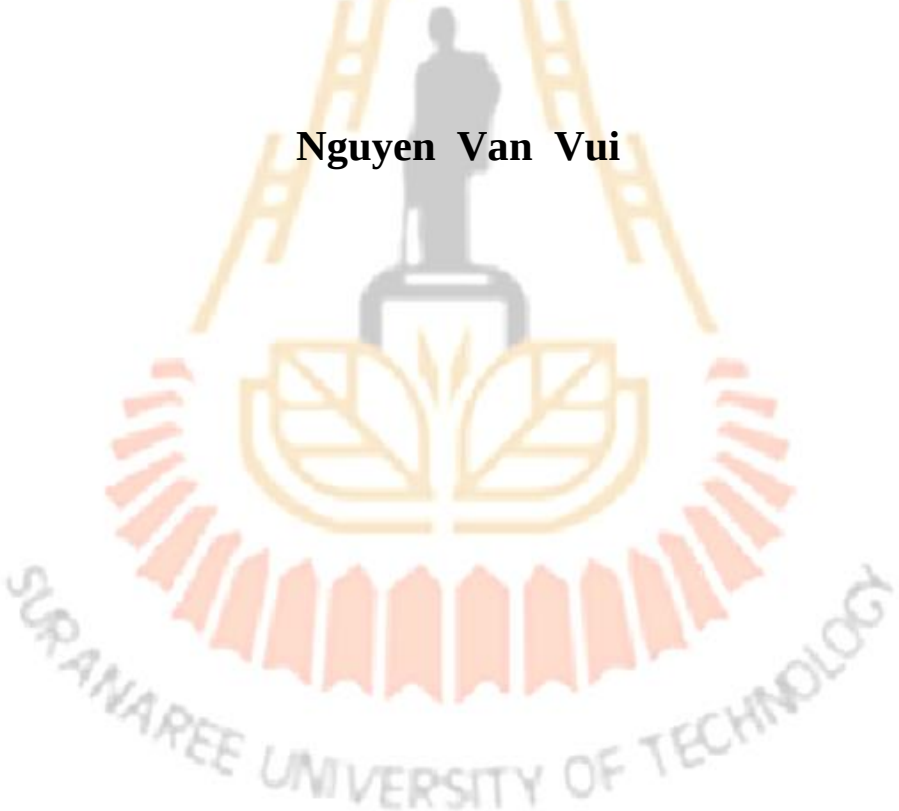
EFFECTS OF *OCIMUM GRATISSIMUM* LEAF ESSENTIAL OILS, VITAMIN E, AND GREEN TEA POLYPHENOLS AS A SUPPLEMENT TO SEMEN EXTENDER ON CHILLED AND FROZEN CANINE SPERM QUALITY

Nguyen Van Vui



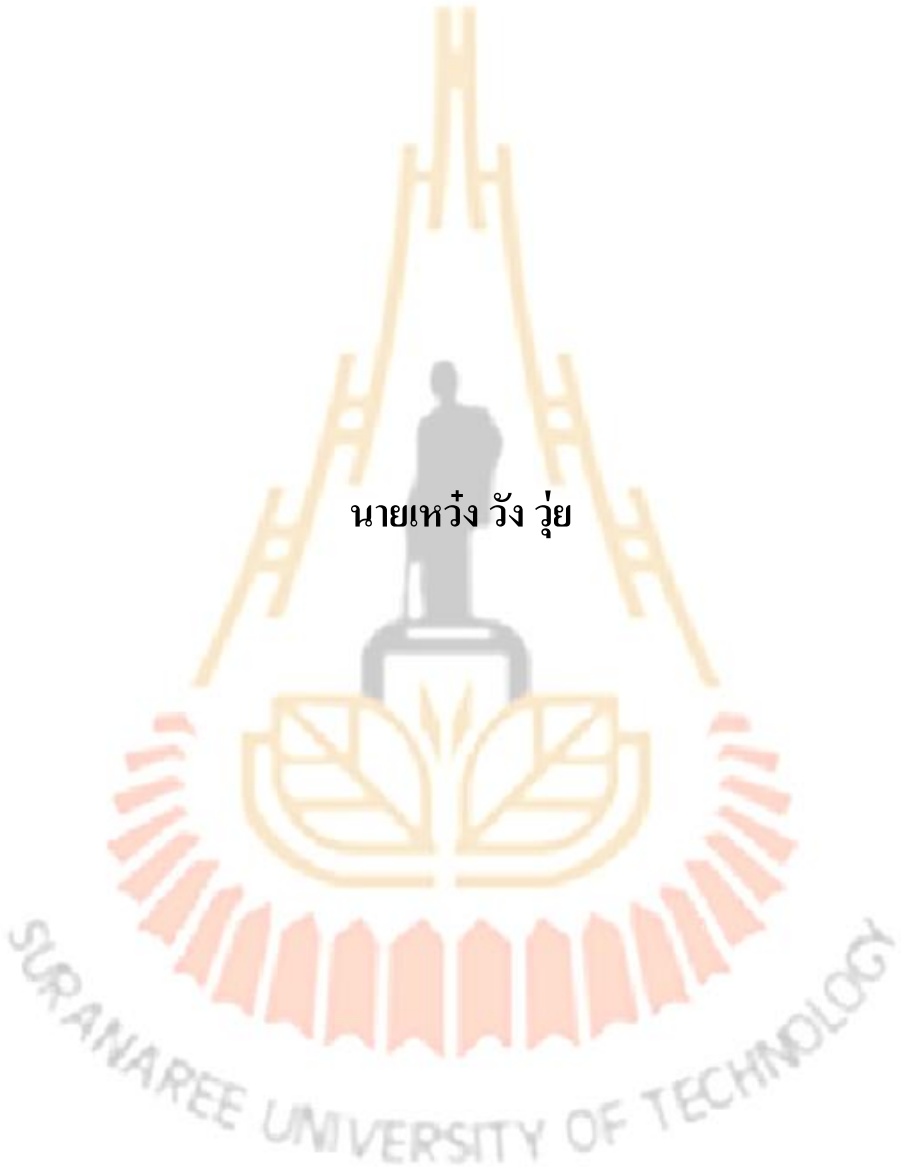
A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Animal Production Technology

Suranaree University of Technology

Academic Year 2019

ผลของน้ำมันหอมระเหยจากใบกะเพรา วิตามินอี และโพลีฟีนอลจากชาเขียว ที่เสริมในสารรักษาสภาพน้ำเชื้อต่อคุณภาพตัวอสุจิสุนัข ในแบบแช่เย็น และแช่แข็ง

นายเหว่ง วัง วุ่ย



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาปรัชญาดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีการผลิตสัตว์
มหาวิทยาลัยเทคโนโลยีสุรนารี
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EFFECTS OF *OCIMUM GRATISSIMUM* LEAF ESSENTIAL OILS, VITAMIN E, AND GREEN TEA POLYPHENOLS AS A SUPPLEMENT TO SEMEN EXTENDER ON CHILLED AND FROZEN CANINE SPERM QUALITY

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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เหว่ง วัง วุ่ย : ผลของน้ำมันหอมระเหยจากใบกะเพรา วิตามินอี และโพลีฟีนอลจากชาเขียวที่เสริมในสารรักษาสภาพน้ำเชื้อต่อคุณภาพตัวอสุจิสุนัขในแบบแช่เย็น และแช่แข็ง (EFFECTS OF *OCIMUM GRATISSIMUM* LEAF ESSENTIAL OILS, VITAMIN E, AND GREEN TEA POLYPHENOLS AS A SUPPLEMENT TO SEMEN EXTENDER ON CHILLED AND FROZEN CANINE SPERM QUALITY) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ นายสัตวแพทย์ ดร. ภคนิจ คุปพิทยานันท์, 186 หน้า.

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อทดลองหาสารรักษาสภาพน้ำเชื้อที่เหมาะสมสำหรับการเจือจาง และการเก็บรักษาในรูปแบบการแช่เย็น และแช่แข็งอสุจิของสุนัข การทดลองที่ 1 เป็นการประเมินผลของเลซิตินจากถั่วเหลือง และไข่แดงในสารรักษาสภาพน้ำเชื้อ Tris-citric-fructose หรือ Tris-citric-fructose-mineral salts ต่อการแช่เย็นอสุจิของสุนัข พบว่าสารรักษาสภาพน้ำเชื้อทั้ง Tris-citric-fructose และ Tris-citric-fructose-mineral salts ที่มีไข่แดงเป็นองค์ประกอบให้ผลดีกว่าเลซิตินจากถั่วเหลืองสำหรับการรักษาคุณภาพอสุจิสุนัขแบบแช่เย็นได้ภายใน 10 วันของการเก็บรักษา ($p<0.05$) นอกจากนี้ไข่แดงในสารรักษาสภาพน้ำเชื้อ Tris-citric-fructose สามารถรักษาการเคลื่อนที่ของอสุจิได้ดีกว่าสารรักษาสภาพน้ำเชื้ออื่นๆ แต่สารรักษาสภาพน้ำเชื้อ Tris-citric-fructose-mineral salts ที่มีการผสมไข่แดงให้ผลดีที่สุดในการรักษาเยื่อหุ้มเซลล์ และเยื่อหุ้มอะโครโซมไม่ให้ถูกทำลาย และยังช่วยเพิ่มศักยภาพการทำงานของเยื่อหุ้มไมโทคอนเดรีย ($p<0.05$) ในทางตรงกันข้ามผลการใช้เลซิตินจากถั่วเหลืองในสารรักษาสภาพน้ำเชื้อ Tris-citric-fructose ทำให้คุณภาพอสุจิลดลงต่ำกว่าการใช้เลซิตินจากถั่วเหลืองในสารรักษาสภาพน้ำเชื้อ Tris-citric-fructose-mineral salts ($p<0.05$) การทดลองที่ 2 การศึกษาผลของน้ำมันหอมระเหยจากใบกะเพราเพื่อเสริมเป็นสารต้านอนุมูลอิสระในสารรักษาสภาพน้ำเชื้อ Tris-citric-fructose-mineral salts ที่มีไข่แดง ต่อคุณภาพอสุจิสุนัขเมื่อถูกแช่เย็น พบว่าความเข้มข้นของน้ำมันหอมระเหยจากใบกะเพราที่ระดับต่ำ (25 50 และ 100 ไมโครกรัม/มิลลิลิตร) ให้ผลดีต่อคุณภาพอสุจิ ในขณะที่น้ำมันหอมระเหยจากใบกะเพราที่ระดับความเข้มข้นสูง (200 ไมโครกรัม/มิลลิลิตร) มีผลเชิงลบ โดยการเสริมน้ำมันหอมระเหยจากใบกะเพราที่ระดับ 100 ไมโครกรัม/มิลลิลิตร ส่งผลดีที่สุดในการปรับปรุงคุณภาพการแช่เย็นอสุจิสุนัขในทุกพารามิเตอร์ ยกเว้นการเคลื่อนที่เมื่อเทียบกับกลุ่มควบคุม ($p<0.05$) การทดลองที่ 3 และ 4 เป็นการประเมินผลการเสริมน้ำมันหอมระเหยจากใบกะเพรา วิตามินอี และโพลีฟีนอลจากชาเขียวในสารรักษาสภาพน้ำเชื้อ Tris-citric-fructose-mineral salts ที่มีไข่แดง ต่อคุณภาพอสุจิสุนัขในการเก็บรักษาแบบแช่เย็น และแช่แข็ง จากผลการลองพบว่า การเสริมน้ำมันหอมระเหยจากใบกะเพรา วิตามินอี และโพลีฟีนอลจากชาเขียวในสารรักษาสภาพน้ำเชื้อไม่ส่งผลต่อการเคลื่อนที่ของอสุจิ ($p<0.05$) แม้ว่าสารรักษาสภาพน้ำเชื้อที่มีการเสริมโพลีฟีนอลจากชาเขียว และวิตามินอีมี

การเกิดปฏิกิริยาลิพิดเปอร์ออกซิเดชันในอสุจิต่ำกว่าน้ำมันหอมระเหยจากใบกะเพรา แต่การเสริมน้ำมันหอมระเหยจากใบกะเพราในสารรักษาสภาพน้ำเชื้อสามารถช่วยป้องกันการถูกทำลายบริเวณเยื่อหุ้มเซลล์อสุจิ เยื่อหุ้มอะโครโซมบริเวณหัวอสุจิ และเพิ่มศักยภาพการทำงานของเยื่อหุ้มไมโทคอนเดรียได้คล้ายกับสารรักษาสภาพน้ำเชื้อที่มีการเสริมวิตามินอี และโพลีฟีนอลจากชาเขียว และมีความแตกต่างอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มควบคุม ($p<0.05$) สรุปได้ว่าน้ำมันหอมระเหยจากใบกะเพราในสารรักษาสภาพน้ำเชื้อ Tris-citric-fructose-mineral salts ที่มีไข่แดงเป็นสารรักษาสภาพน้ำเชื้อที่ดีที่สุดสำหรับการรักษาคุณภาพอสุจิสุนัขในกระบวนการเก็บรักษาได้ทั้งในรูปแบบแช่เย็นและแช่แข็ง

สาขาเทคโนโลยีและนวัตกรรมทางสัตว์ ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2562 ลายมือชื่ออาจารย์ที่ปรึกษา P. Kupattayanant

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม Samorn P.

NGUYEN VAN VUI : EFFECTS OF *OCIMUM GRATISSIMUM* LEAF ESSENTIAL OILS, VITAMIN E, AND GREEN TEA POLYPHENOLS AS A SUPPLEMENT TO SEMEN EXTENDER ON CHILLED AND FROZEN CANINE SPERM QUALITY. THESIS ADVISOR : ASST. PROF. PAKANIT KUPITTAYANANT, Ph.D., 186 PP.

OCIMUM GRATISSIMUM/ESSENTIAL OIL/VITAMIN E/GREEN TEA POLYPHENOLS/EXTENDER/CANINE SPERM/CHILLED/FROZEN

The objective of this study was to find the appropriate semen extender for diluting and preserving chilled and frozen canine sperm. The first experiment was to evaluate the effects of soybean lecithin and egg yolk in Tris-citric-fructose or Tris-citric-fructose-mineral salts extender on chilled canine sperm quality. Egg yolk was found to be better than soybean lecithin in Tris-citric-fructose or Tris-citric-fructose-mineral salts extender for maintaining the quality of chilled canine sperm within 10 days of storage ($p<0.05$). Although egg yolk in the Tris-citric-fructose extender could maintain the motility better than other extenders, egg yolk in the Tris-citric-fructose-mineral salts extender was the highest in the intact plasma membrane, intact acrosome membrane, and high mitochondrial membrane potential ($p<0.05$). In contrast, the sperm quality of soybean lecithin in the Tris-citric-fructose-mineral salts extender was lower than that of soybean lecithin in the Tris-citric-fructose extender ($p<0.05$). Experiment 2 was conducted to investigate the effects of essential oils from *Ocimum gratissimum* leaves as antioxidant supplement in the Tris-citric-fructose-mineral salts egg-yolk extender on chilled canine sperm quality. Low concentrations of *Ocimum gratissimum*

essential oils (25, 50, and 100μg/mL) have beneficial effects on sperm quality, whereas *Ocimum gratissimum* essential oils at high levels (above 200μg/mL) have harmful effects. Specifically, the addition of 100μg/mL of *Ocimum gratissimum* essential oils to the semen extender had the most beneficial effect in improving the quality of chilled canine sperm, and had a significant difference in all sperm quality parameters except motility compared to the control group ($p<0.05$). Experiments 3 and 4 were carried out to estimate the effects of the adding *Ocimum gratissimum* essential oils, vitamin E, and green tea polyphenols to the Tris-citric-fructose-mineral salts egg-yolk extender on chilled and frozen canine sperm quality. The sperm motility parameters of chilled and frozen canine sperm among all the semen extenders were not significantly different ($p>0.05$). Although the percentages of sperm lipid peroxidation in the green tea polyphenols and the vitamin E extenders were lower than those in the *Ocimum gratissimum* essential oils extender, the proportion of the intact plasma membrane, intact acrosome membrane, and high mitochondrial membrane potential parameters in the *Ocimum gratissimum* essential oils extender were similar to those in the vitamin E and green tea polyphenols extenders and had a significant difference as compared to the control group ($p<0.05$). In conclusion, *Ocimum gratissimum* essential oils supplementation in the Tris-citric-fructose-mineral salts egg-yolk extender is the best semen extender for improving chilled and frozen canine sperm quality during chilling and freezing.

School of Animal Technology and Innovation

Academic Year 2019

Student's Signature _____

Advisor's Signature P. Kupttavanart

Co-advisor's Signature Samorn P.

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Nguyen Van Vui

CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF TABLES (Continued)

Table **Page**

LIST OF TABLES (Continued)

Table **Page**

LIST OF TABLES (Continued)

Table **Page**

LIST OF TABLES (Continued)

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF ABBREVIATIONS

AI	=	Artificial insemination
ANOVA	=	Analysis of variance
ATP	=	Adenosine triphosphate
CASA	=	Computer assisted sperm analysis
DPBS	=	Dulbecco's phosphate-buffered saline
DPPH	=	1, 1-diphenyl-2-picrylhydrazyl
DMSO	=	Dimethyl-sulfoxide
DMF	=	Dimethyl-formamide
FITC	=	Fluorescein isothiocyanate–conjugated *Pisum sativum* agglutinin
H342	=	Hoechst 33342
JC-1	=	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide
MIN	=	Minute
MDA	=	Malondialdehyde
PI	=	Propidium iodide
PM	=	Progressive motility
ROS	=	Reactive oxygen species
SEC	=	Second
SD	=	Standard deviation

LIST OF ABBREVIATIONS (Continued)

TM	=	Total motility
VAP	=	Velocity average pathway
VSL	=	Velocity straight line
VCL	=	Velocity curvilinear

CHAPTER I

INTRODUCTION

1.1 Rationale of the study

Semen collection and artificial insemination (AI) techniques are currently widespread and play a crucial role for breeding dogs. To prepare sperm for the AI techniques, canine sperm can be preserved by chilling (preservation) or freezing (cryopreservation) (Thomassen and Farstad, 2009). The major limitation for the use of preservation and cryopreservation in canine sperm are the survival time and quality of sperm after thawing. To prolong canine sperm longevity and increase quality of sperm at low metabolism, sperm must be diluted with appropriate extenders to provide energy, maintain pH and osmolarity, and support cryoprotectants for protecting sperm plasma membrane during freezing and thawing. Previously, the Tris-citric-fructose extender with 20% egg yolk was proposed one of the most common extenders for chilled and frozen canine sperm that best protects the canine sperm quality during storage (Rota et al., 1995; Ponglowhapan et al., 2004; Verstegen et al., 2005; Shahiduzzaman and Linde-Forsberg, 2007; Michael et al., 2007; Bencharif et al., 2010; Goericke-Pesch et al., 2012; Batista et al., 2012; Santana et al., 2013; Rodenas et al., 2014; Pezo et al., 2017). Recently, soybean lecithin used as an alternative to egg yolk in canine semen extenders to avoid hygiene problems from bacterial contamination has obtained equal or superior results (Beccaglia et al., 2009; Kmenta et al., 2011; Kasimanickam et al., 2012; Axnér and Lagerson, 2016; Dalmazzo et al., 2018).

Canine seminal plasma is a complex biological fluid containing ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-), energy substrates (fructose, sorbitol, glycerylphosphocholine), and organic compounds (citric acid, amino acids, peptides, proteins, lipids, hormones, cytokines) (Wales and White, 1965; Juyena and Stelletta, 2012). It has essential functions in sperm ejaculation and sperm survival in the female genital tract. The role of mineral ions is particularly valuable for maintaining osmotic balance, forming parts of primary enzymes relating to sperm metabolism and sperm function (Çevik et al., 2007; Juyena and Stelletta, 2012; Smith et al., 2018). In previous studies, despite canine seminal plasma has been found to be beneficial for canine sperm plasma membrane, acrosome membrane, and mitochondrial membrane potential, it has negative effects on sperm motility (Rota et al., 1995; Treulen et al., 2012; Hori et al., 2017). The reduction in sperm motility is due to the decreasing of adenosine triphosphate (ATP) concentration in seminal plasma by acid and alkaline phosphatase activity (Günzel-Apel and Ekrod, 1991). Moreover, the centrifugation and removal of seminal plasma before diluting with extenders has been conducted, and no detrimental effects on the function of canine sperm have been investigated (Rota et al., 1995; Peña and Linde-Forsberg, 2000; Rijsselaere et al., 2002; Shahiduzzaman and Linde-Forsberg, 2007; Goericke-Pesch et al., 2012). Hence, creating a semen extender by adding mineral ions may enhance the longevity of sperm as well as improving the quality of canine sperm without seminal plasma.

During sperm storage, canine sperm is highly susceptible to oxidative stress which induce biochemical and functional damages to the sperm. Since canine sperm plasma membrane involve a rich amount of polyunsaturated fatty acids (Darin Bennett et al., 1974), they are prone to lipid peroxidation in the presence of reactive oxygen

species [ROS] during the process of chilling and freezing (Lamirande et al., 1997; Vieira et al., 2017). In physiological concentrations, ROS can assist sperm function in hyper-activation, acrosome reaction, capacitation, and zona pellucida binding (Lamirande et al., 1997; Aitken, 2017), while the high concentration of ROS can induce sperm lipid peroxidation which leads to changes in membrane fluidity and damage to sperm structures as well as subsequent sperm death (Moustafa et al., 2004; Lucio et al., 2016; Aitken, 2017). Fortunately, the enzymatic antioxidants in seminal plasma of canine semen including superoxide dismutase, glutathione peroxidase, phospholipid hydro-peroxide glutathione peroxidase, and catalase (Cassani et al., 2005; Strzezek et al., 2009; Neagu et al., 2011; Angrimani et al., 2014), can prevent the injurious effects of oxidation caused by ROS (Birben et al., 2012; Ighodaro and Akinloye, 2017). However, the functional effects of these enzymatic antioxidants are no longer available because the canine seminal plasma must be removed during sperm preservation and cryopreservation process (Rota et al., 1995; Treulen et al., 2012; Hori et al., 2017). This may reduce the antioxidant capability and contribute to the high susceptibility of canine sperm to oxidative stress. Thus, the adding of antioxidant molecules in the semen extenders may inhibit free radicals or the attack of ROS during the sperm storage process, and improve sperm quality. Several studies were carried out to evaluate the effects of various enzymatic and non-enzymatic antioxidants on canine sperm (Michael et al., 2007; Beccaglia et al., 2009; Neagu et al., 2009; 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), but various outcomes were found depending on the type and concentration of antioxidants as well as the semen extenders.

Therefore, finding the appropriate antioxidant substances for chilled and frozen canine sperm is required.

Vitamin E (α-Tocopherol) is a lipophilic antioxidant which plays a major role in scavenging the free radical-induced lipid peroxidation by functioning as a chain-breaking antioxidant (Wang and Quinn, 1999; Dad et al., 2006). Previously, several studies have revealed that the supplementation of vitamin E in the semen extenders had positive effects on sperm quality of stallion (Almeida and Ball, 2005; Vasconcelos Franco et al., 2013; Vasconcelos Franco et al., 2014; Vasconcelos Franco et al., 2016), boar (Cerolini et al., 2000; Jeong et al., 2009; Satorre et al., 2012), bull (Asadpour, 2011), ram (Abdi-Benemar et al., 2015), canine (Michael et al., 2009), and rooster (Moghbeli et al., 2016).

Moreover, green tea polyphenols are hydrophilic antioxidants in which contain a great variety of bioactive compounds including epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate. These bioactive compounds have a powerful antioxidant activity by inhibiting the free radicals from the ROS and the chain reaction of lipid peroxidation (Coyle et al., 2008; Lamberta and Eliasa, 2010; Forester and Lambert, 2013). The adding of green tea polyphenols in the semen extenders to improve the sperm quality have been found in stallion (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 aromatic medicinal herb that have a rich essential oils from the leaves (3.5%) (Trevisan et al., 2006). Many studies have found that the composition of *Ocimum gratissimum* essential oils contain the main substance of eugenol (30-70%), and the other phytochemicals such as thymol, α-bisabolene, β-selinene, 1,8-cineole, and γ-terpinene (Silva et al., 2004; Lemos et al.,

2005; Trevisan et al., 2006; Prabhu et al., 2009; Monga et al., 2017; Kumar et al., 2019). These bioactive compounds almost have an amphiphilic characteristic and a strong function of antioxidant activity (Trevisan et al., 2006; Ouyang et al., 2012; Chiu et al., 2013; Mahapatra and Roy, 2014). Therefore, *Ocimum gratissimum* essential oils supplementation in the semen extenders can improve the quality of canine sperm by reducing sperm lipid peroxidation during sperm storage.

With these backgrounds, adding antioxidants as vitamin E, green tea polyphenols, and *Ocimum gratissimum* leaf essential oils in Tris-citric-fructose-mineral salts egg-yolk or soybean lecithin extender is a potential and promising of a perfect semen extender for canine sperm preservation and cryopreservation.

1.2 Research objectives

1.2.1 To investigate the effects of egg yolk and soybean lecithin in Tris-citric-fructose or Tris-citric-fructose-mineral salts extender on chilled canine sperm quality.

1.2.2 To evaluate the effects of *Ocimum gratissimum* leaf essential oils as a supplement in semen extender on chilled canine sperm quality.

1.2.3 To study the effects of adding *Ocimum gratissimum* essential oils, vitamin E, and green tea polyphenols to semen extender on chilled and frozen canine sperm quality.

1.3 Research hypotheses

1.3.1 Tris-citric-fructose-mineral salts extender was better than Tris-citric-fructose extender and soybean lecithin could replace egg yolk for improving chilled canine sperm quality.

1.3.2 *Ocimum gratissimum* essential oils could improve chilled canine sperm quality against oxidative stress.

1.3.3 *Ocimum gratissimum* essential oils was superior to vitamin E and green tea polyphenols in protecting chilled and frozen canine sperm quality.

1.4 Scope and limitation of this study

American Bullies dogs were used to collect semen for this study.

The canine sperm quality was evaluated using CASA for the motility parameter and using confocal laser scanning microscope for the plasma membrane integrity, acrosome membrane integrity, and mitochondrial membrane potential parameters.

Since the limitation of the time and budgets, the sperm fertilization parameter and the synergetic effects between vitamin E, green tea polyphenols, and *Ocimum gratissimum* essential oils on canine sperm quality were not conducted.

1.5 Expected results

1.5.1 Tris-citric-fructose-mineral salts with adding egg yolk or soybean lecithin extender can use for chilled and frozen canine sperm.

1.5.2 *Ocimum gratissimum* essential oils, vitamin E, and green tea polyphenols are useful for improving chilled and frozen canine sperm quality.

1.5.3 The outcomes of this study not only provide a valuable formulation for canine semen extender, but also propose for further study to investigate the synergetic effects among these antioxidants on canine sperm quality.

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CHAPTER II

LITERATURE REVIEW

2.1 Canine semen collection

Although the presence of a bitch would improve the ejaculates, canine semen can be collected in a quiet room without a teaser. For reluctant males, using a bitch in estrus or using frozen-thawed swabs or gauze sponges taken from vaginal secretions of canine estrus females should be provided (Joni, 2002; Kutzler, 2005).

Semen collection should be prepared in advance and registered the interval among collections. The ideal interval among collections is 2 to 5 days, while the interval over 10 days may result in a reducing of sperm motility and an increasing of sperm abnormality. In longer periods, it should be performed one previous collection before chilling or freezing. Semen extender should be prepared before semen collection (Joni, 2002). Digital manipulation is the most common method for canine semen collection (Linde-Forsberg, 1991; Payan-carreira et al., 2011).

Semen collection is started with a manipulation of the canine prepuce at the bulbus glandis position until developing partial erection, and then the rapid retraction of the prepuce and penile expose. When the pelvic stop thrusting and the rear leg of dog lifts, the canine penis should be rotated 180^{o} backward and the erectile penis should be put directly into the collection tube or funnel. During collection, some pressure of the thumb on the apex of the glans penis should be applied to stimulate ejaculation (Linde-Forsberg, 1991; Payan-carreira et al., 2011).

There are 3 fractions of canine ejaculation with the first and third fraction containing of prostatic fluid, and the second fraction consisting of the rich sperm. The first fraction is a pre-sperm portion which is colorless and has a volume of 1-5mL. The second fraction is a sperm-rich portion which is quickly completed around 1-2 minutes with a volume of 1-3mL, and grayish-white in color. The third fraction has a volume of 30-40mL which comes from the prostate; it may take around 5 to 30 minutes to be completed (Joni, 2002; Payan-carreira et al., 2011).

2.2 The composition of canine semen

Table 2.1 The composition of canine semen (Wales and White, 1965).

Constituent or property	**1st Fraction**	**2nd Fraction**	**3rd Fraction**
Sperm count (10^6/mL)	-	505±206	-
pH	-	6.3±0.2	6.8±0.3
Sodium (m-equiv./l)	145±6	122±8	156±11
Potassium (m-equiv./l)	14.6±2.1	12.9±1.8	7.0±3.2
Magnesium (m-equiv./l)	0.62±0.2	0.46±0.26	0.17±0.09
Calcium (m-equiv./l)	0.63±0.37	0.37±0.07	0.24±0.07
Chloride (m-equiv./l)	141±2	125±3	148±4
Total orcinol reactive carbohydrate (mg%)	97±52	341±109	189±67
Total reducing sugar (mg%)	14±7	9±11	4±2
Fructose (mg%)	2	1	1
Lactic acid (mg%)	7±3	5±1	8±6

Table 2.1 Continue.

Constituent or property	**1st Fraction**	**2nd Fraction**	**3rd Fraction**
Citric acid (mg%)	3±4	4±2	1±0.02
Total protein (mg%), ethanol precipitate	1.3±0.7	3.7±1.4	2.8±1.1
Urea (mg%)	68.8±24.2	69.4±25.1	72.0±17.3
Ammonia (mg%)	9.8±2.1	14.7±1.0	18.6±5.1
Total phosphorus (mg%)	18.8±3.2	92.7±14.8	7.8±2.0
Acid insoluble phosphorus (mg%)	1.7±0.8	7.8±0.8	4.8±1.3
Lipid phosphorus (mg%)	0.4±0.2	0.5±0.2	0.1±0.2
Inorganic phosphorus (mg%)	3.9±2.4	6.5±1.5	1.6±0.5
Acid soluble phosphorus (mg%)	16.2±3.0	82.3±22.0	5.4±3.9
Glycerylphosphorylcholine (mg%)	5±6	176±31	20±9

2.3 Canine sperm evaluation

2.3.1 Volume

Canine semen is ejaculated in three fractions. The volumes of the first and third fractions are variable. It depends on the canine size, the size of the prostate gland, the age of dog, the frequency of semen collection, and the volume of the third fraction. The third fraction volume can be controlled by the collector who would like to collect more or less prostatic fluid. The semen volume is not an indicator for semen quality. However, the semen volume indicate the number of spermatozoa which is one of indicator for semen quality (Root Kustritz, 2007). Moreover, the reducing of semen volume can cause of prostatic hyperplasia, prostatic cysts, inflammatory lesions of

prostate and testicles, inflammation of epididymis, vas deferens or urethra, and weak libido (Payan-carreira et al., 2011).

2.3.2 Color

The color of canine semen depends on the volume of the third fraction of ejaculate collected, on the sperm concentration, and the presence of contamination. The normal color of the whole ejaculate in canine semen is greyish-white. There are some adnormal colors including green-greyish typical for the presence of the pus; red or pink for erythrocytes contamination cause of haemorrhages from urethra or corpora cavernosa; yellow specific for urine contamination (Root Kustritz, 2007; Payan-carreira et al., 2011; Anderson, 2013).

2.3.3 pH

Seminal plasma has a normal pH range of 6.3 – 6.8 (Wales and White, 1965; Root Kustritz, 2007).

2.3.4 Motility

Motility is one of the important parameter for a fertile sperm and also an indicator for structural and functional competence of spermatozoa. Sperm motility is expressed as the total motility or progressive motility. The percentage of total motility in normal ejaculates is from 70 to 90%. It has been suggested that the fertile dogs should have at least 70% of the total motility (Peña Martínez, 2004; Payan-carreira et al., 2011; Anderson, 2013). However, the progressive motility and morphological normal sperm may be more correlated with the fertility than the total motility parameter (Mickelsen et al., 1993; Freshman, 2001). The minimum number of healthy sperm required for optimal fertility has not been determined in dogs. In previous studies, for using the fresh semen by vaginal insemination, the motile sperm at least 200 x 10^6 sperm were needed

for fertility, while for frozen-thawed sperm deposited into the uterus, the dose of insemination around 150-200 x 10^6 sperm is recommended (Tsutsui et al., 2003; Payan-carreira et al., 2011)

Sperm motility is usually evaluated using visual examination under a phase contrast microscope. The semen with high concentration of sperm is diluted with a warm saline or Tris-fructose buffer before assessing (Payan-carreira et al., 2011). This method is the most simple, rapid, and cheap. However, it depend on the technician and unreliable assay for predicting fertility (Peña Martínez, 2004). Besides that, computer assisted sperm analysis (CASA) system has been used to evaluate the sperm motility characteristics. This system allow technicians to control the weakness of visual examination (Verstegen et al., 2002; Domosławska et al., 2013). Many studies have evaluated the sperm motility using CASA in canine sperm (Ponglowhapan et al., 2004; Michael et al., 2007; Kmenta et al., 2011; Kasimanickam et al., 2012; Lucio et al., 2016; Andersen et al., 2018).

2.3.5 Sperm concentration

Sperm concentration is a parameter which presents the number of sperm in the volume of semen and an indicator for semen quality. The sperm concentration depends on the volume of collected semen. The total sperm in the ejaculate is the result of the sperm concentration multiply by semen volume and depends on the testicular size (Root Kustritz, 2007). The canine sperm concentration in the whole normal ejaculate is usually around 80 x 10^6 sperm/mL. If the sperm-rich fraction is collected separately, the sperm concentration is usually from 200 to 600 x 10^6 sperm/mL. It is suggested that the number of motile sperm require for AI should be over 150 x 10^6 sperm/mL (Tsutsui et al., 2003; Thomassen and Farstad, 2009). The canine sperm

concentration varies according to the dog breeders, age, testicular weight, sexual activity, the size of dog (Root Kustritz, 2007). The sperm concentration is usually evaluated by cytometric method on the haemocytometer such as Neubauer chambers. Currently, several modern methods have been used to identify the sperm concentration such as CASA, spectrophotometer, flow-cytometer (Rijsselaere et al., 2005).

2.3.6 Morphology

Sperm morphology indicates the normal and abnormal spermatozoa in semen. The percentage of the normal sperm in canine semen should be higher than 80%. The sperm morphology may evaluate under a contrast-phase microscope or light microscope on the stained slides. The stains have been used such as Giemsa (DiffQuik), Spermac, or nigrosin-eosin. The sperm morphology evaluation should be completed using oil immersion with an objective of 100x or 125x. At least 200 sperm should be counted and evaluated for normal and abnormal sperm (Tamuli and Watson, 1994; Payan-carreira et al., 2011).

2.3.7 Viability

Sperm viability is a parameter that indicates the percentage of live and dead sperm. Nigrosin-eosin staining has been used to evaluate the sperm viability. Base on the sperm plasma membrane in the dead sperm can allow nigrosin-eosin penetration. Thus, the percentage of sperm with positive nigrosin-eosin stained is considered as the percentage of the dead sperm. In the normal canine semen, the proportion of the dead sperm allows maximum of 30% (Tamuli and Watson, 1994; Payan-carreira et al., 2011).

2.3.8 Sperm plasma membrane integrity

Sperm plasma membrane integrity is one of the most important parameter of sperm quality. It has the function in sperm capacitation, acrosome reaction, and sperm-egg fusion (Lenzi et al., 1996; Mandal et al., 2014). Hypo-osmotic swelling test is a popular method to evaluate the sperm plasma membrane integrity (Kumi-Diaka, 1993; Peña Martínez, 2004). In this method, sperm is incubated with hypo-osmotic solutions for 30 minutes at 37 ^{o}C. As a result, sperm with intact plasma membrane become swelled and show coiled tails. In addition, the combination of several fluorescent dyes such as propidium iodide (PI) with carboxyfluorescein diacetate, SYBR-14 or Hoechst 33342 (H342) allows the identification of alive sperm, death sperm as well as sperm plasma membrane integrity. The dead sperms are stained with red color in the nucleus due to the influx of PI through the damaged plasma membrane (Ponglowhapan et al., 2004; Rijsselaere et al., 2005; Silva and Gadella, 2006).

2.3.9 Sperm acrosome membrane integrity

Sperm acrosome membrane is an important parameter of sperm quality. It has the function in protecting the sperm acrosome which contains high amount of acrosin. This enzyme is necessary for gamete fusion, particularly for binding to, penetration of zona pellucida. In addition, it participates in the acrosome reaction, capacitation, and chromatin decondensation in the oocyte (Schill et al., 1988; Hirohashi and Yanagimachi, 2018). A fluorescein isothiocyanate–conjugated *Pisum sativum* agglutinin (FITC-PSA) or isothiocyanate–conjugated Peanut agglutinin (FITC-PNA) has been used to detect the sperm acrosome membrane integrity. PNA labeling is specific for the outer acrosome membrane whereas PSA is labeling acrosome matrix.

For FITC-PSA, the sperm damaged acrosome membrane show the green color in the acrosome cap. For FITC-PNA, the sperm intact acrosome membrane show the right fluorescent over the acrosome cap (Silva and Gadella, 2006; Celeghini et al., 2007; Chankitisakul et al., 2013).

2.3.10 Sperm mitochondrial membrane potential

Mitochondria are important cellular organelles which have a principal role in maintaining the cellular bio-energetic and ion-homeostasis, and producers of reactive oxygen species. This organelle is key in many crucial sperm function such as motility, hyperactivation, acrosome reaction, and fertilization. Mitochondrial membrane potential is a major index of the bio-energetics state of spermatozoa (Srivastava and Pande, 2016; Moraes and Meyers, 2018). To evaluate the mitochondrial membrane potential, lipophilic cationic fluorochrome JC-1 (0.15 mM in DMSO) has been used. Under a fluorescent microscope, the sperm with high mitochondrial membrane potential has orange fluorescence in the midpiece, and the sperm with low mitochondrial membrane potential has green fluorescence (Celeghini et al., 2007; Silva et al., 2012).

2.4 Canine sperm preservation

2.4.1 The benefit and processing of canine sperm preservation

The use of sperm preservation is easier and cheaper than sperm cryopreservation when the sperm is transported over the short distances (Linde-Forsberg, 1991; Eilts, 2005). In addition, the fertile capacity of chilled sperm is higher than that of frozen sperm when compared to the same condition of estrous cycle time and AI techniques (Linde-Forsberg, 1995).

To preserve a sperm sample at chilling temperature, the canine semen collected from the second sperm-rich fraction is centrifuged to remove the seminal plasma at 720 x g, 5 minutes (Rijsselaere et al., 2002). After centrifuging, the sperm is diluted with an appropriate extender in a rate of 1:3 to 1:4 (1 mL of semen with 3 to 4 mL of the semen extender). Then the extended sperm is cooled down gradually (0.3^{o}C/min) to 5^{o}C by manual and stored at 5^{o}C for up to 10 days (Bouchard et al., 1990).

2.4.2 Semen extenders for preservation

Because sperm are metabolically relatively inert, the extracellular environment plays a key role in sperm survival. The seminal plasma have negative effects for prolong the longevity of canine sperm during storage at 5^{o}C (Rota et al., 1995; Yamashiro et al., 2009; Treulen et al., 2012). To prolong the sperm quality during storage, sperm must be diluted with a suitable semen extenders before and preserved by chilling or freezing (Thomassen et al., 2006). Many different the semen extenders have been investigated. They have been revealed to be appropriate in both in vitro and in vivo studies. In general, the composition of semen extenders are included the buffers to maintain the sperm at a constant pH and osmolarity such as Tris, citric; the energy substances as fructose or glucose to provide energy for sperm activity; the lipoprotein as egg yolk (20%) to protect sperm from cold shock during cooling; and antibiotics to prevent the growth of bacteria such as penicillin, streptomycin or gentamycin (Ponglowhapan et al., 2004; Verstegen et al., 2005; Dorado et al., 2011; Kmenta et al., 2011). In addition, the adding of antioxidants in extender to prevent oxidative stress is necessary to prolong the sperm life span (Michael et al., 2007; Neagu et al., 2009; Michael et al., 2009; Monteiro et al., 2009; Sahashi et al., 2011; Thiangtum et al., 2012).

Moreover, since egg yolk is a substance of animal origin, studies are conducted to replace egg yolk by phospholipids or vegetable lecithin to avoid hygiene problems from bacterial contamination (Farstad, 2009; Kasimanickam et al., 2012; Kmenta et al., 2011).

2.5 Canine sperm cryopreservation

Sperm cryopreservation is a process by which sperm are preserved at subzero temperatures (-196^{o}C) resulting in a radical decrease in the rate of metabolic processes and the ability to store samples for extended periods.

2.5.1 Semen extenders for cryopreservation

Semen extenders are required for diluting canine sperm before cryopreservation and extending sperm motility after thawing. The composition of semen extenders for sperm cryopreservation is similar to that of semen extenders for sperm preservation. However, semen extenders for sperm cryopreservation must to add cryoprotective agents (Michael et al., 2007; Lopes et al., 2009; Martins et al., 2012).

2.5.2 Cryoprotectant

Cryoprotectants can reduce the physical and chemical stress on sperm during the freezing process. They are necessary to lower the freezing point of the medium and thus allows sperm cells vitrification. The purpose of vitrification is to protect sperm plasma membrane destruction caused by ice formation during freezing and thawing. Cryoprotectants are divided into permeating (glycerol, ethylene glycol, DMSO, MeOH) and nonpermeating agents (sugars, starch). The most popular cryoprotectant for canine sperm cryopreservation is glycerol which can act both in intracellular and extracellular. Glycerol can lower the freezing point of intracellular and

extracellular medium and induce the dehydration at low temperature. This leads to reduce the intracellular ice crystal formation and protect sperm from cryogenic injury. Depend on the composition of the semen extenders, different concentrations of glycerol have been used (4-11%) (Rota et al., 1998; Cardoso et al., 2003), but most protocols recently use 5% glycerol (Álamo et al., 2005; Michael et al., 2007; Bencharif et al., 2010; Kim et al., 2010; Kim et al., 2012; Varesi et al., 2014; Pezo et al., 2017; Caturla-Sánchez et al., 2018). Although the using glycerol as a cryoprotectant was successful for canine sperm cryopreservation, the other cryoprotectants such as dimethyl-sulfoxide (DMSO), dimethyl-formamide (DMF), and ethylene glycol have been tried. However, these cryoprotectants were not better than glycerol to protect canine sperm during freezing and thawing (Martins-Bessa et al., 2006; Lopes et al., 2009; Futino et al., 2010; Rota et al., 2010; Mota Filho et al., 2011).

2.5.3 Freezing and thawing

Canine cryopreservation processing is followed by the main steps including centrifugation, dilution, equilibration, packing, freezing and thawing.

Before diluting with the semen extenders, the canine semen is centrifuged to remove the seminal plasma which have negative effects on frozen-thawed sperm motility and viability. The most centrifugation protocol used in canine sperm is at 720 x g for 5 minutes (Rijsselaere et al., 2002).

After centrifuging, canine sperm must to dilute with an appropriate extender to support energy and protect sperm from the negative effects during freezing and thawing, and obtain the suitable final sperm concentration. In frozen canine sperm, the final concentration of sperm is 50-100 x 10^6 sperm per 0.5 mL straw and 2 to 4 straws are used for one insemination. The optimal of sperm concentration per

insemination is 200 x 10^6 sperm (Peña and Linde-Forsberg, 2000; Payan-carreira et al., 2011).

The dilution of canine sperm can be done in one or two steps. If canine sperm is diluted in two steps, the first step is conducted at room temperature and the second step is taken after chilling or equilibration and just before freezing (Linde-Forsberg et al., 1999; Peña and Linde-Forsberg, 2000). There was no significant difference in frozen-thaw sperm quality between one step and two step of the adding glycerol (Peña and Linde-Forsberg, 2000; Brito et al., 2016).

After equilibration, the extended sperm is stored in straws, pellets, or tubes. The most common packing form for frozen canine sperm is the straws because they have several advantages over the other methods of packing such as low volume, uniform freezing. Two kind of straws with small volume (0.25 mL) and large volume (0.5 mL) have been used in canine sperm cryopreservation (Linde-Forsberg, 1991).

Before freezing, the extended sperm is cooled down gradually to 5^{o}C and equilibrated for a variable time depending on the protocol (1-4 hours). Then the sperm straws are frozen with different freezing rates. Several canine sperm freezing rates have been tested and the optimal one is the freezing at a rate of 5^{o}C/min from 5^{o}C to -15^{o}C, and 20-50^{o}C/min from -15^{o}C to -100^{o}C (Olar et al., 1989; Rota et al., 1998; Michael et al., 2007). The freezing can be done with a programmable freezer (Olar et al., 1989; Michael et al., 2007), or by putting the sperm straws at different distances over the liquid nitrogen (Martins-Bessa et al., 2006; Silva et al., 2006; Bencharif et al., 2010; Kim et al., 2012; Varesi et al., 2014; Caturla-Sánchez et al., 2018). The last stage of freezing is the plunge the sperm straws into the liquid nitrogen (-196^{o}C). For thawing, the sperm straws are immersed in a water bath at 70°C for 8 sec or 37°C for 15 to 60

sec. It was proposed that the thawing of 0.5 mL straws in a water bath at 70°C for 8 sec is greater than the thawing at 37°C for 15 to 60 sec in post-thaw sperm survival (Olar et al., 1989; Rota et al., 1998; Peña and Linde-Forsberg, 2000).

2.5.4 In vitro sperm test in predicting pregnancy success after AI with fresh, chilled and frozen canine sperm

In dogs, the ovulation of bitch is very important to guarantee adequate fertility in natural mating. It is also more essential to determine the ovulation period when to inseminate bitches with fresh, chilled or frozen sperm. For fresh and chilled sperm, the insemination should be performed on the day of ovulation and the second insemination should be conducted after 2 days later. For frozen sperm, the insemination should be performed 2 days after ovulation for canine oocytes mature in the oviducts and the second insemination 48h later. However, the schedule for insemination may be adjusted following the experience of the operator, the position of sperm deposition, and the number of insemination (Payan-carreira et al., 2011). The artificial insemination schedules for fresh, chilled and frozen canine sperm are present in Table 2.2. The success of AI for fresh semen depends on the semen quality and the moment for AI, while chilled and frozen sperm are related to both the quality of sperm and the site of sperm deposition (Table 2.3).

Table 2.2 The artificial insemination schedules for fresh, chilled and frozen canine sperm (Payan-carreira et al., 2011).

Sperm	Doses	Expected survival	AI schedule	Expected fertility
Fresh	150-200x10^6 spz/mL (extended)	4-6 days	- Every other day, when P_4 rise above 4ng/mL, up to 3 times - Day 1 to 4 post-ovulation	80-90% (either with transcervical or vaginal deposition)

Table 2.2 Continue.

Sperm	Doses	Expected survival	AI schedule	Expected fertility
Chilled	150-200x10^6 spz/mL (extended)	24-72hrs	- Breeding once or twice 2-4 days post ovulation (P_4 = 4-10ng/mL) - Day 2 to 4 post-ovulation - P_4 levels between 8 and 15ng/mL	80-90% (either with transcervical or vaginal deposition)
Frozen	50-300x10^6 spz/mL (extended)	12-24hrs	- Twice, at P_4 levels above 8ng/mL and estrus vaginal cytology - Day 5 to 7 post-ovulation - P_4 levels between 18 and 28ng/mL	- 45% if vaginal deposition - 67-84% if transcervical or intrauterine

Table 2.3 The whelping rate (%) of fresh, chilled and frozen canine sperm with intra-vaginal and intra-uterine inseminations

Sperm	Whelping rate (%) (n)		References
	Intra-vaginal	Intra-uterine	
Fresh	83.8	-	Linde-Forsberg (1991)
Frozen	69.3	-	
Frozen	58.9 (161)	84.4 (167)	Linde-Forsberg et al. (1999)
Fresh	47.7 (1212)	62.0 (121)	Payan-carreira et al. (2011)
Chilled	45.4 (348)	65.0 (40)	
Frozen	36.7 (30)	55.5 (290)	
Frozen	10.0 (20)	75.0 (665)	Thomassen et al. (2006)
Frozen	-	65.0 (78)	Mason and Rous (2014)

2.6 Antioxidant agents

2.6.1 Vitamin E

Vitamin E is the generic term used to identify various molecules: α-, β-, γ-, and δ-tocopherol; and α-, β-, γ-, and δ-tocotrienol. Among them, α-tocopherol is the only isomer related to the main function of vitamin E. It is a lipid-soluble antioxidant which plays a major role in inhibit the free radical-induced lipid peroxidation by functioning as a chain-breaking antioxidant (Wang and Quinn, 1999; Dad et al., 2006). The supplementation of vitamin E in the semen extenders has been evaluated to protect the sperm quality of stallion (Almeida and Ball, 2005; Vasconcelos Franco et al., 2013; Vasconcelos Franco et al., 2014; Vasconcelos Franco et al., 2016), boar (Cerolini et al., 2000; Jeong et al., 2009; Satorre et al., 2012), bull (Asadpour, 2011), ram (Abdi-Benemar et al., 2015), canine (Michael et al., 2009), and rooster (Moghbeli et al., 2016).

2.6.2 Green tea polyphenols

Green tea polyphenols are widely considered to be the bioactive compounds with the main component of catechins. The major catechins in green tea include (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), and (-)-epigallocatechin gallate (EGCG). These catechins have been proved to have an antioxidant activity based on their catechol hydroxyl groups. Several mechanisms have been determined to explain the antioxidant activity of green tea polyphenols. Firstly, they can directly inhibit the free radicals through hydrogen or electron donation. Secondly, they can interrupt the propagation of lipid peroxidation. Finally, they can chelate transition metal ions, inhibit the formation and propagation of iron-mediated free radical reactions. (Kumamoto et al., 2001; Coyle et al., 2008; Lamberta and Eliasa, 2010; Forester and Lambert, 2013). Several previous studies have demonstrated that

the adding of green tea polyphenols in the semen extenders had positive effects on sperm quality in stallion (Nouri et al., 2018), boar (Gadani et al., 2017), ram (Mehdipour et al., 2016), and canine (Wittayarat et al., 2013).

2.6.3 *Ocimum gratissimum* leaf essential oils

Ocimum gratissimum, also known as aromatic medicinal herb, is a species of Ocimum that are rich in essential oils (3.5%) (Trevisan et al., 2006). Plant extracts and essential oils of *Ocimum gratissimum* are known to have the function of ovicidal (Pessoa et al., 2002), antibacterial (Matias et al., 2011), antifungal (Matasyoh et al., 2007), anticarcinogenic (Nangia-Makker et al., 2007), antidiarrheal (Offiah and Chikwendu, 1999), phototoxic (Matias et al., 2010), anticonvulsant and anxiolytic (Okoli et al., 2010), and radio-protective activities (Monga et al., 2011). All the ethanolic extracts from this plant showed good antioxidant activity (Akinmoladun et al., 2007). Many studies have found that the composition of essential oils extract from *Ocimum gratissimum* contained eugenol (30-70%), and the other phytochemicals such as thymol, α-bisabolene, β-selinene, 1,8-cineole, and γ-terpinene (Silva et al., 2004; Lemos et al., 2005; Trevisan et al., 2006; Prabhu et al., 2009; Monga et al., 2017; Kumar et al., 2019). These bioactive compounds almost have a strong antioxidant activity (Trevisan et al., 2006; Ouyang et al., 2012; Chiu et al., 2013; Mahapatra and Roy, 2014). Therefore, essential oils extract from *Ocimum gratissimum* leaves is a potential source of antioxidants for animal science.

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CHAPTER III

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

ABSTRACT

The study evaluated the effects of 8 treatments with different levels of soybean lecithin concentration (1, 3, and 5%) and egg yolk (20%) in Tris-citric-fructose or Tris-citric-fructose-mineral salts extender on chilled canine sperm quality during 10 days of storage. The sperm motility was analyzed by computer-assisted sperm analysis (CASA), whereas plasma membrane integrity, acrosome membrane integrity, and mitochondrial membrane potential parameters were determined using 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) by confocal laser scanning microscope. Egg yolk was found to be better than soybean lecithin in Tris-citric-fructose or Tris-citric-fructose-mineral salts extender for maintaining the quality of chilled canine sperm within 10 days of storage (p<0.05). Although egg yolk

in Tris-citric-fructose extender could maintain the motility better than other extenders, egg yolk in Tris-citric-fructose-mineral salts extender was the highest in intact plasma membrane, intact acrosome membrane, and high mitochondrial membrane potential (p <0.05). In contrast, the sperm quality of soybean lecithin in Tris-citric-fructose-mineral salts extender was lower than that of soybean lecithin in Tris-citric-fructose extender, and soybean lecithin 1% was greater than soybean lecithin 3% and 5% in plasma membrane integrity, acrosome membrane integrity, and mitochondrial membrane potential (p<0.05). In conclusion, soybean lecithin cannot replace egg yolk in Tris-citric-fructose or Tris-citric-fructose-mineral salts extenders, and egg yolk in Tris-citric-fructose-mineral salts extender is superior to other extenders in chilling canine sperm.

Keywords: Canine, sperm, egg yolk, soybean, extender

3.1 Introduction

The estrous cycle of bitches is different from the estrous cycle of other domestic animals with a long interestrous interval of 6 to 7 months (Concannon, 2011), while the estrous cycle of sows, cows, and mares is around 21 days (Frandson et al., 2009). In natural mating, however, the semen of 1 ejaculation only fertilizes 1 bitch. Although the volume of canine semen is less than that of swine semen, canine sperm concentration is higher than swine sperm concentration with averages of 600 x 10^6 sperm/mL (Payan-carreira et al., 2011) and 360 x 10^6 sperm/mL (Bajena et al., 2016), respectively. In addition, the uterus in bitches is similar in sows which have a long horn-shaped uterus (Frandson et al., 2009). Thus, sperm dilution and artificial insemination (AI) are applicable in dogs.

To maintain the diluted sperm quality for AI techniques, sperm must be preserved by chilling or freezing (Thomassen and Farstad, 2009). However, sperm cryopreservation involves more special equipment and complicated processing operation than sperm chilling (Linde-Forsberg, 1991; Eilts, 2005). In addition, the fertile capacity of chilled sperm is higher than that of frozen sperm when compared to the same condition of estrous cycle time and AI techniques (Linde-Forsberg, 1995). Hence, chilled sperm is more popular than frozen sperm in AI techniques.

The major weakness of chilled sperm is a limitation of spermatozoa survival time. To improve the quality of chilled sperm during storage time, spermatozoa are diluted with an appropriate extender to provide energy, maintain pH and osmolality, and protect the plasma membrane integrity, acrosome membrane integrity, mitochondrial membrane potential, and DNA fragmentation against damage. In previous studies, the Tris-citric-fructose or glucose extender with 20% egg yolk was considered one of the most common extenders for chilled canine sperm that best maintains the quality of sperm during cooling storage (Rota et al., 1995; Ponglowhapan et al., 2004; Verstegen et al., 2005; Shahiduzzaman and Linde-Forsberg, 2007; Goericke-Pesch et al., 2012; Batista et al., 2012; Rodenas et al., 2014). Recently, soybean lecithin used as an alternative to egg yolk in extender to avoid hygiene problems from bacterial contamination has obtained equal or superior results (Beccaglia et al., 2009; Kmenta et al., 2011; Kasimanickam et al., 2012).

Furthermore, seminal plasma is a complex biological fluid containing ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-), energy substrates (fructose, sorbitol, glycerylphosphocholine), and organic compounds (citric acid, amino acids, peptides, proteins, lipids, hormones, cytokines) (Wales and White, 1965; Juyena and Stelletta, 2012). It has crucial functions

in sperm ejaculation and sperm survival in the female genital tract. The role of mineral ions is particularly essential for maintaining osmotic balance, forming parts of principal enzymes relating to sperm metabolism and sperm function (Çevik et al., 2007; Juyena and Stelletta, 2012; Smith et al., 2018). In previous studies, although canine seminal plasma has been found to be beneficial for chilled sperm plasma membrane integrity, acrosome membrane integrity, and mitochondrial membrane potential, it has detrimental effects on sperm motility (Rota et al., 1995; Treulen et al., 2012; Hori et al., 2017). The reduction in sperm motility is due to the decreasing of adenosine tryphosphate (ATP) concentration in seminal plasma by acid and alkaline phosphatase activity (Günzel-Apel and Ekrod, 1991). Moreover, the centrifugation and removal of seminal plasma before diluting with extenders has been used, and no harmful effects on the function of chilled canine sperm have been found (Rota et al., 1995; Peña and Linde-Forsberg, 2000; Rijsselaere et al., 2002; Shahiduzzaman and Linde-Forsberg, 2007; Goericke-Pesch et al., 2012). Thus, creating a new extender by adding mineral ions may increase the sperm survival time as well as improving the quality of chilled canine sperm without seminal plasma.

Therefore, the aim of the present study was to investigate the effects of egg yolk and soybean lecithin in Tris-citric-fructose or Tris-citric-fructose-mineral salts extender on motility, plasma membrane integrity, acrosome membrane integrity, and mitochondrial membrane potential in chilled canine sperm during 10 days of storage.

3.2 Materials and methods

3.2.1 Animals

A total of 5 healthy male American Bully dogs aged 2 to 5 years were used. All dogs with proven fertility after natural mating were trained to ejaculate by

digital manipulation before studying. The experiments were performed in accordance with the advice of the Institutional Animal Care and Use Committee of the Suranaree University of Technology, Nakhon Ratchasima, Thailand.

3.2.2 Semen collection and evaluation

Twenty ejaculates from 5 dogs were collected once per week by digital manipulation, and the 3 fractions were separated as described by Linde-Forsberg (1991). The sperm-rich fraction of ejaculates was deposited into pre-warmed polypropylene-calibrated tubes and placed in a water bath at 38ºC. Immediately, each ejaculate was analyzed to determine the semen volume, motility, concentration, viability, and abnormal morphology before ejaculates of the 5 dogs were pooled. Only ejaculates with progressive motility ≥70%, sperm concentration $\geq 200 \times 10^6$ spermatozoa/mL, sperm abnormal morphology ≥5%, and sperm viability ≥90% were included in this study. The percentage of sperm progressive motility and sperm concentration were estimated using computer-assisted sperm analysis (CASA). Sperm morphology and viability were determined using eosin-nigrosin staining (Tamuli and Watson, 1994).

3.2.3 Preparation of extenders

All chemicals used in this study were purchased from Sigma-Aldrich (Singapore), and all solutions were prepared using sterile distilled water.

Tris-citric-fructose and Tris-citric-fructose-mineral salts extenders added to 20% egg yolk or 1, 3, or 5% soybean lecithin (P3644 from Sigma) were used for canine sperm chilling in this study. The Tris-citric-fructose-egg yolk extender was used as a control and prepared as previously described (Kmenta et al., 2011), while the Tris-citric-fructose-soybean lecithin extenders used the same recipe, but egg yolk was

replaced by soybean lecithin. The Tris-citric-fructose-mineral salts extenders were made from the result of canine seminal fluid composition analysis (Table 3.1). Therefore, there were 8 extenders, including the Tris-citric-fructose-egg yolk (T-EY), Tris-citric-fructose-soybean lecithin 1% (T-SL1%), Tris-citric-fructose-soybean lecithin 3% (T-SL3%), Tris-citric-fructose-soybean lecithin 5% (T-SL5%), Tris-citric-fructose-mineral salts-egg yolk (T-M-EY), Tris-citric-fructose-mineral salts-soybean lecithin 1% (T-M-SL1%), Tris-citric-fructose-mineral salts-soybean lecithin 3% (T-M-SL3%), and Tris-citric-fructose-mineral salts-soybean lecithin 5% (T-M-SL5%). The composition of these extenders is shown in Table 3.2. Regarding the soybean lecithin extenders, the process of preparing the extenders was conducted with centrifuging and filtering as described by Axnér and Lagerson (2016).

Table 3.1 The composition of canine seminal fluid from American Bully dogs and analysis methods

Components	**Values (Mean±SD)**	**Analysis methods**
pH	6.45±0.20	pH meter
Osmolality (mOsmol/kg)	324.50±4.50	Osmometer
Sodium (m-equiv./L)	155.00±5.00	
Potassium (m-equiv./L)	13.20±1.50	Inductively coupled plasma-
Magnesium (m-equiv./L)	0.42±0.01	optical emission spectrometry
Calcium (m-equiv./L)	0.39±0.01	(ICP-OES)
Phosphorus (mg%)	7.15±0.75	
Chloride (mg/L)	5466.50±58.50	Ion-chromatograph (Anino/Conduct)

Table 3.1 Continue.

Components	**Values (Mean±SD)**	**Analysis methods**
Fructose (mg/L)	2.00±0.10	Ion-chromatograph (Electrochem)
Lactic acid (mmol/L)	3.52±0.48	High performance liquid
Citric acid (mmol/L)	3.63±0.37	chromatograph (HPLC)

Table 3.2 The composition, pH, and osmolality of the extenders

Ingredients	Extenders							
	T-EY	T-SL1%	T-SL3%	T-SL5%	T-M-EY	T-M-SL1%	T-M-SL3%	T-M-SL5%
Tris (mg)	3025	3025	3025	3025	900	900	900	900
Citric acid (mg)	1700	1700	1700	1700	500	500	500	500
Fructose (mg)	1250	1250	1250	1250	1250	1250	1250	1250
NaCl (mg)	-	-	-	-	450	450	450	450
$KHPO_4$ (mg)	-	-	-	-	60	60	60	60
KCl (mg)	-	-	-	-	60	60	60	60
$CaHPO_4$ (mg)	-	-	-	-	20	20	20	20
$MgCl_2$ (mg)	-	-	-	-	10	10	10	10
Egg yolk (mL)	20	-	-	-	20	-	-	-
Soybean lecithin (mg)	-	1000	3000	5000	-	1000	3000	5000
Gentamycin (mg)	200	200	200	200	200	200	200	200
Distilled water (mL)	To 100	To 100	To 100	To 100	To 100	To 100	To 100	To 100
pH	6.44	6.49	6.47	6.45	6.43	6.47	6.44	6.41
Osmolality (mOsmol/kg)	326	332	333	338	324	324	332	333

Tris-citric-fructose (T) buffer: pH 6.50, Osmolality: 328; Tris-citric-fructose-mineral salts (T-M) buffer: pH: 6.49, Osmolality: 325; EY: egg yolk; SL: soybean lecithin

3.2.4 Semen processing and experimental design

To study the effects of extenders on chilled canine sperm during a period of time, the experimental design was a repeated measurement in the completely randomized design of the effects of 8 extenders on sperm quality during 10 days of storage. Four replicates, with each being a pool of 5 ejaculates, were conducted.

Pooled semen was divided into 8 equal aliquots and placed in sterile tubes. The tubes were then centrifuged at 720×g for 5 min, and the supernatants were discarded (Rijsselaere et al., 2002). Sperm pellets were resuspended in 8 extenders to achieve the final sperm concentration of 100 x 10^6 spermatozoa/mL (Nizański et al., 2009; Batista et al., 2012). After that, equal volumes of 0.5 mL of every extended sperm were collected and put into 10 microcentrifuge tubes (1.5 mL). Then, they were placed in a plastic box containing water at 25ºC. Next, extended sperm was cooled down gradually (0.3ºC /min) to ºC for up to 1 hour (Bouchard et al., 1990) and stored at 5ºC during 10 days.

Sperm quality in all parameters were evaluated every day in the first 4 days, and once every 2 days after that over a period of 10 days.

3.2.5 Sperm evaluation

3.2.5.1 Evaluation of sperm motility

The sperm motility was evaluated using computer-assisted sperm analysis (CASA) by Hamilton Thorne Sperm Analyser (USA), version IVOS 14.0 (HTR-IVOS 14.0). The technical settings of CASA for canine sperm as the following were used in this study: frames per sec. (Hz), 60; no. of frames, 30; minimum contrast, 30; minimum cell size (pix), 7; cell size (pix), 6; cell intensity, 75; path velocity (VAP) (µm/s), 20; straightness (STR) (%), 40; VAP cutoff (µm/s), 9; and VSL

cutoff (μm/s), 20. A volume of 5 μL of the chilled sperm samples was mounted in a 2X-CEL counting chamber and was allowed to settle on the minitherm heating stage (38ºC) before the analysis. For each sample, at least 200 spermatozoa from 4 randomly selected fields were evaluated. The percentage of total motility (TM%), the percentage of progressive motility (PM%), velocity average pathway (VAP), velocity straight line (VSL), and velocity curvilinear (VCL) parameters were recorded.

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

The plasma membrane integrity, acrosome membrane integrity, and mitochondrial membrane potential were evaluated by incubating spermatozoa with 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). A stock and work solution of PI, H342, FITC-PSA, and JC-1 were prepared as described by Celeghini et al. (2007). A 100-μL chilled sperm sample was put into a warmed microcentrifuge tube, and 10 μL of H342 (40 μg/mL in Dulbecco's phosphate-buffered saline (DPBS)) was added. The mixture was incubated for 10 minutes at 38ºC. After the incubation, 2 μL of PI (0.5 mg/mL in DPBS), 15 μL of JC-1 (153 μM JC-1 in dimethyl sulfoxide (DMSO)), and 20 μL of FITC-PSA (100 μg/mL in DPBS) were added to the sample. The sample was then incubated for 8 minutes at 38ºC. To reduce background fluorescence, unbound H342, PI, FITC-PSA, and JC-1 were removed by adding 200 μL of DPBS, and spermatozoa were washed by centrifugation at 800×*g* for 2 minutes (Chelucci et al., 2015). The supernatant was removed, and the pellet was resuspended in 100 μL of DPBS. After washing, an 8-μL sample of stained spermatozoa was put on a slide and

coverslipped. The slide was immediately examined by a confocal laser scanning microscope (CLSM) (Nikon/Ni-E, Japan). To evaluate the stained spermatozoa, at least 200 cells were identified in duplicate for each sample with a 60x objective lens. 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). The staining standard of canine sperm in the fluorescent combination of H342, PI, FITC-PSA, and JC-1 can be seen in Figure 3.1.

3.2.6 Statistical analysis

Statistical analyses were performed with SPSS software version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). All data are provided as mean ± standard deviation (SD). The Kolmogorov-Smirnov test was used for normality analysis of the parameters. Differences were examined by a two-factor mixed analysis of variance (ANOVA) with interaction including time and extender as the main effects, followed by the post hoc analysis using Tukey test. When the results had a statistically significant interaction, the difference between groups at each level of each factor (time, extender) was determined. In this case, a modification of the repeated measurement command in the syntax was conducted by adding compare simple main effects for both time and extender factors. Pairwise comparisons were performed using a confidence interval

adjustment by the Bonferroni method. A difference of $p<0.05$ was considered significant.

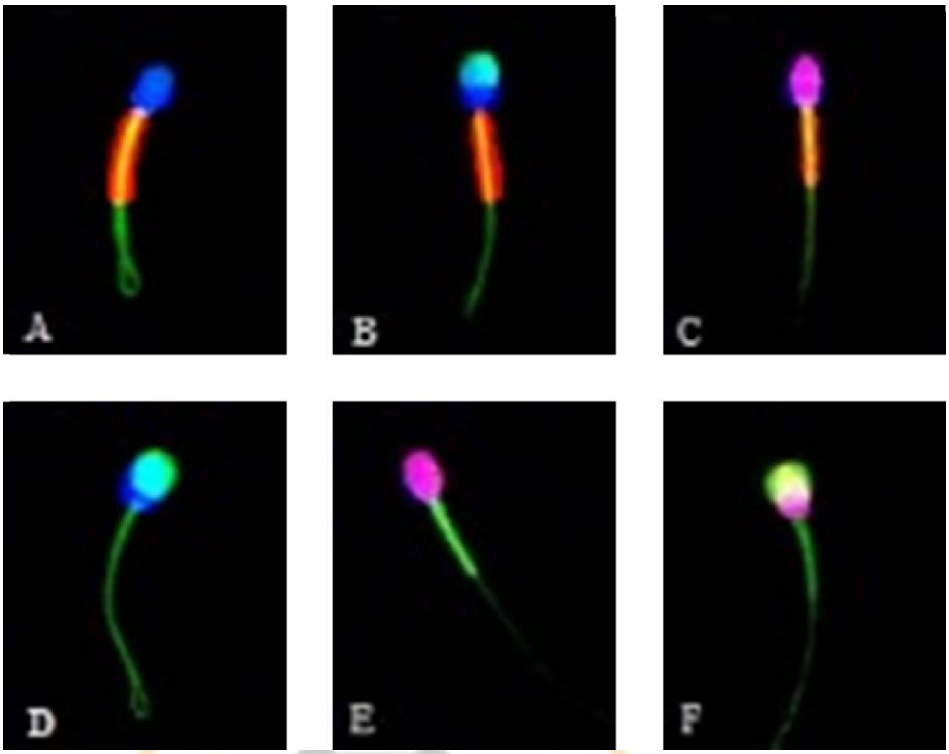

Figure 3.1 Canine spermatozoa stained with the association of fluorescent probes, H324, PI, FITC-PSA, and JC-1 under a confocal laser scanning microscope (600x magnification).

(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.

3.3 Results

3.3.1 Sperm motility

The total motility (TM) and progressive motility (PM) of spermatozoa in the 8 extenders are shown in Table 3.3. Overall, spermatozoa in T-EY and T-M-EY extenders were high, and decreased gradually in TM (from 89.4±1.9% to 65.2±5.1% in T-EY, and from 85.9±3.8% to 13.0±2.3 % in T-M-EY) and in PM parameters (from 66.1±3.3% to 32.2±2.9% in T-EY, and from 70.5±4.8% to 3.6±1.3% in T-M-EY) during the whole experimental period (10 days), while the percentage of TM and PM were reduced significantly in T-SL (from 92.2±0.8% (day 1) to <5.2±2.0% (day 10) in TM, from 82.0±2.3% (day 1) to <4.4±1.1 (day 8) in PM), in T-M-SL extenders (from 85.7±3.2% (day 1) to <1.8±0.5% (day 6) in TM, and from 61.1±6.7% (day 1) to 0% (day 4) in PM). However, the sperm in T-SL3% extender was the highest in TM during the first 4 days of storage (92.2±0.8% (day 1) and 82.8±1.4% (day 4)) and in PM during the first 3 days storage (82.0±2.3% (day 1), and 60.9±4.6% (day 3)) when compared to the rest extenders. Yet, there was no significant difference when it was compared to T-EY, T-SL1%, and T-M-EY extenders ($p>0.05$). In addition, from days 6 to 10, the percentage of TM and PM in T-EY extender were the highest and had a significant difference when compared to that of other extenders ($p<0.05$). Although T-M-EY extender was lower than T-EY extender in TM and PM after day 6, it was still significantly higher than that in T-SL and T-M-SL extenders ($p<0.05$). Moreover, for T-M-SL extenders, TM and PM of the sperm decreased dramatically after day 3 and stopped rapidly on day 8 in TM as well as obtained a zero value on day 4 in the PM parameter.

Another evolution that was observed in sperm motility characteristics was sperm velocity (VAP, VSL, and VCL). Changes in the sperm velocity parameter during the storage period are given in Table 3.4. As TM and PM parameters, the sperm velocity parameters in T-EY and T-M-EY extenders declined steadily and were highest among the extenders during 10 days storage. In particular, although T-M-EY extender was lower than T-EY extender in VAP and VCL parameters, it was higher than that in the VSL parameter during the storage process without significant difference (p>0.05). Furthermore, during the first 4 days, there was a slow change with no significant difference among T-EY, T-SL, and T-M-EY extenders in these parameters. In contrast, the sperm velocity parameters in T-M-SL were only maintained for 3 days and decreased suddenly afterward.

3.3.2 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 presented in Table 3.5. These parameters in all the extenders decreased gradually during the chilling storage. For plasma membrane integrity, the high values of intact plasma membrane were shown in both T-EY (from 49.5±4.3% to 22.5±8.7%) and T-M-EY (from 47.2±7.5% to 14.9±3.5%) extenders during 10 days of storage. However, the percentage of intact plasma membrane in T-SL1% and T-M-SL1% extenders were not significantly lower than that in T-EY and T-M-EY extenders during the first 4 days (p>0.05). In addition, there was a similar value of this parameter in T-SL1% and T-SL3% extenders during the whole storage period (p>0.05). In contrast, T-M-EY extender had the highest value and was not significantly different from T-EY extender in both the intact acrosome

membrane (63.5±7.2% vs. 47.7±4.9% on day 1 and 22.7±3.9% vs. 22.6±1.6% on day 9, respectively) and high mitochondrial membrane potential parameters (66.1±3.8% vs. 62.5±5.9% on day 1, and 43.4±3.6% vs. 39.4±2.0% on day 6, respectively) (p<0.05). Moreover, T-SL1% was higher in the intact acrosome membrane and in high mitochondrial membrane potential values than T-SL3%, but it was not significantly different (p>0.05). Specifically, the proportion of these parameters in the extenders with high levels of soybean lecithin (T-SL3%, T-SL5%, T-M-SL3%, and T-M-SL5%) reduced quickly and had a significant difference when compared to T-EY and T-M-EY extenders during the whole storage period (p<0.05).

On the other hand, the percentage of healthy sperm with the intact plasma membrane, intact acrosome membrane, and high mitochondrial membrane potential is summarized in Table 3.6. In general, like the previous parameters, the proportion of healthy sperm achieved with the intact plasma membrane, intact acrosome membrane, and high mitochondrial membrane potential in T-M-EY and T-EY extenders were the highest when compared with that in other extenders during 10 days. However, during the first 6 days, the percentage of healthy sperm in T-M-EY (from 46.8±7.9% to 20.8±2.5%) was significantly higher than that in T-EY extenders (from 42.2±7.0% to 13.6±3.2%) (p<0.05), but it decreased suddenly after day 6, and obtained a similar value as in T-EY extender on day 10 (5.4±1.8% and 4.6±0.8%, respectively) (p>0.05). Furthermore, although the healthy sperm in T-SL1% extender was not significantly different from that in T-M-SL1% extender, it was significantly higher than that in the other extenders (T-SL3%, T-SL5%, T-M-SL3%, and T-M-SL5%).

Table 3.3 Effects of egg yolk (EY) 20% and soybean lecithin (SL) at different concentrations (1%, 3%, and 5%) in Tris-citric-fructose (T) or Tris-citric-fructose-mineral salts (T-M) extender on total motility (TM) and progressive motility (PM) parameters of chilled canine sperm during a storage period of 10 days at 5 ºC

Parameters	Extenders	Day 1	Day 2	Day 3	Day 4	Day 6	Day 8	Day 10
TM (%)	T-EY	89.4±1.9^{aA}	85.4±1.7^{aAB}	83.4±2.7^{aAB}	81.1±2.6^{aB}	77.7±4.5^{aB}	74.9±4.7^{aBC}	65.2±5.1^{aD}
	T-SL1%	90.6±2.5^{aA}	87.5±0.8^{aAB}	84.9±2.8^{aAB}	79.5±3.3^{aB}	71.6±1.3^{aC}	15.0±4.4^{dE}	2.1±0.9^{cdF}
	T-SL3%	92.2±0.8^{aA}	89.3±1.8^{aAB}	85.2±2.2^{aAB}	82.8±1.4^{aB}	74.1±1.8^{aC}	26.1±5.5^{cE}	5.2±2.0^{cG}
	T-SL5%	88.8±3.5^{aA}	85.0±5.2^{aA}	77.6±4.7^{aB}	72.2±4.3^{bB}	46.8±4.9^{bD}	5.9±1.3^{deF}	0.0±0.0^{dF}
	T-M-EY	85.9±3.8^{abA}	83.7±2.5^{aAB}	81.6±3.1^{aAB}	79.1±2.7^{aB}	71.8±3.4^{aCD}	37.5±8.0^{bE}	13.0±2.3^{bG}
	T-M-SL1%	85.7±3.2^{abA}	58.9±8.7^{bB}	48.2±6.5^{bC}	12.3±2.4^{cD}	1.8±0.5^{cE}	0.0±0.0^{eE}	0.0±0.0^{dE}
	T-M-SL3%	82.6±4.0^{bA}	44.9±7.5^{cB}	27.5±4.2^{cC}	7.5±1.4^{cdD}	1.1±0.4^{cE}	0.0±0.0^{eE}	0.0±0.0^{dE}
	T-M-SL5%	80.2±2.4^{bA}	37.8±2.8^{cB}	23.5±3.3^{cC}	2.4±1.0^{dD}	0.0±0.0^{cD}	0.0±0.0^{eD}	0.0±0.0^{dD}
PM (%)	T-EY	66.1±3.3^{cdeA}	61.8±5.0^{bA}	58.5±5.8^{aAB}	56.7±6.5^{aAB}	48.9±4.6^{aCD}	43.4±6.3^{aD}	32.2±2.9^{aF}
	T-SL1%	80.5±2.2^{abA}	68.9±1.1^{abB}	62.7±1.2^{aB}	42.5±4.9^{bC}	24.8±6.1^{bcE}	3.5±1.5^{cG}	0.0±0.0^{cG}
	T-SL3%	82.0±2.3^{aA}	73.9±2.2^{aB}	60.9±4.6^{aC}	44.8±5.8^{bD}	27.6±5.0^{bF}	4.4±1.1^{cH}	0.0±0.0^{cH}
	T-SL5%	74.3±1.6^{abcA}	65.3±1.8^{bB}	45.5±5.6^{bC}	38.6±5.3^{bD}	13.4±2.6^{cF}	1.7±0.3^{cG}	0.0±0.0^{cG}
	T-M-EY	70.5±4.8^{bcdA}	67.3±5.0^{abAB}	62.3±4.1^{aABC}	59.5±3.3^{aBC}	44.2±9.1^{aD}	22.0±3.3^{bF}	3.6±1.3^{bH}
	T-M-SL1%	61.1±6.7^{deA}	25.9±3.0^{cB}	11.1±3.0^{cC}	0.0±0.0^{cD}	0.0±0.0^{dD}	0.0±0.0^{cD}	0.0±0.0^{cD}
	T-M-SL3%	57.5±2.6^{eA}	18.7±2.5^{cdB}	11.6±1.7^{cC}	0.0±0.0^{cD}	0.0±0.0^{dD}	0.0±0.0^{cD}	0.0±0.0^{cD}
	T-M-SL5%	58.2±6.6^{eA}	12.6±2.3^{dB}	5.4±2.0^{cC}	0.0±0.0^{cC}	0.0±0.0^{dC}	0.0±0.0^{cC}	0.0±0.0^{cC}

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

Lowercase superscript letters (a, b, c, d or e) in the same column indicates significant difference among extenders ($p<0.05$) and uppercase superscript letters (A, B, C, D, E, F, G or H) in the same row indicates significant difference within extenders with different storage time ($p<0.05$).

Table 3.4 Effects of egg yolk (EY) 20% and soybean lecithin (SL) at different concentrations (1%, 3% and 5%) in Tris-citric-fructose (T) or Tris-citric-fructose-mineral salts (T-M) extender on average pathway velocity (VAP), straight line velocity (VSL) and curvilinear velocity (VCL) parameters of chilled canine sperm during a storage period of 10 days at 5 ^{o}C

Parameters	Extenders	Day 1	Day 2	Day 3	Day 4	Day 6	Day 8	Day 10
VAP (µm/s)	T-EY	89.6 ± 0.5^{abA}	76.3 ± 5.2^{aB}	71.9 ± 3.5^{aB}	67.6 ± 6.5^{aB}	64.8 ± 7.6^{aB}	60.8 ± 7.5^{aBC}	55.6 ± 8.4^{aC}
	T-SL1%	93.5 ± 0.8^{aA}	76.1 ± 5.0^{aB}	67.8 ± 7.2^{aBC}	56.5 ± 6.5^{aC}	47.1 ± 6.4^{bE}	41.4 ± 8.0^{bE}	0.0 ± 0.0^{cG}
	T-SL3%	91.6 ± 1.6^{abA}	76.9 ± 2.9^{aB}	67.6 ± 8.7^{aBC}	56.3 ± 7.0^{aCD}	46.9 ± 4.7^{bE}	35.7 ± 1.6^{bcF}	0.0 ± 0.0^{cG}
	T-SL5%	$90.8\pm4,4^{abA}$	78.4 ± 5.8^{aA}	64.1 ± 6.7^{aB}	55.9 ± 5.9^{aB}	43.6 ± 2.9^{bC}	27.1 ± 2.3^{cE}	0.0 ± 0.0^{cF}
	T-M-EY	92.9 ± 4.0^{aA}	78.2 ± 4.3^{aAB}	71.6 ± 7.0^{aB}	64.9 ± 6.0^{aBC}	60.5 ± 6.1^{aC}	53.3 ± 7.2^{abD}	43.1 ± 7.8^{bE}
	T-M-SL1%	82.3 ± 7.0^{abA}	59.7 ± 9.4^{bB}	38.6 ± 9.0^{bC}	0.0 ± 0.0^{bD}	0.0 ± 0.0^{cD}	0.0 ± 0.0^{dD}	0.0 ± 0.0^{cD}
	T-M-SL3%	79.6 ± 6.3^{bA}	47.2 ± 6.9^{bB}	37.1 ± 6.0^{bB}	0.0 ± 0.0^{bC}	0.0 ± 0.0^{cC}	0.0 ± 0.0^{dC}	0.0 ± 0.0^{cC}
	T-M-SL5%	79.7 ± 9.8^{bA}	55.0 ± 8.9^{bB}	35.6 ± 5.5^{bC}	0.0 ± 0.0^{bD}	0.0 ± 0.0^{cD}	0.0 ± 0.0^{dD}	0.0 ± 0.0^{cD}
VSL (µm/s)	T-EY	77.0 ± 2.8^{abA}	62.1 ± 7.2^{abAB}	52.1 ± 4.0^{abBC}	47.4 ± 8.1^{abBCD}	44.3 ± 6.9^{abCDE}	40.3 ± 4.4^{aCDE}	36.3 ± 3.3^{aE}
	T-SL1%	74.0 ± 2.2^{abA}	59.4 ± 4.4^{abAB}	46.9 ± 5.7^{bBC}	41.4 ± 6.8^{bC}	33.8 ± 3.4^{bcD}	29.7 ± 2.6^{bD}	0.0 ± 0.0^{cF}
	T-SL3%	72.6 ± 5.8^{abA}	58.1 ± 6.3^{abAB}	47.2 ± 7.1^{bBC}	39.4 ± 4.7^{bC}	31.2 ± 4.2^{cD}	24.2 ± 2.0^{bcE}	0.0 ± 0.0^{cF}
	T-SL5%	72.1 ± 8.5^{abA}	56.4 ± 6.2^{abB}	44.8 ± 6.4^{bBC}	36.6 ± 3.9^{bC}	29.1 ± 3.9^{cD}	15.6 ± 2.2^{cF}	0.0 ± 0.0^{cG}
	T-M-EY	86.1 ± 4.6^{aA}	69.1 ± 3.7^{aB}	63.2 ± 7.7^{aB}	55.5 ± 8.2^{aB}	49.2 ± 8.1^{aC}	41.3 ± 1.0^{aD}	31.6 ± 4.0^{bE}

Table 3.4 Continue.

Parameters	Extenders	Day 1	Day 2	Day 3	Day 4	Day 6	Day 8	Day 10
VSL (µm/s)	T-M-SL1%	71.6 ± 8.4^{abA}	51.1 ± 7.6^{bB}	28.0 ± 4.4^{cC}	0.0 ± 0.0^{cD}	0.0 ± 0.0^{dD}	0.0 ± 0.0^{dD}	0.0 ± 0.0^{cD}
	T-M-SL3%	71.2 ± 6.2^{abA}	45.6 ± 7.9^{bcB}	29.3 ± 4.2^{cC}	0.0 ± 0.0^{cD}	0.0 ± 0.0^{dD}	0.0 ± 0.0^{dD}	0.0 ± 0.0^{cD}
	T-M-SL5%	65.8 ± 9.4^{bA}	32.6 ± 8.2^{cB}	25.5 ± 6.4^{cB}	0.0 ± 0.0^{cC}	0.0 ± 0.0^{dC}	0.0 ± 0.0^{dC}	0.0 ± 0.0^{cC}
VCL (µm/s)	T-EY	148.0 ± 6.5^{bcA}	142.1 ± 7.4^{bcAB}	135.8 ± 8.6^{aABC}	127.2 ± 15.3^{aABCD}	121.8 ± 19.5^{aBCD}	115.7 ± 17.6^{aBCD}	108.3 ± 14.4^{aD}
	T-SL1%	181.1 ± 8.8^{aA}	168.4 ± 10.9^{aAB}	144.9 ± 13.8^{aB}	116.1 ± 19.0^{aC}	98.6 ± 19.0^{aD}	58.6 ± 27.0^{bF}	0.0 ± 0.0^{cG}
	T-SL3%	172.3 ± 1.6^{aA}	165.0 ± 7.6^{abAB}	143.6 ± 18.9^{aBC}	118.7 ± 17.6^{aC}	97.5 ± 15.0^{aE}	62.2 ± 8.6^{bF}	0.0 ± 0.0^{cG}
	T-SL5%	164.3 ± 5.3^{abA}	158.7 ± 8.7^{abAB}	134.6 ± 17.5^{aBC}	108.7 ± 25.8^{aC}	89.4 ± 21.1^{aD}	42.2 ± 10.2^{bF}	0.0 ± 0.0^{cG}
	T-M-EY	133.5 ± 3.2^{cA}	129.3 ± 3.9^{cdAB}	123.1 ± 6.1^{aABC}	117.1 ± 2.8^{aABC}	110.1 ± 4.0^{aABC}	101.7 ± 9.6^{aBCD}	84.4 ± 12.7^{bD}
	T-M-SL1%	134.2 ± 8.7^{cA}	121.8 ± 7.5^{cA}	72.7 ± 19.0^{bB}	0.0 ± 0.0^{bC}	0.0 ± 0.0^{bC}	0.0 ± 0.0^{cC}	0.0 ± 0.0^{cC}
	T-M-SL3%	133.2 ± 7.2^{cA}	114.7 ± 16.8^{dB}	78.9 ± 14.4^{bC}	0.0 ± 0.0^{bD}	0.0 ± 0.0^{bD}	0.0 ± 0.0^{cD}	0.0 ± 0.0^{cD}
	T-M-SL5%	131.9 ± 9.3^{cA}	87.9 ± 10.5^{eB}	59.4 ± 9.1^{bC}	0.0 ± 0.0^{bD}	0.0 ± 0.0^{bD}	0.0 ± 0.0^{cD}	0.0 ± 0.0^{cD}

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

Lowercase superscript letters (a, b, c, d or e) in the same column indicates significant difference among extenders ($p<0.05$) and uppercase superscript letters (A, B, C, D, E, F or G) in the same row indicates significant difference within extenders with different storage time ($p<0.05$).

Table 3.5 Percentage of intact plasma membrane, intact acrosome membrane, and high mitochondrial membrane potential in chilled canine sperm diluted in Tris-citric-fructose (T) or Tris-citric-fructose-mineral salts (T-M) extender plus 20% egg yolk (EY) or soybean lecithin (SL) at different concentrations (1%, 3%, and 5%) during a storage period of 10 days at 5 ^{o}C

Parameters	Extenders	Day 1	Day 2	Day 3	Day 4	Day 6	Day 8	Day 10
Plasma membrane (%)	T-EY	49.5±4.3^{aA}	43.6±7.0^{aAB}	38.6±6.5^{abB}	34.0±3.3^{aC}	30.2±5.8^{aC}	26.7±5.9^{aCD}	22.5±8.7^{aD}
	T-SL1%	35.0±3.7^{cA}	31.5±2.8^{abA}	29.1±1.8^{bcdA}	27.3±2.7^{abAB}	20.8±5.6^{abcB}	4.3±2.3^{bC}	1.3±0.4^{bC}
	T-SL3%	36.0±1.2^{bcA}	29.0±3.3^{bB}	25.5±2.7^{cdBC}	22.1±2.3^{bcdC}	19.1±3.7^{bcC}	6.3±6.7^{bD}	0.7±0.3^{bE}
	T-SL5%	28.7±2.8^{cA}	27.8±2.8^{bA}	25.7±3.7^{cdA}	16.8±3.9^{cdB}	12.2±1.7^{cB}	2.2±1.0^{bC}	0.1±0.0^{bC}
	T-M-EY	47.2±7.5^{abA}	42.6±5.3^{aA}	40.3±4.5^{aA}	34.4±5.7^{aB}	27.2±3.2^{abBC}	20.4±1.1^{aC}	14.9±3.5^{aD}
	T-M-SL1%	39.4±8.2^{abcA}	35.2±8.0^{abAB}	29.9±5.3^{abcB}	24.9±5.0^{abcC}	15.5±3.8^{cD}	1.8±0.7^{bE}	0.3±0.1^{bE}
	T-M-SL3%	35.3±2.4^{cA}	29.6±5.8^{bA}	23.7±4.1^{cdB}	19.6±2.3^{bcdBC}	15.2±3.7^{cC}	1.8±0.7^{bD}	0.1±0.0^{bD}
	T-M-SL5%	28.2±2.7^{cA}	22.8±2.9^{bAB}	18.6±5.2^{dBC}	15.1±4.8^{dC}	13.3±3.4^{cC}	1.5±0.2^{bD}	0.1±0.0^{bD}
Acrosome membrane (%)	T-EY	47.7±4.9^{bA}	42.0±4.6^{abAB}	38.0±4.0^{aB}	32.2±5.3^{abC}	26.1±4.0^{abD}	22.6±1.6^{aD}	16.5±4.2^{aE}
	T-SL1%	40.9±6.3^{bA}	31.9±3.8^{bcB}	28.2±3.5^{bB}	25.7±5.5^{bcB}	19.5±4.0^{bC}	18.1±3.9^{aC}	9.6±4.7^{bcD}
	T-SL3%	36.7±3.2^{bcA}	25.2±5.3^{cB}	20.6±4.6^{bcBC}	17.0±5.8^{cdC}	7.9±2.2^{cD}	6.0±1.7^{bD}	3.2±1.7^{cD}
	T-SL5%	25.1±2.5^{cA}	19.1±4.3^{cB}	14.1±4.4^{cdBC}	11.0±3.7^{dCD}	7.5±3.0^{cDE}	5.7±3.3^{bDE}	3.6±2.5^{cE}
	T-M-EY	63.5±7.2^{aA}	56.1±11.1^{aB}	45.8±5.7^{aC}	40.2±5.7^{aD}	33.8±4.1^{aE}	22.7±3.9^{aF}	16.0±1.6^{abG}

Table 3.5 Continue.

Parameters	Extenders	Day 1	Day 2	Day 3	Day 4	Day 6	Day 8	Day 10
Acrosome membrane (%)	T-M-SL1%	39.3 ± 8.9^{bA}	29.0 ± 7.4^{bcB}	16.4 ± 1.8^{cdC}	14.0 ± 3.3^{dCD}	9.9 ± 3.0^{cD}	3.5 ± 2.6^{bE}	2.0 ± 0.9^{cE}
	T-M-SL3%	25.1 ± 2.9^{cA}	21.0 ± 2.1^{cAB}	13.5 ± 3.4^{cdB}	8.8 ± 2.7^{dC}	5.1 ± 2.5^{cCD}	2.5 ± 1.6^{bCD}	1.6 ± 1.0^{cD}
	T-M-SL5%	24.5 ± 3.7^{cA}	16.8 ± 1.7^{cB}	8.8 ± 2.7^{dC}	6.4 ± 1.6^{dCD}	5.2 ± 1.6^{cCDE}	1.7 ± 0.5^{bDE}	0.8 ± 0.5^{cE}
Mitochondrial membrane potential (%)	T-EY	62.5 ± 5.9^{aA}	54.2 ± 2.5^{abB}	49.1 ± 2.1^{aC}	42.3 ± 2.1^{aD}	39.4 ± 2.0^{aD}	36.0 ± 3.0^{aD}	21.3 ± 2.6^{aE}
	T-SL1%	58.0 ± 6.7^{abA}	44.5 ± 4.6^{bcB}	36.8 ± 3.2^{bC}	32.1 ± 4.3^{bD}	29.2 ± 3.5^{bD}	10.2 ± 2.2^{cE}	3.6 ± 2.6^{bF}
	T-SL3%	41.4 ± 4.4^{cdA}	34.6 ± 3.1^{cdB}	28.9 ± 1.9^{cC}	24.1 ± 4.0^{bD}	19.2 ± 2.3^{cE}	5.6 ± 1.7^{cdF}	1.7 ± 0.8^{bG}
	T-SL5%	33.7 ± 3.3^{deA}	25.2 ± 4.3^{deB}	17.0 ± 2.5^{deC}	10.3 ± 1.7^{cdD}	6.3 ± 1.5^{dDE}	2.1 ± 1.6^{dEF}	0.8 ± 0.7^{bF}
	T-M-EY	66.1 ± 3.8^{aA}	57.7 ± 5.3^{aB}	49.1 ± 3.6^{aC}	46.5 ± 3.8^{aCD}	43.4 ± 3.6^{aD}	29.9 ± 2.9^{bE}	17.9 ± 2.0^{aF}
	T-M-SL1%	47.2 ± 4.1^{bcA}	42.2 ± 3.9^{cA}	33.2 ± 2.8^{bcB}	28.0 ± 4.1^{bC}	20.0 ± 1.7^{cD}	5.6 ± 1.7^{cdE}	1.5 ± 0.3^{bF}
	T-M-SL3%	32.7 ± 4.1^{deA}	25.4 ± 5.2^{deB}	19.1 ± 2.9^{dC}	12.7 ± 1.4^{cD}	7.5 ± 2.8^{dE}	4.3 ± 3.6^{dEF}	0.7 ± 0.5^{bF}
	T-M-SL5%	25.7 ± 3.2^{eA}	17.3 ± 2.4^{eB}	11.0 ± 2.4^{eC}	4.3 ± 3.5^{dD}	2.1 ± 1.3^{dD}	1.0 ± 0.1^{dD}	0.1 ± 0.0^{bD}

Values are mean±SD 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 3.6 Percentage of healthy sperm with intact plasma membrane, intact acrosome membrane, and high mitochondrial membrane potential in chilled canine sperm diluted in Tris-citric-fructose (T) or Tris-citric-fructose-mineral salts (T-M) extender plus 20% egg yolk (EY) or soybean lecithin (SL) at different concentrations (1%, 3%, and 5%) during a storage period of 10 days

Extenders	Day 1	Day 2	Day 3	Day 4	Day 6	Day 8	Day 10
T-EY	42.2 ± 7.0^{aA}	31.9 ± 3.6^{bB}	27.4 ± 3.6^{bC}	22.2 ± 5.1^{bD}	13.6 ± 3.2^{bE}	11.4 ± 2.9^{aF}	5.4 ± 1.8^{aG}
T-SL1%	25.9 ± 4.7^{bcA}	18.8 ± 1.2^{cB}	13.7 ± 2.2^{cC}	6.9 ± 1.5^{cD}	1.3 ± 0.5^{cE}	0.1 ± 0.0^{cE}	0.0 ± 0.0^{bE}
T-SL3%	17.5 ± 2.5^{cdA}	8.0 ± 0.6^{deB}	4.9 ± 1.8^{dBC}	2.2 ± 0.4^{cdBC}	1.3 ± 0.5^{cC}	0.3 ± 0.1^{cC}	0.0 ± 0.0^{bC}
T-SL5%	14.8 ± 1.8^{dA}	5.2 ± 1.7^{eB}	3.3 ± 2.1^{dBC}	2.2 ± 1.4^{cdCD}	0.8 ± 0.2^{cD}	0.1 ± 0.0^{cD}	0.0 ± 0.0^{bD}
T-M-EY	$46.8\pm7.\ 9^{aA}$	39.0 ± 4.2^{aB}	36.2 ± 4.0^{aB}	29.1 ± 2.3^{aC}	20.8 ± 2.5^{aD}	6.9 ± 1.6^{bE}	4.6 ± 0.8^{aE}
T-M-SL1%	30.6 ± 2.0^{bA}	16.3 ± 0.8^{cB}	7.8 ± 1.6^{cdC}	6.8 ± 1.3^{cdC}	1.7 ± 1.0^{cD}	0.3 ± 0.1^{cD}	0.0 ± 0.0^{bD}
T-M-SL3%	20.2 ± 1.6^{bcdA}	13.7 ± 3.4^{cdB}	6.9 ± 1.5^{dC}	2.4 ± 0.8^{cdD}	0.45 ± 0.1^{cD}	0.0 ± 0.0^{cD}	0.0 ± 0.0^{bD}
T-M-SL5%	20.0 ± 1.7^{bcdA}	8.6 ± 2.1^{deB}	2.2 ± 1.0^{dC}	0.8 ± 0.3^{dC}	0.1 ± 0.0^{cC}	0.0 ± 0.0^{cC}	0.0 ± 0.0^{bC}

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

Lowercase superscript letters (a, b, c, d or e) in the same column indicates significant difference among extenders ($p<0.05$) and uppercase superscript letters (A, B, C, D, E, F or G) in the same row indicates significant difference within extenders with different storage time ($p<0.05$).

3.4 Discussion

The study investigated the effects of egg yolk and soybean lecithin in Tris-citric-fructose or Tris-citric-fructose-mineral salts extender on canine sperm quality. The obtained results clearly demonstrated that egg yolk is superior to soybean lecithin in Tris-citric-fructose or Tris-citric-fructose-mineral salts extender in motility, plasma membrane integrity, acrosome membrane integrity, and mitochondrial membrane potential parameters during 10 days of chilling storage. The results of the current study are in agreement with the previous reports in dogs (Axnér and Lagerson, 2016), rams (Ustuner et al., 2016), and bulls (Crespilho et al., 2012). They have confirmed that egg yolk extender was more efficient on sperm quality than soybean lecithin extender. This may be explained by the fact that both low-density lipoproteins in egg yolk and soybean lecithin, known as a protective factor for sperm plasma membranes, are mainly composed of phospholipids. However, there is a difference in their mechanisms of action by their composition. Soybean lecithin is comprised almost entirely of phospholipids, whereas low-density lipoprotein in egg yolk has both phospholipids and proteins. Proteins are necessary to keep phospholipid fractions in a solubilized form, and closely associated with the plasma membrane (Watson, 1981). This leads to the interaction between low-density lipoprotein in egg yolk and sperm plasma membrane (Belala et al., 2016). In addition, low-density lipoprotein in egg yolk can decrease the binding of proteins in seminal plasma to sperm as well as reduce the phospholipid efflux from sperm membranes (Manjunath et al., 2002; Bergeron, 2003). They can also prevent premature sperm capacitation and acrosome reaction (Witte et al., 2009). It is noteworthy that egg yolk is a conventional but powerful extender for canine sperm preservation and cryopreservation (Silva et al., 2002; Ponglowhapan et al., 2004;

Verstegen et al., 2005; Shahiduzzaman and Linde-Forsberg, 2007; Witte et al., 2009; Goericke-Pesch et al., 2012; Batista et al., 2012; Treulen et al., 2012; Rodenas et al., 2014; Hori et al., 2017).

However, the results also indicated that although soybean lecithin extenders were lower than egg yolk extenders in almost all sperm quality parameters during 10 days of storage, they were similar to egg yolk extenders in the total motility and plasma membrane parameters during the first 6 days (in T-SL1% and T-SL3% extenders). Similar findings on chilled canine sperm were reported by Beccaglia et al. (2009). They found that there was no significant difference between Tris-citric-fructose-soybean lecithin (0.04%) and Tris-citric-fructose-egg yolk (20%) extenders in motility, capacitation, and zona pellucida binding during 4 days of chilling storage. In contrast to our results, Kmenta et al. (2011) reported that 0.8% lecithin extender was better than Tris-citric-fructose-egg yolk (20%) extender in motility and viability of chilling canine sperm during 8 days when adding an enhancer (Tris buffer). In another study on chilled canine sperm, motility, plasma membrane integrity, and mitochondrial membrane potential in soybean lecithin (0.4%) extender were found to be greater than those in 20% egg yolk extender during 10 days of storage (Kasimanickam et al., 2012). Furthermore, several investigations have proven that soybean lecithin could replace egg yolk in extender to protect sperm during cryopreservation in dogs (Beccaglia et al., 2009; Dalmazzo et al., 2018), goats (Salmani et al., 2014; Yotov, 2015; Chelucci et al., 2015), bulls (Aires, 2003; El-sisy et al., 2016), rams (Masoudi et al., 2016), stallions (Nouri et al., 2013), rabbits (Nishijima et al., 2015), and fishes (Yildiz et al., 2013). The difference between our results and previous studies may be due to the difference in soybean lecithin sources, the preparation for soybean lecithin extenders and the

concentrations of soybean lecithin. Paz et al. (2010) have found that various soybean lecithin sources have different compositions and effects on sperm quality. In addition, because soybean lecithin has amphiphilic characteristics, there are large lipid droplets in extender after diluting. Consequently, soybean lecithin is unsuitable for protecting sperm during storage. Thus, centrifuging and filtering are necessary to prepare soybean lecithin extenders as described earlier (Vick et al., 2012; Paz et al., 2010; Axnér and Lagerson, 2016). However, Paz et al. (2010) have also reported that the centrifugation-filtration process can reduce the quantity of phospholipids in extenders by 50%.

The study suggested that high concentrations of soybean lecithin have negative effects on sperm quality. Our results are in agreement with those reported by Forouzanfar et al. (2010) and Salmani et al. (2014). Their works have reported that the reduction of all sperm quality parameters at high levels of soybean lecithin may be due to the high viscosity of soybean lecithin in extenders. We also observed that there was more particle debris on 3% and 5% soybean lecithin extenders, as described by Forouzanfar et al. (2010). Moreover, Dalmazzo et al. (2018) have explained that high concentrations of soybean lecithin (phosphatidylcholine) can cause higher concentrations of exogenous phosphatidylchonline inside the mitochondria and lead to an imbalance between intracellular and extracellular as the result of reducing mitochondria activity as well as motility.

The most important finding of our study is that T-EY extender was inferior to T-M-EY extender in VSL parameter and in the percentage of healthy sperm with intact plasma membrane, intact acrosome membrane, and high mitochondrial membrane potential during 10 days of storage. From a clinical point of view, VSL is most likely the most important parameter in the CASA system in which the average velocity of the

sperm heads through a straight line connecting to the first point of the last track. In a previous study, the researchers demonstrated that the decline in VSL was highly correlated with the outcome of fertilization *in vitro* in rat spermatozoa (Harry and Mehdi, 1996). In addition, healthy sperm are defined as spermatozoa which have a good quality of plasma membrane, acrosome membrane, and mitochondrial membrane potential. These spermatozoa also have a high survival potential in the female reproductive track as well as fertility ability (Grunewald et al., 2008). Moreover, although the proportion of healthy sperm in T-M-EY was significantly higher than that in T-EY during 6 days, it reduced suddenly after day 6 and gained a similar value with T-EY extender on day 10. These factors may help to explain that T-M-EY extenders contain several mineral cations, such as Na^+, K^+, Mg^{2+}, Ca^{2+}, which are the main ions of seminal plasma, whose important functions are to maintain osmotic balance, form parts of principal enzymes relating to sperm metabolism and sperm function (Çevik et al., 2007; Juyena and Stelletta, 2012; Smith et al., 2018). In particular, Mg^{2+} has a vital function in modulating the regulation of K^+ (Na-K pump) and Ca^{2+} (Owczarzy et al., 2008; Smith et al., 2018) as well as plays an essential role in enzymatic reactions involving anaerobic glycolysis and energy release from ATP for sperm activities (Wong et al., 2001; Asghari et al., 2016). Furthermore, Ca^{2+} ions also have an important role in intramitochondrial metabolism and energy production in cells (McCormack and Denton, 1989). Mitochondria can import Ca^{2+} from cytosol into mitochondrial matrix via the mitochondrial uniporter (Walsh et al., 2009). When the concentration of free Ca^{2+} increases within the mitochondrial matrix, it activates several dehydrogenases and carriers. As a result, it increases the respiratory rate, H^+ extrusion, and ATP production as well as supports energy for cell activities (McCormack and Denton, 1989; Hansford,

1994; Santo-Domingo and Demaurex, 2010). Nevertheless, when the concentration of Ca^{2+} is overloaded, it can open the mitochondrial permeability transition pore (PTP) and deplete ATP. This leads to mitochondrial swelling, cytochrome C release, and subsequently apoptosis (Demaurex and Distelhorst, 2003; Giorgi et al., 2008). Therefore, sperm in T-M-EY extender have good quality in motility and mitochondrial membrane potential as well as plasma and acrosome membrane integrity during the former period of storage time and reduced quality in the last period of the storage time. Moreover, Baumgartner et al. (2009) and Voccoli et al. (2014) have shown that the apoptosis was not only the result of increased Ca^{2+} within the mitochondrial matrix, but also a powerful synergism of the combination between reactive oxygen species (ROS) production and mitochondrial Ca^{2+} overload. Thus, to optimize the effect of T-M-EY extender on sperm quality, the addition of antioxidant agents into this extender to reduce oxidative stress as well as apoptosis is necessary in the future.

In contrast, our results also indicated that T-SL extender is greater than T-M-SL extender in all sperm quality parameters during storage. We have found that there is no synergy in the combination of soybean lecithin and Tris-citric-fructose-mineral salts extender. The negative effects of T-M-SL extenders on sperm quality may be due to several non-organic salts in these extenders, including NaCl, KCl, $KHPO_4$, $CaHPO_4$, and $MgCl_2$. These non-organic salts can induce a transition from spherical to long cylindrical micelles of soybean lecithin micelles by binding cations to the phosphate portion of lecithin headgroups (Lee et al., 2010; Markina et al., 2017). This results in an increase in the viscosity of soybean lecithin extenders as well as loss of cations and phospholipids after the centrifugation-filtration processing.

Our results indicate that the healthy sperm are more correlated to intact sperm plasma membrane, intact acrosome membrane, and high mitochondrial membrane potential than to sperm motility. These results are similar to those reported by Volpe et al. (2009) in that the functional integrity of canine mitochondria is more strongly correlated to plasma membrane than to sperm motility. Nascimento et al. (2015) also demonstrated that there was no correlation between motility and mitochondrial membrane potential in canine sperm and suggested that when oxidative phosphorylation was inhibited, the energy from glycolysis in the sperm tail supported motility. Moreover, our results propose that the T-M-EY extender is more stable and suitable than the other extenders for protecting chilled canine sperm during 10 days of storage with a high motility and healthy sperm parameters, whereas T-SL and T-EY extenders are most productive in motility but less productive in healthy sperm parameters.

3.5 Conclusions

In conclusion, the results of our investigation revealed that egg yolk is greater than soybean lecithin in Tris-citric-fructose or Tris-citric-fructose-mineral salts extenders for chilling canine sperm. Egg yolk in Tris-citric-fructose-mineral salts extender was superior to egg yolk in Tris-citric-fructose extender, whereas soybean lecithin in Tris-citric-fructose-mineral salts extenders was inferior to Tris-citric-fructose-soybean lecithin extenders in motility, plasma membrane integrity, acrosome membrane integrity, and mitochondrial membrane potential. Further studies are necessary to study the addition of antioxidant into Tris-citric-fructose-egg yolk or Tris-

citric-fructose-mineral salts-egg yolk extender and evaluate more sperm quality parameters as DNA fragmentation and fertility ability.

3.6 References

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CHAPTER IV

EFFECTS OF *OCIMUM GRATISSIMUM* LEAF ESSENTIAL OILS AS A SUPPLEMENT TO SEMEN EXTENDER ON CHILLED CANINE SPERM QUALITY

ABSTRACT

Oxidative stress during chilled storage is a major problem with canine sperm. To improve the quality of chilled canine sperm during storage, the study evaluated the effects of essential oils from *Ocimum gratissimum* as antioxidant supplement in extender on chilled canine sperm during 12 days of storage. *Ocimum gratissimum* leaves were extracted by alcohol. Sperm samples were obtained from American Bullies dogs (n=5). Semen was pooled and separated into eight treatments contained Tris-citric-fructose-mineral salts extender with different concentrations of *Ocimum gratissimum* supplementation. Sperm evaluation was performed for motility, plasma membrane integrity, acrosome membrane integrity, and mitochondrial membrane potential during 12 day-storage at 5^{o}C. The results showed that low concentrations of *Ocimum gratissimum* essential oils (25, 50, and 100µg/mL) have beneficial effects on sperm quality, whereas *Ocimum gratissimum* essential oils at high levels (above 200µg/mL) have harmful effects. Specifically, the addition of 100µg/mL of *Ocimum gratissimum* essential oils 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). In conclusion, *Ocimum gratissimum* essential oils have 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 100µg/mL.

Keywords: *Ocimum gratissimum,* essential oils, antioxidant, canine sperm, chilled

4.1 Introduction

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 (Thomassen and Farstad, 2009). However, chilling is used more frequently than freezing for its convenience and high fertilizing capacity (Linde-Forsberg, 1991; Linde-Forsberg, 1995; Eilts, 2005). In addition, canine sperm could be maintained at a chilling temperature without any deleterious effects before freezing (Santana et al., 2013). In our previous studies, we found that Tris-citric-fructose-mineral salts egg-yolk extender was the best extender for canine sperm chilling (Vui et al., 2019).

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] (Lamirande et al., 1997; Vieira et al., 2017). 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 (Moustafa et al., 2004;

Lucio et al., 2016). Fortunately, living organisms can produce a considerable amount of antioxidants physiologically that may prevent or minimise the effect of oxidative stress (Birben et al., 2012; Ighodaro and Akinloye, 2017). In canine semen, almost all enzymatic antioxidants including superoxide dismutase, glutathione peroxidase, phospholipid hydro-peroxide glutathione peroxidase, and catalase, are from seminal plasma (Cassani et al., 2005; Strzezek et al., 2009; Neagu et al., 2011; Angrimani et al., 2014). Nevertheless, due to the negative effects of seminal plasma during preservation, canine semen is centrifuged to remove seminal plasma before diluting with extenders (Rota et al., 1995; Treulen et al., 2012; Hori et al., 2017). 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 (Michael et al., 2007; Neagu et al., 2009; Michael et al., 2009; Monteiro et al., 2009; Sahashi et al., 2011; Thiangtum et al., 2012), 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 (Trevisan et al., 2006). 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 (Silva et al., 2004; Lemos et al., 2005; Trevisan et al., 2006; Prabhu et al., 2009). These phytochemicals are known to have antioxidant activity (Trevisan et al., 2006; Akinmoladun et al., 2007; Ouyang et al., 2012; Huang et al., 2013; Mahapatra and Roy, 2014). 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.

4.2 Materials and methods

4.2.1 Plant material and preparation of *Ocimum gratissimum* ethanol extract

Ocimum gratissimum leaves were collected in the morning and dried in a hot-air oven at 40^{o}C for three days. The dried leaves were finely powdered using a grinder. The extraction was conducted at room temperature by soaking 100g 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^{o}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.

4.2.2 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 (1958).

Vitamin E was used as standard. The diluted working solutions of essential oils and standard (100µL) were prepared in methanol with amounts ranging from 25 to 600µg/mL and from 0.5 to 50µg/mL in 96 wells micro plate, respectively. 100µL DPPH (200µM in methanol) was added to the essential oils and standard solution. Methanol (100µL) with DPPH solution (200µM, 100µL) was used as a control, while methanol (100µL) with essential oils or standard (100µL) was used as a blank. These solution mixtures were kept in the dark for 30 minutes and optical density was measured at 517nm using a spectrophotometric plate reader. The percentage of antioxidant activity (AA%) was determined using the following formula (Mensor et al., 2001):

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

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.

4.2.3 Animals

Sperm samples were obtained from five mature American Bullies 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.

4.2.4 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 (1991). 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;

>200×10^6 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 (Tamuli and Watson, 1994).

4.2.5 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 800µg/mL). The composition of these extenders is shown in Table 4.1. 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 4.1 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

Table 4.1 Continue.

Ingredients	**Extenders**							
	T0	**T25**	**T50**	**T100**	**T200**	**T400**	**T600**	**T800**
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 *Ocimum gratissimum* leaves.

4.2.6 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×*g*) (Rijsselaere et al., 2002). The sperm pellets were diluted with a sufficient volume of the extenders to reach the sperm concentration of 100 x 10^6 sperm/mL. After that, the extended sperm was slowly cooled (0.3ºC/min) to 5ºC by manual (Bouchard et al., 1990), and stored at 5ºC for 12 days. A 12 day-storage was chosen to cover the fertile period of the female dogs which taken around 4-21days. Experimental design in this study was presented by a repeated measurement in the completely randomised design with four replicate trials.

4.2.7 Sperm evaluation

4.2.7.1 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 38ºC in a water bath for 15 minutes. Tris buffer dilution was made to distinguish between the molecules of egg yolk in extenders and those of sperms as they have the same size. The parameters of sperm motility were recorded including total motility (TM %), progressive motility (PM %), velocity average pathway (VAP, µm/s), velocity straight line (VSL, µm/s), and velocity curvilinear (VCL, µm/s).

4.2.7.2 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 (Celeghini et al., 2007) and modified by the method of (Vui et al., 2019). 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 Figure 3.1. 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).

4.2.7.3 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 (1978) and modified by Maia et al. (2010). The MDA concentration of each sample was measured immediately after inducing sperm lipid peroxidation with 0.24mM $FeSO_4$ at 37^{o}C in a water bath for 15 minutes. Then, 1mL TBA reagent (trichloroacetic acid 15% (w/v), thiobarbituric acid 0.375% (w/v) in 0.25N hydrochloric acid) was added to 0.5mL of each sample. The mixture was treated in a boiling water bath for 15 minutes. After cooling, the suspension was centrifuged at 1,000×*g* for ten minutes. The supernatant was separated and the absorbance was measured at 535nm using a spectrophotometric plate reader. The MDA concentration was determined by comparing the sample's absorbance at 535nm with an MDA standard curve. The results were expressed in nmol MDA/50x10^6 sperm.

4.2.8 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 $p<0.05$.

4.3 Results

4.3.1 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 263.63µg/mL and 11.03µg/mL, respectively.

4.3.2 Sperm motility

The results of the total motility (TM) and progressive motility (PM) are presented in Table 4.2. 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 (0-200µg/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 400µg/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.

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 4.3. 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 additions of up to

600µg/mL essential oils (p<0.05) on day 9 and 400µg/mL essential oils (p<0.05) on day 12.

4.3.3 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 4.4. 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 100µg/mL) and then decreased gradually with the high levels of essential oils (from 200 to 800µg/mL). In particular, the treatment of 100µg/mL essential oils had the highest values and was significantly 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). Specifically, the percentage of intact acrosome membrane in treatment of 100µg/mL essential oil supplementation was greatest and had a significant difference when compared to the control (70.2±1.6 vs. 57.1±4.6 on day 1 (p<0.001), 66.4±2.8 vs. 48.3±3.9 on day 6 (p<0.001), and 60.1±2.8 vs. 39.4±4.4 on day 9 (p<0.001), respectively) and the treatments with a higher concentration of essential oils, 400µg/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 4.2 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

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}

Table 4.2 Continue.

Parameters	Extenders	Day1	Day3	Day6	Day9	Day12
PM (%)	T100	74.5 ± 1.2^{A}	72.8 ± 2.1^{A}	68.9 ± 1.4^{B}	59.5 ± 6.1^{aB}	26.8 ± 7.4^{aC}
	T200	74.7 ± 3.9^{A}	69.6 ± 4.2^{B}	66.7 ± 2.4^{B}	50.9 ± 9.5^{aC}	23.6 ± 8.2^{aD}
	T400	73.0 ± 1.9^{A}	69.4 ± 3.7^{B}	65.9 ± 2.1^{B}	30.1 ± 8.4^{bC}	4.0 ± 0.8^{bD}
	T600	72.9 ± 1.8^{A}	69.2 ± 2.0^{B}	65.4 ± 2.5^{B}	23.2 ± 5.8^{bC}	0.0 ± 0.0^{bD}
	T800	72.6 ± 3.1^{A}	68.7 ± 1.7^{B}	64.6 ± 2.6^{C}	20.1 ± 4.1^{bD}	0.0 ± 0.0^{bE}

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

Table 4.3 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

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

Table 4.3 Continue.

Parameters	Extenders	Day1	Day3	Day6	Day9	Day12
VSL(µm/s)	T400	79.6±1.2^{A}	77.0±2.1^{A}	68.1±1.6^{B}	46.8±4.5^{abC}	30.3±8.2^{bD}
	T600	79.2±1.1^{A}	73.5±7.3^{AB}	66.6±9.9^{B}	45.7±7.3^{abC}	0.0±0.0^{cD}
	T800	78.3±3.4^{A}	72.0±7.1^{A}	63.8±7.5^{B}	44.6±7.4^{bC}	0.0±0.0^{cD}
VCL(µm/s)	T0	116.5±6.1^{A}	111.9±3.3^{AB}	110.5±3.7^{B}	108.6±5.4^{aB}	100.9±6.8^{aC}
	T25	117.7±5.5^{A}	111.4±6.5^{B}	109.6±5.9^{BC}	107.6±4.8^{aBC}	102.5±6.6^{aC}
	T50	117.0±5.3^{A}	112.2±2.0^{AB}	110.3±2.0^{BC}	104.4±6.0^{abCD}	102.1±5.2^{aD}
	T100	118.0±6.0^{A}	113.7±3.2^{A}	112.3±2.6^{AB}	111.2±2.4^{aAB}	105.7±4.5^{aB}
	T200	116.9±7.1^{A}	111.9±2.5^{AB}	109.0±3.9^{B}	107.6±3.6^{aB}	99.7±2.6^{aC}
	T400	116.7±4.3^{A}	111.2±3.7^{A}	104.6±3.5^{B}	100.7±1.5^{abB}	84.2±3.3^{bC}
	T600	116.3±1.7^{A}	111.2±5.1^{A}	106.7±3.2^{B}	93.4±6.5^{bcC}	0.0±0.0^{cD}
	T800	116.1±1.1^{A}	112.5±1.1^{A}	106.4±2.4^{B}	86.3±4.3^{cC}	0.0±0.0^{cD}

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

Table 4.4 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

Parameters	Extenders	Day1	Day3	Day6	Day9	Day12
Plasma membrane (%)	T0	72.4 ± 5.6^{abA}	67.1 ± 5.8^{bcB}	59.2 ± 9.2^{bC}	52.1 ± 0.9^{bCD}	45.5 ± 5.6^{abD}
	T25	73.2 ± 2.1^{abA}	70.2 ± 4.3^{abcA}	63.8 ± 5.6^{abB}	56.7 ± 5.1^{abB}	48.4 ± 6.0^{abC}
	T50	78.5 ± 5.6^{aA}	74.9 ± 3.2^{abA}	65.7 ± 5.3^{abB}	60.9 ± 6.4^{abB}	52.3 ± 3.3^{aC}
	T100	80.0 ± 3.5^{aA}	77.5 ± 2.2^{aAB}	74.3 ± 3.7^{aB}	65.1 ± 5.1^{aC}	56.4 ± 3.5^{aD}
	T200	73.4 ± 2.0^{abA}	71.4 ± 1.2^{abcAB}	66.9 ± 2.1^{abBC}	59.7 ± 3.7^{abC}	50.2 ± 6.0^{abD}
	T400	71.5 ± 2.3^{abA}	69.1 ± 2.4^{abcAB}	64.1 ± 1.9^{abBC}	56.9 ± 3.0^{abC}	39.0 ± 3.2^{bcD}
	T600	69.1 ± 2.9^{bA}	67.2 ± 2.2^{bcA}	60.5 ± 5.8^{bB}	55.4 ± 2.6^{abB}	33.3 ± 3.7^{cC}
	T800	68.7 ± 3.3^{bA}	64.4 ± 3.8^{cAB}	58.9 ± 2.8^{bBC}	50.5 ± 4.9^{bC}	31.8 ± 4.9^{cD}
Mitochondrial membrane potential (%)	T0	78.7 ± 1.3^{aA}	70.3 ± 4.3^{bB}	62.5 ± 5.9^{cC}	54.1 ± 3.2^{cD}	47.9 ± 1.7^{abD}
	T25	79.6 ± 4.8^{aA}	76.2 ± 3.9^{abA}	68.5 ± 6.1^{abcB}	61.9 ± 6.1^{abcC}	52.9 ± 8.6^{abD}
	T50	82.4 ± 5.9^{aA}	80.0 ± 6.7^{abA}	73.3 ± 4.5^{abB}	67.6 ± 5.5^{abB}	58.8 ± 5.7^{aC}
	T100	86.2 ± 1.6^{aA}	82.2 ± 2.5^{aB}	78.0 ± 3.0^{aC}	73.7 ± 3.0^{aC}	62.5 ± 3.5^{aD}
	T200	81.6 ± 3.2^{aA}	78.3 ± 4.5^{abA}	73.2 ± 2.8^{abcB}	65.9 ± 5.0^{abcC}	53.9 ± 7.2^{abD}

Table 4.4 Continue.

Parameters	Extenders	Day1	Day3	Day6	Day9	Day12
	T400	78.1 ± 4.2^{aA}	72.9 ± 4.0^{abB}	69.1 ± 3.3^{abcC}	63.3 ± 3.8^{abcC}	40.1 ± 3.8^{bcD}
	T600	78.7 ± 1.9^{aA}	72.0 ± 3.4^{abB}	67.6 ± 4.0^{abcC}	60.7 ± 3.0^{bcD}	28.6 ± 9.1^{cE}
	T800	77.6 ± 1.9^{aA}	70.0 ± 2.7^{bB}	66.0 ± 3.5^{bcC}	57.0 ± 7.1^{bcD}	25.3 ± 4.4^{cE}
	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}
Acrosome	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}
membrane (%)	T200	67.8 ± 1.5^{abcA}	65.8 ± 1.8^{aA}	60.4 ± 1.9^{bB}	52.1 ± 1.9^{abC}	36.4 ± 6.1^{abcD}
	T400	62.5 ± 1.4^{cA}	59.0 ± 1.3^{bcAB}	57.9 ± 1.3^{bcB}	48.2 ± 1.6^{bcC}	26.8 ± 6.8^{cdD}
	T600	62.4 ± 4.3^{cA}	57.9 ± 0.8^{cB}	54.0 ± 1.7^{cdB}	45.1 ± 1.9^{bcC}	22.2 ± 4.9^{cdD}
	T800	61.4 ± 3.8^{cA}	54.3 ± 2.5^{cB}	50.1 ± 1.7^{deC}	39.8 ± 4.1^{cD}	17.3 ± 5.6^{dE}

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

Table 4.5 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

Extenders	Day1	Day3	Day6	Day9	Day12
T0	60.0 ± 3.6^{cdA}	52.9 ± 9.3^{cB}	43.0 ± 6.5^{eC}	36.3 ± 5.4^{cD}	21.9 ± 4.5^{cdE}
T25	64.7 ± 2.2^{bcA}	60.9 ± 5.6^{abcA}	51.9 ± 2.4^{bcdB}	43.0 ± 5.4^{bcC}	33.0 ± 6.7^{abcD}
T50	68.1 ± 3.7^{abA}	64.3 ± 4.1^{abA}	56.2 ± 3.6^{bB}	46.5 ± 3.6^{bC}	39.9 ± 4.9^{aC}
T100	71.4 ± 1.6^{aA}	68.7 ± 0.5^{aAB}	65.6 ± 2.2^{aB}	56.3 ± 3.5^{aC}	44.3 ± 5.9^{aD}
T200	65.4 ± 2.2^{abcA}	64.4 ± 1.9^{abA}	59.3 ± 2.7^{abB}	49.4 ± 1.7^{abC}	34.1 ± 7.4^{abD}
T400	61.7 ± 1.0^{cdA}	59.1 ± 0.7^{abcAB}	55.7 ± 1.4^{bcB}	44.8 ± 1.3^{bcC}	22.8 ± 3.3^{bcdD}
T600	61.2 ± 1.8^{cdA}	56.7 ± 0.6^{bcA}	50.8 ± 2.5^{cdeB}	42.2 ± 2.8^{bcC}	15.6 ± 3.4^{dD}
T800	57.8 ± 2.1^{dA}	52.5 ± 3.1^{cB}	46.6 ± 2.3^{deC}	37.1 ± 3.5^{cD}	10.4 ± 2.0^{dE}

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

Table 4.6 Effects of different concentrations of *Ocimum gratissimum* essential oils supplementation in extender on the level of malondialdehyde (MDA) (nmol/50x10^6 sperm) of chilled canine sperm during 12 days

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 ($p<0.05$).

Table 4.5 presents the proportion of healthy sperm with intact plasma membrane, high mitochondrial membrane potential, and intact acrosome membrane. 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 100µg/mL before going down at the greater levels of essential oils (200µg/mL).

Although the values of those sperm in the treatment of 100µg/mL essential oils were not significantly different from that in the treatments of 50 and 200µg/mL essential oils, they were superior and had a significant difference when compared to the other treatments (p<0.05).

4.3.4 Sperm lipid peroxidation

Table 4.6 summarises the level of malondialdehyde (MDA) (nmol/50x10^6 sperm) of chilled canine sperm during 12 days. 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 no significant difference in this parameter among the treatments with essential oil supplementation, the level of MDA was lowest and had a significant difference from the control in both the treatments of 100µg/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 200µg/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.

4.4 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 (Lamirande et al., 1997; Vieira et al., 2017). 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 (Michael et al., 2007; Neagu et al., 2009; Michael et al., 2009; Monteiro et al., 2009) or

sometimes limited (Sahashi et al., 2011; Thiangtum et al., 2012). Our previous study demonstrated the benefits of Tris-citric-fructose-mineral salts egg-yolk extender in chilled canine sperm (Vui et al., 2019). The present study, we 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 100µg/mL) have positive effects on sperm quality, while *Ocimum gratissimum* essential oils at high levels (above 200µg/mL) have a harmful effect. A supplement of 100µg/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 (Silva et al., 2004; Lemos et al., 2005; Trevisan et al., 2006; Prabhu et al., 2009), which may subsequently assist the intercellular antioxidant system, including superoxide dismutase, glutathione peroxidase, phospholipid hydro-peroxide glutathione peroxidase, and catalase (Cassani et al., 2005; Strzezek et al., 2009; Neagu et al., 2011; Angrimani et al., 2014). Our results are similar to those reported by Motlagh et al. (2014). 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. (2014) have also illustrated that adding a maximum clove bud extract of 75µg/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 (Chikhoune et al., 2015). 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 (Matias et al., 2011; Prakash et al., 2011; Aguiar et al., 2015). 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 (Visconti et al., 2002; Nazzaro et al., 2013). These results are in agreement with previous reports on rams (Baghshahi et al., 2014; Arando et al., 2019), bulls (Shoae and Zamiri, 2008), and boars (Roca et al., 2004). 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) (Buege and Aust, 1978), which is an important indicator for oxidative damage in sperm (Maia et al., 2010; Toker et al., 2016; Vieira et al., 2017). Our results have found that the treatments of 100µg/mL and 200µg/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 (Cassani et al., 2005; Kasimanickam et al., 2006; Kao et al., 2008; Motlagh et al., 2014). 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.

4.5 Conclusions

In conclusion, the results of our investigation revealed that supplementation of *Ocimum gratissimum* essential oils in an extender at an appropriate level (100µg/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. Future study, it is worth studying more on sperm quality characteristics such plasma membrane integrity, acrosome integrity. Apart from protection from oxidative stress, it is also worth examining what other benefits could have the extract provided to the sperm; e.g. energy source.

4.6 References

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CHAPTER V

EFFECTS OF *OCIMUM GRATISSIMUM* LEAF ESSENTIAL OILS, VITAMIN E, AND GREEN TEA POLYPHENOLS AS A SUPPLEMENT TO THE TRIS-CITRIC-FRUCTOSE-MINERAL SALTS EGG-YOLK EXTENDER ON CHILLED CANINE SPERM QUALITY

ABSTRACT

During chilled storage oxidative stress is the main reason for reducing the longevity of canine sperm. To prolong the chilled canine sperm quality, the study investigated the effects of the adding *Ocimum gratissimum* essential oils, vitamin E, and green tea polyphenols to the Tris-citric-fructose-mineral salts egg-yolk extender on chilled canine sperm during 12 days of storage. Twenty ejaculates from five American Bullies dogs were used. The sperm quality was determined by computer-assisted sperm analysis (CASA) for sperm motility parameters, and by confocal laser scanning microscope for plasma membrane integrity, acrosome membrane integrity, and mitochondrial membrane potential parameters. Sperm lipid peroxidation was also analysed through the malondialdehyde production. The study found that there was a gradual decrease in all sperm quality parameters of all the semen extenders during the whole storage time of 12 days. In addition, the sperm motility parameters of chilled

canine sperm among all the semen extenders were not significantly different at each period time of evaluation (p>0.05). Although the percentages of sperm lipid peroxidation in the green tea polyphenols and the vitamin E extenders were lower than those in the *Ocimum gratissimum* essential oils extender, the proportion of the intact plasma membrane, intact acrosome membrane, and high mitochondrial membrane potential parameters in the *Ocimum gratissimum* essential oils extender were higher than those in the rest extenders and had a significant difference as compared to the control group (p<0.05). In conclusion, the *Ocimum gratissimum* essential oils is superior to the vitamin E and green tea polyphenols for improving the chilled canine sperm during 12 days storage.

Keywords: *Ocimum gratissimum*, essential oils, vitamin E, green tea polyphenols, canine sperm, chilled.

5.1 Introduction

The use of artificial insemination (AI) technique is currently popular in dog reproduction. Before using for AI technique, canine sperm must be diluted with the suitable semen extenders to maintain the sperm quality. In our previous study, we have illustrated that the Tris-citric-fructose-mineral salts egg-yolk extender had the useful effects on chilled canine sperm quality during storage (Vui et al., 2019). After diluting, the extended sperm was preserved by chilling or freezing (Thomassen and Farstad, 2009). However, the use of chilled canine sperm results in more convenience and high fertilization as compared to the use of frozen canine sperm (Linde-Forsberg, 1991; Linde-Forsberg, 1995; Eilts, 2005). A major problem of chilled canine sperm is the

reducing of sperm quality during a long duration of storage. Thus, studies to prolong the chilled canine sperm quality is necessary.

During the chilling storage, canine sperm must to suffer the deleterious effects from oxidative stress. Because canine sperm plasma membrane contains a low ratio of phospholipid-bound polyunsaturated : saturated fatty acids (Darin Bennett et al., 1974), they are susceptible to lipid peroxidation as exposed to reactive oxygen species [ROS] (Lamirande et al., 1997; Vieira et al., 2017). Although the ROS has physiological roles in normal sperm function, such as hyper-activation, acrosome reaction, capacitation, and zona pellucida binding (Lamirande et al., 1997; Aitken, 2017), the high concentration of ROS can induce the damage of sperm structures encompassing DNA, lipids, proteins, and carbohydrates (Moustafa et al., 2004; Lucio et al., 2016; Aitken, 2017). With normal conditions, the antioxidant defence mechanisms of living organisms can counteract the generation of ROS to prevent or minimise the negative effects of oxidation (Birben et al., 2012; Ighodaro and Akinloye, 2017). Almost the enzymatic antioxidants present in canine semen are from seminal plasma, including glutathione peroxidase, phospholipid hydro-peroxide glutathione peroxidase, catalase, and superoxide dismutase (Cassani et al., 2005; Strzezek et al., 2009; Neagu et al., 2011; Angrimani et al., 2014). However, canine seminal plasma is removed by centrifuging before diluting with the semen extenders due to its negative effects on canine sperm quality (Rota et al., 1995; Treulen et al., 2012; Hori et al., 2017). This may reduce the antioxidant defence mechanisms of canine sperm against the attack of ROS. Hence, the adding antioxidant substances in the semen extenders may improve the canine sperm quality. Several studies were carried out to evaluate the effects of various enzymatic and non-enzymatic antioxidants on canine sperm with the positive outcomes (Michael

et al., 2007; Neagu et al., 2009; Michael 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; Thiangtum et al., 2012; Andersen et al., 2018). Therefore, finding the appropriate antioxidant substance for chilled canine sperm may be interesting.

In addition, vitamin E (α-Tocopherol) is a lipid-soluble antioxidant which plays a major role in inhibit the free radical-induced lipid peroxidation 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 stallion (Almeida and Ball, 2005; Vasconcelos Franco et al., 2013; Vasconcelos Franco et al., 2014; Vasconcelos Franco et al., 2016), boar (Cerolini et al., 2000; Jeong et al., 2009; Satorre et al., 2012), bull (Asadpour, 2011), ram (Abdi-Benemar et al., 2015), canine (Michael et al., 2009), and rooster (Moghbeli et al., 2016).

Moreover, green tea polyphenols are water-soluble antioxidants in which contains 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 the 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 adding of green tea polyphenols in the semen extenders had useful effects on sperm quality in stallion (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 aromatic medicinal herb in which the leaf contains high level of essential oils (3.5%) (Trevisan et al., 2006). Many studies

have found that the essential oils extract from *Ocimum gratissimum* were constituent of eugenol (30-70%), and the other phytochemicals such as thymol, α-bisabolene, β-selinene, 1,8-cineole, and γ-terpinene (Silva et al., 2004; Lemos et al., 2005; Trevisan et al., 2006; Prabhu et al., 2009; Monga et al., 2017; Kumar et al., 2019). These bioactive substances are almost amphiphilic compounds which have a strong antioxidant activity (Trevisan et al., 2006; Ouyang et al., 2012; Chiu et al., 2013; Mahapatra and Roy, 2014). Thus, *Ocimum gratissimum* essential oils supplementation in the semen extenders can protect chilled canine sperm against sperm lipid peroxidation during storage.

With these backgrounds, the objective of the present study was to assess the effects of adding *Ocimum gratissimum* essential oils, vitamin E, and green tea polyphenols to the Tris-citric-fructose-mineral salts egg-yolk extender on canine sperm quality during chilled storage.

5.2 Materials and methods

5.2.1 Animals

Semen samples were collected from five healthy and sexually mature American Bullies dogs, aged 2-5 years. All dogs were trained to ejaculate by digital manipulation method and proven fertility after natural mating. The study was implemented with the recommendation of the Institutional Animal Care and Use Committee of the Suranaree University of Technology, Nakhon Ratchasima, Thailand.

5.2.2 Semen collection and evaluation

A total of twenty ejaculates from five dogs were used in this study. Semen of five dogs were collected once a week from each dog. The sperm-rich fraction

was separated as described by Linde-Forsberg (1991). Immediately after semen collection, each ejaculate was evaluated the sperm quality before pooling. Only ejaculates with high sperm quality as the following criteria were included in this study: progressive motility ≥70%, sperm concentration ≥200×10^6 sperm/mL, sperm abnormal morphology ≤5%, and sperm viability ≥90%. For sperm progressive motility and sperm concentration parameters, they were evaluated using computer-assisted sperm analysis (CASA), while eosin-nigrosin staining was used to detect sperm morphology and viability (Tamuli and Watson, 1994).

5.2.3 Preparation of extenders

All chemicals in this study were purchased from Sigma-Aldrich (Singapore). Essential oils, vitamin E ((±)-α-Tocopherol, T3251), green tea polyphenols (Polyphenon 60, P1204), and eugenol (E51791) were used as various antioxidants. Especially, essential oils were extracted from *Ocimum gratissimum* leaves by soaking 10% of leaf powder in ethanol (98%) for five days at room temperature**.** The solution was filtered through filter paper (No.1) and condensed using a rotary evaporator. Then, the condensed solution was separated into layer by centrifuging at 3,500 x *g* for 15 minutes, and the bottom layer was used as essential oils in this study.

Table 5.1 The component of the semen extenders

Extender ingredients	**Extenders**				
	Control	**Essential oils**	**Vitamin E**	**Green tea polyphenols**	**Eugenol**
Tris (mg)	900	900	900	900	900
Citric acid (mg)	500	500	500	500	500
Fructose (mg)	1250	1250	1250	1250	1250
NaCl (mg)	450	450	450	450	450
$KHPO_4$ (mg)	60	60	60	60	60

Table 5.1 Continue.

Extender ingredients	Extenders				
	Control	**Essential oils**	**Vitamin E**	**Green tea polyphenols**	**Eugenol**
KCl (mg)	60	60	60	60	60
$CaHPO_4$ (mg)	20	20	20	20	20
$MgCl_2$ (mg)	10	10	10	10	10
Egg yolk (mL)	20	20	20	20	20
Essential oils (mg)*	-	10	-	-	-
Vitamin E (mg)	-	-	5	-	-
Green tea polyphenols (mg)	-	-	-	5	-
Eugenol (mg)	-	-	-	-	0.26
Gentamycin (mg)	200	200	200	200	200
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.

Tris-citric-fructose-mineral salts egg-yolk extender which had the 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 (100µg/mL), vitamin E (50µg/mL), green tea polyphenols (50µg/mL), and eugenol (2.6µg/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 (not show data). In particular, the concentration of eugenol using in this study was equal to the level of eugenol in essential oils extraction (2.6%). The level

of eugenol was detected using GC-MS. The extender without adding antioxidant was as a control. The component of these extenders is presented in Table 5.1. All antioxidants were diluted in DMSO before adding to extenders, and each extender contained 0.8% DMSO.

5.2.4 Semen processing and experimental design

After semen collection and initial evaluation, the ejaculates from five dogs was pooled and divided into five equal aliquots. 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 extenders to reach the final sperm concentration of 100 x 10^6 sperm/mL. After that, each extended sperm (0.5 mL) was separated into microcentrifuge and placed in a styrofoam box containing water at 25^{o}C. Next, the extended sperm was cooled down steadily to 5^{o}C by adding the ice at a cooling rate of 0.3^{o}C/min (Bouchard et al., 1990). After cooling, samples were stored at 5^{o}C. Sperm quality was evaluated once every three days over a period of 12 days.

A repeated measurement in the completely randomized design with four replicates and each replicate being a pool of five ejaculates was conducted for experimental design in this study.

5.2.5 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 (Blois, 1958). The solutions of various antioxidants (100µL) were diluted in methanol and placed in 96 wells micro plate. 100µL DPPH (200µM in methanol) was added to various antioxidant solutions. Methanol (100µL) with DPPH solution (200µM, 100µL) was used as a control. Methanol (100µL) with various antioxidants (100µL) was used as a

blank. After 30 minutes keeping in the dark, the absorbance of these solution mixtures was measured at 517nm using a spectrophotometric plate reader. The percentage of antioxidant activity (AA) was calculated using the following formula:

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

The concentration of various antioxidants providing 50% inhibition (IC50) of DPPH activity were calculated from the graph plotting between the percentage of antioxidant activity and the concentration of various antioxidants.

5.2.6 Sperm evaluation

5.2.6.1 Evaluation of sperm motility

The sperm motility was performed using computer-assisted sperm analysis (CASA; HTR-IVOS 14.0; Hamilton Thorne, USA). Before analysing, extended sperm was diluted with Tris buffer and incubated at 38^{o}C in a water bath for 15 minutes. Briefly, a volume of 5 µL of the chilled sperm samples was deposited into a warmed (38^{o}C) 2X-CEL counting chamber and covered by coverslips. At least 5 randomly selected fields for each chamber were evaluated. The following sperm motility parameters were recorded: the percentage of total motility (TM%); the percentage of progressive motility (PM%); velocity average pathway (VAP, µm/s); velocity straight line (VSL, µm/s); and velocity curvilinear (VCL, µm/s).

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

To evaluate the plasma membrane integrity, acrosome membrane integrity, and mitochondrial membrane potential of canine sperm, sperm was stained with a fluorescent 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). The staining was performed according to the previous technique (Vui et al., 2019). Briefly, chilled sperm sample (100 µL) was added by 10 µL of H342 (40 µg/mL in DPBS) and placed in a water bath for 10 minutes at 38°C. Then, 2 µL of PI (0.5 mg/mL in DPBS), 15 µL of JC-1 (153 µM JC-1 in DMSO), and 20 µL of FITC-PSA (100 µg/mL in DPBS) were added continuously into the sample and incubated at 38°C for 8 minutes. Next, 200 µL of DPBS was put in the stained sperm sample and centrifugation at 800×*g* for 2 minutes for washing. The stained sperm pellet was re-diluted in 100 µL of DPBS. After that, the stained sperm sample was immediately evaluated using a confocal laser scanning microscope (Nikon/Ni-E, Japan). At least 200 sperms were determined for each sample. The stained sperm with the intact plasma membrane, intact acrosome membrane, and high mitochondrial membrane potential had blue-stained in nucleus (H342-positive) and bright red-orange in mid-piece region (JC-1-positive), whereas the stained sperm with the damaged plasma membrane, damaged acrosome membrane, and low mitochondrial membrane potential had red-stained in nucleus (PI-positive), yellow-green in acrosome region (FITC-PSA-positive), and bright green in mid-piece region (JC-1-negative). The identification of the stained sperm under a confocal laser scanning microscope can be seen in Figure 3.1.

5.2.6.3 Evaluation of sperm lipid peroxidation

The canine sperm lipid peroxidation was evaluated using the thiobarbituric acid (TBA) assay to measure the malondialdehyde (MDA) production, according to the previous method (Maia et al., 2010). Before measuring the MDA concentration, the sperm sample was induced lipid peroxidation by adding 0.24mM $FeSO_4$ and incubated in a water bath at 38°C for 15 minutes. Then, 0.5 mL of the sperm

sample was combined with 1mL TBA reagent (trichloroacetic acid 15% (w/v), thiobarbituric acid 0.375% (w/v) in 0.25N hydrochloric acid) and 1% (v/v) butylated hydroxytoluene solution (50mM). The mixture was boiled in a water bath (95^{o}C) for 20 minutes. After that, the mixture was cooled and centrifuged at 1,000×*g* for 10 minutes to separate the supernatant. The absorbance of supernatant was measured using a spectrophotometric plate reader at 535nm. The MDA concentration was calculated by comparing the absorbance of sample at 535nm with a MDA standard curve. The results were represented in nmol MDA/50x10^{6} sperm.

5.2.7 Statistical analysis

Statistical analyses were performed using IBM SPSS statistics for Windows, version 20 (IBM Corp., Armonk, N.Y., USA). Two-factor mixed analysis of variance (ANOVA) was used to determine the interaction between period and treatment as the main effects, and the Tukey method was applied for multiple comparisons of means among groups of each factor (period, treatment). All data are provided as mean ± standard deviation (SD). A difference of $p<0.05$ was considered significant.

5.3 Results

5.3.1 Antioxidant activity (DPPH radical scavenging activity) of various antioxidants

The antioxidant activity of the various antioxidants is presented in Table 5.2. The IC50 value of the essential oils from *Ocimum gratissimum* (263.63±11.52 µg/mL) was the highest and had a significant difference compared to 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.

Table 5.2 The antioxidant activity (DPPH radical scavenging activity) of 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 providing 50% inhibition of DPPH activity. Values are mean ± standard deviation. Superscript letters (a or b) in the same row indicates significant difference (p<0.05).

5.3.2 Sperm motility

The total motility (TM) and progressive motility (PM) of chilled canine sperm are shown in Table 5.3. In general, the percentages of the TM and PM parameters in all the treatments decreased gradually during the whole storage time (12 days). In addition, there were no significant differences among the treatments at each period time in these parameters.

Another parameter that was used to evaluate the sperm motility characteristics was the sperm velocity (VAP, VSL, and VCL). The results of the sperm velocity are given in Table 5.4. The trend values of the VAP, VSL, and VCL parameters were consistent with those of the TM and PM parameters during 12 days storage. These parameters also reduced steadily during storage and there were parallel values among the treatments at each period of storage time.

5.3.3 Plasma membrane integrity, acrosome membrane integrity and mitochondrial membrane potential

Table 5.5 summarises the results of the plasma membrane integrity, acrosome membrane integrity, and mitochondrial membrane potential parameters in chilled canine sperm during 12 days storage. Overall, the percentages of the intact plasma membrane, intact acrosome membrane, and high mitochondrial membrane potential in the essential oils (extract from *Ocimum gratissimum*) extender were the highest and had a significant difference as compared with those in the control group (p<0.05). For the plasma membrane integrity, although the essential oils extender was superior to the other extenders, the values of the intact plasma membrane in the essential oils extender were not significantly different compared to those in the vitamin E, green tea polyphenols, and eugenol extenders. Moreover, the proportions of intact plasma membrane in the vitamin E, green tea polyphenols, and eugenol extenders were not significantly higher than those in the control group. For the acrosome integrity, the value of this parameter in the essential oils extender on day 9 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). Especially, the percentages of this parameter in the all antioxidant extenders were significantly different as compared to those in the control group 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 significantly lower than those in the rest extenders (p<0.05), except on day 9 in the green tea polyphenols and eugenol extenders.

On the other hand, the proportions of the sperm with intact plasma membrane, intact acrosome membrane, and high mitochondrial membrane potential are presented in Table 5.6. As with the former parameters, the values of the sperm with intact plasma membrane, intact acrosome membrane, and high mitochondrial membrane potential in the essential oils extender were the greatest and significantly higher than those in the control group ($p<0.05$), whereas the percentages of this parameter in the other antioxidant extenders were significantly different compared to those in the control group from day 9 to day 12 ($p<0.05$).

Table 5.3 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 ± standard deviation. 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 5.4 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 ± standard deviation. Uppercase superscript letters (A, B, C, or D) in the same row indicates significant difference within extenders with different storage time ($p<0.05$).

Table 5.5 The percentages of intact plasma membrane, intact acrosome membrane, and high mitochondrial membrane potential 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}
	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 ± standard deviation. 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, D or E) in the same row indicates significant difference within extenders with different storage time ($p<0.05$).

Table 5.6 The percentages of the sperm with intact plasma membrane, high mitochondrial membrane potential, and intact acrosome membrane in the extended canine sperm with the addition of various antioxidants during 12 days at 5ºC

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 ± standard deviation. Lowercase superscript letters (a or b) 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 ($p<0.05$).

5.3.4 Sperm lipid peroxidation

Table 5.7 represents the concentration of the malondialdehyde (MDA) (nmol/50x10^6 sperm) of chilled canine sperm with the addition of various antioxidants during 12 days. The levels of the MDA in all the extenders reduced gradually from day 1 to day 6 and then increased steadily to day 12. The MDA values in all the antioxidant extenders, except the eugenol extender on day 1 and day 12, were significantly lower than those in the control group. Although the concentrations of the MDA in the vitamin E and green tea polyphenols extenders were the lowest, they were no significant difference compared to those in the essential oils extender.

Table 5.7 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^{o}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 ± standard deviation. 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).

5.4 Discussion

The excessive production of reactive oxygen species (ROS) in canine sperm during storage can cause oxidative stress, which leads to damage the sperm structures as well as reduce the sperm longevity (Moustafa et al., 2004; Lucio et al., 2016; Aitken, 2017). In previous studies, several enzymatic and non-enzymatic antioxidants have been used to improve the quality of canine sperm during storage against the harmfulness of oxidative stress with the various outcomes (Michael et al., 2007; Beccaglia et al., 2009; Neagu et al., 2009; 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). Especially, vitamin E and green tea polyphenols have been had positive effects on sperm quality of animals (Cerolini et al., 2000; Almeida and Ball, 2005; Jeong et al., 2009; Michael et al., 2009; Asadpour, 2011; Satorre et al., 2012; Vasconcelos Franco et al., 2013; Wittayarat et al., 2013; Vasconcelos Franco et al., 2014; Abdi-Benemar et al., 2015; Vasconcelos Franco 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 mammalian sperm as a natural antioxidant. Our previous study have revealed that the Tris-citric-fructose-mineral salts egg-yolk extender could significantly improve the quality of chilled canine sperm during storage (Vui et al., 2019). This study was conducted to investigate the effects of *Ocimum gratissimum* essential oils, vitamin E, and green tea polyphenols supplementation in Tris-citric-fructose-mineral salts-egg yolk extender on chilled canine sperm.

The present study showed that although there was not significantly difference among the treatments in the sperm motility parameters, the antioxidant treatments could

improve the plasma membrane integrity, acrosome membrane integrity, and mitochondrial membrane potential parameters of chilled canine sperm during 12 days storage compared with the control group. Interestingly, the essential oils extract from *Ocimum gratissimum* extender was superior to the rest extenders, including the eugenol extender (as a control for the level of eugenol in *Ocimum gratissimum* essential oils), and significantly improved the quality of chilled canine sperm as compared to the control group. This means that the composition of *Ocimum gratissimum* essential oils contain not only eugenol, but also the other substances which have the useful influences on the quality of chilled canine sperm. The primary constituents of *Ocimum gratissimum* essential oils are known to contain the various bioactive compounds such as eugenol, thymol, α-bisabolene, β-selinene, 1,8-cineole, and γ-terpinene (Silva et al., 2004; Lemos et al., 2005; Trevisan et al., 2006; Prabhu et al., 2009; Monga et al., 2017; Kumar et al., 2019), which have the function of antioxidant activity (Trevisan et al., 2006; Ouyang et al., 2012; Chiu et al., 2013; Mahapatra and Roy, 2014). These compounds may contribute not only their antioxidant activity individually, but also a cocktail and synergistic antioxidant activity among these compounds (Sonam and Guleria, 2017). As a result, these antioxidant properties may assist the intercellular antioxidant system, including superoxide dismutase, catalase, glutathione peroxidase and phospholipid hydro-peroxide glutathione peroxidase to protect sperm against oxidation (Cassani et al., 2005; Strzezek et al., 2009; Neagu et al., 2011; Angrimani et al., 2014).

In addition, the antioxidant activity of vitamin E and green tea polyphenols were significantly higher than that of *Ocimum gratissimum* essential oils, but the plasma membrane integrity, the acrosome membrane integrity, and the mitochondrial

membrane potential parameters of chilled sperm in the *Ocimum gratissimum* essential oils extender were greater than those in the vitamin E and green tea polyphenols extenders. 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 against ROS and lipid membrane peroxidation (Wang and Quinn, 1999; Aitken, 2017), whereas green tea polyphenols are limited. Besides antioxidant properties, the valuable effects 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 sodium, potassium, calcium, magnesium, iron, zinc, phosphorus, manganese (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 thus the oxidative damage level in sperm (Toker et al., 2016; Vieira et al., 2017). The present study resulted that the addition of *Ocimum gratissimum* essential oils, vitamin E, and green tea polyphenols in the extender could inhibit the formation of MDA in sperm compared to the control group. In addition, although the sperm quality in the *Ocimum gratissimum* essential oils extender was greater than that in the vitamin E and green tea polyphenols extenders,

the level of MDA in the vitamin E and green tea polyphenols extenders were lower than that of the essential oils extender. These results 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. Especially, green tea polyphenols are water-soluble compounds which may have a restricted activity in sperm plasma membrane, but the level of oxidative damage in this treatment was the lowest compared to the rest treatments. This may be explained that the green tea polyphenols had a perfect antioxidant activity in the semen extender, but it was limited in the sperm. Because the lipid peroxidation parameter in the present study was conducted to evaluate the lipid peroxidation of the extended sperm. This means that the evaluation was performed in both the sperm and the semen extender. However, the lipid peroxidation occurred not only on the sperm, but also on the semen extender (Maia et al., 2010). Consequently, the results of sperm lipid peroxidation were the total concentration of MDA in both the sperm and the semen extender. Hence, the level of MDA produced from the semen extender could have an important influence in the sperm lipid peroxidation results. This study may suggest that the combination of the water-soluble antioxidant compounds with the lipid-soluble or amphiphilic antioxidant compounds may have a potential antioxidant activity for both the sperm and semen extender.

5.5 Conclusions

In conclusion, the supplementation of *Ocimum gratissimum* essential oils, vitamin E, and green tea polyphenols in the Tris-citric-fructose-mineral salts egg-yolk extender have positive effects on chilled canine sperm quality. Especially, the *Ocimum*

gratissimum essential oils extender is the best and has a significant improvement in chilled canine sperm quality during 12 days storage.

5.6 References

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CHAPTER VI

EFFECTS OF *OCIMUM GRATISSIMUM* LEAF ESSENTIAL OILS, VITAMIN E, AND GREEN TEA POLYPHENOLS AS A SUPPLEMENT TO THE TRIS-CITRIC-FRUCTOSE-MINERAL SALTS EGG-YOLK EXTENDER ON FROZEN CANINE SPERM QUALITY

ABSTRACT

Canine sperm are susceptible to oxidation due to the unbalance between the high level of ROS and inadequate antioxidant protection during cryopreservation. To improve the quality of frozen-thawed canine sperm, the supplementation of *Ocimum gratissimum* essential oils, vitamin E, and green tea polyphenols in the Tris-citric-fructose-mineral salts egg-yolk extender was conducted to evaluate the effects of them on canine sperm quality during cryopreservation. Twelve ejaculates from three American Bullies dogs were used. The sperm straws were frozen in a programmable freezer at a freezing rate of 5^{o}C/min from 4 to -15^{o}C, and of 20^{o}C/min from -15 to -100^{o}C. The sperm motility parameters were analysed by using computer-assisted sperm analysis (CASA), whereas the plasma membrane integrity, acrosome membrane integrity, and mitochondrial membrane potential parameters were identified by using a confocal laser scanning microscope. Thiobarbituric acid (TBA) assay was used to determine the level of sperm lipid peroxidation. The results presented that there was no

significant difference in the values of frozen-thawed canine sperm motility parameters among all the treatments (p>0.05). In addition, despite the antioxidant activity of the vitamin E and green tea polyphenols were higher than that of the *Ocimum gratissimum* essential oils, the percentage of the intact plasma membrane, intact acrosome membrane, and high mitochondrial membrane potential parameters in the *Ocimum gratissimum* essential oils treatment were better than those in the rest treatments and had a significant difference compared with the control group (p<0.05). In conclusion, the *Ocimum gratissimum* essential oils is greater than the vitamin E and green tea polyphenols to protect canine sperm during the process of freezing and thawing.

Keywords: *Ocimum gratissimum*, essential oils, vitamin E, green tea polyphenols, canine sperm, frozen.

6.1 Introduction

Canine sperm cryopreservation is an indispensable tool for breeding dogs by allowing a long-term preservation and transportation to distant locations. During sperm cryopreservation, canine sperm is highly sensitive to oxidative stress which induce biochemical and functional damages to the sperm. Because canine sperm plasma membrane involve a rich amount of polyunsaturated fatty acids (Darin Bennett et al., 1974), they are prone to lipid peroxidation as exposed to reactive oxygen species [ROS] during the process of freezing and thawing (Lamirande et al., 1997; Vieira et al., 2017). In physiological concentrations, ROS can assist sperm function in hyper-activation, acrosome reaction, capacitation, and sperm oocyte penetration (Lamirande et al., 1997; Aitken, 2017), while the high concentration of ROS can induce sperm lipid peroxidation which leads to changes in membrane fluidity and damage to sperm

structures as well as subsequent sperm death (Moustafa et al., 2004; Lucio et al., 2016; Aitken, 2017). Fortunately, the enzymatic antioxidants in seminal plasma of canine semen including superoxide dismutase, glutathione peroxidase, phospholipid hydro-peroxide glutathione peroxidase, and catalase (Cassani et al., 2005; Strzezek et al., 2009; Neagu et al., 2011; Angrimani et al., 2014), can prevent or minimise the harmful effects of oxidation caused by ROS (Birben et al., 2012; Ighodaro and Akinloye, 2017). However, the useful effects of these enzymatic antioxidants are no longer available because the seminal plasma of canine semen must be removed during sperm cryopreservation process (Rota et al., 1995; Treulen et al., 2012; Hori et al., 2017). This may reduce the available antioxidants for sperm protection. Thus, the supplementation of antioxidant substances in the semen extenders may improve the quality of frozen canine sperm. In previous studies, several enzymatic and non-enzymatic antioxidants have been added to improve the quality of canine sperm during storage (Michael et al., 2007; Beccaglia et al., 2009; Neagu et al., 2009; 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), but varied results were found depending on the type and concentration of antioxidants as well as the kind of semen extenders. Therefore, finding the appropriate antioxidant substances for frozen canine sperm is required.

In addition, vitamin E (α-Tocopherol) is a lipophilic antioxidant that not only scavenges the oxygen radicals, but also inhibit lipid peroxyl radicals which appear in the propagation of the chain reaction of lipid peroxidation (Wang and Quinn, 1999; Dad et al., 2006). Several previous studies have revealed that the adding of vitamin E in the semen extenders had positive effects on sperm quality of stallion (Almeida and Ball,

2005; Vasconcelos Franco et al., 2013; Vasconcelos Franco et al., 2014; Vasconcelos Franco et al., 2016), boar (Cerolini et al., 2000; Jeong et al., 2009; Satorre et al., 2012), bull (Asadpour, 2011), ram (Abdi-Benemar et al., 2015), canine (Michael et al., 2009), and rooster (Moghbeli et al., 2016).

Moreover, green tea polyphenols are natural antioxidants in which consists of hydrophilic compounds including epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate. These bioactive compounds have a strong antioxidant activity by quenching the free radicals from the ROS and the chain reaction of lipid peroxidation (Coyle et al., 2008; Lamberta and Eliasa, 2010; Forester and Lambert, 2013). The supplementation of green tea polyphenols in the semen extenders to improve the sperm quality have been investigated in stallion (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 have a rich essential oils from the leaves (3.5%) (Trevisan et al., 2006). It has been demonstrated that the composition of *Ocimum gratissimum* essential oils contains the main substance of eugenol (30-70%), and the other bioactive constituents following by thymol, α-bisabolene, β-selinene, 1,8-cineole, and γ-terpinene (Silva et al., 2004; Lemos et al., 2005; Trevisan et al., 2006; Prabhu et al., 2009; Monga et al., 2017; Kumar et al., 2019). These bioactive compounds almost have an amphiphilic characteristic and a powerful function of antioxidant activity (Trevisan et al., 2006; Ouyang et al., 2012; Chiu et al., 2013; Mahapatra and Roy, 2014). Therefore, adding *Ocimum gratissimum* essential oils to the semen extenders can improve the quality of frozen canine sperm by reducing sperm lipid peroxidation during cryopreservation.

In our previous study, we have revealed that the Tris-citric-fructose-mineral salts egg-yolk extender had the beneficial effects on chilled canine sperm quality during storage (Vui et al., 2019). Therefore, the aim of the present study was to investigate the effects of *Ocimum gratissimum* essential oils, vitamin E, and green tea polyphenols as a supplement in the Tris-citric-fructose-mineral salts egg-yolk extender on frozen canine sperm quality during cryopreservation.

6.2 Materials and methods

6.2.1 Reagents

All chemicals were purchased from Sigma-Aldrich (Singapore), and sterile distilled water was used to prepare all solution. The various antioxidants were used in this study 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, 300g powder of *Ocimum gratissimum* leaves was soaked in 3L ethanol (98%) at room temperature for five days. Then, the mixture solution was filtered using filter paper (No.1) and concentrated using a rotary evaporator. The essential oils using in this study were the bottom layer after centrifuging the concentrated solution at 3,500 x *g* for 15 minutes.

6.2.2 Animals and semen collection

Twelve ejaculates from three healthy male American Bully dogs aged 2 to 5 years were collected to use in this study. Ejaculates were obtained once a week from each dog by digital manipulation according to the previous technique (Linde-Forsberg, 1991). All dogs were trained to ejaculate and proven fertility before studying.

The study was performed with the advice of the Institutional Animal Care and Use Committee of the Suranaree University of Technology, Nakhon Ratchasima, Thailand.

6.2.3 Initial evaluation of semen quality

Before pooling, each ejaculate was analysed to determine the semen quality including volume, concentration, progressive motility, viability and abnormal morphology. Sperm progressive motility and sperm concentration were evaluated using computer-assisted sperm analysis (CASA). Sperm morphology and viability were estimated using eosin-nigrosin staining (Tamuli and Watson, 1994). The ejaculates were used in this study with the quality criteria of progressive motility ≥70%, sperm concentration $\geq 200 \times 10^6$ sperm/mL, sperm abnormal morphology ≤5%, and sperm viability ≥90%.

6.2.4 Preparation of extenders

Different extenders in this study contained the basis extender of Tris-citric-fructose-mineral salts-egg yolk extender (Vui et al., 2019) and one of the following various antioxidants: essential oils (100µg/mL), vitamin E (50µg/mL), green tea polyphenols (50µg/mL), and eugenol (2.6µg/mL). The level of essential oils adding to extender (100µg/mL) was the results of our previous study, while the concentration of vitamin E and green tea polyphenols (50µg/mL) were based on our preliminary study (not show data). Especially, the level of eugenol using in this study was the same as the number of eugenol in essential oils extraction (2.6%). The percentage of eugenol in essential oils was detected using GC-MS. The basis extender without antioxidants was as a control. DMSO was used as a solvent to dilute essential oils and eugenol, whereas vitamin E was diluted in ethanol before diluting continuously in DMSO. The final level

of DMSO in each extenders was 0.2%. Moreover, glycerol was added to extenders as a cryoprotectant. All extenders were prepared for two groups with group 1 (3% glycerol) and group 2 (7% glycerol). The final concentration of glycerol after diluting with sperm was 5% for every extenders. The composition of these extenders is given in Table 6.1.

Table 6.1 The composition of the five semen extenders used to dilute canine sperm

Extender components	**Extenders**				
	Control	**Essential oils**	**Vitamin E**	**Green tea polyphenol**	**Eugenol**
Tris (mg)	900	900	900	900	900
Citric acid (mg)	500	500	500	500	500
Fructose (mg)	1250	1250	1250	1250	1250
NaCl (mg)	450	450	450	450	450
$KHPO_4$ (mg)	60	60	60	60	60
KCl (mg)	60	60	60	60	60
$CaHPO_4$ (mg)	20	20	20	20	20
$MgCl_2$ (mg)	10	10	10	10	10
Egg yolk (mL)	20	20	20	20	20
Essential oils (mg)*	-	10	-	-	-
Vitamin E (mg)	-	-	5	-	-
Green tea polyphenols (mg)	-	-	-	5	-
Eugenol (mg)	-	-	-	-	0.26
Gentamycin (mg)	200	200	200	200	200
DMSO (mL)	0.2	0.2	0.2	0.2	0.2
Glycerol (mL)	5	5	5	5	5
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

* Essential oils extract from *Ocimum gratissimum* leaves.

6.2.5 Semen cryopreservation and experimental design

Canine semen cryopreservation was carried out as previously described procedure with modifications (Michael et al., 2007). After semen collection and initial evaluation, the pooled semen was divided into five sterile tubes. Then, semen was centrifuged at 720×*g* for 5 minutes and the seminal plasma was discarded. The sperm pellets were diluted in five extenders of group 1 (3% glycerol) to reach a sperm concentration of 200 x 10^6 sperm/mL. After that, sperm samples were placed in a styrofoam box containing water at 25ºC and cooled down gradually (0.3ºC/min) to 4ºC by adding the ice for 1 hour. Next, sperm samples were rediluted (1:1 v/v) in five extenders of group 2 (7% glycerol) at the same condition of 4ºC to obtain the final concentration of 100 x 10^6 sperm/mL. After 30 minutes at 4ºC, extended sperm samples were filled into 0.5mL 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 5ºC/min from 4 to -15ºC, and of 20ºC/min from -15 to -100ºC. After that, the sperm straws were immediately plunged into liquid nitrogen and stored in liquid nitrogen container at least two weeks before being thawed for evaluation.

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

Experimental design in this study was a completely randomized design of five treatments with the effects of five semen extenders (various antioxidants) on frozen-thawed canine sperm quality. Four replicates and each replicate being a pool of three ejaculates was conducted.

6.2.6 Antioxidant activity

The antioxidant activity of the various antioxidants using in this study was performed using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical method as previously described (Blois, 1958). The various antioxidants were diluted in methanol to be the different concentration solutions. The volume of 100µL of various antioxidant solutions was placed in 96 wells micro plate. Then, 100µL DPPH (200µM in methanol) was added to the wells. A control group was a mixture of methanol (100µL) with DPPH solution (200µM, 100µL), while a blank group was a combine of methanol (100µL) with various antioxidant solutions (100µL). After 30 minutes, the absorbance of these solution mixtures was determined using a spectrophotometric plate reader at 517nm. The percentage of antioxidant activity (AA) was measured using the following formula:

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

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.

6.2.7 Sperm evaluation

6.2.7.1 Evaluation of sperm motility

Automated analysis of sperm motility was evaluated using computer-assisted sperm analysis (CASA; HTR-IVOS 14.0; Hamilton Thorne, USA). The technical settings of CASA for canine sperm as the following were used in this study: frames per sec. (Hz), 60; no. of frames, 30; minimum contrast, 30; minimum cell size (pix), 7; cell size (pix), 6; cell intensity, 75; path velocity (VAP) (µm/s), 20; straightness (STR) (%), 40; VAP cutoff (µm/s), 9; and VSL cutoff (µm/s), 20. Before

analysing, thawed sperm was diluted with a warmed (38^{o}C) Tris buffer at a rate of 1:1. Then, 5 µL of each thawed sperm samples was mounted into a warmed (38^{o}C) 2X-CEL counting chamber and covered by coverslips. Each sperm sample in 2X-CEL counting chamber was evaluated at least 5 randomly selected fields. The percentage of total motility, the percentage of progressive motility, velocity average pathway (VAP), velocity straight line (VSL), and velocity curvilinear (VCL) parameters were collected.

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

The plasma membrane integrity, acrosome membrane integrity, and mitochondrial membrane potential parameters of canine sperm were determined using 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) according to the method described by Celeghini et al. (2007) and modified by Vui et al. (2019). Briefly, 10 µL of H342 (40 µg/mL in DPBS) was added to 100 µL thawed sperm sample and incubated in a water bath at 38^{o}C for 10 minutes. After that, the mixture sample was added continuously by 2 µL of PI (0.5 mg/mL in DPBS), 15 µL of JC-1 (153 µM JC-1 in DMSO), and 20 µL of FITC-PSA (100 µg/mL in DPBS). The mixture sample was then incubated for 8 minutes at 38^{o}C. Next, the stained sperm sample was washed by adding 200 µL of DPBS and centrifugation at 800×*g* for 2 minutes. The stained sperm pellet was re-suspended in 100 µL of DPBS and immediately examined using a confocal laser scanning microscope (Nikon/Ni-E, Japan) with a 60x objective lens. For each stained sperm sample, at least 200 sperms were identified. The stained sperm with blue-stained in nucleus (H342-positive) and bright

red-orange in mid-piece region (JC-1-positive) was intact plasma membrane, intact acrosome membrane, and high mitochondrial membrane potential, while the stained sperm with red-stained in nucleus (PI-positive), yellow-green in acrosome region (FITC-PSA-positive), and bright green in mid-piece region (JC-1-negative) was damaged plasma membrane, damaged acrosome membrane, and low mitochondrial membrane potential. The stained sperm classification under a confocal laser scanning microscope is shown in Figure 3.1.

6.2.7.3 Evaluation of sperm lipid peroxidation

The lipid peroxidation of frozen canine sperm was determined using thiobarbituric acid (TBA) assay to measure the concentration of malondialdehyde (MDA) production as described by Maia et al. (2010). Briefly, thawed sperm sample was incubated with 0.24mM $FeSO_4$ to induce lipid peroxidation in a water bath at 38^{o}C for 15 minutes. Then, 1mL TBA reagent (trichloroacetic acid 15% (w/v), thiobarbituric acid 0.375% (w/v) in 0.25N hydrochloric acid) and 1% (v/v) butylated hydroxytoluene solution (50mM) were added to 0.5 mL of the thawed sperm sample. The mixture sample was placed in a water bath (95^{o}C) for 20 minutes and then cooled down immediately. After cooling, the mixture was centrifuged at 1,000×*g* for 10 minutes to separate the supernatant. The absorbance of sample was calculated using a spectrophotometric plate reader at 535nm. The concentration of MDA in each sample was determined by converting the absorbance of sample with a MDA standard curve. The level of MDA was expressed in nmol MDA/50x10^6 sperm.

6.2.8 Statistical analysis

Statistical analyses were represented using IBM SPSS statistics for Windows, version 20 (IBM Corp., Armonk, N.Y., USA). A one-way analysis of

variance (ANOVA) with the post hoc analysis using Tukey test was used to determine the difference among means of antioxidant groups. All data are performed as mean ± standard deviation (SD). A difference was considered significant for $p<0.05$.

6.3 Results

6.3.1 Antioxidant activity of the various antioxidants

The antioxidant activity of the various antioxidants using in this study is shown in Figure 6.1. 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*.

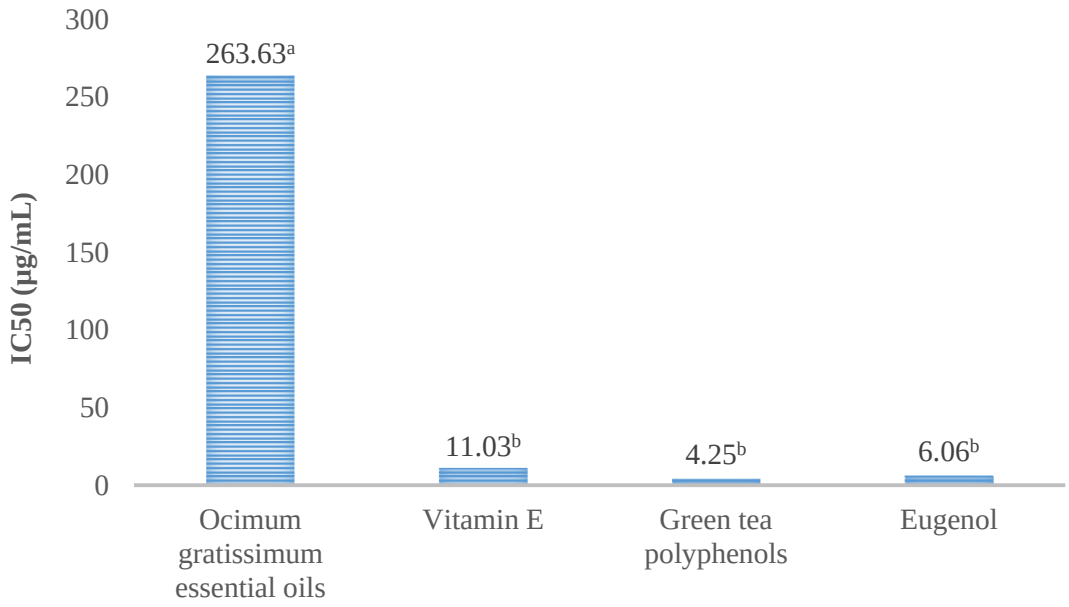

Figure 6.1 The concentration of the various antioxidants inhibit 50% DPPH activity (IC50). Values are mean ± standard deviation. Superscript letters (a or b) in the same row indicates significant difference ($p<0.05$).

6.3.2 Sperm motility

The results of the sperm motility of frozen-thawed canine sperm are represented in Table 6.2. On the whole, the values of the total motility (TM), progressive motility (PM), average pathway velocity (VAP), straight line velocity (VSL), and curvilinear velocity (VCL) parameters of frozen-thawed canine sperm were not significantly different among the treatments (p>0.05).

Table 6.2 Effects of the various antioxidants supplementation in semen extender on the total motility (TM), progressive motility (PM), average pathway velocity (VAP), straight line velocity (VSL), and curvilinear velocity (VCL) parameters of the frozen-thawed canine sperm

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 ± standard deviation for four replicates, each being a pool of three ejaculates.

6.3.3 Plasma membrane integrity, acrosome membrane integrity and mitochondrial membrane potential

The results of the plasma membrane integrity, acrosome membrane integrity, and mitochondrial membrane potential of frozen-thawed canine sperm are given in Table 6.3. In general, the proportions of the intact plasma membrane, intact acrosome membrane, and high mitochondrial membrane potential in the essential oils

(extract from *Ocimum gratissimum*) treatment were superior to those in the rest treatments, and had 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 plasma membrane integrity and the mitochondrial membrane potential, although the percentages of these parameters of frozen-thawed sperm in the essential oils extender were the highest, they were not significantly different compared with those in the vitamin E, green tea polyphenols, and eugenol extenders (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 group 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.

Moreover, Figure 6.2 presents the percentage of the sperm with intact plasma membrane, intact acrosome membrane, and high mitochondrial membrane potential. The results of this parameter were parallel with those of the intact plasma membrane and the high mitochondrial membrane potential parameters. This means that the essential oils extender was the greatest in this parameter with being significantly higher than the control group (48.86±3.01 vs. 37.41±4.61, p=0.015), whereas the rest antioxidant extenders including the vitamin E, green tea polyphenols, and eugenol were not significantly different as compared with the control group (p>0.05).

Table 6.3 Effects of the various antioxidants supplementation in semen extender on the plasma membrane, acrosome membrane, and mitochondrial membrane potential parameters of the frozen-thawed canine sperm

Extenders	Plasma membrane (%)	Acrosome membrane (%)	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 ± standard deviation for four replicates, each being a pool of three ejaculates. Superscript letters (a or b) in the same column indicates significant difference among extenders (p<0.05).

6.3.4 Sperm lipid peroxidation

The levels of the malondialdehyde (MDA) (nmol/50x10^{6} sperm) of frozen-thawed canine sperm with the addition of various antioxidants are summarized in Figure 6.3. The concentration of the MDA of frozen-thawed canine sperm in the control group 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) extenders. Although the level of the MDA of frozen-thawed canine sperm in the green tea polyphenols extender was the lowest, it was not significantly different compared with the other antioxidant extenders (p>0.05).

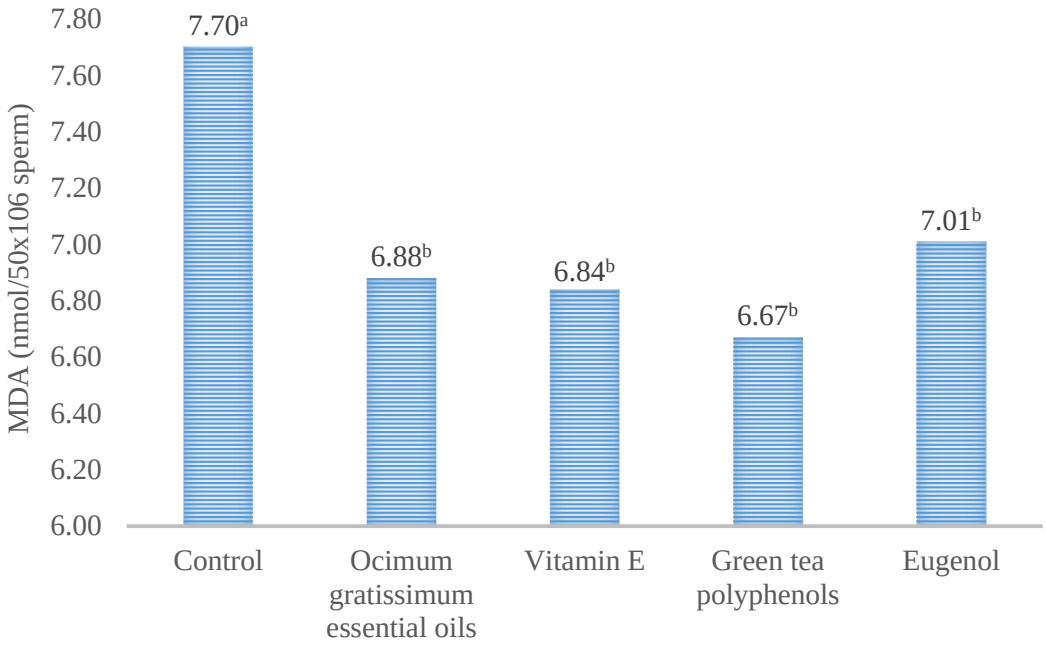

Figure 6.2 Effects of the various antioxidants supplementation in semen extender on the sperm with intact plasma membrane, intact acrosome membrane, and high mitochondrial membrane potential of frozen-thawed canine sperm. Values are mean ± standard deviation for four replicates, each being a pool of three ejaculates. Superscript letters (a or b) in the same column indicates significant difference among extenders (p<0.05).

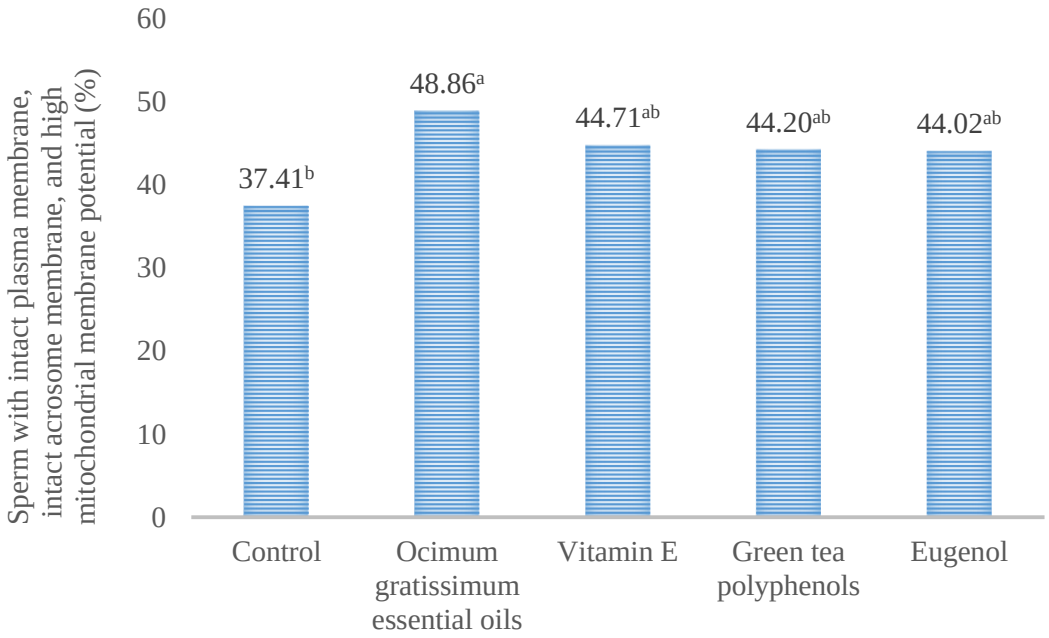

Figure 6.3 Effects of the various antioxidants supplementation in semen extender on the level of the malondialdehyde (MDA) (nmol/50x10^6 sperm) of frozen-thawed canine sperm. Values are mean ± standard deviation for four replicates, each being a pool of three ejaculates. Superscript letters (a or b) in the same column indicates significant difference among extenders (p<0.05).

6.4 Discussion

During cryopreservation, canine sperm have to encounter with the deleterious effects of oxidative stress due to the high production of reactive oxygen species (ROS). These effects can damage the 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 investigate the effects of various enzymatic and non-enzymatic antioxidants on canine sperm against the deleterious of oxidation with the

useful outcomes (Michael et al., 2007; Neagu et al., 2009; Michael et al., 2009; Monteiro et al., 2009; Wittayarat et al., 2013; Ogata et al., 2015; Lucio et al., 2016), or inefficient results (Beccaglia et al., 2009; Sahashi et al., 2011; 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 (Cerolini et al., 2000; Almeida and Ball, 2005; Jeong et al., 2009; Michael et al., 2009; Asadpour, 2011; Satorre et al., 2012; Vasconcelos Franco et al., 2013; Wittayarat et al., 2013; Vasconcelos Franco et al., 2014; Abdi-Benemar et al., 2015; Vasconcelos Franco et al., 2016; Moghbeli et al., 2016; Mehdipour et al., 2016; Gadani et al., 2017; Nouri et al., 2018), while no study has evaluated the effect of *Ocimum gratissimum* essential oils on mammalian sperm as a natural antioxidant. Our previous study have illustrated that the Tris-citric-fructose-mineral salts egg-yolk extender had positive effects on chilled canine sperm quality during storage (Vui et al., 2019). This study was implemented to evaluate the effects of the addition of *Ocimum gratissimum* essential oils, vitamin E, and green tea polyphenols to Tris-citric-fructose-mineral salts egg-yolk extender on frozen canine sperm.

The present study resulted that the supplementation of *Ocimum gratissimum* essential oils, vitamin E, and green tea polyphenols in Tris-citric-fructose-mineral salts egg-yolk extender could not influence in the sperm motility parameters, but the antioxidant treatments could enhance the quality of frozen-thawed canine sperm in the plasma membrane integrity, acrosome membrane integrity, and mitochondrial membrane potential parameters as compared to the control group. In particular, the extender with adding of *Ocimum gratissimum* essential oils 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 *Ocimum gratissimum* essential oils extender, was not greater than the *Ocimum gratissimum* essential oils extender in all sperm quality parameters. This means that the beneficial effects of *Ocimum gratissimum* essential oils were not only from eugenol function, but also from the other substances. Many studies have showed that the major composition of *Ocimum gratissimum* essential oils contained the various phytochemicals comprising eugenol, 1,8-cineole, thymol, α-bisabolene, β-selinene, and γ-terpinene (Silva et al., 2004; Lemos et al., 2005; Trevisan et al., 2006; Prabhu et al., 2009; Monga et al., 2017; Kumar et al., 2019). These bioactive compounds have been proven with strong antioxidant activity (Trevisan et al., 2006; Ouyang et al., 2012; Chiu et al., 2013; Mahapatra and Roy, 2014). In the present study, the antioxidant activity of these compounds may be represented not only from their antioxidant activity individually, but also from the combination of these compounds to being a cocktail and synergistic antioxidant activity (Sonam and Guleria, 2017). These antioxidant properties of *Ocimum gratissimum* essential oils may consequently contribute directly or cooperate with the intercellular antioxidant system, including catalase, glutathione peroxidase, phospholipid hydro-peroxide glutathione peroxidase, and superoxide dismutase against oxidative stress (Cassani et al., 2005; Strzezek et al., 2009; Neagu et al., 2011; Angrimani et al., 2014).

Besides antioxidant activity properties, the energy and mineral sources of *Ocimum gratissimum* essential oils 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 oils had high carbohydrates and mineral elements such as sodium, potassium, calcium, magnesium, phosphorus, iron,

manganese, zinc (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 metabolism and function (Juyena and Stelletta, 2012; Smith et al., 2018).

In addition, although the antioxidant activity of *Ocimum gratissimum* essential oils was lower than that of vitamin E and green tea polyphenols in the same concentration, vitamin E and green tea polyphenols extenders were not better than *Ocimum gratissimum* essential oils extender in protecting the plasma membrane integrity, the acrosome membrane integrity, and the mitochondrial membrane potential. On explanation could be the fact that almost the bioactive compounds in *Ocimum gratissimum* essential oils have amphiphilic characteristic (Prabhu et al., 2009; Kumar et al., 2019), whereas vitamin E is a lipid-soluble substance and green tea polyphenols are water-soluble compounds (Prasanth et al., 2019). Thus, *Ocimum gratissimum* essential oils and vitamin E are prone to 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 level of malondialdehyde (MDA) was determined using thiobarbituric acid (TBA) assay which was an important indicator for sperm lipid peroxidation during cryopreservation (Toker et al., 2016; Vieira et al., 2017). The present study indicated that the supplementation of the various antioxidants in the extender could prevent the sperm lipid peroxidation by inhibiting the MDA production. In addition, the level of MDA in the vitamin E and green tea polyphenols extenders were lower than that in the *Ocimum gratissimum* essential oils extender. These results were consistent with antioxidant activity results of these various antioxidants in which

the antioxidant activity of *Ocimum gratissimum* essential oils was significantly lower than that of vitamin E and green tea polyphenols. However, the plasma membrane integrity, the acrosome membrane integrity, and the mitochondrial membrane potential parameters in the *Ocimum gratissimum* essential oils extender was better than that in the vitamin E and green tea polyphenols extenders. Especially, although the green tea polyphenols are hydrophilic compounds which may have limited contact with sperm plasma membrane, the MDA value in this extender was the lowest as compared to that in the other extenders. This may be explained by the fact that the antioxidant ability of the green tea polyphenols may only have the function in the semen extender and not act in the sperm. In this study, the sperm lipid peroxidation parameter was carried out to investigate the lipid peroxidation of the frozen-thawed extended sperm. This may indicate that the evaluation was represented in both the semen extender and the sperm. Nevertheless, during cryopreservation both the sperm and the semen extender could have lipid peroxidation (Maia et al., 2010). As a result, the MDA concentration results in this study were the sum of lipid peroxidation occurring in both the sperm and the semen extender. Therefore, the lipid peroxidation in the semen extender could significantly influence in the results of the sperm lipid peroxidation parameter. The present study may suggest that the combination of the hydrophilic antioxidants with the lipophilic or amphiphilic antioxidants supplementation in the semen extender may support a positive interaction between the antioxidants for the sperm and the semen extender.

6.5 Conclusions

In conclusion, the adding of *Ocimum gratissimum* essential oils, vitamin E, and green tea polyphenols to the Tris-citric-fructose-mineral salts egg-yolk extender have protective effects on frozen-thawed canine sperm quality. In addition, the *Ocimum gratissimum* essential oils is superior to the vitamin E and green tea polyphenols, and has a significant protection for canine sperm during cryopreservation.

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CHAPTER VII

CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

In conclusion, the Tris-citric-fructose-mineral salts egg-yolk extender is useful for improving chilled canine sperm quality. In addition, *Ocimum gratissimum* essential oils have an impact on chilled canine sperm quality in a dose-dependent manner, and the best results in sperm protection are achieved with 100µg/mL of *Ocimum gratissimum* essential oils in the Tris-citric-fructose-mineral salts egg-yolk extender. Moreover, the adding of *Ocimum gratissimum* essential oils, vitamin E, and green tea polyphenols in the Tris-citric-fructose-mineral salts egg-yolk extender have beneficial effects on chilled and frozen canine sperm quality. Especially, the *Ocimum gratissimum* essential oils extender is superior to vitamin E and green tea polyphenols extenders and has a significant improvement in chilled and frozen canine sperm quality.

7.2 Recommendations

The Tris-citric-fructose-mineral salts egg-yolk extender with the adding of *Ocimum gratissimum* essential oils is appropriate for canine sperm diluting and preserving by chilling or freezing. The interval storage of chilled canine sperm diluted with this semen extender is recommended for a maximum of 9 days to guarantee the adequate quality of canine sperm for fertilization.

The outcomes of this study are also suggested that further studies should evaluate more sperm quality parameters as DNA fragmentation and fertility ability. Especially, the combination of *Ocimum gratissimum* essential oils with vitamin E and green tea polyphenols may have a synergetic antioxidant activity for improving canine sperm quality in the future.

BIOGRAPHY

Mr Nguyen Van Vui was born on 20th October 1985 in Vinh Long province, Vietnam. In 2008, he received his Bachelor degree in Veterinary Science from Can Tho University, Vietnam. He started his work on May 2009 as a lecturer in Animal Science Department, Agriculture and Aquaculture Faculty, Tra Vinh University, Vietnam. In 2014, he got a scholarship from Suranaree University of Technology, Thailand for Ph.D degree in the program "SUT-Ph.D scholarship for ASEAN countries". He studied in the field of Animal Physiology from July 2014 to September 2019 at School of Animal Technology and Innovation, Institute of Agricultural Technology, Suranaree University of Technology, Thailand. He studied with the thesis title: "Effects of *Ocimum gratissimum* leaf essential oils, vitamin E, and green tea polyphenols as a supplement to semen extender on chilled and frozen canine sperm quality". He has published two research articles in Veterinary Medicine and Science Journal and Reproduction in Domestic Animals Journal. He also finished to prepare more two manuscripts to submit for the other International Journals.