# ผลของการเสริมโคเลสเตอรอล ไซโคลเด็กตรินต่อคุณภาพอสุจิจากอิพิดิไดมิสม้าภายหลังการแช่แข็งและการทำละลาย

นาย ธีระพร ภมรศักดา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

# EFFECTS OF CHOLESTEROL-LOADED CYCLODEXTRIN ON QUALITY OF EQUINE EPIDIDYMAL SPERM AFTER FREEZING AND THAWING

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for the Degree of Master of Science Program in Theriogenology
Department of Obstetrics, Gynaecology and Reproduction
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| Field of Study | Theriogenology |
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ธีระพร ภมรศักดา : ผลของการเสริมโคเลสเตอรอล ไซโคลเด็กตรินต่อคุณภาพอสุจิจากอิพิดิไดมิสม้าภายหลังการแช่แข็งและการทำละลาย (EFFECTS OF CHOLESTEROL-LOADED CYCLODEXTRIN ON QUALITY OF EQUINE EPIDIDYMAL SPERM AFTER FREEZING AND THAWING) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.น.สพ.ดร.ธีรวัฒน์ ธาราศานิต 39 หน้า

การศึกษานี้มีวัตถุประสงค์เพื่อทราบปริมาณและการกระจายตัวของโคเลสเตอรอลภายหลังการเสริมโคเลสเตอรอล ไซโคลเด็กตรินให้กับเยื่อหุ้มอสุจิ และศึกษาเปรียบเทียบคุณภาพของอสุจิจากอิพิดิไดมีสม้าที่มีการเสริมโคเลสเตอรอล ไซโคเด็กตรินในระหว่างขั้นตอนต่างๆ ของการแช่แข็ง

การทดลองที่ 1 เก็บอสุจิจากอิพิดิไดมีสจากพ่อม้าพันธุ์ผสม อายุระหว่าง 3-12 ปี จำนวน 3 ตัว ทำการเสริมโคเลสเตอรอล ไซโคลเด็กตรินในน้ำเชื้อที่ปริมาณและระยะเวลาที่แตกต่างกัน ได้แก่ การเสริม 1.5 3.0 และ 6.0 มิลลิกรัมโคเลสเตอรอล ไซโคลเด็กตรินต่ออสุจิ 120 ล้านตัว ในช่วงเวลา 15 30 และ 45 นาที กลุ่มควบคุมไม่มีการเสริมโคเลสเตอรอล ไซโคลเด็กตริน พบว่าปริมาณโคเลสเตอรอลของเยื่อหุ้มอสุจิมีปริมาณเพิ่มขึ้นตามความเข้มข้นและระยะเวลาการเสริมโคเลสเตอรอล โคเลสเตอรอลไซโคลเด็กตริน 1.5 มิลลิกรัมให้อัตราการเคลื่อนที่และอสุจิมีชีวิตไม่แตกต่างกับกลุ่มควบคุม ส่วนการเสริมโคเลสเตอรอลไซโคลเด็กตรินในปริมาณสูงโดยเฉพาะขนาด 6.0 มิลลิกรัม ให้ผลลบต่อคุณภาพอสุจิ

การทดลองที่ 2 ทำการเก็บอสุจิจากอิพิดิไดมีสพ่อม้าพันธุ์ผสม อายุระหว่าง 3-12 ปี จำนวน 3 ตัว ทำการตรวจการกระจายตัวของโคเลสเตอรอลของเยื่อหุ้มอสุจิ ด้วยการยอมสีเรืองแสง(ฟิลิปิน) ภายหลังการบ่มอสุจิด้วยโคเลสเตอรอล ไซโคเด็กตรินขนาด 1.5 มิลลิกรัม นาน 15 นาที กลุ่มควบคุมไม่มีการเสริมโคเลสเตอรอล ไซโคลเด็กตริน พบว่าอสุจิกลุ่มควบคุม มีการกระจายตัวของโคเลสเตอรอลค่อนข้างน้อยที่ส่วนหัวของอสุจิ หลังการเสริมโคเลสเตอรอล ไซโคลเด็กตริน พบว่าร้อยละ 64.3±2.8 มีโคเลสเตอรอลการกระจายตัวหนาแน่นทั่วทั้งส่วนหัวอสุจิ และร้อยละ 27.7±3.2 มีการกระจายตัวของโคเลสเตอรอล น้อยหรือไม่มี อสุจิร้อยละ 8.0±4.6 มีการกระจายของโคเลสเตอรอลเหมือนกลุ่มควบคุม

การทดลองที่ 3 ทำการเก็บอสุจิจากอิพิดิไดมีสพ่อม้าพันธุ์ผสม อายุระหว่าง 3-12 ปี จำนวน 7 ตัว แบ่งอสุจิออกเป็น 4 กลุ่มเหมือนการทดลองที่ 1 นำอสุจิไปแช่แข็งและทำการตรวจคุณภาพตัวอสุจิภายหลังการแช่เย็นอสุจิที่อุณหภูมิ 4 อาศาเซลเซียส นาน 1 ชั่วโมง และภายหลังการแช่แข็งและการทำละลาย ที่ 10 นาที 2 ชั่วโมง และ 4 ชั่วโมง พบว่าการเสริมโคเลสเตอรอล ไซโคลเด็กตริน 1.5 มิลลิกรัมต่ออสุจิ 120 ล้านตัว ให้คุณภาพของอสุจิ ในระหว่างขั้นตอนต่างๆ ของการแช่แข็ง ดีกว่ากลุ่มควบคุมและกลุ่มที่มีการเสริมโคเลสเตอรอล ไซโคลเด็กตรินความเข้มข้นอื่นๆ อย่างมีนัยสำคัญทางสถิติ ($p<0.05$)

การศึกษานี้สรุปว่า การเสริมโคเลสเตอรอลไซโคลเด็กตรินในอสุจิด้วยปริมาณที่เหมาะสม ช่วยลดความไวรับต่อความเย็น และเพิ่มคุณภาพของอสุจิภายหลังการแช่แข็ง

ภาควิชา สูติศาสตร์ เธนุเวชศาสตร์ วิทยาการสืบพันธุ์ ลายมือชื่อนิสิต............................................

สาขาวิชา วิทยาการสืบพันธุ์สัตว์ ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.................

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KEYWORDS: SPERM/ CHOLESTEROL CYCLODEXTRINS/ STALLION/ CRYOPRESERVATION / EPIDIDYMIS

TEERAPORN PAMORNSAKDA: EFFECTS OF CHOLESTEROL-LOADED CYCLODEXTRINS ON QUALITY OF EQUINE EPIDIDYMAL SPERM AFTER FREEZING AND THAWING

THESIS ADVISOR: ASST. PROF. THEERAWAT THARASANIT, Ph.D., 39 pp.

The objectives of this study were to examine the effects of cholesterol-loaded cyclodextrins (CLCs) on amount and distribution of cholesterol in equine epididymal sperm (EXP.1, EXP.2), and to study the effect of CLCs on quality of epididymal sperm during cryopreservation.

EXP.1 Sperm from Thai crossbreed stallions (n=3, aged 3-12 years old) were loaded with three concentrations of CLCs: 1) 1.5 mg, 2) 3 mg, and 3) 6 mg of CLCs per $120x10^6$ sperm. The CLCs loaded sperm were then analyzed for the amount of total cholesterol in the sperm plasma membrane at 15, 30 and 45 min of CLC incubation using gas chromatography. Sperm incubated in extender without CLCs served as a control. The cholesterol contents in epididymal sperm were increased in a manner of dose and time dependence. Incubating sperm with 1.5 mg CLCs/120 x $10^6$ sperm preserved sperm quality similar to non-CLC treated control (P>0.05). However, high CLC concentration, especially at 6 mg/ 120 x $10^6$, significantly decreased sperm motility and viability in all incubation times.

EXP.2 Sperm from Thai crossbreed stallions (n=3, aged 3-12 years old) were treated with 1.5 mg CLCs/ $120x10^6$ sperm. After 15 min, the sperm were fixed and stained with filipin to examine the cholesterol distribution. Filipin-sterol complexes were distributed throughout the sperm head with a lower intensity at apical and pre-equatorial subdomains of non-CLC treated sperm. In CLC treated sperm, two patterns of filipin-sterol complexes were predominantly detected. A large proportion of CLCs treated sperm (64.3 ± 2.8%) had a marked increase of filipin-sterol complex intensity over the sperm head compared to non-CLC treated sperm. However, 27.7 ± 3.2% of sperm demonstrated an absent or patchy labeling of filipin at apical and pre-equatorial parts. Small number of sperm (8.0 ± 4.6%) had pattern of filipin-sterol complexes similar to that of non-treated control.

EXP.3 Epididymal sperm from Thai crossbreed stallions (n=7, aged 3-12 years old) were treated with CLCs as similar to EXP.1 and then submitted to cryopreservation. The sperm quality was examined at before cryopreservation, post-equilibration and 10 min, 2 h and 4 h after freezing and thawing. CLCs at 1.5 mg/120 x $10^6$ sperm significantly improved sperm quality during sperm equilibration and cryopreservation compared to CLCs at 3 and 6 mg/120 x $10^6$ sperm and non-CLC treated sperm.

It is concluded that cholesterol loading to the sperm plasma membrane by CLCs at optimal concentration decreases chilling sensitivity and also improves epididymal sperm cryopreservability.

Department: Obstetrics, Gynaecology and Reproduction Student's Signature ..........................

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

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AI | Artificial insemination |
| APES | Aminopropyltriethoxysilane |
| CLCs | Cholesterol-loaded cyclodextrins |
| CGTase | Cyclodextrin glucanotransferase |
| DAPI | 4′, 6-diamidino-2-phenylindole, dihydrochloride |
| DMF | Dimethylformamide |
| EthD-1 | Ethidium homodimer-1 |
| EY | Egg yolk |
| FITC-PNA | Fluorescien isothiocyanate-labeled peanut agglutinin |
| FT | Frozen-thawed |
| GC-FID | Gas Chromatograph Flame Ionized Detector |
| HMDS | Substance Hexamethyldisilane |
| $LN_2$ | Liquid nitrogen |
| kg | Kilogram |
| M | Molar |
| min | Minute |
| mL | Millititer |
| mM | Millimolar |
| NFDM | Non-fat dry milk extender |
| nm | Nanometer |
| PBS | Phosphate buffered saline |
| SEM | Standard error of the mean |
| sHost | Short hypo-osmotic swelling test |
| SV | Sperm viability |
| TCMS | Trimethylchlorosilane |
| TALP | Tyrode's albumin lactate pyruvate |
| TUNEL | Terminal deoxyribonucleotidyl transferase-mediated dUTP nick end-labeling |

CHAPTER I

INTRODUCTION

## Important and Rationale

Artificial insemination using ejaculated semen has become a major tool to accelerate genetic potential of desired stallion. This technique has been well integrated in the equine industry worldwide. Sperm collected by artificial vagina can be maintained their viability and fertilizabilty when the semen will only be extended with semen extender and slowly cooled to 5-8°C. However, the quality of stallion semen is gradually decreased over time of cold storage. It is therefore recommended to use the cooled semen within 2-3 days. Although stallion semen can be “long-term” cryopreserved, viability of frozen-thawed stallion semen markedly decreases and, as a result, pregnancy rate is generally poor or highly viable when compared to those obtained from fresh or cooled semen. Alternatively, mature sperm that are capable of fertilization can also be collected from epididymis of particular stallion by retrograde flushing of caudal epididymis after elective castration or unexpected death of the stallion. The number of stored epididymal sperm has been reported to be higher when compared to a single collection with artificial vagina (Garde, 1994; Murad´as et al., 2006). This is of importance because many top-performance stallions may need to be euthanized after sever injury during the competition, and one would therefore require storing his reproductive potentials by means of freezing the epididymal sperm for subsequent use.

Stallion sperm are fairly sensitive to cold stress as compared to other domestic species such as bull, dog and man. There is also a large variation of semen’s freezability among individual stallion. It has been estimated that only 20-40% of stallions will provide semen that well tolerate to cryopreservation (Amann and Pickett, 1987). Also, the pregnancy rate of epididymal sperm after artificial insemination is significantly lower than that of ejaculated sperm (Morris et al., 2002). This cold sensitivity of the sperm has become a major obstacle for the utilization of both cooled and frozen epididymal sperm in artificial insemination program. Modification and/or optimization of freezing procedures are thus required in order to improve cryopreservability and pregnancy rate after artificial insemination. To date, although the mechanism of cryoinjury that occurs during freezing and thawing is not entirely studied,

non-optimized freezing technique induces irreversibly damage to sperm plasma membrane, DNA integrity and also organelles (Aurich, 2005). When sperm are cooled below 18°C, the bilayer membrane phospholipids undergo a phase transition from a liquid to gel state that may render a transient loss of phospholipids from the plasma membrane resulting in a marked decrease of plasma membrane fluidity promptly for plasma membrane disruption (Amann and Pickett, 1987; Parks and Lynch, 1992; Crockett et al., 2001; Purdy and Graham, 2004). Cholesterol/phospholipid ratios of sperm plasma membrane thus play a central role in determining the cryopreservability of the sperm. The concentration of cholesterol in the sperm plasma membrane varies considerably among species. The cholesterol/phospholipid molar ratios are about 0.20, 0.36, 0.40, 0.43 and 0.83 in boar, stallion, bovine, ram and human sperm respectively (Parks et al., 1987; Parks and Hammerstedt, 1985; Parks and Lynch, 1992; Mack et al., 1986). The low cholesterol/phospholipid molar ratio in stallion sperm may contribute to the increased-cold sensitivity of stallion sperm because increasing cholesterol/phospholipid ratios reduces the transition temperature of the plasma membrane and thereby maintaining membrane fluidity.

Cholesterol can be incorporated into or extracted from the sperm plasma membrane using cyclodextrins, a cyclic heptasaccharides consisting of $\beta$ (1-4) glucopyranose units. Cholesterol- loaded cyclodextrins (CLCs) have been used prior to cryopreservation in order to increase cholesterol/phospholipid ratios of sperm plasma membrane. Interestingly, this technique has been demonstrated to improve cryosurvival of bull and stallion sperm, in terms of higher proportions of motile and membrane intact sperm compared to non-CLCs loaded sperm (Purdy and Graham, 2004; Moore et al, 2005). Owing that the epididymal sperm are less tolerant to cryopreservation, it is of great interest to study the effect of cholesterol-loaded cyclodextrins on epididymal sperm quality following cryopreservation. Until recently, this technique has not been studied for equine epididymal sperm.

Hypothesis

Cholesterol loading into sperm plasma membrane can improve the quality of frozen-thawed epididymal sperm.

**Keywords (Thai):** โคเลสตอรอล ไซโคลเด็กตริน การแช่แข็ง อิพิดิไดมิส อสุจิ พ่อม้า

**Keywords (English):** Cholesterol cyclodextrins, Cryopreservation, Epididymis, Sperm, Stallion

# CHAPTER II

# LITERATURE REVIEWS

Reproductive organs of stallion are composed of testes, reproductive ducts (epididymis, vas deferens, ejaculatory duct and urethra), accessory glands (seminal vesicles, prostate gland and bulbourethral glands) and other supporting structures (Figure 1). Spermatogenesis (consisting of spermatocytogenesis and spermiogenesis) takes place in lobular structure (seminiferous tubules) within testicular parenchyma after stallion has entered the puberty at approximately 18-24 months of age. This process continues throughout the adult life (Clay and Clay, 1992). Within testis, microenvironment conducted from several transduction pathways such as endocrine, paracrine and also autocrine precedes the production of testicular sperm. The spermatogenic cycle that occurs as a wave-like fashion requires approximately 57 days. The sperm are then transported from the seminiferous tubular lumen to the caput epididymis via the rete testis (Meyers, 2000; Figure 2). The mean daily sperm production of mature stallion is about 7-8 x $10^9$ sperm, and it takes 8-10 days for sperm to pass from the testis to the exterior (Johnson et al., 1997; Davies Morel, 1999, 2003)

Sperm structure can be broadly divided into 3 major parts (Figure 2): 1) the sperm head containing the DNA and acrosome, 2) the midpiece which contains the mitochondria and 3) the tail. Sperm plasma membrane is mainly composed of phospholipids and proteins that arrange as a bilayer. Following the spermatogenesis, sperm release from the seminiferous tubule and transport to the epididymis where they are stored and the final maturation of sperm takes place. Mature sperm finally will be expelled during ejaculation through the vas deferens and urethra.

Although the testicular sperm are mature according to the morphological description of fully developed sperm as described above, they are actually immotile and require further modifications in order to attain their fertilizabilty (Merkies and Buhr, 1998).

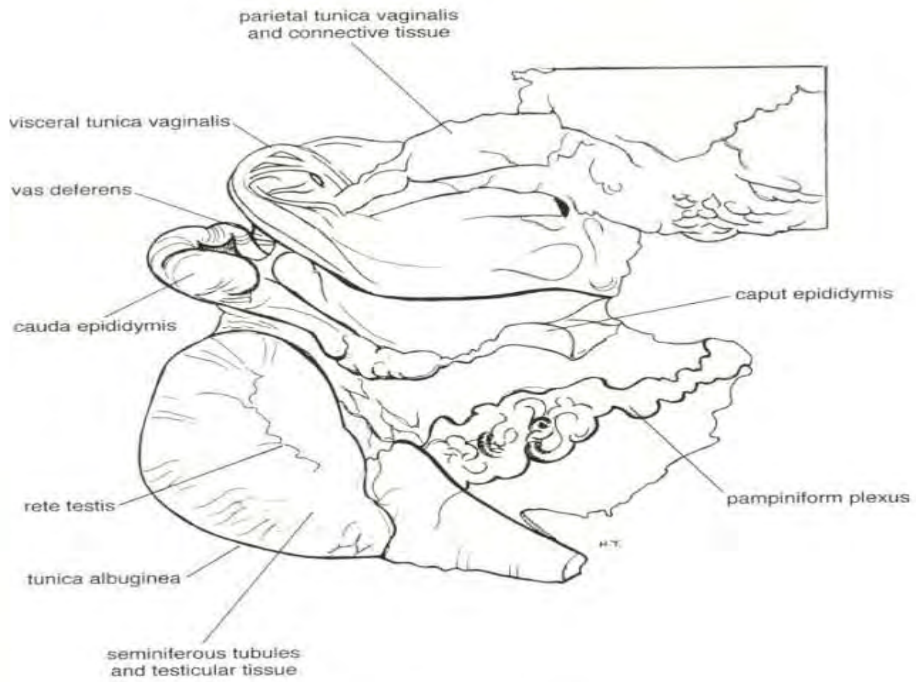


Figure 1: Anatomy of reproductive organs of stallion (Davies Morel, 2003)

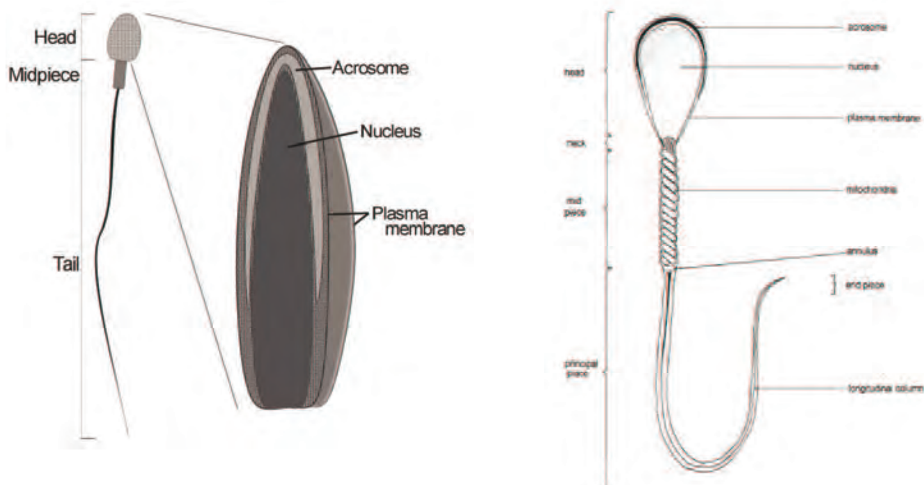


Figure 2: Structural details of stallion spermatozoon. (Davies Morel, 2003)

Structure and function of the epididymis

In the stallion, the epididymis has a total length of approximately 70 meters (Nickel et al., 1979) and can be divided into three segments including caput (head), corpus (body) and cauda (tail) (Figure 3). Within the epididymis, specialized cells in each tubular segment play an essential role for epididymal function responsible for transport, maturation and storage of the sperm along the epididymis (França et al., 2005). Regional specificity of epididymis is actively maintained by a combination of secretion and reabsorption of several molecules.

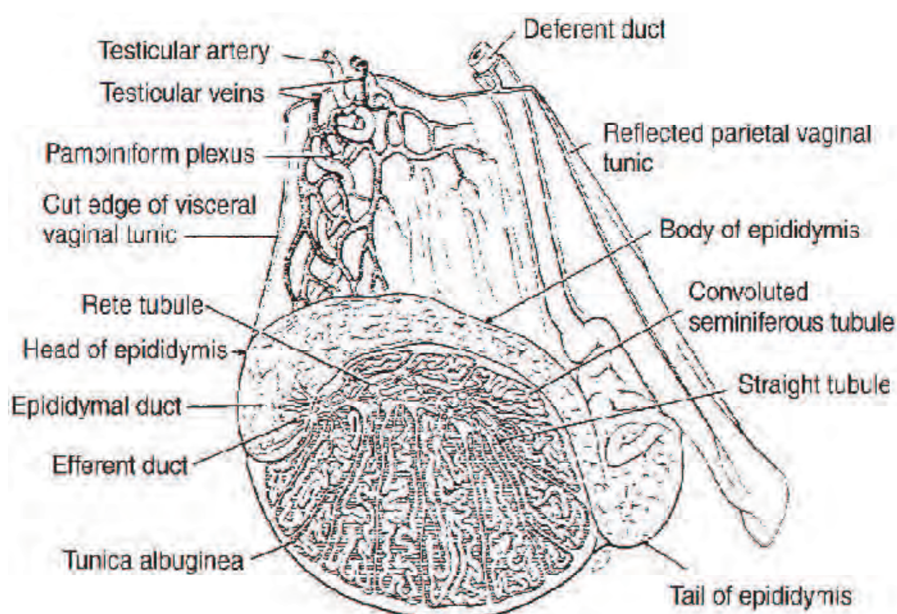


Figure 3: Overview structures of the testicle and epididymis (Meyers, 2000)

At the caput of epididymis, the rete fluid that facilitate the transportation of testicular sperm is absorbed and replaced by secretions from its epithelium essentially by an active transport of the apical membrane of the epididymal cells (Moore, 1995). Indeed, although these sperm attain their maturity as defined by the sperm structure, they are usually immotile and are not capable of fertilization. The alterations in the epididymal fluid cause changes in the surface of the sperm plasma membrane during the epididymal transit. Therefore, sperm that arrive and store in cauda epididymis are functionally mature (Flesch and Gadella, 2000). Sperm obtained from cauda epididymis have been reported to be fertilizable as pregnancy after insemination of mares with epididymal sperm has been reported (Barker and Gandier, 1957). This evidence

therefore becomes clear that sperm maturity occurs in this specific part of epididymis. It is anticipated that the transit of epididymal sperm takes place around 5 to 14 days in stallion (França et al., 2005).

Epididymis is known to actively express several steroid hormones receptors such as androgen (Bilinska et al., 2005) and estrogen (Parlevliet et al., 2006), and thus functions of epididymis is therefore regulated by steroid predominantly secreted by testicular parenchyma. Relative recently, bidirectional communication between luminal epithelium of epididymis and also sperm has been demonstrated (Reyes-Moreno et al., 2008).

During the transition of sperm through epididymal epithelium, plasma membrane of sperm is modified (Olson et al., 2003). Major modifications include the changes in the plasma membrane compositions of glycoproteins and sterols (Retamal et al., 2000). The mechanism that controls the transfer of epididymal proteins to the sperm plasma membrane is still unclear, small membranous vesicles, so called 'epididymosomes' have however been known to involve this. Epididymosomes are rich in cholesterol and have sphingomyelin as their major phospholipid (Rejraji et al., 2006). When epididymosomes cooperates to sperm plasma membrane, it results in marked increase of cholesterol composition (Jones, 1998). This has been believed to stabilize plasma membrane of the sperm in the cauda epididymis (Saether et al., 2003).

One of specific features of sperm morphology during the epididymal transit is the ability of the sperm to shed the cytoplasmic droplet containing all packed RNA and also organelles such as endoplasmic reticulum and Golgi apparatus. Sperm therefore present in quiescent stage within the epididymis, essentially because the compositions of the plasma membrane can not be newly synthesized (Flesch and Gadella, 2000). Compositions of sperm plasma membrane have been variable among species studied, but it is generally composed of approximately 70% of phospholipids, 25% neutral lipids (cholesterol) and 5% glycolipids (Mann and Lutwak-Mann, 1981). However, these compositions can be different among species such as human sperm has high amount of cholesterols compared to stallion and boar sperm.

Characteristics and fertilizing ability of stallion epididymal sperm

Epididymal sperm are different from ejaculated sperm by the fact that they are more difficult to undergo *in vitro* hyperactivation (capacitation) and acrosome reaction essentially required for normal fertilization, although these sperm are capable of fertilization *in vivo*. Epididymal transit has evolved the morphological and functional changes of sperm, making epididymal sperm differ from ejaculated sperm particularly at the levels of gene expression and plasma membrane composition. This difference may also render the membrane stability and ability to withstand the cold and osmotic stress that usually occurs during cryopreservation procedure (Hewitt et al., 2001).

In many mammals, sperm capacitation requires calcium and bicarbonate, which induce a reorganization of the membrane lipid architecture such as the cholesterol efflux from the plasma membrane (for review see: Flesch and Gadella, 2000). In horses, sperm from the caput and corpus epididymides are unable to undergo the acrosome reaction in response to acrosome reaction inducers (progesterone or calcium ionophore), while only a small proportion of sperm from cauda epididymis can become acrosome reacted sperm compared to ejaculated sperm (Rathi et al., 2003). This is likely to involve an inappropriate exposure of progesterone receptors (Cheng et al., 1998) or sperm plasma membrane needs an extensive plasma membrane modification just after ejaculation probably when exposes to seminal plasma or uterine fluid.

Pregnancy of mare inseminated with fresh epididymal sperm was firstly reported by Barker and Gandier (1957). Since then, studies on fertility of epididymal sperm has been limited (Morris et al., 2002 and 2004). When fresh epididymal sperm was used to inseminate mare via endoscopic- guided technique, the pregnancy rates of these epididymal sperm was lesser than that obtained from fresh ejaculated sperm (46% versus 80%) (Morris et al., 2002). These evidences suggest that the fertility of epididymal sperm may be lower when compared to ejaculated sperm. However, given that epididymal sperm may need more care during sperm handling, it is, up to date, not possible to draw a conclusion that epididymal sperm are far less fertilizability compared to the ejaculated sperm.

Cryopreservation of stallion sperm: fundamental and applied aspects

Stallion sperm are most susceptible to cold shock between 19°C and 8°C (Amann and Pickett, 1987; Parks and Lynch, 1992; Crockett et al., 2001; Purdy and Graham, 2004) where phase transition of liquid to a gel stage takes place. During cooling and cryopreservation, sperm undergo rapid changes in thermal and osmotic stresses that render sperm becoming injured at levels of plasma membrane, mitochondria and also DNA. Improper freezing protocol increases the incidence of sperm death (Aurich, 2005). When semen is cooled below their physical temperature, the intracellular water become supercooled (referred as temperature is lower than water melting/freezing point: 0 °C) without ice formation. Intracellular ice is typically formed somewhat lower than -15 °C. As a result, intracellular water gradually moves across plasma membrane, and, subsequently, concentration of intracellular solute rapidly increases (Figure 4). If cooling rate is too fast, a large number of intracellular ice crystals form, resulting in cellular injury and death (Amann and Pickett, 1987). By contrast, if the freezing rate is too slow, sperm will suffer from severe dehydration and concentrated intracellular solutes become toxic to the sperm. Taken together, sperm need an optimal freezing procedure in order to maintain sperm viability following cryopreservation and thawing. To decrease intracellular ice, permeating cryoprotectants such as glycerol must be added into freezing extender.

Several factors have been postulated to impair the membrane integrity, structure and function of the sperm such as lipid phase transition, intracellular ice formation and osmotic-induced excessive water influxes (Amann and Pickett, 1987; Samper et al., 1991). Differences in fatty acid compositions and sterol are associated with its tolerance to cold shock and freezing-thawing process. This can be varied among species and individual stallion (Loomis and Graham, 2008).

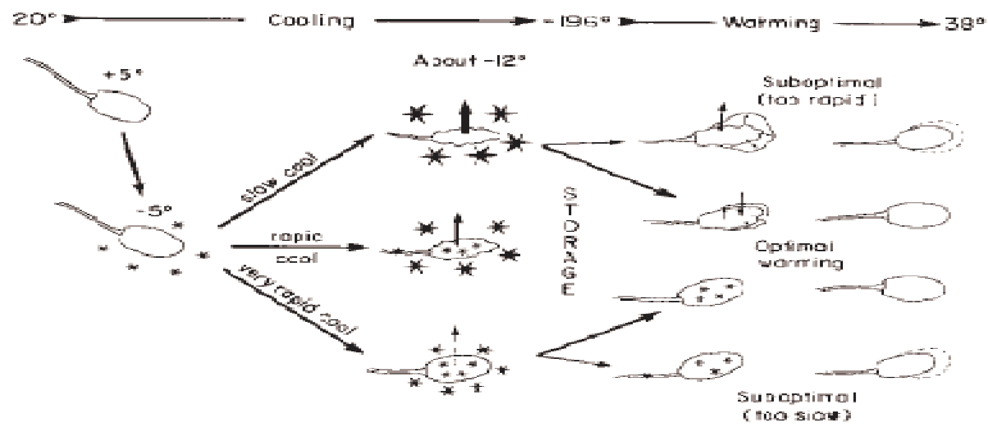


Figure 4: Diagram representing the interaction among physical changes in equine sperm and its viability during freezing and thawing (Amann and Pickett, 1987)

Pregnancy rates of mares bred with frozen–thawed semen are highly variable range 0-100% per-cycle (Samper and Morris, 1998). To date, current freezing procedures for stallion semen have been variable among the laboratory. However, it is well documented that frozen-thawed semen to be used for artificial insemination should have more than 30% progressive motility. Because a large variation of sperm quality after freezing and thawing, modification and standardization of freezing techniques are therefore required. For example, adding 5-20% seminal plasma back to the centrifuged semen prior to cryopreservation improved sperm quality post-thawing (Katila et al., 2002). Cryoprotectants also play essential role in maintaining sperm viability following cryopreservation. It is worth to note that most techniques to improve cryopreservability are relied on the additives that proceed against cold and osmotic shock. The membrane of stallion sperm contain low cholesterol to phospholipid ratios compared to those of human and bull, and high cholesterol contents in the sperm have been demonstrated to increase membrane fluidity, thereby improving sperm cryosurvival. Recently, sperm plasma membrane from a number of domestic species has been modified either adding or removal of cholesterols prior to cryopreservation. Cholesterol can be loaded into sperm plasma membrane using cholesterol loaded cyclodextrins in order to increase the cholesterol to phospholipid ratio.

Unlikely to ejaculated sperm, equine epididymal sperm are far more sensitive to freezing and thawing. Pregnancy rates after insemination of frozen-thawed epididymal semen has been poor with variable results: 17% (Barcker and Gandier, 1957), 24% (Morris et al., 2002).

It there for become clear that stallion sperm (both ejaculated and epididymal sperm) are sensitive to cold stress and many factors are contributed to the success of semen cryopreservation. It is essential to further investigate factors involving the cryopreservability of sperm and also to generate optimal procedures for freezing stallion semen.

## Beta-cyclodextrins

Cyclodextrins are cyclic oligosaccharides (Figure 5) consisting of six α-cyclodextrin, seven β-cyclodextrin, eight γ-cyclodextrin. β-cyclodextrin is the most accessible, the lowest-priced and generally the most useful (Martin Del Valle, 2004). The properties of cyclodextrins are given in Table 1. They are produced by an intramolecular transglycosylation reaction from degradation of starch by cyclodextrin glucanotransferase (CGTase) enzyme (Szetjli, 1998).

Table 1: property of cyclodextrins (Martin Dell Valle, 2004)

| Property | α-cyclodextrins | β-cyclodextrins | γ-cyclodextrins |
|---|---|---|---|
| Number of glucopyranose units | 6 | 7 | 8 |
| Molecular weight (g/mol) | 972 | 1135 | 1297 |
| Solubility in water at 25°C (%, w/v) | 14.5 | 1.85 | 23.2 |
| Outer diameter (Â) | 14.6 | 15.4 | 17.5 |
| Cavity diameter (Â) | 4.7-5.3 | 6.0-6.5 | 7.5-8.3 |
| Height of torus (Â) | 7.9 | 7.9 | 7.9 |
| Cavity volume ($Â^3$) | 174 | 262 | 427 |

The most important feature of cyclodextrin is the ability to form inclusion complexes with several compounds (Pitha et al., 1988; Hedges, 1998). Because they have hydrophilic property outside, which can be dissolved in water, and an apolar cavity, which provides a hydrophobic matrix, described as a 'micro heterogeneous environment'.

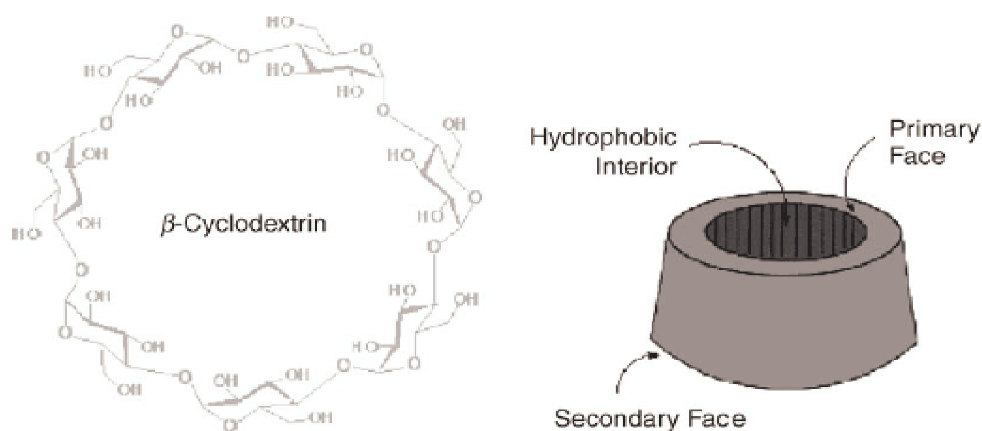


Figure 5: structure of beta-cyclodextrins (Martin Dell Valle, 2004)

As a result, cyclodextrins are capable of integrating to form inclusion complexes with a wide variety of hydrophobic guest molecules. One or two guest molecules can be entrapped by one, two or three cyclodextrins. Cytodextrin strongly interacts with cholesterol to form a complex resulting in cholesterol efflux and a disruption of lipid rafts on the plasma membrane (Yancey et al., 1996; Keller and Simons, 1998). These β-Cyclodextrins have been demonstrated to improve cryopreservability and also fertility of sperm in a wide range of species such as mouse (Takeo et al., 2008), Bull (Purdy and Graham, 2004) and stallion (Moore et al., 2005).

## Objectives

1. To study the effects of cholesterol-loaded cyclodextrins on amount and distribution of cholesterol in epididymal stallion sperm
2. To study the effect of cholesterol-loaded cyclodextrins on quality of epididymal sperm following freezing and thawing

## Expected output

1. To gain more knowledge regarding the response of stallion epididymal sperm during exposure to cholesterol-loaded cyclodextrins
2. To improve cryopreservability of frozen-thawed epididymal sperm in horse
3. To publish the results in an international journal

# CHAPTER III

# MATERIALS AND MEDTHODS

All chemicals used in this study were purchased from Sigma Aldrich, St Louis, USA, unless otherwise specified.

**Recovery of epididymal sperm**

Epididymal sperm were collected from the epididymides of Thai crossbreed stallions (aged 3-12 years old) following routine castration. The spermatic cord was ligated and the testicles/epididymides were then transported in an insulating box at 4-6 °C to the laboratory within 6 h. The cauda epididymis excluding the vas deferens was dissected from extraneous tissues. For sperm flushing, an indwelling intravenous catheter (22G) was gently inserted into the lumen of the convoluted tubule of the cauda epididymis. Retrograde flushing was performed at room temperature (25°C) using Modified Tyrode's medium (TALP, pH 7.4, 290 mOsm/kg). Epididymal sperm from two epididymides of each stallion were pooled and evaluated for motility, viability, acrosome integrity, morphology and plasma membrane function. Only epididymal sperm that had more than 50% progressive motility, viability and normal morphology were only used in the experiment.

**Preparation and loading of CLCs**

Cyclodextrins was prepared as described by Purdy and Graham (2004). Briefly, one gram of methyl- β-cyclodextrin (dissolved into methanol) was mixed with 200 mg of cholesterol (dissolved in chloroform). The remaining solvents were removed by nitrogen gas. A stock solution of 50 mg CLCs/ml in modified Tyrode's medium without bovine serum albumin, calcium and magnesium was incubated at 37 °C prior to use.

**Cryopreservation and thawing of epididymal sperm**

Following CLC loading, epididymal sperm were centrifuged at 400 g for 10 min at 25°C, and the sperm pellet was resuspended and washed with glucose-EDTA solution (Solution I: 333.0 mM, glucose monohydrate, 12.6 mM Tri-sodium citrate, 9.9 mM Di-sodium EDTA, 14.3 g sodium hydrogencarbonate, 100 IU/ml penicillin and 100 µg/ml streptomycin; pH 6.7 ± 0.1 and

osmolality 400 ± 10 mOsm./kg.). The freezing extender consisting of 50 ml of 11% (w/v) lactose, 26 ml of solution I, 20 ml of egg yolk, 0.5 ml Equex STM and 4 ml glycerol, pH 7.0 ± 0.1 and osmolality 300 ± 10 mOsm./kg.) was slowly added into the sperm and then equilibrated at 4 °C for 60 min before loading into 0.5 ml freezing straw (IMV Technologies, Avenue North Maple Grove, USA). Cryopreservation of epididymal sperm was performed using a controlled-rate freezing technique. In short, the straws containing sperm was placed vertically into a cryochamber of freezing apparatus (Cryologic system, Australia). The freezing curve was controlled by Cryogenesis freezing program (version 5, Australia). The initial temperature was set at 4 °C, at which the temperature was reduced to -20°C at a rate of -10 °C/min and then gradually reduced from -20°C down to -120 °C at a rate of -8 °C/min. The straws containing with sperm were finally plunged into liquid nitrogen. Thawing of frozen semen was carried out at 37 °C in water bath for 30 sec. Frozen-thawed sperm was then diluted (1:1) with non-fat dry milk extender (NFDM). Diluted sperm was centrifuged and fresh NFDM was subsequently added in order to remove cryoprotectant that may render cytotoxic effect on sperm viability during sperm incubation.

### Visualization of cholesterol in sperm plasma membrane

Distribution of cholesterol in sperm plasma membrane was visualized after staining epididymal sperm with filipin (Friend, 1982). Following sperm treatment with CLCs, sperm were fixed with 4% (w/v) paraformaldehyde for 60 min. After washing and incubation with 0.15% (w/v) glycine for 10 min to quench the paraformaldehyde, the sperm were washed and stained with filipin at a final concentration of 0.05 mg/ml in PBS (without $Ca^{2+}$ /$Mg^{2+}$) for 2 h at room temperature (approximately 25 °C). The fluorescently labeled sperm was examined under UV light excitation using an epifluorescent microscope (BX51, Olympus, Shinjuku, Japan) equipped with a UV filter (340-380 nm excitation filter).

### Determination of cholesterol contents

To determine cholesterol contents within epididymal sperm following CLC treatment, the sperm were first layered onto a 45% (v/v) Percoll gradient density and then centrifuged at 600 g for 25 min at 25 °C to remove any free cholesterol. The sperm pellet was then washed with phosphate buffered saline (pH 7.4) and then stored at -20 °C until cholesterol analysis.

When required, 60% (v/v) potassium hydroxide and 95% (v/v) ethanol were added into the sperm samples. The mixture was then mixed and heated to 95-100°C for 1 h. The lipid was extracted with hexane for four times (Lindenthal et al., 2001). The upper part of this solution was collected and then evaporated with a rotary evaporator (BÜCHI Labortechnik AG, Flawil, Switzerland) at 40 °C. After adding dimethylformamide (DMF), the samples were subsequently derivatized with hexamethyldisilane (HMDS) and trimethylchlorosilane (TCMS) and analyzed in duplicate with gas chromatograph flame ionized detector (GC-FID) using Hewlett-Packard GC system (HP Agilent 6890N, Foster City, CA, USA) as previously described (Ohshima, 2001).
The cholesterol was quantified against the peak areas of the internal standards (5α-cholestane solution).

**Assessment of sperm quality**

*Sperm morphology*

Head morphology was examined after William's staining using a light microscope at 1000x magnifications. For sperm tail morphology, 5-10 µl sperm diluted with a formal saline at 1:10 dilution ratios was placed on a grass microscopic slide and then examined under a phase contrast microscope (Olympus, Shinjuku, Japan) at 400x magnifications. A total of 200 sperm were examined for each morphological test.

*Progressive motility*

Sperm motility was examined at before freezing (post-equilibration) and 10 min, 2 and 4 hours after thawing (semen was maintained at room temperature about 25 °C). Progressive motility of sperm was subjectively examined by placing a 10 µl of the sperm suspension onto a pre-warmed glass microscopic slide at 37°C and examined under light microscope (Olympus, Japan) at 200x and 400x magnifications. Motility is expressed as the percentage of progressively motile sperm.

*Viability and plasma membrane integrity*

Sperm viability was evaluated using multiple fluorescent probes labeling. Integrity of sperm plasma membrane was assessed by a non-membrane permeable DNA staining (Ethidium homodimer-1, EthD-1, Molecular Probes™, Oregon, USA), while esterase enzyme activity was tested by staining the sperm with calcein AM (Molecular Probes™, Oregon, USA). Sperm was stained with a mixture of EthD-1 and Calcein AM at a final concentration of 2 µM and

4 μM, respectively. After incubation of sperm with vital dyes for 15 min, total of 200 sperm were evaluated and classified into 2 categories as either viable (intact plasma membrane: positive to calcein AM and negative to EthD-1 negative) or dead sperm (damaged plasma membrane, EthD-1 positive).

*Acrosome integrity*

The integrity of the sperm acrosome was evaluated by fluorescein isothiocyanate-labeled peanut (*Arachis hypogaea*) agglutinin (FITC-PNA) combined with EthD-1 staining as described by Cheng et al. (1996). Briefly, the semen sample (10 μl) was mixed with 10 μl of 4 μM EthD-1 in PBS and incubated for 15 min at 37 ºC. Salmon sperm DNA (10 mg/ml) was added into the sperm suspension in order to neutralize the EthD-1. Five microliters of solution was then smeared on a glass slide and air dried. The sample was fixed with 96 % (v/v) ethanol for 30 sec and allowed air dry. To label the acrosomal membrane of the sperm, 20 μl of 100 μM FITC-PNA was spread over the slide and incubated in a moist chamber at 4ºC for 30 min. The sample was subsequently rinsed with cold PBS and allowed air dry. Only sperm that were negative to EthD-1 staining (alive sperm) were examined under a fluorescent microscope (BX51, Olympus, Japan) at 1000x magnifications. The sperm was classified as either intact acrosome or damaged/loss acrosome.

*Functional integrity of plasma membrane*

Functional integrity of sperm plasma membrane was assessed using a short hypoosmotic swelling test (sHOST) as described by Neild et al. (1999). Sperm were incubated, at 37 °C for 30 min, with 100 mOsm/kg, a hypo-osmotic solution that consisted of 0.49% (w/v) Na3-citrate and 0.9% (w/v) fructose (Merck, Germany) in distilled water. Following this incubation, the semen was fixed in a hypoosmotic solution supplemented with 5% (v/v) formaldehyde (Merck, Germany). Typical tail abnormalities indicative of swelling (sHOST positive) were evaluated under light microscopy at 400 x magnifications. Coiled tail (sHOST positive) sperm found following incubation was classified as functional intact sperm plasma membrane.

*Sperm DNA integrity*

Following recovery of epididymal sperm either before or 10 min after thawing, sperm were first stained with 2 μM EthD-1 at 37 °C for 10 min. The salmon sperm DNA (10 mg/ml) was added to bind any excess of EthD-1 in sperm suspension. Five microliters of sperm suspension

were smeared onto a glass microscope slide coated with aminopropyltriethoxysilane (APES). The slide containing with sperm was then fixed and stored in 4% (w/v) paraformaldehyde in dark for 30 min. Detection of DNA fragmentation was performed by TUNEL assay (*In Situ* Cell Death Detection Kit, Roche, Mannheim, Germany) following the manufacturer's instructions. In brief, the slides containing sperm were washed twice in PBS and incubated with a mixture of TUNEL reaction (TdT enzyme and nucleotide mix) for 1 h at 37 °C in a humidified chamber. The sperm were counter-stained with 0.1 µg/mL DAPI. Sperm positive to only TUNEL was classified as DNA fragmented sperm, while sperm positive to ethidium-homodimer-1 or both ethidium-homodimer-1 and TUNEL was classified as dead cells. A total of 200 sperm per treatment were randomly counted under an epifluorescent microscope at wavelength of 380, 488 and 568 nm for DAPI, TUNEL and Ethd-1, respectively.

*Longevity test*

After freezing and thawing, sperm diluted with NFDM extender as described previously were incubated at room temperature and assessed for sperm quality at 10 min, 2 and 4 h post-thawing.

## Experimental Design

***Experiment I:*** *effects of CLC loading on the sperm cholesterol contents*

The experiment was performed to examine the amount of cholesterol in plasma membrane of epididymal sperm before and after treatment with CLCs. Epididymides were collected from 3 stallions (6 epididymides). Epididymal sperm pooled from 2 epididymides of each stallion were divided and loaded with CLCs at different concentrations: 1) 1.5 mg, 2) 3 mg, and 3) 6 mg of CLCs per $120 \times 10^6$ sperm. The cholesterol within the sperm plasma membrane was determined using GC-FID over the times of incubation (15, 30 and 45 min). This was performed simultaneously with the examination of sperm motility and viability. Sperm incubated without CLCs were used as controls.

*Experiment II: effects of CLC loading on the distribution of cholesterol*

To demonstrate the localization and distribution of cholesterol within sperm plasma membrane, epididymal sperm from 3 stallions were loaded with 1.5 mg CLCs per $120x10^6$ sperm for 15 min at room temperature (25°C) and then fixed. The sperm were then stained with filipin and visualized the filipin-sterol complexes with an epifluorescent microscope. A total of 200 CLC-treated sperm were categorized according to the observed patterns of cholesterol distribution over the sperm head.

*Experiment III: effects of CLC loading on sperm cryopreservability*

A total of 14 epididymides (7 stallions) were used in this study. Sperm recovered from each pair of epididymides were pooled, diluted with Tyrode's medium and then divided into 4 aliquots. Each sperm aliquot was treated with CLCs at concentrations of 1.5, 3.0, 6.0 mg of CLCs per $120x10^6$ sperm for 15 min at room temperature (25°C). Epididymal sperm without CLC loading was used as control group. Sperm quality, in terms of motility, viability, plasma membrane functionality and DNA integrity, was assessed at post-equilibration and 10 min, 2 h and 4 h after freezing and thawing.

Statistical analysis

Values are present as means ± standard error of the mean (SEM). The statistical analysis was performed using SPSS statistical program (version 16.0). The amounts of cholesterol at each time point were compared between cholesterol loaded and unloaded groups. Descriptive statistic was used to describe the pattern of cholesterol distribution on sperm plasma membrane. Comparisons between percentages of motility, viability, head and tail morphology, acrosome integrity, functional integrity of plasma membrane and DNA integrity from different CLC concentrations were analyzed by one-way analysis of variance (ANOVA) after logarithm transformation. $P$-values < 0.05 were considered statistical significance.

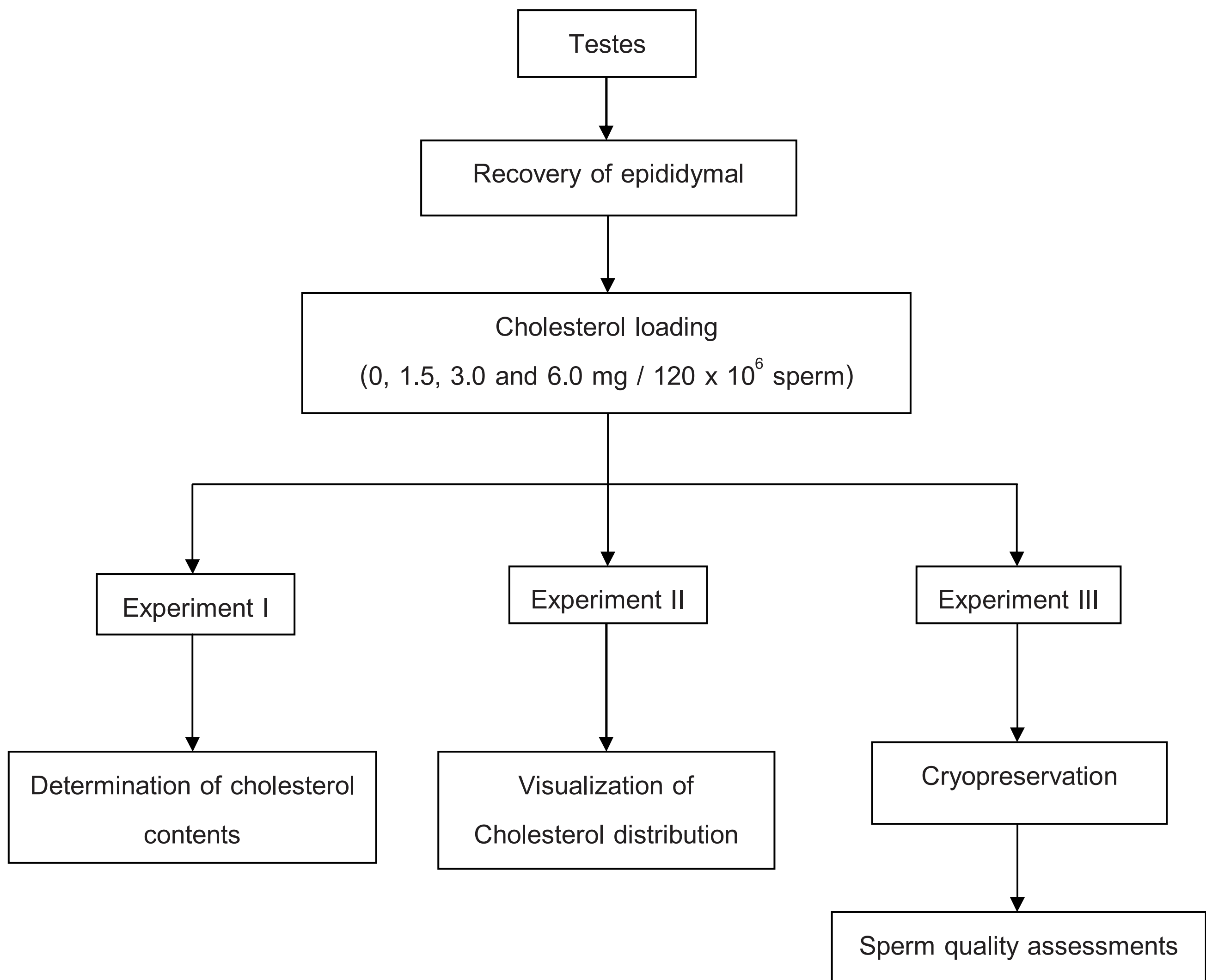


Figure 6: Diagram representing the experimental design

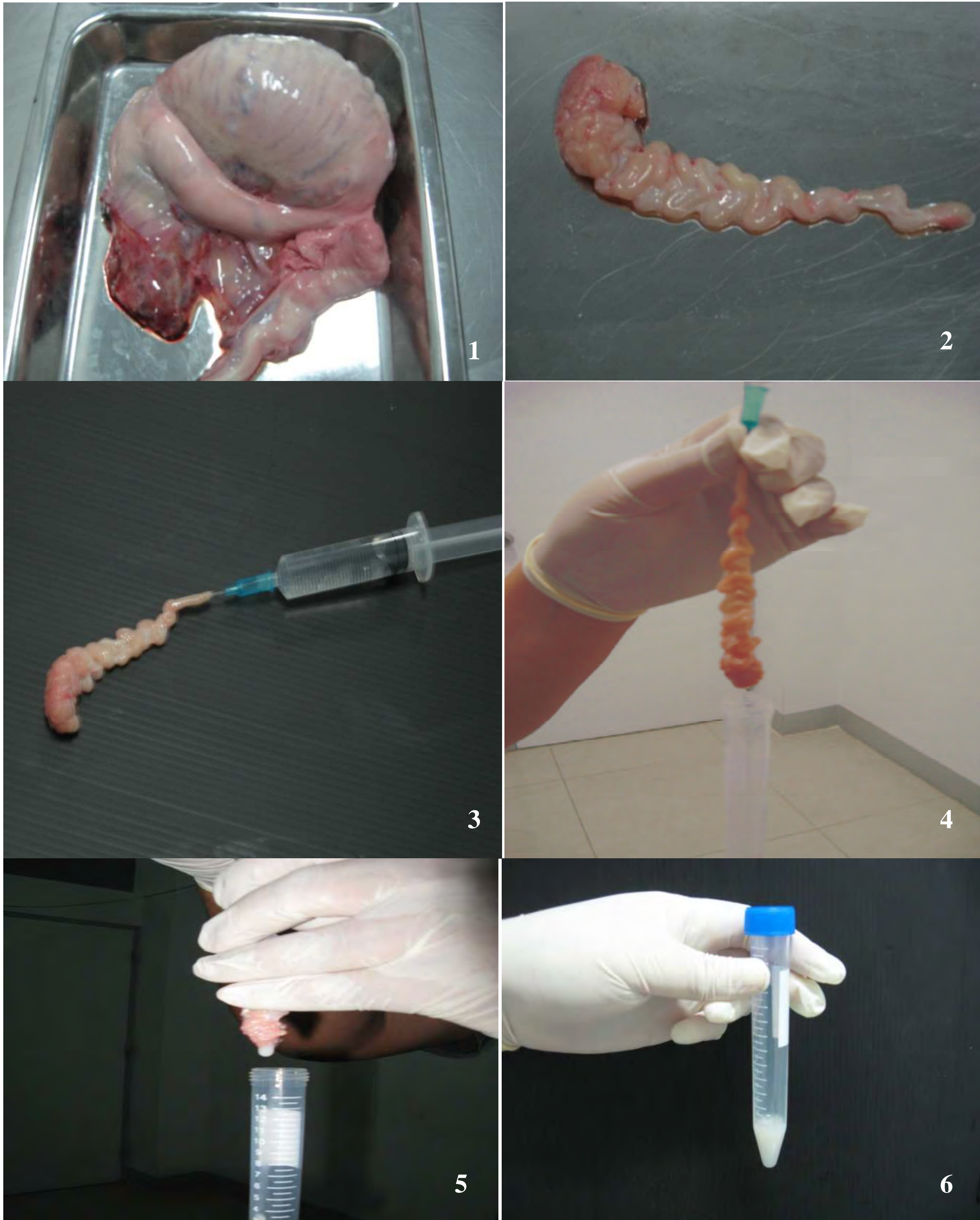


Figure 7: Equine epididymis after castration (1, 2) and recovery of epididymal sperm by retrograde flushing technique (3, 4, 5, 6)

CHAPTER IV

RESULTS

*Experiment I*

Concentrations of cholesterol and quality (motility and viability) of epididymal sperm treated with CLCs are shown in Figure 8 and Table 2, respectively. The cholesterol contents in epididymal sperm were increased in a manner of dose and time dependence (Fig. 8). Incubating sperm with 1.5 mg CLCs/120 x $10^6$ sperm preserved sperm quality similar to non-CLC treated control (P>0.05). Incubating sperm with 3.0 mg CLCs/120 x $10^6$ sperm for 15 and 30 min preserved sperm quality similar to non-CLC treated control and 1.5 mg CLCs treated sperm (P>0.05). In contrast, higher CLC concentrations especially at 6 mg/120 x $10^6$ and prolonged incubation times significantly decreased the sperm motility and viability, (Table 2), suggesting the toxicity of CLCs for sperm viability. Because sperm quality and cholesterol contents of sperm treated with 1.5 mg CLCs/120 x $10^6$ did not significantly differ among incubation times (15, 30 and 45 min). This CLC concentration was therefore used in the following experiments.

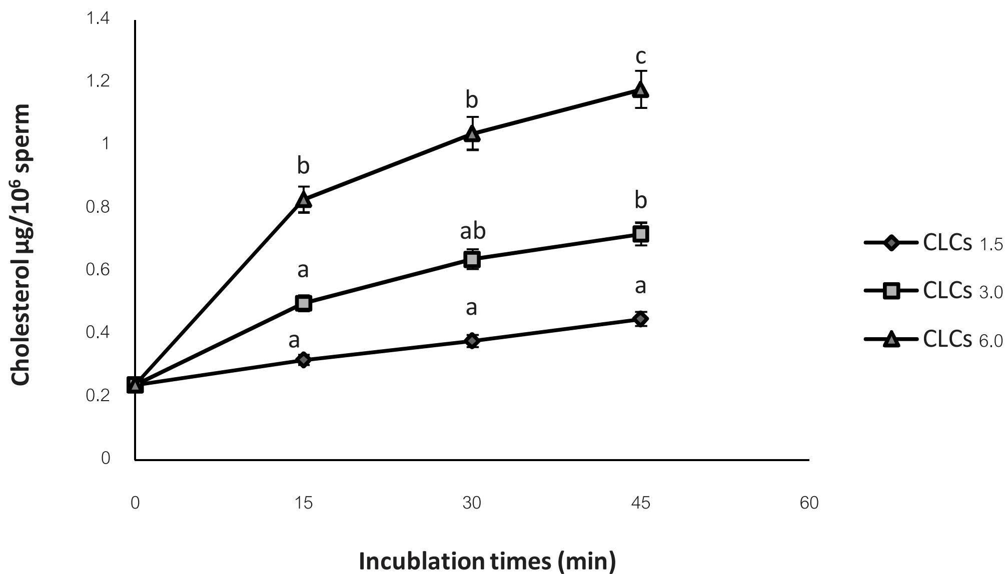


Figure 8: The concentration of cholesterol (µg/$10^6$ sperm) within the sperm plasma membrane when adding CLCs with different concentrations during incubation times (15, 30 and 45 min).
[a, b, c] within time, different superscripts denote values that differ significantly ($P < 0.05$).

**Table 2:** Mean ± SEM of motility and viability, of equine epididymal sperm pretreated with different concentrations of cholesterol-loaded cyclodextrins (CLCs) at 15, 30 and 45 min of CLC incubation.

| | Times of CLC incubation | | | |
|---|---|---|---|---|
| | 0 min | 15 min | 30 min | 45 min |
| %Motility | | | | |
| Control | $78.3 \pm 1.7^{A}$ | $76.7 \pm 1.7^{Aa}$ | $73.3 \pm 1.7^{Aa}$ | $70.0^{Aa}$ |
| CLCs 1.5 mg | $78.3 \pm 1.7^{A}$ | $81.7 \pm 3.3^{Aa}$ | $75.0^{Aa}$ | $73.3 \pm 1.7^{Aa}$ |
| CLCs 3.0 mg | $78.3 \pm 1.7^{A}$ | $71.7 \pm 4.4^{Aa}$ | $65 \pm 2.9^{Aa}$ | $53.3 \pm 3.3^{Bb}$ |
| CLCs 6.0 mg | $78.3 \pm 1.7^{A}$ | $56.7 \pm 3.3^{Bb}$ | $36.7 \pm 3.3^{Cb}$ | $20.0^{Dc}$ |
| %Viability | | | | |
| Control | $83.0 \pm 2.1^{A}$ | $80.3 \pm 1.3^{Aa}$ | $75.0 \pm 1.5^{Aa}$ | $72.7 \pm 0.9^{Aa}$ |
| CLCs 1.5 mg | $83.0 \pm 2.1^{A}$ | $80.7 \pm 1.7^{Aa}$ | $76.7 \pm 0.3^{Aa}$ | $74.0 \pm 2.0^{Aa}$ |
| CLCs 3.0 mg | $83.0 \pm 2.1^{A}$ | $75.0 \pm 1.5^{Aa}$ | $72.7 \pm 1.7^{Ba}$ | $68.3 \pm 2.0^{Cb}$ |
| CLCs 6.0 mg | $83.0 \pm 2.1^{A}$ | $69.3 \pm 0.3^{Bb}$ | $56.7 \pm 2.4^{Cb}$ | $52.7 \pm 3.7^{Dc}$ |

$^{a, b, c}$ within in a column, $^{A, B, C, D}$ within in a row, different superscripts denote values that differ significantly ($P<0.05$).

*Experiment II*

The cholesterol distribution in epididymal sperm plasma membrane was visualized using filipin staining. In all CLC non-treated sperm, filipin-sterol complexes were distributed throughout the sperm head with a lower intensity at apical and pre-equatorial subdomains (Fig. 9A). Two patterns of filipin complexes were predominantly detected in CLC treated sperm. A large proportion of CLC treated sperm (64.3 ± 2.8%) had a marked increase of filipin sterol complex intensity over the sperm head compared to non-CLC treated sperm (pattern A, Fig. 9B). However, 27.7 ± 3.2% of sperm demonstrated an absent or patchy labeling of filipin at apical and pre-equatorial parts (Fig. 9C). Small number of sperm (8.0 ± 4.6%) had pattern of filipin sterol complexes similar to that of non-treated control.

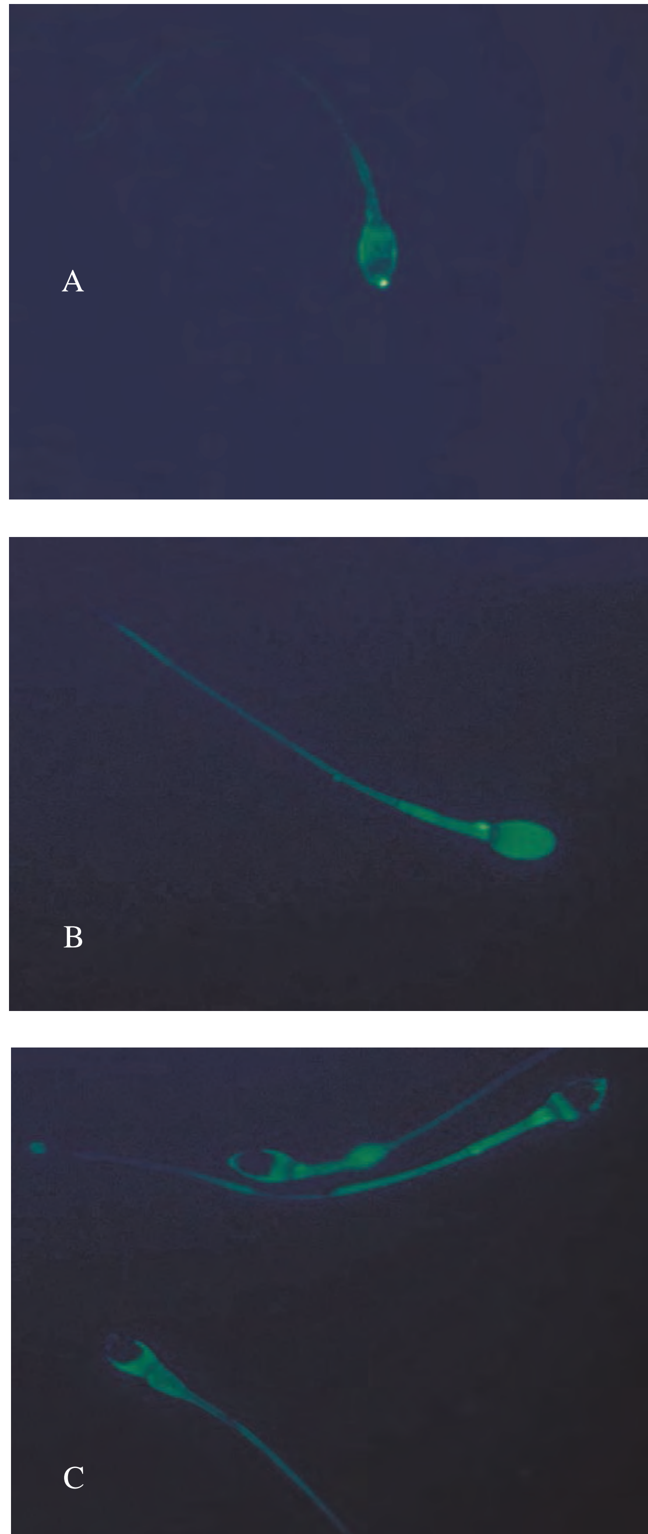


Figure 9: The cholesterol distribution of epididymal sperm before and after loading with CLCs. Cholesterol (filipin sterol complexes) distribution was visualized under a fluorescent microscope at 1000 magnifications. Cholesterol was unevenly distributed in non-CLC treated sperm head (A). Two patterns of flipin complexes (B and C) were observed after CLC treatment.

*Experiment III*

From pooled data, the epididymal sperm prior to cryopreservation had 82.5 ± 4.3, 87.7 ± 2.3, 83.4 ± 2.6 and 83.1 ± 4.6 for the percentages of motility, viability, intact acrosome and functional membrane integrity, respectively. Epididymal sperm were typified by a high proportion of abnormal tail morphology, predominantly with proximal and distal droplet sperm. Sperm morphology (head and tail) was, however, not affected by sperm equilibration and cryopreservation (Table 3). Overall, quality of equine epididymal sperm, in terms of viability, motility, acrosomal and functional membrane integrity was gradually decreased following sperm equilibration at 4°C for 1 h and cryopreservation. During sperm equilibration, CLCs at 1.5 mg/120 x $10^6$ sperm significantly improved sperm motility and viability compared to non-CLCs treated control and to other concentrations of CLCs (3 or 6 mg/120 x $10^6$ sperm) (Table 4). Similarly, the effect of CLCs in protecting sperm from cyroinjury during freezing and thawing was in a manner dose-dependence. Only 1.5 mg CLCs demonstrated an improvement of sperm quality post-thawing (viability, motility and acrosome integrity) compared to non-CLC treated frozen-thawed control. Higher concentrations (3 or 6 mg) of CLCs had no or adverse effect on the sperm quality. For example, sperm motility and viability of 1.5 mg CLCs treated sperm at 10 min post-thawing were 50.7± 3.5 and 69.0 ± 2.5, respectively. These results were significantly higher than those obtained from non-CLC treated (30.0 ± 6.5 and 56.7 ± 3) or 3 mg (35.0 ± 5.0 and 49.7 ± 4) and 6 mg (30.0 ± 3.8 and 46.0 ± 3.5) CLC treated groups ($P<0.05$, Table 4). Furthermore, 1.5 mg/120 x $10^6$ sperm also improved sperm longevity following cryopreservation by means of sperm viability and motility across all incubation times (2 and 4 h) compared with control and other concentrations of CLC treated sperm. The effect of CLC pretreatment on DNA integrity of frozen-thawed sperm as determined by TUNEL assay, however, did not differ among CLC concentrations and non-CLC treated sperm. There were only <4 % of frozen-thawed equine epididymal sperm exhibited DNA fragmentation (Table 3).

Table 3: Mean ± SEM of sperm morphology and DNA integrity of epididymal stallion sperm after retrograde flushing (fresh), post-equilibration and 10 min post-thawing.

| | % normal sperm morphology | | DNA integrity |
|---|---|---|---|
| | Head | Tail | |
| Fresh | 89.3 ± 2.5 | 59.1 ± 3 | 98.0 ± 0.5 |
| Post-equilibration | | | |
| 0 mg CLCs | N/A | 52.4 ± 2.2 | N/A |
| 1.5 mg CLCs | N/A | 57.1 ± 2.2 | N/A |
| 3.0 mg CLCs | N/A | 55.6 ± 2.1 | N/A |
| 6.0 mg CLCs | N/A | 55.0 ± 2.3 | N/A |
| 10 min after freezing and thawing | | | |
| 0 mg CLCs | 85.9 ± 2.7 | 52.6 ± 2.7 | 96.4 ± 0.4 |
| 1.5 mg CLCs | 89.6 ± 2.6 | 54.4 ± 1.9 | 98.8 ± 0.4 |
| 3.0 mg CLCs | 87.0 ± 2.2 | 54.3 ± 2.5 | 97.5 ± 0.3 |
| 6.0 mg CLCs | 87.4 ± 2.3 | 55.1 ± 3.3 | 96.9 ± 0.6 |

N/A = not applicable

**Table 4:** Mean ± SEM of motility, viability, acrosomal membrane integrity and plasma membrane functionality (sHost positive sperm) of equine epididymal sperm pretreated with different concentrations of cholesterol- loaded cyclodextrins (CLCs). Sperm quality was assessed after equilibration at 4 °C for 1 h and after thawing for 10 min, 2 and 4 h.

| Parameter | Motility(%) | Viability(%) | Intact Acrosome(%) | sHost(%) |
|---|---|---|---|---|
| Post-equilibration | | | | |
| Control | 65.0 ± 3.9[a] | 74.5 ± 3.4[a] | 72.3 ± 1.3[ab] | 70.2 ± 3.4[a] |
| CLCs 1.5 mg | 72.9 ± 1.5[b] | 82.8 ± 1.4[b] | 75.5 ± 1.2[b] | 69.9 ± 2.7[a] |
| CLCs 3.0 mg | 65.7 ± 2.3[ab] | 68.4 ± 2.0[a] | 68.3 ± 1.9[a] | 57.7 ± 4.1[b] |
| CLCs 6.0 mg | 58.6 ± 2.0[a] | 60.2 ± 2.6[c] | 60.8 ± 3[c] | 50.7 ± 4.8[b] |
| 10 min post-thawing | | | | |
| Control | 30.0 ± 6.5[a] | 56.7 ± 3[a] | 51.9 ± 5.3[ab] | 33.6 ± 5[a] |
| CLCs 1.5 mg | 50.7± 3.5[b] | 69.0 ± 2.5[b] | 63.1 ± 4.9[b] | 36.4 ± 6.4[a] |
| CLCs 3.0 mg | 35.0 ± 5.0[a] | 49.7 ± 4[ac] | 50.7 ± 5.0[ab] | 24.4 ± 6.2[ab] |
| CLCs 6.0 mg | 30.0 ± 3.8[a] | 46.0 ± 3.5[c] | 44.4 ± 4.4[a] | 16.7 ± 4.3[b] |
| 2 h post-thawing | | | | |
| Control | 15.7 ± 5.7[a] | 50.4 ± 2.9[a] | 44.3 ± 4.0[a] | 25.9 ± 5.6[ab] |
| CLCs 1.5 mg | 30.0 ± 5.8[b] | 62.0 ± 3.1[b] | 56.2 ± 4.4[b] | 30.6 ± 7[a] |
| CLCs 3.0 mg | 24.3± 5.6[ab] | 45.9 ± 2.9[ac] | 46.3 ± 4.5[ab] | 19.9 ± 6.7[ab] |
| CLCs 6.0 mg | 13.6 ± 3.9[a] | 41.50 ± 3.6[c] | 36.3 ± 3.9[a] | 14.1 ± 3.4[b] |
| 4 h post-thawing | | | | |
| Control | 5.7 ± 3[a] | 42.28 ± 4[a] | 35.8 ± 5.9[a] | 24.1 ± 5.5[a] |
| CLCs 1.5 mg | 15.7 ± 4.9[b] | 55.0 ± 4.0[b] | 47.3± 5.4[b] | 27.4 ± 7.1[a] |
| CLCs 3.0 mg | 6.4 ± 2.8[a] | 38.2 ± 4.7[a] | 36.7 ± 5.6[ab] | 16.5 ± 7.1[a] |
| CLCs 6.0 mg | 2.1 ± 1.5[a] | 32.6 ± 4.3[a] | 28.6 ± 3.1[a] | 12.0 ± 3.9[a] |

[a, b c] within in a column, different superscripts denote values that differ significantly at each time points ($P<0.05$)

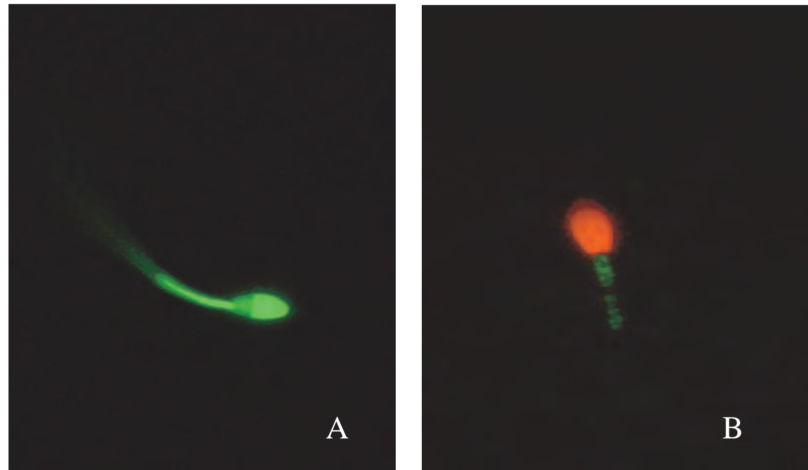


Figure 10: Photomicrographs of stallion sperm stained with Calcein-AM combined with Ethidium homodimer-1. The sperm with intact plasma membrane were stained green with calcein-AM (A; alive sperm), while EthD-1 (red) positive sperm indicated the damage of plasma membrane (B; dead sperm).

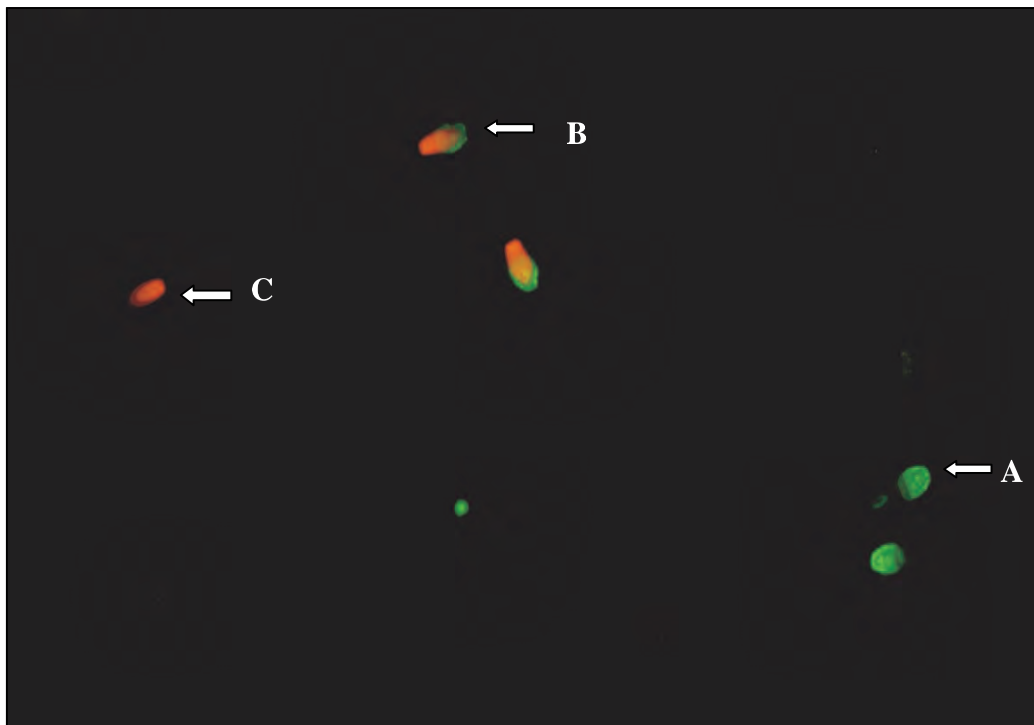


Figure 11: Photomicrographs of stallion epididymal sperm stained with FITC-PNA /Ethidium homodimer-1. The sperm with intact acrosome (A) are stained green with FITC-PNA, while dead sperm with damage acrosome (B) or loss acrosome (C) were stained red with EthD-1 (1000x magnification).

# CHAPTER V

# DISCUSSION

In the present study, incorporation of cholesterol to the sperm plasma membrane of equine epididymal sperm using cholesterol-loading cyclodextrins (CLCs) technique prior to cryopreservation improved sperm quality during cryoprotectant equilibration and after freezing and thawing. Up to our knowledge, the effects of CLCs on sperm cryopreservability have only been reported in ejaculated sperm.

Mammalian sperm have varying degrees of cold shock susceptibility depending on the membrane phospholipid composition as well as the membrane cholesterol to phospholipid molar ratio (Holt, 2000). Stallion sperm have low cholesterol/phospholipid ratios and are very susceptible to cold shock especially when they expose to critical temperatures between 8°C and 19 °C (Amann and Pickett, 1987; Parks and Lynch, 1992; Crockett et al., 2001; Purdy and Graham, 2004).

Cyclodextrins (cyclic heptasaccharides consisting of beta (1-4) glucopyranose units), which have a hydrophobic center, effectively remove cholesterol from sperm plasma membranes into their hydrophobic core (Christian et al., 1997; Visconti et al., 1999, (Gitler, 1972; Klein et al.1995). In the present study, the cholesterol content of stallion epididymal sperm were measured, using gas chromatograph flame ionized detector (Ohshima, 2001). We found that the cholesterol content of non-CLC treated sperm (0.24 µg cholesterol/$1x10^{6}$ sperm) was similar to that reported in stallion ejaculated sperm (Moor et al., 2005). After CLC loading, the amounts of cholesterol contents of epididymal sperm plasma membrane as measured at 15 min post incubation, were increased over the time of CLC incubation, and these amounts were similar to a report in ejaculated sperm (Moore et al., 2005). The results also indicated that the rate of cholesterol transfer from CLCs to the plasma membrane was rapid because the levels of cholesterols in all chosen concentrations were rapidly increased by 15 min after CLC loading. This short period of CLCs is therefore suitable to modify the sperm plasma membrane since longer incubation times (30 and 45 min) were detrimentally affected the sperm motility and viability. We found the different patterns of cholesterol at the sperm head between our study and previous reports. The cholesterol concentration was high at anterior region of head (apical, acrosome, pre-equatorial and equatorial sub domains) and less at post- equatorial subdomain

of head and tail (Suzuki, 1988). In our study, the intensity of cholesterol was high at post-equatorial subdomain. The different pattern of cholesterol between the two types of sperm may cause by the fact the ejaculated sperm need to be exposed with cholesterol enriched seminal plasma during ejaculation. After 1.5 mg CLC loading for 15 min and visualization of filipin-sterol complexes under an fluorescent microscope, significant numbers of CLC-loaded sperm (64.3 ± 2.8%) demonstrated an increase filipin-sterol complex intensity compared to non-CLC loaded sperm (Figs. 9A and 9B). However, a small proportion of sperm (27.7 ± 3.2%) had patchy pattern or weakly labeled the filipin-sterol complexes at apical and pre-equatorial subdomains. This could cause by the cholesterol-free β cyclodextrin that removed cholesterol from the sperm plasma membrane. It is of interested that poor viability of sperm after long-term CLC incubation would be associated with an excessive efflux of cholesterol from the sperm plasma membrane. It is well documented that removal of cholesterol by methyl-beta cyclodextrin induced capacitation via tyrosine phosphorylation, thereby shortening sperm longevity (Osheroff et al., 1999).

Until recently, the relationship between amounts of cholesterol in the sperm plasma membrane on cryopreservability of sperm has been contradictory. Several reports demonstrated that removal of cholesterol from the sperm plasma membrane prior to cryopreservation using methyl-beta-cyclodextrin, an acceptor of cholesterol, improved frozen-thawed sperm quality in pig (Zeng and Terada, 2001) compared to non-treated controls, possibly because the sperm plasma membrane fluidity increased when cholesterol was depleted (Companyó et al., 2007). In the present study, we increased the levels of cholesterol in the equine sperm plasma membrane because freezing and thawing cause a rapid loss of cholesterol that induces premature capacitation-like, thereby shortening the sperm longevity (Cerolini et al., 2001, Cormier and Bailey, 2003). It is therefore preferable to increase the amount of cholesterol rather than removing it from sperm plasma membrane.

We demonstrated the positive effects of CLCs treatment (1.5 mg/$120\times10^6$ sperm) on sperm viability and motility of epididymal sperm (Table 4) during sperm equilibration compared with non-CLC treated group. It is likely possible that adding cholesterol to the epididymal sperm plasma membrane could maintain membrane fluidity and also increased membrane permeability of glycerol and thereby reducing the membrane osmotic stress (Glazar et al., 2009). Our also results indicated that the effect of adding cholesterol to the sperm plasma

membrane by means of CLCs was in a manner of dose-dependence. CLC at $1.5 mg/120 \times 10^6$ improved sperm quality both post-equilibration and post-thawing. Similar results have been reported for ejaculated sperm of bull (Purdy and Graham, 2004; Mocé and Graham, 2006), stallion (Combes et al., 2000; Moore et al., 2005) and ram (Morrier et al., 2004). It was however clearly evident that increasing concentration of CLCs to four-fold CLCs (6 mg) had become detrimental to sperm quality similar to previous report in bulls (Purdy and Graham, 2004). Although the reason remains unclear, plasma membrane of sperm seems likely to require an optimal ratio of cholesterol and phospholipids in order to sustain the normal membrane fluidity necessary for sperm function. Excessive water efflux occurred during removal of cholesterol from the sperm plasma membrane could also be the cause of sperm death (Atger et al., 1997).

In addition to the sperm viability and motility, our result is in agreement with a report indicating that incidence of sperm DNA fragmentation following cryopreservation was low (Table 3). DNA of mammalian sperm differ from somatic cells in that the histone protein is replaced by protamine, with disulfide cross linkages between the protamines. Tightly packed sperm chromatin may responsible to protect the DNA fragmentation during cryopreservation in this species (Corzett et al., 2002; Aoki et al., 2005).

Conclusion

We firstly demonstrated that incorporation of cholesterol by cholesterol-loaded methyl- β cyclodextrin (CLCs) decrease chilling sensitivity and also improve equine epididymal sperm cryopreservability. This protective effect of CLCs on post-thawing sperm quality, however, is in a manner of dose-dependence. The optimal concentration of CLCs for equine epididymal sperm is 1.5 mg/$120\times10^6$ sperm.

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# VITAE

Teeraporn Pamornsakda, the 2nd son of Kosol and Supratra Pamornsakda, was born in February 1977 in Bangkok, Thailand. Following his graduation from Bodindeacha1, he studied veterinary medicine at the Faculty of Veterinary Medicine, Kasetsart University. After his graduation, he worked as veterinarian at private 'animal farm hospital' and frequently lecturer in equine clinic at Faculty of Veterinary Medicine, Technology Mahanakorn University. At present, he is a lecturer in equine clinic at Faculty of Veterinary Science, Mahidol University. He began the master degree of science (Theriogenology) program at the Department of Obstetrics, Gynaecology and Reproduction at the Faculty of Veterinary Science, Chulalongkorn University in 2007. During the study, he has focused on semen cryopreservation techniques and assisted reproductive technology in the horse.