Research Article

The Potential of Using *Ocimum gratissimum* Leaf Essential Oils as a Supplement in Extender to Improve Chilled Canine Sperm Quality by Assessing Its Antioxidant Effects

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Abstract | The preservation of dog sperm is impeded by oxidative stress, leading researchers to investigate the impact of incorporating essential oils derived from *Ocimum gratissimum* leaves in order to prolong the viability of chilled dog sperm. The sperm's characteristics were assessed using an automated sperm analyzer and a confocal microscope to analyze motility and membrane properties, respectively. Additionally, malondialdehyde production was measured to evaluate lipid peroxidation capacity. The results revealed that incorporating essential oils at concentrations under 100 µg/ml in semen extenders had a positive impact on sperm quality. Nonetheless, employing essential oils at elevated concentrations of 200 µg/ml exhibited detrimental impacts on the sperm. The optimal dosage for maintaining the integrity of the dog sperm's membrane and enhancing its property parameters was determined to be a supplementation of 100 µg/ml essential oils, which demonstrated a significant difference from the control treatment throughout the 12-day storage period ($P<0.05$). In summary, the results indicate that the properties of dog sperm during preservation are influenced by the dosage of *Ocimum gratissimum* leaf essential oils in a manner that depends on the concentration. The most favorable outcomes were observed when using a maximum essential oil concentration of 100 µg/ml.

Keywords | Canine sperm, Preservation, *Ocimum gratissimum,* Essential oils, Antioxidants.

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INTRODUCTION

Sperm preservation and cryopreservation are prevalent practices in canine breeding and play a significant role. Before being stored through cooling or freezing, appropriate semen extenders are mixed with the sperm (Thomassen and Farstad, 2009). Cooling is more popular due to its convenience and effectiveness compared to freezing (Linde-Forsberg, 1991; Linde-Forsberg, 1995; Eilts, 2005), and canine sperm is capable of being maintained at cooling temperatures without adverse effects before undergoing cryopreservation (Santana et al., 2013). According to our study, the incorporation of egg yolk in Tris buffer and mineral salts extender yielded high-quality chilled dog sperm (Vui et al., 2019). However, the presence of oxidative stress poses a significant risk to the viability of chilled dog sperm due to the abundance of polyunsaturated fatty acids in their plasma membrane, which makes them vulnerable to lipid peroxidation upon exposure to reactive oxygen species (ROS) (Lamirande et al., 1997; Vieira et al., 2017). This lipid peroxidation can impair sperm cell structures and cause apoptosis (Moustafa et al., 2004; Lucio et al., 2016). Fortunately, there is a constant production of antioxidant enzymes in the living organisms to combat

oxidative stress (Ighodaro and Akinloye, 2017). In canine semen, almost antioxidant enzymes are mainly found in seminal plasma (Angrimani et al., 2014), but it is typically removed before mixing with semen extenders to avoid harmful impacts during preservation (Hori et al., 2017). This creates a decreased antioxidant capacity and increased sensitivity to oxidative effects. Hence, supplementing with antioxidants is necessary to neutralize free radicals and inhibit the effects of ROS during preservation, prolonging sperm lifespan. In addition, various antioxidant substances have been studied in canine sperm to prevent lipid oxidation during storage time (Neagu et al., 2009; Michael et al., 2009; Monteiro et al., 2009; Thiangtum et al., 2012), however, the outcomes were variable depending on the level and type of antioxidant substances.

Furthermore, the medicinal plant *Ocimum gratissimum* is known for its leaves that possess a substantial concentration of essential oils, accounting for 3.5% of their composition (Trevisan et al., 2006). Studies have identified eugenol, β-selinene, thymol, 1,8-cineole, and α-bisabolene as constituents of *Ocimum gratissimum* essential oils (Trevisan et al., 2006; Prabhu et al., 2009), all of which possess potent antioxidant properties (Ouyang et al., 2012; Mahapatra and Roy, 2014). However, there have been no studies assessing the impact of these essential oils on animal sperm quality. Incorporating essential oils into canine semen extenders might prevent sperm oxidation during chilling preservation, thus improving sperm lifespan. The aim of this investigation was to assess the effects of *Ocimum gratissimum* essential oils on the quality of chilled dog sperm over a 12-day period of storage in Tris buffer and mineral salts extender.

MATERIALS AND METHODS

Extraction Of Essential Oils

To acquire the essential oils, fully grown leaves of *Ocimum gratissimum* were subjected to drying at a temperature of 40°C in a hot air oven for an entire night. Subsequently, 100 g of the dried leaf powder was immersed in 1 liter of absolute ethanol for a duration of five days. The resultant solution underwent filtration, and the ethanol was removed by employing a rotary evaporator. The concentrated mixture was then subjected to centrifugation at 3500 x g for a duration of 10 minutes, leading to the separation and preservation of the essential oils present in the lower layer for utilization in this study.

Essential Oils Antioxidant Capacity

The antioxidant activity of the essential oils was assessed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, as previously described by Blois (1958). Vitamin E served as the reference standard. In this process, the essential oils and standard solutions (100 µl) were dissolved in methanol and placed in a 96-well microplate. Subsequently, 100 µl of DPPH (200 µM in methanol) was added to the mixture of essential oils and standard solutions. The control group consisted of methanol and DPPH, while the blank group contained methanol with the essential oils or standard. After a 30-minute incubation period in darkness, the absorbance of the solution mixtures was measured using a spectrophotometric plate reader. By plotting the inhibition proportion against the concentration, a linear graph was utilized to calculate the IC50 value of the essential oils and standard, indicating the concentration at which 50% inhibition of DPPH activity was achieved.

Semen Collection

Over a period of one week, the digital manipulation technique outlined by Linde-Forsberg (1991) was employed to collect canine semen samples from five American Bully dogs. The research exclusively focused on semen samples of exceptional quality, adhering to specific criteria such as a sperm concentration of $\geq 0.2 \times 10^7$ sperm/ml, progressive motility of ≥70%, sperm viability of ≥90%, and sperm abnormal morphology of ≤5%. The assessment of sperm progressive motility and concentration was conducted using an automated sperm analyzer system. Furthermore, the viability and morphology of the sperm were evaluated using eosin-nigrosin staining, following the method outlined by Tamuli and Watson (1994).

Preparation Of Extenders

Sigma-Aldrich (Singapore) provided all the chemicals required for semen extender preparation. In this study, the semen extender employed consisted of Tris buffer and mineral salts supplemented with 20% egg yolk. Various treatments were examined by incorporating different concentrations of essential oils extracted from *Ocimum gratissimum* leaves into the base semen extender. The essential oil concentrations ranged from 0 to 800 µg/ml. Table 1 shows the main components of the semen extender solutions used. To dilute the essential oils, dimethyl sulfoxide (DMSO) was used as a solvent, and each extender consisted of 0.8% DMSO.

Semen Processing And Experimental Design

A repeated measures design was employed in this study for a period of 12 days, with four replicates and five ejaculates for each replicate. Following semen collection, the collected semen was divided into eight groups, and the seminal plasma was separated through centrifugation, as described by Rijsselaere et al. (2002). Subsequently, the sperm was diluted with extender solutions in the appropriate ratio to achieve a concentration of 100×10^6 cells/ml. The extended sperm samples were then subjected to chilling in cold water until reaching a temperature of 5°C, following the proced-

Table 1: The composition, pH, and osmolality of the extenders

Ingredients	Extenders							
	T0	T25	T50	T100	T200	T400	T600	T800
Tris (mg)	900	900	900	900	900	900	900	900
Citric acid (mg)	500	500	500	500	500	500	500	500
Fructose (mg)	1250	1250	1250	1250	1250	1250	1250	1250
NaCl (mg)	450	450	450	450	450	450	450	450
$KHPO_4$ (mg)	60	60	60	60	60	60	60	60
KCl (mg)	60	60	60	60	60	60	60	60
$CaHPO_4$ (mg)	20	20	20	20	20	20	20	20
$MgCl_2$ (mg)	10	10	10	10	10	10	10	10
Egg yolk (mL)	20	20	20	20	20	20	20	20
Essential oils (µg)*	0	25	50	100	200	400	600	800
Gentamicin (mg)	200	200	200	200	200	200	200	200
DMSO (µl)	80	80	80	80	80	80	80	80
Distilled water (mL)	To 100	To 100	To 100	To 100	To 100	To 100	To 100	To 100
pH	6.57	6.56	6.55	6.54	6.54	6.54	6.53	6.53
Osmolality (mOsmol/kg)	453	454	455	457	460	469	472	476

T0: control; T25, T50, T100, T200, T400, T600, and T800: 25, 50, 100, 200, 400, 600, and 800 µg/ml essential oils, respectively.* Essential oils extract from *Ocimum gratissimum* leaves.

-ure outlined by Bouchard et al. (1990). The samples were maintained at this temperature throughout the evaluation period of sperm quality, with assessments conducted once every three days over a total duration of 12 days.

Sperm Motility

For the assessment of sperm motility parameters, the automatic sperm analyzer system (CASA; HTR-IVOS 14.0; USA) was employed. Prior to analysis, the sperm samples were incubated in a water bath at 38°C for a duration of 15 minutes. Five randomly selected fields were chosen, and approximately 200 sperm were evaluated from each chamber. The analysis involved recording several sperm motility parameters, including the total motility proportion (TM%), progressive motility proportion (PM%), curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s), and average pathway velocity (VAP, µm/s).

Sperm Lipid Peroxidation

The evaluation of canine sperm lipid peroxidation was conducted by measuring the production of malondialdehyde (MDA) using a thiobarbituric acid reactive substance (TBARS) assay, as described by Maia et al. (2010). To induce lipid peroxidation, the sperm samples were treated with 0.24 mM FeSO4 and incubated for 15 minutes at a temperature of 38°C in warm water. Subsequently, 0.5 ml of the sperm sample was mixed with 1 ml of TBA reagent, followed by boiling for 20 minutes. After cooling and centrifugation to remove the supernatant, the absorbance of the supernatants was measured at 535 nm using a microplate spectrophotometer. The MDA level was determined by comparing the absorbance of each sample to a standard curve of MDA, and the resulting values were presented in nmol/50x10^6 sperm.

Sperm Membrane Integrity

The evaluation of canine sperm membrane integrity was conducted using a fluorescent staining technique that involved a combination of Pisum sativum agglutinin, Hoechst 33342, 5,5',6,6'-tetrachloro-11',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide, and propidium iodide, following the protocol outlined by Vui et al. (2019). The stained sperm samples were immediately examined using a confocal microscope. Sperm exhibiting an intact acrosome and plasma membrane, along with a high mitochondrial membrane potential, displayed a distinct bright red-orange color in the mid-piece region and appeared blue-stained in the nucleus. Conversely, sperm with damaged acrosomes and plasma membranes, along with a low mitochondrial membrane potential, exhibited a bright green color in the mid-piece area, a yellow-green color in the acrosome region, and a red color in the nucleus. The stained sperm samples were identified and presented in Figure 1.

Statistical Analysis

The statistical analysis was performed using IBM SPSS Statistics, version 22, and the results were presented as mean ± standard deviation. To assess the impact of individual factors and the interaction between two factors (period and extender), a two-factor mixed analysis of variance

(ANOVA) was conducted. For comparing means among groups within each factor, the Tukey method was applied. A significance level of P<0.05 was considered statistically significant.

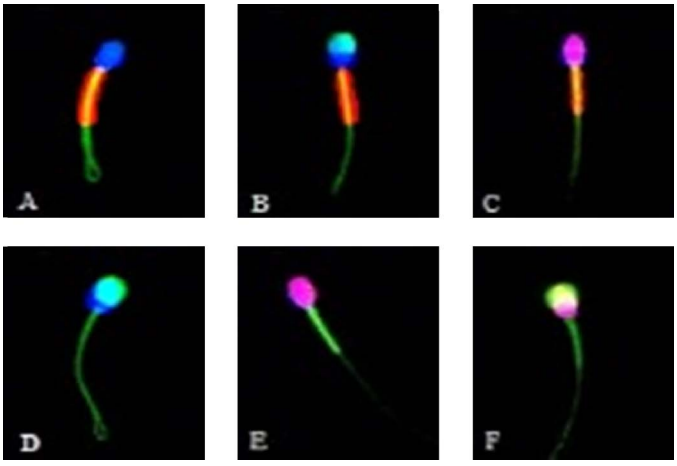

Figure 1: Stained canine sperm under a confocal microscope (600x magnification). (A) High mitochondrial membrane potential, integral plasma and acrosome membrane. (B) High mitochondrial membrane potential, integral plasma membrane, and broken acrosome membrane. (C) High mitochondrial membrane potential, broken plasma membrane, and integral acrosome membrane. (D) Low mitochondrial membrane potential, integral plasma membrane, and broken acrosome membrane. (E) Low mitochondrial membrane potential, broken plasma membrane, and integral acrosome membrane. (F) Low mitochondrial membrane potential, broken plasma and acrosome membrane.

RESULTS

Essential Oils Antioxidant Capacity

At a level of 203.00 µg/ml, the essential oils derived from *Ocimum gratissimum* leaves were found to inhibit 50% of DPPH radical, whereas vitamin E exhibited the same inhibitory capacity at a concentration of 11.03 µg/ml. Based on the findings, it can be concluded that the antioxidant effectiveness of vitamin E, at an equivalent concentration level, exceeded that of the essential oils derived from *Ocimum gratissimum* leaves.

Sperm Motility

Table 2 and Table 3 present the outcomes of the canine sperm motility analysis. In general, sperm quality in all extenders reduced gradually in total motility and progressive motility indicators during 12 days storage. There was no significant distinction observed in total motility and progressive motility measures among the extenders containing essential oil concentrations below 200 µg/ml during the chilling storage period. However, it should be noted that beyond day 9 of storage, the total motility and progressive motility indicators of sperm exposed to extenders with essential oil concentrations exceeding 400 µg/ml displayed a substantial decrease and exhibited a noticeable disparity compared to the other extenders (P<0.05).

In addition, canine sperm velocity served as a crucial parameter for assessing sperm motility characteristics. The results of sperm velocity values exhibited a similar pattern to that of total motility and progressive motility indicators. Notably, a significant decline in sperm velocity values was observed on day 12 for essential oil supplementation exceeding 400 µg/ml and on day 9 for supplementation exceeding 600 µg/ml (P<0.05).

Sperm Membrane Integrity

Table 4 presents the data on sperm membrane integrity. In general, these parameters showed a progressive decrease during sperm preservation in all essential oil concentrations. Sperm quality demonstrated a progressive enhancement in extenders containing essential oil concentrations below 100 µg/ml, whereas a consistent decline was observed in extenders with essential oil concentrations exceeding 200 µg/ml. Particularly noteworthy is that the extender supplemented with 100 µg/ml of essential oils exhibited the highest level of sperm membrane integrity. This difference was statistically significant compared to the extender without essential oils, as evidenced by both intact plasma and mitochondrial membrane indicators from day 3 to day 9 of preservation (P<0.05). Specifically, the sperm acrosome membrane values were highest in the extender with 100 µg/ml of essential oils, and this was significantly different from the extender without essential oils as well as the extenders with higher 400 µg/ml levels of essential oils during the 12-day preservation period (P<0.05).

Table 5 presents the percentages of healthy sperm, categorized based on the integrity of their acrosome, mitochondrial membrane, and plasma membrane. Similar to the aforementioned indicators, a gradual increase in the rates of healthy sperm was observed with the addition of essential oils to the semen extender, with the optimal outcomes achieved at a concentration of 100 µg/ml. However, it should be noted that the rates of healthy sperm declined at higher essential oil concentrations of 200 µg/ml. While the healthy sperm rates at 100 µg/ml did not show significant differences compared to the rates at 50 and 200 µg/ml, they were notably higher compared to the other extenders (P<0.05).

Sperm Lipid Peroxidation

Table 6 presents the concentrations of malondialdehyde (MDA) in canine sperm that underwent chilling and storage. The MDA levels in all extenders exhibited a consistent

Table 2: Effects of various levels of essential oils supplementation in extender on total motility (TM) and progressive motility (PM) parameters of chilled canine sperm.

Parameters	Extenders	Day1	Day3	Day6	Day9	Day12
TM (%)	T0	92.9±0.7^{A}	91.4±0.9^{A}	87.0±2.0^{B}	83.2±1.0^{aB}	61.2±4.9^{aC}
	T25	93.7±0.8^{A}	92.2±1.5^{A}	88.7±1.5^{B}	86.1±1.9^{aB}	69.2±6.8^{aC}
	T50	93.8±1.4^{A}	92.2±0.8^{AB}	90.2±1.0^{B}	85.5±4.2^{aB}	71.6±3.7^{aC}
	T100	94.4±1.3^{A}	92.3±1.5^{B}	90.2±2.5^{B}	88.0±3.3^{aB}	64.5±6.7^{aC}
	T200	94.1±1.0^{A}	91.5±1.9^{B}	89.0±2.1^{B}	84.5±2.4^{aB}	43.4±9.7^{bC}
	T400	94.0±1.2^{A}	91.2±1.2^{B}	88.5±2.9^{B}	66.0±8.4^{bC}	8.3±1.1^{cD}
	T600	93.6±0.5^{A}	91.0±0.6^{B}	88.8±1.5^{B}	53.6±5.8^{bcC}	4.9±0.9^{cD}
	T800	93.2±0.8^{A}	90.9±0.9^{B}	86.3±2.4^{C}	48.3±8.5^{cD}	3.4±0.9^{cE}
PM (%)	T0	70.1±0.7^{A}	68.4±4.7^{A}	63.6±2.2^{B}	55.9±9.7^{aB}	27.9±3.6^{aC}
	T25	70.7±3.1^{A}	68.7±3.5^{A}	64.4±2.3^{B}	59.2±7.1^{aB}	31.2±4.9^{aC}
	T50	72.4±2.0^{A}	69.9±3.6^{AB}	66.3±3.0^{B}	62.7±3.2^{aB}	33.2±4.8^{aC}
	T100	74.5±1.2^{A}	72.8±2.1^{A}	68.9±1.4^{B}	59.5±6.1^{aB}	26.8±7.4^{aC}
	T200	74.7±3.9^{A}	69.6±4.2^{B}	66.7±2.4^{B}	50.9±9.5^{aC}	23.6±8.2^{aD}
	T400	73.0±1.9^{A}	69.4±3.7^{B}	65.9±2.1^{B}	30.1±8.4^{bC}	4.0±0.8^{bD}
	T600	72.9±1.8^{A}	69.2±2.0^{B}	65.4±2.5^{B}	23.2±5.8^{bC}	0.0±0.0^{bD}
	T800	72.6±3.1^{A}	68.7±1.7^{B}	64.6±2.6^{C}	20.1±4.1^{bD}	0.0±0.0^{bE}

T0: control; T25, T50, T100, T200, T400, T600, and T800: 25, 50, 100, 200, 400, 600, and 800 µg/ml essential oils, respectively. Values are mean ± standard deviation for four replicates, each being a pool of five ejaculates. Lowercase superscript letters (a, b, or c) in the same column indicates significant difference among extenders (P<0.05) and uppercase superscript letters (A, B, C, or D) in the same row indicates significant difference within extenders with different storage time (P<0.05).

Table 3: Effects of various levels of essential oils supplementation in extender on average pathway velocity (VAP), straight line velocity (VSL) and curvilinear velocity (VCL) parameters of chilled canine sperm.

Parameters	Extenders	Day1	Day3	Day6	Day9	Day12
VAP (µm/s)	T0	84.0±3.0^{A}	78.5±2.6^{B}	73.5±3.4^{BC}	67.7±5.9^{abCD}	57.9±8.2^{aD}
	T25	84.4±3.0^{A}	80.9±2.2^{A}	73.6±4.2^{B}	68.8±3.3^{abBC}	62.0±6.9^{aC}
	T50	86.0±2.5^{A}	80.8±2.3^{AB}	75.6±4.7^{BC}	69.8±2.7^{abCD}	62.3±4.0^{aD}
	T100	86.1±2.5^{A}	81.3±1.4^{AB}	76.9±1.6^{BC}	70.1±4.3^{aCD}	60.3±7.1^{aD}
	T200	86.9±2.8^{A}	83.7±1.1^{A}	74.3±4.2^{B}	70.5±0.6^{aB}	57.1±8.1^{aC}
	T400	86.4±1.2^{A}	82.3±1.7^{AB}	76.6±3.3^{B}	58.9±2.8^{abcC}	38.8±8.4^{bD}
	T600	86.3±1.0^{A}	82.2±2.0^{A}	72.7±7.2^{B}	57.9±7.3^{bcC}	0.0±0.0^{cD}
	T800	86.1±2.3^{A}	81.8±2.1^{A}	71.9±4.4^{B}	51.3±8.6^{cC}	0.0±0.0^{cD}
VSL (µm/s)	T0	78.0±2.3^{A}	72.2±3.9^{AB}	67.7±4.9^{BC}	59.6±7.3^{abC}	45.4±5.6^{abD}
	T25	78.7±3.3^{A}	72.7±1.0^{AB}	67.3±5.4^{BC}	61.4±4.4^{aC}	51.4±6.0^{aD}
	T50	79.0±3.2^{A}	73.0±1.7^{AB}	67.7±3.1^{B}	62.6±4.5^{aB}	49.9±6.8^{aC}
	T100	79.2±3.2^{A}	74.8±2.9^{A}	70.5±4.2^{AB}	61.6±5.6^{aB}	46.8±7.9^{aC}
	T200	82.3±2.0^{A}	76.7±1.3^{A}	68.9±2.3^{B}	60.7±8.9^{aB}	44.4±8.4^{abC}
	T400	79.6±1.2^{A}	77.0±2.1^{A}	68.1±1.6^{B}	46.8±4.5^{abC}	30.3±8.2^{bD}
	T600	79.2±1.1^{A}	73.5±7.3^{AB}	66.6±9.9^{B}	45.7±7.3^{abC}	0.0±0.0^{cD}
	T800	78.3±3.4^{A}	72.0±7.1^{A}	63.8±7.5^{B}	44.6±7.4^{bC}	0.0±0.0^{cD}
VCL (µm/s)	T0	116.5±6.1^{A}	111.9±3.3^{AB}	110.5±3.7^{B}	108.6±5.4^{aB}	100.9±6.8^{aC}
	T25	117.7±5.5^{A}	111.4±6.5^{B}	109.6±5.9^{BC}	107.6±4.8^{aBC}	102.5±6.6^{aC}
	T50	117.0±5.3^{A}	112.2±2.0^{AB}	110.3±2.0^{BC}	104.4±6.0^{abCD}	102.1±5.2^{aD}
	T100	118.0±6.0^{A}	113.7±3.2^{A}	112.3±2.6^{AB}	111.2±2.4^{aAB}	105.7±4.5^{aB}
	T200	116.9±7.1^{A}	111.9±2.5^{AB}	109.0±3.9^{B}	107.6±3.6^{aB}	99.7±2.6^{aC}

	T400	116.7±4.3^{A}	111.2±3.7^{A}	104.6±3.5^{B}	100.7±1.5^{abB}	84.2±3.3^{bC}
	T600	116.3±1.7^{A}	111.2±5.1^{A}	106.7±3.2^{B}	93.4±6.5^{bcC}	0.0±0.0^{cD}
	T800	116.1±1.1^{A}	112.5±1.1^{A}	106.4±2.4^{B}	86.3±4.3^{cC}	0.0±0.0^{cD}

T0: control; T25, T50, T100, T200, T400, T600, and T800: 25, 50, 100, 200, 400, 600, and 800 µg/ml essential oils, respectively. Values are mean ± standard deviation for four replicates, each being a pool of five ejaculates. Lowercase superscript letters (a, b, or c) in the same column indicates significant difference among extenders (P<0.05) and uppercase superscript letters (A, B, C, or D) in the same row indicates significant difference within extenders with different storage time (P<0.05).

Table 4: Effects of various levels of essential oils supplementation in extender on high mitochondrial membrane potential, intact plasma and acrosome membrane of chilled canine sperm.

Parameters	Extenders	Day1	Day3	Day6	Day9	Day12
Plasma membrane (%)	T0	72.4±5.6^{abA}	67.1±5.8^{bcB}	59.2±9.2^{bC}	52.1±0.9^{bCD}	45.5±5.6^{abD}
	T25	73.2±2.1^{abA}	70.2±4.3^{abcA}	63.8±5.6^{abB}	56.7±5.1^{abB}	48.4±6.0^{abC}
	T50	78.5±5.6^{aA}	74.9±3.2^{abA}	65.7±5.3^{abB}	60.9±6.4^{abB}	52.3±3.3^{aC}
	T100	80.0±3.5^{aA}	77.5±2.2^{aAB}	74.3±3.7^{aB}	65.1±5.1^{aC}	56.4±3.5^{aD}
	T200	73.4±2.0^{abA}	71.4±1.2^{abcAB}	66.9±2.1^{abBC}	59.7±3.7^{abC}	50.2±6.0^{abD}
	T400	71.5±2.3^{abA}	69.1±2.4^{abcAB}	64.1±1.9^{abBC}	56.9±3.0^{abC}	39.0±3.2^{bcD}
	T600	69.1±2.9^{bA}	67.2±2.2^{bcA}	60.5±5.8^{bB}	55.4±2.6^{abB}	33.3±3.7^{cC}
	T800	68.7±3.3^{bA}	64.4±3.8^{cAB}	58.9±2.8^{bBC}	50.5±4.9^{bC}	31.8±4.9^{cD}
Mitochondrial membrane potential (%)	T0	78.7±1.3^{aA}	70.3±4.3^{bB}	62.5±5.9^{cC}	54.1±3.2^{cD}	47.9±1.7^{abD}
	T25	79.6±4.8^{aA}	76.2±3.9^{abA}	68.5±6.1^{abcB}	61.9±6.1^{abcC}	52.9±8.6^{abD}
	T50	82.4±5.9^{aA}	80.0±6.7^{abA}	73.3±4.5^{abB}	67.6±5.5^{abB}	58.8±5.7^{aC}
	T100	86.2±1.6^{aA}	82.2±2.5^{aB}	78.0±3.0^{aC}	73.7±3.0^{aC}	62.5±3.5^{aD}
	T200	81.6±3.2^{aA}	78.3±4.5^{abA}	73.2±2.8^{abcB}	65.9±5.0^{abcC}	53.9±7.2^{abD}
	T400	78.1±4.2^{aA}	72.9±4.0^{abB}	69.1±3.3^{abcC}	63.3±3.8^{abcC}	40.1±3.8^{bcD}
	T600	78.7±1.9^{aA}	72.0±3.4^{abB}	67.6±4.0^{abcC}	60.7±3.0^{bcD}	28.6±9.1^{cE}
	T800	77.6±1.9^{aA}	70.0±2.7^{bB}	66.0±3.5^{bcC}	57.0±7.1^{bcD}	25.3±4.4^{cE}
Acrosome membrane (%)	T0	63.6±3.4^{bcA}	57.1±4.6^{cB}	48.3±3.9^{eC}	39.4±4.4^{cD}	24.8±3.7^{cdE}
	T25	66.7±2.1^{abcA}	64.7±1.9^{abA}	53.8±1.7^{cdeB}	45.1±5.0^{bcC}	34.7±7.3^{bcD}
	T50	70.1±2.5^{abA}	67.2±2.5^{aA}	59.0±2.1^{bcB}	50.5±5.5^{bC}	43.7±7.0^{abC}
	T100	73.0±1.8^{aA}	70.2±1.6^{aAB}	66.4±2.8^{aB}	60.1±2.8^{aC}	50.2±3.4^{aD}
	T200	67.8±1.5^{abcA}	65.8±1.8^{aA}	60.4±1.9^{bB}	52.1±1.9^{abC}	36.4±6.1^{abcD}
	T400	62.5±1.4^{cA}	59.0±1.3^{bcAB}	57.9±1.3^{bcB}	48.2±1.6^{bcC}	26.8±6.8^{cdD}
	T600	62.4±4.3^{cA}	57.9±0.8^{cB}	54.0±1.7^{cdB}	45.1±1.9^{bcC}	22.2±4.9^{cdD}
	T800	61.4±3.8^{cA}	54.3±2.5^{cB}	50.1±1.7^{deC}	39.8±4.1^{cD}	17.3±5.6^{dE}

T0: control; T25, T50, T100, T200, T400, T600, and T800: 25, 50, 100, 200, 400, 600, and 800 µg/ml essential oils, respectively. Values are mean ± standard deviation for four replicates, each being a pool of five ejaculates. Lowercase superscript letters (a, b, c or d) in the same column indicates significant difference among extenders (P<0.05) and uppercase superscript letters (A, B, C, D or E) in the same row indicates significant difference within extenders with different storage time (P<0.05).

Table 5: Effects of various levels of essential oils supplementation in extender on healthy sperm with high mitochondrial membrane potential, intact plasma and acrosome membrane of chilled canine sperm.

Extenders	Day1	Day3	Day6	Day9	Day12
T0	60.0±3.6^{cdA}	52.9±9.3^{cB}	43.0±6.5^{eC}	36.3±5.4^{cD}	21.9±4.5^{cdE}
T25	64.7±2.2^{bcA}	60.9±5.6^{abcA}	51.9±2.4^{bcdB}	43.0±5.4^{bcC}	33.0±6.7^{abcD}
T50	68.1±3.7^{abA}	64.3±4.1^{abA}	56.2±3.6^{bB}	46.5±3.6^{bC}	39.9±4.9^{aC}
T100	71.4±1.6^{aA}	68.7±0.5^{aAB}	65.6±2.2^{aB}	56.3±3.5^{aC}	44.3±5.9^{aD}
T200	65.4±2.2^{abcA}	64.4±1.9^{abA}	59.3±2.7^{abB}	49.4±1.7^{abC}	34.1±7.4^{abD}
T400	61.7±1.0^{cdA}	59.1±0.7^{abcAB}	55.7±1.4^{bcB}	44.8±1.3^{bcC}	22.8±3.3^{bcdD}

T600	61.2±1.8^{cdA}	56.7±0.6^{bcA}	50.8±2.5^{cdeB}	42.2±2.8^{bcC}	15.6±3.4^{dD}
T800	57.8±2.1^{dA}	52.5±3.1^{cB}	46.6±2.3^{deC}	37.1±3.5^{cD}	10.4±2.0^{dE}

T0: control; T25, T50, T100, T200, T400, T600, and T800: 25, 50, 100, 200, 400, 600, and 800 µg/ml essential oils, respectively. Values are mean ± standard deviation for four replicates, each being a pool of five ejaculates. Lowercase superscript letters (a, b, c or d) in the same column indicates significant difference among extenders (P<0.05) and uppercase superscript letters (A, B, C, D or E) in the same row indicates significant difference within extenders with different storage time (P<0.05).

Table 6: Effects of various levels of essential oils supplementation in extender on the concentration of malondialdehyde (MDA) (nmol/50x10^6 sperm) of chilled canine sperm.

Extenders	Day1	Day6	Day12
T0	7.03±0.47^{aA}	6.34±0.38^{aB}	6.85±0.20^{aA}
T25	6.55±0.48^{abA}	6.02±0.28^{abB}	6.46±0.19^{abA}
T50	6.15±0.22^{abAB}	5.76±0.16^{abB}	6.31±0.27^{abA}
T100	6.02±0.24^{bAB}	5.61±0.08^{bB}	6.04±0.24^{bA}
T200	5.93±0.33^{bAB}	5.51±0.26^{bB}	6.03±0.39^{bA}
T400	6.06±0.32^{bAB}	5.74±0.32^{abB}	6.30±0.37^{abA}
T600	6.24±0.40^{abAB}	5.83±0.28^{abB}	6.37±0.30^{abA}
T800	6.46±0.48^{abA}	5.98±0.37^{abB}	6.31±0.26^{abAB}

T0: control; T25, T50, T100, T200, T400, T600, and T800: 25, 50, 100, 200, 400, 600, and 800 µg/ml essential oils, respectively. Values are mean ± standard deviation for four replicates, each being a pool of five ejaculates. Lowercase superscript letters (a or b) in the same column indicates significant difference among extenders (P<0.05) and uppercase superscript letters (A or B) in the same row indicates significant difference within extenders with different storage time (P<0.05).

decrease from day 1 to day 6, followed by a gradual increase from day 6 to day 12. Although there were no significant differences in this parameter among the extenders supplemented with essential oils, the extender with 100 µg/ml and 200 µg/ml of essential oils demonstrated the lowest MDA values. These values differed significantly from the control group extender throughout the 12-day storage period (P<0.05).

DISCUSSION

Overall, the findings of this study indicated that the effects of *Ocimum gratissimum* essential oils on chilled canine sperm quality followed a dose-dependent pattern. Lower concentrations of essential oils (25-100 µg/ml) exhibited positive effects on sperm quality, whereas higher concentrations (200-800 µg/ml) had detrimental effects. The optimal level of essential oil to protect chilled canine sperm during storage was found to be 100 µg/ml. The positive impacts of essential oils on sperm quality may be due to their antioxidant activities. Several bioactive compounds have been identified in the leaves of *Ocimum gratissimum*, including β-selinene, α-bisabolene, thymol, 1,8-cineole, and eugenol (Trevisan et al., 2006; Prabhu et al., 2009). These compounds have been found to support the activity of intercellular enzyme antioxidants such as catalase, glutathione peroxidase, phospholipid hydro-peroxide glutathione peroxidase, and superoxide dismutase (Strzezek et al., 2009; Neagu et al., 2011; Angrimani et al., 2014). These study findings are similar to previous research on rosemary essential oils and clove bud extract in extender for ram and ovine sperm, respectively (Motlagh et al., 2014; Baghshahi et al., 2014). In contrast, thymol and essential oils from *Thymus munbyanus* had negative impacts on human sperm (Chikhoune et al., 2015). The potential harmful effects of high concentrations of essential oils in the extender could be attributed to their antimicrobial properties. In addition to their antioxidant characteristics, the bioactive compounds present in *Ocimum gratissimum* essential oils have been shown to possess antimicrobial properties, effectively inhibiting bacterial growth (Prakash et al., 2011; Aguiar et al., 2015). Due to their lipophilic nature, these bioactive compounds can interact with the phospholipids and proteins in sperm membranes, impacting their potential, permeability, and ion influx (Visconti et al., 2002; Nazzaro et al., 2013). These findings are consistent with previous studies conducted on bulls (Shoae and Zamiri, 2008), boars (Roca et al., 2004), and rams (Arando et al., 2019). Therefore, while the use of essential oils in semen extenders can have positive effects on sperm quality, careful consideration of the concentration level is essential to prevent any adverse impacts on sperm quality.

Furthermore, the process of sperm storage can lead to the production of malondialdehyde (MDA) through lipid peroxidation (Buege and Aust, 1978). MDA serves as a crucial marker for oxidative damage in canine sperm, as demonstrated in previous studies (Maia et al., 2010; Toker et al., 2016; Vieira et al., 2017). The results of this study support the notion that extenders supplemented with 100

and 200 µg/ml essential oils exhibited lower MDA levels compared to other extenders, resulting in improved sperm quality. These findings align with the research conducted by Cassani et al. (2005), Kao et al. (2008), and Motlagh et al. (2014), which have all reported a negative association between lipid peroxidation levels and sperm quality. Additionally, the study observed that MDA concentrations were higher on day 1 compared to day 6, suggesting that the antioxidant substances derived from essential oils may not fully bind to sperm membranes during the initial day of preservation, thereby rendering the sperm more susceptible to oxidation induced by $FeSO_4$ prior to reacting with thiobarbituric acid.

CONCLUSIONS

In conclusion, the addition of essential oils derived from *Ocimum gratissimum* leaves at a concentration of 100 µg/ml to a canine semen extender has shown beneficial effects on sperm motility, membrane integrity, and lipid peroxidation during the cooling storage process. Further research is required to examine DNA integrity, sperm fertility, and frozen canine sperm.

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CONFLICT OF INTEREST

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NOVELTY STATEMENT

This study examines a new method involving the use of Ocimum gratissimum leaf essential oil in extending canine semen. The results of the research highlight the positive impact of these essential oils on dog sperm, suggesting their potential usefulness in real-world breeding practices

AUTHORS CONTRIBUTION

The experiments were conceived and designed by P.K. and carried out by V.V.N. and P.K. Data analysis was performed by V.V.N. and P.K. S.P. provided materials for the study. The paper was written by V.V.N., S.K., and P.K. All authors reviewed and approved the final version of the manuscript.

REFERENCES

Aguiar JS, Sousa CPB, Araruna MKA, Silva MKN, Portelo AC, Lopes JC, Carvalho VRA, Figueredo FG, Bitu VCN, Coutinho HDM, Miranda TAS, Matias EFF, (2015). Antibacterial and modifying-antibiotic activities of the essential oils of *Ocimum gratissimum* L. and *Plectranthus amboinicus* L. Eur. J. Integr. Med., 7: 151–156. https://doi.org/10.1016/j.eujim.2014.10.005.

Angrimani DSR, Lucio CF, Veiga GAL, Silva LCG, Regazzi FM, Nichi M, Vannucchi CI, (2014). Sperm maturation in dogs: sperm profile and enzymatic antioxidant status in ejaculated and epididymal spermatozoa. Andrologia, 46: 814–819. https://doi.org/10.1111/and.12154.

Arando A, Delgado J V, Fernández-prior A, León JM, Bermúdez-oria A, Nogales S, (2019). Effect of different olive oil-derived antioxidants (hydroxytyrosol and 3 , 4-dihydroxyphenylglycol) on the quality of frozen-thawed ram sperm. Cryobiology., 86: 33–39. https://doi.org/10.1016/j.cryobiol.2019.01.002.

Baghshahi H, Riasi A, Mahdavi AH, Shirazi A (2014). Cryobiology Antioxidant effects of clove bud (*Syzygium aromaticum*) extract used with different extenders on ram spermatozoa during cryopreservation. Cryobiology., 69: 482–487. https://doi.org/10.1016/j.cryobiol.2014.10.009.

Blois MS, (1958). Antioxidant Determinations by the Use of a Stable Free Radical. Nature, 181: 1199–1200. https://doi.org/10.2307/3280933.

Bouchard GF, Morris JK, Sikes JD, Youngquist RS (1990). Effect of Storage Temperature , Cooling Rates and Two Different Semen Extender on Canine Spermatozoal Motility. Theriogenology., 34: 147–157.

Buege JA, Aust SD (1978). Microsomal Lipid Peroxidation. Methods Enzymol.., 52: 302–310. https://doi.org/10.1016/S0076-6879(78)52032-6.

Cassani P, Beconi MT, O'Flaherty C (2005). Relationship between total superoxide dismutase activity with lipid peroxidation, dynamics and morphological parameters in canine semen. Anim. Reprod. Sci., 86: 163–173. https://doi.org/10.1016/j.anireprosci.2004.06.006.

Chikhoune A, Stouvenel L, Iguer-ouada M, Hazzit M, Schmitt A, Lorès P, Philippe J, Aissat K, Auger J, Vaiman D, Touré A, (2015). In-vitro effects of *Thymus munbyanus* essential oil and thymol on human sperm motility and function. Reprod. Biomed. Online, 31: 411–420. https://doi.org/10.1016/j.rbmo.2015.06.011.

Eilts BE (2005). Theoretical aspects of canine semen cryopreservation. Theriogenology., 64: 692–697. https://doi.org/10.1016/j.theriogenology.2005.05.019.

Hori T, Masuda T, Kobayashi M, Kawakami E (2017). Role of prostatic fluid in cooled canine epididymal sperm. Reprod. Domest. Anim., 52: 655–660. https://doi.org/10.1111/rda.12963.

Ighodaro OM, Akinloye OA (2017). First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. Alexandria J. Med., 54: 287–293. https://doi.org/10.1016/j.ajme.2017.09.001.

Kao S, Ph D, Chao H, Ph D, Chen H, Ph D (2008). Increase of oxidative stress in human sperm with lower motility. Fertil. Steril., 89: 1183–1190. https://doi.org/10.1016/j.fertnstert.2007.05.029.

Lamirande E De, Zini A, Lamirande E De, Jiang H, Zini A,

Kodama H, Gagnon C (1997). Reactive oxygen species and sperm physiology. Reprod. Fertil., 48–54. https://doi.org/10.1530/ror.0.0020048.

Linde-Forsberg C (1991). Achieving canine pregnancy by using frozen or chilled extended semen. Vet. Clin. North Am. Small Anim. Pract., 21: 467–485. https://doi.org/10.1016/S0195-5616(91)50054-1.

Linde-Forsberg C (1995). Artificial insemination with fresh, chilled extended, and frozen- thawed semen in the dog. Semin. Vet. Med. Surg. (Small Anim)., 10: 48–58.

Lucio CF, Regazzi FM, Silva LCG, Angrimani DSR, Nichi M, Vannucchi CI (2016). Oxidative stress at different stages of two-step semen cryopreservation procedures in dogs. Theriogenology,. https://doi.org/10.1016/j.theriogenology.2016.01.016.

Mahapatra SK, Roy S (2014). Phytopharmacological approach of free radical scavenging and anti-oxidative potential of eugenol and *Ocimum gratissimum* Linn. Asian Pac. J. Trop. Med., 7: S391–S397. https://doi.org/10.1016/S1995-7645(14)60264-9.

Maia S, Dimas S, Cecilia C, Rodello L, Cristina I, Gallego S, (2010). Lipid peroxidation and generation of hydrogen peroxide in frozen-thawed ram semen cryopreserved in extenders with antioxidants. Anim. Reprod. Sci., 122: 118–123. https://doi.org/10.1016/j.anireprosci.2010.08.004.

Michael AJ, Alexopoulos C, Pontiki E a, Hadjipavlou-Litina DJ, Saratsis P, Ververidis HN, Boscos CM (2009). Effect of antioxidant supplementation in semen extenders on semen quality and reactive oxygen species of chilled canine spermatozoa. Anim. Reprod. Sci., 112: 119–135. https://doi.org/10.1016/j.anireprosci.2008.04.007.

Monteiro J, Silva L, Lúcio C, Gonçalves J, Assumpção M, Rodrigues J, Vannucchi C, (2009). Influence of Ascorbic Acid and Glutathione Antioxidants on Frozen-Thawed Canine Semen. Reprod. Domest. Anim., 44: 359–362. https://doi.org/10.1111/j.1439-0531.2009.01434.x.

Motlagh MK, Sharafi M, Zhandi M, Mohammadi-Sangcheshmeh A, Shakeri M, Soleimani M, Zeinoaldini S, (2014). Antioxidant effect of rosemary (*Rosmarinus officinalis* L.) extract in soybean lecithin-based semen extender following freeze-thawing process of ram sperm. Cryobiology., 69: 217–222. https://doi.org/10.1016/j.cryobiol.2014.07.007.

Moustafa MH, Sharma RK, Thornton J, Mascha E, Abdel-hafez MA, Thomas AJ, Agarwal A, (2004). Relationship between ROS production , apoptosis and DNA denaturation in spermatozoa from patients examined for infertility. Hum. Reprod., 19: 129–138. https://doi.org/10.1093/humrep/deh024.

Nazzaro F, Fratianni F, Martino L De, (2013). Effect of Essential Oils on Pathogenic Bacteria. Pharmaceuticals., 6: 1451–1474. https://doi.org/10.3390/ph6121451.

Neagu VR, García BM, Rodríguez AM, Ferrusola CO, Bolaños JMG, Fernández LG, Tapia JA, Peña FJ, (2011). Determination of glutation peroxidase and superoxide dismutase activities in canine seminal plasma and its relation with sperm quality and lipid peroxidation post thaw. Theriogenology., 75: 10–16. https://doi.org/10.1016/j.theriogenology.2010.07.004.

Neagu VR, García BM, Rodríguez AM, Tapia JA, Sandoval CS, Fernández LG, Ferrusola CO, Peña FJ (2009). Freezing dog semen in presence of the antioxidant butylated hydroxytoluene improves postthaw sperm membrane integrity. Theriogenology., 73: 645–650. https://doi.org/10.1016/j.theriogenology.2009.10.021.

Ouyang X, Wei L, Pan Y, Huang S, Wang H, Begonia GB, Ekunwe SIN (2012). Antioxidant properties and chemical constituents of ethanolic extract and its fractions of *Ocimum gratissimum*. Med. Chem. Res., 22: 1124–1130. https://doi.org/10.1007/s00044-012-0113-z.

Prabhu KS, Lobo R, Shirwaikar a. a., Shirwaikar a. (2009). *Ocimum gratissimum*: A Review of its Chemical, Pharmacological and Ethnomedicinal Properties. Open Complement. Med. J., 1: 1–15. https://doi.org/10.2174/1876391X00901010001.

Prakash B, Shukla R, Singh P, Kumar P, Kishore N, Nath R, (2011). Efficacy of chemically characterized *Ocimum gratissimum* L . essential oil as an antioxidant and a safe plant based antimicrobial against fungal and a flatoxin B1 contamination of spices. Food Res. Int., 44: 385–390. https://doi.org/10.1016/j.foodres.2010.10.002.

Rijsselaere T, Van Soom A, Maes D, De Kruif A, (2002). Effect of centrifugation on in vitro survival of fresh diluted canine spermatozoa. Theriogenology., 57: 1669–1681. https://doi.org/10.1016/S0093-691X(02)00663-5.

Roca J, Gil MA, Hernandez M, Parrilla I, Vazquez JM, Martinez EA, (2004). Survival and Fertility of Boar Spermatozoa After Freeze-Thawing in Extender Supplemented With Butylated Hydroxytoluene. Andrology., 25: 397–405.

Santana M, Batista M, Alamo D, Lez FG, Nin˜o T, Cabrera F, Gracia A (2013). Influence of Cool Storage before Freezing on the Quality of Frozen – Thawed Semen Samples in Dogs. Reprod. Domest. Anim., 48: 165–170. https://doi.org/10.1111/j.1439-0531.2012.02124.x.

Shoae A, Zamiri MJ (2008). Effect of butylated hydroxytoluene on bull spermatozoa frozen in egg yolk-citrate extender. Anim. Reprod. Sci., 104: 414–418. https://doi.org/10.1016/j.anireprosci.2007.07.009.

Strzezek R, Koziorowska-Gilun M, Kowalówka M, Strzezek J (2009). Characteristics of antioxidant system in dog semen. Pol. J. Vet. Sci., 12: 55–60.

Tamuli MK, Watson PF (1994). Use of a simple stating technique to distinguish acrosomal changes in the live sperm sub-population. Anim. Reprod. Sci., 35: 247–254.

Thiangtum K, Hori T, Kawakami E (2012). Effect of catalase and superoxide dismutase on motility, viability and acrosomal integrity of canine spermatozoa during storage at 5°c. Thai J. Vet. Med., 42: 447–453. https://doi.org/10.1111/j.1439-0531.2009.01420.x.

Thomassen R, Farstad W, (2009). Artificial insemination in canids: A useful tool in breeding and conservation. Theriogenology., 71: 190–199. https://doi.org/10.1016/j.theriogenology.2008.09.007.

Toker MB, Alcay S, Gokce E, Ustuner B (2016). Cryobiology Cryopreservation of ram semen with antioxidant supplemented soybean lecithin-based extenders and impacts on incubation resilience. Cryobiology., 72: 205–209. https://doi.org/10.1016/j.cryobiol.2016.05.001.

Trevisan MTS, Silva MGV, Pfundstein B, Spiegelhalder B, Owen RW (2006). Characterization of the volatile pattern and antioxidant capacity of essential oils from different species of the genus *Ocimum*. J. Agric. Food Chem., 54: 4378–4382. https://doi.org/10.1021/jf060181.

Vieira N de M, Góes, Kawai GKV, Assumpção MEOD, Rui BR, Angrimani D de SR, Losano JD de A, Bicudo L de C, Nichi M, da Silva B do CS (2017). Induced sperm oxidative stress in dogs: Susceptibility against different reactive

oxygen species and protective role of seminal plasma. Theriogenology., 108: 39–45. https://doi.org/10.1016/j.theriogenology.2017.11.020.

Visconti PE, Westbrook VA, Chertihin O, Demarco I, Sleight S, Diekman AB (2002). Novel signaling pathways involved in sperm acquisition of fertilizing capacity. Reprod. Immunol., 53: 133–150.

Vui N V., Samorn P, Sajeera K, Pakanit K (2019). Effects of egg yolk and soybean lecithin on sperm quality determined by computer-assisted sperm analysis and confocal laser scanning microscope in chilled canine sperm. Vet. Med. Sci., 5: 345–360. https://doi.org/10.1002/vms3.158.