



Influence of *Perilla Frutescens* Essential Oil Added to Semen Extenders on Frozen-Thawed Canine Sperm Quality

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Abstract | Oxidative stress has a significant impact on canine sperm preservation, highlighting the importance of antioxidant supplementation to maintain sperm quality. *Perilla frutescens* essential oil, rich in bioactive compounds with strong antioxidant properties, can enhance sperm quality when added to semen extenders during storage. This study investigated the effects of *Perilla frutescens* essential oil (20, 25 and 30 µg/ml) in extenders on frozen-thawed sperm. The oil was extracted via steam distillation and sperm quality was assessed by motility, viability, membrane integrity and lipid peroxidation. The results indicated that concentrations below 25 µg/ml improved sperm quality, while 30 µg/ml had a negative impact. The 25 µg/ml extender showed the best sperm quality ($P < 0.05$). In conclusion, *Perilla frutescens* essential oil positively affects sperm quality, with 25 µg/ml being the optimal concentration for reducing oxidative stress and enhancing preservation.

Keywords | Canine sperm, Frozen-thawed, *Perilla frutescens*, Essential oils, Antioxidants, Cryopreservation

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INTRODUCTION

Cryopreservation of canine sperm is a vital method in dog breeding, allowing for the storage and transportation of sperm to remote locations. Nevertheless, the process of freezing sperm can induce oxidative stress, which harms both its structure and function. Canine sperm cells are especially susceptible to oxidative damage from reactive oxygen species (ROS) during freezing and thawing (Vieira *et al.*, 2017) due to the high levels of polyunsaturated fatty acids in their membranes (Darin Bennett *et al.*, 1974). At physiological levels, they play crucial roles in sperm functions such as maturation, acrosome reaction, hyperactiva-

tion and fertilization with oocytes (Aitken, 2017). However, excessive ROS production triggers lipid peroxidation in sperm membranes, resulting in reduced membrane fluidity, structural damage and sperm cell death (Lucio *et al.*, 2016). Canine seminal plasma naturally contains antioxidant enzymes (Angriman *et al.*, 2014) that help neutralize the damaging effects of ROS (Ighodaro and Akinloye, 2017). Nevertheless, during the sperm freezing process, seminal plasma is removed prior to mixing with diluents (Hori *et al.*, 2017). This removal eliminates the antioxidant enzymes, leaving the sperm unprotected. Incorporating antioxidants into the sperm diluent formulation could potentially enhance the quality of frozen sperm. Different antioxidants

have been evaluated for this purpose, but the outcomes have been inconsistent, depending on the type and concentration of the antioxidant used (Lucio *et al.*, 2016; Andersen *et al.*, 2018). Moreover, the application of naturally sourced antioxidants has recently attracted considerable interest from researchers and has been utilized to enhance the quality of canine sperm during storage (Calabria *et al.*, 2023; Vui *et al.*, 2024; Partyka *et al.*, 2024).

Perilla frutescens is an aromatic herb widely utilized as a food source. Beyond its common use as a spice in various culinary dishes, it is also widely used for extracting essential oils for medicinal applications. The essential oil of *Perilla frutescens* contains key bioactive compounds, including α -terpineol, D-limonene, eugenol and β -caryophyllene (Baser *et al.*, 2003; Verma *et al.*, 2015). These compounds exhibit amphiphilic properties and possess potent antioxidant activity (Ahmed, 2018; Vanit *et al.*, 2022). Despite its potential, no studies have been conducted to investigate the effects of these essential oils on animal sperm quality. With the rising interest among dog breeders and the growing use of canine sperm cryopreservation, the quest for optimal preservation techniques and effective additives continues. Thus, our aim was to assess whether incorporating *Perilla frutescens* essential oil into semen extenders can protect canine sperm from freezing damage and preserve their fertility. By thoroughly analysing sperm quality factors such as motility, membrane integrity, viability and lipid peroxidation, we seek to uncover the protective mechanisms of this essential oil.

MATERIALS AND METHODS

EXTRACTION OF ESSENTIAL OILS

The plant used in this study was obtained in June 2024 from an herb garden, where it was grown without pesticides or growth stimulants. Its identification and verification were carried out by the Crop Science Department at Tra Vinh University, Vietnam, before being utilized. Essential oils were extracted through steam distillation. After harvesting, the leaves were cut into pieces approximately 3 cm long and dried at 40°C. A total of 150 g of the dried leaves were combined with 500 ml of water in a 1000 ml round-bottom flask connected to a Clevenger distillation apparatus. The mixture was heated to 100°C for 5 hours. The resulting *Perilla frutescens* essential oils were separated from water using Na₂SO₄ and stored at a temperature of 4°C until further use. Gas chromatography-mass spectrometry (GC-MS) was used to identify the composition of bioactive compounds in the *Perilla frutescens* essential oils.

ANTIOXIDANT POTENTIAL OF ESSENTIAL OILS

The antioxidant potential of *Perilla frutescens* essential oils was evaluated using the 1,1-diphenyl-2-picrylhydrazyl

(DPPH) assay, following the method described by Blois (1958). The standard for antioxidant capacity was vitamin E. During the procedure, essential oil or the standard solution, prepared in methanol, was added to a 96-well microplate. Next, DPPH solution was introduced into the above mixture. The samples were incubated in darkness for 30 minutes, after which their absorbance was measured with a spectrophotometer. IC₅₀ values, representing the concentration required to inhibit 50% of DPPH activity, were determined by plotting the inhibition percentage versus concentration and analysing the resulting linear graph.

ANIMALS

The study involved three healthy male Rottweiler dogs, aged 2-5 years, all tested for reproductive health, raised at the same kennel in Tra Vinh City, Vietnam and trained for semen collection. The dogs were given dry food and had unrestricted access to water every day. Sperm was collected weekly. The study was carried out with the approval of the Institutional Animal Care and Use Committee at Tra Vinh University, Vietnam.

SEMEN COLLECTION AND PRELIMINARY ASSESSMENT OF SEMEN QUALITY

The digital manipulation technique described by Linde-Forsberg (1991), was applied to collect semen samples from dogs over the course of one week. Following collection, sperm quality from each ejaculate was evaluated. Sperm motility was examined directly under a microscope, while sperm morphology and viability were assessed using Eosin-nigrosin staining (Tamuli and Watson, 1994). In this study, canine semen with progressive sperm motility greater than 70%, sperm viability above 90%, sperm concentration higher than 200×10⁶ sperm/ml and abnormal sperm morphology under 5% was used.

PREPARATION OF EXTENDERS

All chemicals required for preparing the semen diluent were supplied by Sigma-Aldrich (Singapore). The semen diluent in this study consisted of a Tris buffer with citric acid and fructose, supplemented with 20% egg yolk. Different treatments were tested by adding essential oil extracted from *Perilla frutescens* leaves at concentrations of 0, 20, 25 and 30 µg/ml to the base diluent. The essential oils were diluted in dimethyl sulfoxide (DMSO), with each semen extender ultimately containing 0.8% DMSO. Glycerol was also included as a cryoprotectant. Two experimental groups were prepared: group 1 contained 3% glycerol, while group 2 contained 7% glycerol. After dilution with sperm, the final glycerol concentration in each diluent was 5%. Details of the diluent composition were presented in Table 1.

SEMEN PROCESSING AND EXPERIMENTAL DESIGN

The process of sperm cryopreservation in dogs was performed based on a previously established protocol with

some modifications (Michael *et al.*, 2007). After collection, semen from three dogs was combined for homogeneity and divided into four portions, each placed in sterile test tubes. The semen was then centrifuged to separate the seminal plasma and sperm. The sperm were diluted with group 1 semen diluent containing 3% glycerol to achieve a concentration of 200×10^6 sperm/ml. The sperm solution was gradually cooled to 4°C by adding ice. Then, the sperm solution was further diluted with group 2 semen diluent containing 7% glycerol in a 1:1 ratio, resulting in a concentration of 100×10^6 sperm/ml. The sperm solution was held at 4°C for 30 minutes before being loaded into 0.5 ml straws and sealed with a heat sealer. The straw containing semen was manually pre-cooled by positioning it 7 cm above the surface of liquid nitrogen for 5 minutes before being immersed in liquid nitrogen (Bucci *et al.*, 2019). The straw semen was stored in liquid nitrogen for at least two weeks before being thawed for sperm quality evaluation. Thawing was performed by immersing the straws in a 70°C water bath for 8 seconds, followed by dilution in Tris buffer (38°C) at a 1:1 ratio for 15 minutes to evaluate sperm quality post-thaw.

Table 1: The composition of the four semen extenders used to dilute canine sperm.

Extender components	Extenders			
	Control	PF20	PF25	PF30
Tris (mg)	3025	3025	3025	3025
Citric acid (mg)	1700	1700	1700	1700
Fructose (mg)	1250	1250	1250	1250
Egg yolk (ml)	20	20	20	20
<i>Perilla frutescens</i> essential oils (mg)	0	2.0	2.5	3.0
Streptomycin (mg)	100	100	100	100
Penicillin (mg)	60	60	60	60
DMSO (ml)	0.8	0.8	0.8	0.8
Glycerol (ml)	5	5	5	5
Distilled water (ml)	To 100	To 100	To 100	To 100
pH	6.62	6.63	6.65	6.65
Osmolality (mOsmol/kg)	1589	1603	1657	1626

PF20, PF25 and PF30: 20, 25 and 30 µg/ml of *Perilla frutescens* essential oils, respectively.

The experiment was designed in a completely random manner with four treatments, each corresponding to different concentrations of essential oils added to the semen dilution medium. The experiment was conducted in four repetitions.

SPERM MOTILITY

To evaluate the progressive motility parameter of spermatozoa, a microscope with a magnification of 400x was used.

Before evaluation, the sperm solution was warmed to 38°C degrees in a thermostatic bath for 15 minutes. Five random locations were selected and approximately 200 spermatozoa were evaluated from each location. Progressive motility of spermatozoa was calculated by counting the number of progressively motile spermatozoa out of the total number of spermatozoa observed (Shah *et al.*, 2011).

SPERM VIABILITY

The viability of sperm is assessed using the Eosin-nigrosin staining method (Tamuli and Watson, 1994). Dead sperm are those that allow the dye to penetrate into the cell, staining them pink - the colour of the dye. In contrast, live sperm do not permit the dye to enter the cell, retaining their natural white colour.

SPERM MEMBRANE INTEGRITY

The integrity of the sperm cell membrane after thawing was assessed using the hypo-osmotic swelling test. To conduct this test, 10 µl of sperm was added to 100 µl of a hypoosmotic solution with a concentration of 150 mOsm. The mixture was incubated at 38°C for 30 minutes. Then, 0.2 µl of the solution was examined under a microscope with a 40x objective lens to evaluate the sperm membrane integrity. A total of 200 spermatozoa were observed for swelling or non-swelling. Swelling indicated an intact sperm plasma membrane, while the absence of swelling signifies a compromised membrane (Goericke-Pesch *et al.*, 2012).

SPERM LIPID PEROXIDATION

The oxidative capacity of sperm was measured by determining the malondialdehyde (MDA) content using the thiobarbituric acid (TBA) method (Maia *et al.*, 2010). Sperm oxidation was induced by exposure to FeSO₄ before being treated with the TBA solution. The absorbance of each solution, after the reaction, was recorded at 535 nm using a 96-well plate spectrophotometer to quantify the MDA content. The results were reported as nmol MDA per 50×10^6 spermatozoa.

STATISTICAL ANALYSIS

Statistical analysis was carried out using SPSS software version 22. ANOVA and the Tukey test were used to compare the differences between treatments with essential oil added to the semen dilution medium. A difference was deemed statistically significant when $P < 0.05$.

RESULTS AND DISCUSSION

EXTRACTION AND CHEMICAL COMPOSITION ANALYSIS OF ESSENTIAL OIL

The steam distillation method extracted 0.55 ml of essential oil from 150g of dried basil powder, resulting in a yield of 0.37%. The biological compound composition of the es-

sential oils is shown in Table 2. GC-MS analysis revealed 23 compounds, representing 98.42% of the total components in the essential oils. The major components of *Perilla frutescens* essential oil included perilla aldehyde (65.23%), β -caryophyllene (8.32%), (3Z,6E)- α -farnesene (7.77%), D-limonene (6.54%), estragole (1.17%), α -linalool (1.30%) and perillyl alcohol (1.33%).

Table 2: Composition of *Perilla frutescens* essential oils.

Components	Retention time (min)	Relative percentage
3-Octenol	10.080	0.40
3-Octanol	10.917	0.22
D-Limonene	12.635	6.54
α -Linalool	17.076	1.30
α -Terpineol	21.699	0.45
Estragole	22.240	1.17
Perilla aldehyde	25.177	65.23
Bornyl acetate	25.467	0.25
Perillyl alcohol	25.901	1.33
Eugenol	27.852	0.70
α -Copaene	28.437	0.13
β -Elemene	28.949	0.13
Isocaryophyllene	29.398	1.12
β -Caryophyllene	29.765	8.32
Humulene	30.761	0.79
Germacrene D	31.521	0.67
(3Z,6E)- α -Farnesene	31.841	7.77
Bicyclogermacrene	31.916	0.10
α -Farnesene	32.142	0.64
δ -Cadinene	32.522	0.15
Nerolidol	33.333	0.30
Caryophyllene oxide	33.804	0.57
Phytol	39.634	0.14
Total		98.42

ANTIOXIDANT POTENTIAL OF ESSENTIAL OILS

The assessment of antioxidant activity showed that the IC₅₀ value, representing the concentration required to neutralize 50% of DPPH compounds, was 581 μ g/ml for the essential oil derived from *Perilla frutescens* leaves and 45.18 μ g/ml for vitamin E, based on the standard curve equation. These results indicate that vitamin E has a higher antioxidant potential compared to *Perilla frutescens* essential oil at the same concentration. However, despite being lower than the vitamin E standard, the antioxidant capacity of *Perilla frutescens* essential oil was still noteworthy.

SPERM PROGRESSIVE MOTILITY, PLASMA MEMBRANE INTEGRITY AND VIABILITY

The results for the mean proportions of sperm progressive motility, viability and plasma membrane integrity are

shown in Table 3. The findings reveal an improvement in sperm quality as the essential oil concentration increased from 20 to 25 μ g/ml, followed by a decline at the higher concentration of 30 μ g/ml. The treatment containing 25 μ g/ml of essential oil showed the highest mean percentages of progressive motility, viability and plasma membrane integrity, with values significantly higher than those of the control group and the treatments supplemented with 20 and 30 μ g/ml ($P < 0.05$). Interestingly, the treatment with the highest essential oil concentration of 30 μ g/ml showed average percentages of progressive motility, viability and plasma membrane integrity comparable to those of the 20 μ g/ml treatment and considerably greater than those of the control treatment ($P < 0.05$).

Table 3: Effects of the various *Perilla frutescens* essential oils supplement in semen extender on the frozen-thawed canine sperm progressive motility, plasma membrane integrity and viability parameters.

Parameters	Control	PF20	PF25	PF30	P-value
Progressive motility (%)	47.31 $\pm$ 0.95 ^c	52.17 $\pm$ 0.57 ^b	55.61 $\pm$ 0.63 ^a	52.27 $\pm$ 0.77 ^b	<0.001
Plasma membrane integrity (%)	47.71 $\pm$ 0.64 ^c	52.24 $\pm$ 0.51 ^b	55.05 $\pm$ 1.77 ^a	52.66 $\pm$ 0.97 ^b	<0.001
Viability (%)	47.52 $\pm$ 0.85 ^c	52.30 $\pm$ 0.60 ^b	55.78 $\pm$ 0.68 ^a	52.51 $\pm$ 0.76 ^b	<0.001

PF20, PF25 and PF30: 20, 25 and 30 μ g/ml of *Perilla frutescens* essential oils, respectively. Values are mean $\pm$ SD for four replicates, each being a pool of three ejaculates. Superscript letters (a, b, or c) in the same row indicate significant difference among extenders ($P < 0.05$).

Table 4: Effects of the various *Perilla frutescens* essential oils supplement in semen extender on the malondialdehyde (MDA) (nmol/50x10⁶ sperm) production of frozen-thawed canine sperm.

Parameters	Control	PF20	PF25	PF30	P-value
MDA	18.28 $\pm$ 0.14 ^a	10.56 $\pm$ 0.57 ^b	8.02 $\pm$ 0.29 ^c	11.04 $\pm$ 0.31 ^b	<0.001

PF20, PF25 and PF30: 20, 25 and 30 μ g/ml of *Perilla frutescens* essential oils, respectively. Values are mean $\pm$ SD for four replicates, each being a pool of three ejaculates. Superscript letters (a, b, or c) in the same row indicate significant difference among extenders ($P < 0.05$).

SPERM LIPID PEROXIDATION

The malondialdehyde (MDA) concentrations produced by sperm after cryopreservation are presented in Table 4. The results show a gradual decrease in MDA levels from the control group to the treatment supplemented with 25 μ g/ml of essential oil, followed by an increase at the higher concentration of 30 μ g/ml. The treatment with 20 μ g/ml of essential oil had an average MDA concentration comparable to that of the treatment with 30 μ g/ml of essential oil

and both were substantially lower than the control treatment ($P < 0.05$). It is worth noting that the treatment with 25 $\mu\text{g/ml}$ of essential oil showed the lowest average MDA concentration, which was significantly different from the control group and the treatments supplemented with 20 and 30 $\mu\text{g/ml}$ ($P < 0.05$).

Overall, this study revealed a dose-dependent relationship between *Perilla frutescens* essential oils and the quality of frozen canine sperm. Concentrations below 25 $\mu\text{g/ml}$ had beneficial effects on sperm quality, while concentrations exceeding 25 $\mu\text{g/ml}$ were harmful. The ideal concentration for preserving frozen canine sperm during storage was determined to be 25 $\mu\text{g/ml}$. The essential oil of *Perilla frutescens* in this study demonstrated protective effects on canine sperm during cryopreservation. This protective capacity is attributed to the antioxidant compounds present in the essential oil, such as D-limonene, eugenol, β -linalool, β -caryophyllene, estragole, δ -cadinene, phytol, isocaryophyllene, perilla aldehyde, trans-nerolidol and octanol. D-limonene and eugenol have been shown to enhance sperm motility and reduce oxidative damage by donating a hydrogen atom or an electron, thus stabilizing free radicals and slowing oxidative processes (Barboza *et al.*, 2018; Mahmoud *et al.*, 2020). β -linalool directly interacts with free radicals, such as hydroxyl ($\text{OH}^\cdot$) and peroxy ($\text{ROO}^\cdot$) radicals, by donating a hydrogen atom or an electron to stabilize them. This helps protect cellular structures, such as membranes, proteins and DNA, from oxidative damage, maintaining the structural integrity and functionality of cells (Cheng *et al.*, 2022). β -caryophyllene has been proven to improve sperm quality in mice, enhancing motility, viability and plasma membrane integrity parameters by combating oxidative stress and neutralizing free radicals ($\text{OH}^\cdot$ and $\text{ROO}^\cdot$) during sperm oxidation (Francomano *et al.*, 2019). Estragole exhibits antioxidant activity by neutralizing free radicals, including hydroxyl ($\text{OH}^\cdot$) and superoxide ($\text{O}_2^\cdot$), through electron or hydrogen atom donation, thus reducing their harmful effects on cellular components (Júniora *et al.*, 2020). Furthermore, δ -cadinene, phytol, isocaryophyllene, perilla aldehyde, trans-nerolidol and octanol were compounds with demonstrated antioxidant properties that contribute to the protection of cell membranes (Legault *et al.*, 2013; Hobbs *et al.*, 2016; Dong *et al.*, 2021). The study results are consistent with the findings of Partyka *et al.* (2024), who supplemented the canine sperm preservation medium with the bioactive compounds flavone and 3-hydroxyflavone, contributing to improved sperm viability and reduced oxidative stress during storage. Similarly, the addition of crocin to the preservation medium has been shown to protect the sperm membrane and DNA while enhancing resistance to oxidative stress (Calabria *et al.*, 2023). Moreover, these findings align with prior studies investigating the use of rosemary, clove bud and *Ocimum gratissimum* essential oils in extenders for ram, ovine and canine sperm,

respectively (Motlagh *et al.*, 2014; Baghshahi *et al.*, 2014; Nguyen *et al.*, 2023). However, contrasting results were observed with thymol and *Thymus munbyanus* essential oils, which negatively affected human sperm (Chikhoun *et al.*, 2015). The study results highlight the potential application of *Perilla frutescens* essential oil in canine sperm preservation and its possible extension to other livestock species.

At high concentrations, *Perilla frutescens* essential oil negatively impacts sperm quality. This effect is attributed to perilla aldehyde, a compound with pungent and heating properties that, in large amounts, can damage cell membranes (Hobbs *et al.*, 2016). Caryophyllene oxide has been shown to inhibit mitochondrial membrane potential by targeting positively charged centers in protein transport channels and altering ion concentrations around the cell membrane. It increases extracellular K^+ ion concentrations, thereby reducing mitochondrial membrane potential via its impact on K^+ ion channels (Monzote *et al.*, 2009). Isocaryophyllene, which exhibits anesthetic properties, induces cytotoxicity at high concentrations by increasing cell membrane permeability, leading to enhanced lipid peroxidation and oxidative damage to the membrane (Legault *et al.*, 2013). Other compounds, such as D-limonene, eugenol, β -linalool, estragole, phytol and β -caryophyllene, have also been shown to exert cytotoxic effects on cell membranes and intracellular components when administered at high levels (Mahmoud *et al.*, 2020; Júniora *et al.*, 2020; Pinheiro *et al.*, 2021).

Furthermore, sperm cryopreservation can lead to the production of malondialdehyde (MDA) as a result of lipid peroxidation (Buege and Aust, 1978). MDA has been recognized in earlier research as a significant indicator of oxidative damage in canine sperm (Vieira *et al.*, 2017). The findings of this study indicate that extenders containing 25 $\mu\text{g/ml}$ of essential oils effectively lowered MDA levels, thereby improving sperm quality. These results align with those of Kao *et al.* (2008) and Motlagh *et al.* (2014), both of which demonstrated an inverse relationship between lipid peroxidation and sperm quality. The reduction in MDA levels from low to high concentrations of essential oils is attributed to the gradual increase in antioxidant concentrations in *Perilla frutescens* essential oil, which helps protect sperm cells from oxidative damage. However, when antioxidant concentrations are excessively high, they can compromise the integrity of the plasma membrane, contributing to higher sperm mortality and increased MDA levels. Our research emphasizes the impact of *Perilla frutescens* essential oil on canine sperm quality, focusing on parameters like motility, plasma membrane integrity, viability and lipid peroxidation. However, aspects such as DNA integrity and fertility potential require further investigation in future studies to ensure the production of high-quality sperm for livestock breeding.

In conclusion, adding 25 µg/ml of *Perilla frutescens* essential oils to a canine semen extender has been shown to enhance sperm progressive motility, membrane integrity, viability and reduce lipid peroxidation during cryopreservation. These findings suggest the potential protective role of *Perilla frutescens* essential oil in enhancing canine sperm resilience during cryopreservation. Additional research is required, particularly focusing on parameters like DNA integrity and fertility potential, to validate these findings and investigate the wider applications of this essential oil in improving assisted reproductive techniques.

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NOVELTY STATEMENTS

This study explores a novel approach utilizing *Perilla frutescens* leaf essential oil for the cryopreservation of canine sperm. The findings demonstrate the beneficial effects of this essential oil on frozen-thawed dog sperm, highlighting its potential use in breeding programs.

AUTHOR'S CONTRIBUTIONS

The experiments were conceived and designed by Nguyen Van Vui. Huynh Thanh Tan and Nguyen Van Vui carried out the experimental setup, data collection, data analysis, and manuscript writing.

CONFLICT OF INTEREST

Authors declared no conflict of interest.

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