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Research article

# Effects of vitamin E, green tea polyphenols, and *Ocimum gratissimum* leaf essential oil as a supplement to extender on frozen-thawed canine sperm quality

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## Abstract

During cryopreservation, oxidative stress is a principal cause of reducing the quality of frozen canine sperm. To prolong the longevity of canine sperm, adding vitamin E, green tea polyphenols, and *Ocimum gratissimum* essential oil in the semen extender was fulfilled to assess the effects of these antioxidant substances on frozen canine sperm quality. A total of twelve ejaculates were collected from three American Bully dogs in this study. The sperm straws were frozen in a programmable freezer at a freezing rate of 5°C/min from 4 to -15°C and 20°C/min from -15 to -100°C. The sperm motility, mitochondrial membrane potential, plasma membrane, and acrosome membrane integrity parameters were used to evaluate the quality of canine sperm using computer-assisted sperm analysis and confocal laser scanning microscope. The sperm lipid peroxidation was conducted using a Thiobarbituric acid assay. The results presented that the canine frozen-thawed sperm motility parameters in all the treatments showed no remarkable difference ($P>0.05$). In addition, although the antioxidant activity of the vitamin E and green tea polyphenols were higher than that of the *Ocimum gratissimum* essential oil, the value of mitochondrial membrane potential, plasma membrane, and acrosome membrane parameters of frozen-thawed sperm in the *Ocimum gratissimum* essential oil extender were higher than those in the rest extenders and had a noticeable difference compared to the control group ($P<0.05$). In conclusion, *Ocimum gratissimum* essential oil is superior to vitamin E and green tea polyphenols in protecting canine sperm during the process of freezing and thawing.

**Keywords**: Antioxidants, Canine sperm, Frozen-thawed, *Ocimum gratissimum* essential oil.

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# INTRODUCTION

Canine sperm cryopreservation is an essential tool for breeding dogs, as it allows long-term preservation and transportation to distant locations. During sperm freezing, oxidative stress can damage sperm structure and function. Because the sperm cell membrane of dogs is high in polyunsaturated fatty acids (Darin Bennett et al., 1974), they are easily oxidized by reactive oxygen species (ROS) during the freezing and thawing (Lamirande et al., 1997; Vieira et al., 2017). At physiologically normal concentrations, ROS can support sperm function, including sperm maturation, hyper-activation, capacitation, acrosome reaction, and fertilization of the oocyte (Lamirande et al., 1997; Aitken, 2017), whereas overproduction of ROS can stimulate sperm lipid peroxidation which results in sperm membrane fluidity changes, sperm structure damage, and sperm death (Moustafa et al., 2004; Aitken, 2017). Fortunately, canine seminal plasma involves a number of enzymatic antioxidants comprising phospholipid hydro-peroxide glutathione peroxidase, glutathione peroxidase, catalase, and superoxide dismutase (Cassani et al., 2005; Angrimani et al., 2014), which can minimize the negative effects of ROS (Birben et al., 2012; Ighodaro and Akinloye, 2017). However, during the sperm cryopreservation process, the canine seminal plasma must be removed before diluting with semen extenders (Treulen et al., 2012; Hori et al., 2017). As a result, the enzymatic antioxidants are no longer available for sperm protection. Thus, adding antioxidants in semen extender ingredients may increase the canine frozen sperm quality. Several antioxidant substances have been used to expand the frozen canine sperm quality, but the outcome results were very fluctuated depending on the concentration and type of antioxidants (Michael et al., 2009; Monteiro et al., 2009; Sahashi et al., 2011; Thiangtum et al., 2012; Wittayarat et al., 2013; Ogata et al., 2015; Lucio et al., 2016; Andersen et al., 2018).

In addition, vitamin E (α-tocopherol) is a fat-soluble antioxidant that not only neutralizes the oxygen radicals but also inhibits lipid peroxyl radicals occurring in the chain reaction of lipid peroxidation (Wang and Quinn, 1999; Dad et al., 2006). Several studies have found that adding vitamin E in the semen extender ingredients had beneficial effects on sperm quality of canine (Michael et al., 2009), bull (Asadpour, 2011), stallion (Almeida and Ball, 2005; Vasconcelos Franco et al., 2016), ram (Abdi-Benemar et al., 2015), rooster (Moghbeli et al., 2016), and boar (Jeong et al., 2009; Satorre et al., 2012).

Moreover, green tea polyphenols are natural antioxidants that consist of hydrophilic compounds, including epicatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate. These bioactive compounds have a strong antioxidant activity by reducing the free radicals from the ROS and the chain reaction of lipid peroxidation (Forester and Lambert, 2013). The supplementation of green tea polyphenols in the semen extenders to improve sperm quality has been investigated in stallions (Nouri et al., 2018), boar (Gadani et al., 2017), ram (Mehdipour et al., 2016), and canine (Wittayarat et al., 2013).

Furthermore, *Ocimum gratissimum* is an herbaceous plant that has rich essential oils from the leaves (3.5%) (Trevisan et al., 2006). It has been demonstrated that the composition of *Ocimum gratissimum* essential oil contains the main substance of eugenol (30-70%) and the other bioactive constituents, followed by α-bisabolene, thymol, β-selinene, γ-terpinene, and 1,8-cineole (Monga et al., 2017; Kumar et al., 2019). These bioactive compounds almost have an amphiphilic characteristic and a powerful function of antioxidant activity (Chiu et al., 2013; Mahapatra and Roy, 2014). Therefore, adding *Ocimum gratissimum* essential oil to the semen extenders can improve the quality of frozen canine sperm by inhibiting sperm lipid peroxidation during cryopreservation.

The previous study demonstrated that the Tris-citric-fructose-mineral salts egg-yolk extender had powerful effects on canine sperm quality during chilling

storage (Vui et al., 2019). Therefore, the aim of this study was to evaluate the effects of *Ocimum gratissimum* essential oil, vitamin E, and green tea polyphenols as a supplement in the Tris-citric-fructose-mineral salts egg-yolk extender on frozen canine sperm quality.

# MATERIALS AND METHODS

## Reagents

Sterile distilled water and chemicals from Sigma-Aldrich were used in this study. Various antioxidants were used, including essential oils, vitamin E (T3251), green tea polyphenols (P1204), and eugenol (E51791). Only essential oils were extracted from *Ocimum gratissimum* leaves by ethanol extraction. To extract essential oils, 300 g powder of *Ocimum gratissimum* leaves was soaked in 3L ethanol (98%) at room temperature for five days to achieve a mixture solution after filtering. A rotary evaporator was used to evaporate ethanol from the mixture solution. The essential oils used in this study were the bottom layer after centrifuging the concentrated solution.

## Animals and semen collection

Three American Bully dogs were used to collect semen by digital manipulation technique following the previous method of Linde-Forsberg (1991). All dogs were proven to have fertility before studying. Semen from each dog was exploited once a week, and a total of twelve ejaculates were used in this study. The study was conducted with the guide of the Institutional Animal Care and Use Committee of the Suranaree University of Technology, Thailand.

## Initial evaluation of semen quality

After collecting, the semen from each ejaculation was evaluated for sperm quality. A computer-assisted sperm analysis (CASA) was used to analyze sperm concentration and motility. Eosin-nigrosin staining was used to estimate sperm morphology and viability parameters (Tamuli and Watson, 1994). The semen was used with the sperm quality following the percentage of sperm progressive motility ≥70%, sperm viability ≥90%, sperm concentration ≥$200\times10^6$ sperm/mL, and sperm abnormal morphology ≤5% (Linde-Forsberg, 1995).

## Preparation of extenders

Tris-citric-fructose-mineral salts-egg yolk extender was used as a basic extender (Vui et al., 2019), and one of the following various antioxidants: essential oils (100 µg/mL), vitamin E (50 µg/mL), green tea polyphenols (50 µg/mL), and eugenol (2.6 µg/mL). The level of essential oils added to the extender (100 µg/mL) was based on our previous study, while the concentration of vitamin E and green tea polyphenols (50 µg/mL) were the results of our preliminary study. Especially, the level of eugenol used in this study was the same as the number of eugenol in essential oils extract (2.6%). The percentage of eugenol in essential oils was detected by using GC-MS. A control extender was the basic extender without antioxidants. Moreover, glycerol was added to extenders as a cryoprotectant. All extenders were prepared for two groups, group 1 (3% glycerol) and group 2 (7% glycerol). The final concentration of glycerol after diluting with sperm was 5% for every extender. The extender composition is shown in Table 1.

## Semen cryopreservation and experimental design

Canine semen cryopreservation was carried out as the previously described procedure with modifications (Michael et al., 2007). After collection and initial evaluation, the pooled semen was divided into five sterile tubes. After that, semen

was centrifuged for 5 minutes at 720×*g*, and the seminal plasma was separated. The sperm pellets were diluted in five extenders of group 1 (3% glycerol) to reach $200\times10^6$ sperm/mL. Then, extended sperm samples were cooled down gradually (0.3°C/min) to 4°C for 1 hour by adding the ice. Next, sperm samples were re-diluted (1:1 v/v) in five extenders of group 2 (7% glycerol) at the same condition of 4°C to obtain $100\times10^6$ sperm/mL. After 30 minutes at 4°C, extended sperm samples were filled into 0.5 mL French straws and sealed using a heat sealer. The sperm straws were frozen in a programmable freezer (Planer Kryo 360 – 1.7, England) at a freezing rate of 5°C/min from 4 to -15°C and 20°C/min from -15 to -100°C. After that, the sperm straws were immediately plunged into liquid nitrogen and stored in a liquid nitrogen container for at least two weeks before being thawed for evaluation.

The sperm straws were thawed at 70°C in a water bath for 8s and then diluted in Tris buffer (38°C) at a rate of 1:1 before post-thaw evaluation.

The experimental design was a completely randomized design of five treatments with the effects of five sperm extenders (various antioxidants) on frozen-thawed canine sperm quality. Four replicates were conducted.

**Table 1** The composition of semen extenders

| Extender components | Extenders | | | | |
|---|---|---|---|---|---|
| | Control | Essential oils | Vitamin E | Green tea polyphenols | Eugenol |
| Tris (g) | 0.90 | 0.90 | 0.90 | 0.90 | 0.90 |
| Citric acid (g) | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 |
| Fructose (g) | 1.25 | 1.25 | 1.25 | 1.25 | 1.25 |
| NaCl (g) | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 |
| $KHPO_4$ (g) | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 |
| KCl (g) | 0.06 | 0.06 | 0.06 | 0.06 | 0.06 |
| $CaHPO_4$ (g) | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 |
| $MgCl_2$ (g) | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| Egg yolk (mL) | 20.00 | 20.00 | 20.00 | 20.00 | 20.00 |
| Essential oils (mg)* | - | 10.00 | - | - | - |
| Vitamin E (mg) | - | - | 5.00 | - | - |
| Green tea polyphenols (mg) | - | - | - | 5.00 | - |
| Eugenol (mg) | - | - | - | - | 0.26 |
| Gentamicin (g) | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 |
| DMSO (mL) | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 |
| Glycerol (mL) | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 |
| Distilled water (mL) | To 100 | To 100 | To 100 | To 100 | To 100 |
| pH | 6.52 | 6.53 | 6.55 | 6.55 | 6.54 |
| Osmolality (mOsmol/kg) | 1489 | 1503 | 1557 | 1526 | 1566 |

* *Ocimum gratissimum* essential oil

## Antioxidant activity evaluation

The antioxidant activity of the various antioxidants used in this study was performed using a free radical of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) (Blois, 1958). The various antioxidants were diluted in methanol to create different concentration solutions. A volume of 100µl of various antioxidant solutions was placed in a microplate. Then, 100 µl DPPH (200 µM in methanol) was added to the wells. The control group was a mixture of methanol (100 µl) and DPPH solution (200 µM, 100 µl), while the blank group was a combination of methanol (100 µl) with various antioxidant solutions (100 µl). After 30 minutes, the absorbance of these solution mixtures was determined using a microplate spectrophotometer at 517 nm.

The IC50 values (the inhibitory concentration of various antioxidants needed to inhibit 50% of DPPH free radicals) were calculated from the standard curve between the concentration of various antioxidants and the percentage of antioxidant activity.

## Sperm motility evaluation

Sperm motility was analyzed using computer-assisted sperm analysis (CASA; HTR-IVOS 14.0; Hamilton Thorne, USA). All sperm motility parameters, including total motility, progressive motility, straight-line velocity, curvilinear velocity, and average pathway velocity, were recorded.

## Sperm membrane integrity evaluation

Canine sperm mitochondrial membrane potential, plasma membrane, and acrosome membrane parameters were examined using combined fluorescent staining as described by Celeghini et al. (2007) and modified by Vui et al. (2019). The thawed sperm samples were stained by Hoechst 33342 (H342), propidium iodide (PI), 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1), and fluorescein isothiocyanate–conjugated *Pisum sativum* agglutinin (FITC-PSA). After staining, sperm samples were evaluated using a confocal laser scanning microscope (Nikon/Ni-E, Japan). The sperm with high mitochondrial membrane potential, intact plasma membrane, and acrosome membrane was bright red-orange in the mid-piece region and blue-stained in the nucleus, whereas the sperm with low mitochondrial membrane potential, damaged plasma membrane, and acrosome membrane was bright green in the mid-piece region, red-stained in the nucleus, and yellow-green in acrosome region. The stained sperm classification is presented in Figure 1.

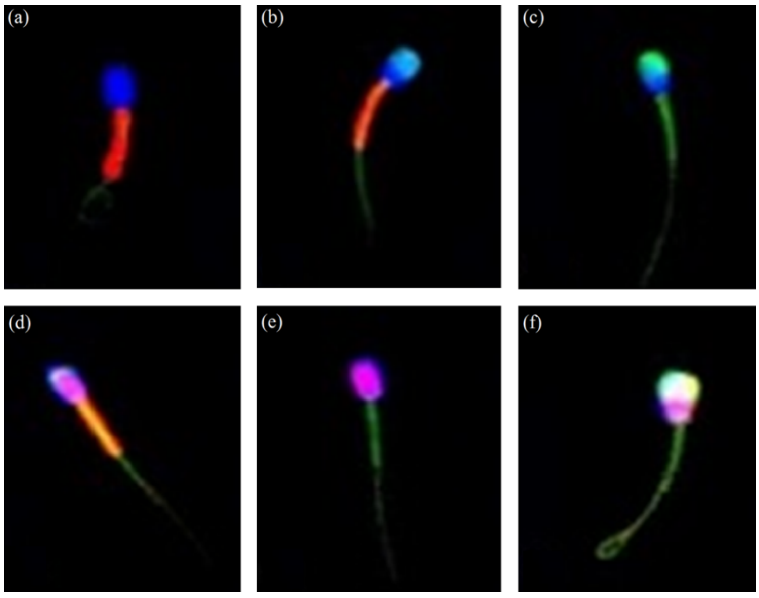


**Figure 1** Frozen-thawed canine sperm under a confocal laser scanning microscope (60x). (a) Sperm with high mitochondrial membrane potential, complete plasma, and acrosome membrane. (b) Sperm with high mitochondrial membrane potential, complete plasma membrane, and broken acrosome membrane. (c) Sperm with low mitochondrial membrane potential, complete plasma membrane, and broken acrosome membrane. (d) Sperm with high mitochondrial membrane potential, broken plasma membrane, and complete acrosome membrane. (e) Sperm with low mitochondrial membrane potential, broken plasma membrane, and complete acrosome membrane. (f) Sperm with low mitochondrial membrane potential, broken plasma, and acrosome membrane.

## Sperm lipid peroxidation evaluation

The thiobarbituric acid (TBA) assay was used to calculate sperm lipid peroxidation through the concentration of malondialdehyde (MDA) (Maia et al., 2010). Sperm samples were induced lipid peroxidation before adding with TBA reagent by $FeSO_4$. The absorbance of the sperm sample was determined at 535nm using a microplate spectrophotometer to measure the level of MDA. The value of MDA was shown in nmol MDA/50×$10^6$ sperm.

## Statistical analysis

Statistical analyses were performed using IBM SPSS, version 20 (IBM Corp., Armonk, N.Y., USA). A one-way analysis of variance (ANOVA) with the post hoc analysis using the Tukey test was used to determine the difference among means of antioxidant groups. All data are expressed as mean±SD. $P \leq 0.05$ was set as a significant difference.

# RESULTS

## Antioxidant activity of the various antioxidants

The antioxidant activity of the various antioxidants used in this study is represented in Table 2. The essential oils from *Ocimum gratissimum* had the highest value in the IC50 (263.63±11.52 µg/mL) and was significantly different from the vitamin E (11.03±0.55 µg/mL), eugenol (6.06±0.30 µg/mL), and green tea polyphenols (4.25±0.33 µg/mL). This means that the antioxidant activity of the vitamin E, eugenol, and green tea polyphenols were superior to that of the essential oils from *Ocimum gratissimum*.

**Table 2** The concentration of the various antioxidants inhibit 50% DPPH activity (IC50).

| Antioxidants | Essential oils | Vitamin E | Green tea polyphenols | Eugenol |
|---|---|---|---|---|
| IC50 (µg/ml) | 263.63±11.52[a] | 11.03±0.55[b] | 4.25±0.33[b] | 6.06±0.30[b] |

Values are mean ± standard deviation. Superscript letters (a or b) in the same row indicates significant difference ($p<0.05$).

## Sperm motility

The results of frozen-thawed canine sperm motility are shown in Table 3. On the whole, the values of total motility, progressive motility, straight-line velocity, curvilinear velocity, and average pathway velocity parameters of frozen-thawed canine sperm were not noticeably different among the extenders ($P>0.05$).

**Table 3** Effects of the various antioxidants supplement in semen extender on the frozen-thawed canine sperm total motility (TM), progressive motility (PM), average pathway velocity (VAP), straight-line velocity (VSL), and curvilinear velocity (VCL) parameters.

| Extenders | TM (%) | PM (%) | VAP (µm/s) | VSL (µm/s) | VCL (µm/s) |
|---|---|---|---|---|---|
| Control | 69.95±2.64 | 44.10±1.81 | 71.97±1.87 | 63.76±2.17 | 118.36±4.31 |
| Essential oils | 71.23±2.29 | 44.58±2.27 | 72.48±0.90 | 64.28±1.42 | 119.05±2.60 |
| Vitamin E | 72.50±2.09 | 44.71±3.61 | 73.39±2.49 | 64.76±1.13 | 129.71±7.84 |
| Green tea polyphenols | 68.70±1.43 | 43.25±1.23 | 73.93±5.14 | 64.52±5.29 | 121.82±8.64 |
| Eugenol | 71.35±1.00 | 44.20±2.30 | 74.98±0.97 | 64.79±5.04 | 126.83±3.17 |

Values are mean±SD for four replicates, each being a pool of three ejaculates.

## Sperm membrane integrity

The results of canine sperm mitochondrial membrane potential, plasma membrane, and acrosome membrane integrity are performed in Table 4. In general,

the percentages of the high mitochondrial membrane potential, intact plasma membrane, and acrosome membrane in the *Ocimum gratissimum* essential oil extender were superior to those in the other extenders and had a significant difference as compared to the control group (50.65±1.55 vs. 41.93±4.17 ($P$=0.031), 53.12±2.41 vs. 41.51±3.75 ($P$=0.002), and 55.31±1.24 vs. 45.63±1.31 ($P$=0.043), respectively). For the sperm mitochondrial membrane potential and plasma membrane integrity, although the proportions of these parameters of canine frozen-thawed sperm in the essential oils treatment were the highest, they were not evidently different compared with those in the eugenol, vitamin E, and green tea polyphenols treatments ($P$>0.05). In addition, there were no significant differences in these parameters between the control group, vitamin E, green tea polyphenols, and eugenol extenders ($P$>0.05). For the acrosome membrane integrity, the value of the intact acrosome membrane in the control extender was significantly lower than that in the essential oils (41.51±3.75 vs. 53.12±2.41, $P$=0.002), vitamin E (41.51±3.75 vs. 50.53±2.76, $P$=0.016), green tea polyphenols (41.51±3.75 vs. 49.11±5.13, $P$=0.048), and eugenol (41.51±3.75 vs. 52.45±2.43, $P$=0.003) extenders.

**Table 4** Effects of the various antioxidants supplement in semen extender on the frozen-thawed canine sperm mitochondrial membrane potential, plasma membrane, and acrosome membrane parameters.

| Extenders | Plasma membrane integrity (%) | Acrosome membrane integrity (%) | Mitochondrial membrane potential (%) |
|---|---|---|---|
| Control | 41.93±4.17$^{b}$ | 41.51±3.75$^{b}$ | 45.63±1.31$^{b}$ |
| Essential oils | 50.65±1.55$^{a}$ | 53.12±2.41$^{a}$ | 55.31±1.24$^{a}$ |
| Vitamin E | 47.75±2.65$^{ab}$ | 50.53±2.76$^{a}$ | 54.49±1.82$^{ab}$ |
| Green tea polyphenols | 46.41±5.66$^{ab}$ | 49.11±5.13$^{a}$ | 52.18±6.10$^{ab}$ |
| Eugenol | 45.55±3.08$^{ab}$ | 52.45±2.43$^{a}$ | 53.40±7.04$^{ab}$ |

Values are mean±SD for four replicates, each being a pool of three ejaculates. Superscript letters (a or b) in the same column indicate significant difference among extenders ($P$≤0.05).

Moreover, Table 5 gives the proportion of healthy sperm with high mitochondrial membrane potential, intact plasma membrane, and acrosome membrane. The values of this parameter corresponded to those of the high mitochondrial membrane potential and the intact plasma membrane parameters. This means that the essential oils treatment was the greatest in this parameter, being significantly higher than the control extender (48.86±3.01 vs. 37.41±4.61, $P$=0.015), whereas the rest of the antioxidant extenders, including the vitamin E, green tea polyphenols, and eugenol were not obviously different compared with the control extender ($P$>0.05).

**Table 5** Effects of the various antioxidants supplementation in semen extender on the sperm with intact plasma membrane, intact acrosome membrane, and high mitochondrial membrane potential of frozen-thawed canine sperm.

| Extenders | Control | Essential oils | Vitamin E | Green tea polyphenols | Eugenol |
|---|---|---|---|---|---|
| Sperm (%) | 37.41±4.61$^{b}$ | 48.86±3.01$^{a}$ | 44.71±3.84$^{ab}$ | 44.20±5.97$^{ab}$ | 44.02±3.65$^{ab}$ |

Values are mean±SD for four replicates, each being a pool of three ejaculates. Superscript letters (a or b) in the same row indicates significant difference among extenders ($P$<0.05).

## Sperm lipid peroxidation

The malondialdehyde concentration of frozen-thawed canine sperm with the addition of various antioxidants is presented in Table 6. The level of the MDA of frozen-thawed canine sperm in the control treatment was significantly higher than that in the essential oils (7.70±0.20 vs. 6.88±0.01, $P$<0.001), vitamin E (7.70±0.20

vs. 6.84±0.03, $P$<0.001), green tea polyphenols (7.70±0.20 vs. 6.67±0.24, $P$<0.001), and eugenol (7.70±0.20 vs. 7.01±0.23, $P$<0.001) treatments. Although the value of the MDA of frozen-thawed canine sperm in the green tea polyphenols treatment was the smallest, it was not noticeably different compared with the rest antioxidant treatments ($P$>0.05).

**Table 6** Effects of the various antioxidants supplementation in semen extender on the level of the malondialdehyde (MDA) (nmol/50x10$^6$ sperm) of frozen-thawed canine sperm.

| Extenders | Control | Essential oils | Vitamin E | Green tea polyphenols | Eugenol |
|---|---|---|---|---|---|
| MDA | 7.70±0.20$^a$ | 6.88±0.01$^b$ | 6.84±0.03$^b$ | 6.67±0.24$^b$ | 7.01±0.23$^b$ |

Values are mean±SD for four replicates, each being a pool of three ejaculates. Superscript letters (a or b) in the same row indicates significant difference among extenders ($P$<0.05).

# DISCUSSION

During cryopreservation, canine sperm have to encounter the deleterious effects of oxidative stress due to the high production of reactive oxygen species (ROS). These effects can damage sperm structures such as lipids, proteins, and DNA (Lamirande et al., 1997; Aitken, 2017). The supplementation of antioxidants in semen extenders can protect canine sperm against the oxidation effects (Vieira et al., 2017). Several studies were conducted to evaluate the effects of various antioxidants on canine sperm against the deleterious of oxidation with useful outcomes (Monteiro et al., 2009; Neagu et al., 2009; Wittayarat et al., 2013; Ogata et al., 2015; Lucio et al., 2016), or inefficient results (Thiangtum et al., 2012; Andersen et al., 2018). Vitamin E and green tea polyphenols have been used to improve sperm quality in animals and had positive effects (Michael et al., 2009; Satorre et al., 2012; Wittayarat et al., 2013; Vasconcelos Franco et al., 2016; Gadani et al., 2017; Nouri et al., 2018), while no study has investigated the impact of essential oils from *Ocimum gratissimum* on sperm as a herbal antioxidant. Our study has found that the Tris-citric-fructose-mineral salts egg-yolk extender had a positive influence on canine sperm quality during chilling storage (Vui et al., 2019). This study was implemented to determine the outcome of the supplement of *Ocimum gratissimum* essential oil, green tea polyphenols, and vitamin E to Tris-citric-fructose-mineral salts egg-yolk extender on frozen canine sperm.

The present study showed that the addition of *Ocimum gratissimum* essential oil, vitamin E, and green tea polyphenols in semen extender could not impact the sperm motility parameters, but the antioxidant extenders could improve the quality of frozen-thawed canine sperm in the mitochondrial membrane potential, plasma membrane, and acrosome membrane integrity parameters as compared to the control extender. In particular, the treatment with adding *Ocimum gratissimum* essential oil was superior to the other extenders and significantly better than the control group in improving the quality of frozen-thawed canine sperm. In addition, the eugenol extender, as a control for the concentration of eugenol in the *Ocimum gratissimum* essential oil extender, was not greater than the *Ocimum gratissimum* essential oil extender in all sperm quality parameters. This means that the beneficial influence of *Ocimum gratissimum* essential oil was not only from eugenol function but also from the other substances. Many studies have shown that the major composition of *Ocimum gratissimum* essential oil contained the various phytochemicals comprising eugenol, 1,8-cineole, thymol, α-bisabolene, β-selinene, and γ-terpinene (Monga et al., 2017; Kumar et al., 2019). These bioactive compounds have been proven to have strong antioxidant activity (Chiu et al., 2013; Mahapatra and Roy, 2014). In the present study, the antioxidant activity of these compounds may be represented not only by their antioxidant activity individually but also by the combination of these compounds to be a synergistic

antioxidant activity (Sonam and Guleria, 2017). These antioxidant properties of *Ocimum gratissimum* essential oil may consequently contribute directly or cooperate with the intercellular antioxidant system, including catalase, phospholipid hydro-peroxide glutathione peroxidase, glutathione peroxidase, and superoxide dismutase against oxidative stress (Neagu et al., 2011; Angrimani et al., 2014).

Besides antioxidant activity properties, the energy and mineral sources of *Ocimum gratissimum* essential oil may contribute to the improvement of frozen-thawed canine sperm quality during cryopreservation. In previous studies, it was found that the composition of *Ocimum gratissimum* essential oil had high carbohydrates and mineral elements such as calcium, sodium, potassium, manganese, magnesium, zinc, phosphorus, and iron (Idris et al., 2011; Igbinosa et al., 2013). These substances could support energy for sperm activities and maintain osmotic balance, as well as form parts of primary enzymes relating to sperm function and metabolism (Juyena and Stelletta, 2012; Smith et al., 2018).

In addition, although *Ocimum gratissimum* essential oil antioxidant activity was lower than that of vitamin E and green tea polyphenols in the same concentration, green tea polyphenols and vitamin E extenders were not better than *Ocimum gratissimum* essential oil extender in protecting the sperm mitochondrial membrane potential, the sperm plasma membrane and acrosome membrane integrity parameters. One explanation could be the fact that almost all bioactive compounds in *Ocimum gratissimum* essential oil have amphiphilic characteristics (Prabhu et al., 2009; Kumar et al., 2019), while vitamin E is a fat-soluble substance and green tea polyphenols are water-soluble compounds (Prasanth et al., 2019). Thus, *Ocimum gratissimum* essential oil and vitamin E can absorb in the sperm plasma membranes against lipid peroxidation during cryopreservation (Wang and Quinn, 1999; Aitken, 2017), while green tea polyphenols are restricted.

Moreover, the value of MDA was a principal indicator for lipid peroxidation during sperm cryopreservation (Toker et al., 2016; Vieira et al., 2017). The results demonstrated that adding the various antioxidants in the semen extender could protect frozen canine sperm to decrease lipid peroxidation by restraining MDA production. In addition, the concentration of MDA in the vitamin E and green tea polyphenols extenders were lower than that in the *Ocimum gratissimum* essential oil extender. These results were consistent with the antioxidant activity results of these various antioxidants in which the antioxidant activity of *Ocimum gratissimum* essential oil was significantly lower than that of vitamin E and green tea polyphenols. However, the sperm mitochondrial membrane potential, the sperm plasma membrane, and acrosome membrane integrity parameters in the *Ocimum gratissimum* essential oil treatment were better than those in the vitamin E and green tea polyphenols treatments. Especially, although the green tea polyphenols are hydrophilic compounds that may have limited contact with the sperm plasma membrane, the MDA value in this treatment was the lowest as compared to that in the rest treatments. This result may be explained by the fact that the antioxidant ability of the green tea polyphenols may only have a function in the semen extender and not act in the sperm. In this study, a canine sperm lipid peroxidation indicator was conducted to evaluate the lipid peroxidation of the frozen-thawed extended sperm. Thus, the results of this parameter may be shown in both the semen extender and inside the sperm. In fact, lipid peroxidation could occur within the sperm cell membrane and also within the dilution medium (Maia et al., 2010). As a result, the lipid peroxidation results were the total MDA production occurring in both the semen extender and inside the sperm. Therefore, the oxidation in the sperm dilution medium could noticeably impact the outcome of the sperm lipid peroxidation indicator. This study suggests that the combination of the water-soluble antioxidants with the fat-soluble or amphoteric antioxidants supplement in the canine semen extender may improve sperm lipid peroxidation during cryopreservation.

## CONCLUSIONS

In conclusion, the results showed that adding green tea polyphenols, vitamin E, and *Ocimum gratissimum* essential oil to semen extender has a protective impact on frozen-thawed canine sperm quality. In addition, the *Ocimum gratissimum* essential oil is superior to the vitamin E and green tea polyphenols, and has a significant protection for canine sperm during freezing storage.

## AUTHOR 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.

## CONFLICT OF INTEREST

We have no conflict of interest.

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