ผลของการใช้สารคัดหลั่งจากต่อมลูกหมากที่ผ่านการเก็บรักษาด้วยความเย็นเป็นน้ำยาชะล้างและตัวทำละลายต่อคุณลักษณะของอสุจิจากอิพิดิไดมิสสุนัขในกระบวนการแช่แข็ง

นางสาว สิตานันต์ กรลักษณ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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EFFECT OF USING PRESERVED HOMOLOGOUS PROSTATIC FLUID AS FLUSHING AND THAWING MEDIUM ON CANINE EPIDIDYMAL SPERM CHARACTERISTICS DURING FREEZING-THAWING PROCESS

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Theriogenology
Department of Obstetrics Gynaecology and Reproduction
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Thesis Title	EFFECT OF USING PRESERVED HOMOLOGOUS PROSTATIC FLUID AS FLUSHING AND THAWING MEDIUM ON CANINE EPIDIDYMAL SPERM CHARACTERISTICS DURING FREEZING-THAWING PRECESS
By	Miss Sitanant Karalak
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สิดานันต์ กรลักษณ์ : ผลของการใช้สารคัดหลั่งจากต่อมลูกหมากที่ผ่านการเก็บรักษาด้วยความเย็นเป็นน้ำยาชะล้างและตัวทำละลายต่อคุณลักษณะของอสุจิจากอิพิดิไดมิสสุนัขในกระบวนการแช่แข็ง. (EFFECT OF USING PRESERVED HOMOLOGOUS PROSTATIC FLUID AS FLUSHING AND THAWING MEDIUM ON CANINE EPIDIDYMAL SPERM CHARACTERISTICS DURING FREEZING-THAWING PRECESS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.น.สพ.ดร.สุดสรร ศิริไวทยพงศ์, 36 หน้า.

การทดลองนี้ประเมินผลของสารคัดหลั่งจากต่อมลูกหมากที่ผ่านการเก็บรักษาด้วยความเย็นเมื่อสัมผัสก่อนกระบวนการลดความเย็น หลังกระบวนการละลายหรือทั้งสองช่วงเวลาของกระบวนการแช่แข็งน้ำเชื้อต่อคุณลักษณะของอสุจิจากท่ออิพิดิไดมิสส่วนท้ายสุนัข รวมทั้งพิจารณาว่าสารคัดหลั่งจากต่อมลูกหมากที่ผ่านการเก็บรักษาด้วยความเย็นมีผลต่ออสุจิจากท่ออิพิดิไดมิสส่วนท้ายของสุนัขหลังการละลายและทำการเก็บรักษาที่อุณหภูมิห้อง (ประมาณ 25 องศาเซลเซียส) และ 4 องศาเซลเซียสหรือไม่ อสุจิจากท่ออิพิดิไดมิสส่วนท้ายทำการเก็บจากสุนัขจำนวน 20 ตัว ภายหลังจากการชะล้างและละลายจะทำการประเมินการเคลื่อนที่ไปข้างหน้าของอสุจิ อสุจิมีชีวิตและความสมบูรณ์ของเยื่อหุ้มอสุจิ เมื่อทำการเก็บรักษาน้ำเชื้อหลังการละลายไว้เป็นเวลา 30 นาที 60 นาที และ 120 นาที จะทำการประเมินการเคลื่อนที่ไปข้างหน้าของอสุจิและอสุจิมีชีวิต ผลการทดลองหลังการชะล้างพบว่าการเคลื่อนที่ไปข้างหน้าของอสุจิ ($P = 0.001$) และความสมบูรณ์ของเยื่อหุ้มอสุจิ ($P = 0.004$) ในกลุ่มที่ทำการชะล้างโดยสารคัดหลั่งจากต่อมลูกหมากที่ผ่านการเก็บรักษามีค่ามากกว่ากลุ่มที่ทำการชะล้างโดยสารเลี้ยงเชื้อเอ็กยอล์คทริสอย่างมีนัยสำคัญในขณะที่ไม่พบว่ามีความแตกต่างอย่างมีนัยสำคัญระหว่างกลุ่มภายหลังการละลายน้ำเชื้อ การเคลื่อนที่ไปข้างหน้าของอสุจิและอสุจิมีชีวิตของทุกกลุ่มทดลองลดลงอย่างมีนัยสำคัญภายหลังการเก็บรักษาเป็นเวลา 30 นาที ($P < 0.05$) และเมื่อทำการเก็บรักษาไว้ 120 นาทีภายหลังการละลายพบว่า ไม่มีความแตกต่างอย่างมีนัยสำคัญของปริมาณอสุจิมีชีวิตเมื่อทำการเปรียบเทียบระหว่างกลุ่ม อย่างไรก็ตามการเคลื่อนที่ไปข้างหน้าของอสุจิลดลงอย่างมีนัยสำคัญ ($P < 0.05$) การทดลองนี้แสดงให้เห็นว่าสารคัดหลั่งจากต่อมลูกหมากที่ผ่านการเก็บรักษาด้วยความเย็นเหมาะสมที่จะนำมาใช้เป็นน้ำยาชะล้างมากกว่าสารเลี้ยงเชื้อเอ็กยอล์คทริส รวมทั้งยังสามารถนำมาใช้เป็นตัวทำละลายน้ำเชื้อแทนทริสบัฟเฟอร์ในกระบวนการแช่แข็งอสุจิจากท่ออิพิดิไดมิสส่วนท้ายของสุนัข

ภาควิชา สูติศาสตร์-เธนุเวชวิทยาและวิทยาการสืบพันธุ์ ลายมือชื่อนิสิต สิดานันต์ กรลักษณ์

สาขาวิชา วิทยาการสืบพันธุ์สัตว์ ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก สุดสรร

ปีการศึกษา 2553

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KEYWORDS : PROSTATIC FLUID / CANINE / FROZEN EPIDIDYMAL SPERM

SITANANT KARALAK: EFFECT OF USING PRESERVED HOMOLOGOUS PROSTATIC FLUID AS FLUSHING AND THAWING MEDIUM ON CANINE EPIDIDYMAL SPERM CHARACTERISTICS DURING FREEZING-THAWING PROCESS. ADVISOR: ASSOC. PROF. SUDSON SIRIVAIDYAPONG, D.V.M., Ph.D., 36 pp.

This study had investigated the effects of preserved homologous prostatic fluid (PPF) to canine caudal epididymal sperm characteristics which exposed prior to cooling, after thawing or at both times of cryopreservation. This study also determined whether PPF had any influence on post-thawed canine caudal epididymal sperm after stored at room temperature (approximately 25°C) or 4°C. The caudal epididymal spermatozoa were collected from 20 dogs. After recovery and thawing, sperm progressive motility, sperm viability and sperm plasma membrane integrity were evaluated. Sperm progressive motility and sperm viability of post-thawed epididymal sperm were evaluated after 30, 60 and 120 minutes storage. After recovery, the sperm progressive motility ($P = 0.001$) and sperm plasma membrane integrity ($P = 0.004$) were significantly higher in the PPF group compared to egg yolk-tris extender (EE-I). While, they were not significantly different between groups after thawing. Sperm progressive motility and sperm viability of all groups significantly decreased after 30 minutes storage ($P < 0.05$). At 120 minutes after thawing, sperm viability was not significantly different among all groups, however, sperm progressive motility declined significantly ($P < 0.05$). This study showed that preserved homologous prostatic fluid was more suitable to be used as a flushing medium than the egg yolk-tris extender. Also, it can be used as a thawing medium instead of the tris buffer for canine caudal epididymal sperm cryopreservation.

Department : Obstetrics, Gynaecology and Reproduction Student's Signature สิทธินันท์ กรลักษ์

Field of Study : Theriogenology Advisor's Signature .....

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LIST OF ABBREVIATIONS

AI	Artificial insemination
PPF	Preserved homologous prostatic fluid
EE-I	Egg-yolk tris, 3% glycerol extender
EE-II	Egg-yolk tris, 7% glycerol extender
HOST	Hypoosmotic swelling test

CHAPTER I

INTRODUCTION

Importance and Rationale

Cryopreservation is the important tool to conserve valuable genetic materials for indefinite time (Silva *et al*., 2005). Moreover, cryopreservation of epididymal sperm can conserve the gametes when the valuable animal accidentally died, need to be castrated and natural mating cannot be done. Unfortunately, there are many factors that damage spermatozoa during cryopreservation (Rota *et al*., 2005). The semen extender has been developed to provide a source of energy, buffer and to protect spermatozoa during freezing and thawing process (Linde-Forsberg, 1991). However, the preparation of extender is complicated and expensive. Prostatic fluid (canine seminal plasma) is a natural semen extender. Canine prostatic fluid is about 1 to 30 ml. in volume per ejaculation (Sirivaidyapong, 2000). However it is not used in the artificial insemination. Many previous studies have shown several properties of seminal plasma. The seminal plasma are source of energy, buffer (Brown, 1992), cell membrane protection (Maxwell and Johnson, 1999), delay sperm capacitation (Sirivaidyapong *et al*., 1999), improving sperm transport, advance ovulation time (Weitze *et al.,* 1990; Willmen *et al*., 1991; Waberski *et al.,* 1997), immunological role (Rozeboom *et al.,* 1999; O'Leary *et al.,* 2004) and uterine remodeling in preparation for conceptus arrival (Robertson, 2007). Prostatic fluid is an interesting choice to replace or reduce commercial semen extender in the cryopreservation process. This is a remnant fluid from semen collection, easier to collect, more practical and cheaper when compared to commercial semen extender. The usage of prostatic fluid can be divided into autologous prostatic fluid and homologous prostatic fluid. The autologous prostatic fluid is fluid from the same dog that collected spermatozoa. The homologous prostatic fluid is fluid from other dogs. The autologous prostatic fluid might not able to be collected from every dog. Thus, homologous prostatic fluid is more practical to use. Moreover, homologous prostatic fluid was proved to be used in canine cryopreservation (Nöthling *et al*., 2005; Shuttleworth and Nöthling, 2002 and Nöthling *et al.,* 2007). In this study, homologous prostatic fluid has been

proceeded to preserved at -20˚C (Nöthling *et al*., 2007) before using. The purpose of prostatic fluid preservation is for instantly use whenever it is needed.

There were some studies related to the applying of prostatic fluid as canine sperm diluents or extender. Hori *et al.* (2005) found that using homologous prostatic fluid as flushing medium before freezing improved the conception rate in dog. Moreover, epididymal sperm that exposed with prostatic fluid before freezing had higher motility, viability and more mature spermatozoa than egg yolk tris-fructose citrate. Nöthling *et al.* (2007) have demonstrated that addition of homologous prostatic fluid before cooling and after thawing can reduce bent principle pieces and improve sperm motility until 1 hour after thawing. Rota *et al*. (2007) have found that initial motility and straight line velocity of frozen-thawed spermatozoa treated with autologous prostatic fluid were higher than tris buffer. Nöthling *et al.* (2005) have demonstrated that the addition of homologous prostatic fluid prior to intravaginal insemination resulted in an increased odds of conception compared to albumin-free Tyrode's albumin lactate pyruvate. To our knowledge, no study used homologous prostatic fluid as a flushing and a thawing medium in canine epididymal sperm cryopreservation. The present study was presumed to use preserved homologous prostatic fluid as a flushing and a thawing medium in cryopreservation process of canine caudal epididymal sperm when using egg yolk-tris extender. In addition, the effects of preserved homologous prostatic fluid on the post-thawed caudal epididymal sperm characteristics were also determined after storage at room temperature or 4˚C for 120 minutes.

Objectives of Study

1. To evaluate the effects of preserved homologous prostatic fluid exposed prior to cooling, after thawing or at both times to sperm progressive motility, sperm viability and sperm plasma membrane integrity of canine caudal epididymal sperm in comparison to egg yolk-tris extender and tris buffer.
2. To determine whether preserved homologous prostatic fluid has influence on sperm progressive motility and sperm viability of post-thawed canine caudal epididymal sperm compare to tris buffer after storage at room temperature (approximately 25˚C) or 4˚C.

Hypothesis

1. Preserved homologous prostatic fluid improved sperm progressive motility, sperm viability and sperm plasma membrane integrity of frozen-thawed canine caudal epididymal sperm when exposed prior to cooling and after thawing.
2. Preserved homologous prostatic fluid could be used as a thawing medium and maintained sperm quality of chilled canine caudal epididymal sperm after- thawing.

Scope of research

This study evaluated the effects of preserved homologous prostatic fluid as a flushing and a thawing medium on canine caudal epididymal sperm characteristics during freezing process and after storage at 4° C. To evaluate the property as a flushing medium, sperm progressive motility, sperm viability, sperm plasma membrane integrity and sperm morphology of canine caudal epididymal sperm were evaluated after recovery in preserved homologous prostatic fluid compared to egg yolk-tris extender. After canine caudal epididymal sperm were frozen at least 1 week, they were thawed in preserved homologous prostatic fluid compared with tris buffer. Sperm progressive motility, sperm viability, sperm plasma membrane integrity and sperm morphology were evaluated. After thawing, each sample was divided to storage at room temperature or 4°C. Sperm progressive motility and sperm viability were evaluated after storage for 30, 60 and 120 minutes.

Initial commitment

This study investigated the effects of preserved homologous prostatic fluid as a flushing and a thawing medium on canine caudal epididymal sperm characteristics during freezing-thawing process and after storage.

Definition of words

- Homologous prostatic fluid is third fraction of ejaculated semen that collected from other dogs.

Advantages of Study

1. To decrease the expense and the complication of preparing flushing and thawing medium in the cryopreservation process of caudal epididymal sperm.
2. To improve cryopreservation technique for the better sperm quality and the practically use in clinic.
3. To maintain characteristics of chilled cuadal epididymal sperm after- thawing.

Research methodology

Action plans	Months											
	1	2	3	4	5	6	7	8	9	10	11	12
Review literature and research design	x	x	X									
Prepare equipments and chemicals			X	X								
Experimental and data collection				X	x	x	x	x	X			
Data analysis									X	X		
Writing paper									X	x		
Presentation											x	X

Chapter II

LITERATURE REVIEW

The goal of breeding is to produce the high conception rate and litter size with lowest cost and labour. There are two main mating techniques, natural insemination and artificial insemination. Artificial insemination (AI) facilitates the use of one stud dog to several female dogs by using semen dilution, increases breeding hygiene, allows crossing between the two species and the use of male that natural mating cannot be done (Thomassen and Farstad, 2009). There are three types of semen using for AI which are fresh, chilled and frozen semen. Fresh semen should be used immediately or within 3-4 hours after ejaculation, however obtains 85% conception rate. Chilled semen insemination results in 65% conception rate and can be used 48 hours after kept at 4˚C (England and Lofstedt, 2000). Briefly, fresh semen and chilled semen are usually used when time is short and transport distances are not too far (Thomassen and Farstad, 2009). While, insemination by frozen semen results in 40% conception rate but the semen can be kept for indefinite time (England and Lofstedt, 2000).

Cryopreservation is an important tool for long-term storage of genetic materials from valuable stud dog (Silva *et al.,* 2005). This technique reduces cost, dangerous and time-consuming animal transportation. Moreover, it allows breeders to collect the semen from genetically superior dogs even if natural mating is impossible and to storage the semen until breeding time (Michael *et al*., 2007). The site of sperm deposition can be intravaginal and intrauterine insemination (Seager, 1969). The source of spermatozoa can be from an ejaculation, the testis or the epididymis. Epididymis can be divided anatomically into head, body and tail. During epididymal transit, spermatozoa become motile, membrane changes and loss of the cytoplasmic droplet. The mature spermatozoa are stored in the tail of epididymis (Hewitt, 1998). Sperm quality of sperm from caudal part of epididymal was significantly higher than from caput and corpus part (Hori *et al*., 2004). The difference between ejaculated sperm and epididymal sperm is exposure to seminal plasma (Nöthling *et al*., 2007). Seminal plasma modulates epididymal sperm surface and function during ejaculation (Fickel *et al*., 2007). Epididymal sperm can be collected from post-mortem stud dog or after castration (Thomassen

and Farstad, 2009). There are commonly two methods to recover epididymal sperm, which are perfusion (Marks *et al*., 1994) method and mincing (Hewitt *et al*., 2001) method. The problem of mincing method is contamination with blood and tissue fragments comparison to perfusion recovery method (Hori *et al*., 2003). On the other hand, Sirivaidyapong (2002) reported that no significant difference of semen quality between two methods. Many previous reports have been demonstrated that sperm freezability of epididymal sperm is lower than ejaculated sperm (Hewitt *et al*, 2001, Hori *et al*, 2005 and Nöthling *et al*., 2007). Frozen-thawed sperm quality of epididymal sperm is lower than that ejaculated sperm (Hori *et al*., 2003). The number of sperm maturation is lower in the epididymis than in frozen-ejaculated sperm, however, there is no difference in oocyte penetrating ability (Hewitt *et al*, 2001). Nevertheless, many studies have reported the successful of using frozen-thawed epididymal sperm for artificial insemination (Marks *et al*., 1994, Hori *et al*., 2005). Unfortunately, there are many factors that damage spermatozoa during freezing process (Rota *et al*., 2005). Such as, spermatozoa is damaged by cooling from physiological temperatures to the freezing point is called cold shock (Watson, 1981). Cooling and freezing may cause irreversible alteration in the sperm membrane such as decreased membrane fluidity, increased membrane permeability, acrosome damage, dehydration, enzyme and phospholipid liberation, reduced metabolic activity and diminished consumption of ATP. The effect of these changes is loss of sperm motility (Farstad, 1996). Thus semen extender has been developed to protect spermatozoa during freezing-thawing process. Anyhow, the preparation is complicate and quite expensive.

The canine ejaculation can be separated into three fractions. The first, pre-sperm, fraction has a small volume. This fraction contains few to no sperm and prostatic fluid. The second, sperm-rich, fraction mainly consists of caudal epididymal sperm and little prostatic fluid. The third, post-sperm, fraction contributes the large volume to the seminal plasma during ejaculation. This fraction is predominately made up of prostatic fluid. Seminal plasma is a physiological secretion from multiple sex glands (Yamashiro *et al*., 2009). The prostate is the only accessory gland of the uro-genital tract of the dog therefore prostatic fluid is practically the only component of seminal plasma (Rota *et al*., 2007). Canine prostatic fluid is clear fluid that is released in pulses over a period of 1 to 45 minutes from prostate gland. It can be found in the

first and third fractions of ejaculated semen (England *et al.,* 1990). Total volume is normally between 1 to 30 ml. (Sirivaidyapong, 2000) with the range of pH approximately 6-7 (England and Allen, 1992).

Components of seminal plasma

There are many components in seminal plasma which support spermatozoa. Seminal plasma consists of low level of fructose (Bartlett, 1962) which is a source of energy for spermatozoa (Brown, 1992; Hori *et al*., 2004). Lipid, polypeptide and protein coat sperm plasma membrane (Maxwell and Johnson, 1999) and sperm progesterone receptor cause delay capacitation (Sirivaidyapong, *et al*., 1999). Moreover, heparin-binding protein acts as acrosomal reaction modulator (de Souza *et al.,* 2006). There are some hormones such as prostaglandin and estrogen which play direct role in sperm transport (Troedsson, 2005). Prostaglandins support sperm motility and transport by stimulating female uterine muscular contraction. In addition, this hormone also has a role in an inflammatory-inducing agent to promote sperm and embryogenic survival by synergist with other seminal cytokines (Robertson, 2005). Antioxidative system in seminal plasma composed of antioxidative enzymes and non-enzymatic antioxidants. Antioxidative enzymes are superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx). Non-enzymatic antioxidants are α-tocopherol (vitamin E), ascorbic acid (vitamin C), uric acid, retinol (vitamin A), taurine and thiols (Luciana, 2006). This system is scavenging property to protect mammal spermatozoa from free radical damage (Agarwal *et al*., 2003). Besides, there are many kinds of enzymes in seminal plasma especially proteolytic enzymes such as arginine esterase, acid phosphatase (Dube *et al.,* 1985), glycosidase, choline esterase and lactic dehydrogenase (Hamner, 1970). Furthermore, organic and inorganic compounds in seminal plasma maintain spermatozoa membrane integrity and able to increase fertility in mammal (Maxwell et al, 2007). Sodium bicarbonate, inorganic compound, supports activation of sperm motility (Okamura *et al*., 1985). There are many electrolytes in seminal plasma such as Na^+ K^+ Ca^{2+} Mg^{2+} and Cl^- (Hamner, 1970). Moreover, cytokines implicate in conditioning the female reproductive tract, through the inflammatory response, to tolerate and facilitate embryo development and implication (Robertson, 2005). Even though, there have been several reports about the important elements of prostatic fluid, all components are still unknown.

As mentioned above, seminal plasma is the beneficial portion to spermatozoa. Using seminal plasma instead of commercial semen extender is interesting. Nonetheless, using autologous prostatic fluid or fresh homologous prostatic fluid is almost impossible in emergency case. Preserved homologous prostatic fluid is more practical. Some studies proved that preserved homologous prostatic fluid by storage at -18°C (Nöthling *et al*., 2005) and -40°C (Hori *et al*., 2005) can be used.

Application of canine seminal plasma

From previous study, Mann (1964) has shown that seminal plasma action as a diluter and vehicle for spermatozoa. Moreover, it exerts a distinct stimulative effect on sperm motility at the ejaculation time. Catt *et al*. (1997) suggested simple metabolites or ionic components might be the most important components of seminal plasma for the maintenance of cell viability. Moreover, presence of canine seminal plasma rendered the sperm membrane structures less susceptible to osmotic stress during cryopreservation. This might because of canine seminal plasma is the source of variation in the osmotolerance (Strazežek and Fraser, 2009).

Post-thawed sperm diluted with prostatic fluid had higher total motility and straight line velocity compared to tris extender (Rota *et al*., 2007). Corresponding to Hori *et al*. (2005), using prostatic fluid as thawing medium improved motility and viability of after-thawed epididymal sperm. Besides, prostatic fluid improved freezability and post-thaw longevity of epididymal spermatozoa frozen in BilEq (Nöthling *et al.,* 2007). Whereas, positive effects on motility and viability had been shown only in short period after sperm exposed to prostatic fluid. Different studies demonstrated that motility and viability of spermatozoa decreased when added prostatic fluid to semen. England and Allen (1992) reported that mixing of the second fraction with prostatic fluid for short incubation at 37°C adversely affect on the mean percentage of motility. Also, sperm motility had already decreased to 0% by day2 after extended in autologous seminal plasma and preserved at 4°C (Rota *et al*., 1995). Besides, addition of prostatic fluid to ejaculated sperm results in a more rapid decrease in a percentage progressive motility of sperm comparison to not added and egg yolk-Tris wxrender (Günzel-Apel and Ekrod, 1991). In addition, Sirivaidyapong *et al*. (2001) showed that addition of prostatic fluid during semen processing adversely affects the motility and viability of frozen-thawed spermatozoa. However,

prostatic fluid does not appear to affect the motility and viability of chilled spermatozoa or to alter acrosome integrity in either system of preservation. While, some studies showed that prostatic fluid had no effects on motility and viability of post-thawed sperm (Nöthling *et al.,* 2005 and Rota *et al.,* 2007).

On morphology, epididymal sperm that exposed to prostatic fluid before freezing had lower incidence of immature sperm compared to egg-yolk tris fructose citrate (Hori *et al*., 2005). While, England (1992) reported that normal sperm morphology decreased after diluted with prostatic fluid and incubated at 37°C. However, most of studies showed positive effects of prostatic fluid on state of acrosome and fertility. After thawing, epididymal sperm that exposed to prostatic fluid before freezing had higher percentage of intact acrosome than EYT-FC (Hori *et al*., 2005). In addition, prostatic fluid prevented progesterone binding to the acrosomal region and might postponed acrosomal reaction (Sirivaidyapong *et al*., 1999 and Nöthling *et al.,* 2005). Including, addition and sensitization of prostatic fluid increased number of newborn and conception rate (Nöthling, 1993, Nöthling and Volkmann, 1993, Hori *et* al., 2005 and Nöthling *et al.,* 2005).

CHAPTER III

MATERIALS AND METHODS

Animals

- Animal used for collection of prostatic fluid: 5 healthy male dogs, age 2-4 years with a good breeding soundness by general examination.
- Animal used for collection of epididymal sperm: 20 healthy male dogs, aged 1-7 years with a good breeding soundness by general examination, process to castration at Small Animal Teaching Hospital of Chulalongkorn University.

Table1 Extender for freezing and thawing

Extender	EE-I	EE-II	Tris
Tris (g)	2.4	2.4	2.4
Citric acid (g)	1.4	1.4	1.4
Glucose (g)	0.8	0.8	0.8
Glycerol (ml)	3	7	0
Egg yolk (ml)	20	20	0
Na benzyl penicillin (g)	0.06	0.06	0.06
Streptomycin sulphate (g)	0.1	0.1	0.1
Equex STM Paste (ml)	0	1	0
Distilled water (ml)	To 100 ml.	To 100 ml.	To 100 ml.
pH	6.4	6.5	6.5
Osmolarity (mOsm)	877	1374	256

The semen extender I (EE-I) and II (EE-II) were prepared according to Axnér *et al*. (2004). All solutions were kept at -20ºC until used.

Preparation and Collection of prostatic fluid

Third fraction fluid was collected by digital manipulation (Linde-Forsberg, 1991) from healthy male dogs. Prostatic fluid of each ejaculate was separated from spermatozoa by centrifugation (600xg for 10 min), then supernatants from each sample was pooled in a flask. Pooled prostatic fluid was evaluated for pH (6-7) (England, 1992) and osmolarity (294.1-337.8 mOsm/L) (Rota *et al*., 1995) before transferred to a 1.5 ml. eppendorf and storage at -20˚C (Nöthling *et al*., 2007).

Collection of epididymal sperm and freezing methods

Preserved prostatic fluid (PPF) and egg yolk, Tris, 3% glycerol extender (EE-I) were thawed in water bath at 37˚C, after that keep at room temperature (25-28˚C). After routine orchidectomy, testes were prepared on sterile plate. Tunica vaginalis was opened by using tissue scissors. Epididymis was separated from testis by cutting caudal ligament of epididymis. After that, caudal part epididymis was collected by cutting of caput and corpus epididymis. Blunted needle of number 21, 22 or 23 was inserted into vas deference for releasing spermatozoa. One epididymis was flushed by 1 ml preserved prostatic fluid (Group 1) to release epididymal sperm, while the another one was flushed by egg yolk, Tris, 3% glycerol extender (EE-I) (Group 2) into sterile plate (Sirivaidyapong and Swangchan-uthai, 2003). Then, centrifuge at 600 x g for 5 minutes to remove the diluents. The time excision of the caudal epididymis to transmigration of sperm was about 30 minutes. Each spermatozoa pellet was first diluted with an egg yolk, Tris, 3% glycerol extender (EE-I). Spermatozoa were equilibrated at 4˚C for 1 hour and then second extender was added (EE-II). Each of chilled semen was frozen in 0.5 ml straws and sperm concentration was adjusted to 1 x 10^8/ml. The straws were vertically suspended in liquid nitrogen tank containing 15-18 cm of liquid nitrogen. They were suspended 7 cm lower the opening of liquid nitrogen tank for 2 minutes, 13 cm lower the opening of liquid nitrogen tank for 2 minutes and 20 cm lower the opening of liquid nitrogen tank for 1 minute before immersion (Linde-Forsberg *et al*., 1999).

Thawing methods

All straws were thawed in a water-bath at 37˚C for 15 seconds (Linde-Forsberg *et al*., 1999). Cut the tip of the straws to release thawed semen in to thawing medium. One straw of each group was diluted 1:2 with preserved homologous prostatic fluid (Group 1.1 and 2.1), while another one was diluted 1:2 with tris buffer (T) (Group 1.2 and 2.2). After that, each group was separated into two aliquots. One of each was kept at 4˚C, while another one was kept at room temperature (approximately 25˚C).

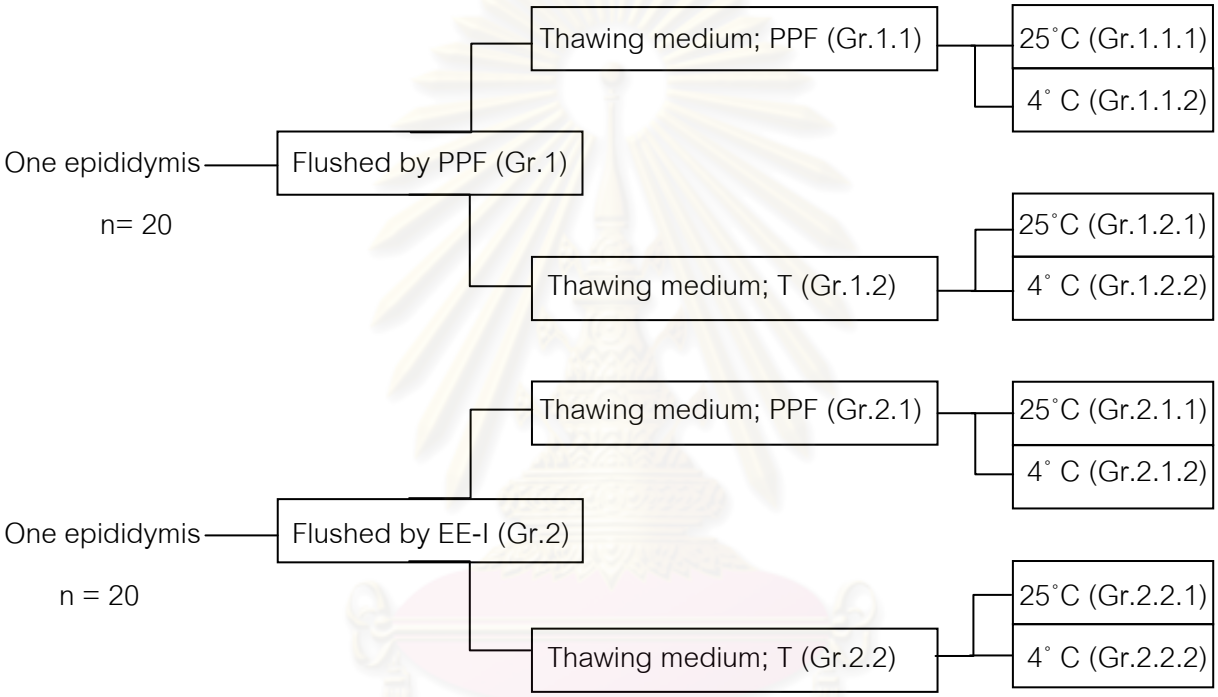

Fig.1 The allocation of the spermatozoa from each epididymis of dog to PPF and EE-I, then thawed into PPF and Tris buffer before storage at room temperature (approximately 25°C) and 4°

Semen evaluation

Sperm progressive motility

Sperm progressive motility was evaluated after release sperm from caudal epididymis and frozen-thawed semen at immediately, 30, 60 and 120 minutes. A 5 µl aliquot of the sperm sample was placed on a pre-warmed glass slide. Cover with a warmed glass slip and

subjectively assess under a light microscope at 100×. The motility was recorded in the percentage of progressive motile spermatozoa (Bearden and Fuquay, 1997).

Viability assessment

Viability was evaluated after release sperm from caudal epididymis and frozen-thawed semen at immediately, 30, 60 and 120 minutes. A 5 µl aliquot of the sperm sample was mixed with 5 µl aliquot of Eosin-Analene blue. They were smeared on the glass slide and allowed to air dry. Stained spermatozoa represented dead spermatozoa, while non-stained spermatozoa represented live spermatozoa. Two hundred spermatozoa were evaluated by using light microscopic examination at 1000×. The viability was recorded in the percentage of live spermatozoa (Parlevliet *et al*., 1994).

Morphology

Head abnormalities were evaluated after release sperm from caudal epididymis and after thaw semen immediately. A 5 µl aliquot of sperm sample was smeared on the glass slide and allowed to air dry. The smeared slides were fixed for 3 minutes in absolute alcohol at room temperature. The fixed slides were dipped in 0.5% Chloramine solution for 1-2 minutes, then were washed by distilled water. The slides were dipped in 96% alcohol, then were fixed by Carbo fushin-Eosin for 6-8 minutes. Then, the slides were washed by distilled water and allowed to air dry. Five hundred spermatozoa were evaluated by using light microscopic examination (magnification 1000×).

Midpiece and tail were evaluated after release sperm from caudal epididymis and after thaw semen immediately. A 10 µl aliquot of sperm sample was diluted with 400 µl formal saline. After that, drop 5 µl of mixed solution on glass slide and cover with glass slip. Two hundred spermatozoa were evaluated by using light microscopic examination (magnification 1000×). Percentage of morphologically normal sperm and sperm with primary and secondary abnormalities were determined (Johnston, 1991).

Sperm membrane integrity by Hypoosmotic swelling test (HOST)

Sperm membrane integrity was evaluated after release sperm from caudal epididymis and after thaw semen immediately. A 50 µl aliquot of sperm sample was mixed with 1 ml hypoosmotic solution (Sodium citrate 4.9 g., Fructose 9 g., water 1000 ml; 100 mOsm/l). The sample was incubated at 37˚C for 60 minutes. Two hundred spermatozoa were counted in at least five different fields by using phase-contrast microscope (400x). (Dobranić *et al*., 2005)

Experimental design

This study was a prospective observation research.

Statistical analyses

To evaluate sperm motility, sperm viability, sperm morphology and sperm plasma membrane integrity before cooling process, Paired T-test was used. The analysis of variance (ANOVA) was used to evaluate sperm motility, sperm viability, sperm morphology and sperm plasma membrane integrity after thawing process immediately, 30, 60 and 120 minutes at room temperature (approximately 25˚C) and 4˚C. Values were presented as mean ± SE. The level of significance is set at $P \leq 0.05$.

CHAPTER IV

RESULTS

Quality of canine caudal epididymal sperm recovered in PPF and EE-I

The sperm quality recovered in PPF and EE-I was shown in the Table 2. The sperm progressive motility (89.3 $\pm$ 1.4% versus 80.3 $\pm$ 2.1%, P = 0.001) and sperm plasma membrane integrity (91.0 $\pm$ 1.1% versus 86.7 $\pm$ 1.5%, P = 0.004) of recovered caudal epididymal sperm were significantly higher in the group recovered in PPF. Sperm viability and mean percentage of normal sperm head did not show any significant difference between groups. Mean percentage of normal sperm tail of the group recovered in PPF was significantly higher than the group recovered in EE-I (91.0 $\pm$ 1.7% versus 86.3 $\pm$ 2.3%, P = 0.002). Sperm with proximal droplet and bent tail of group recovered in PPF were significantly lower than group recovered in EE-I group (5.8$\pm$1.7% versus 10.0$\pm$2.4%, P = 0.007 and 1.3$\pm$0.3% versus 1.9$\pm$0.3%, P = 0.034, respectively).

Table 2 Semen quality (mean ± SE) after recovered from canine caudal epididymis in PPE and EE-I (*n* = 20)

Characteristics	Flushing medium	
(%)	PPF (Gr.1)	EE-I(Gr.2)
Motility	89.3 ± 1.4[a]	80.3 ± 2.1[b]
Viability	91.3 ± 0.7	91. ± 0.8
HOST	91.0 ± 1.1[a]	86.7 ± 1.5[b]
Normal head	97.9±1.5	97.7±1.5
Normal Tail	91.0 ± 1.7[a]	86.3 ± 2.3[b]
Proximal droplet	5.8±1.7[a]	10.0±2.4[b]
Distal droplet	0.03±0.03	0.13±0.08
Bent tail	1.3±0.3[A]	1.9±0.3[B]
Coil tail	1.7±0.3	1.6±0.3

The different superscript letters between columns were significantly different ($P < 0.01$)

The different superscript capital letters between columns were significantly different ($P < 0.05$)

Quality of frozen-thawed canine caudal epididymal sperm

Characteristics of thawed sperm were shown in the Table 3. Sperm progressive motility, sperm viability and sperm plasma membrane integrity had no significant difference among all groups (P = 1.000). Mean percentages of normal tail sperm that was flushed with PPF was significantly higher than in sperm that was flushed with EE-I (81.2±2.8% and 82.1±3.0% versus 66.2±3.2% and 66.5±3.4% in group 1.1, 1.2, 2.1 and 2.2 respectively, $P < 0.01$). Mean of percentages of proximal droplet in sperms flushed with PPF was significantly lower than sperms

flushed with EE-I (3.7±1.1% and 4.1±51.3% versus 14.6±2.7% and 17.3±3.0% in group 1.1, 1.2, 2.1 and 2.2 respectively, $P < 0.01$).

Table 3 Semen quality (mean ± SE) after thawing in PPF and Tris buffer (n =20)

Sperm	Recover in PPF		Recover in EE-I	
(%)	PPF(Gr.1.1)	T(Gr.1.2)	PPF(Gr.2.1)	T(Gr.2.2)
Motility	36.6±4.4	37.0±4.0	34.5±3.3	29.0±2.6
Viability	48.6±4.5	49.4±4.3	45.4±3.3	44.7±3.4
HOST	31.6±3.5	25.4±3.5	25.7±2.7	24.5±2.5
Normal head	97.5± 0.7	97.5±0.9	96.9±1.2	96.8±1.4
Normal Tail	81.2±2.8[a]	82.1±3.0[a]	66.2±3.2[b]	66.5±3.4[b]
Proximal droplet	3.7±1.1[a]	4.1±1.3[a]	14.6±2.7[b]	17.3±3.0[b]
Distal droplet	0.03±0.03	0.03±0.03	0.06±0.04	0
Bent tail	6.3±1.0	6.5±1.0	9.8±1.8	7.9±1.4
Coil Tail	8.1±2.2	6.8±2.5	6.6±1.2	7.2±2.2

The different superscript letters between columns were significantly different ($P < 0.01$)

Comparison of tail abnormalities after recovered from caudal epididymis in PPF and EE-I and after thawing in PPF and Tris buffer

Values of tail abnormalities were mentioned above and shown in Table 4. In Mean percentage of bent tail and coil tail sperms after thawing were significantly higher before recovering in all groups ($P < 0.001$). Mean percentage of proximal droplet in Group 2.2 which caudal epididymal sperms were flushed with EE-I and thawed in tris buffer was significantly higher after thawing than after recovery from caudal epididymis (17.3±3.0 % versus 10.4±2.7%, $P < 0.05$). Mean percentage of proximal droplet in Group 1.1 which caudal epididymal sperm

were flushed and thawed with and in PPF was tended to be lower after thawing than after recovery from caudal epididymis (3.7±1. % versus16.2±1.8%, $P = 0.058$).

Table 4 Sperm tail abnormalities (mean ± SE) after recovery in PPF and EE-I and after thawing in PPF and Tris buffer (n =18)

Group		After recovery	After thawing
PP (Gr.1.1)	Proximal droplet (%)	6.2±1.8	3.7±1.1
	Distal droplet (%)	0.03±0.03	0.03±0.03
	Bent tail (%)	1.2±0.3^{a}	6.3±1.0^{b}
	Coil tail (%)	1.6±0.3^{a}	8.1±2.2^{b}
PT (Gr.1.2)	Proximal droplet (%)	6.2±1.8	4.1±1.3
	Distal droplet (%)	0.03±0.03	0.03±0.03
	Bent tail (%)	1.2±0.3^{a}	6.5±1.0^{b}
	Coil tail (%)	1.6±0.3^{a}	6.8±2.5^{b}
EP (Gr.2.1)	Proximal droplet (%)	10.4±2.7	14.6±2.7
	Distal droplet (%)	0.03±0.03	0.06±0.04
	Bent tail (%)	1.9±0.3^{a}	9.8±1.8^{b}
	Coil tail (%)	1.5±0.3^{a}	6.6±1.2^{b}
ET (Gr.2.2)	Proximal droplet (%)	10.4±2.7^{a}	17.3±3.0^{b}
	Distal droplet (%)	0.03±0.03	0
	Bent tail (%)	1.9±0.3^{a}	7.9±1.4^{b}
	Coil tail (%)	1.5±0.3^{a}	7.2±2.2^{b}

The different superscript letters between columns were significantly different (P<0.05)

Comparison of sperm qualities immediately after thawing and after storaging at room temperature and 4˚C

Sperm progressive motility

Sperm progressive motility after thawing and during storaging was shown in Table 5, Figure 2 and 3. Sperm progressive motility of all groups were significantly decreased after 30, 60 and 120 minutes after storaging ($P < 0.05$), respectively. After 30 minutes of storage at 4°C, progressive motility of sperm that were flushed with EE-I then recovered in tris buffer was significantly lower than other groups ($P < 0.05$). After 60 and 120 minutes of storaging at room temperature and 4°C, progressive motility of sperms that was flushed with EE-I then recovered in tris buffer was significantly lower than other groups ($P < 0.05$).

Table 5 Sperm progressive motility (mean ± SE) after thawing in PPF and Tris buffer (n =20)

Group	Temp.	T0	T30 mins	T60 mins	T120mins
PP	Troom (Gr.1.1.1)	$36.6\pm4.4^{a\,A}$	$31.1\pm4.9^{a\,B}$	$21.3\pm3.6^{a\,C}$	$16.0\pm3.6^{a\,D}$
	T4°C (Gr.1.1.2)	$36.6\pm4.4^{a\,A}$	$20.0\pm4.4^{a\,B}$	$11.6\pm2.9^{a\,C}$	$6.1\pm1.8^{a\,D}$
PT	Troom (Gr.1.2.1)	$37.0\pm4.0^{a\,A}$	$25.4\pm4.1^{a\,B}$	$15.5\pm3.6^{a\,C}$	$10.1\pm2.9^{a\,D}$
	T4°C (Gr.1.2.2)	$37.0\pm4.0^{a\,A}$	$18.3\pm4.0^{a\,B}$	$13.2\pm3.2^{a\,C}$	$8.5\pm2.6^{a\,D}$
EP	Troom (Gr.2.1.1)	$34.5\pm3.3^{a\,A}$	$23.5\pm3.1^{a\,B}$	$12.8\pm2.7^{a\,C}$	$11.1\pm2.5^{a\,C}$
	T4°C (Gr.2.1.2)	$34.5\pm3.3^{a\,A}$	$15.8\pm2.7^{a\,B}$	$9.4\pm2.1^{a\,C}$	$5.0\pm1.3^{b\,D}$
ET	Troom (Gr.2.2.1)	$29.0\pm2.6^{a\,A}$	$15.5\pm2.5^{a\,B}$	$7.5\pm1.4^{b\,C}$	$3.6\pm1.1^{b\,D}$
	T4°C (Gr.2.2.2)	$29.0\pm2.6^{a\,A}$	$9.9\pm2.2^{b\,B}$	$5.8\pm1.5^{b\,C}$	$2.4\pm0.7^{b\,D}$

The different superscript capital letters between columns were significantly different ($P<0.05$)

The different superscript letters between rows were significantly different ($P<0.05$)

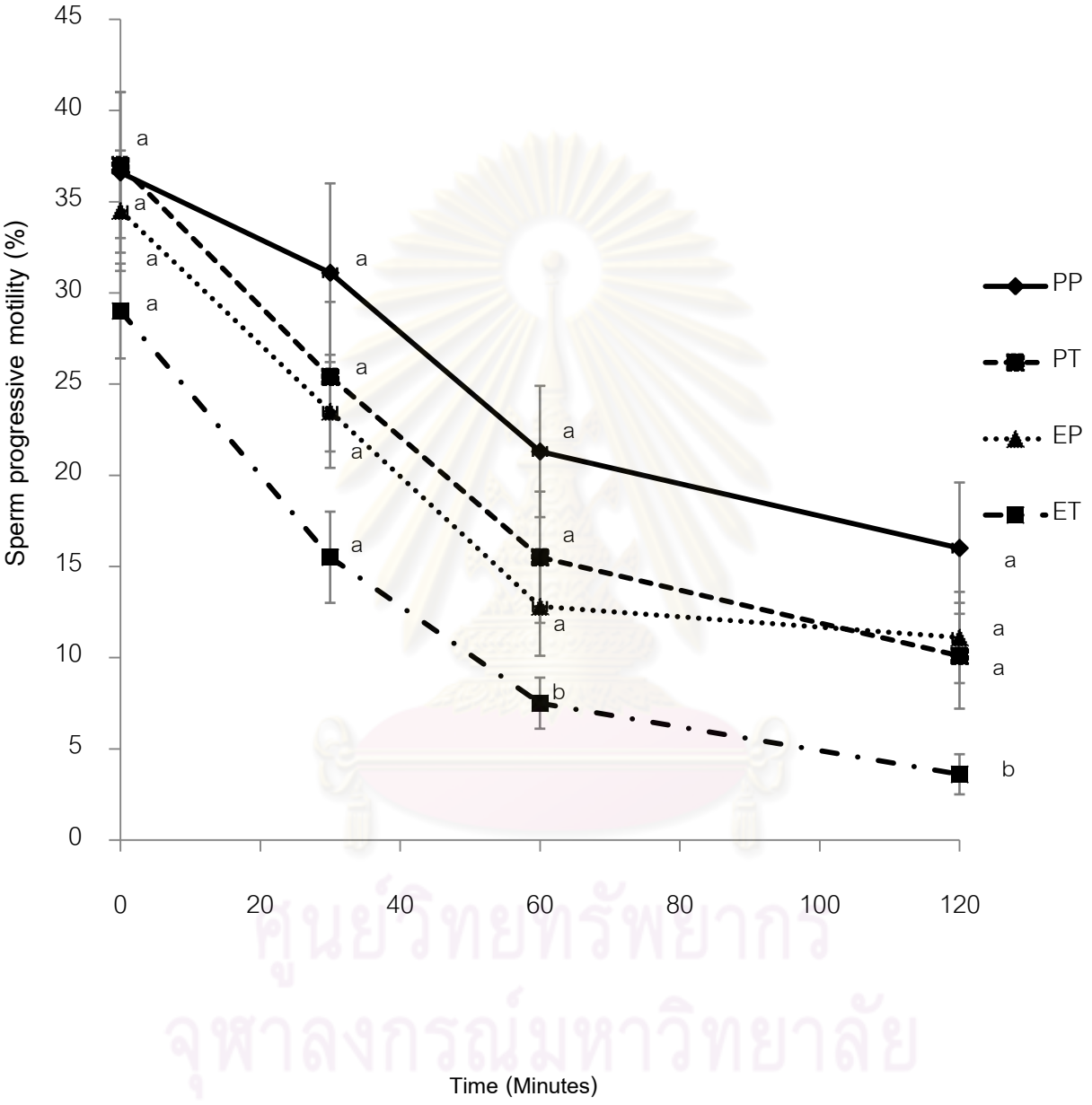

Fig.2 Sperm progressive motility after thawing and storaging at room temperature. Means marked with (a) was significantly different from the mean marked with (b) (P <0.05).

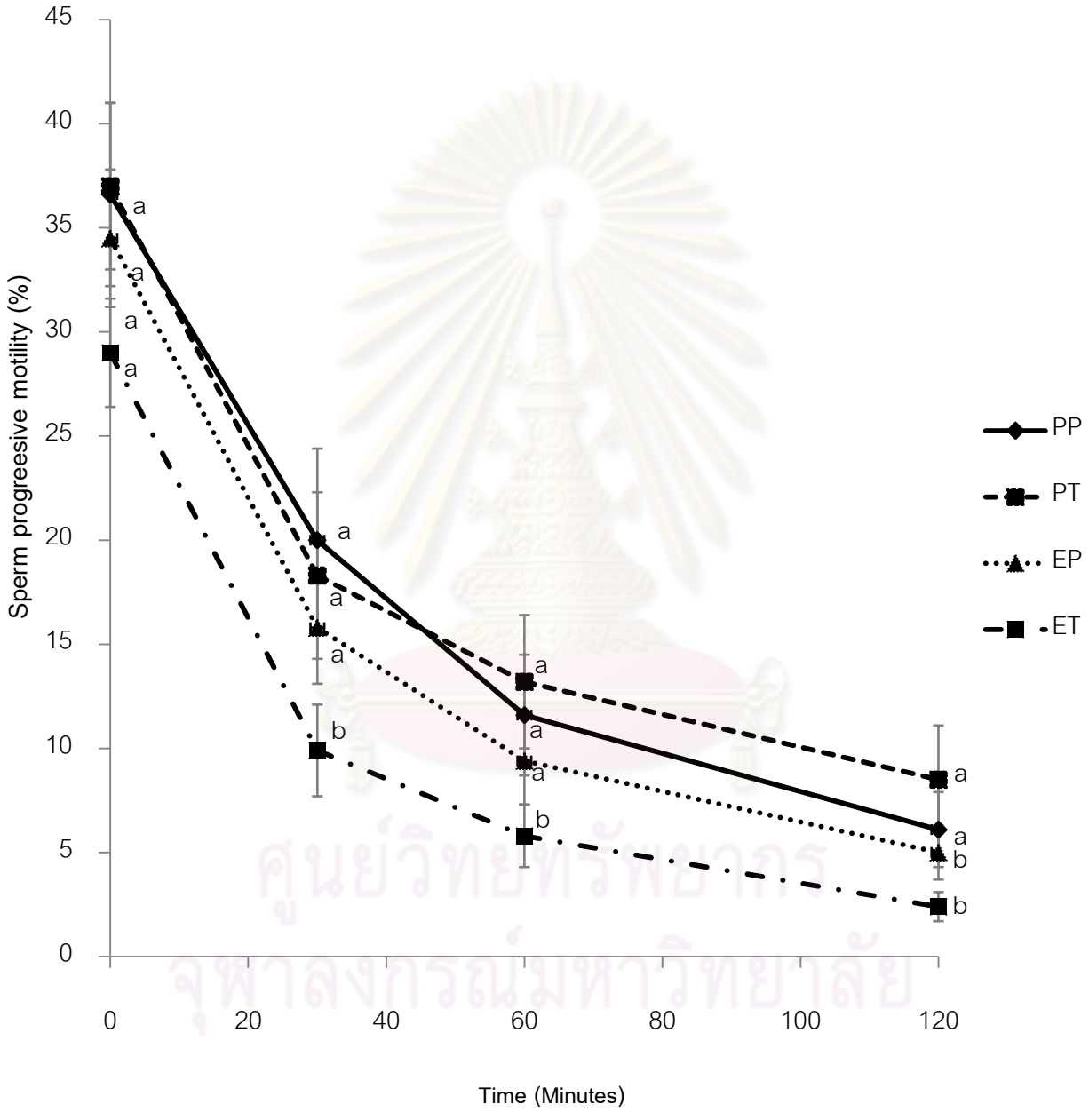

Fig.3 Sperm progressive motility after thawing and storaging at 4°C. Means marked with (a) were significantly different from means marked with (b) (P <0.05).

Sperm viability

Sperm viability after thawing and during storaging was shown the Table 6, Figure 4 and 5. No significant difference among groups were found in any period of storaging time. After caudal epididymal sperm were flushed with PPF then thawed in PPF and tris buffer, the mean percentage of sperm viability significantly decreased after 30 minutes of storaging ($P < 0.05$). Although the mean percentage of sperm viability tended to decrease after 120 minutes of storaging, no significant difference were found among groups at 30, 60 and 120 minutes of storage. In group 2.1.1, caudal epididymal sperm were recovered in EE-I, thawed in PPF then was stored at room temperature, the mean of percentage of sperm viability significantly decreased after 30, 60 and 120 minutes of storage ($P < 0.05$), respectively. In group 2.1.2, caudal epididymal sperm were recovered in EE-I, thawed in PPF then kept at 4°C, the mean of percentage of sperm viability significantly decreased after 30 minutes of storage ($P < 0.05$). However, no significant difference was found between the mean of percentage of sperm viability after storaging at 4°C for 60 minutes and storaging for 0 and 30 minutes. In group 2.2.1, caudal epididymal sperm were flushed with EE-I, thawed in tris buffer then was stored at room temperature, mean percentage of sperm viability after 60 minutes of storage was significantly lower than sperms that were immediately thawed ($P < 0.05$), however no significant difference were found from 30 minutes of storage. At 120 minutes of storaging, the mean percentage of sperm viability was significantly lower than other groups ($P < 0.05$). In group 2.2.2, caudal epididymal sperm were flushed with EE-I, thawed in tris buffer then were stored at 4°C, mean percentage of sperm viability at 120 minutes of storaging was significantly lower than at 0, 30 and 60 minutes of storaging ($P < 0.05$).

Table 6 Sperm viability (mean ± SE) after thawing in PPF and Tris buffer (*n* =20)

Group	Temp.	T0	T30 mins	T60 mins	T120mins
PP	Troom (Gr.1.1.1)	48.6±4.5^{A}	42.5±4.6^{B}	44.0±4.8^{AB}	39.6±4.8^{B}
	T4°C (Gr.1.1.2)	48.6±4.5 ^{A}	41.2±5.4^{B}	37.9±4.7^{B}	36.8±4.9^{B}
PT	Troom (Gr.1.2.1)	49.4±4.3^{A}	38.6±5.0^{B}	36.2±4.8^{B}	35.9±5.0^{B}
	T4°C (Gr.1.2.2)	49.4±4.3 ^{A}	39.3±5.6^{B}	36.9±4.6^{B}	34.7±4.7^{B}
EP	Troom (Gr.2.1.1)	45.4±3.3 ^{A}	40.5±4.1^{B}	40.1±4.6^{C}	37.8±3.8^{D}
	T4°C (Gr.2.1.2)	45.4±3.3^{A}	38.2±3.6^{B}	39.2±4.3^{AB}	35.0±3.7^{B}
ET	Troom (Gr.2.2.1)	44.7±3.4^{A}	40.1±4.0^{AB}	37.3±3.4^{B}	33.0±4.2^{C}
	T4°C (Gr.2.2.2)	44.7±3.4^{A}	39.7±3.8^{A}	39.9±4.2^{A}	34.4±3.9^{B}

The different superscript capital letters between columns were significantly different (P<0.05)

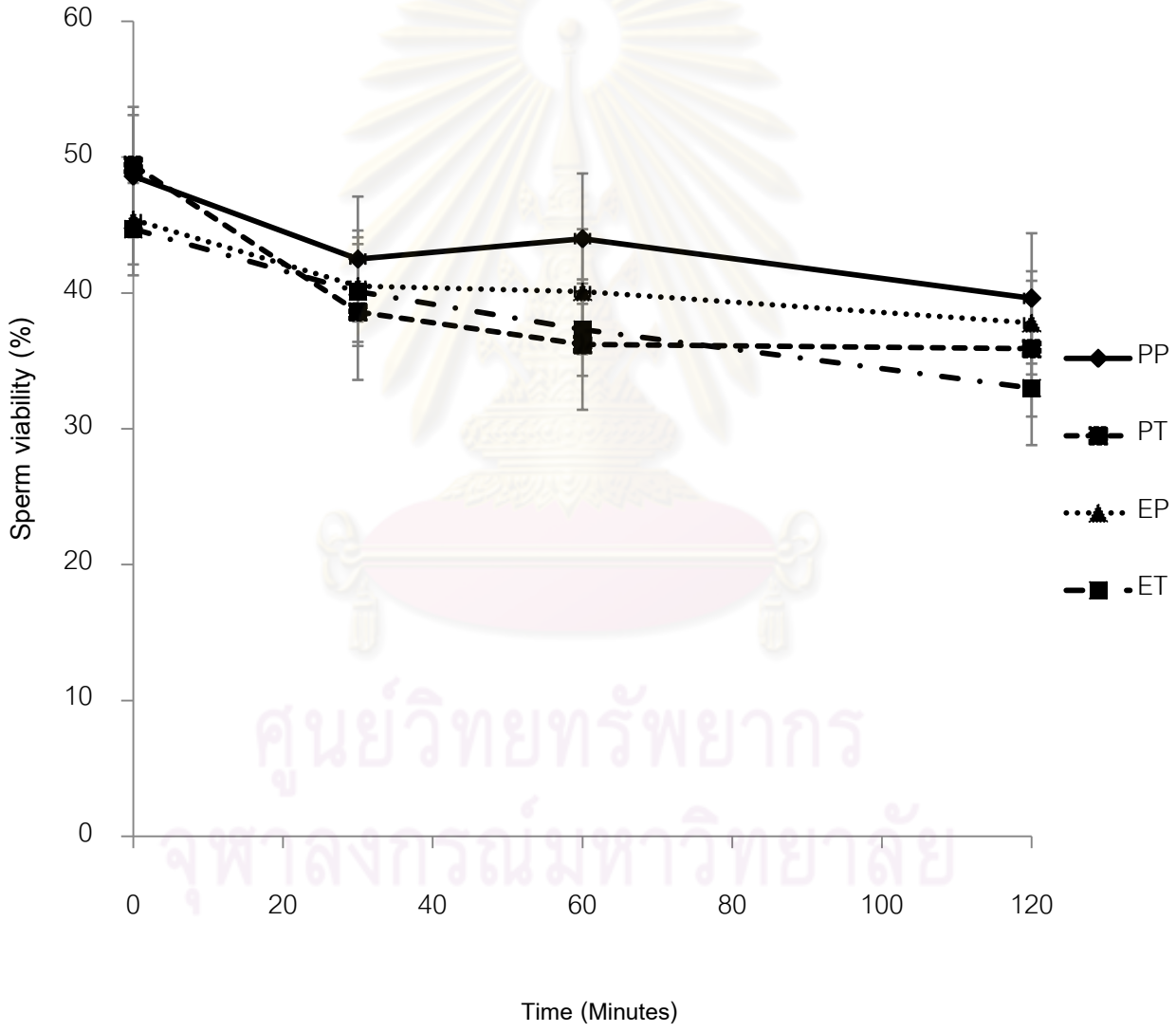

Fig.4 Sperm viability after thawing and storaging in room temperature.

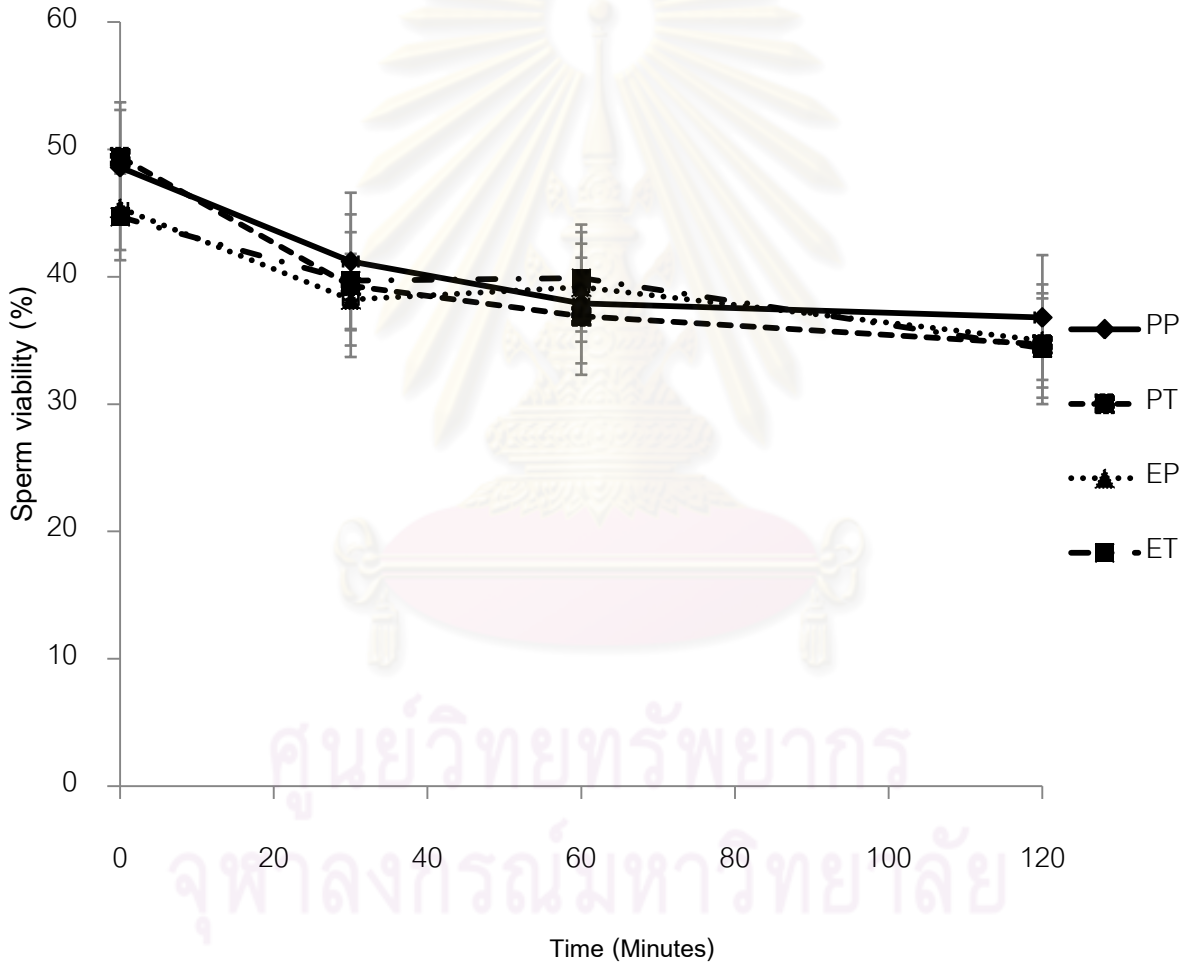

Fig.5 Sperm viability after thawing and storaging in 4°C.

CHAPTER V

DISCUSSION

The aim of this study was to evaluate the effects of preserved homologous prostatic fluid (PPF) exposed prior to cooling, after thawing or at both times on sperm progressive motility, sperm viability and sperm plasma membrane integrity of canine caudal epididymal spermatozoa compared to those exposed with egg yolk-tris extender (EE-I) or tris buffer. Furthermore, it was to determine whether preserved homologous prostatic fluid had influence on sperm progressive motility and sperm viability of post-thawed canine caudal epididymal sperm compared to tris buffer after storage at room temperature (approximately 25˚C) and at 4˚C.

This study showed that preserved homologous prostatic fluid was more suitable for applying as a flushing medium than egg yolk-tris extender. After caudal epididymal sperm were recovered in PPF and EE-I, sperm morphology was in normal range (Johnston, 1991). The sperm progressive motility of caudal epididymal sperm recovered in PPF was significantly higher than in EE-I (P = 0.001). This might because of many components in canine seminal plasma. Fructose in PPF supported energy for spermatozoa (Brown, 1992). This sugar also had strong influence on sperm motility and sperm movement pattern. According to a previous study, fructose maintained higher sperm motility than glucose did (Ponglowhapan *et al.,* 2004). In this study, egg yolk-tris extender consisted of glucose instead of fructose. Sodium bicarbonate, inorganic compound, supported activation of sperm motility (Okamura *et al*., 1985). In addition, Prostaglandin and Estrogen played direct role in sperm transport (Troedsson *et al*., 2005). Sperm plasma membrane integrity assessed by Hypoosmotic swelling test (HOST) in PPF group was significantly higher than EE-I group (P = 0.004). When spermatozoa were exposed to seminal plasma, sperm plasma membrane was coated by lipid, polypeptide and protein (Maxwell and Johnson, 1999). Besides, there were many kinds of inorganic and organic compounds to maintain sperm plasma membrane integrity of spermatozoa. In contrast, Hori *et al*. (2005) reported that there was no significant difference of sperm motility and sperm viability between recovered epididymal sperm in homologous prostatic fluid and egg yolk tris-fructose citrate. This might be due to the different recovery method that in our study the caudal

epididymal sperm were recovered by flushing method whereas in the study of Hori *et al*. (2005) the epididymal sperm were recovered by mincing method. The contamination with blood and tissue fragments was the problem of mincing method (Hori *et al*., 2004). England and Allen (1992) demonstrated that contamination of blood in canine semen affected to the mean percentage of motility in short incubation period at 37°C.

After thawing, the mean percentage of immature sperms characterized by cytoplasmic droplets that exposed to PPF before freezing was significantly lower than to EE-I ($P < 0.01$). Moreover, the immature sperm of caudal epididymal sperm that flushed and thawed in PPF after thawing were tended to lower than after recovery ($P = 0.058$). This result supports Nöthling *et al*. (2007) and Hori *et al*. (2005) that addition of prostatic fluid prior to cooling resulted in lower mean percentages of cytoplasmic droplets compared to sperm without prostatic fluid. Hori *et al*. (2005) reported that sensitization epididymal sperm with prostatic fluid induced the loss of cytoplasmic droplet. Comparison of sperm tail abnormality after recovery with that after thawing, the mean percentage of bent tail and coil tail after thawing was significantly higher than after recovery in all groups ($P < 0.001$). This might be the effect of cryopreservation (Woolley and Richardson, 1978).

The post-thawed caudal epididymal sperm quality in this study was in accordance with previous study. The mean percentage of sperm progressive motility immediately after thawing was ranging from 29.0% to 37.0%. The mean percentage of sperm viability immediately after thawing ranged from 44.7% to 49.4%. From Hori *et* al. (2005) studied, frozen-thawed motility of epididymal sperm varied from 20% to 40% and sperm viability was ranging from 37.8 to 66.9%. Their result of intrauterine unilateral artificial insemination with frozen-thawed epididymal sperm obtained 80% conception rate. After caudal epididymal sperm were thawed, there was no significant difference of sperm progressive motility, sperm viability and sperm plasma membrane integrity among all groups ($P = 1.000$). Frozen-thawed caudal epididymal sperm were stored at room temperature and at 4°C. Sperm progressive motility of all groups significantly decreased after 30, 60 and 120 minutes storage ($P < 0.05$), respectively. This might because sperm continued using energy, while the source of energy in PPF and tris buffer were limited. The frozen-thawed caudal epididymal sperm that exposed to PPF had more ability to

maintain sperm progressive motility than the unexposed sperm. The sperm motility activation might be supported by inorganic compound (Okamura *et al*., 1985), Prostaglandin and Estrogen (Troedsson *et al*., 2005) in PPF. Sperm viability significantly decreased after 30 minutes storage. However, sperm viability of post-thawed caudal epididymal sperm that exposed to PPF before freezing did not significantly decrease after storage from 30 minutes until 120 minutes. This study confirmed previous study that sensitized epididymal sperm with prostatic fluid before freezing maintained sperm quality at 20°C for 6 hours (Hori *et* al., 2005). The mean percentage of sperm viability was ranging from 33.0% to 40.0% after storage for 120 minutes. Sperm viability was no significant difference among all groups in any period of storage time. This study supported study of Rota *et al.* (2007) that addition of autologous prostatic fluid to frozen-thawed canine ejaculated sperm had no effect on semen longevity. Furthermore, the qualities of sperm stored at room temperature was not different from those stored at 4° C. This result showed that PPF could maintain sperm quality similar to tris buffer. Moreover, post-thawed caudal epididymal sperm could be stored for 120 minutes at room temperature or at 4° C.

This study suggested that PPF could be used as a flushing and a thawing medium for canine caudal epididymal sperm cryopreservation for substitution of egg-yolk tris extender or tris buffer. The post-thawed caudal epididymal sperm also could be stored at room temperature or 4°C for a short period prior to using. For artificial insemination, the sperm progressive motility was essential for sperm to interact the reproductive epithelium (England *et al*., 2006). Post-thawed caudal epididymal sperm should be used within 30 minutes. The insemination should be undertaken during fertilization period of the bitch which is 2 to 3 days after ovulation (Thomassen and Farstad, 2009). Moreover, the semen should be deposited in the uterus to facilitate sperm migration. On the other hand, sperm viability was more than 30 % in all groups, post-thawed epididymal sperm could be kept until 120 minutes at either room temperature or at 4°C. Therefore, they could be used to fertilize with oocytes by other techniques which need no sperm progressive motility of sperm.

Conclusion

This study shows that preserved homologous prostatic fluid is more suitable for using as a flushing medium than egg yolk-tris extender. Also, it can be used as a thawing medium instead of tris buffer for canine caudal epididymal cryopreservation, which can reduce the expense and complications of preparing the flushing and thawing medium in the canine epididymal sperm cryopreservation process. In addition, as thawing medium, preserved homologous prostatic fluid maintained applicable sperm progressive motility within 30 minutes and sperm viability until 120 minutes storage.

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