Antioxidant Effects of Ocimum Gratissimum Leaf Essential Oils as a Supplement to Extender on Chilled Canine Sperm Quality

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Research article

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Abstract

Background: Oxidative stress during chilled storage is a major problem with canine sperm. To improve the quality of chilled canine sperm during storage, many synthetic antioxidants have been examined, but different outcomes were investigated depending on antioxidant properties. The bioactive compounds of essential oils from *Ocimum gratissimum* leaves are known as a natural antioxidant source. This study aimed to evaluate the antioxidant effects of essential oils from *Ocimum gratissimum* leaves as a supplement in extender on chilled canine sperm during 12 days of storage.

Results: The results showed that low concentrations of *Ocimum gratissimum* essential oils (25, 50, and 100 µg/mL) have beneficial effects on sperm quality, whereas *Ocimum gratissimum* essential oils at high levels (above 200 µg/mL) have harmful effects. Specifically, the addition of 100 µg/mL of *Ocimum gratissimum* essential oil to the extender had the greatest beneficial effect in improving the quality of chilled canine sperm, and had a significant difference in all sperm quality parameters except motility when compared to the control group ($p < 0.05$).

Conclusions: *Ocimum gratissimum* essential oil has an impact on chilled canine sperm quality in a dose-dependent manner, and the best results are achieved with a maximum dose of *Ocimum gratissimum* essential oils of 100 µg/mL.

Background

Semen collection and artificial insemination (AI) techniques are currently widespread and play an essential role for breeding dogs. To prepare sperm for the AI technique, it must be diluted with appropriate extenders and preserved by chilling or freezing [1]. However, chilling is used more frequently than freezing for its convenience and high fertilizing capacity [2, 3]. In addition, canine sperm could be maintained at a chilling temperature without any deleterious effects before freezing [4]. In our previous studies, we found that tris-citric-fructose-mineral salts egg-yolk extender was the best extender for canine sperm chilling [5].

Reducing sperm quality during storage is a principal limitation of chilled canine sperm. One of the reasons for the decrease in chilled canine sperm quality is oxidative stress. Since sperm plasma membrane contains rich polyunsaturated fatty acids, they are susceptible to lipid peroxidation in the presence of reactive oxygen species [ROS] [6, 7]. Sperm lipid peroxidation can lead to changes in membrane fluidity and damage to plasma membrane, acrosome membrane, mitochondria, and DNA as well as subsequent sperm death [8, 9]. Fortunately, living organisms can produce a considerable amount of antioxidants physiologically that may prevent or minimise the effect of oxidative stress [10]. In canine semen, almost all enzymatic antioxidants including superoxide dismutase, glutathione peroxidase, phospholipid hydro-peroxide glutathione peroxidase, and catalase, are from seminal plasma [11, 12]. Nevertheless, due to the negative effects of seminal plasma during preservation, canine semen is centrifuged to remove seminal plasma before diluting with extenders [13, 14]. This may reduce the antioxidant capability and contribute to the high susceptibility of canine sperm to oxidative stress. Thus,

adding antioxidant molecules may inhibit free radicals or the attack of ROS during the sperm storage process, and improve sperm quality. Moreover, different synthetic antioxidants have been tested on canine sperm to improve sperm quality during storage against oxidative stress [15–20], but varied effects were found depending on the type and concentration of antioxidants.

Furthermore, *Ocimum gratissimum*, also known as an aromatic medicinal plant, is a species of *Ocimum* that is rich in essential oils (3.5%) from the leaves [21]. Studies had also shown that the essential oils from *Ocimum gratissimum* leaves contain bioactive components made up of eugenol, α -bisabolene, β -selinene, 1,8-cineole, and thymol [22–23]. These phytochemicals are known to have antioxidant activity [24–27]. However, until now, no study has investigated the effect of *Ocimum gratissimum* essential oils on mammalian sperm as a natural antioxidant. Hence, adding essential oil extract from *Ocimum gratissimum* leaves to the extender can improve chilled canine sperm quality by reducing sperm lipid peroxidation during storage.

Therefore, the aim of the present study was to investigate the antioxidant effects of essential oils from *Ocimum gratissimum* leaves as a supplement in tris-citric-fructose-mineral salts egg-yolk extender on motility, plasma membrane integrity, acrosome membrane integrity, and mitochondrial membrane potential in chilled canine sperm during 12 days of storage.

Results

Antioxidant activity (DPPH radical scavenging activity) of essential oils from *Ocimum gratissimum*

The concentration of *Ocimum gratissimum* essential oils providing 50% inhibition (IC₅₀) of DPPH activity was higher than that of vitamin E with 263.63 $\mu\text{g/mL}$ and 11.03 $\mu\text{g/mL}$, respectively [dataset] [51].

Sperm motility

The results of the total motility (TM) and progressive motility (PM) are presented in Table 2 [dataset] [51]. Overall, sperm in all treatments decreased gradually in TM and PM parameters during the whole experimental period (12 days). However, the treatments with low concentrations of *Ocimum gratissimum* essential oils (0–200 $\mu\text{g/mL}$) were similar in TM and PM parameters during 12 days, while the TM and PM of sperm in the high level of essential oils (up to 400 $\mu\text{g/mL}$) reduced drastically and had a significant difference when compared to that of the low level of essential oils ($p < 0.05$) from day 9 to day 12.

Table 2

Effects of different concentrations of *Ocimum gratissimum* essential oils supplementation in extender on total motility (TM) and progressive motility (PM) parameters of chilled canine sperm during 12 days at 5°C.

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 ($p < 0.05$).						

Besides TM and PM parameters, sperm velocity (VAP, VSL, and VCL) was also an important parameter in evaluating the sperm motility characteristics. The sperm velocity parameters are shown in Table 3 [dataset] [51]. The results of the VAP, VSL, and VCL were parallel with that of TM and PM parameters. It was found that there was a significant decrease in the VAP, VSL, and VCL parameters after the addition of up to 600 µg/mL essential oils ($p < 0.05$) on day 9 and 400 µg/mL essential oils ($p < 0.05$) on day 12.

Table 3

Effects of different concentrations of *Ocimum gratissimum* essential oils supplementation in extender on average pathway velocity (VAP), straight line velocity (VSL) and curvilinear velocity (VCL) parameters of chilled canine sperm during 12 days at 5°C.

Parameters	Extenders	Day1	Day3	Day6	Day9	Day12
VAP($\mu\text{m/s}$)	T0	84.0 $\pm$ 3.0 ^A	78.5 $\pm$ 2.6 ^B	73.5 $\pm$ 3.4 ^{BC}	67.7 $\pm$ 5.9 ^{abCD}	57.9 $\pm$ 8.2 ^{aD}
	T25	84.4 $\pm$ 3.0 ^A	80.9 $\pm$ 2.2 ^A	73.6 $\pm$ 4.2 ^B	68.8 $\pm$ 3.3 ^{abBC}	62.0 $\pm$ 6.9 ^{aC}
	T50	86.0 $\pm$ 2.5 ^A	80.8 $\pm$ 2.3 ^{AB}	75.6 $\pm$ 4.7 ^{BC}	69.8 $\pm$ 2.7 ^{abCD}	62.3 $\pm$ 4.0 ^{aD}
	T100	86.1 $\pm$ 2.5 ^A	81.3 $\pm$ 1.4 ^{AB}	76.9 $\pm$ 1.6 ^{BC}	70.1 $\pm$ 4.3 ^{aCD}	60.3 $\pm$ 7.1 ^{aD}
	T200	86.9 $\pm$ 2.8 ^A	83.7 $\pm$ 1.1 ^A	74.3 $\pm$ 4.2 ^B	70.5 $\pm$ 0.6 ^{aB}	57.1 $\pm$ 8.1 ^{aC}
	T400	86.4 $\pm$ 1.2 ^A	82.3 $\pm$ 1.7 ^{AB}	76.6 $\pm$ 3.3 ^B	58.9 $\pm$ 2.8 ^{abcC}	38.8 $\pm$ 8.4 ^{bD}
	T600	86.3 $\pm$ 1.0 ^A	82.2 $\pm$ 2.0 ^A	72.7 $\pm$ 7.2 ^B	57.9 $\pm$ 7.3 ^{bcC}	0.0 $\pm$ 0.0 ^{cD}
	T800	86.1 $\pm$ 2.3 ^A	81.8 $\pm$ 2.1 ^A	71.9 $\pm$ 4.4 ^B	51.3 $\pm$ 8.6 ^{cC}	0.0 $\pm$ 0.0 ^{cD}
VSL($\mu\text{m/s}$)	T0	78.0 $\pm$ 2.3 ^A	72.2 $\pm$ 3.9 ^{AB}	67.7 $\pm$ 4.9 ^{BC}	59.6 $\pm$ 7.3 ^{abC}	45.4 $\pm$ 5.6 ^{abD}
	T25	78.7 $\pm$ 3.3 ^A	72.7 $\pm$ 1.0 ^{AB}	67.3 $\pm$ 5.4 ^{BC}	61.4 $\pm$ 4.4 ^{aC}	51.4 $\pm$ 6.0 ^{aD}
	T50	79.0 $\pm$ 3.2 ^A	73.0 $\pm$ 1.7 ^{AB}	67.7 $\pm$ 3.1 ^B	62.6 $\pm$ 4.5 ^{aB}	49.9 $\pm$ 6.8 ^{aC}
	T100	79.2 $\pm$ 3.2 ^A	74.8 $\pm$ 2.9 ^A	70.5 $\pm$ 4.2 ^{AB}	61.6 $\pm$ 5.6 ^{aB}	46.8 $\pm$ 7.9 ^{aC}
	T200	82.3 $\pm$ 2.0 ^A	76.7 $\pm$ 1.3 ^A	68.9 $\pm$ 2.3 ^B	60.7 $\pm$ 8.9 ^{aB}	44.4 $\pm$ 8.4 ^{abC}
	T400	79.6 $\pm$ 1.2 ^A	77.0 $\pm$ 2.1 ^A	68.1 $\pm$ 1.6 ^B	46.8 $\pm$ 4.5 ^{abC}	30.3 $\pm$ 8.2 ^{bD}
	T600	79.2 $\pm$ 1.1 ^A	73.5 $\pm$ 7.3 ^{AB}	66.6 $\pm$ 9.9 ^B	45.7 $\pm$ 7.3 ^{abC}	0.0 $\pm$ 0.0 ^{cD}
	T800	78.3 $\pm$ 3.4 ^A	72.0 $\pm$ 7.1 ^A	63.8 $\pm$ 7.5 ^B	44.6 $\pm$ 7.4 ^{bcC}	0.0 $\pm$ 0.0 ^{cD}

T0: control; T25, T50, T100, T200, T400, T600, and T800: 25, 50, 100, 200, 400, 600, and 800 $\mu\text{g/mL}$ essential oils, respectively. Values are mean $\pm$ 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 ($p < 0.05$).

Parameters	Extenders	Day1	Day3	Day6	Day9	Day12
VCL($\mu\text{m/s}$)	T0	116.5 $\pm$ 6.1 ^A	111.9 $\pm$ 3.3 ^{AB}	110.5 $\pm$ 3.7 ^B	108.6 $\pm$ 5.4 ^{aB}	100.9 $\pm$ 6.8 ^{aC}
	T25	117.7 $\pm$ 5.5 ^A	111.4 $\pm$ 6.5 ^B	109.6 $\pm$ 5.9 ^{BC}	107.6 $\pm$ 4.8 ^{aBC}	102.5 $\pm$ 6.6 ^{aC}
	T50	117.0 $\pm$ 5.3 ^A	112.2 $\pm$ 2.0 ^{AB}	110.3 $\pm$ 2.0 ^{BC}	104.4 $\pm$ 6.0 ^{abCD}	102.1 $\pm$ 5.2 ^{aD}
	T100	118.0 $\pm$ 6.0 ^A	113.7 $\pm$ 3.2 ^A	112.3 $\pm$ 2.6 ^{AB}	111.2 $\pm$ 2.4 ^{aAB}	105.7 $\pm$ 4.5 ^{aB}
	T200	116.9 $\pm$ 7.1 ^A	111.9 $\pm$ 2.5 ^{AB}	109.0 $\pm$ 3.9 ^B	107.6 $\pm$ 3.6 ^{aB}	99.7 $\pm$ 2.6 ^{aC}
	T400	116.7 $\pm$ 4.3 ^A	111.2 $\pm$ 3.7 ^A	104.6 $\pm$ 3.5 ^B	100.7 $\pm$ 1.5 ^{abB}	84.2 $\pm$ 3.3 ^{bcC}
	T600	116.3 $\pm$ 1.7 ^A	111.2 $\pm$ 5.1 ^A	106.7 $\pm$ 3.2 ^B	93.4 $\pm$ 6.5 ^{bcC}	0.0 $\pm$ 0.0 ^{cD}
	T800	116.1 $\pm$ 1.1 ^A	112.5 $\pm$ 1.1 ^A	106.4 $\pm$ 2.4 ^B	86.3 $\pm$ 4.3 ^{cC}	0.0 $\pm$ 0.0 ^{cD}
T0: control; T25, T50, T100, T200, T400, T600, and T800: 25, 50, 100, 200, 400, 600, and 800 $\mu\text{g/mL}$ essential oils, respectively. Values are mean $\pm$ 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 ($p < 0.05$).						

Plasma membrane integrity, acrosome membrane integrity and mitochondrial membrane potential

The results of the plasma membrane integrity, acrosome membrane integrity, and mitochondrial membrane potential are given in Table 4 [dataset] [51]. In general, these parameters in all the concentrations of essential oils declined steadily during chilled storage. In addition, the quality of sperm increased progressively in the treatments with the low levels of essential oils (from 0 to 100 $\mu\text{g/mL}$) and then decreased gradually with the high levels of essential oils (from 200 to 800 $\mu\text{g/mL}$). In particular, the treatment of 100 $\mu\text{g/mL}$ essential oils had the highest values and was markedly different from the control (without essential oils) from day 3 to day 9 in both intact plasma membrane (77.5 ± 2.2 vs. 67.1 ± 5.8 on day 3 ($p = 0.008$), 74.3 ± 3.7 vs. 59.2 ± 9.2 on day 6 ($p = 0.01$), and 65.1 ± 5.1 vs. 52.1 ± 0.9 on day 9 ($p = 0.007$), respectively) and high mitochondrial membrane potential parameters (82.2 ± 2.5 vs. 70.3 ± 4.3 on day 3 ($p = 0.013$), 78.0 ± 3.0 vs. 62.5 ± 5.9 on day 6 ($p = 0.001$), and 73.7 ± 3.0 vs. 54.1 ± 3.2 on day 9 ($p < 0.001$), respectively). Notably, the percentage of intact acrosome membrane in treatment of 100 $\mu\text{g/mL}$ essential oil supplementation was greatest and had a substantial difference when compared

to the control (73.0 ± 1.8 vs. 63.6 ± 3.4 on day 1 ($p = 0.002$), 66.4 ± 2.8 vs. 48.3 ± 3.9 on day 6 ($p < 0.001$), and 50.2 ± 3.4 vs. 24.8 ± 3.7 on day 12 ($p < 0.001$), respectively) and compared to the treatments with a higher concentration of essential oils, 400 $\mu\text{g/mL}$ (73.0 ± 1.8 vs. 62.5 ± 1.4 on day 1 ($p = 0.001$), 66.4 ± 2.8 vs. 57.9 ± 1.3 on day 6 ($p = 0.001$), and 50.2 ± 3.4 vs. 26.8 ± 6.8 on day 12 ($p < 0.001$), respectively) during the whole storage period of 12 days.

Table 4

Effects of different concentrations of *Ocimum gratissimum* essential oils supplementation in extender on intact plasma membrane, high mitochondrial membrane potential, and intact acrosome membrane of chilled canine sperm during 12 days at 5°C.

Parameters	Extenders	Day1	Day3	Day6	Day9	Day12
Plasma membrane (%)	T0	72.4 ± 5.6 ^{abA}	67.1 ± 5.8 ^{bcB}	59.2 ± 9.2 ^{bcC}	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 ^{baA}	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 ^{baA}	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}

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 ($p < 0.05$).

Parameters	Extenders	Day1	Day3	Day6	Day9	Day12
	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 ^{abcdD}
	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 presents the proportion of healthy sperm with intact plasma membrane, high mitochondrial membrane potential, and intact acrosome membrane [dataset] [51]. As with the previous parameters, the percentage of healthy sperm increased regularly after adding essential oils to the extender and reached the top value at the level of 100 µg/mL before going down at the greater levels of essential oils (200 µg/mL). Although the values of those sperm in the treatment of 100 µg/mL essential oils were not evidently different from that in the treatments of 50 and 200 µg/mL essential oils, they were superior and had a significant difference when compared to the other treatments ($p < 0.05$).

Table 5

Effects of different concentrations of *Ocimum gratissimum* essential oils supplementation in extender on healthy sperm with intact plasma membrane, high mitochondrial membrane potential, and intact acrosome membrane of chilled canine sperm during 12 days at 5°C.

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 ^{abcdD}
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 ($p < 0.05$).					

Sperm lipid peroxidation

Table 6 summarises the level of malondialdehyde (MDA) (nmol/50 × 10⁶ sperm) of chilled canine sperm during 12 days [dataset] [51]. The concentration of MDA in all the treatments decreased slowly from day 1 to day 6 and then went up gradually to day 12. Moreover, although there was not notable difference in this parameter among the treatments with essential oil supplementation, the level of MDA was lowest and had a substantial difference from the control in both the treatments of 100 µg/mL (6.02 ± 0.24 vs. 7.03 ± 0.47 on day 1 ($p = 0.029$), 5.61 ± 0.08 vs. 6.34 ± 0.38 on day 6 ($p = 0.035$), and 6.04 ± 0.24 vs. 6.85 ± 0.02 on day 12 ($p = 0.015$), respectively), and 200 µg/mL (5.93 ± 0.33 vs. 7.03 ± 0.47 on day 1 ($p = 0.012$), 5.51 ± 0.26 vs. 6.34 ± 0.38 on day 6 ($p = 0.010$), and 6.03 ± 0.39 vs. 6.85 ± 0.02 on day 12 ($p = 0.012$), respectively) essential oils during 12 days' storage.

Table 6

Effects of different concentrations of *Ocimum gratissimum* essential oils supplementation in extender on the level of malondialdehyde (MDA) (nmol/50 × 10⁶ sperm) of chilled canine sperm during 12 days at 5°C.

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 ($p < 0.05$).			

Discussion

During chilled storage, canine sperm are susceptible to oxidative stress due to the high amounts of reactive oxygen species (ROS), which can cause damage to all sperm components [6, 7]. In previous studies, different synthetic antioxidants have been tested on chilled canine sperm to improve sperm quality during storage against oxidative stress, and the results were useful [15–18] or sometimes limited [19, 20]. Our previous study demonstrated the benefits of tris-citric-fructose-mineral salts egg-yolk extender in chilled canine sperm [5]. The present study, into applying an antioxidant substance of plant origin, was carried out to evaluate the effects of essential oils from *Ocimum gratissimum* leaves supplementation in tris-citric-fructose-mineral salts egg-yolk extender on chilled canine sperm.

In general, *Ocimum gratissimum* essential oils supplementation affected all parameters studied in a dose-dependent manner. The study suggested that low concentrations of *Ocimum gratissimum* essential oils (25, 50, and 100 µg/mL) have positive effects on sperm quality, while *Ocimum gratissimum* essential oils at high levels (above 200 µg/mL) have a harmful effect. A supplement of 100 µg/mL of *Ocimum gratissimum* essential oil in the extender was the best treatment to improve the quality of chilled canine sperm. The beneficial effects of *Ocimum gratissimum* essential oils in canine sperm quality may be due

to its antioxidant properties. *Ocimum gratissimum* essential oil is known to contain important phenolic compounds comprising eugenol, α -bisabolene, β -selinene, 1,8-cineole and thymol [21–23], which may subsequently assist the intercellular antioxidant system, including superoxide dismutase, glutathione peroxidase, phospholipid hydro-peroxide glutathione peroxidase, and catalase [24–27]. Our results are similar to those reported by [28]. They found that a rosemary aqueous extract supplement in soybean lecithin extender influenced ram sperm quality in a dose-dependent manner. In the same way, Baghshahi et al. [29] have also illustrated that adding a maximum clove bud extract of 75 $\mu\text{g/mL}$ in semen extender could improve sperm motility, viability, and plasma membrane integrity of ovine sperm. In contrast, *Thymus munbyanus* essential oils and thymol had no protective effects on human sperm, instead acting as potent immobilising and spermicidal agents [30]. In addition, the negative effects on chilled canine sperm quality at high levels of *Ocimum gratissimum* essential oils may be explained by its antimicrobial activities. Besides antioxidant activities, the phenolic compounds of *Ocimum gratissimum* essential oils are also known to inhibit microbial growth [31–33]. Based on hydrophobic properties, these phenolic compounds can interact with phospholipids and proteins in sperm plasma membranes. As a result, they affect membrane permeability, membrane potential and ion fluxes [34, 35]. These results are in agreement with previous reports on rams [29, 36], bulls [37], and boars [38]. They have confirmed that the high antioxidant concentrations could induce a higher plasma membrane fluidity and lead to increased sperm susceptibility.

Furthermore, one of the main by-products of lipid peroxidation is malondialdehyde (MDA) [39], which is an important indicator for oxidative damage in sperm [7, 40, 41]. Our results have found that the treatments of 100 $\mu\text{g/mL}$ and 200 $\mu\text{g/mL}$ *Ocimum gratissimum* essential oils have a lower concentration of MDA, while these treatments have a higher sperm quality when compared to the other treatments. These results are consistent with the previous studies [28, 42–44]. They have demonstrated that there was a negative correlation between sperm quality and rate of lipid peroxidation. Moreover, the results of sperm lipid peroxidation in the present study have also shown that the levels of MDA on day 1 were higher than that on day 6. This may be explained by the fact that on the first day of storage, the antioxidant substrates in the extender may absorb incompletely into the sperm plasma membrane. Thus, spermatozoa are prone to lipid peroxidation by FeSO_4 inducing before using thiobarbituric acid (TBA) assay.

Conclusions

In conclusion, the results of our investigation revealed that supplementation of *Ocimum gratissimum* essential oils in an extender at an appropriate level (100 $\mu\text{g/mL}$) has protective effects on chilled canine sperm without any adverse effects on sperm motility, plasma membrane, acrosome membrane, and mitochondrial membrane potential parameters, as well as a decrease in MDA concentration. Further studies are necessary in frozen canine sperm to evaluate more sperm quality parameters such as DNA fragmentation and fertility.

Methods

Plant material and preparation of *Ocimum gratissimum* ethanol extract

The source of *Ocimum gratissimum* trees was supported by School of Crop Production Technology, Suranaree University of Technology, Thailand. The formal identification was performed by Khanittha Kuboran, Head of Plant Science Unit, Suranaree University of Technology Farm - Thailand and voucher specimens, herbarium No. PK-012018, was recorded and deposited at the Centre for Scientific and Technological Equipment, Suranaree University of Technology – Thailand for future reference. *Ocimum gratissimum* leaves were collected in the morning and dried in a hot-air oven at 40°C for three days. The dried leaves were finely powdered using a grinder. The extraction was conducted at room temperature by soaking 100 g of leaf powder in one litre of ethanol (98%) for five days. The mixture was passed through filter papers (No. 1) and concentrated using a rotary evaporator at 40°C. Then, this concentrated solution was centrifuged at 3,500 x *g* for 15 minutes, and the bottom layer was freeze-dried to obtain the essential oils.

Antioxidant activity (DPPH radical scavenging activity) of essential oils from *Ocimum gratissimum*

The antioxidant activity of essential oils was carried out using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay according to the method of Blois [45]. Vitamin E was used as standard. The diluted working solutions of essential oils and standard (100 µL) were prepared in methanol with amounts ranging from 25 to 600 µg/mL and from 0.5 to 50 µg/mL in 96 wells micro plate, respectively. 100 µL DPPH (200 µM in methanol) was added to the essential oils and standard solution. Methanol (100 µL) with DPPH solution (200 µM, 100 µL) was used as a control, while methanol (100 µL) with essential oils or standard (100 µL) was used as a blank. These solution mixtures were kept in the dark for 30 minutes and optical density was measured at 517 nm using a spectrophotometric plate reader. The percentage of antioxidant activity (AA%) was determined using the following formula [46]:

$$AA\% = 100 - \{[(\text{Absorbance}_{\text{sample}} - \text{Absorbance}_{\text{blank}}) \times 100] / \text{Absorbance}_{\text{control}}\}$$

The concentration of essential oils and standard providing 50% inhibition (IC₅₀) of DPPH activity were calculated from the graph plotting between the percentage inhibition and concentration.

Animals

All dogs were supported by America Bully Dogs groups in Nakhon Ratchasima Province, Thailand. Sperm samples were obtained from five mature American Bully dogs (2–5 years old) of proven fertility. All dogs

were trained to ejaculate by digital manipulation for semen collection before studying. This study was performed under the guidelines of the Institutional Animal Care and Use Committee of the Suranaree University of Technology, Nakhon Ratchasima, Thailand. Animals were returned to the groups after sample collection.

Semen collection and evaluation

Ejaculates were collected once a week from each dog by digital manipulation according to the technique as described by Linde-Forsberg [2]. The collections were performed from all 5 dogs at the same time concurrently. Sperm with the following quality criteria was used in this study: >70% progressive motility; $>200 \times 10^6$ sperm/mL; <5% sperm abnormal morphology; and > 90% sperm viability. Computer-assisted sperm analysis (CASA) was used to determine sperm progressive motility and sperm concentration, while sperm morphology and viability were estimated using eosin-nigrosin staining [47].

Preparation of extenders

The basis extender in this study was tris-citric-fructose-mineral salts extender added to 20% egg yolk. Different extenders were prepared with different concentrations of *Ocimum gratissimum* essential oils supplementation (0, 25, 50, 100, 200, 400, 600, and 800 µg/mL). The composition of these extenders is shown in Table 1. All chemicals were purchased from Sigma-Aldrich. Before being added to the extenders, the *Ocimum gratissimum* essential oils were diluted in DMSO, and the final concentration of DMSO in each extender was 0.8%. Sterile distilled water was used to prepare solutions.

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 (mg)*	0	2.5	5	10	20	40	60	80
Gentamycin (mg)	200	200	200	200	200	200	200	200
DMSO (mL)	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
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 <i>Ocimum gratissimum</i> leaves.								

Semen processing and experimental design

After collection, semen from five dogs was pooled and separated into eight sterile tubes. Then, the seminal plasma was removed by centrifuging (5 minutes, $720 \times g$) [48]. The sperm pellets were diluted with a sufficient volume of the extenders to reach the sperm concentration of 100×10^6 sperm/mL. After that, the extended sperm was slowly cooled ($0.3^\circ\text{C}/\text{min}$) to 5°C by manual [49], and stored at 5°C for 12 days. Experimental design in this study was presented by a repeated measurement in the completely randomised design with four replicate trials.

Sperm evaluation

Sperm quality was evaluated every three days (Day1, Day3, Day6, Day9, and Day12).

Evaluation of sperm motility

Computer-assisted sperm analysis (CASA; Hamilton Thorne, USA), version IVOS 14.0 (HTR-IVOS 14.0) was used to evaluate the sperm motility. Before analysing, extended sperm was diluted with tris buffer and incubated at 38°C in a water bath for 15 minutes. The parameters of sperm motility were recorded including total motility (TM %), progressive motility (PM %), velocity average pathway (VAP, $\mu\text{m/s}$), velocity straight line (VSL, $\mu\text{m/s}$), and velocity curvilinear (VCL, $\mu\text{m/s}$).

Evaluation of plasma membrane integrity, acrosome membrane integrity, and mitochondrial membrane potential

A fluorescent staining combination of propidium iodide (PI), Hoechst 33342 (H342), fluorescein isothiocyanate–conjugated *Pisum sativum* agglutinin (FITC-PSA), and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) was carried out to evaluate the plasma membrane integrity, acrosome membrane integrity, and mitochondrial membrane potential. The fluorescent staining process was prepared as described by [50] and modified by the method of [5]. The stained sperm was identified by a confocal laser scanning microscope (CLSM; Nikon/Ni-E, Japan). The stained canine sperm under a confocal laser scanning microscope can be seen in Fig. 1. The spermatozoa with the intact plasma membrane, intact acrosome membrane, and high mitochondrial membrane potential were PI- and FITC-PSA-negative, and H342- and JC-1-positive, while the spermatozoa with the damaged plasma membrane, damaged acrosome membrane, and low mitochondrial membrane potential were PI- and FITC-PSA-positive, and H342 and JC-1 negative (PI-positive (+) = red-stained nucleus; H342-positive (+) = blue-stained nucleus; FITC-PSA positive (+) = yellow-green acrosome region; JC-1-positive (+) = bright red-orange in midpiece region; JC-1 negative (-) = bright green in midpiece region).

Evaluation of sperm lipid peroxidation

The lipid peroxidation of spermatozoa was determined by measuring the malondialdehyde (MDA) production, using thiobarbituric acid (TBA) assay according to the method described by Buege and Aust [39] and modified by Maia et al. [40]. The MDA concentration of each sample was measured immediately after inducing sperm lipid peroxidation with 0.24 mM FeSO_4 at 37°C in a water bath for 15 minutes. Then, 1 mL TBA reagent (trichloroacetic acid 15% (w/v), thiobarbituric acid 0.375% (w/v) in 0.25N hydrochloric acid) was added to 0.5 mL of each sample. The mixture was treated in a boiling water bath for 15 minutes. After cooling, the suspension was centrifuged at $1,000 \times g$ for ten minutes. The supernatant was separated and the absorbance was measured at 535 nm using a spectrophotometric plate reader. The

MDA concentration was determined by comparing the sample's absorbance at 535 nm with an MDA standard curve. The results were expressed in nmol MDA/50 × 10⁶ sperm.

Statistical analysis

Two-factor mixed analysis of variance (ANOVA) was used to examine the interaction between time and extender as the main effects, and the Tukey test was applied for multiple comparisons of means among groups of each factor (time, extender). Statistical analyses were performed with SPSS software version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). All data are presented as mean ± standard deviation (SD). A difference was considered significant for $p < 0.05$.

Abbreviations

AI: artificial insemination; ROS: reactive oxygen species; DNA: deoxyribonucleic acid; TM: total motility; PM: progressive motility; VAP: velocity average pathway; VSL: velocity straight line; VCL: velocity curvilinear; DPPH: 1,1-diphenyl- 2 picrylhydrazyl; MDA: malondialdehyde; CASA: computer assisted sperm analysis; DMSO: dimethyl sulfoxide; PI: propidium iodide; FITC-PSA: fluorescein isothiocyanate–conjugated *Pisum sativum* agglutinin; JC-1: 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide; H342: Hoechst 33342; TBA: thiobarbituric acid

Declarations

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Authors' contributions

P.K.conceived and designed the experiments; V.V.N. and P.K. performed the experiments; V.V.N. and P.K. analysed the data; S.P. contributed materials; V.V.N., S.K. and P.K.wrote the paper; all authors reviewed and approved the final manuscript.

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Availability of data and materials

Most of the analyses that were carried out in this investigation were made with the data that can be found in the link “<https://figshare.com/s/5d6d45c8238f5615e503>” or provided by the corresponding author upon request.

Ethics approval and consent to participate

This study was performed with the approval of Ethics Committee for experiments on animals of School of Animal Production Technology and Innovation, Suranaree University of Technology, Nakhon Ratchasiam, Thailand.

Informed written consent was obtained from America Bully Dogs groups in Nakhon Ratchasima Province, Thailand. This study complied with the Institutional Animal Care and Use Committee of the Suranaree University of Technology, Nakhon Ratchasima, Thailand.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests

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Figures

Figure 1

Canine spermatozoa stained with the association of fluorescent probes, H324, PI, FITC-PSA, and JC-1 under a confocal laser scanning microscope (600x magnification). (A) Intact plasma and acrosome membrane, and high mitochondrial membrane potential. (B) Intact plasma membrane, damaged acrosome membrane, and high mitochondrial membrane potential. (C) Damaged plasma membrane, intact acrosome membrane, and high mitochondrial membrane potential. (D) Intact plasma membrane, damaged acrosome membrane, and low mitochondrial membrane potential. (E) Damaged plasma membrane, intact acrosome membrane, and low mitochondrial membrane potential. (F) Damaged plasma and acrosome membrane, and low mitochondrial membrane potential.

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