EFFECT OF FISH ANTIFREEZE PROTEIN TYPE III SUPPLEMENTATION TO SEMEN EXTENDER ON CRYOPRESERVED DOG SPERMATOZOA

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ผลของการเติมโปรตีนต้านการเยือกแข็งชนิดสามที่สกัดได้จากปลาในสารเจือจางน้ำเชื้อต่อคุณภาพน้ำเชื้อแช่แข็งสุนัข

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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รูดอล เจทต้า บายูสาลัคสา : ผลของการเติมโปรตีนต้านการเยือกแข็งชนิดสามที่สกัดได้จากปลาในสารเจือจางน้ำเชื้อต่อคุณภาพน้ำเชื้อแช่แข็งสุนัข. (EFFECT OF FISH ANTIFREEZE PROTEIN TYPE III SUPPLEMENTATION TO SEMEN EXTENDER ON CRYOPRESERVED DOG SPERMATOZOA) อ.ที่ปรึกษาหลัก : ศุภวิวัธน์ พงษ์เลาหพันธุ์

โปรตีนต่อต้านการเกิดผลึกน้ำแข็งชนิดที่ 3 (antifreeze protein type III; AFPIII) ถูกนำมาใช้เป็นสารป้องกันการเกิดผลึกน้ำแข็ง (cryoprotectant) ในการเก็บน้ำเชื้อแช่แข็งและได้รับการยืนยันว่าช่วยเพิ่มอัตราการรอดชีวิตของอสุจิ (sperm viability) ในบางสปีชีส์ยกเว้นในสุนัข สำหรับในสุนัขนั้นการใส่ EquexSTM ในสารละลายสำหรับแช่แข็งอสุจิ (semen freezing medium) ช่วยให้เกิดผลดีต่ออสุจิที่ผ่านกระบวนการ แช่แข็งวัตถุประสงค์ของวิทยานิพนธ์ฉบับนี้คือ 1. เพื่อประเมินประสิทธิภาพของ AFP ต่อคุณภาพน้ำเชื้อแช่แข็งในสุนัข (การทดลองที่ 1) 2. เพื่อประเมินประสิทธิภาพของ AFP เมื่อใช้ร่วมกับ Equex STM ต่อคุณภาพน้ำเชื้อแช่แข็งในสุนัข (การทดลองที่ 2) โดยศึกษาน้ำ เชื้อสุนัขที่มีการเคลื่อนไหวของอสุจิทั้งหมด (total motility; TM) มากกว่า 70 เปอร์เซ็นต์ นำตัวอย่างน้ำเชื้อจากสุนัขทั้ง 3 ตัวมารวมกัน (pooled semen) แล้วแบ่งใส่หลอดทดลองในปริมาณเท่าๆกัน สำหรับการทดลองที่ 1 ใช้วิธีการเจือจางน้ำเชื้อแช่เข็งด้วยวิธี Two steps-dilution freezing โดยใช้สารละลายเจือจางน้ำเชื้อ (extender) พื้นฐาน tris-egg yolk ที่มีการเติม AFP ในปริมาณต่างๆ ดังนี้ 1. กลุ่ม P0 ไม่ใส่ AFP (กลุ่มควบคุม) 2. กลุ่ม P1 ใส่ AFP 0.01 ug/ml 3. กลุ่ม P2 ใส่ AFP 0.1 ug/ml 4. กลุ่ม P3 ใส่ AFP 1 ug/ml สำหรับการทดลองที่ 2 แบ่งกลุ่มดังนี้ 1. กลุ่ม E1 ใส่ AFP 0.1 ul/ml 2. กลุ่ม E2 ใส่ AFP 0.1 ul/ml ร่วมกับ Equex STM 1 เปอร์เซ็นต์โดยปริมาตร 3. กลุ่ม E3 ใส่ Equex STM 1 เปอร์เซ็นต์โดยปริมาตร สำหรับการละลายน้ำเชื้อแช่แข็ง (thawing) ทำได้โดยการจุ่มหลอดบรรจุน้ำเชื้อแช่แข็งลงในน้ำอุณหภูมิ 37 องศาเซลเซียส เป็นเวลา 60 วินาที มีการตรวจประเมินการเคลื่อนไหว (sperm motility) และความเร็ว (sperm velocity) ในการเคลื่อนที่ของอสุจิด้วยเครื่อง Sperm Class Analyzer® มีการตรวจวัด sperm viability ด้วยการย้อมสี SBYR-PI fluorescent ตรวจวัดความสมบูรณ์ของอะโครโซมอสุจิ (acrosome integrity) ด้วยการย้อมสี FITC-PNA และ PI มีการตรวจวัดศักย์เยื่อหุ้ม เซลล์ของไมโตคอนเดรีย (mitochondrial membrane potential) โดยการย้อมสี JC-1 ตรวจวัดความสมบูรณ์ของเยื่อหุ้มตัวอสุจิ (Plasma membrane functional integrity; PMFI) ด้วยวิธี hypo-osmotic swelling test (HOST) ผลจากการทดลองที่ 1 พบว่าในกลุ่ม P3 (AFP 0.1 ug/ml) ส่งผลให้เกิดการลดลงอย่างมีนัยสำคัญของ TM, การเคลื่อนไหวไปด้านหน้า (progressive motility; PM) และ sperm velocity เมื่อเปรียบเทียบกับกลุ่มควบคุม (P0) กลุ่ม P1 และ P2 สำหรับกลุ่มที่ใช้ extender ที่ใส่ AFP 0.01 และ 0.1 ug/ml พบว่าไม่ได้ช่วยเพิ่มคุณภาพของน้ำเชื้อแช่ แข็งอย่างมีนัยสำคัญสำหรับการประเมินคุณภาพน้ำเชื้อแช่แข็งทันทีหลังจากการละลายพบว่าไม่มีความแตกต่างอย่างมีนัยสำคัญ (P>0.05) ระหว่างกลุ่มทดลองทั้ง 3 กลุ่มเมื่อเปรียบเทียบกับกลุ่มควบคุม ยกเว้นค่า PM ซึ่งพบว่าค่า PM ของกลุ่ม P3 น้อยกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ (P<0.05) อย่างไรก็ตามพบว่า sperm velocity หลังจากละลายน้ำเชื้อแช่แข็งเป็นเวลา 1 ชั่วโมง ในกลุ่ม P2 ไม่ได้มีค่าลดลงอย่างมีนัยสำคัญ สำหรับการทดลองที่ 2 พบว่า extender ในกลุ่ม E2 และ E3 ทำให้คุณภาพของอสุจิหลังจากการล ะลายน้ำเชื้อแช่แข็งดีขึ้นอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับกลุ่มE1 (P<0.0001) การศึกษานี้แสดงให้เห็นว่าการใช้ AFP ร่วมกับ Equex STM ในสารละลายเจือจางน้ำเชื้อแช่แข็งช่วยให้ sperm viability ดีขึ้นทันทีอย่างมีนัยสำคัญ (P<0.05) หลังจากละลายน้ำเชื้อแช่เข็ง สรุปได้ว่าการใส่ AFPIII ที่ความเข้มข้น 0.01 ug/ml ส่งผลให้เกิดการเพิ่มขึ้นของ sperm velocity ในสุนัข การใส่ AFPIII ที่ความเข้มข้น 1 ug/ml ส่งผลให้เกิดการลดลงของคุณภาพน้ำเชื้อหลังจากการละลายน้ำเชื้อแช่แข็ง และการใส่ AFPIII ที่ความเข้มข้น 0.01 ug/ml ร่วมกับ Equex STM ช่วยรักษา sperm viability หลังจากการละลายน้ำเชื้อแช่แข็งได้

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Rudal Jetta Bayusalaksa : EFFECT OF FISH ANTIFREEZE PROTEIN TYPE III SUPPLEMENTATION TO SEMEN EXTENDER ON CRYOPRESERVED DOG SPERMATOZOA. Advisor: Asst. Prof. Dr SUPPAWIWAT PONGLOWHAPAN

Ice binding protein such as antifreeze protein (AFP) type III have been used as a cryoprotectant and proven to improve sperm survival in some species but dog. Addition of Equex STM paste to semen freezing medium provides beneficial effects on cryopreserved dog sperm. Hence, the objectives of this study were to (a) evaluate the effects of AFP (Experiment I) and (b) its combination with Equex STM paste on cryopreserved dog spermatozoa (Experiment II). The semen samples were pooled to allow for a sufficient number of sperm and to reduce an individual variation. Only sperm-rich fractions with total motility more than 70% were used. Pooled semen from three dogs was split in equal portions according to the number of tested extenders in Experiment I or II. Two steps-dilution freezing methods with 4 different tris egg-yolk based extender were used in experiment I, e.g. P0; no AFP added (control), P1; 0.01 µg/mL, P2; 0.1 µg/mL, P3; 1 µg/mL (w/v) and experiment II, e.g. E1; 0.1µl/mL AFP), E2; 0.1µl/ml AFP plus 1% (v/v) Equex STM paste and E3; 1% (v/v) Equex STM paste. Sperm evaluation was done one week after the freezing. Thawing was performed by immerse freeze-straw into 37°C water for 60 second. Sperm motility and velocity were evaluated by Sperm Class Analyzer® (SCA Microptic SL, Barcelona, Spain). Post-thawed samples were stained for sperm viability (SBYR-PI fluorescent staining), acrosome integrity (FITC-PNA and PI) and mitochondrial membrane potential (JC-1), and were evaluated. Plasma membrane functional integrity (PMFI) was evaluated by hypo-osmotic swelling test (HOST). In Experiment I, overall, regardless of time post thawing, addition of AFP type III at a concentration of 1µg/ml (P3) significantly deteriorated total motility, progressive motility and sperm velocity compared to P0 (control), P1 and P2. Semen extenders supplemented with 0.01 or 0.1µg/ml AFP yielded no significantly better quality of frozen-thawed dog sperm than those of the control. Immediately post-thawing, there are no significant differences among 3 treatments and control groups (P>0.05) except for the progressive motility. The progressive motility was significantly lower in P3 (P>0.05). In general, all parameters decreased after 1h incubation at 37°C (P>0.05). However, post-thawed sperm velocity (VCL, VSL and VAP) did not significantly decline at 1h in P2. In Experiment II, overall, Equex-contained extenders (E2, E3) significantly improved sperm quality post-thawing compared to E1 (P<0.0001). A combination of AFP and Equex STM paste provided a beneficial effect on sperm viability immediately after thawing (P<0.05). In conclusion, supplementation of AFP type III at a concentration of 0.01µg/ml provided a beneficial effect on dog sperm velocity. Higher concentration of AFP (1µg/ml) deteriorated post-thawed sperm quality. Addition of 0.01 µg/ml into Equex-contained semen extenders was essential for maintaining sperm viability post-thawing.

Field of Study:	Veterinary Science and technology	Student's Signature .....
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Rudal Jetta Bayusalaksa

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CHAPTER I

INTRODUCTION

Importance and Rationale

Breeding technology in the dog has been developed for almost a century. Two decades ago, natural mating was very popular among the breeder to breed domestic animals. Although this method obtains satisfactory results, it highly spreads some diseases and does not allow long-term semen storage (Moran, 2012). When male animals are limited, different estrus time of the bitch, and boundary constricted, application of artificial insemination (AI) are needed. High quality of frozen semen is essential for successful AI. The greatest advantage of frozen semen is the only way to retain potential genetics of valuable male animals indefinitely. Other advantages of using frozen semen include preserving and wider distributing genetic materials and disease prevention (Feldman and Nelson, 2003; Nelson and Couto, 2008). However, the effectivity of this procedure still low, resulting in low pregnancy rate in the bitches inseminated with frozen semen (Suwa et al., 2009). Study conducted by Linde-Forsberg show that the successful pregnancies obtained from bitches inseminated with frozen semen remains low (pregnancy rates 69.3%) compared with the one inseminated with fresh semen (pregnancy rates 83.8%). Liter size also corresponded with pregnancy rate with smaller liter size in bitches that inseminated with frozen sperm compared with fresh sperm (Linde-Forsberg and Forsberg, 1989).

Since conception rate is depended on post thawed sperm quality and the results vary among the individual, optimal protocol for sperm freezing is imperative. Syrups like substance such as glycerol is essential to protect the sperm in freezing processes, but this substance is cytotoxic parallel with its dose or exposure duration (Peña and Linde-Forsberg, 2000). Until now, two-steps dilution methods using Uppsala Equex extender is still the most popular protocol used and believed as the best methods for the dog sperm cryopreservation.

Ice crystal formation on cryopreservation process can damage the sperm and decrease the post-thawed sperm quality. To minimize negative effect of cryopreservation, extender that contains cryoprotective agent (CPA) is needed. The CPA plays an important role to maintain semen quality during the freezing process until it is thawed and used (Van den Berghe et al., 2018). Cryoprotective agents such as glycerol, lecithin and sodium dodecyl sulfate such as Equex STM paste are widely used to prevent cryoinjuries in canine sperm cryopreservation (Sánchez-Calabuig et al., 2017). Certain concentrations are needed for cryoprotectants to be able to protect sperm cells from cryoinjuries (Pinto et al., 1999). Penetrating cryoprotectants such as glycerol work by increasing total solute concentration of solution, resulting in decreasing of water ice crystal formation (Pegg, 2007). This cryoprotective agent has been used in semen freezing of many species (Silva et al., 2003a). One of cryoprotective non-penetrating agent that can inhibit natural water crystal formation is antifreeze protein (AFP) (Wang, 2000). This protein is classified into ice binding protein (IBP). The IBP will bind into ice nuclei (Davies, 2014) and inhibit normal ice growth, ice re-crystallization, and shaping the ice crystal formation into less harming shape (Wang, 2000; Nishimiya et al., 2003). In buffalo sperm, adding AFP in semen freezing extender along with glycerol works synergistically, thus improving the post-thawed sperm quality in term of total motility. Similar result was obtained when AFP added into chimpanzee sperm extender (Younis et al., 1998). Although, the beneficial effects of Equex STM paste (Nova Chemical Sales, Scituate, Inc., MA, USA), a detergent, that can decrease the surface tension and stabilize the membrane plasma of sperm cell, on improving dog semen quality post-thawed have been demonstrated for many years (Peña and Linde-Forsberg, 2000), this product is no longer available commercially. Alternatively, other cryoprotective agents are needed.

Recently, AFP is widely applied as a cryoprotective agent to preserve tissue or cells in many species such as mouse ovarian tissues, human red blood cells and spermatozoa (Kim et al., 2017). Many AFP types have been documented such as AFP I, AFP II and AFP III. The AFP from fish sera extract have been commercialized. However, the effect of AFP III on cryopreservability of canine spermatozoa has not yet been demonstrated. The concentration of AFP supplementation to semen

extender is also different among species. The effects of different concentrations of AFP and its protective effects on cryopreserved dog sperm warrant further studies. Moreover, the effect of combination of AFP and Equex STM paste supplemented into semen extender on frozen-thawed dog sperm remained an interesting subject to be elucidated.

Objectives

1. To study the effect of antifreeze protein III (AFP III) supplementation to semen extender on the quality of post-thawed dog sperm
2. To find the optimal concentration of AFP III that yield the better sperm characteristics as evaluated by sperm motility, plasma membrane integrity, functional membrane integrity, acrosome integrity and mitochondrial membrane potential
3. To study effect of combination of AFP and Equex STM paste supplemented into semen extender on frozen-thawed dog sperm

Keywords (in Thai) : โปรตีนต้านการเยือกแข็ง การแช่แข็ง สุนัข อสุจิ

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Hypothesis

1. It is hypothesized that adding AFP III at certain concentration into the semen extender improves the post-thaw quality of dog sperm.
2. It is hypothesized that Equex STM paste and AFP type III work synergically and improves the post-thaw quality of dog sperm.

CHAPTER II

LITERATURE REVIEW

Canine spermatozoa

Spermatozoa are produced in the seminiferous tubule. The canine spermatogenesis consists of spermatocytogenesis, which starts from formation of spermatids from spermatogonia and spermiogenesis, where spermatid will be differentiated into spermatozoa. This whole process will take about 62 days long (Johnston et al., 2001). Normal morphology of canine spermatozoa consists of acrosomal cap, head, neck, mid piece and tail. Acrosome is cap-like structure covering almost half of spermatozoa's head (Feldman and Nelson, 2003). Acrosome contains some enzymes; the main two are acrosin and hyaluronidase. Head sperm also contains dens nucleus. This enzyme work during acrosome reaction for disperse cumulus oophorous and local lysis of zona pelucida (Arthur et al., 2001) . The middle piece of sperm starts from the head until the end of the helically arranged mitochondrial sheath. The 9+2 microtubular axoneme lay in the center of mid-piece until the tail of spermatozoa (Kierszenbaum and Tres, 2015).

Spermatozoa will be exposed to various osmolarities when they are transported in the male reproductive tract until being ejaculated (Damm and Cooper, 2010). Further, sperm will be exposed in high osmolarities in extender. Cryoprotective agent such as glycerol, egg yolk or other components added onto extender will increase its osmolarity. Dog spermatozoa motility is sensitive to osmotic stress. A study conducted by Songsasen showed that sperm motility will decrease in sugar-contained extender with 750 mOsm and will completely lose its motility in 1500 mOsm osmolarity. In contrast, dog sperm plasma membrane integrity is more tolerance in osmotic stress compared with the plasma membrane integrity will significant different when exposed with sugar-contained extender in 1000 mOsm. Despite that dog spermatozoa are sensitive to osmotic stress, they can tolerate to be

exposed in hypertonic semen extender (Songsasen et al., 2002). This finding is similar with sperm osmotic resistance in human. Sperm survival is depended on duration of sperm exposed with hypertonic solution; sperm survival is higher in short exposure time. This indicates spermatozoa have limitation time in hyperosmotic solution due to cell shrinkage. Cells will be re-expansion after permeable cryoprotectant enter the cell and will increase the sperm survival rates. In addition, human sperm can maintain 50% survival up to 2000 mOsm hypertonic extender (Gao et al., 1993).

Cryopreservation and extender

Spermatozoa lose its cytoplasm during spermatogenesis, make it less contains of water and more suitable for cryopreservation than egg, embryo or somatic cells (Suzuki and Donnez, 2016). However, cells are not always viable and normal function after thawing. Freezing and thawing process in canine sperm can decrease its viability and impact on sperm functional such as motility, acrosome integrity and morphology (Silva et al., 2003b; Sánchez-Calabuig et al., 2017). During freezing process, cells will be exposed with high osmolarity environment due to water crystallization and leaves salt dissolved with unfrozen fraction. This phenomenon leads intracellular water move into extracellular, leading on cell dehydration. Intracellular ice crystal formation from retained water when dehydration process is not optimum will be formed and can damage the cell. Eventually, further extracellular ice crystal growth also believed to be detrimental to cell (Kim et al., 2017). Sperm morphology will be decreased 37% by freezing-thawing process. It shows that sperm morphology are susceptible for cryopreservation-induced damage (O'Connell et al., 2002).

Cryopreservation is an important technique to keep various cells, organs or even tissues in very low temperature. This technique conventionally uses liquid nitrogen in -196^{o}C (Mazur, 1984). Implementation this technique in sperm cells has given a big impact on clinical reproduction field and become one important tool to aid assisted reproduction (Naresh and Atreja, 2015). However, cryopreserving processes expose sperm cells to stress, resulting in lower quality of spermatozoa after thawing (Pezo et al., 2017). The quality of frozen semen is important to determine the conception rate. Various improvement in semen extender, freezing and thawing method are always developed to improve the quality of freezing semen (Gordon, 2004). Almost all cryopreservation methods rely on preventing ice crystal formation. This ice crystal formation may damage cell (Silber, 2018), making the cell lost its permeability and will result in the death of the cell (Eilts, 2005). Recently, two steps of dilution along with vitrification result in high post-thawing motility in canine sperm cryopreservation, this preservation method also supported by the suitable extender and CPA (Peña and Linde-Forsberg, 2000).

Many different extenders have been developed in many species. Semen extenders comprise some substances that maintain sperm survival outside of the reproductive tract (Brinsko et al., 2010). Factors that must be fulfilled in sperm extender for sperm cryopreservation are nutrient, buffer, antibiotics and cryoprotectant. In general, nutrient such as fructose (Yildiz et al., 2000), sucrose or glucose, are added into the extender. These various sugar provide energy to sperm and prevent sperm to use its own intracellular phospholipid (Mulhall et al., 2013). Energy sources such as glucose and fructose are mainly used for canine sperm cells to flagellar movement along with movement pattern. A study conducted in 2004 showed that in canine sperm, the usage of fructose with 70mM concentration on semen extender is more effective for cold storage (Ponglowhapan et al., 2004). To avoid and eliminate bacterial contamination, antibiotics are added into extender (Brinsko et al., 2010). According to European Council Directive 90/429/EEC, Annex C2,

antibiotics that are non-toxic and not harmful to sperm are essential. Antibiotics contains in semen extender must be able to fight against bacteria and mycoplasma without affecting the motility and fertility. Following combinations are recommended to produce on effect, at least equivalent to the following dilution such as 500 µg per mL streptomycin final dilution, 500 IU per mL penicillin final dilution, 150 µg per mL lincomycin final dilution, 300 µg per mL spectinomycin final dilution (Morrell and Wallgren, 2014). The extenders also must contain cryoprotective agent (CPA) that protect cells from the low freezing. Addition of cryoprotectants into semen extenders can change cellular osmolarity, decrease in osmotic stress and change intracellular water volume (Eilts, 2005).

After cryopreservation, not many sperm cells are viable and normally functioned compare with the fresh ejaculate sperm. This reducing of sperm quality mainly is caused by cryodamage (Pezo et al., 2017). There are many kinds of substance and CPA that have been used in canine sperm cryopreservation, such as milk, egg yolk, lecithin, glycerol, sodium dodecyl sulfate (SDS), dimethylformamide and antifreeze protein (Peña and Linde-Forsberg, 2000; Mota Filho et al., 2011; Qadeer et al., 2014; Axner and Lagerson, 2016; Nouri Gharajelar et al., 2016). Some of CPA work together as combinations on semen extender and give better results in comparison with a sole CPA in extender (Oldenhof et al., 2017). Cryoprotective agent works by many ways to protect the sperm depend on type of CPA, such as ice binding CPA and surfactant CPA. Ice binding CPA work by inhibit ice crystal growth that can be harmful to the sperm like AFP and Surfactant CPA work by changing the surface tension such as SDS (Equex STM paste) (Peña and Linde-Forsberg, 2000; Prathalingam et al., 2006). Equex STM Paste which have main active substance is sodium dodecyl sulfate (SDS), reported can increase sperm motility, plasma membrane integrity and acrosome integrity in epididymal (Ponglowhapan and Chatdarong, 2008) and ejaculated dog sperm (Peña and Linde-Forsberg, 2000).

Antifreeze protein

Antifreeze protein (AFP) were first extracted and published in 1969 from blood sera of polar fish and were recognized as macromolecule that can attach and inhibit ice crystal growth (DeVries and Wohlschlag, 1969). Since its discovery, AFP become a subject of many studies because of its ability in preventing or reducing the damage caused by freezing to living organisms (Fletcher et al., 2001). AFP have ability to inhibit normal ice growth, ice recrystallization and protecting plasma membrane by inhibit ion leakage at hypothermic temperature (Wang, 2000). Because its properties, AFP have attract interest of scientist as a candidate of CPA which is less toxic and non-penetrating (Buzzini and Margesin, 2013).

This polypeptide protein has two important properties to against cryodamage. Those properties are change thermal hysteresis (TH) and ice re-crystallization inhibition (Prathalingam et al., 2006). The TH is referred to gap of freezing point and melting point of liquid solution. This temperature gap are resulted by irreversible AFP binding into ice crystal and series of inhibition activities during their grow until temperature reach to nonequilibrium freezing point (Kristiansen and Zachariassen, 2005). AFP will bind to specific ice crystal formation and shape it into unique ice morphology (Raymond and DeVries, 1977). Following picture will explain about AFP works during TH gap. The second property that maybe the most powerful tool of AFP is ice recrystallization inhibition (IRI). AFP can bind to the boundary of ice grains and inhibit the growth process (Raymond and Knight, 2003). Ice recrystallization inhibition is more likely to be an important property for an organism to survive in cold environment and can preserve membrane from freezing injuries (Hew and Yang, 1992). Because its capability to inhibits ice crystal formation, ice crystal grains

formation in solution contains AFP is smaller in size compared with ice crystal grain in solution without AFP (Kim et al., 2017).

Based on amino acid sequence and tertiary structure, AFP is categorized into AFPs I-IV and Antifreeze glycoproteins (AFG) (Harding et al., 2003; Graether and Sykes, 2004). The AFG has strong recrystallisation properties, but this protein is hard to be synthesized and natural polar fish sources are not enough to produce large quantities of AFG. In contrast with AFG, mass production of AFP is possible due to application of recombinant protein expression techniques. Commercial issue cause by low production of AFG is one of the reasons that AFP is widely used for studies and industry (Kim et al., 2017). Antifreeze protein type I is extracted from winter flounder *Pseudopleuronectes americanus*. This fish can be founded in pole region. Ala-rich hydrophobic faces are suggested as an ice active binding site of this protein. Antifreeze protein type two II is another protein that can inhibit ice crystal formation. Disulfide bound structure makes this protein stabilize its structure. Type two AFP only poses tree or two active sides of ice binding surface which consist of Thr96, Leu97, Thr98, and Thr115 residues (Liu et al., 2007). Antifreeze protein III (AFP III) is one type of antifreeze proteins family with 7 kDa molecular weight in average, this protein can be founded and extracted from pole fish serum (Yeh and Feeney, 1996; Salvay et al., 2010). Antifreeze Protein III mainly has several loops in structure, those loops are formed from stable hydrophobic interaction and hydrogen bound in inner structure. (Chao et al., 1994). Making this protein extremely stable and tolerance in pH (ranged 1-11). This protein still can be worked after being heated at 80°C for 1 hour. Short pressure up to 400MPa had no influence on AFP III works (Leiter et al., 2016). The differences between each AFP type briefly are showed in Table 1.

Table 1 Antifreeze protein type I, II, III, IV and AFG differences

Protein	Structural type	Ice binding site	Species	References
AFP I	single alpha-helix	Alanine face and residues associated.	righteye flounders, Shorthorn sculpins	(Chao et al., 1996b; Baardsnes et al., 2001)
AFP II	Globular (Ca2+/-dependent)	Thr residues	sea raven, rainbow smelt	(Slaughter et al., 1981; Ewart and Fletcher, 1990)
AFP III	Globular with single flattened surface	Residues in flanking flat surface	eel pouts	(Yu et al., 2005)
AFP IV	Antiparallel helix	Lys residues	longhorn sculpin	(Baardsnes et al., 2001)
AFG	Polymer Ala-Ala-Thr and variant. With disaccharide in each Thr	Gal-GalNAc	cods and Antarctic notothenioids	(Chen et al., 1997)

Because its ice binding properties are not affected by temperature or any other typical substantial parameters such as pH or pressure, AFP type III is widely used as a CPA (Table 2) compared with other AFP types. Antifreeze Protein III has been reported to increase the number of motility rabbit sperm and rabbit embryo survival following freezing and thawing (Nishijima et al., 2014). Addition of 0.01 µg/mL AFP III in semen extender increases buffalo sperm motility and plasma membrane integrity compared with extender with no AFP III supplementation (Qadeer et al., 2014). Extenders contained AFP increase osmotic resistant and reduce mechanical stress in the membrane of bovine sperm (Prathalingam et al., 2006).

Efficiency of AFP III as a CPA is depended on its concentration in the solution, high concentration of AFP can lead ice formation into needle like shape and damage the cells. Different concentrations in different samples, species or preservation methods have to be established (Lee et al., 2015a). Addition of AFP III in extender maintains the viability, linearity of movement and velocity of teleost sperm (Beirao et al., 2012). To our knowledge, there is no study on the effect of AFP III as a CPA on cryopreserved canine sperm.

Table 2 List of AFP type III used in cryopreservation agent in biological samples

Sample types	Organism	AFP III quantities used	Freezing methods	References
Ovarian tissue	Mouse	0, 5 and 20 µg/mL	Vitrification	(Lee et al., 2015b)
Oocyte	Bovine	20mg/ ml	Hypothermic	(Rubinsky et al., 1991)
Embryo	Turbot	10 mg/ml	Hypothermic	(Robles et al., 2006)
Spermatozoa	Seabream	0,1, 1 and 10 µg/mL	Cryopreservation	(Zilli et al., 2014)
Heart	Rat	3, 5 and 15 mg/ml	Vitrification	(Amir et al., 2002)
Red blood cell	Human	0–1.54 mg/ml	Cryopreservation	(Chao et al., 1996a)

Sperm evaluation techniques

Sperm motility is the most important assessment to determine its fertile-ability. Motility assessment can give brief image how sperm ability to penetrate into, and pass through cervical mucus and oocyte (Mortimer and

Mortimer, 2013). Conventionally, this assessment is conducted and judged by a person to determine its motility percentage (Qadeer et al., 2014; Tesi et al., 2018). However, subjective motility assessment is rather imprecise. Computer-aided semen analysis (CASA) is needed to increase its accuracy (Hoflack et al., 2007). This computer based analysis also can give detail in motility such as velocity average pathway (VAP): the average velocity of the smoothed cell path (μm/s); velocity straight line (VSL) (Rijsselaere et al., 2003). In contrast, sperm concentration and several parameters will influence CASA outcome (Verstegen et al., 2002). To avoid this inaccuracy, sperm concentration and some parameter have to be adjusted. Concentration 50 x 10^6 spermatozoa/ml has been recommended in canine sperm CASA study with 30 frame rate image (Rijsselaere et al., 2003).

To understand the damage resulted from sperm cryopreservation, advance sperm evaluation technique is needed other than motility test. Antibody-based fluorescent staining technique now widely used for post-thawed sperm quality. This antibody-based fluorescent staining technique is more reliable compare with conventional staining procedure such as eosin-nigrosine staining and could be interpreted by flow cytometry (Nagy et al., 2003; Kunkitti et al., 2017). By using FC, one major problem in sperm quality assessment could be well handled, which is increasing the sperm counted number in the short period of the time and increase accuracy on represented sperm quality feature in sample that contain billions of sperm. The degree of objectivity is also provided during the process. Because of this feature, the result is more reliable because can avoid subjectivity (Love, 2018). FC works by drive the labeled spermatozoa within laminar flow. Spermatozoa one-by-one passing through the wall where every cell will be illuminated with one or more lasers. The emitted light or scatters that produced by illuminating are filtered by mirror and filter. Reaching several photodetectors and then be amplified. Finally, data obtained will be digitalized and will be presented in different fluorescent intensity unit. The data that obtained from each spermatozoon in each detector further are recovered and analyzed to interpret the result (Martinez-Pastor et al., 2010). Sperm evaluation by using FC has been done in some species such as boar

semen, cat semen, and dog semen to evaluate plasma membrane integrity, acrosome integrity and mitochondrial membrane potential (Torres et al., 2016; Kunkitti et al., 2017; Pezo et al., 2017).

Certain staining is used to stain the sperm's organelles before it assayed by flow cytometry. Combination of Propidium Iodine (Salmon et al., 2017) and SYBR-14 have been proved effectively identified living, dying and dead sperm in dog, rabbit, mouse, ram and man spermatozoa (Garner and Johnson, 1995; Pezo et al., 2017). The SYBR-14 will emit green color when exposed with 488 nm wavelength. This fluorescent dye is cell permeable and stains only the nuclei of living cell. PI are usually used for counter staining. The PI is non-permeable dye and would not be able to cross intact sperm membrane. It gains access into sperm nuclei when sperm membrane are damage and non-viable (Samper, 2009). The main advantage of this staining is had a same targeting point which is sperm DNA. But it becomes its weakness as well since DNA only lays in the head of the sperm. This technique only concern in plasma membrane integrity of sperm head. It cannot asses plasma membrane integrity of mid-peace and tail of spermatozoa (Hossain et al., 2011).

Acrosome integrity assay is considered an important fertility assessment in post-thawed dog semen. Acrosome enzymes allow sperm to penetrate the zona pellucid of egg by series of reaction. The most reliable method to assess acrosome integrity is based on use dyes or fluorescent markers. The standard fluorescent staining of acrosome integrity is by using fluorescently labeled agglutinin with plant-based peas (PSA) or peanut (PNA). PNA is lecithin from peanut plants that bind to beta galactose moieties, which associated with outer acrosomal membrane. FITC-PNA in 488/515 nm wavelength excitation has been prove successfully to determine acrosome integrity of canine dog sperm (Samper, 2009; Pezo et al., 2017).

Mitochondrial membrane potential (MMP) is one of parameter that routinely perform for post-thawed semen, which has positive correlation with motility of the sperm (Hallap et al., 2005). Mitochondria provides ATP for

axonemal function and sperm motility. The mitochondrial stain 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) has been use to determine MMP in post-thawed dog semen combined with FC. In high functional mitochondria, JC-1 concentration inside the mitochondria increased and stain from aggregates will show fluorescent orange with 488 nm wavelength. In low mitochondria potential, JC-1 form as monomer and fluorescent green (Kasai et al., 2002; Samper, 2009; Hossain et al., 2011).

Sperm functional plasma membrane integrity is considered an important parameter for sperm analysis, which have correlation with sperm fertile-ability (Samper, 2009). One study conducted to determine canine sperm resistant in osmotic stress, show that canine sperm with high motility and high progressive motility have high tolerant when exposed in serve osmotic stress. This test basically is exposed sperm in low osmotic buffer solution or usually called hypo osmotic swelling test (HOST). This finding shows that correlation of motility, osmotic stress level and also give raw image of sperm survival inside the female reproductive tract (Peña et al., 2012). It becomes an important issue because sperm had been exposed in various osmolarities in male reproduction tract and further in female reproductive tracts, which give osmotic stress. Sperm survival is essentials in order to maintain its fertile-ability and its number will decrease time by time (Cooper and Yeung, 2003).

CHAPTER III

MATERIALS AND METHODS

In this study, experiments were divided into two parts. First experiment was to find out the effect of AFP type III and the optimal concentration on frozen-thawed dog sperm. The second experiment was to evaluate the synergism between Equex STM paste and AFP type III on the quality of dog sperm post-thawing.

Experiment I

Semen collection, extension and cryopreservation

The method of collection, extension and cryopreservation used in this study were adapted and modified from the two-steps dilution method (Peña and Linde-Forsberg 2000). Different concentrations of AFP type III (0.01, 0.1 and 1 microgram/L) were tested (Table 3). Semen was obtained from 12 healthy male dogs by digital manipulation. Only semen with sperm motility more than 70% were included. Match pair experimental design was chosen; semen samples were collected from three healthy, fertile dogs and then samples were pooled to get the sufficient number of spermatozoa (final $75x10^6$ spz/straw). Four replications of experiment were performed. Pooled semen then divided into four aliquots. Semen aliquots were centrifuged 700 x g. After 10 min of centrifugation, supernatant was discarded. Sperm pellets were diluted with extender I (P0, P1, P2 or P3) and sperm concentrations were adjusted to get 300 x 10^6 spz/mL. Diluted sperm then were cooled in controlled cooling chamber at 4°C for 1h. After 1 hour in the cooler, the second dilution was carried out. The aliquots were diluted accordingly with extender II (P0, P1, P2 or P3) to get the final concentration of 150 x 10^6 spz/mL. The compositions of semen extender I and II were shown in Table 3. The sperm suspensions were loaded into 0.5 mL straws and placed

horizontally 4 (approximately -180°C) cm above liquid nitrogen for 10 mins before plugging them into liquid nitrogen for freezing.

Freeze-thaw procedure

After one week of storage, the samples were thawed in water bath at 37°C for 60 seconds. The samples were diluted with thaw media with 1:1 ratio and kept in an incubator at 37°C for 5 min until being evaluated at 0 h (immediately post-thawing) and 1 h after thawing.

Thawing media

Thawing media consists of 3.025 g tris, 1.7 g citric acid, 1.25 g fructose, 0.06 benzyl penicillin, 0.1 g streptomycin sulfate, distilled water up to 100 ml.

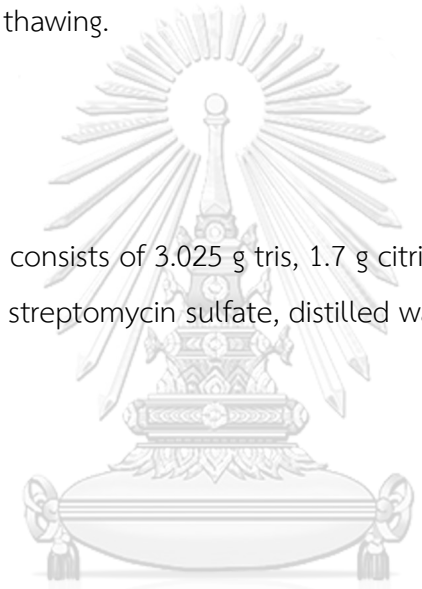
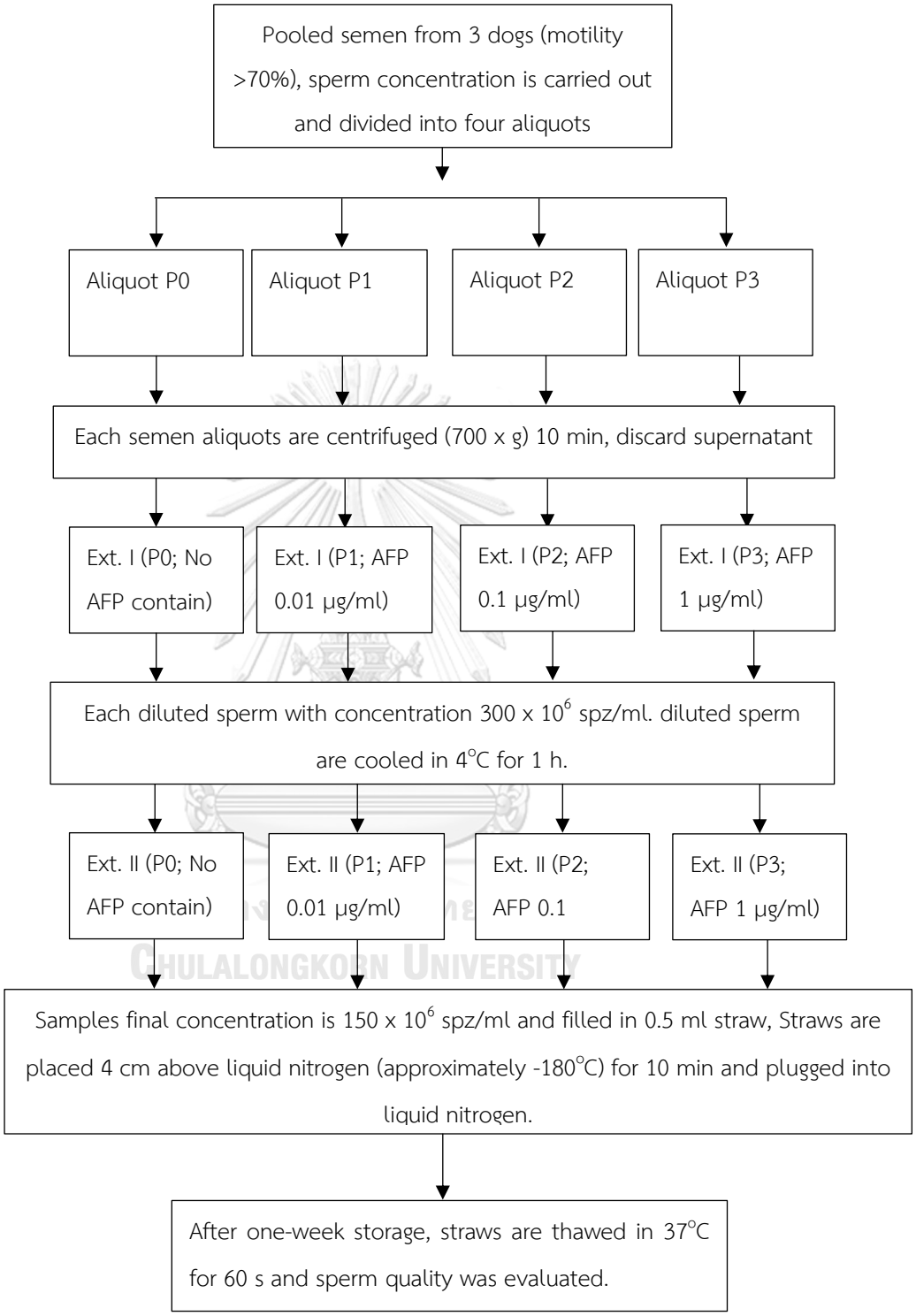


Table 3. Extender composition consists of P0 as a control, P1 contains 0.01µg/mL of AFP III, P2 contains 0.1µg/mL of AFP III and P3 contains 1µg/mL of AFP III (modified Uppsala semen extender)

Substance	**Extender I**				**Extender II**			
	P0	P1	P2	P3	P0	P1	P2	P3
Tris (g)	3.025	3.025	3.025	3.025	3.025	3.025	3.025	3.025
Citric acid (g)	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7
Fructose (g)	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25
Benzyl penicillin (g)	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
Streptomycin sulphate (g)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Egg yolk (ml)	20	20	20	20	20	20	20	20
Glycerol (ml)	3	3	3	3	7	7	7	7
AFPs III (g)	**0**	**0.000001**	**0.00001**	**0.0001**	**0**	**0.000001**	**0.00001**	**0.0001**
Distilled water	up to 100mL	up to 100mL	up to 100mL	up to 100mL	up to 100mL	up to 100mL	up to 100mL	up to 100mL
pH	6.8	6.8	6.8	6.8	6.8	6.8	6.8	6.8
Osmolarity (mOsm/kg)	977	978	976	1016	1784	1893	1742	1800

Experimental Framework:



Sperm quality assessment

a. Assessment of sperm motility

After semen collection, subjective motility was conducted by placing thawed semen onto a warm slide under the cover glass and sperm samples with more than 70% total motility was included in this study. Percent motility was recorded from 5 different fields under a light microscope at 100x magnification. After thawing, sperm motility was evaluated by computer assisted sperm analysis (CASA). The motility evaluation by CASA (SCA, Microptic S.L Spain) was performed by loading 7 µl of the sample in a pre-warmed slide (37ºC). The device captured motile and non-motile sperm under 100x magnification (at least 4 – 5 fields per examination). The computer analyzed and reported the result for total motility, progressive motility and sperm velocity. CASA settings were : frame rate, 25 frames per s; minimum contrast, 75; straightness threshold, 75%; low velocity average pathway (VAP) cutoff, 10; and medium VAP cutoff, 55 (Mota Filho et al., 2011).

b. Assessment of plasma membrane integrity (viability)

Plasma membrane integrity (sperm viability) was assessed using SYBR-14/PI. According to previous studies (Kunkitti et al., 2017; Pezo et al., 2017) with some modifications, 250 µl of sperm solution in PBS (2 x 10^6/ml), 0.5 µl of SYBR-14 (10 µM) and 1 µl of propidium iodine (15.58 µl 2,4mM PI stock diluted with 84.42 µl PBS) were mixed and incubated for 20 mins, 37 ^{o}C (Salmon et al., 2017). For washing, 1 ml of PBS was added and centrifuged at 300 x g for 5 min. Supernatant was discarded and sperm cells were re-suspended in 400 µl of PBS for analysis by flow cytometry. Excitation was induced by argon ion 488 nm laser. SYBR-14 fluorescence (green) was detected via channel FL1 (525 nm), PI fluorescence (red: spermatozoa with permeable plasma membrane) was detected via channel FL3 (620 nm). Total of 30,000 spermatozoa were assessed by flowcytometry and categorized into

tree population based on degree of plasma membrane integrity: living sperm (%) (SYBR14-positive/PI-negative), dying sperm (SYBR14-positive/PI-positive) and dead sperm (SYBR14-negative/PI-positive).

c. Acrosome integrity status

Acrosomal status was assessed by using fluorescein isothiocyanate (FITC) – labelled with Peanut agglutinin (PNA) and assessed by flowcytometry to determine intact spermatozoa. The Two hundred µl of sperm solution in PBS (2 x 10^6/ml) will mixed with 0.5 µl FITC-PNA and 2 µl of PI (250 µg/ml) and sample was incubated for 15 min at 37°C. Sample then washed by adding 1 ml of PBS and centrifuged at 300 x g for 5 min. Supernatant was discarded and sperm cell re-suspended in 400 µl of PBS. The cells further were analyzed by using flow cytometry to evaluate the acrosomal intact or damage. Flow cytometric analysis was performed with 620nm beam-splitting filter and a 525nm band-pass filter for FITC-PNA detection. Within the population, a subpopulation of Acrosome Reacted cells bound PNA, resulting in a sharp fluorescent emission in forward scatter (Nagy et al., 2003). Total 30,000 spermatozoa cells were counted and categorized into two groups: intact acrosome (no color) and lose acrosome (green fluorescent) (Pezo et al., 2017).

d. Assessment of mitochondrial membrane potential status

Mitochondrial membrane potential (MMP) will be assessed using JC-1 fluorescent dye. Mixing 250 µl of sperm solution in PBS (2 x 10^6/ml) and 1 µl 3mm JC-1 working solution (19.7 µl JC-1 stock (3,8 mM) in 5.27 µl DMSO) was incubated at 37°C for 40 min. Finally, 1 ml of PBS was added, and sample was centrifuged at 300 x g for 5 min. Supernatant was discarded and sperm cell re-suspended in 400 µl of PBS. Sperm cells further were analyzed by using flow cytometry to evaluate MMP. Excitation was induced by argon laser ion 488 nm laser. FL1 (525 nm) and FL2 (575 nm) filters were used. Total of 30,000 spermatozoa cell were counted and categorized into two groups: spermatozoa with high mitochondrial membrane

potential (orange fluorescence) and with low mitochondrial membrane potential (green fluorescence) (Pezo et al., 2017).

d. Assessment of plasma membrane fluidity (hypo-osmotic swelling test)

Sperm plasma membrane integrity (PMI) are assessed by hypo-osmotic swelling assay (HOS). HOS solutions are composed of 13.51 g fructose, 7.53 g tri-sodium citrate, 1000 mL distilled water (100 mOsm/kg). The sperm sample (25 µl) was incubated at 38 ℃ with HOS solution (25 µl) for 30 min. HOS solution with 5% formaldehyde was used for inactive the action of HOS medium. After incubation, a drop of mixture is placed on warm slide (38°C), cover slipped and evaluate under light microscope with 400x magnification. A total of 1000 sperm were examined per treatment (200 per each of treatment). Swollen tails indicated intact, while un-swollen tails indicate disrupted.

Statistical analysis

Percent motility (total motility, progressive motility and sperm velocity), plasma membrane integrity, acrosomal integrity, mitochondrial membrane potential and functional membrane integrity were analyzed by multivariate analysis of variance and Turkey's Multiple Comparison Test were used for multiple comparison among different AFP III concentrations. A P-value < 0.05 was taken to indicate statistical significance and all data were presented as the mean ± standard error of the mean.

Experiment II

Animals and semen collection

Twelve ejaculates from 12 healthy dogs were collected from beagle dogs, by digital manipulation. The ejaculates immediately being evaluated for volume, concentration, viability, morphology and motility. Sperm motility were assessed subjectively under light microscope with warm stage at 38°C. Sperm concentration was assessed with hemocytometer. Only ejaculates with minimum 70% total motility were included in the experiment.

Semen freezing and extender composition

Three ejaculates then pooled together and divided into three aliquots. Samples were centrifuged (700g for 10 min), and supernatants were discarded. Dilution steps by 2 steps-dilution methods with tris-egg yolk extender based (Table 1). First diluted sperm then cooled in 5°C for 1 hour then second dilution was carried out. Cool-diluted sperm then loaded into 0.5 mL straw, evaporated 4 cm above liquid nitrogen for 10 mins then plugged into liquid nitrogen.

In this study, we labeled 3 extenders as E1, extender that contain AFP type III with 0.1 µg/mL as a final concentration. This final concentration was chosen based on Experiment I and as comparation with another two extenders; E2, extender that contain both AFP type III with 0.1 µg/mL as final concentration and Equex STM Paste. This extender was to evaluate the combination of both substance when added into semen extender; E3, extender that contain Equex STM paste, this extender was used as positive control. The composition of three extender are shown in table 4.

Table 4. Extender compositions; E1 contains AFP 0.1 µg/mL, E2 contains AFP 0.1 µg/mL + Equex STM paste in extender, E3 contains Equex STM paste in extender.

Substance	Extender I	Extender II		
		E1	E2	E3
Tris (g)	3.025	3.025	3.025	3.025
Citric acid (g)	1.7	1.7	1.7	1.7
Fructose (g)	1.25	1.25	1.25	1.25
Benzyl penicillin (g)	0.06	0.06	0.06	0.06
Streptomycin sulphate (g)	0.1	0.1	0.1	0.1
Egg yolk (Ozkavukcu et al.)	20	20	20	20
Glycerol (ml) (Ozkavukcu et al.)	3	7	7	7
AFPs III (g)	-	0.00002	0.00002	0
Equex STM paste (ml)	-	-	1	1
Distilled water	up to 100mL	up to 100mL	up to 100mL	up to 100mL
pH	6.8	6.8	6.8	6.8
Osmolarity (mOsm/kg)	971	1789	1798	1791

Semen evaluation

Evaluation was done one weeks after freezing. Thawing was done by plugging freeze strawed sperm into water with temperature 37°C for 60 seconds. Thawed sperm samples then diluted with thawing media (1-fold dilution). Diluted sperm were incubated in 37°C for 5 min and sperm assessments were performed immediately at 0, 1 and 2 h.

Sperm motility

Sperm motility was done by Sperm Class Analyzer® (SCA Microptic SL, Barcelona, Spain). The 7µL diluted sperm was dropped upon pre-warmed slide glass the covered with prewarmed deck-glass. Sperm was observed with 100x magnificent using negative phase contrast lenses. Total 1000 spermatozoa from 5 different fields was captured. Progressive motility, curvilinear velocity (CLV), Straight line velocity (VSL) and average path velocity (VAP) were recorded.

Plasma membrane integrity

Plasma membrane integrity (PMI) was assessed by using dual station fluorescent technique SYBR and EthD-1. Ten microliter diluted sperm was mixed with 2.7 µl SYBR-14 (concentration 0.01 mM) and 10 µl working EthD-1 (4.65 µM). Stained sperm then incubated in 37°C for 15 mins. Two hundred sperm were counted under fluorescent microscope. The fluorescent labelled sperm were examined using a fluorescent microscope with 400x magnificent. The red colored sperm head indicates the leakage or disruption of sperm plasma membrane (dead sperm), while green colored sperm indicted the presence of metabolism (alive sperm) (Strzezek et al., 2015)

Acrosome integrity

Acrosome integrity (FITC-PNA and PI) were evaluated by double fluorescent staining's. Ten microlite diluted sperm were spread into slide glass. Mixture of FITC-PNA and PI then poured covering dried diluted sperm upon the slide glass. Sample then incubated in dark cooled chamber 4°C for 30 mins and washed by cooled distilled water. Samples were dried in room temperature and 200 sperm was evaluated under fluorescent microscope 1000x magnification. The sperm were classified as intact acrosome, reacted and completely loss acrosome. The sperm with

intact acrosome were presented as bright green color over an acrosomal region. The sperm with patchy pattern of the acrosome indicate the damage of acrosomal membrane, while the sperm only red colored nucleus (without FITC PNA staining) indicate the complete loss of acrosome (Ponglowhapan and Chatdarong, 2008)

Plasma membrane functional integrity

Plasma membrane functional integrity (PMFI) was evaluated by hypo osmotic swelling test (HOST). Ten microlites diluted sperm were mixed with 100 µl HOS solution 100 mOsm and incubated for 30 mins. The 50 µl of formalin solution was added to stop the working solution. Two hundred sperm in total were recorded and showed as percentage. Sperm with swollen or coiled tails indicate the functions of sperm plasma membrane to response to the hypoosmotic condition (Dobranić et al., 2005)

Statistical analysis

Statistical Analyses System (SAS Version 9.0, SAS Institutes, Cary, NC, U.S.A.) were used in this study and General linear model (GLM) repeated measurement was chosen. P value less than 0.05 indicated significant different. Total Motility, progressive motility, viability, acrosome integrity, mitochondrial membrane potential and HOST are represented as percentage ±SEM and velocity parameter represented as µm/s ±SEM.

CHAPTER IV
RESULTS

Experiment I

Overall, regardless of time post thawing, addition of AFP type III at a concentration of 1µg/ml (P3) significantly deteriorated total motility, progressive motility and sperm velocity compared to control (P0), 0.01µg/ml AFP (P1) or 0.1µg/ml AFP (P2) extenders (Table 5). Semen extenders supplemented with 0.01 or 0.1µg/ml AFP yielded no significantly better quality of frozen-thawed dog sperm than those of control group (P0) (Table 5). No significant differences between P1 and P2 were observed in all parameters.

Table 5post-thawed pooled data regardless of the time of total, progressive motility (PM), plasma membrane integrity (PMI), acrosome integrity (AI), mitochondrial membrane potential (MMP), hypo-osmotic solution test (HOST), curvilinear velocity (VCL), straight line velocity (VSL) and average path velocity (VAP).

	TM	PM	PMI	AI	MMP	HOST	VCL	VSL	VAP
P0	40.1±4.5^{ab}	21.9±2.8^{a}	63.8±4.7^{a}	54.2±4.4^{a}	75.4±7.0^{a}	26.6±3.3^{a}	115.6±8.8^{a}	35.6±3.4^{a}	60.1±4.7^{a}
P1	37.2±4.5^{ab}	18.5±2.8^{ab}	69.0±4.7^{a}	48.6±4.4^{a}	76.2±7.0^{a}	23.5±3.3^{a}	109.2±8.8^{a}	32.0±3.4^{a}	54.9±4.7^{a}
P2	46.1±4.5^{a}	25.4±2.8^{a}	61.9±4.7^{a}	55.6±4.4^{a}	72.7±7.0^{a}	25.8±3.3^{a}	115.4±8.8^{a}	32.8±3.4^{a}	58.6±4.7^{a}
P3	29.9±4.5^{b}	13.9±2.8^{b}	67.8±4.7^{a}	48.8±4.4^{a}	78.1±7.0^{a}	20.5±3.3^{a}	80.8±8.8^{b}	20.1±3.4^{a}	38.8±4.7^{a}

Different superscripts (a and b) indicate significance among evaluations within the column.

All post-thawed motility and velocity parameters are shown in Table 6. Immediately post-thawing, there are no significant differences among 3 treatments and control groups (P>0.05) except for the progressive motility. The progressive motility was significantly lower in P3 (P>0.05). In general, all parameters decreased after 1h incubation at 37°C (P>0.05) (Table 6). However, post-thawed sperm velocity, i.e. VCL, VSL and VAP, did not significantly differ between 0h and 1h in the extender contained 0.1µg/ml AFP (Table 6).

Table 6. Mean±SEM of total motility (TM%), progressive motility (PM%), VCL, VSL and VAP at 0h and 1h post-thawing.

Time	Treatment	TM	PM	VCL	VSL	VAP
0h	P0	$55.5\pm6.4^{a,A}$	$33.4\pm4.0^{a,A}$	$134.8\pm12.5^{a,A}$	$44.5\pm4.8^{a,A}$	$71.3\pm6.6^{a,A}$
	P1	$55.5\pm6.4^{a,A}$	$30.6\pm4.0^{ab,A}$	$129.1\pm12.5^{a,A}$	$41.9\pm4.8^{a,A}$	$68.2\pm6.6^{a,A}$
	P2	$62.4\pm6.4^{a,A}$	$37.8\pm4.0^{a,A}$	$129.1\pm12.5^{a,A}$	$38.7\pm4.8^{a,A}$	$66.3\pm6.6^{a,A}$
	P3	$43.8\pm6.4^{a,A}$	$21.5\pm4.0^{b,A}$	$112.8\pm12.5^{a,A}$	$28.5\pm4.8^{a,A}$	$54.9\pm6.6^{a,A}$
1h	P0	$24.6\pm6.4^{a,B}$	$10.5\pm4.0^{a,B}$	$96.4\pm12.5^{a,B}$	$26.7\pm4.8^{a,B}$	$48.9\pm6.6^{a,B}$
	P1	$19.07\pm6.4^{a,B}$	$6.5\pm4.0^{a,B}$	$89.4+12.5^{a,B}$	$22.2\pm4.8^{a,B}$	$41.8\pm6.6^{a,B}$
	P2	$29.9\pm6.4^{a,B}$	$12.9\pm4.0^{a,B}$	$101.8\pm12.5^{a,A}$	$26.9\pm4.8^{a,A}$	$51.0\pm6.6^{a,A}$
	P3	$16.0\pm6.4^{a,B}$	$6.3\pm4.0^{a,B}$	$48.8\pm12.5^{a,B}$	$11.7\pm4.8^{a,B}$	$22.6\pm6.6^{a,B}$

Within a column, different superscripts (a, b) show significant differences ($P<0.05$) among extenders (P0; no AFP added (control), P1; 0.01 µg/mL, P2; 0.1 µg/mL, P3; 1 µg/mL (w/v)) within each time point post-thawing (0h or 1h) and superscripts (A,B) show significant differences ($P<0.05$) between times post-thawing (0h and 1h) in each tested extenders (P0, P1, P2 or P3).

Sperm viability (PMI), acrosome integrity, functional plasma membrane integrity (HOST) and mitochrondrial membrane potential (MMP) showed no significant differences in all treated groups (Table 7). As examples, the results of flowcytometry scatter plots are shown in Figures 1-3.

Table 7. Percentage (mean±SEM) of PMI, intact acrosome, HOST and MMP at 0h and 1h post-thawing.

Time	Treatment	PMI	Acrosome	HOST	MMP
0h	P0	$75.5\pm6.6^{a,A}$	$57.5\pm6.3^{a,A}$	$33.7\pm4.7^{a,A}$	$88.7\pm9.9^{a,A}$
	P1	$79.5\pm6.6^{a,A}$	$56.4\pm6.3^{a,A}$	$30.7\pm4.7^{a,A}$	$77.7\pm9.9^{a,A}$
	P2	$75.0\pm6.6^{a,A}$	$61.4\pm6.3^{a,A}$	$32.5\pm4.7^{a,A}$	$79.38\pm9.9^{a,A}$
	P3	$72.6\pm6.6^{a,A}$	$58.7\pm6.3^{a,A}$	$26.0\pm4.7^{a,A}$	$87.1\pm9.9^{a,A}$
1h	P0	$52.2\pm6.6^{a,B}$	$50.9\pm6.3^{a,B}$	$19.5\pm4.7^{a,B}$	$62.1\pm9.9^{a,A}$
	P1	$58.5\pm6.6^{a,B}$	$40.8\pm6.3^{a,B}$	$16.2\pm4.7^{a,A}$	$74.6\pm9.9^{a,A}$
	P2	$48.7\pm6.6^{a,B}$	$49.8\pm6.3^{a,B}$	$19.2\pm4.7^{a,AB}$	$58.6\pm9.9^{a,A}$
	P3	$63.0\pm6.6^{a,B}$	$39.0\pm6.3^{a,B}$	$15.0\pm4.7^{a,B}$	$69.0\pm9.9^{a,A}$

Within a column, different superscripts (a, b) show significant differences ($P<0.05$) among extenders (P0-P4) within each time point post-thawing (0h or 1h) and superscripts (A, B) show significant differences ($P<0.05$) between times post-thawing (0h and 1h) in each tested extenders (P0, P1, P2 or P3).

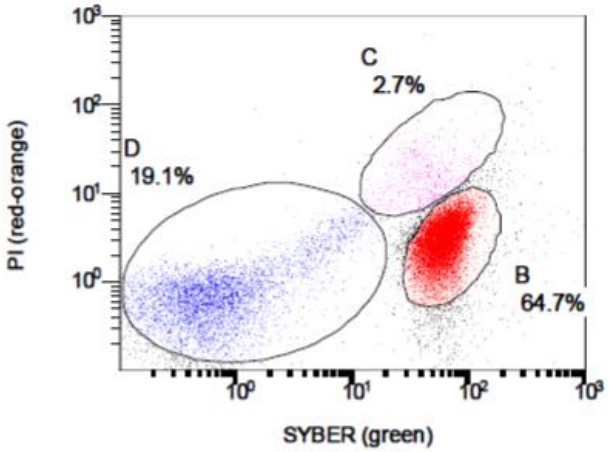


Figure 1. Sperm viability test using SBYR/PI fluorescent staining. A cell population in B gate are cells that emit green fluorescent colour; cells are intact and sybr can penetrate inside the cell. A cell population in gate C are the cell emitting green and red colour (SYBR and PI positive), indicating that cells are in the process of dying. A cell population in D gate are the cells that not stained with sybr; cells are not viable and cannot transport sybr into the cell.

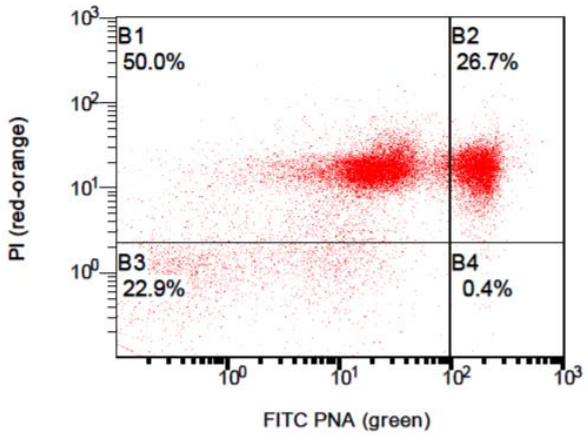


Figure 2. Plasma membrane integrity and acrosome integrity after freezing-thawing in P1 immediately post thawing. Cells in B1 are stained with PI (PI+/PNA-), these cells are damaged-plasma membrane sperm cells (non-viable cell) with intact acrosome. B2 represents damaged-plasma membrane integrity (non-viable cell) with damaged acrosome integrity; positive in dual staining (PI+/PNA+). B3 represents live sperm with intact acrosome integrity (PI-/PNA-) and B4 represents intact plasma membrane integrity (viable cells) with damaged acrosome integrity (PI-/PNA+).

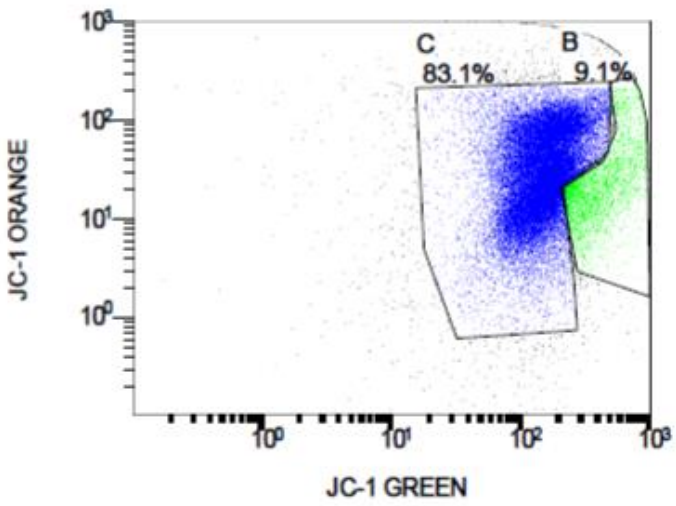


Figure 3. Mitochondrial Membrane Potential (MMP) was evaluated by JC-1 staining. Dots in scatter plots show cell with high MMP (C gate) and low MMP (B gate).

Experiment II

Detergent-contained in Equex STM paste increased post-thawed sperm quality in all parameters (P<0.05). A combination of AFP and Equex STM paste provided a beneficial effect on plasma membrane integrity (PMI) immediately after thawing. The results were shown in Table 8.

When data were pooled across observation times, regardless of time post-thaw, semen extenders containing Equex STM paste (E2, E3) showed better (P<0.0001) sperm quality compared to E1 in all parameters except for acrosome integrity (Table 8). Semen extender containing both Equex STM paste and AFP (E2) significantly improved sperm viability compared to semen extender containing Equex alone (E3) when data were pooled across the times (P=0.02) or immediately after thawing (P=0.02).

Table 8. Mean±SEM of total motility (TM%), PMI, HOST, acrosome integrity and sperm velocity (VCL, VSL and VAP) in different extenders (E1, E2 and E3) at 0h, 1h, 2h and 3h.

	Time	TM	PMI	HOST	Acrosome	VCL	VSL	VAP
E1	0 h	$24.3\pm5.0^{a,A}$	$28.2\pm4.5^{a,A}$	$34.2\pm5.1^{a,A}$	$62.5\pm3.6^{a,A}$	$110.8\pm8.9^{a,A}$	$33.2\pm5.5^{a,A}$	$55.6\pm6.1^{a,A}$
	1 h	$10.3\pm3.5^{b,A}$	$16.5\pm3.6^{b,A}$	$26.1\pm5.3^{ab,A}$	$58.2\pm6.8^{ab,A}$	$63.5\pm20.5^{b,A}$	$13.4\pm4.4^{b,A}$	$28.4\pm9.3^{b,A}$
	2 h	$1.9\pm1.2^{b,A}$	$8.5\pm1.5^{b,A}$	$17.7\pm1.1^{b,A}$	$45.1\pm7.3^{ab,A}$	$25.3\pm16.3^{bc,A}$	$5.1\pm3.3^{bc,A}$	$11.2\pm7.2^{bc,A}$
	3 h	$0.0\pm0.0^{b,A}$	$7.7\pm1.4^{b,A}$	$17.6\pm4.8^{b,A}$	$40.7\pm8.0^{b,A}$	$0.0\pm0.0^{c,A}$	$0.0\pm0.0^{c,A}$	$0.0\pm0.0^{c,A}$
E2	0 h	$54.6\pm4.1^{a,B}$	$61.7\pm4.4^{a,B}$	$57.1\pm0.8^{a,B}$	$77.6\pm3.4^{a,A}$	$156.5\pm5.4^{a,B}$	$57.7\pm3.7^{a,B}$	$87.8\pm4.25^{a,B}$
	1 h	$35.1\pm4.2^{b,B}$	$44.6\pm4.2^{b,B}$	$43.7\pm1.0^{b,B}$	$62.4\pm7.4^{ab,A}$	$123.2\pm6.1^{ab,B}$	$37.8\pm2.5^{b,B}$	$64.9\pm3.2^{b,B}$
	2 h	$23.3\pm6.4^{bc,B}$	$37.4\pm4.2^{bc,B}$	$42.8\pm4.1^{b,B}$	$54.4\pm9.1^{b,A}$	$98.9\pm20.7^{b,B}$	$29.1\pm6.0^{bc,B}$	$50.7\pm10.5^{b,B}$
	3 h	$19.8\pm4.5^{c,B}$	$31.0\pm3^{c,B}$	$34.3\pm3.2^{b,B}$	$48.7\pm6.0^{b,A}$	$91.4\pm19.7^{b,B}$	$23.6\pm5.6^{c,B}$	$44.6\pm9.8^{b,B}$
E3	0 h	$53.3\pm2.9^{a,B}$	$50.5\pm3.3^{a,C}$	$57.2\pm5.4^{a,B}$	$74.7\pm1.7^{a,A}$	$154.9\pm3.4^{a,B}$	$58.3\pm0.9^{a,B}$	$87.5\pm2.0^{a,B}$
	1 h	$28.0\pm3.5^{b,B}$	$35.7\pm4.5^{b,B}$	$43.6\pm5.0^{b,B}$	$60.7\pm5.4^{ab,A}$	$119.4\pm7.6^{ab,B}$	$37.4\pm3.7^{b,B}$	$62.1\pm4.7^{b,B}$
	2 h	$25.0\pm6.3^{b,B}$	$34.2\pm4.7^{b,B}$	$30.4\pm1.7^{c,C}$	$52.83\pm7.0^{b,A}$	$102.9\pm21.7^{b,B}$	$32.3\pm6.9^{bc,B}$	$54.2\pm11.4^{b,B}$
	3 h	$18.1\pm4.1^{b,B}$	$31.9\pm3.0^{b,B}$	$33.7\pm5.1^{cb,B}$	$45.8\pm6.0^{b,A}$	$87.7\pm18.0^{b,B}$	$22.6\pm4.7^{c,B}$	$42.8.2\pm8.8^{b,B}$

Different superscripts (a, b, c, d) between times in same extender and superscript (A, B, C) between semen extenders in the same time point indicate significant differences (P<0.05).

CHAPTER V
DISCUSSION AND CONCLUSION

Experiment I

Cryopreservation is multi-disciplinary science methods to prolong cell survival in extremely low temperature to slowing down the biological metabolism of the cell. Despite from the advantages of cryopreservation such as; allow to banking massive number of the cells and keeping biodiversity enrichment, but this method always exposes the cell from cryoinjuries suffer in the processes. Some substances need to be included into extender prior preservation, one of substance that attracts many scientists interest is AFP. Antifreeze proteins can inhibit normal ice crystal formation. When this protein added into semen extender, some protein molecule reminds outside the cell (Buzzini and Margesin, 2013) and some interact with the cell membrane plasma (Prathalingam et al., 2006).

Extracellular protein molecules are responsible on extracellular ice crystal formation in semen extender. A study conducted by Nishimiya showed that different concentrations of AFP affect on the shape ice crystal formation. It shows that AFP is concentration-dependent substance when added into freezing semen extenders (Nishimiya et al., 2003). Previous studies prove that the combination of proper AFP concentration, freezing methods and extender formulation give protection on Chimpanzee sperm. Higher or lower concentration will give negative effects on post-thawed sperm quality. In buffalo, only extender with a final concentration of 0.1 µg/ml AFP give beneficial effects on post-thawed sperm motility (Younis et al., 1998; Prathalingam et al., 2006; Qadeer et al., 2014). In our study, adding AFP as an extracellular CPA in modified Uppsala extender (Egg Yolk-Glycerol extender) with final concentration 0.01, 0.1 or 1 µg/ml did not improve post-thawed sperm quality. These results corresponded with a study that conducted in mouse sperm, adding AFP type III with final concentrations of 0.01, 0.1, 1, 10 or 100 µg/ml in mouse sperm extender tend to decrease sperm survival post-thawing (Koshimoto and Mazur, 2002). It is believed that AFP will change the ice crystal structure depending on concentration.

We suggested that negative effect as also shown in our study is caused by those changes resulting on mechanical stress on sperm. Moreover, some factors such as freezing method and thawing rate are also essential on sperm survival (Koshimoto and Mazur, 2002). In our study, 2-step freezing and thawing techniques have been published and successfully used (Peña and Linde-Forsberg, 2000; Ponglowhapan and Chatdarong, 2008).

Cells will be exposed in many forms of stress along the freezing/thawing periods, including cold shock, osmotic stress, heat stress and destabilizing the membrane (Meyers, 2005; Oldenhof et al., 2013). Antifreeze protein Type III tends to interact with polyunsaturated fatty acid both in head of plasma membrane and flagellar membrane of teleost sperm (Fletcher et al., 2001) when interaction only occur with head of plasma membrane of bovine sperm (Prathalingam et al., 2006) Proteins that interacted with membrane plasma of the cell will stabilizing the membrane, resulting in increasing the survival rate in teleost (Fletcher et al., 2001; Prathalingam et al., 2006). In adverse, using 0.01, 0.1, 1 µg/ml of AFP type III gave no beneficial effects on canine sperm plasma membrane integrity, acrosome integrity, sperm plasma membrane functional integrity and mitochondrial membrane potential. The similar results are obtained by adding AFP type III in buffalo and bull semen extender (Prathalingam et al., 2006; Qadeer et al., 2014). Antifreeze protein does not give any beneficial on maintaining sperm viability and acrosome integrity after exposed by AFP type III. Composition of CPA in the extender can affect the freezing behaviour of the specific cell, which effect on the rate of transmembrane water transport, ice nucleation and ice crystal growth. By the mean, differences in composition of extender have to be followed by specific optimum freezing protocols (Le et al., 2019; Pabon et al., 2019; Varkonyi et al., 2019). The adverse result in our study can be related with those factors, which are related with the ice crystal formation in freezing or thawing processes.

Sperm motility is complex processes in viable sperm that require continuous work between organelles in intact plasma membrane. Induced and non-induced stimuli generate complex mechanism and activate flagellar movement (de Ziegler et al., 1987), it expels sperm with various movement patterns (Yun et al., 2013). The patterns and characteristic movement are corelated with its fertile-ability and the chance of conception. A study conducted in human shows that there is based line on VCL value to be able to reach successful fertilization and data from prospective follow up study show that pregnancies are obtained from sperm with high VSL compared with sperm showing low VSL (Shibahara et al., 2004). Along with total motility, movement pattern such as VAP have to be concerned to optimize successful assisted reproduction (Shibahara et al., 2004; Fréour et al., 2010). In our study, adding AFP prior to preservation did not give positive effects on sperm movement and patterns compared to each sample (VCL, VSL and VAP). But, sperm velocity parameter in extender contain 0.1 µg/ml AFP can maintain its velocity after 1h of incubation. It seems like adding 0.1 µg/ml AFP into semen extender is beneficial in maintaining durability of sperm velocity (VCL, VSL, VAP). Moreover, negative effects showed in extender that contain 1 µg/ml of AFP; sperm progressive motility was lower in this extender compared with the control. The osmolality of extender P3 (1 µg/ml of AFP) was slightly higher compared with other extenders but it should not cause low sperm motility and velocity of spermatozoa frozen in P3 post-thawing. Studies showed that adding AFP into semen extender with a final concentration of 0.1 µg/ml increase buffalo post-thawed sperm motility, while 1 µg/ml concentration is the optimal concentration of the rabbit sperm extender (Nishijima et al., 2014; Qadeer et al., 2014). By adding several concentrations of AFP type III into dog semen extender, we would therefore find the optimal concentration to be added into dog semen extender and have similar positive result with previous study. Instead, the different result might be caused by different of phospholipid

structure of each species. is one of the reasons and this rift need to be studied furthermore.

Sperm face extreme conditions changed since ejaculated until being frozen. Osmotic, temperature, gravity in centrifugation process can damage the cell, resulting in low quality of the sperm including its plasma membrane functional integrity (Ramu and Jeyendran, 2013). Intact plasma membrane is needed to repeal its self to move forward. It plays an integral role on successful fertilization. To determine its function, low osmolarity medium is commonly used (Zubair et al., 2015). This method is easy, practical and time efficient, although subjectivity of the examiner still involves. There are several osmolality options used, such as 75, 100 or 150 mOsm depended on species, purpose and the incubation time (Zubair et al., 2015). In our study, to understand the effect of AFP in protecting dog sperm and maintain the functional plasma membrane, we used 100 mOsm based on a previous study (Dobranić et al., 2005). Two hundred sperm was counted to identify functionally intact sperm or the opposite. Sperm with functionally intact membrane will maintain its equilibrium between cell and the environment. Low osmolarity in environment will force fluid goes inside the cell, resulting swelling and coiling the cell. The results obtained from our study showed that adding AFP prior to cryopreservation did not improve plasma functional integrity. It could be due to stress from the freezing-thawing result. Antifreeze protein did not give enough protection and due to ice crystal; cell membrane are rupture and resulting on cell are not be able to maintain its equilibrium

Massive energy sources are needed for sperm to generate expulsive movement. Mitochondria is believed as a powerhouse of the sperm energy, which provides energy to flagellar movement (Miki, 2007). Approximately 90% of energy provided by mitochondria are relied on extracellular substrates monosaccharides, lactic acid and amino acid (Van Hall, 2000; Ponglowhapan et al., 2004; Ariyoshi et al., 2017). This organelle is located on the midpiece of sperm and thought to be an

important parameter as sperm quality assessment due to its mitochondrial membrane potential (MMP) such as in the cat and boar sperm, which can easily be examined in-vitro study by using fluorescent staining (Lee et al., 2015c; Kunkitti et al., 2017). In our study, MMP was examined with single stain fluorescent staining with JC-1 and used flowcytometry as a device to count sperm based on its stain. There is no difference on sperm MMP between extenders tested and after incubation. The results showed that motility of sperm starts to drop after one-hour post-thawing but the MMP still well maintained after one-hour post-thawing. It is supported by the newest finding that there is no correlation between MMP and motility nor membrane integrity (Cheuquemán et al., 2012).

Sperm need several enzymatic reactions to penetrate zona pelucida of the oocyte. This penetration is important to successful fertilization. These enzymes are stored in head of the sperm especially in acrosome. Sperm acrosome is cap-like organelle containing some enzymes such as hyaluronic acid (Gadella, 2010). The time of acrosome reaction is crucial and can be a parameter of sperm quality assessment (Tello-Mora et al., 2018). In our study, a combination of FITC-PNA and PI was used. Fluorescent staining FITC-PNA will stain outer membrane of the acrosome, resulting in a relative low emission of light. This intact acrosome membrane will keep the enzymatic contains inside the acrosome and will release nearly fertilization by subsequent acrosome reaction; and it will emit high emission value when the acrosome is reacted. In the same time, PI was used to determine intact or non-intact plasma membrane integrity. This fluorescent staining only able to go inside the cell and will stain the sperm DNA, resulting in red emission when cells are non-intact. In the present study, adding AFP type III into dog semen extender had no beneficial on maintaining acrosome integrity of the dog sperm. There is no difference between extenders nor time incubation post-thawing. The result of this study demonstrated no differences between the treatments but after one hour of incubation, the percentage of sperm with intact acrosome in P3 dropped significantly. It suggested

that AFP does not give any beneficial on maintaining dog sperm acrosome integrity. A study conducted in buffalo shows that adding AFP into semen extenders give no protective effect on acrosome integrity (Qadeer et al., 2014). Correlated, adding AFP type I, type III and antifreeze glycoprotein also do not alter the number of acrosome integrity value (Prathalingam et al., 2006). It seems like some types of AFP do not give direct effects on protecting dog sperm acrosome integrity because it stays outside the cell or interact with plasma membrane integrity.

For more than decade, fluorescent staining has been used to evaluate sperm quality parameter such as plasma membrane integrity or even sperm organelle-like acrosome integrity (V. Rajaram, 2017). Conventionally, stained cells will be smeared onto the slide and counted one by one (Prathalingam et al., 2006). This method is laborious, time consuming and limited cell counted. Flowcytometry, however, can be used to avoid the subjectivity on sperm counting. This method allows massive number of cells counting, resulting in high accuracy with more time efficient. Several probes can work simultaneously in one sample in the flowcytometry protocol, allowing evaluation many sperm characteristics in the same time (Nagy et al., 2003). In this study, the total numbers of 30,000 sperm cells were counted in each sample, with a counting rate was two hundred until five hundred sperm per second. This number are significant compared to subjective counting; that mostly only 200-400 sperm cells are counted. Two different probes FITC-PNA/PI was use in the same time to evaluate the plasma membrane integrity and acrosome integrity. For mitochondrial membrane potential assessment, JC-I staining was used, and the same cell number was counted. Using dual probe for sperm cell plasma membrane integrity and acrosome integrity in the same time also has been conducted in other species and many other cells (Nagy et al., 2003). In our study, Flowcytometry was used in order to avoid subjectivity, time benefit, huge number of cells were counted to make the evaluation more precise and this method is more sensitive.

In freezing/thawing processes, sperm expose two opposite stress. In this study, sperm facing drastic temperature from 5°C after equilibration to temperature below 0°C when sperm are frozen 4 cm above liquid nitrogen for 10 mins. Opposite, thawing method that chosen in this study was 37 °C for 60 seconds. It means that sperm cell was warmed from -196°C to ambient temperature. Freezing and thawing rate are crucial steps on freeze-thaw sperm (Shah et al., 2016). Freezing process will freeze water inside and outside the cell. In the process between, water is transported outside the cell across plasma membrane during cooling. Reducing intracellular water is essential to avoid intracellular ice formation. The combination between solution effects and intracellular freezing are really important on sperm survival, collaborated with cooling and freezing processes (Mazur, 1963). In the end, sperm are in the environment surrounded by frozen material and unfrozen material. Making sperm cell vulnerable from cryodamage due to ice crystal (Mazur, 1963). In the other hand, thawing mechanism are likely the opposite. There are so many methods to freeze-thaw sperm for sperm cryopreservation when AFP III added into semen extender, resulting in diverse of the result (Appendix 1). The species and freeze-thawed method are crucial on sperm cryopreservation. Performing different freezing protocol or temperature of thawing might give better result on dog sperm cryopreservation when AFP presented. The optimum protocol and thawing temperature can not be explained in this study and further study need to be conducted.

Experiment II

Equex STM paste has long been included in freezing dog semen extenders because of its great benefits to post-thawed dog sperm survival (Peña and Linde-Forsberg, 2000). The main and active ingredient is sodium dodecyl sulphate (SDS). Sodium dodecyl sulphate usage as a CPA in sperm cryopreservation has been used widely in many species (Ponglowhapan and Chatdarong, 2008; Buranaamnuay et al., 2013). From our result, adding SDS in the extender yields higher post-thaw motility compared with non-SDS supplementation. Antifreeze protein can shape and inhibit ice crystal formation (Rahman et al., 2019) when sodium dodecyl sulphate works into cell membrane permeability (Ponglowhapan and Chatdarong, 2008), resulting in sperm membrane permeability resistant into cold shock. Sodium dodecyl sulphate theoretically can desaturate antifreeze protein by interference protein hydrogen bound and hydrophobic interaction (Bhuyan, 2010). Based on this theory, mixing these two kinds of CPA in the same extender can decrease its individual function as CPA even thought both extenders have its individual positive ability on protecting sperm from cold shock. In contrast, adding AFP and SDS in one extender did not react each other.

Sperm Plasma membrane integrity immediately after thawing in AFP and SDS contained extender (E2) were superior compared with sperm PMI in other extenders tested in our study. Cryopreservation leads ice crystal formation intra- and extra-cellular. Resulting in less viable sperm after this process. Adding AFP in extender can shape the ice crystal formation from sharp-shape ice crystal into round formation which is less harmful to the sperm (Prathalingam et al., 2006). This action of AFP was favour from binding its active side into ice crystal nuclei. Sodium dodecyl sulphate works by increasing sperm plasma membrane fluidity, thus increasing plasma membrane integrity providing a positive impact on sperm survival (Peña and Linde-

Forsberg, 2000). The combinations of both CPA were suggested to improve sperm PMI post-thawed immediately, compared with single CPA alone. Sperm PMI in all extenders significantly dropped after one hour of incubation. Decreasing PMI after incubation could be related with intracellular Ca^{2+} (Alhaider and Watson, 2009), limited energy sources, free-radical produced or other factor related (Gibb and Aitken, 2016).

The present of AFP type III and SDS in Equex STM paste did not have a significant effect on proportion of intact acrosome–in dog sperm. This result was in agreement with a study conducted by Prathalingam et al. (Prathalingam et al., 2006) that AFP type III did not have correlation with acrosome intact in bull following freeze-thawed process. In contrast, study conducted by Younis et al. (Younis et al., 1998) adding AFP type III into chimpanzee sperm extender can increase percentage of acrosome integrity. Antifreeze protein suggested stabilized chimpanzee sperm membrane, resulting in inhibiting induced-capacitation and subsequence acrosome reaction. Our results was along with a study that conducted in bull semen show that maybe there is interaction of AFP type III with sperm plasma membrane in both species. The different results from Younis et al. (Younis et al., 1998) need to be studied further to better understand the functional and interaction differences between dog and chimpanzee sperm toward AFP in the extender. Supplementations of SDS or a combination of SDS and AFP in semen extender were not improving acrosomal intact on dog sperm. This result was similar with study that conducted in alpaca sperm. Adding Equex STM paste as a CPA in alpaca epididymal sperm extender do not give beneficial effects on maintaining acrosome integrity (Morton et al., 2010), but give positive impact on maintaining acrosome integrity prior to freezing in cat epididymal sperm (Axner et al., 2004), dog epididymal sperm (Ponglowhapan and Chatdarong, 2008) and dog ejaculate sperm (Peña and Linde-Forsberg, 2000).

Source of sperm (ejaculates or epididymal) and differences on species lead different results when those exposed in the same extender, which can be caused by different structure and seminal plasma exposure.

Advantage

The outcomes obtained from this study is even thought AFP type III alone does not improve dog sperm quality after thawing, but when combinded AFP type III with Equex STM paste, it can maintain quality of plasma membrane integrity on cryopreserved dog sperm.

Limitation

There were some limitations in this study. Firstly, we were not able to measure the ice crystal formation using cryomicroscope to find out in which stage and temperature were crucial on this protocol. Secondly, different freezing and thawing rates could be done to test the AFP extenders in dog sperm because there is an interaction between freezing/thawing rate and the formation of ice crystal.

Conclusion

In conclusion, the presence of AFP type III (0.1, 0.01 and 1 μg/ml) in Modified-Uppsala semen extender and the 2-step freezing method used in the study did not improve post-thawed sperm quality compared to the control. However, sperm velocity was significantly improved after 1h incubation with the presence of 0.1 μg/ml AFP. Furthermore, the higher concentration of AFP (1 μg/ml) significantly deteriorated sperm quality post-thawing. The beneficial effects of AFP in dog semen extender is suggested to be concentration-dependent. A combination of AFP type III and Equext STM paste significantly improved dog sperm viability as evaluated by plasma membrane integrity.

Appendix

Table 9. Antifreeze protein type III usage in sperm extender in different species, freezing protocol, thawing temperature and result.

Sample	Freezing protocol	Thawing rate	Result	Reference
Cyprinus caprio sperm	put extended sperm 3cm above liquid nitrogen for 15 mins	40 °C, 5s	Good protection in motility	(Shaliutina-Kolešová et al., 2019)
Buffalo sperm	Liquid nitrogen vapor, 10 mins	37 °C, 30s	Improve progressive motility and PMI	(Qadeer et al., 2014)
Rabbit sperm	3.5 cm Liquid N2 Vapor, 15 mins	37 °C, 30s	Increase rapid motile	(Nishijima et al., 2014)
Human sperm	Liquid nitrogen vapor, 10 mins	37 °C, 30s	Beneficial on progressive motility, PMI and DNA fragment index	(Zandiyeh et al., 2018)
Chimpanzees sperm	1.5-inch liquid N2 Vapor, 5 mins	37 °C for 60s	Beneficial on motility, PMI, acrosome integrity	(Younis et al., 1998)
Mouse sperm	23 °C/min (0.57 °C/s)	1875 °C/min, 126 °C/min, 11.5 °C/min	No beneficial, low rate thawing rate, low survival.	(Koshimoto and Mazur, 2002)

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