ผลของไฮดรอกซีเอทิลสตาร์ชต่อคุณภาพอสุจิม้าภายหลังการแช่เย็นและแช่แข็ง

นางสาวอรวรรยา นุชนารถ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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The effects of Hydroxyethyl starch (HES) on quality of equine sperm following cold storage and cryopreservation

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Theriogenology
Department of Obstetrics Gynaecology and Reproduction
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อรวรรยา นุชนารถ : ผลของไฮดรอกซีเอทิลสตาร์ชต่อคุณภาพอสุจิม้าภายหลังการแช่เย็นและแช่แข็ง (The effects of Hydroxyethyl starch (HES) on quality of equine sperm following cold storage and cryopreservation) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. น.สพ. ดร. ธีรวัฒน์ ธาราศานิต, หน้า.

การศึกษาในครั้งนี้มีวัตถุประสงค์เพื่อทราบผลของการเสริมไฮดรอกซีเอทิลสตาร์ชที่ความเข้มข้นต่างกันต่อคุณภาพอสุจิม้าภายหลังการแช่เย็นและการแช่แข็ง

การทดลองที่ 1 ทำการรีดเก็บน้ำเชื้อจากพ่อม้าทั้งหมด 6 ตัว ตัวละ 3 ครั้ง จากนั้นเติมสารละลายเจือจางน้ำเชื้อสำหรับแช่เย็น ซึ่งมีร้อยละความเข้มข้นของไฮดรอกซีเอทิลสตาร์ช (HES) ที่แตกต่างกัน จำนวน 5 กลุ่ม (2.5%, 5%, 10% และ 15% โดยสารละลายเจือจางน้ำเชื้อที่ไม่เติมไฮดรอกซีเอทิลสตาร์ช (HES 0%) เป็นกลุ่มควบคุม ทำการลดอุณหภูมิช้าๆ จนถึง 4 องศาเซลเซียส ทำการตรวจสอบคุณภาพของอสุจิ โดยทดสอบการการเคลื่อนที่ไปข้างหน้าและอัตราการรอดชีวิต หลังจากแช่เย็นเป็นเวลา 6, 24 และ 48 ชั่วโมง พบว่าภายในระยะเวลาไม่เกิน 24 ชั่วโมง อสุจิในกลุ่ม 2.5% และ 5% HES มีร้อยละอัตราการเคลื่อนที่ไม่แตกต่างจากกลุ่มควบคุม แต่เมื่อตรวจคุณภาพที่ 48 ชั่วโมงขึ้นไป พบว่ากลุ่ม 2.5% HES ช่วยเพิ่มร้อยละอัตราการเคลื่อนที่อสุจิได้ดีกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ ($P<0.05$) นอกจากนี้ HES ที่ความเข้มข้นร้อยละ 2.5 มีร้อยละการรอดชีวิตสูงกว่ากลุ่มควบคุมอย่างมีนัยสำคัญเมื่อทำการแช่เย็นนานไม่เกิน 24 ชั่วโมง ($P<0.05$) แต่ไม่พบความแตกต่างที่ 48 ชั่วโมง การเพิ่มความเข้มข้นของ HES ในระดับร้อยละ 10 และ 15 สำหรับการแช่เย็นอสุจิมีผลในทางลบต่อคุณภาพอสุจิ

การทดลองที่ 2 ทำการรีดเก็บน้ำเชื้อจากพ่อม้าทั้งหมด 6 ตัว ตัวละ 3 ครั้ง ทำการแช่แข็งน้ำเชื้อด้วยสารละลายสำหรับแช่แข็งอสุจิที่มีการเติม HES ที่ความเข้มข้นแตกต่างกัน จำนวน 5 กลุ่ม (2.5%, 5%, 10% และ 15%) การแช่แข็งด้วยสารละลายแช่แข็งน้ำเชื้อที่ไม่เติม HES เป็นกลุ่มควบคุม ภายหลังการละลายน้ำเชื้อแช่แข็ง ทำการตรวจคุณภาพอสุจิ โดยการทดสอบการการเคลื่อนที่ไปข้างหน้า การรอดชีวิต ความสมบูรณ์ของดีเอ็นเอ ความสมบูรณ์ของเยื่อหุ้มอะโครโซม การทำงานของเยื่อหุ้มเซลล์ และระยะเวลาการอยู่รอดของอสุจิ การศึกษาครั้งนี้พบว่า HES มีผลต่อคุณภาพอสุจิม้าภายหลังการแช่แข็งและการทำละลาย โดยร้อยละความเข้มข้นของ HES ในสารละลายแช่แข็งน้ำเชื้อมีผลต่อคุณภาพอสุจิ อสุจิม้าที่แช่แข็งด้วยสารละลายแช่แข็งน้ำเชื้อที่ผสม HES ร้อยละความเข้มข้น 2.5 ให้ผลการเคลื่อนที่ การอยู่รอด และมีความสมบูรณ์ของเยื่อหุ้มอะโครโซมมากกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ ($P<0.05$)

นอกจากนี้ HES ยังส่งผลให้คุณภาพอสุจิในส่วนของการเคลื่อนที่อยู่ในระดับที่มากกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ ($P<0.05$) เมื่อทำการตรวจที่ 2 และ 4 ชั่วโมงภายหลังการทำละลาย การเพิ่มความเข้มข้นของ HES ในระดับที่สูงขึ้นไม่สามารถเพิ่มคุณภาพอสุจิภายหลังการทำละลายได้ ซึ่งสอดคล้องไปในทิศทางเดียวกันกับผลในการทดลองที่ 1 HES ที่ความเข้มข้นร้อยละ 2.5 ให้ผลเพิ่มอัตราการมีชีวิตของอสุจิม้าในระดับที่สูงกว่ากลุ่มควบคุมอย่างนัยสำคัญเมื่อทำการตรวจน้ำเชื้อที่ 6 ชั่วโมงภายหลังการทำละลาย ($P<0.05$) แต่ไม่พบความแตกต่างที่ชั่วโมงที่ 0 ถึง 4

การศึกษาครั้งนี้สรุปได้ว่าการเสริม HES ในสารละลายสำหรับแช่เย็นและแช่แข็งอสุจิม้า ช่วยเพิ่มคุณภาพอสุจิม้าในขณะทำการแช่เย็นและแช่แข็งได้ แต่จำเป็นต้องเสริม HES ในความเข้มข้นที่เหมาะสม การศึกษาครั้งนี้พบว่าการเสริม HES ที่ความเข้มข้นร้อยละ 2.5 มีประสิทธิภาพสูงที่สุดการป้องกันความเสียหายของอสุจิม้าขณะทำการแช่เย็นและการแช่แข็งน้ำเชื้อ ควรมีการศึกษาเพิ่มเติมในด้านการตรวจความสัมพันธ์ของ HES และองค์ประกอบอื่นๆ ของ สารละลายสำหรับแช่แข็ง รวมถึงการนำน้ำเชื้อที่แช่แข็งด้วยการเสริม HES ไปผสมเทียมเพื่อยืนยันความสามารถในการปฏิสนธิของอสุจิแช่แข็ง

ภาควิชา สูติศาสตร์-เธนุเวชวิทยาและวิทยาการสืบพันธุ์ ลายมือชื่อนิสิต .....

สาขาวิชา วิทยาการสืบพันธุ์สัตว์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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5875326131 : MAJOR THERIOGENOLOGY
KEYWORDS: CRYOPRESERVATION / HYDROXYETHYL STARCH (HES) / SPERM / STALLION

ORNWANYA NUCHANART: The effects of Hydroxyethyl starch (HES) on quality of equine sperm following cold storage and cryopreservation. ADVISOR: ASST. PROF. THEERAWAT THARASANIT, pp.

This study aimed at examining the effects of different concentrations of hydroxyethyl starch (HES) on quality of equine sperm following cold storage and cryopreservation.

In experiment 1, the semen was collected from 6 stallions (3 replicates). The semen was diluted with extender for cold storage containing different concentrations of HES (2.5%, 5%, 10% and 15%). Extender without HES (0% HES) served as a control group. The temperature of extended semen was gradually decreased to 4 ºC. The quality of sperm in terms of progressive motility and viability was evaluated after cold storage for 6, 24 and 48 hours. This study revealed that equine sperm maintained in extender with 2.5% and 5% HES had similar progressive motility to that observed in a control group when examined within 24 hours of cold storage. However, the 2.5% HES significantly improved progressive motility at 48 hours of cold storage when compared with the control ($P<0.05$). In addition, this concentration of HES also resulted in a significant improvement of sperm viability only if examined within 24 hours ($P<0.05$). There was no significant different between the control and 2.5% HES at 48 hours of cold storage ($P>0.05$). Increasing concentrations of HES to 10% and 15% negatively affected to sperm quality during cold storage.

In experiment 2, the semen was collected from 6 stallions (3 replicates). The semen was diluted with a freezing extender containing different concentrations of HES (2.5%, 5%, 10% and 15%). Equine sperm frozen without HES served as a control group. Sperm quality in terms of progressive motility, viability, DNA integrity, acrosomal integrity, membrane functionality (hypo-osmotic swelling test, HOS test) and sperm longevity was examined after cryopreservation and thawing at 0 (10 min), 1, 2, 4 and 6 hours post-thaw. This study revealed that HES influenced on freezing ability of equine sperm. This effect was found to be a concentration dependency. Equine sperm frozen with 2.5% HES had significantly higher progressive motility, viability and acrosomal integrity when compared with a control group ($P<0.05$). In addition, this concentration of HES also significantly improved progressive motility at 2 and 4 hours post-thaw when compared with the control group ($P<0.05$). There was no further protective effect of HES against progressive motility if the concentrations of HES were increased over 5%, similarly to the observation in the experiment 1. The 2.5% HES also positively improved sperm viability at 6 hours post-thaw when compared with the control. However, no significant effect was observed at 0 and 4 hours post-thaw.

This study concluded that HES supplement in semen extender improved sperm quality during cold storage and cryopreservation. However, optimal concentration of HES was required. The current study indicated that the 2.5% HES was the optimal concentration that yielded the highest efficiency to protect equine sperm during cold storage and cryopreservation. Further study to examine the interactions of HES and other compositions in extender will need to be studied. In addition, it is necessary to use the equine sperm frozen with HES for insemination in order to test their fertilizing ability.

Department: Obstetrics Gynaecology and Reproduction
Field of Study: Theriogenology
Academic Year: 2017

Student's Signature .....
Advisor's Signature .....

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LIST OF ABBREVIATIONS

AI	Artificial insemination
AO	Acridine orange
AV	Artificial vagina
BSA	Bovine serum albumin
ºC	Degree Celsius
cm	Centimeter
CPA	Cryoprotective agent
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
FITC-PNA	Fluorescien isothiocyanate-labeled peanut agglutinin
g	Gram
h	Hour
HES	Hydroxyethyl starch
HOS Test	Hypo-osmotic swelling test
kDa	Kilodalton
kg	Kilogram
M	Molar
mg	milligram
min	minute
ml	milliliter
mOsm	milliosmole
MS	Molar substitution
MW	Molecular weight
NFDM	Non-fat dry milk extender
sec	Second
SEM	Standard error of the mean

v/v	Volume/volume
w/v	Weight/volume
µg	Microgram
µl	Microliter
µM	Micromolar

CHAPTER I

INTRODUCTION

Importance and rationale

Breeding technology is economically important for equine industry worldwide. Natural mating has been used in several stud farms because this technique is cost effective with high pregnancy rate. However, this breeding technique do not allow short- and long-term semen storage. Natural cover is therefore unsuitable for realistically breeding strategies when a large number of mares will need to be mated. Artificial breeding by mean of artificial insemination has thus become a tool of choice for equine industry. Semen to be used for artificial insemination can be divided into three types according to the techniques used, including fresh, cooled and frozen-thawed semen. Fresh semen and cooled semen can be used to inseminate mares with high pregnancy rates (around 60 – 70%). In contrast, pregnancy rates of mares can significantly low with large variability of pregnancy results (10 – 40%). This results from poor quality of equine sperm following cryopreservation and thawing. For example, sperm viability and motility decrease immediately after thawing, and sperm will need be inseminated within 6 h of ovulation (Samper, 2007) . In addition, two-third of the stallion populations will give sperm that are intolerant to cryopreservation. This has become a large obstacle of equine breeding since we may not be able to cryopreserve sperm even if the stallions have high genetic background stallions with good performance at competition.

Equine sperm are fairly sensitive to cold stress especially during cryopreservation. The membrane fluidity of sperm membrane decreases rapidly when temperature is reduced under their physiological temperature (Loomis and Squires, 2005; Nicolson, 2014). Cryoinjury induces several changes at sperm structure and functions such as plasma membrane disruption and poor fertilizing ability. This

cryoinjury can be occurred at any steps of freezing and thawing of the sperm (Parks and Graham, 1992). To decrease cryoinjury during cryopreservation steps, freezing extender with cryoprotective agents (CPA) will need to be used. Two types of CPAs, including penetrating and non-penetrating CPA are commonly supplemented in the semen extender (Mottershead, 2000). It is worth nothing that only penetrating CPA is insufficient to protect sperm against cryoinjuries, and therefore both penetrating and non-penetrating CPAs are synergistically required. Of non-penetrating CPA used for improved cryopreservation, Hydroxyethyl starch (HES) has been demonstrated to protect cells against cryoinjuries. The large molecule of carbohydrate which is composed of glucose core and several branches of hydroxyethyl group modify the outflow rate of water from the sperm during freezing, thereby preventing osmotic stress and minimizing intercellular ice formation (Stolzing et al., 2012). The cryoprotective effect of HES has been originally demonstrated for red blood cells. However, the effect of HES on cryopreservability of sperm has yet to be demonstrated in the stallion. The effects of different concentrations of HES and the protective effects of HES during cryopreservation will be studied in order to improve sperm quality following freezing, thawing and insemination.

Objectives

1. To study the effects of different concentrations of HES added in semen extender on sperm quality during cold storage.
2. To study the effects of different concentrations of HES added in semen extender on sperm quality following cryopreservation and thawing.

Hypothesis

1. Supplementation of HES in chilled and freezing extenders increased quality of equine sperm.
2. Different concentrations of HES affected quality of chilled and frozen-thawed equine sperm.

Expected output

1. The study provides information on an appropriate concentration of HES used for cold storage and cryopreservation.
2. Improved quality of equine semen following cooing and cryopreservation can be applied in field practice for increasing pregnancy rates of mares after insemination.
3. The results obtained from this study can be published in an international journal.

Keywords (English): cryopreservation, hydroxyethyl starch (HES), sperm, stallion

Keywords (Thai): การแช่แข็ง, ไฮดรอกซีเอทิล สตาร์ช, อสุจิ, พ่อม้า

CHAPTER II

LITERATURE REVIEW

Characteristics of stallion sperm

The spermatogenesis cycle in stallion is around 57 – 60 days. This composes of three different spermatogenic phases including spermatocytogenesis, meiosis and spermiogenesis for 19.4, 19 and 18.6 days, respectively. In the stallion, the daily sperm production is 7 – 8 x 10^9 sperm/day (Johnson et al., 1997). As similar to other domestic species, the sperm are produced within the seminiferous tubules under the control of several factors such as endocrine, paracrine and autocrine. The mature stallions have ability to produce sperm since they reach the mean age of 18 months (puberty). The processes of spermatogenesis occur in a wave like fashion, starting from the spermatogonium (diploid, n=2) resided at the basement membrane of the seminiferous tubules. The junctions of Sertoli cells, spermatogenic nursing cells, form tight junctions that protect the spermatogonium as the blood-testis-barrier. The primary spermatocytes are released from the blood testes barriers and develop into spermatids (haploid, n=1). The spermatids are further developed to form structurally mature sperm and released into the lumen of the seminiferous tubules. The structure of mature sperm consists of head, midpiece and tail which is similar to other species (Figure 1). The head of sperm contains the nucleus that is covered by acrosomal cap. The midpiece is the position of mitochondria that serve as the energy producer. The tail is responsible for movement and is critical to determine pattern of motility during sperm transit and fertilizing ability.

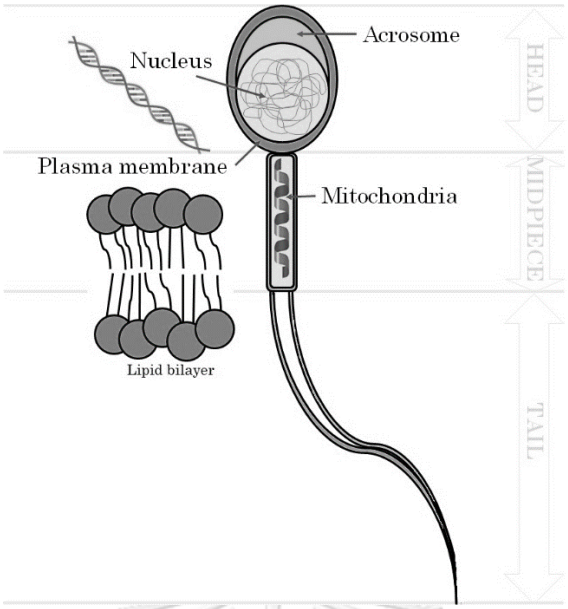

Figure 1 Structure of equine sperm. The spermatozoa are divided into 3 parts, head, midpiece and tail. Each part of the sperm essentially contributes a unique function during sperm maturation and fertilization.

(modified from http://projects.nfstc.org/pdi/Subject03/pdi_s03_m02_01_b.htm)

Of the structures of sperm, plasma membrane plays an important role for sperm viability and fertilizing ability. The lipid bilayers cover all part of the sperm as similar to other somatic cells. However, the sperm plasma membrane is distinct from the somatic cells in that the membrane is reorganized by several factors during hyperactivation and capacitation within the female reproductive tract. More specifically, the lateral and the 'flip-flop' movement of cholesterols between the inner and outer membrane cause membrane dynamic which often refers to as membrane fluidity. When the sperm are inseminated into the female reproductive tract, the sperm undergo capacitation that renders the remodeling of plasma membrane such as the cholesterol efflux and re-localization of several other proteins (López-González et al., 2014). It is therefore essential to preserve sperm plasma membrane integrity and its function during semen storage prior to the insemination, especially when cold or cryopreservation is applied. Poor semen handling and cryopreservation results in abnormal sperm morphology and disruption of sperm viability and functions.

Semen cryopreservation

Stallion sperm are among several species that are highly sensitive to cold temperature especially during the transition phase from liquid to gel states. Due to its high cold sensitivities, the results obtained following semen cryopreservation, in terms of sperm quality and pregnancy after insemination are generally poor, ranged between 0 – 50%. In equine practice, three categories of stallion population have been classified, including good, moderate and poor freezers (Samper, 2007). The post-thaw sperm of good freezing group is 40 – 60% and the pregnancy rate is around 60 – 70%. While the post-thaw sperm poor freezer is 10 – 15% and the pregnancy rate is less than 30% (Cristanelli et al., 1984).

During cryopreservation process, sperm will expose to extremely unusual environments during cooling and freezing that potentially render the cryoinjury. The cryoinjury or cryo-damage affected the sperm at several levels, such as plasma membrane integrity, mitochondrial and DNA (Aurich, 2005; Yeste, 2016).

When the sperm are exposed to freezing temperature, extracellular water will first form ice crystal resulting in an increase solute concentration. The osmolality of the solution will change and make the cell membrane exposing to the osmotic stress. Intracellular water will move across the plasma membrane in order to get cellular equilibrium. If penetrating CPA is added in the semen extender, the cryoprotective agent will replace the intracellular fluid and acts to reduce intracellular ice crystal formation. The formation of lethal intracellular ice formation also depends on other factors, especially the cooling rate of cryopreservation process. Improper cooling rate during cryopreservation of stallion semen such as too slow or too fast freezing is critically harmful to stallion sperm. If the cooling rate is too rapid, intracellular fluid remains at high amount upon freezing point. This condition causes extensive formation of intracellular ice crystals that potentially induce cellular damage. By contrast, too slow cooling induced dehydration of cells which enormously induces chemically cytotoxic condition. For sufficient protection of equine sperm during cryopreservation, it would need an optimal process during

cryopreservation in particular the extender composition and cooling rate (Mottershead, 2000).

In order to minimize cryoinjuries that can occur during cryopreservation process, the freezing extender is needed. The extender provides nutrients, buffer, antibiotics and cryoprotective agents. Cryoprotective agents can be generally divided into two types according to their property to pass across the plasma membrane, penetrating and non-penetrating cryoprotective agent (CPA). In most protocol of cryopreservation, the permeable CPAs such as glycerol, DMSO and ethylene glycol are required, and each particular CPA has different molecular size and indeed the transmembrane properties. In horse, glycerol is the most common CPA used for semen cryopreservation. The optimal concentration of this CPA can be variable from 2 to 5 %. However, the exact concentration CPA to be added in the semen extender is still unknown especially for horses where there are some interaction of CPA and other compositions of the extender. In addition, it is worth nothing that each particular stallion gives semen that differently reacts to semen extender and thus a large variation in cryo-survival of sperm following cryopreservation is frequently observed. Other components within the freezing also play a crucial role in determining the success of semen cryopreservation. The non-permeable CPA is frequently added in the freezing extender. Both permeable and non-permeable CPAs acts synergistically to reduce cryoinjuries such as to reduce intracellular and extracellular ice formation and osmotic buffer (Stolzing et al., 2012).

Hydroxyethyl starch

Hydroxyethyl starch is a synthetic modified polymer based on purified starch of corn or potatoes (Treib et al., 1999; Boldt, 2009). The structure is composed of glucose core with hydroxyethylation at carbon position C2, C3 or C6 (Treib et al., 1999; Boldt, 2009).

Hydroxyethyl starch or HES is a non-penetrate cryoprotective agent due to its large molecule. The action of HES is like natural absorbent that absorbs water molecule around 0.5 g water per 1 g of HES (Körber and Scheiwe, 1980) . The quality

of HES is dependent on their molecular weight, molar substitution and C2/C6 ratio in their structure (Kozek-Langenecker, 2005). HES can be classified into three molecular weights; the high molecular weight is >450 kDa; the medium one is 200 – 400 kDa and the low molecular weight is <200 kDa. The stability of HES depends on molecular weight, the higher form has low degradation rate due to its complex structure. Degree of molar substitution or MS is the number of hydroxyethyl group on the glucose core, the high MS is 0.62 – 0.75 or Heptastarch, the medium MS is 0.5 or Pentastarch and the low MS is 0.4 or Tetrastarch. Moreover, the C2/C6 ratio can be considered as high ratio is >8 and Low ratio is <8.

The natural degradation of HES occurs in blood stream which is caused by the alpha-amylase in serum. When the complex structures of HES have been destroyed, they will loss of osmotic support property. They will lose of viscosity and will be excreted by renal system.

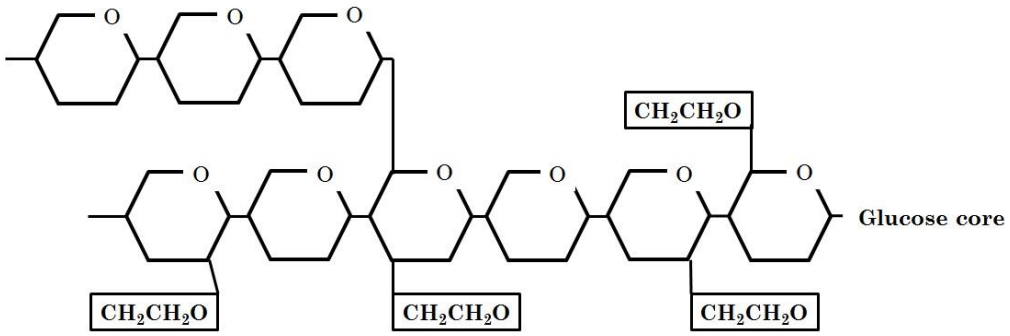

Figure 2 Structure of HES (modified from Stolzing et al., 2012) there are some hydroxyethyl groups on glucose core that connect with covalent bonds. Hydroxyethyl groups can deposit on carbon atom at C2, C3 or C6.

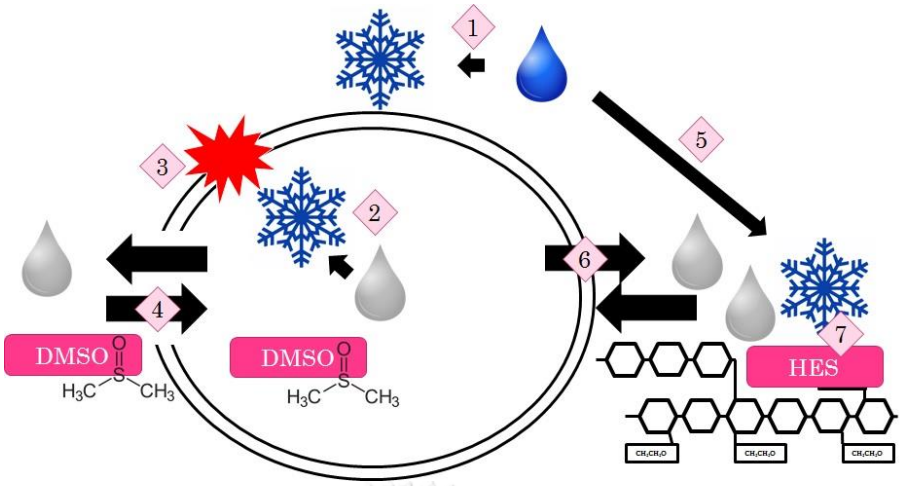

Figure 3 Cryoprotective mechanism of HES in cryopreservation compared with DMSO during cooling (modified from Stolzing et al., 2012).

HES principally protects cells by stabilization of extracellular osmolarity changes during cooling. HES, as large molecule, also maintains its structure around cells resulting water to diffuse across plasma membrane. The reduction of intracellular water tremendously decreases intracellular ice formation during freezing. The HES synergistically acts with the action of penetrating cryoprotectants such as DMSO. HES is commonly used as plasma substitutes in order to stabilize the blood osmotic pressure and blood pressure. However, the HES has also beneficially been used for cryopreservation of somatic cells and sperm. The experimental studies on cryopreservation is summarized in Table 1. It has been used to substitute animal biological product such as bovine serum albumin (BSA) that may carry infection agents such as hepatitis virus. The effect of HES (10 mg/ml) was similar to BSA when incubated with human sperm in terms of the percentage of sperm recovery, acrosome integrity and sperm motility (Matson and Tardif, 2012). HES has also been used in combination of permeable CPA (glycerol) for cryopreservation of ram sperm. The results indicated that HES (16% of saturate HES with egg yolk and glycerol) had beneficial effects on post-thawed sperm quality (Schmehl et al., 1986). However, the data of HES on quality of equine sperm is limited. Oldenhof et al. (2013) demonstrated that HES at increasing concentration (20 %) could improve freezing

ability of equine sperm. Nevertheless, the effect of HES on fertility has not been tested.

Table 1. Summarizing data contributing the use of HES on cryopreservation of somatic cells and sperm

Cell type		Effective concentration and property	Source of HES	Combination CPA	Result	Reference	Remark
Red blood cell	Human	14%(w/v) HES MW=150 kDa DS=0.75	Mc- Gaw Laboratories, Glendale, CA, Lot #PIP005	-	High cell recovery and saline stability in small (25ml) and large volume (405ml). The recovery cell is 99.2% and 97.2% respectively	(Lionetti and Hunt, 1975)	No comparison to DMSO
	Canine	2.5, 7.5, 12.5, 17.5, 22.5%(w/v) HES MW=200 kDa	Ajinomoto Co.Inc., Tokyo	-	12.5% HES has low post-thawed hemolysis, high saline stability and good osmotic fragility (compared to 20% glycerol)	(Heejaung et al., 2004; Kim et al., 2005)	
	Human Monocyte	0, 10, 20, 30, 40, 50% (w/w) HES MW=450 kDa	CV Labs, Riverside, CA	-	70% monocytes survived with 20% HES.	(Takahashi et al., 1988)	
White blood cell	Human Granulocyte	4%(w/v) HES MW=150 kDa DS=0.75		5%DMSO	4%HES give 50% cell recovery compared to 4%HES+5%DMSO	(Kenmochi et al., 2008)	
Other cells	Chinese hamster cell	10, 15, 20%(w/v) HES MW=450 kDa & 150 kDa	-	10% FCS	Not different to 10% DMSO	(Ashwood-Smith et al., 1972)	

Table 1. Summarizing data contributing the use of HES on cryopreservation of somatic cells and sperm (continue)

Cell type		Effective concentration and property	Source of HES	Combination CPA	Result	Reference	remark
Sperm	Human	10mg/ml HES	Sigma (H6382)	-	HES is not different with BSA supplement (control) when measure the percentage of sperm recovery. the total motility of sperm after incubating in 37C for 18 hours is slightly decrease but not significantly when compare to the first 2 hours of incubation period	(Matson and Tardif, 2012)	Not cryopre-servation
	Ram	3.9g/100ml for chilled 16% of saturation HES for frozen	Sigma (H6382)	4% Glycerol Egg yolk	motility of sperm in 3.9g/100ml HES was not decrease when chill in 5C 16% of saturate HES increase the post-thawed motility compare with control group. HES with glycerol is better than extender without glycerol.	(Schmehl et al., 1986)	freezing sperm by dry ice pellet technique
	Horse	10% HES MW=70 kDa	Leopold, Graz, Australia	-	Post-thaw motility increased with increasing of HES, reaching 20% with 10% HES	(Oldenhof et al., 2013)	

CHAPTER III

MATERIALS AND METHODS

Experimental Design

Experiment 1: effect of HES on sperm quality during cold storage

The semen collected from six stallions was divided to five aliquots and incubated with non-fat dry milk extender with different concentrations of HES (2.5%, 5%, 10% and 15%, w/v). The semen incubated with NFDM without HES served as a control group. The sperm were maintained in different conditions at 4 °C for 48 h. The sperm quality was examined at 0, 24 and 48 h after cold storage. The parameters for sperm evaluation included the total and progressive motility, viability, acrosomal integrity and functional membrane integrity and DNA integrity

Experiment 2: effect of HES on sperm quality following cryopreservation and thawing

The semen was collected from six stallions, aliquot to five groups and then randomly allocated to freeze with freezing extender containing different concentrations of HES (2.5%, 5%, 10%, and 15%, w/v). The semen frozen without HES served as control. After cryopreservation, the sperm were thawed and analyzed for sperm quality at 0 hour (within 10 min after thawing, 1 hour, 2 hours and 4 hours after thawing. The parameters for quality analysis included the total and progressive motility, viability, acrosomal integrity, functional membrane integrity, DNA integrity and longevity test.

Materials and methods

Animals

Stallions

The samples were collected from six fertility-proven stallions at the First Livestock and Agriculture Division, Veterinary and Remount Department, Thai army, Kanchanaburi province. The breeds of the stallions were Oldenburg, Warmblood, Irish Sport Horse and Holsteiner. Their ages were ranged between 5 – 15 years old and the weights were approximately 400 – 500 kg. The stallions were fed with concentrate food for three times a day with water and pangola grass ad libitum.

Semen collection

The stallion's semen was collected by artificial vagina (AV) (Missouri model, Manufactured by: NASCO). The phantom mare was used. The AV was filled with approximately 1 liter of 45 – 50 °C water. The amount of water added in the AV was adjusted for individual stallion. The semen collection from each stallion was performed at least 3 replicates with at least 2 days intervals. After semen collection, the semen was observed for volume and color. The semen was filtered through sterile gauze to remove gel fraction of the semen. The gel free fraction was extended with non-fat dry milk extender prior to semen evaluation. The evaluations of the semen included sperm morphology and motility. The semen samples having less than 70% normal head of sperm morphology, 50% normal tail morphology and 50% progressive motility were excluded from this study. These parameters are based on previous reports that fertile stallions should have around 50% normal sperm morphology (Parks and Lynch, 1992). In addition, high percentages of abnormal sperm (>60%) or head defect (>30%) critically affect to fertility (Parks and Lynch, 1992; Stolzing et al., 2012).

The semen to be used for the study was centrifuged at 26 °C for 10 min in order to remove excessive seminal plasma. The sperm pellet was resuspended with non-fat dry milk extender and transported to the laboratory at 4 °C using Equitainer. This device gradually decreases temperature of the samples to 4 °C at a cooling rate of -0.05 °C/min. The semen samples were delivered to the laboratory within 4 – 6 hours post semen collection.

Preparation of semen extender

The non-fat dry milk glucose-based semen extender (NFDM) was used for semen washing and cold storage. This extender composed of glucose monohydrate 49 g, non-fat dry milk powder 24 g, sodium bicarbonate 1.5 g, penicillin (100 unit/ml) and streptomycin (100 µg/ml) in 1,000 ml deionized water. The osmolalities of NFDM measured by micro-osmometer (The FISKE® Micro-Osmometer model 210, USA) were 350 to 360 mOsm/kg. The extender used for experiment 1 was NFDM extender supplemented with different concentrations of HES 0, 2.5%, 5%, 10% and 15%. The HES was purchased from Sigma-Aldrich chemicals.

The freezing extender used was prepared using glucose EDTA based medium supplemented with 20% (v/v) egg yolk. The primary extender was composed of 6 g sorbitol, 0.37g Na acetate, 0.37g Na EDTA, 0.12g sodium bicarbonate, penicillin (100 unit/ml) and streptomycin (100 µg/ml) in 100 ml. The secondary extender composed of primary extender, 11% (v/v) lactose EDTA, 20% (w/v) egg yolk plasma, 0.5% (v/v) Equex STM and 4% (v/v) glycerol. This freezing extender was supplemented with 0%, 2.5%, 5%, 10% and 15%, respectively.

Hydroxyethyl starch (HES)

This study used hydroxyethyl starch from Sigma Aldrich. The Product Number is H6382 (CAS Number: 9005-27-0 MDL: MFCD00131356). The appearance of chemical was white powder, pH 7.0 – 8.0 in 20% slurry solution. It was prepared from corn starch. The Degree of substitution is average of 1 -OH group per 10 glucose units converted to -OCH2CH2OH. The solubility in water has been reported as less than 20 mg/mL and a 100 mg/mL preparation in water forms an opalescent suspension. There is no measured molecular weight but based on the starch starting material, it is assumed to be greater than 1 million.

Semen cryopreservation and thawing

The semen to be cryopreserved was diluted with semen extender (NFDM). The semen was centrifuged, and the pellet was resuspended with freezing extender

to make a final concentration of 200 x10^6 sperm/ml. The semen was equilibrated with freezing extender for 60 min. The post-equilibrated sperm was examined for motility prior to cryopreservation. The semen was then loaded into a 0.5 ml freezing straw and layered in the nitrogen vapors, 4 cm above liquid nitrogen level. The sperm was frozen in this condition for 10 min and the straws was plunged into liquid nitrogen. The frozen semen was stored in liquid nitrogen for approximately 1 week prior to thawing. The frozen semen was thawed in 37 ºC water bath for 30 sec. The liquefied semen was released into a 1.5 ml microtube and resuspended with NFDM extender prior to semen evaluation.

Sperm evaluation

Sperm morphology

Head morphology

The semen was smeared on a glass slide and air-dried. William's staining was performed. The head morphology of the sperm was examined under a light microscope at 1000x magnification. A total of 200 sperm was assessed and classified as either normal and abnormal morphology (Brito, 2007).

Tail morphology

The semen was diluted and fixed in formal saline solution at a ratio of 1:20 (sperm: formal saline). A droplet of fixed sample (10 µl) was dropped onto a glass microscopic slide. Tail morphology was classified under a light phase-contrast microscope at 400x – 1000x magnification. The normal and abnormal sperm tail was identified from 200 sperm (Brito, 2007).

Motility

The sperm motility was evaluated by subjective evaluation. The sample was placed on a microscopic slide under the cover glass. The motility was examined for at least four to five fields under a light microscope at 100x and 400x magnification. The progressive motility was recoded as percentage of sperm that moved straightforward in relation to the total sperm analyzed (Parks, 1997).

Sperm concentration

The semen samples were first fixed and diluted with appropriate amount of formal saline (1:20 – 1:100 (v/v)). The diluted sample was loaded into a chamber of hemocytometer. The sperm heads were counted (5 small squares) at 400x magnification and recorded. The sperm concentration was calculated and expressed as the number of sperm $x10^6$per ml (Aurich et al., 1997).

Sperm viability

The sperm viability (integrity of sperm plasma membrane) was assessed using dual fluorescence probes. The mixture of fluorescent probes consists of 4 μM calcein and of 2 μM ethidium homodimer-1. The probes were mixed with the sperm sample at a ratio of 1: 1. Then the samples were incubated at 37ºC for 10 min. The fluorescent labelled sperm were examined using a fluorescent microscope. The red colored sperm head indicated the leakage or disruption of sperm plasma membrane (dead sperm), while green colored sperm indicted the presence of esterase enzyme activity (alive sperm) (Douglas-Hamilton et al., 1984). In this study, the only green sperm represented intact plasma membrane, while the green-red and red sperm indicated non-intact plasma membrane.

Functional membrane integrity (hypo-osmotic swelling test)

The plasma membrane function integrity was measured by hypoosmotic swelling test (HOS test). The sperm sample (25 μl) was incubated at 37 °C with sucrose-based HOS solution (250 μl, 100 mOsm/kg) for 30 min. The HOS solution with 5% formaldehyde used for inactive the action of HOS medium. Sperm with swollen or coiled tails indicated the functions of sperm plasma membrane to respond to the hypoosmotic condition. The number of swollen tailed sperm were counted and expressed as percentage relative to the total sperm numbers (Pamornsakda et al., 2011).

Acrosomal integrity

The sperm sample was smeared as small circle area on a glass microscopic slide. After air-drying, the sperm plasma membrane was permeabilized with 95%

ethyl alcohol for 30 seconds and air dried. The fluorescein (FITC) conjugated the peanut agglutinin used to localize the outer acrosomal membrane. The Ethidium homodimer-1 used as a counter staining. After staining, the sperm were examined using an epidfluorescent microscope at 1000 magnification. The sperm were classified as intact acrosome, partial damage, severe damage and completely loss. The sperm with intact acrosome presented a bright green color over an acrosomal region. The sperm with patchy pattern of the acrosome indicated the damage of acrosomal membrane, while the sperm only red colored nucleus (without FITC PNA staining) indicated the complete loss of acrosome (Medeiros et al., 2002).

DNA integrity

The sperm was evaluated the DNA integrity by Acridine Orange (AO) fluorescent staining under the fluoromicroscope. The semen was smeared on a glass slide and air dried. The slide containing sperm was fixed in Carnoy's solution (30 ml methanol and 10 ml glacial acetic acid) for overnight. Then, the slide was soaked into the Acridine Orange mixure (10 ml of 1% (w/v) AO solution, 40 ml of 0.1 M citric acid and 2.5 ml of 0.3 M $Na_2HPO_4.7H_2O$) for 10 min and then rinsed with distilled water. A total of 200 sperm was examined with a fluorescence microscope at 1000 magnification. The normal sperm was demonstrated in green color and fragmented DNA was shown in yellow to red color.

Longevity test

Longevity test of sperm used to assess ability of sperm to survive during a time-course of incubation. The frozen-thawed sperm were washed free from cryoprotectant. Then resuspended the sperm sample with non-fat dry milk extender and evaluated for sperm viability and motility at 0 (10 min), 1, 2 and 4 h post-thawing. The progressive motility and viability were recorded as percentage. (Aurich et al., 1997; Cocchia et al., 2011)

Statistical analysis

Data analysis was performed by SPSS statistic program (version 22). Descriptive statistic was described the specific data of each stallion including age and

quality of semen. The results are presented by means and standard error of the mean (SEM). The effects of HES on semen quality (motility, viability, acrosome integrity, DNA integrity, functional membrane integrity (HOS test) and longevity test) were statistically analyzed by One Way Analysis of Variances (ANOVA). Logarithm transformation was performed when the data were not normally distributed. The Kruskal-Wallis One Way ANOVA and Mann-Whitney U Test were combined to analyze the non-parametric data. In all cases, P-value <0.05 was considered statistical significant.

The scope of study

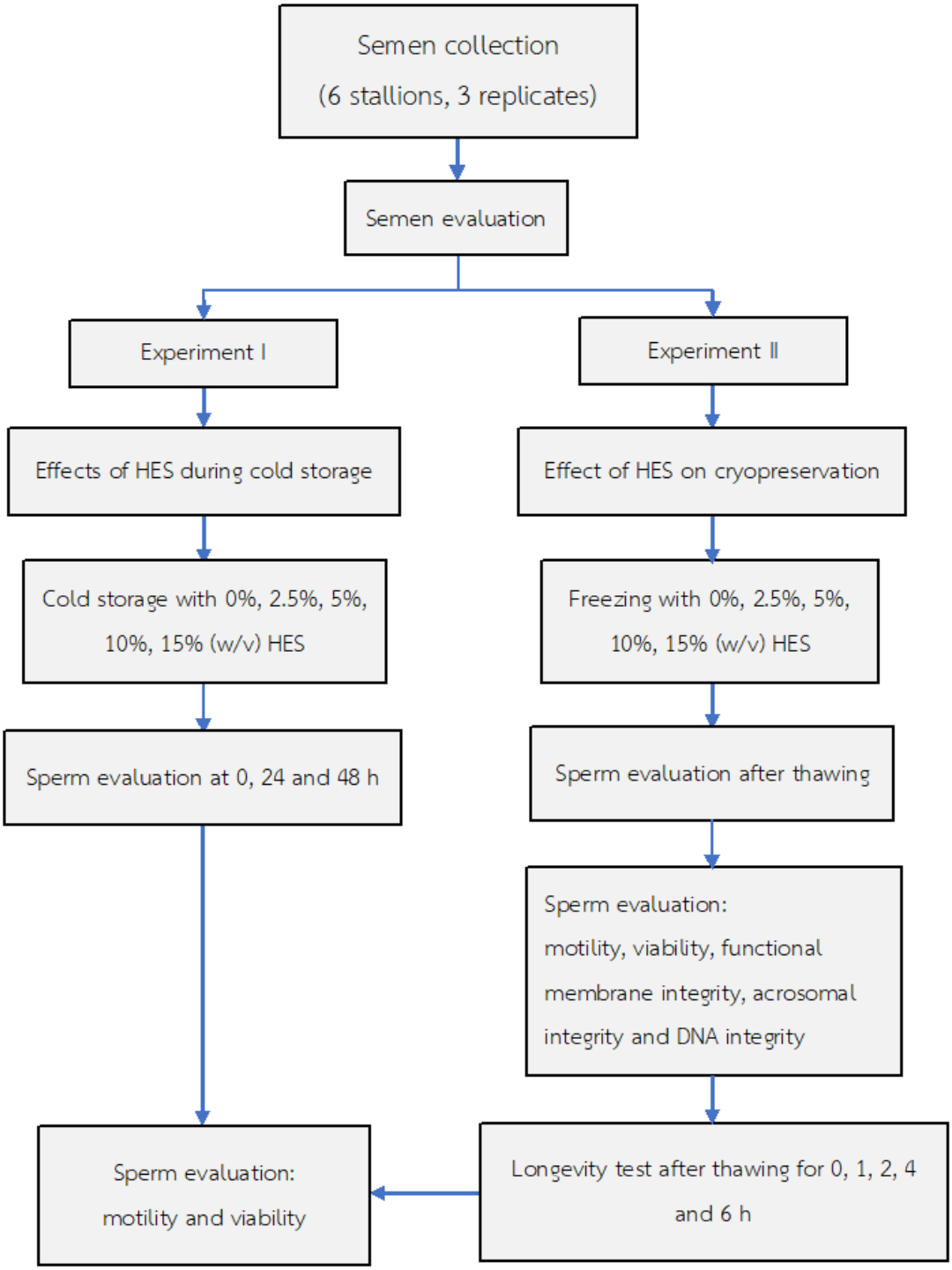

Figure 4 Diagram representing the experimental design

CHAPTER IV

RESULTS

The 18 semen samples from six stallions (3 ejaculates per stallion) were used in experiment 1 and experiment 2. The quality of ejaculated semen is descriptively described in Table 2. The quality of stallion semen used in the current study was within range of semen normally obtained from fertile stallions and acceptably for semen cryopreservation.

Table 2: Semen characteristics and quality of fresh stallion semen (mean ± SEM) (n = 6 stallions).

Horse No.	Age (year)	Volume (ml)	Motility (%)	Concentration ($x10^6$/ml)	Total sperm per ejaculation ($x10^6$/ml)	Normal morphology	
						Head (%)	Tail (%)
1	11	32.50±4.33	65.00±2.89	294.00±60.35	8.97±1.30	85.00±2.46	61.63±3.07
2	7	14.25±3.61	83.75±2.39	262.50±74.51	20.74±6.70	92.13±0.66	87.25±3.31
3	12	27.50±6.61	85.00±1.25	281.25±12.15	11.96±2.49	91.38±3.14	73.63±3.82
4	5	11.33±2.33	90.00±0.00	699.75±264.63	30.55±7.32	87.75±5.74	80.67±0.88
5	8	34.00±19.00	70.00±10	304.00±4.50	12.92±7.08	87.75±4.25	61.50±7.50
6	7	23.38±5.38	73.75±5.54	129.63±74.84	47.83±12.06	87.63±5.74	64.38±6.59
Average	8.33±1.09	23.45±2.85	78.10±2.43	391.71±61.16	21.82±3.71	88.78±1.49	72.02±2.71

The semen was extended in NFDM extender and transported to the laboratory within 6 hours. Table 2 demonstrates that short-term cold storage did not significantly affect to semen quality as the averages of sperm motility before and after transportation were comparable (78.10±2.43% versus 76.39±5.47%, respectively).

Table 3: The average quality (mean ± SEM) of stallion semen (n = 18 replicates) chilled for 6 hours

Horse No.	Motility (%)	Viability (%)	Membrane functionality (%)	Acrosome integrity (%)	DNA integrity (%)
1	62.50±2.50	68.38±2.07	57.75±4.66	62.25±2.17	83.63±7.02
2	80.00±4.08	77.25±4.58	66.38±1.28	80.88±6.12	96.13±1.31
3	82.50±2.50	74.50±3.58	49.88±5.78	72.88±7.24	90.88±2.95
4	70.00±7.07	73.38±3.48	58.25±5.14	80.13±2.10	92.00±4.14
5	75.00±15.00	78.00±4.50	75.00±3.00	73.00±6.00	90.25±1.06
6	88.33±1.67	84.67±0.60	70.33±2.45	89.5±4.09	92.67±3.33
Average	76.39±5.47	76.03±3.14	62.93±3.72	76.44±4.45	90.92±1.73

The experiment 1: effects of different concentrations of HES on sperm quality during cold storage

The semen collected from six stallions were divided to five aliquots and incubated with non-fat dry milk extender with different concentrations of HES supplement (0, 2.5%, 5%, 10% and 15% w/v). The sperm motility and viability quality were examined at 6, 24 and 48 hours respectively.

The motility and viability of chilled semen (Tables 4 and 5) was gradually decreased over time of cold storage. Although HES could not maintain sperm quality during cold storage (6 to 48 hours) to the levels similar to that of non-chilled semen (0 hour), HES supplementation in the NFDM extender improved sperm quality in terms of motility and viability (Table 4 and 5). HES supplementation at concentrations of 2.5 and 5% (w/v) into NFDM extender had no effect on sperm motility during cold storage for 24 hours compared with control (without HES, $P>0.05$). The HES at 2.5% significantly improved sperm motility compared with the controls when examined at 48 hours of cold storage. However, these HES concentrations only tended to improve sperm motility at 24 hours of cold storage (50.00±3.76 and 46.92±4.29 vs. 43.08±3.98 for 2.5%, 5% and control, respectively, $P=0.482$). In all examination times, high concentrations of HES (10 and 15% HES) decreased sperm motility over times of cold storage (Table 4, $P<0.05$).

Table 4: The progressive motility (mean ± SEM) of chilled sperm incubated with non-fat dry milk extender and different concentrations of HES (n = 18 replicates).

HES (%)	progressive motility (%)			
	0 hour (10 min)	6 hours	24 hours	48 hours
0	79.33 ± 1.82^{Aab}	62.86 ± 3.04^{Ba}	43.08 ± 3.98^{Ca}	8.93 ± 3.06^{Dac}
2.5	82.67 ± 1.53^{Aa}	72.14 ± 2.99^{Ba}	50.00 ± 3.76^{Ca}	20.71 ± 4.32^{Db}
5	78.00 ± 2.23^{Aa}	63.57 ± 3.08^{Ba}	46.92 ± 4.29^{Ca}	16.43 ± 4.34^{Dab}
10	69.33 ± 2.06^{Abc}	50.71 ± 3.05^{Bb}	30.77 ± 3.99^{Cb}	5.36 ± 2.43^{Dcd}
15	66.67 ± 1.87^{Ac}	42.86 ± 3.70^{Bc}	11.92 ± 2.86^{Cc}	1.07 ± 0.57^{Dd}

Within column lowercase (a,b,c,d) and within row uppercase (A,B,C,D) superscript letters indicate values that differ statistically ($P<0.05$)

The sperm viability during 24 hours of cold storage improved when HES was added at concentration of 2.5% compared with the control ($P>0.05$, Table 5). Increasing HES concentrations in the semen extender, however, negatively affected to sperm viability (Table 5).

Table 5: The plasma membrane integrity (viability) (mean ± SEM) of equine semen cooled with non-fat dry milk extender and different concentrations of HES (n = 18 replicates).

HES (%)	Mean viability (%)		
	6 hours	24 hours	48 hours
0	64.25 ± 3.91^{Aa}	46.33 ± 3.86^{Ba}	18.75 ± 4.31^{Cabc}
2.5	78.87 ± 4.23^{Ab}	58.83 ± 2.78^{Bb}	30.63 ± 4.29^{Ca}
5	66.88 ± 4.36^{Aab}	48.42 ± 2.61^{Ba}	26.50 ± 4.53^{Cab}
10	62.75 ± 3.69^{Aa}	43.92 ± 2.75^{Ba}	18.50 ± 4.12^{Cbc}
15	60.70 ± 2.23^{Aa}	23.25 ± 2.81^{Bc}	8.18 ± 2.03^{Cc}

Within column lowercase (a,b,c,d) and within row uppercase (A,B,C,D) superscript letters indicate values that differ statistically ($P<0.05$).

The experiment 2: effect of HES on sperm quality following cryopreservation and thawing

The semen was randomly aliquoted into five groups and were frozen with freezing extender containing different concentrations (w/v) of HES (2.5%, 5%, 10%, and 15%). The sperm frozen without HES (0 HES) served as a control group. After cryopreservation, the sperm were thawed and analyzed for sperm quality within 10 minutes (0h) and the longevity of post-thaw sperm was performed for 6 hours.

Cryopreservation and thawing of equine sperm generally decreased sperm quality in all parameter analyzed. Concentrations of HES added in the freezing medium significantly influenced the post-thaw sperm quality. The HES at 2.5% was found to be optimal as this concentration significantly improve sperm quality in terms of motility (41.54±2.49 vs. 33.85±2.66), viability (51.86±3.42 vs. 37.91±3.72) and also acrosome integrity 40.33±4.91 vs. 29.13±2.46) when compared with the control ($P<0.05$). In addition, membrane functionality (HOS-test) and DNA integrity tended to be improved ($P= 0.768$ and $P= 0.163$, respectively). As similar to the results obtained from cold storage semen, increasing concentrations above 2.5% had no further improvement in sperm quality (Table 6). High concentrations of HES especially the 10% and 15% HES adversely affected to sperm quality.

Table 6: The quality (mean ± SEM) of post-thaw semen with different concentrations of HES supplemented extender (n = 18 replicates).

HES (%)	Motility (%)	Viability (%)	Membrane functionality (%)	DNA integrity (%)	Acrosome integrity (%)
0	33.85±2.66^{a}	37.91±3.72^{ac}	31.13±4.14^{ab}	87.94±1.66^{abc}	29.13±2.46^{a}
2.5	41.54±2.49^{b}	51.86±3.42^{b}	32.69±3.25^{a}	91.19±1.52^{a}	40.33±4.91^{b}
5	33.85±3.50^{a}	43.05±3.88^{ab}	27.81±2.77^{a}	88.34±2.49^{ab}	29.05±3.74^{a}
10	19.23±2.88^{c}	36.27±3.53^{ac}	25.06±2.53^{ab}	87.25±2.17^{abc}	19.04±2.01^{c}
15	5.77±2.03^{d}	29.45±3.38^{c}	18.31±2.22^{bc}	84.38±1.79^{c}	10.27±1.52^{cd}

Within column, lowercase (a,b,c,d) superscript letters denote values that differ statistically (P<0.05).

The results for longevity during storage for 6 hours after thawing are shown in tables 7 and 8. Overall, sperm longevity decreased over period of sperm incubation for 6 hours. At 1 hour post-thawing, sperm frozen with 2.5% HES tended to have higher progressive motility when compared with control and 5% HES group (Table 7). The advantage of 2.5% HES in protecting sperm against cryoinjury during freezing and thawing by means of motility was more pronounced at 2 and 4 hours post-thawing. At these time points, the 2.5% HES resulted in significant higher motility compared with the control non-HES group (P<0.05). However, the means motility obtained from sperm frozen with 2.5% HES were not significantly different from 5% HES group (P>0.05). Although no significant improvement of sperm motility of 2.5 and 5% HES groups was obtained, the sperm had higher motility 17.50±4.91 and 13.13±3.89 than the control (9.38±2.74, P>0.05). In all periods of examination, high concentrations of HES (>10%) were detrimental to sperm motility (Table 7).

Table 7: The longevity test by mean of progressive motility (mean ± SEM) of equine sperm frozen with different concentrations of HES (n = 18 replicates).

HES (%)	Mean Motility (%)			
	1 hour	2 hours	4 hours	6 hours
0	33.08 ± 2.37^{Aab}	25.71 ± 1.72^{Bac}	12.50 ± 2.50^{Cac}	9.38 ± 2.74^{Cabc}
2.5	44.62 ± 2.68^{Aa}	37.86 ± 2.80^{b}	24.17 ± 2.88^{Bb}	17.50 ± 4.91^{Ba}
5	37.69 ± 3.23^{Aa}	33.57 ± 3.87^{Aab}	19.17 ± 3.58^{Bab}	13.13 ± 3.89^{Babc}
10	25.38 ± 2.15^{Ab}	20.36 ± 2.13^{Ac}	11.67 ± 1.98^{Bac}	5.00 ± 1.64^{Bbc}
15	14.23 ± 2.93^{Ac}	11.07 ± 1.83^{Aad}	6.25 ± 2.23^{BCcd}	3.13 ± 1.62^{Ccd}

Within column lowercase (a,b,c,d) and within row uppercase (A,B,C,D) superscript letters indicate values that differ statistically ($P<0.05$).

As similar to the results obtained for motility assessment, the viability of sperm gradually decreased over time of examination. The equine sperm frozen with 2.5% and 5% had higher viability than the control when examined during 1 to 4 hours post-thaw ($P>0.05$, Table 8). At 6 hours post-thawing, sperm cryopreserved with 2.5% HES had significantly higher viability than of the control (37.19±3.50 vs. 27.88±3.30, $P<0.05$). Increasing HES concentrations to 5 and 10% had no further effect to protect sperm viability ($P>0.05$).

Table 8: The longevity test by mean of viability (mean ± SEM) of equine sperm frozen with different concentrations of HES (n = 18 replicates).

HES (%)	Mean viability (%)			
	1 hour	2 hours	4 hours	6 hours
0	38.75 ± 4.61^{ABab}	41.73 ± 4.45^{Aab}	31.50 ± 3.60^{BCab}	27.88 ± 3.30^{Cac}
2.5	46.28 ± 4.42^{Aa}	50.62 ± 2.89^{Aa}	37.53 ± 4.50^{Ba}	37.19 ± 3.50^{Bb}
5	43.66 ± 4.06^{Aa}	43.46 ± 4.03^{Aab}	36.53 ± 3.10^{ABa}	33.75 ± 2.64^{Bab}
10	39.03 ± 2.62^{Aab}	39.19 ± 2.84^{Ab}	33.13 ± 2.11^{Bab}	33.86 ± 1.95^{Bab}
15	31.50 ± 3.03^{ABb}	33.92 ± 2.72^{Ab}	26.30 ± 2.87^{Bb}	28.88 ± 2.80^{ABabc}

Within column lowercase (a,b,c,d) and within row uppercase (A,B,C,D) superscript letters indicate values that differ statistically ($P<0.05$).

CHAPTER V

DISCUSSION

It has been well accepted that equine sperm are sensitive to cold stress in particular the cryopreservation and thawing (Amann and Pickett, 1987). These shortcomings result in poor sperm quality and subsequently low pregnancy rate after insemination (Amann and Pickett, 1987; Gibb and Aitken, 2016). It is therefore necessary to understand cryoinjury that occurs during cryopreservation and thawing. Furthermore, study the effect of cryoprotective agents added into freezing medium has become a practical way for improving post-thaw quality of equine sperm. This study demonstrated the protective effects of HES on sperm quality following cold storage and cryopreservation. However, the effects were found to be in a manner of dose-dependence. It was clearly demonstrated in the experiment 1 that equine sperm could withstand the cold stress for a short period of time (6 hours) as the sperm quality did not significantly affect to sperm quality. This result is similar to that observed in a previous study (Douglas-Hamilton et al., 1984). However, long-term cold storage of equine sperm negatively affected to sperm quality (Medeiros et al., 2002). The factors that contribute to cold induced sperm damage have been demonstrated including individual variation of animals (Amann and Pickett, 1987), cooling rate (Brinsko et al., 2000), compositions of extender (Medeiros et al., 2002; Cocchia et al., 2011) and duration of semen storage (Aurich, 2008; Gibb and Aitken, 2016). It seemed likely that the sperm obtained from stallions used in the study were fairly sensitive to cold stress as the sperm quality in terms of motility and viability at 48 hours was poor (Tables 4 and 5). Although sperm quality and pregnancy rates after insemination with 48 hours cold stored are generally acceptable in practice (Jasko et al., 1992; Aurich, 2008), semen used in the study may suffer from temperature fluctuations from time-lapse examination. The mammalian sperm are sensitive to abrupt change of temperature (Brinsko et al., 2000). The

damages caused by low temperature is known as cold shock, which affects to the quality of sperm including motility and structures of the sperm (Parks, 1997; Medeiros et al., 2002).In addition, it has also been recommended to rotate the extended semen in order to prevent sperm precipitation that may potentially generate or accumulate the oxidative stress (Ball, 2008).

In the current study, the results indicated that HES concentrations affected to quality of sperm during cold storage even the extender with different concentrations of HES had no different in osmolarity. The HES at concentrations of 2.5 and 5% had no detrimental to sperm motility and viability during 24 hours of cold storage. The 2.5% HES significantly improved sperm viability when compared with control (no HES). However, the motility did not significantly differ. The results are in an agreement with previous report in chilled ram semen indicating that HES could help to maintain sperm quality (Schmehl et al., 1986). It is likely that HES appear to protect the sperm plasma membrane (viability) by modulating fluid surrounding the sperm cells. This protective effect of HES was found to be in a manner of dose dependence as high concentrations of HES negatively affected to sperm quality. The HES used in the study have high molecular weight with high stability (Körber and Scheiwe, 1980; Stolzing et al., 2012). Although the HES added into the medium did not affect to medium osmolarity, it could form a large complex molecule with water. The medium therefore becomes more viscous (Oldenhof et al., 2013) and sperm may require more energy necessary for progressive motility. In addition, HES molecules naturally absorb water around the cells (Körber and Scheiwe, 1980) and subsequently cover the cell plasma membrane (Stolzing et al., 2012). It is therefore possible that extensive water loss may have occurred in the sperm maintained with high concentrations of HES (HES 10% and 15%). This condition potentially alters sperm hydration status or generates excessive osmotic stress, thereby damaging sperm membrane structure.

For cryopreservation, equine sperm have been demonstrated to sensitive to cold shock due principally to the low ratio of phospholipids and cholesterols within the plasma membrane when compared with other species (Parks and Lynch, 1992;

Pamornsakda et al., 2011). The damage caused by the cold stress or cryopreservation frequently results in disruption of sperm structures and alters the sperm functions. The present study revealed the positive effect of HES on sperm quality post-thaw. However, the similar results of concentration dependency were observed. HES at concentration of 2.5% in the freezing medium was optimal for cryopreservation of stallion sperm, while high concentrations of HES (more than 10% w/v) were detrimental to sperm quality. Although the results obtained in this study are in an agreement with other studies that the HES at optimal concentration could protect sperm from cryoinjuries, the optimal concentration (2.5% HES) used for cryopreservation of stallion sperm in this study was however different from other previous reports. For examples, 10% HES was found to be optimal for maintaining phase transition of equine sperm plasma membrane during cooling (Oldenhof et al., 2013). In other species, the concentrations of HES have been reported to be between 5 to 16% in ram sperm (Schmehl et al., 1986; Oldenhof et al., 2013). It is therefore likely that several factors interplay with the HES molecules and the effectiveness and toxicity of the HES can be variable depending on species and the molecular weights (types) of the HES. Furthermore, interaction of HES and other medium compositions such as intracellular cryoprotective agents play a predominant role in determining the HES effectiveness (Schmehl et al., 1986).

For longevity test, the equine sperm collected from 6 stallions had post-thaw sperm quality within the international standard (>30% post-thaw motility). The results also clearly demonstrated that HES supplement in the freezing medium could also influence on sperm longevity for up to 6 hours. This 6-hour interval is normally used in practice for efficient insemination with an acceptable pregnancy rate (WOODS et al., 1990). The current study found that supplement 2.5% HES in the freezing extender was found to be best to maintain sperm longevity for at least 6 hours (Table 8) because the sperm viability was significantly higher than the control (no HES). The improvement of quality of frozen-thawed sperm is important to increase fertility rate after insemination. However, it is still necessary to use the sperm frozen with HES for insemination in order to test the sperm functionality. From the previous

study in equine sperm indicated that the optimum concentration of HES was 10% with INRA82 extender. They used HES molecular weight of 70 kDa (Leopold, Graz, Austria) (Oldenhof et al., 2013). However, this experiment used >1,000 kDa HES in conventional freezing extender. The different optimum concentrations found in the two studies were likely due to confounding factors such as different molecular weight HES and different compositions of the base extender. In addition, as previous mentioned, the interaction between penetrating cryoprotectant such as glycerol and HES also play a key role to determine HES efficacy or toxicity. The various combinations of HES and different concentrations of glycerol for sperm freezing are of interest in the future. These studies should perform simultaneously with the examination of extracellular and intracellular ice formation as the HES combines with water molecules outside the cells. This binding, in principle, reduces extracellular and intracellular ice formation during cryopreservation and thawing. However, these types of studies have yet to be performed.

In conclusion, equine sperm are sensitive to cold stress during cold storage and cryopreservation. Modifications of cold storage and freezing extenders by supplementation with HES improved sperm quality. However, the protective effects of HES against sperm damage were dependent on concentrations of HES added. HES at a concentration of 2.5% (w/v) demonstrated the highest effectiveness when compared with other concentrations and therefore is the recommened dose for equine sperm. High concentrations of HES between 10 to 15% HES negatively affected to sperm quality during cold storage and cryopreservation. Studies to reveal interactions of other extender compositions and also to determine pregnancy rate after insemination of sperm frozen with HES will be further examined.

REFERENCES

Amann R and Pickett B. 1987. Principles of cryopreservation and a review of cryopreservation of stallion spermatozoa. Journal of equine veterinary science. 7(3): 145-173.

Ashwood-Smith M, Warby C, Connor K and Becker G. 1972. Low-temperature preservation of mammalian cells in tissue culture with polyvinylpyrrolidone (PVP), dextrans, and hydroxyethyl starch (HES). Cryobiology. 9(5): 441-449.

Aurich C. 2005. Factors affecting the plasma membrane function of cooled-stored stallion spermatozoa. Animal Reproduction Science. 89(1): 65-75.

Aurich C. 2008. Recent advances in cooled-semen technology. Animal reproduction science. 107(3-4): 268-275.

Aurich J, Schönherr U, Hoppe H and Aurich C. 1997. Effects of antioxidants on motility and membrane integrity of chilled-stored stallion semen. Theriogenology. 48(2): 185-192.

Ball BA. 2008. Oxidative stress, osmotic stress and apoptosis: Impacts on sperm function and preservation in the horse. Animal reproduction science. 107(3): 257-267.

Boldt J. 2009. Modern rapidly degradable hydroxyethyl starches: current concepts. Anesthesia & Analgesia. 108(5): 1574-1582.

Brinsko S, Rowan K, Varner D and Blanchard T. 2000. Effects of transport container and ambient storage temperature on motion characteristics of equine spermatozoa. Theriogenology. 53(8): 1641-1655.

Cocchia N, Pasolini M, Mancini R, Petrazzuolo O, Cristofaro I, Rosapane I, Sica A, Tortora G, Lorizio R and Paraggio G. 2011. Effect of sod (superoxide dismutase) protein supplementation in semen extenders on motility, viability, acrosome status and ERK (extracellular signal-regulated kinase) protein phosphorylation of chilled stallion spermatozoa. Theriogenology. 75(7): 1201-1210.

Cristanelli M, Squires E, Amann R and Pickett B. 1984. Fertility of stallion semen processed, frozen and thawed by a new procedure. Theriogenology. 22(1): 39-45.

Douglas-Hamilton D, Osol R, Osol G, Driscoll D and Noble H. 1984. A field study of the fertility of transported equine semen. Theriogenology. 22(3): 291-304.

Gibb Z and Aitken RJ. 2016. Recent Developments in Stallion Semen Preservation. Journal of equine veterinary science. 43: S29-S36.

Heejaung K, TANAKA S, Satoshi U, NAKAICHI M, SUMIDA S and TAURA Y. 2004. A comparative study of the effects of glycerol and hydroxyethyl starch in canine red blood cell cryopreservation. Journal of veterinary medical science. 66(12): 1543-1547.

Jasko D, Hathaway J, Schaltenbrand V, Simper W and Squires E. 1992. Effect of seminal plasma and egg yolk on motion characteristics of cooled stallion spermatozoa. Theriogenology. 37(6): 1241-1252.

Johnson L, Blanchard T, Varner D and Scrutchfield W. 1997. Factors affecting spermatogenesis in the stallion. Theriogenology. 48(7): 1199-1216.

Kenmochi T, Asano T, Maruyama M, Saigo K, Akutsu N, Iwashita C, Ohtsuki K, Suzuki A and Miyazaki M. 2008. Cryopreservation of human pancreatic islets from non-heart-beating donors using hydroxyethyl starch and dimethyl sulfoxide as cryoprotectants. Cell transplantation. 17(1-1): 61-67.

Kim H, Itamoto K, Une S, Nakaichi M, Taura Y and Sumida S. 2005. Application of phosphoenolpyruvate into canine red blood cell cryopreservation with hydroxyethyl starch. CryoLetters. 26(1): 1-6.

Körber C and Scheiwe M. 1980. The cryoprotective properties of hydroxyethyl starch investigated by means of differential thermal analysis. Cryobiology. 17(1): 54-65.

Kozek-Langenecker SA. 2005. Effects of hydroxyethyl starch solutions on hemostasis. The Journal of the American Society of Anesthesiologists. 103(3): 654-660.

Lionetti FJ and Hunt SM. 1975. Cryopreservation of human red cells in liquid nitrogen with hydroxyethyl starch. Cryobiology. 12(2): 110-118.

Loomis PR and Squires EL. 2005. Frozen semen management in equine breeding programs. Theriogenology. 64(3): 480-491.

López-González I, Torres-Rodríguez P, Sánchez-Carranza O, Solís-López A, Santi C, Darszon A and Treviño C. 2014. Membrane hyperpolarization during human sperm capacitation. Molecular human reproduction. 20(7): 619-629.

Matson P and Tardif S. 2012. A preliminary search for alternatives to albumin as a medium supplement for the culture of human sperm. Reproductive biology. 12(3): 329-331.

Medeiros C, Forell F, Oliveira A and Rodrigues J. 2002. Current status of sperm cryopreservation: why isn't it better? Theriogenology. 57(1): 327-344.

Mottershead J. 2000. Frozen semen preparation and use. Canadian morgan magazine.

Nicolson GL. 2014. The Fluid—Mosaic Model of Membrane Structure: Still relevant to understanding the structure, function and dynamics of biological membranes after more than 40years. Biochimica et Biophysica Acta (BBA)-Biomembranes. 1838(6): 1451-1466.

Oldenhof H, Gojowsky M, Wang S, Henke S, Yu C, Rohn K, Wolkers WF and Sieme H. 2013. Osmotic stress and membrane phase changes during freezing of stallion sperm: mode of action of cryoprotective agents. Biology of reproduction. 88(3): 68, 61-11.

Pamornsakda T, Pojprasath T, Suwimonteerabutr J and Tharasanit T. 2011. Effects of cholesterol-loaded cyclodextrins on the quality of frozen–thawed equine epididymal sperm. Cryobiology. 63(2): 90-95.

Parks J and Graham J. 1992. Effects of cryopreservation procedures on sperm membranes. Theriogenology. 38(2): 209-222.

Parks JE. 1997. -Hypothermia and Mammalian Gametes. In: Reproductive tissue banking. ed. (ed.). Elsevier. 229-261.

Parks JE and Lynch DV. 1992. Lipid composition and thermotropic phase behavior of boar, bull, stallion, and rooster sperm membranes. Cryobiology. 29(2): 255-266.

Samper J. 2007. methods of inseminating with frozen semen. Florida Association of Equine Practitioners. 118-121.

Schmehl M, Vazquez I and Graham E. 1986. The effects of nonpenetrating cryoprotectants added to TEST-yolk-glycerol extender on the post-thaw motility of ram spermatozoa. Cryobiology. 23(6): 512-517.

Stolzing A, Naaldijk Y, Fedorova V and Sethe S. 2012. Hydroxyethylstarch in cryopreservation–Mechanisms, benefits and problems. Transfusion and Apheresis Science. 46(2): 137-147.

Takahashi T, Hirsh A, Erbe E and Williams RJ. 1988. Mechanism of cryoprotection by extracellular polymeric solutes. Biophysical journal. 54(3): 509.

Treib J, Baron J-F, Grauer M and Strauss R. 1999. An international view of hydroxyethyl starches. Intensive care medicine. 25(3): 258-268.

WOODS J, Bergfelt D and Ginther O. 1990. Effects of time of insemination relative to ovulation on pregnancy rate and embryonic-loss rate in mares. Equine veterinary journal. 22(6): 410-415.

Yeste M. 2016. Sperm cryopreservation update: Cryodamage, markers, and factors affecting the sperm freezability in pigs. Theriogenology. 85(1): 47-64.

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