Research Article

Effects of Green Tea Polyphenols, Vitamin E, and *Ocimum gratissimum* Leaf Essential Oils as a Supplement to Extender on Chilled Canine Sperm Quality

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Abstract | During chilled storage oxidative stress is the primary cause for the reduction in the longevity of canine sperm. To prolong the chilled canine sperm quality, the study investigated the effects of the adding antioxidants to semen extender on canine sperm during 12 days of chilling storage. Twenty ejaculates from five American Bully dogs were used. The sperm quality was determined by system automatic sperm analyser for sperm motility parameters, and by confocal laser scanning microscope for sperm membrane integrity parameters. Sperm lipid peroxidation was also analysed through malondialdehyde production. The study found that there was a gradual decrease in all sperm quality indicators of all the treatments during 12 days of storage. In addition, the chilled sperm motility parameters among all the semen extenders were not noticeably different at each time period of evaluation (P>0.05). Although sperm lipid peroxidation index in the *Ocimum gratissimum* essential oils treatment was higher than those in the treatment of vitamin E and green tea polyphenols, the percentage of the high mitochondrial membrane potential, the intact plasma membrane and acrosome membrane indicators in the *Ocimum gratissimum* essential oils treatment were higher than those in the rest treatments and had a substantial difference when compared to the control treatment (P<0.05). In concluding, semen extender supplemented with *Ocimum gratissimum* essential oils is appropriate for improving the chilled canine sperm for the duration of 12 days in storage.

Keywords | Essential oils, Antioxidants, Canine sperm, Chilling

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INTRODUCTION

The use of artificial insemination (AI) techniques is currently popular in dog reproduction. Before using AI techniques, canine sperm quality must be maintained with a suitable semen extender during storage. Our study have illustrated that canine sperm have a high quality during chilling storage with adding of egg-yolk in the Tris-mineral salts extender (Vui et al., 2019). After dilution with extenders, preservation or cryopreservation was used to storage extended sperm (Thomassen and Farstad, 2009). However, the use of chilled canine sperm is more convenient and has higher fertilization rates, compared to that of frozen canine sperm (Linde-Forsberg, 1995; Eilts, 2005). A main problem of chilled canine sperm is the reduction of sperm quality during long periods of storage. Thus, studies to preserve the quality of chilled canine sperm is necessary. Due to being chilled in storage, canine sperm suffers from

deleterious effects from oxidative stress. This is because canine sperms' plasma membrane contains a high level of polyunsaturated fatty acids (Darin Bennett et al., 1974), which are susceptible to lipid oxidation when interacted to reactive oxygen species [ROS] (Vieira et al., 2017). Even though the ROS has physiological roles in sperm functions, such as hyperactivation, maturation, acrosome reaction, capacitation, and fertilization (Aitken, 2017), the high level of ROS could induce the damage of sperm structures encompassing DNA, lipids, proteins, and carbohydrates (Lucio et al., 2016; Aitken, 2017). Within normal conditions, the antioxidant defence mechanisms of living organisms can counteract the generation of ROS to prevent or minimise the negative effects of oxidation (Ighodaro and Akinloye, 2017). Almost all enzymatic antioxidants that are present in canine semen are from seminal plasma, including phospholipid hydro-peroxide glutathione peroxidase, catalase, glutathione peroxidase, and superoxide dismutase (Angrimani et al., 2014). However, seminal plasma of canine semen is separated before diluting with the semen extenders due to its negative effects on canine sperm quality (Hori et al., 2017). This may reduce the antioxidant defence mechanisms of canine sperm against the attack of ROS, therefore the addition of antioxidant elements in the canine semen extenders may expand the life of chilled sperm. Several studies have previously been carried out to evaluate the effects of various non-enzymatic and enzymatic antioxidants on canine sperm with positive outcomes (Michael et al., 2009; Neagu et al., 2009; Monteiro et al., 2009; Wittayarat et al., 2013; Ogata et al., 2015; Lucio et al., 2016), or ineffective results (Beccaglia et al., 2009; Sahashi et al., 2011; Andersen et al., 2018). Consequently, determining the appropriate antioxidant substance for canine sperm chilling is imperative for future AI success.

Additives such as vitamin E was considered due to it being a lipid-soluble antioxidant which plays a major role in inhibiting the free radicals by functioning as a chain-breaking antioxidant (Wang and Quinn, 1999; Dad et al., 2006). The supplementation of vitamin E has been conducted to improve the sperm quality of stallions (Almeida and Ball, 2005; Vasconcelos et al., 2013; Vasconcelos et al., 2014; Vasconcelos et al., 2016), boars (Cerolini et al., 2000; Jeong et al., 2009; Satorre et al., 2012), bulls (Asadpour, 2011), rams (Abdi-Benemar et al., 2015), canine studs (Michael et al., 2009), and roosters (Moghbeli et al., 2016).

Another additive such as green tea polyphenols are water-soluble antioxidants which contain a great variety of bioactive compounds including epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate. These compounds have been shown to have a powerful antioxidant by removing free radicals, such as hydroxyl radicals, alkyl radicals, peroxyl radicals (Coyle et al., 2008; Lamberta and Eliasa, 2010; Forester and Lambert, 2013). Several previous studies have illustrated that the addition of green tea polyphenols in semen extenders has had useful effects on sperm quality in various animals such as stallions (Nouri et al., 2018), boar (Gadani et al., 2017), ram (Mehdipour et al., 2016), and canine (Wittayarat et al., 2013).

For further study *Ocimum gratissimum* is a medicinal herb in which the leaf contains high level of essential oils (3.5%) (Trevisan et al., 2006). It has been found that the essential oil extract from *Ocimum gratissimum* were constituents of eugenol (30-70%), and the other phytochemicals such as β-selinene, thymol, α-bisabolene, 1,8-cineole, and γ-terpinene (Prabhu et al., 2009; Monga et al., 2017; Kumar et al., 2019). These bioactive substances are almost amphiphilic compounds which have strong antioxidant functions (Ouyang et al., 2012; Chiu et al., 2013; Mahapatra and Roy, 2014). Thus, it can be inferred that essential oils can protect canine sperm against sperm lipid peroxidation during chilling storage. The objective of the present study was to assess the effects of adding vitamin E, green tea polyphenols, and essential oils to the basic semen extender of Tris-mineral salts on canine sperm quality during preservation.

MATERIALS AND METHODS

Animals

Semen samples were collected from five healthy and sexually mature American Bully dogs. All dogs were demonstrated to be fertile after natural mating. The study was implemented with the guide of the Animal Care and Use Committee Institution of the Suranaree University of Technology, Thailand with license number SUT-IACUC 17/2560.

Canine Semen Collection

Canine semen were collected one a week from five dogs by manual manipulation technique as described by Linde-Forsberg (1991). A total of twenty ejaculates were used for four replications in this study. Before semen pooling, the sperm quality of each ejaculate was determined. Only ejaculates with high sperm quality as the following criteria were included in this study: sperm concentration $\geq 0.2\times10^7$ sperm/ml, sperm viability ≥90%, progressive motility ≥70%, and sperm abnormal morphology ≤5%. For sperm concentration and motility, the samples were evaluated using system automatic sperm analyser, while eosin-nigrosin staining was used to detect sperm morphology and viability (Tamuli and Watson, 1994).

Semen Extender Preparation

All chemicals from Sigma-Aldrich (Singapore) were used for semen extender preparation. Essential oils, vitamin

Table 1: The component of the semen extenders

Extender ingredients	Extenders				
	Control	Essential oils	Vitamin E	Green tea polyphenols	Eugenol
Tris (g)	0.9	0.9	0.9	0.9	0.9
Citric acid (g)	0.5	0.5	0.5	0.5	0.5
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	20	20	20	20
Essential oils (g)*	-	0.01	-	-	-
Vitamin E (g)	-	-	0.005	-	-
Green tea polyphenols (g)	-	-	-	0.005	-
Eugenol (mg)	-	-	-	-	0.26
Gentamycin (g)	0.2	0.2	0.2	0.2	0.2
DMSO (ml)	0.8	0.8	0.8	0.8	0.8
Distilled water (ml)	To 100	To 100	To 100	To 100	To 100
pH	6.57	6.56	6.57	6.58	6.57
Osmolality (mOsmol/kg)	475	484	483	479	486

* Essential oils extract from *Ocimum gratissimum* leaves.

E ((±)-α-Tocopherol, T3251), green tea polyphenols (Polyphenon 60, P1204), and eugenol (E51791) were used as various antioxidants. The essential oils were extracted from the *Ocimum gratissimum* leaves by soaking leaf powder in ethanol at room temperature. The solution was filtered through filter paper (No.1) and condensed using a rotary evaporator. Then, the condensed solution was separated by centrifuging. The bottom layer of solution was used as essential oils for the remainder of the study.

The basic extender egg-yolk in Tris-mineral salts, which has known benefits in chilled canine sperm (Vui et al., 2019), was used as the basis extender in this study. Five different extenders contained one of the various antioxidants, including essential oils (100 µg/ml), vitamin E (50 µg/ml), green tea polyphenols (50 µg/ml), and eugenol (2.6 µg/ml). The concentration of essential oils was the results of our previous study, while the level of vitamin E and green tea polyphenols were the results of our preliminary study (no data given). The concentration of eugenol used in this study was equal to the level of eugenol in essential oils extraction (2.6%), and the level of eugenol was detected using GC-MS. The extender without added antioxidants was used as a control. The components of these extenders are presented in Table 1. All antioxidants were diluted in dimethyl sulfoxide (DMSO) before the addition to the extenders, and each extender contained 0.8% DMSO.

Semen Processing And Experimental Design

After semen collection and initial evaluation, the ejaculates from all dogs were merged and divided into five equal groups. Then, semen was centrifuged at 720 × *g* for 5 minutes to remove seminal plasma (Rijsselaere et al., 2002). The sperm pellets were resuspended with five appreciate extenders to reach 0.1 x 10^7 sperm/ml. After that, each extended sperm (0.5 ml) was separated into microcentrifuge tubes and immersed in a styrofoam box of water at the temperature of 25°C. Next, the sperm samples were cooled down steadily to 5°C by adding ice at a cooling rate of 0.3°C/min (Bouchard et al., 1990). After cooling, samples were stored at 5°C. Sperm quality parameters were determined once every three days over a period of 12 days. Experimental design was a completely randomized design with repeated measurement. Four replicates and five ejaculates for each replicate were conducted.

Antioxidant Activity Of Various Antioxidant Agents

The antioxidant activity of various antioxidant agents was conducted using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay as previously described by Blois (1958). The solutions of various antioxidants (100 µl) were diluted in methanol and placed in a 96 wells micro plate. 100 µl DPPH solution in methanol (200 µM) was added to various antioxidant solutions. A control group was methanol and DPPH solution. Methanol (100 µl) with various antioxidants (100

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µl) was used as a blank. After 30 minutes of being kept in the dark, a spectrophotometric plate reader was used to measure the absorbance of the solution mixtures. The concentration of various antioxidants capable of inhibiting 50% (IC50) of DPPH solution activity were determined by a linear graph using the proportion of antioxidant activity and the concentration of various antioxidants.

Sperm Motility Evaluation

The system automatic sperm analyser (CASA; HTR-IVOS 14.0; Hamilton Thorne, USA) was applied to analyse the sperm motility parameters. The sperm samples were mixed with Tris buffer and incubated at 38°C (15 minutes) in a water bath before evaluation. After which the sperm samples (5 µl) were deposited into a warmed (38°C) 2X-CEL slide. There were 5 randomly selected fields for each chamber which were evaluated. The following sperm motility parameters were recorded: total motility percentage (TM%); progressive motility percentage (PM%); velocity curvilinear (VCL, µm/s); velocity straight line (VSL, µm/s); and velocity average pathway (VAP, µm/s).

Sperm Membrane Integrity Evaluation

To evaluate sperm membrane integrity of canine sperm, samples were stained with a fluorescent combination of fluorescein isothiocyanate–conjugated *Pisum sativum* agglutinin (FITC-PSA), Hoechst 33342 (H342), 5,5',6,6'-tetrachloro-11',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1), and propidium iodide (PI). The staining techniques were performed according to the previous technique (Vui et al., 2019). After staining, at least 200 sperm for each sample were immediately evaluated using a confocal microscope (Nikon/Ni-E, Japan). The stained sperm with high mitochondrial membrane potential, integral acrosome membrane and plasma membrane had bright red-orange in the mid-piece region and blue-stained nucleus, whereas the stained sperm with the low mitochondrial membrane potential, broken acrosome membrane and plasma membrane had bright green in the mid-piece region, yellow-green in the acrosome region, and red-stained nucleus. The identification of the stained sperm can be seen in Figure 1.

Sperm Lipid Peroxidation Evaluation

The canine sperm lipid peroxidation was determined using thiobarbituric acid reactive substance (TBARS) assay to calculate the malondialdehyde (MDA) production, according to the previous method by Maia et al. (2010). Before measuring the level of MDA, the sperm sample was induced into lipid peroxidation by adding 0.24 mM ferric sulphate and incubated for 15 minutes in a warm water (38°C). Then, 0.5 ml of the sperm sample was combined with 1 ml TBA reagent (thiobarbituric acid 0.375% (w/v), trichloroacetic acid 15% (w/v) in hydrochloric acid 0.25N) and 1% (v/v) 50 mM butylated hydroxytoluene. The mixture solution was boiled for 20 minutes. After that, it was cooled and centrifuged to separate the supernatant. The absorbance of supernatant was measured using a microplate spectrophotometer at 535 nm. The MDA level was calculated by comparing the absorbance of each sample at 535nm with a curve of MDA standard. The results were represented in nmol MDA/50x10^6 sperm.

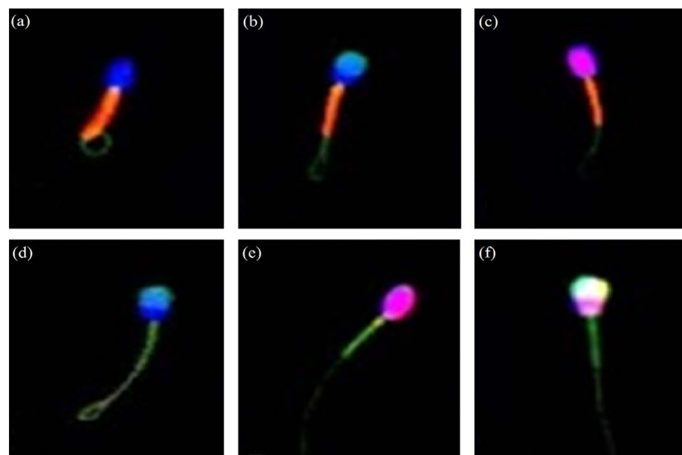

Figure 1: Canine sperm stained with the combination of H324, PI, FITC-PSA, and JC-1 under a confocal laser scanning microscope (600x magnification). (a) Sperm with high mitochondrial membrane potential, intact plasma and acrosome membrane. (b) Sperm with high mitochondrial membrane potential, intact plasma membrane, and damaged acrosome membrane, and. (c) Sperm with high mitochondrial membrane potential, damaged plasma membrane, and intact acrosome membrane. (d) Sperm with low mitochondrial membrane potential, intact plasma membrane, and damaged acrosome membrane. (e) Sperm with low mitochondrial membrane potential, damaged plasma membrane, and intact acrosome membrane. (f) Sperm with low mitochondrial membrane potential, damaged plasma and acrosome membrane.

Statistical Analysis

Statistical analysis was performed using IBM SPSS statistics, version 22. Analysis of variance (ANOVA) of two-factor mixed was used to analyse the effects of each factor and the interaction between 2 factors (period and treatment), and the Tukey method was applied for means multiple comparisons between groups in each factor. All data of the results are provided as mean ± standard deviation. A value of $P<0.05$ was set for significant difference.

RESULTS AND DISCUSSION

Antioxidant Activity Of Various Antioxidants

The antioxidant activity of the various antioxidants is presented in Table 2. The IC50 value of the *Ocimum gratissi*

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Table 2: The antioxidant activity of the various antioxidants

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}

IC50: the concentration of the various antioxidants capable of inhibiting 50% of DPPH activity. Values are mean ± SD. Superscript letters (a or b) in the same row indicates significant difference (P<0.05).

Table 3: The percentages of the total motility (TM%) and progressive motility (PM%) in the extended canine sperm with the addition of various antioxidants during 12 days at 5°C

Parameters	Extenders	Day1	Day3	Day6	Day9	Day12
TM (%)	Control	94.4±0.5^{A}	92.4±0.2^{B}	89.8±2.9^{B}	76.4±2.9^{C}	60.3±8.4^{D}
	Essential oils	95.0±0.8^{A}	94.1±1.1^{AB}	91.5±2.0^{B}	79.9±5.2^{C}	59.9±7.2^{D}
	Vitamin E	95.0±0.7^{A}	93.1±1.2^{B}	91.1±1.8^{B}	78.0±4.9^{C}	58.7±8.8^{D}
	Green tea polyphenols	94.2±0.6^{A}	92.8±1.4^{B}	90.9±3.3^{B}	78.6±6.6^{C}	56.1±9.7^{D}
	Eugenol	94.9±1.4^{A}	92.2±1.2^{B}	89.7±2.4^{B}	80.1±4.1^{C}	58.6±8.5^{D}
PM (%)	Control	72.8±1.5^{A}	68.8±3.1^{B}	62.7±3.0^{C}	43.7±6.7^{D}	23.8±3.2^{E}
	Essential oils	73.1±2.9^{A}	69.0±3.8^{B}	63.4±3.0^{C}	43.2±6.1^{D}	24.3±2.8^{E}
	Vitamin E	70.8±4.0^{A}	67.5±5.1^{AB}	62.7±6.2^{B}	44.6±9.9^{C}	21.9±3.2^{D}
	Green tea polyphenols	71.8±3.8^{A}	67.8±3.3^{B}	60.0±7.5^{C}	46.1±11.1^{D}	20.9±4.4^{E}
	Eugenol	70.9±3.8^{A}	68.6±4.9^{A}	63.1±4.9^{B}	45.2±8.3^{C}	23.8±2.7^{D}

Values are mean ± SD. Uppercase superscript letters (A, B, C, D or E) in the same row indicates significant difference within treatments with different periods (P<0.05).

Table 4: The results of the average pathway velocity (VAP), straight line velocity (VSL), and curvilinear velocity (VCL) in the extended canine sperm with the addition of various antioxidants during 12 days at 5°C

Parameters	Extenders	Day1	Day3	Day6	Day9	Day12
VAP (μm/s)	Control	93.2±7.9^{A}	87.8±6.6^{AB}	79.2±3.4^{B}	72.9±3.8^{C}	62.3±8.5^{D}
	Essential oils	93.2±5.3^{A}	89.4±7.0^{AB}	79.1±4.7^{B}	73.1±6.6^{C}	68.2±8.4^{C}
	Vitamin E	93.6±7.6^{A}	87.8±10.0^{AB}	80.1±5.6^{B}	73.0±4.8^{C}	67.0±6.2^{C}
	Green tea polyphenols	94.3±7.1^{A}	88.9±6.7^{AB}	79.3±6.0^{B}	73.0±5.5^{C}	63.9±8.8^{D}
	Eugenol	96.2±8.0^{A}	92.2±7.5^{AB}	82.7±5.1^{B}	75.4±3.7^{C}	66.8±5.6^{D}
VSL (μm/s)	Control	86.6±7.0^{A}	80.9±7.9^{A}	66.9±5.6^{B}	59.6±3.4^{C}	48.2±7.3^{D}
	Essential oils	86.6±5.5^{A}	80.1±7.7^{B}	68.4±2.9^{B}	60.4±4.6^{C}	54.0±9.9^{C}
	Vitamin E	84.0±6.7^{A}	80.9±6.5^{A}	72.4±3.1^{A}	64.9±4.0^{B}	53.2±5.2^{C}
	Green tea polyphenols	86.0±6.2^{A}	79.3±5.3^{B}	69.5±3.1^{B}	62.0±3.6^{C}	52.5±9.6^{D}
	Eugenol	89.0±8.1^{A}	83.5±5.1^{AB}	71.4±6.5^{B}	60.5±2.6^{C}	54.2±5.1^{C}
VCL (μm/s)	Control	136.7±0.7^{A}	134.6±1.6^{A}	133.3±1.5^{A}	130.1±2.1^{B}	122.6±2.7^{C}
	Essential oils	138.1±2.6^{A}	134.8±4.6^{B}	131.6±5.3^{B}	129.5±5.3^{C}	122.2±6.2^{D}
	Vitamin E	137.7±3.9^{A}	134.7±3.1^{B}	131.5±4.1^{B}	127.8±4.9^{C}	124.8±4.1^{C}
	Green tea polyphenols	136.7±2.9^{A}	134.5±4.3^{AB}	131.3±5.6^{B}	129.0±6.7^{C}	119.6±11.8^{D}
	Eugenol	137.5±4.8^{A}	135.9±4.2^{A}	133.8±4.1^{AB}	131.9±5.0^{B}	126.9±5.3^{B}

Values are mean ± SD. Uppercase superscript letters (A, B, C, or D) in the same row indicates significant difference within treatments with different periods (P<0.05).

Table 5: The percentages of high mitochondrial membrane potential, intact plasma membrane and acrosome membrane parameters in the extended canine sperm with the addition of various antioxidants during 12 days at 5°C

Parameters	Extenders	Day1	Day3	Day6	Day9	Day12
Plasma membrane (%)	Control	76.3±2.9^{bA}	73.4±3.5^{bA}	63.9±2.5^{bB}	53.0±2.9^{bC}	40.4±3.1^{bD}
	Essential oils	81.9±1.4^{aA}	80.4±1.6^{aA}	72.6±3.7^{aB}	61.2±1.8^{aC}	46.7±3.0^{aD}
	Vitamin E	80.7±1.0^{abA}	78.0±2.0^{abA}	71.0±2.8^{abB}	58.3±1.1^{abC}	48.9±1.1^{aD}

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	Green tea polyphenols	80.2±1.4^{abA}	76.1±1.7^{abB}	68.9±3.5^{abC}	57.4±3.9^{abD}	47.8±1.4^{aE}
	Eugenol	78.9±3.3^{abA}	75.2±2.5^{abB}	69.0±3.1^{abC}	54.3±3.9^{bD}	46.9±2.5^{aE}
Acrosome membrane (%)	Control	68.5±1.8^{bA}	64.4±2.7^{bB}	55.3±4.8^{bC}	39.4±3.4^{bD}	24.1±3.6^{cE}
	Essential oils	75.0±2.1^{aA}	73.4±2.4^{aA}	66.7±2.5^{aB}	57.7±2.1^{aC}	42.6±2.1^{aD}
	Vitamin E	73.1±2.1^{aA}	69.2±4.9^{abB}	61.6±3.8^{abC}	46.0±6.3^{bD}	36.2±2.3^{bE}
	Green tea polyphenols	73.9±1.6^{aA}	70.0±2.7^{abB}	62.2±3.7^{abC}	46.3±5.0^{bD}	38.5±2.8^{abE}
	Eugenol	74.3±2.2^{aA}	69.4±2.4^{abB}	61.1±1.5^{abC}	44.5±3.7^{bD}	37.8±2.0^{abE}
Mitochondrial membrane potential (%)	Control	80.7±2.3^{bA}	74.8±3.9^{bB}	65.0±3.0^{cC}	55.5±1.8^{cD}	40.2±0.9^{bE}
	Essential oils	87.2±1.7^{aA}	85.0±2.0^{aA}	78.2±1.4^{aB}	66.6±2.1^{aC}	51.1±2.7^{aD}
	Vitamin E	85.5±1.5^{aA}	82.1±1.9^{aB}	74.5±2.1^{abC}	61.7±1.4^{bD}	55.2±2.5^{aE}
	Green tea polyphenols	85.9±0.7^{aA}	81.5±3.3^{aB}	72.6±2.5^{abC}	59.7±2.5^{bcD}	52.0±2.6^{aE}
	Eugenol	86.0±2.3^{aA}	81.0±1.7^{aB}	70.1±5.1^{bcC}	59.0±2.4^{bcD}	50.0±2.7^{aE}

Values are mean ± SD. Lowercase superscript letters (a, b or c) in the same column indicates significant difference among treatments (P<0.05) and uppercase superscript letters (A, B, C, D or E) in the same row indicates significant difference within treatments with different periods (P<0.05).

Table 6: The percentages of the sperm with high mitochondrial membrane potential, intact plasma membrane and acrosome membrane in the extended canine sperm with the addition of various antioxidants during 12 days at 5°C

Extenders	Day1	Day3	Day6	Day9	Day12
Control	66.5±1.7^{bA}	59.0±3.9^{bB}	48.7±4.2^{bC}	32.1±2.5^{bD}	19.3±1.5^{bE}
Essential oils	73.1±3.3^{aA}	70.1±2.2^{aA}	60.2±5.7^{aB}	46.4±3.4^{aC}	32.2±1.4^{aD}
Vitamin E	71.6±1.9^{abA}	66.1±3.7^{abB}	58.0±3.7^{abC}	41.9±4.1^{aD}	31.1±2.3^{aE}
Green tea polyphenols	71.7±2.2^{abA}	65.9±2.0^{abB}	55.8±4.7^{abC}	40.6±1.8^{aD}	30.0±2.7^{aE}
Eugenol	72.2±2.3^{abA}	64.3±3.1^{abB}	54.7±2.2^{abC}	40.5±1.9^{aD}	31.2±2.7^{aE}

Values are mean ± SD. Lowercase superscript letters (a or b) in the same column indicates significant difference among treatments (P<0.05) and uppercase superscript letters (A, B, C, D or E) in the same row indicates significant difference within treatments with different periods (P<0.05).

Table 7: The levels of the malondialdehyde (MDA) (nmol/50x10^6 sperm) of the extended canine sperm with the addition of various antioxidants during 12 days at 5°C

Extenders	Day1	Day6	Day12
Control	7.38±0.23^{aA}	6.68±0.31^{aB}	7.26±0.51^{aA}
Essential oils	6.29±0.40^{bA}	5.83±0.23^{bB}	6.28±0.46^{bA}
Vitamin E	6.21±0.60^{bA}	5.74±0.25^{bB}	6.21±0.39^{bA}
Green tea polyphenols	6.15±0.52^{bA}	5.69±0.48^{bB}	6.13±0.42^{bA}
Eugenol	6.54±0.38^{abA}	5.93±0.25^{bB}	6.46±0.13^{abA}

Values are mean ± SD. Lowercase superscript letters (a or b) in the same column indicates significant difference among treatments (P<0.05) and uppercase superscript letters (A or B) in the same row indicates significant difference within treatments with different periods (P<0.05).

mum essential oils (263.63±11.52 µg/ml) was noticeably higher than that of 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 essential oils from *Ocimum gratissimum* was lower than that of the vitamin E, eugenol, and green tea polyphenols in the same concentration.

Sperm Motility

Sperm motility of canine sperm are shown in Table 3. On the whole, the results of the sperm total motility and progressive motility parameters in all the antioxidant treatments reduced gradually during the time of storage (12 days). In addition, there were no substantial differences among the treatments at each period time in these parameters. Beside that sperm velocity including velocity curvilinear (VCL), velocity straight line (VSL), and velocity average pathway (VAP) are given in Table 4. The trend values of the VSL, VCL, and VAP parameters were consistent with those of the total motility and progressive motility indicators during 12 days of storage. These parameters also reduced steadily during storage, and there were parallel val-

ues among the treatments at each period of storage time.

Sperm Membrane Integrity

Table 5 summarises the results of mitochondrial membrane potential, plasma membrane and acrosome membrane integrity parameters in canine sperm during 12 days of storage. In general, the percentages of the high mitochondrial membrane potential, integral plasma membrane, and integral acrosome membrane in the essential oils extender were the highest and had a notable difference compared with those in the control extender (P<0.05). For the plasma membrane integrity, although the essential oil extender was superior to the other extenders, the values of the intact plasma membrane in the essential oils extender were not markedly different compared to that in the rest extenders. Moreover, the proportions of intact plasma membrane in the eugenol, green tea polyphenols, and vitamin E extenders were not substantially greater than that in the control treatment. For the acrosome integrity, the value of this parameter in the essential oils extender on day nine was significantly higher than that in the vitamin E (57.7±2.1 vs. 46.0±6.3, P=0.018), in the green tea polyphenols (57.7±2.1 vs. 46.3±5.0, P=0.02), and in the eugenol extender (57.7±2.1 vs. 44.5±3.7, P=0.006). Notably the percentages of this parameter in the all antioxidant extenders were significantly different as compared to that in the control extender on day 1 and day 12 (P<0.05). For the mitochondrial membrane potential, during the whole storage period, the percentages of this parameter in the control group were evidently lower than those in the other extenders (P<0.05), except on day nine in the green tea polyphenols and eugenol extenders.

The proportions of the sperm with an intact membrane are presented in Table 6. As with the former parameters, the values of the sperm with high mitochondrial membrane potential, intact plasma membrane and acrosome membrane in the essential oils extender were sizable and substantially greater than those in the control treatment (P<0.05), the percentages of this parameter in the other antioxidant extenders were also significantly different compared to those in the control group from day 9 to day 12 (P<0.05).

Sperm Lipid Peroxidation

Table 7 presents the concentration of the malondialdehyde (MDA) of canine sperm with the addition of various antioxidants during storage. The levels of the MDA in all the extenders reduced gradually during the first of 6 days and then increased steadily to day 12. The MDA values in all the antioxidant treatments, except the eugenol extender on day 1 and day 12, were significantly less than those in the control extender. Although the concentrations of the MDA in the green tea polyphenols and vitamin E extenders were the lowest, the differences were not prominent compared to those in the essential oils extender.

The overproduction of ROS in canine sperm during storage can cause oxidative stress, which leads to damage of the sperm membrane as well as a reduction in sperm longevity (Moustafa et al., 2004; Lucio et al., 2016; Aitken, 2017). In former studies, some non-enzymatic and enzymatic antioxidant substances have been used to enhance the canine sperm quality during storage against the harmfulness of oxidative stress with various outcomes (Michael et al., 2009; Neagu et al., 2009; Monteiro et al., 2009; Sahashi et al., 2011; Thiangtum et al., 2012; Wittayarat et al., 2013; Ogata et al., 2015; Andersen et al., 2018). In particular, vitamin E and green tea polyphenols usage has shown positive effects on the sperm quality of animals (Jeong et al., 2009; Michael et al., 2009; Asadpour, 2011; Satorre et al., 2012; Vasconcelos et al., 2013; Wittayarat et al., 2013; Vasconcelos et al., 2014; Abdi-Benemar et al., 2015; Vasconcelos et al., 2016; Moghbeli et al., 2016; Mehdipour et al., 2016; Gadani et al., 2017; Nouri et al., 2018), whereas *Ocimum gratissimum* essential oils has never been used for sperm preservation as a herbal antioxidant substance. Our previous study has showed that the basic extender Tris-mineral salts egg-yolk could significantly improve the quality of canine sperm during chilling storage (Vui et al., 2019). This study investigated the effects of these antioxidant substances supplementation in Tris-mineral salts-egg yolk extender on chilled canine sperm.

The present study showed that although there was not a significantly difference among the treatments in the sperm motility parameters, the antioxidant treatments could improve the sperm membrane integrity parameters of canine sperm during 12 days chilling storage compared with the control treatment. Remarkably, the essential oils extract from *Ocimum gratissimum* extender was superior to the rest of the extenders, including the eugenol extender (as a control for the level of eugenol in *Ocimum gratissimum* essential oils), and greatly improved the quality of chilled canine sperm compared to the control group. This means that the composition of *Ocimum gratissimum* essential oils contain not only eugenol, but also other substances which have a useful impact on canine sperm quality. The primary constituents of *Ocimum gratissimum* essential oils are discovered to contain various bioactive compounds such as eugenol, β-selinene, thymol, α-bisabolene, 1,8-cineole, and γ-terpinene (Prabhu et al., 2009; Monga et al., 2017; Kumar et al., 2019), which have the function of antioxidant activity (Ouyang et al., 2012; Chiu et al., 2013; Mahapatra and Roy, 2014). These compounds may contribute not only their antioxidant activity individually, but also create a synergistic antioxidant activity among these compounds (Sonam and Guleria, 2017). As a result, these antioxidant

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properties may support the sperm intracellular antioxidant system to defend sperm against lipid oxidation (Neagu et al., 2011; Angrimani et al., 2014).

In addition, the antioxidant activity of vitamin E and green tea polyphenols were notably higher than that of essential oils, but the mitochondrial membrane potential, the plasma membrane and the acrosome membrane integrity parameters of sperm in the essential oils treatment were greater than those in the green tea polyphenols and vitamin E treatments. This may be explained by the fact that *Ocimum gratissimum* essential oils contains various bioactive compounds which have both hydrophilic and lipophilic characteristics (Prabhu et al., 2009; Kumar et al., 2019), while vitamin E is a lipophilic substance and green tea polyphenols are hydrophilic compounds (Prasanth et al., 2019). Therefore, *Ocimum gratissimum* essential oils and vitamin E can insert themselves in the lipid bilayer of sperm plasma membranes to protect against ROS and lipid membrane peroxidation (Wang and Quinn, 1999; Aitken, 2017), whereas green tea polyphenols are limited in this ability. Besides antioxidant properties, the valuable impacts of *Ocimum gratissimum* essential oils in chilled canine sperm quality may be due to its energy and mineral sources. Previous studies in phytochemical and nutritional properties have illustrated that *Ocimum gratissimum* essential oils contained high carbohydrates and mineral elements such as calcium, sodium, potassium, magnesium, manganese, zinc, iron, phosphorus (Idris et al., 2011; Igbinosa et al., 2013). These compositions could support energy and protect sperm plasma membrane as well as assist sperm metabolism and sperm function (Juyena and Stelletta, 2012; Smith et al., 2018).

Moreover, the malondialdehyde (MDA) is the major by-product of lipid peroxidation, the concentration of MDA indicates the oxidative damage level in sperm (Toker et al., 2016; Vieira et al., 2017). This study summates that the addition of *Ocimum gratissimum* essential oils, green tea polyphenols, and vitamin E in the extender could inhibit the formation of MDA in sperm compared to the control group. Additionally, although the sperm quality in the *Ocimum gratissimum* essential oils group was better than that in the green tea polyphenols and vitamin E groups, the level of MDA in the green tea polyphenols and vitamin E groups were lower than that of the essential oils group. These outcomes were consistent with the results of antioxidant activity, in which the antioxidant activity of the green tea polyphenols and vitamin E were significantly higher than that of the *Ocimum gratissimum* essential oils. In particular, green tea polyphenols are water-soluble which may have restricted the activity in the sperm plasma membrane, but the level of oxidative damage in this extender was the lowest in comparison to the other extenders. This may be explained by the green tea polyphenols having substantial antioxidant activity in the semen extender, but it was limited in the sperm. The lipid peroxidation parameter in the present study was conducted to evaluate the lipid peroxidation of the extended sperm, therefore the evaluation was performed in both the sperm cells and semen extender. However, the lipid peroxidation occurred not only on the sperm, but also on the extender (Maia et al., 2010). Consequently, the sperm lipid peroxidation results were the total concentration of MDA in both the sperm and the extender, and he level of MDA produced from the semen extender could have an important influence in the sperm lipid peroxidation results. This study suggests that the combination of the water-soluble antioxidant compounds with the lipid-soluble or amphiphilic antioxidant compounds may provide potential antioxidant activity for both the sperm cells and semen extenders.

CONCLUSIONS

In conclusion, the results presented that the supplementation of *Ocimum gratissimum* essential oils, green tea polyphenols, and vitamin E in the Tris-mineral salts egg-yolk extender for chilling have positive effects on canine sperm quality. Especially, the *Ocimum gratissimum* essential oils extender produced the best results and has shown a significant improvement in chilled canine sperm quality during 12 days of storage.

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CONFLICT OF INTEREST

Authors declared no conflict of interest.

NOVELTY STATEMENT

The utilization of essential oil derived from *Ocimum gratissimum* leaves in canine semen extenders is a novel approach explored in this study. The research findings demonstrate the beneficial effects of these essential oils on dog sperms, indicating their potential application in practical breeding procedures.

AUTHORS CONTRIBUTION

P.K. and S.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 manuscript; all authors reviewed and approved the final manuscript

REFERENCES

Abdi-Benemar H, Jafaroghli M, Khalili B, Zamiri MJ, Ezazi H, Shadparvar AA (2015). Effects of DHA supplementation of the extender containing egg yolk and α- tocopherol on the freezability and post-thawing fertility of ram semen. Small Rumin. Res., 130: 166–170. https://doi.org/10.1016/j.smallrumres.2015.06.013.

Aitken RJ (2017). Reactive oxygen species as mediators of sperm capacitation and pathological damage. Mol. Reprod. Dev., 84: 1039–1052. https://doi.org/10.1002/mrd.22871.

Almeida J, Ball BA (2005). Effect of α-tocopherol and tocopherol succinate on lipid peroxidation in equine spermatozoa. Anim. Reprod. Sci., 87: 321–337. https://doi.org/10.1016/j.anireprosci.2004.12.004.

Andersen AH, Thinnesen M, Failing K, Goericke-Pesch S (2018). Effect of reduced glutathione (GSH) supplementation to Tris-egg yolk extender on chilled semen variables of dogs. Anim. Reprod. Sci., 198: 145–153. https://doi.org/10.1016/j.anireprosci.2018.09.013.

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.

Asadpour R (2011). Influence of Added Vitamin C and Vitamin E on Frozen-Thawed Bovine Sperm Cryopreserved in Citrate and Tris-Based Extenders. Vet. Res. Forum, 2: 37–44.

Beccaglia M, Anastasi P, Chigioni S, Luvoni GC (2009). Tris-lecithin extender supplemented with antioxidant catalase for chilling of canine semen. Reprod. Domest. Anim., 44: 345–349. https://doi.org/10.1111/j.1439-0531.2009.01410.x.

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.

Cerolini S, Maldjian A, Surai P, Noble R, (2000). Viability, susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. Anim. Reprod. Sci., 58: 99–111. https://doi.org/10.1016/S0378-4320(99)00035-4.

Chiu YW, Lo HJ, Huang HY, Chao PY, Hwang JM, Huang PY, Huang SJ, Liu JY, Lai TJ, (2013). The antioxidant and cytoprotective activity of, *Ocimum gratissimum* extracts against hydrogen peroxide-induced toxicity in human HepG2 cells. J. Food Drug Anal., 21: 253–260. https://doi.org/10.1016/j.jfda.2013.07.002.

Coyle CH, Philips BJ, Morrisroe SN, Chancellor MB, Yoshimura N (2008). Antioxidant Effects of Green Tea and Its Polyphenols on Bladder Cells. Life Sci, 83: 12–18. https://doi.org/10.1038/jid.2014.371.

Dad S, Bisby RH, Clark IP, Parker AW (2006). Formation of singlet oxygen from solutions of vitamin E. Free Radic. Res., 40: 333–338. https://doi.org/10.1080/10715760500491174.

Darin Bennett A, Poulous A, White IG (1974). The phospholipids and phospholipid bound fatty acids and aldehydes of dog and fowl spermatozoa. J. Reprod. Fertil., 41: 471–474. https://doi.org/10.1530/jrf.0.0410471.

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

Forester SC, Lambert JD (2013). Antioxidant effects of green tea. Mol Nutr Food Res, 55: 844–854. https://doi.org/10.1002/mnfr.201000641.Antioxidant.

Gadani B, Bucci D, Spinaci M, Tamanini C, Galeati G (2017). Resveratrol and Epigallocatechin-3-gallate addition to thawed boar sperm improves in vitro fertilization. Theriogenology, 90: 88–93. https://doi.org/10.1016/j.theriogenology.2016.11.020.

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.

Idris S, Iyaka YA, Ndamitso MM, Paiko YB (2011). Nutritional Composition of the Leaves and Stems of *Ocimum gratissimum*. J. Emerg. Trends Eng. Appl. Sci., 2: 801–805.

Igbinosa EO, Uzunuigbe EO, Igbinosa IH, Odjadjare EE, Igiehon NO, Emuedo OA (2013). In vitro assessment of antioxidant, phytochemical and nutritional properties of extracts from the leaves of *Ocimum gratissimum* (Linn). African J. Tradit. Complement. Altern. Med., 10: 292–298.

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.

Jeong YJ, Kim MK, Song HJ, Kang EJ, Ock SA, Mohana Kumar B, Balasubramanian S, Rho GJ (2009). Effect of α-tocopherol supplementation during boar semen cryopreservation on sperm characteristics and expression of apoptosis related genes. Cryobiology, 58: 181–189. https://doi.org/10.1016/j.cryobiol.2008.12.004.

Juyena NS, Stelletta C, (2012). Seminal plasma: An essential attribute to spermatozoa. J. Androl., 33: 536–551. https://doi.org/10.2164/jandrol.110.012583.

Kumar A, Mishra P, Rodrigues V, Baskaran K, Verma RS, Padalia RC, Sundaresan V (2019). Delineation of *Ocimum gratissimum* L. complex combining morphological, molecular and essential oils analysis. Ind. Crops Prod., 139: 1–8. https://doi.org/10.1016/j.indcrop.2019.111536.

Lamberta JD, Eliasa RJ (2010). The antioxidant and pro-oxidant activities of green tea polyphenols: a role in cancer prevention. Arch Biochem Biophys, 50: 65–72. https://doi.org/10.1038/jid.2014.371.

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 (2016a). Oxidative stress at different stages of two-step semen cryopreservation procedures in dogs. Theriogenology, 85: 1568–1575. https://doi.org/10.1016/j.theriogenology.2016.01.016.

Lucio CF, Regazzi FM, Silva LCG, Angrimani DSR, Nichi M, Vannucchi CI (2016b). Oxidative stress at different

Links Researchers

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 antioxidative potential of eugenol and *Ocimum gratissimum* Linn. Asian Pac. J. Trop. Med., 7: 391–397. 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.

Mehdipour M, Daghigh Kia H, Najafi A, Vaseghi Dodaran H, García-Álvarez O (2016). Effect of green tea (*Camellia sinensis*) extract and pre-freezing equilibration time on the post-thawing quality of ram semen cryopreserved in a soybean lecithin-based extender. Cryobiology, 73: 297–303. https://doi.org/10.1016/j.cryobiol.2016.10.008.

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.

Moghbeli M, Kohram H, Zare-Shahaneh A, Zhandi M, Sharafi M, Nabi MM, Zahedi V, Sharideh H, (2016). Are the optimum levels of the catalase and vitamin E in rooster semen extender after freezing-thawing influenced by sperm concentration? Cryobiology, 72: 264–268. https://doi.org/10.1016/j.cryobiol.2016.03.008.

Monga S, Dhanwal P, Kumar R, Kumar A, Chhokar V (2017). Pharmacological and physico-chemical properties of Tulsi (*Ocimum gratissimum* L.): An updated review. Pharma Innov., 6: 181–186.

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.

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.

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.

Nouri H, Shojaeian K, Samadian F, Lee S, Kohram H, Lee JI (2018). Using Resveratrol and Epigallocatechin-3-Gallate to Improve Cryopreservation of Stallion Spermatozoa With Low Quality. J. Equine Vet. Sci., 70: 18–25. https://doi.org/10.1016/j.jevs.2018.07.003.

Ogata K, Sasaki A, Kato Y, Takeda A, Wakabayashi M, Sarentonglaga B, Yamaguchi M, Hara A, Fukumori R, Nagao Y, (2015). Glutathione supplementation to semen extender improves the quality of frozen-thawed canine spermatozoa for transcervical insemination. Reprod. Dev., 61: 116–122.

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 AA, 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.

Prasanth MI, Sivamaruthi BS, Chaiyasut C, Tencomnao T (2019). A review of the role of green tea (*Camellia sinensis*) in antiphotoaging, stress resistance, neuroprotection, and autophagy. Nutrients, 11: 1–24. https://doi.org/10.3390/nu11020474.

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.

Sahashi Y, Otsuki T, Higaki S, Nagano M, Yamashita Y, Hishinuma M (2011). Effect of butylated hydroxytoluene on dog sperm longevity in chilling storage and cryopreservation. J. Vet. Med. Sci., 73: 895–899. https://doi.org/10.1292/jvms.10-0460.

Satorre MM, Breininger E, Beconi MT (2012). Cryopreservation with α-tocopherol and Sephadex filtration improved the quality of boar sperm. Theriogenology, 78: 1548–1556. https://doi.org/10.1016/j.theriogenology.2012.06.023.

Smith AMJ, Bonato M, Dzama K, Malecki IA, Cloete SWP (2018). Mineral profiling of ostrich (*Struthio camelus*) seminal plasma and its relationship with semen traits and collection day. Anim. Reprod. Sci., 193: 98–106. https://doi.org/10.1016/j.anireprosci.2018.04.004.

Sonam KS, Guleria S (2017). Synergistic antioxidant activity of natural products. Ann. Pharmacol. Pharm., 2: 1–6.

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). 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.

Vasconcelos Franco JS, Chaveiro A, Góis A, Moreira da Silva F

(2013). Effects of α-tocopherol and ascorbic acid on equine semen quality after cryopreservation. J. Equine Vet. Sci., 33: 787–793. https://doi.org/10.1016/j.jevs.2012.12.012.

Vasconcelos Franco JS, Chaveiro A, da Silva FM (2014). Effect of freezing rates and supplementation of α-tocopherol in the freezing extender in equine sperm cryosurvival. J. Equine Vet. Sci., 34: 992–997. https://doi.org/10.1016/j.jevs.2014.05.007.

Vasconcelos Franco JS de, Faheem M, Chaveiro A, Silva FM da (2016). Effects of α-tocopherol and freezing rates on the quality and heterologous in vitro fertilization capacity of stallion sperm after cryopreservation. Theriogenology, 86: 957–962. https://doi.org/10.1016/j.theriogenology.2016.03.019.

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.

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.

Wang X, Quinn PJ (1999). Vitamin E and its function in membranes. Prog. Lipid Res., 38: 309–336. https://doi.org/10.1016/S0163-7827(99)00008-9.

Wittayarat M, Ito A, Kimura T, Namula Z, Luu VV, Do LTK, Sato Y, Taniguchi M, Otoi T (2013). Effects of green tea polyphenol on the quality of canine semen after long-term storage at 5°C. Reprod. Biol., 13: 251–254. https://doi.org/10.1016/j.repbio.2013.07.006.