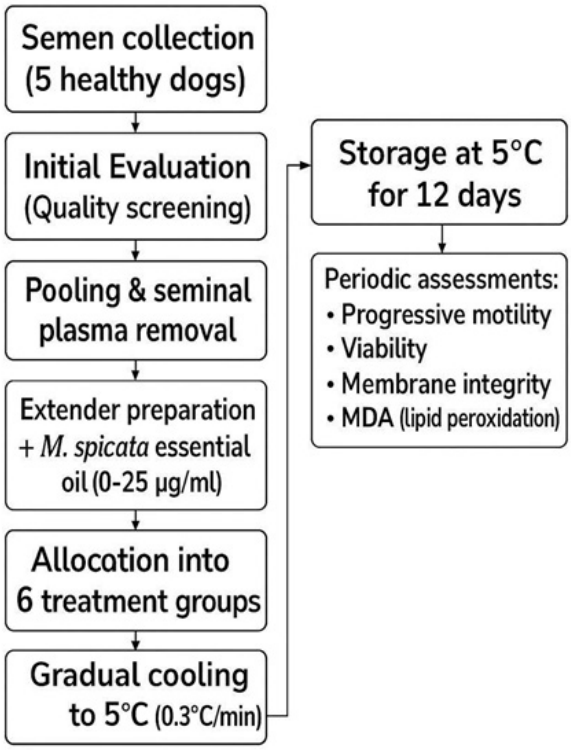

Figure 1: Overview of the experimental workflow.

Sperm motility evaluation

The progressive motility of spermatozoa was assessed using a phase contrast microscope at 400× magnification under strictly controlled laboratory conditions. Prior to the assessment, each semen sample was gently mixed to ensure homogeneity, then equilibrated in a water bath at 38°C for 15 minutes, corresponding to the physiological temperature of the male canine reproductive tract. This warming step helps restore sperm activity and prevents chilling-induced reduction in motility.

A small volume of approximately 10 µL of semen was placed on a pre-warmed glass slide, then covered with a coverslip to create a thin, uniform layer for easy observation. For each experimental group, five random fields were selected for observation, with an average of 200 spermatozoa per field (Shah *et al.*, 2011).

Sperm viability evaluation

Sperm viability was determined by the Eosin–Nigrosin staining technique as described by Tamuli and Watson (1994). This method allows the differentiation of live and dead spermatozoa based on the integrity of the plasma membrane and is widely recognized for its simplicity, high accuracy and the ability to visually observe the degree of membrane damage associated with sperm mortality. Specifically, a small volume (10 µL) of semen was mixed with an equal volume (10 µL) of Eosin–Nigrosin staining solution on a clean glass slide. The mixture was gently mixed to homogenize, then spread thinly on another glass slide tilted at about 45° to create a uniform thin smear. The stained slides were naturally dried at room temperature for a few minutes before observation. Microscopic evaluation was performed in bright-field mode with a magnification of 400×. Sperm with damaged plasma membrane will absorb Eosin dye, causing the sperm head to turn pink or red, and are identified as dead sperm. On the contrary, sperm with intact plasma membrane will not absorb the dye, keeping their natural ivory or light blue color, and are considered live sperm.

Sperm plasma membrane integrity evaluation

The functional integrity of the sperm plasma membrane was assessed by the Hypo-Osmotic Swelling Test (HOST), a reliable method for determining the osmotic responsiveness of the sperm membrane. The principle of this test is based on the fact that spermatozoa with intact plasma membranes can regulate the osmotic movement of water in a hypotonic environment, resulting in the characteristic swelling or coiling of the tail, while spermatozoa with damaged membranes lose this ability and do not change their shape.

During the experiment, 10 µL of semen sample was gently mixed with 100 µL of hypotonic solution (150 mOsm/L), which was freshly prepared by dissolving sodium citrate and fructose in appropriate proportions in distilled water. The mixture was gently mixed to ensure a homogeneous distribution of spermatozoa in the solution. The samples were then incubated in a water bath at 38°C for 30 minutes, corresponding to the physiological temperature of the male canine reproductive tract. This incubation period allowed for osmotic equilibrium to be achieved, allowing spermatozoa with normal membrane activity to exhibit a swelling response. After incubation, 0.2 µL of the mixture was placed on a clean, pre-warmed glass slide, covered

with a coverslip, and immediately observed under a phase contrast microscope with a 40× objective. A total of 200 spermatozoa were evaluated for each sample, observed at various fields of view. Spermatozoa with coiled or swollen tails were recorded as having intact plasma membranes, while spermatozoa with straight, non-swollen tails were considered to have damaged or non-functional plasma membranes (Goericke-Pesch *et al*., 2012).

Sperm lipid oxidative damage evaluation

The level of lipid peroxidation in sperm was determined by measuring malondialdehyde (MDA) content by the thiobarbituric acid reactive substances (TBARS) assay, following the procedure of Maia *et al*. (2010), with some minor adjustments to optimize the analytical conditions. The principle of this method is based on the reaction between MDA the main secondary product of lipid peroxidation and thiobarbituric acid (TBA) in an acidic environment and at high temperature, forming a pink complex that can be measured by absorption spectroscopy. Specifically, the sperm sample was first treated with ferrous sulfate to induce oxidative stress and promote lipid peroxidation of the sperm plasma membrane. After the oxidation reaction, the reaction was stopped, and the sample was added with TBA reagent, then heated at 95–100 °C for 15 min to form the MDA–TBA complex. Next, the reaction mixture was cooled to room temperature and centrifuged to remove the residue and precipitated protein, obtaining a clear supernatant for photometric measurements. The optical absorbance of the supernatant was measured at 535 nm using a 96-well plate spectrophotometer, in which a blank and a standard sample were included in each measurement to ensure accuracy.

The MDA concentration in each sample was calculated based on a standard curve established with 1,1,3,3-tetramethoxypropane, a precursor of MDA. The results were expressed as nmol MDA per 50×10^6 spermatozoa, to standardize the oxidation activity between treatments.

Statistical analysis

All statistical analyses were conducted using IBM SPSS Statistics software, version 22.0. Data were expressed as mean ± standard deviation. To evaluate the effects of experimental factors and their interaction on sperm quality parameters, a two-way mixed analysis of variance (ANOVA) was employed, with storage period and type of extender (essential oil concentration) designated as the main factors. This approach allowed assessment of both the independent (main) effects of each factor and the combined (interaction) effects between them over the storage duration. When significant differences were detected by ANOVA, Tukey's post hoc multiple comparison test was applied to identify specific differences among group means within each factor. A P-value less than 0.05 was considered statistically significant. All analyses were performed under the assumption of normal data distribution and homogeneity of variances, which were verified prior to conducting the ANOVA.

RESULTS

Extraction and analysis of the chemical composition of the essential oil

The essential oil of *Mentha spicata* was obtained by steam distillation, yielding 1.125 mL of essential oil from 150 g of dried leaf powder, corresponding to an extraction yield of 0.75%. This yield reflects the efficiency of the distillation process as well as the natural volatile oil content of the plant material. The chemical composition of the *Mentha spicata* essential oil is summarized in Table 2. Using gas chromatography–mass spectrometry (GC–MS) analysis, a total of 48 distinct compounds were identified, representing 98.88% of the total chemical constituents detected in the essential oil. These results demonstrate the high purity and complexity of the volatile profile of *Mentha spicata*. Among the identified constituents, several compounds were predominant. The major component was perillaldehyde (44.05%), which accounted for nearly half of the total composition and is known for its strong biological properties. Other important compounds include piperitenone oxide (12.53%), cis-cinerolone (5.14%), β-trans-caryophyllene (4.77%), and estragole (4.44%) – all of which contribute to the oil's potential antioxidant activity. In addition, minor but notable compounds include caryophyllene oxide (3.21%), carvone (3.11%), eugenol (2.80%), p-cymene-8-ol (2.14%), α-(Z,E)-farnesene (1.92%), and β-linalool (1.63%). Overall, these compounds form a complex mixture, which contributes to the diverse biological properties of *Mentha spicata* essential oil, which may play an important role in the potential antioxidant activity evaluated in this study.

Antioxidant activity of essential oils

The antioxidant activity of *Mentha spicata* essential oil was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method. In this assay, the partial inhibitory concentration (IC_{50}) value was determined, which represents the sample concentration required to inhibit 50% of the DPPH free radical. Based on the calibration curve equation, the IC_{50} value of *Mentha spicata* leaf extract was 725 µg/mL, while the IC_{50} of vitamin E (used as a reference antioxidant standard) was 42 µg/mL. This result shows that vitamin E has a significantly stronger antioxidant capacity than *Mentha spicata* essential oil when compared at the same concentration. However, although the antioxidant capacity of *Mentha spicata* essential oil was

Table 2: The composition of *Mentha spicata* essential oils.

Components	Retention time (min)	Relative percentage (%)
1-Octen-3-ol	9.470	0.43
Sulcatone	9.583	0.16
3-Octanol	9.933	0.35
D-Limonene	10.750	0.17
β-Linalool	12.477	1.63
Lavandulol	13.897	0.08
Borneol	14.087	0.28
Terpinen-4-ol	14.270	0.11
p-Cymene-8-ol	14.389	2.14
α-Terpineol	14.571	0.56
Estragole	14.625	4.44
trans-Isopiperitenol	14.680	0.07
β-Cyclocitral	15.070	0.08
Nerol	15.158	0.15
Carvone	15.556	3.11
Isopiperitenone	16.070	0.30
Perillaldehyde	16.238	44.05
trans-Shisool	16.434	0.32
Perilla alcohol	16.645	1.44
Piperitenone	17.374	0.85
Eugenol	17.615	2.80
Piperitenone oxide	17.985	12.53
α-Copaene	18.111	0.11
cis-Cinerolone	18.453	5.14
β-cis-Caryophyllene	18.690	0.68
β-trans-Caryophyllene	18.911	4.77
α-cis-Farnesene	19.362	0.17
α-Humulene	19.523	0.65
α-trans-Ionone	19.836	0.82
6-Methyl-6-(5-methyl-2-furyl)-2-heptanone	19.848	0.15
Germacrene-D	19.988	0.44
α-(Z,E)-Farnesene	19.993	1.92
α-(E,E)-Farnesene	20.223	0.23
δ-Cadinene	20.532	0.15
cis-Calamenene	20.580	0.10
trans-Nerolidol	21.142	0.63
Spathulenol	21.510	0.47
Caryophyllene oxide	21.615	3.21
Humulene oxide	22.049	0.27
Epicubenol	22.107	0.24
τ-Cadinol	22.160	0.18
δ-Cadinol	22.744	0.09
α-Cadinol	22.776	0.13
2-Ethylhexyl cyclohexanecarboxylate	22.850	0.37
ent-Germacra-4(15),5,10(14)-trien-1β-ol	25.274	0.31
Platambin	26.692	0.21
Palmitic acid	28.115	0.18
Phytol	30.334	1.21
Total		98.88

lower than that of vitamin E, the essential oil still exhibited significant free radical scavenging activity, suggesting the presence of bioactive plant compounds (phytochemicals) contributing to the antioxidant mechanism of the essential oil. The moderate IC_{50} value of *Mentha spicata* essential oil suggests that it may serve as a natural source of antioxidants, with potential applications in reducing oxidative stress in biological systems.

Progressive sperm motility, viability, and plasma membrane integrity

The average values of sperm progressive motility, sperm viability, and plasma membrane integrity obtained under different treatment conditions are summarized in Tables 3, 4, and 5, respectively. These indicators comprehensively reflect the quality and functional performance of sperm after exposure to different concentrations of *Mentha spicata* essential oil. The results showed that there was a dose-dependent relationship between the essential oil concentration and the sperm quality indicators. When the essential oil concentration increased from 5 μg/mL to 15 μg/mL, all the evaluation indicators including progressive motility, viability, and plasma membrane integrity were significantly improved. This suggests that at medium concentrations, *Mentha spicata* essential oil can exert protective and stimulating effects on sperm cells, and is capable of reducing oxidative damage and stabilizing the membrane structure. However, when the essential oil concentration exceeded 15 μg/mL, especially at 20 μg/mL, sperm quality decreased. Among all treatment groups, the sample supplemented with essential oil at 15 μg/mL showed the highest mean values for progressive motility, viability, and plasma membrane integrity. These values were statistically significantly higher ($P < 0.05$) than the control group as well as the groups treated at 10, 20, and 25 μg/mL, suggesting that 15 μg/mL is the optimal concentration to maintain the functional integrity of sperm. Notably, although sperm quality parameters decreased at 25 μg/mL, this group still had a statistically significant higher mean value ($P < 0.05$) than the control group. This finding suggests that, even at high concentrations, *Mentha spicata* essential oil maintains a certain level of bioactivity that has a positive effect on sperm function, although too high a concentration may lead to diminished efficacy or mild side effects. Overall, these results emphasize that the effect of *Mentha spicata* essential oil on canine sperm quality is concentration-dependent, with 15 μg/mL being identified as the optimal level for improving sperm motility, viability, and plasma membrane stability.

Sperm lipid peroxidation

The extent of lipid peroxidation in sperm cells after preservation was assessed by measuring the concentration of malondialdehyde (MDA), an important end product

Table 3: Impact of different concentrations of essential oils added to the extender on chilled sperm progressive motility (%).

Treatments	Day 1	Day 3	Day 6	Day 9	Day 12
T0	92.99±1.24^{A}	82.69±1.38^{cB}	65.02±1.42^{cC}	52.17±2.09^{bD}	35.82±1.72^{dE}
T1	94.33±0.91^{A}	83.83±1.27^{bcB}	66.73±2.82^{bcC}	52.89±2.23^{bD}	37.84±1.62^{cdE}
T2	92.70±0.94^{A}	85.38±0.95^{abcB}	67.30±2.73^{abcC}	54.50±2.36^{abD}	42.19±1.42^{bE}
T3	94.32±0.96^{A}	87.76±1.80^{aB}	72.03±2.19^{aC}	58.99±1.50^{aD}	49.19±1.04^{aE}
T4	93.80±0.77^{A}	86.25±1.29^{abB}	70.41±2.08^{abC}	56.56±1.58^{abD}	41.12±1.43^{bcE}
T5	94.76±0.99^{A}	85.94±1.10^{abB}	68.03±1.63^{abcC}	55.62±2.95^{abD}	38.73±1.47^{bcdE}

T0: Control; T1, T2, T3, T4, and T5: 5, 10, 15, 20, and 25 µg/mL of *Mentha spicata* essential oil, respectively. Values are presented as mean ± standard deviation for four replicates. Different lowercase letters (a, b, c, or d) in a column indicate treatment differences (P<0.05); different uppercase letters (A, B, C, D, or E) in a row indicate storage-time differences (P<0.05).

Table 4: Impact of different concentrations of essential oils added to extender on chilled sperm viability (%).

Treatments	Day 1	Day 3	Day 6	Day 9	Day 12
T0	96.35±1.57^{A}	87.26±1.59^{cB}	76.89±1.56^{bC}	60.12±4.63^{bD}	37.28±1.98^{dE}
T1	97.41±0.15^{A}	87.55±1.74^{bcB}	79.77±0.88^{aC}	63.88±1.24^{abD}	39.30±2.68^{cdE}
T2	96.35±1.48^{A}	89.59±0.87^{abcB}	80.80±0.73^{aC}	68.79±1.32^{abD}	44.78±2.73^{bE}
T3	96.82±0.37^{A}	91.44±0.44^{aB}	80.33±0.55^{aC}	72.21±7.23^{aD}	52.27±1.90^{aE}
T4	96.63±0.34^{A}	90.60±1.44^{abB}	80.71±1.02^{aC}	68.01±2.50^{abD}	43.61±1.38^{bcE}
T5	97.11±0.41^{A}	89.01±1.34^{abcB}	80.46±0.55^{aC}	63.52±0.83^{abD}	41.75±1.82^{bcdE}

T0: Control; T1, T2, T3, T4, and T5: 5, 10, 15, 20, and 25 µg/mL of *Mentha spicata* essential oil, respectively. Values are presented as mean ± standard deviation for four replicates. Different lowercase letters (a, b, c, or d) in a column indicate treatment differences (P<0.05); different uppercase letters (A, B, C, D, or E) in a row indicate storage-time differences (P<0.05).

Table 5: Impact of different concentrations of essential oils added to extender on chilled sperm membrane integrity (%).

Treatments	Day 1	Day 3	Day 6	Day 9	Day 12
T0	96.01±1.56^{A}	86.16±2.94^{B}	77.99±1.72^{bC}	61.88±2.50^{cD}	38.63±0.86^{cE}
T1	96.36±1.56^{A}	86.67±2.68^{B}	80.21±1.28^{aC}	63.86±3.81^{bcD}	41.55±1.97^{bcE}
T2	95.95±1.36^{A}	88.29±2.06^{B}	81.22±0.80^{aC}	69.58±1.67^{bD}	45.22±2.16^{bE}
T3	95.92±0.95^{A}	90.59±1.56^{B}	82.46±0.75^{aC}	76.20±0.48^{aD}	52.66±1.71^{aE}
T4	95.64±1.35^{A}	89.16±2.14^{B}	80.71±0.85^{aC}	69.20±2.33^{bD}	44.90±1.82^{bE}
T5	96.39±1.46^{A}	88.72±2.15^{B}	80.64±0.79^{aC}	64.78±1.88^{bcD}	42.48±1.50^{bcE}

T0: Control; T1, T2, T3, T4, and T5: 5, 10, 15, 20, and 25 µg/mL of *Mentha spicata* essential oil, respectively. Values are presented as mean ± standard deviation for four replicates. Different lowercase letters (a, b, or c) in a column indicate treatment differences (P<0.05); different uppercase letters (A, B, C, D, or E) in a row indicate storage-time differences (P<0.05).

Table 6: Impact of different concentrations of essential oils added to extender on the concentration of malondialdehyde (MDA) (nmol/50x10^{6} sperm) of chilled sperm.

Treatments	Day 1	Day 3	Day 6	Day 9	Day 12
T0	6.55±0.19^{aE}	7.16±0.47^{aD}	9.24±0.29^{aC}	13.60±0.02^{aB}	16.37±0.18^{aA}
T1	5.96±0.15^{bE}	6.43±0.21^{bD}	8.27±0.23^{bC}	13.51±0.30^{aB}	15.89±0.07^{bA}
T2	5.48±0.17^{cE}	6.05±0.18^{bcD}	7.82±0.24^{cC}	12.91±0.03^{bB}	15.29±0.05^{cA}
T3	4.02±0.17^{dE}	4.56±0.17^{eD}	5.85±0.03^{eC}	9.31±0.08^{eB}	12.14±0.10^{eA}
T4	4.77±0.09^{eE}	5.16±0.21^{deD}	6.90±0.04^{dC}	11.84±0.07^{dB}	15.18±0.27^{cA}
T5	4.92±0.13^{eE}	5.45±0.23^{cdD}	7.47±0.04^{cC}	12.35±0.07^{cB}	15.78±0.19^{bA}

T0: Control; T1, T2, T3, T4, and T5: 5, 10, 15, 20, and 25 µg/mL of *Mentha spicata* essential oil, respectively. Values are presented as mean ± standard deviation for four replicates. Different lowercase letters (a, b, c, d, or e) in a column indicate treatment differences (P<0.05); different uppercase letters (A, B, C, D, or E) in a row indicate storage-time differences (P<0.05).

formed during the oxidation of polyunsaturated fatty acids. The results of this analysis are presented in Table 6, which shows the effect of different concentrations of *Mentha spicata* essential oil on the extent of oxidative damage to

sperm membranes. The data showed a gradual decrease in MDA concentration from the control group to the group supplemented with 15 µg/mL essential oil, followed by an increase again at a higher concentration of 25 µg/mL. This pattern reflects a dose-dependent antioxidant response, in which medium concentrations of essential oil are able to effectively inhibit lipid peroxidation, while too high concentrations may reduce the protective effect or even cause mild oxidative stress. Specifically, sperm samples treated with 5 µg/mL essential oil had similar mean MDA concentrations as the 25 µg/mL group, and both groups had significantly lower MDA levels than the control group ($P < 0.05$). This result suggests that even low doses of *Mentha spicata* essential oil can partially reduce oxidative damage in sperm cells. Notably, the group supplemented with 15 µg/mL essential oil exhibited the lowest mean MDA concentration, which was statistically significantly different ($P < 0.05$) from the control group as well as the 5 µg/mL and 25 µg/mL groups. This result confirms the optimal antioxidant effect of *Mentha spicata* essential oil at this concentration, possibly due to the balance of bioactive compounds in the essential oil that effectively neutralize free radicals and stabilize the sperm plasma membrane. Overall, these results demonstrate that *Mentha spicata* essential oil supplementation at a concentration of 15 µg/mL is effective in reducing lipid peroxidation during preservation, thereby maintaining membrane integrity and enhancing the overall oxidative stability of spermatozoa.

DISCUSSION

This study showed that supplementation of *Mentha spicata* essential oil at appropriate concentrations significantly improved the quality of chilled canine sperm over the course of 12 days of storage. A clear concentration-dependent pattern was observed, with the 15 µg/mL treatment consistently outperforming all other treatments in terms of sperm motility, viability, and membrane integrity, while producing the lowest malondialdehyde (MDA) levels compared to all other treatments and the control group. These findings highlight the important role of antioxidant compounds in maintaining sperm function during chilling preservation, especially under conditions where endogenous antioxidants in seminal plasma are depleted. The protective effects observed at optimal concentrations could be explained by the presence of multiple bioactive compounds identified in the *Mentha spicata* essential oil. The main compounds such as perillaldehyde and piperitenone oxide exhibited strong free radical scavenging and cytoprotective properties (Sitzmann *et al*., 2014; Fuyuno *et al*., 2018). Other compounds including β-caryophyllene, estragole, carvone, eugenol, and linalool further enhance the antioxidant capacity of the essential oil through different but complementary mechanisms. β-caryophyllene has been shown to increase mitochondrial activity and inhibit the accumulation of reactive oxygen species (ROS) (Gushiken *et al*., 2022; Moura *et al*., 2024). Estragole contributes to antioxidant activity by neutralizing highly reactive free radicals such as hydroxyl (OH–) and superoxide (O_2–) through electron or hydrogen atom donation, thereby reducing their harmful effects on cellular structures (Júniora *et al*., 2020). Carvone exhibits strong free radical scavenging ability, which helps maintain sperm membrane motility and integrity (Pombal *et al*., 2017). Eugenol supports membrane structure protection by reducing lipid peroxidation (Barboza *et al*., 2018; Vui *et al*., 2021). Linalool also plays an important role by directly neutralizing hydroxyl and peroxyl radicals, thereby protecting proteins, lipids, and DNA from oxidative damage (Cheng *et al*., 2022). Collectively, these compounds have the potential to synergistically enhance sperm resistance and maintain cellular homeostasis during preservation.

The decline in sperm quality at higher essential oil concentrations (20-25µg/mL) suggests that excessive antioxidant supplementation may disrupt physiological redox signaling, leading to cytotoxic effects. Excessive ROS inhibition has been shown to impair ATP production, reduce sperm motility, and interfere with protein phosphorylation (Sanocka and Kurpisz, 2004). In addition, several components of the essential oil including piperitenone oxide, eugenol, β-caryophyllene, and linalool are potentially cytotoxic at high concentrations due to their lipophilic nature, which causes them to interact strongly with cell membranes and intracellular organelles (Sitzmann *et al*., 2014; Fuyuno *et al*., 2018; Moura *et al*., 2024). These mechanisms are consistent with the observed reduction in sperm motility, viability, and membrane integrity at supra-optimal concentrations.

Malondialdehyde (MDA) is widely recognized as a reliable indicator of oxidative damage and lipid peroxidation in cells (Vieira *et al*., 2017). In this study, supplementation with *Mentha spicata* essential oil at optimal antioxidant concentrations resulted in the lowest MDA levels, while the control without essential oil supplementation showed significantly higher MDA levels. These results demonstrate the ability of *Mentha spicata* essential oil to counteract oxidative stress and protect sperm, thereby limiting MDA formation. These findings are consistent with studies by Motlagh *et al*. (2014) and Vui *et al*. (2025), which also noted a link between increased lipid peroxidation and reduced sperm quality. The observed decrease in MDA with increasing essential oil concentration suggests that the increase in antioxidant compounds in *Mentha spicata* enhances the ability to protect sperm from oxidative damage. However, when antioxidant concentrations

exceed optimal levels, they can destabilize the cytoplasmic membrane, leading to increased sperm mortality and a consequent increase in MDA concentration.

These results are consistent with previous studies that have demonstrated the beneficial effects of antioxidant supplementation on chilled and frozen canine sperm. Previous studies have shown that antioxidants such as catalase, ascorbic acid, and glutathione can improve sperm motility, membrane integrity, and acicular protection by reducing the production of reactive oxygen species (ROS) (Michael *et al*., 2007; Monteiro *et al*., 2009). Recent studies have also highlighted the effectiveness of plant-derived antioxidants and essential oils including *Ocimum gratissimum*, *Perilla frutescens*, and *Ocimum basilicum* in improving the quality of canine sperm after cryopreservation (Vui *et al*., 2021, 2025; Vui and Tan, 2025). Therefore, the present findings add further evidence to the growing trend suggesting that natural plant antioxidants are effective supplements in enhancing canine sperm preservation.

CONCLUSIONS

In conclusion, the addition of *Mentha spicata* essential oil at a concentration of 15 µg/mL to canine semen dilution medium showed significant effects in enhancing progressive motility, maintaining plasma membrane integrity and sperm survival, and reducing lipid peroxidation during cryopreservation. These results highlight the protective and antioxidant properties of *Mentha spicata* essential oil, which may contribute to the improvement of canine sperm stability and function under refrigerated conditions. This study provides promising evidence for the potential application of *Mentha spicata* essential oil as a natural additive in canine semen dilution medium, reducing oxidative stress and improving sperm quality. However, further in-depth studies, particularly focusing on factors such as sperm DNA integrity, acrosome function and actual fertilization capacity, are needed to confirm the observed benefits and assess the potential for widespread application of this essential oil in enhancing the efficacy of assisted reproductive techniques in dogs.

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NOVELTY STATEMENT

This study is the first to define the concentration-dependent effects of *Mentha spicata* essential oil on the preservation of chilled canine sperm. It identifies 15 µg/mL as the optimal dose that significantly reduces lipid peroxidation and maintains key sperm quality parameters. The work also highlights the cytotoxic risks of higher concentrations, providing clear dose–response evidence. These findings introduce a precise and natural antioxidant strategy for improving canine semen preservation.

AUTHOR'S CONSTRIBUTION

NVV and HTT conceived and designed the experiments. HTT and BTP performed the experiments and analysed the data. NVV, HTT and BTP wrote the paper; all authors reviewed and approved the final manuscript.

Generative AI and AI-assisted technology statement

The authors state that ChatGPT was employed only for refining the manuscript's English grammar. All material was thoroughly checked, edited, and verified by the authors to ensure its correctness and originality.

Conflict of interest

The authors have declared no conflict of interest.

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