ผลของทรีฮาโลสต่อความทนต่อแรงดันออสโมติก ความสมบูรณ์ของเยื่อหุ้มเซลล์ และคุณภาพของอสุจิม้าในขณะแช่เย็นและแช่แข็ง

นางสาวดิษยา ศรีนุติยากร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาสรีรวิทยา (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2560

THE EFFECTS OF TREHALOSE ON OSMOTIC TOLERANCE, MEMBRANE INTEGRITY AND QUALITY OF EQUINE SPERM DURING 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 Physiology
(Interdisciplinary Program)
Graduate School
Chulalongkorn University
Academic Year 2017

Thesis Title	THE EFFECTS OF TREHALOSE ON OSMOTIC TOLERANCE, MEMBRANE INTEGRITY AND QUALITY OF EQUINE SPERM DURING COLD STORAGE AND CRYOPRESERVATION
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ดิษยา ศรีนุติยากร : ผลของทรีฮาโลสต่อความทนต่อแรงดันออสโมติก ความสมบูรณ์ของเยื่อหุ้มเซลล์ และคุณภาพของอสุจิม้าในขณะแช่เย็นและแช่แข็ง (THE EFFECTS OF TREHALOSE ON OSMOTIC TOLERANCE, MEMBRANE INTEGRITY AND QUALITY OF EQUINE SPERM DURING COLD STORAGE AND CRYOPRESERVATION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. สพ.ญ. ดร. สุทธาสินี ปุญญโชติ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. น.สพ. ดร. ธีรวัฒน์ ธาราศานิต, 58 หน้า.

การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลการป้องกันของทรีฮาโลสต่อความทนต่อแรงดันออสโมติก และคุณภาพของอสุจิม้าก่อนและหลังการแช่แข็ง น้ำเชื้อม้าถูกเก็บจากพ่อม้าหกตัว (เก็บน้ำเชื้อ 3 ครั้งต่อตัว มีการเคลื่อนที่มากกว่า 50% และสัณฐานวิทยาที่ปกติมากกว่า 70 %) การศึกษาครั้งนี้ ความทนต่อแรงดันออสโมติกของอสุจิม้าต่อการเปลี่ยนแปลงออสโมลาริตีถูกตรวจสอบเป็นอันดับแรก และตามด้วยผลการป้องกันของทรีฮาโลสต่อความทนต่อแรงดันออสโมติกของอสุจิม้า การศึกษานี้ อสุจิได้รับน้ำยา Tyrode's albumin lactate pyruvate (TALP) ที่ออสโมลาริตีแตกต่างกันคือ ออสโมลาริตีที่เท่ากับในเซลล์ (300 มิลลิออสโมลต่อกิโลกรัม; กลุ่มควบคุม) และออสโมลาริตีที่ไม่เท่ากับในเซลล์ (150, 450, 600 และ 750 มิลลิออสโมลต่อกิโลกรัม) และบ่มที่อุณหภูมิ 37 องศาเซลเซียส เป็นเวลา 10 นาที คุณภาพของอสุจิในแง่ของการเคลื่อนที่ การอยู่รอด และการทำงานของเยื่อหุ้มเซลล์ได้รับการประเมิน ผลการทดลองแสดงให้เห็นว่าค่าเฉลี่ยของการเคลื่อนที่ลดลงอย่างมีนัยสำคัญทางสถิติ ในกลุ่มที่มีออสโมลาริตีน้อยกว่าและมากกว่าในเซลล์ ($P<0.05$) เมื่อเทียบกับกลุ่มที่ออสโมลาริตีเท่ากับในเซลล์ ค่าเฉลี่ยของการอยู่รอดและการทำงานของเยื่อหุ้มเซลล์ลดลงในกลุ่มที่มีออสโมลาริตีมากกว่าในเซลล์ แต่ไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติในกลุ่มที่มีออสโมลาริตีน้อยกว่าในเซลล์ ($P>0.05$) นอกจากนี้พารามิเตอร์ดังกล่าวแย่ลงเมื่อออสโมลาริตีเพิ่มขึ้น ข้อมูลข้างต้นชี้ให้เห็นว่าอสุจิม้ามีการตอบสนองต่อการเปลี่ยนแปลงออสโมลาริตี และมีความทนต่อแรงดันออสโมติกที่จำกัด การให้ทรีฮาโลส 100 มิลลิโมลาร์ พบว่าการเคลื่อนที่ของอสุจิ การอยู่รอด และการทำงานของเยื่อหุ้มเซลล์ เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ เมื่อเปรียบเทียบกับกลุ่มที่ไม่มีทรีฮาโลส ($P<0.05$) ยกเว้นการเคลื่อนที่ที่ 650 และ 750 มิลลิออสโมลต่อกิโลกรัม ($P>0.05$) นอกจากนี้การให้ทรีฮาโลสก่อน 100 มิลลิโมลาร์ อสุจิถูกปั่นเหวี่ยงเพื่อนำทรีฮาโลสออกและเผชิญกับออสโมลาริตีที่แตกต่างกัน พบว่าคุณภาพของอสุจิเพิ่มขึ้นในแง่ของการเคลื่อนที่ การอยู่รอด และการทำงานของเยื่อหุ้มเซลล์ ($P<0.05$) ดังนั้นผลการทดลองข้างต้นชี้ให้เห็นว่าทรีฮาโลสช่วยเพิ่มความทนต่อแรงดันออสโมติกของอสุจิม้า ในการศึกษานี้ ผลการป้องกันของทรีฮาโลสต่ออสุจิม้าก่อนและหลังการแช่แข็งถูกศึกษาในลำดับต่อไป ในส่วนแรก กระบวนการแช่เย็น อสุจิม้าได้รับน้ำยา TALP ที่ไม่มีทรีฮาโลส (กลุ่มควบคุม) และมีทรีฮาโลส 100 มิลลิโมลาร์ บ่มที่ 37 องศาเซลเซียส เป็นเวลา 10 นาที ทำให้เย็นลงจนถึง 4 องศาเซลเซียส และคงไว้เป็นเวลา 10 และ 60 นาที เมื่อเปรียบเทียบกับกลุ่มควบคุม (ไม่มีทรีฮาโลส) กลุ่มที่มีทรีฮาโลสเพิ่มคุณภาพของอสุจิ เมื่อคงไว้ที่ 4 องศาเซลเซียส เป็นเวลา 10 นาที ($P<0.05$) อย่างไรก็ตามคุณภาพของอสุจิ (ตัวอย่างเช่นการเคลื่อนที่ การอยู่รอด และการทำงานของเยื่อหุ้มเซลล์) ไม่เปลี่ยนแปลงเมื่อคงไว้เป็นเวลา 60 นาที ($P>0.05$) ส่วนที่สอง กระบวนการแช่แข็ง สารละลายน้ำเชื้อมาตราฐานที่ไม่มี (กลุ่มควบคุม) และมีทรีฮาโลส 100 มิลลิโมลาร์ ถูกใช้เพื่อแช่แข็งอสุจิ อสุจิที่แช่แข็งแล้วถูกละลายที่ 37 องศาเซลเซียส เป็นเวลา 30 วินาที และคุณภาพของอสุจิหลังจากการแช่แข็งถูกประเมินที่เวลา 10 นาที, 2, 4 และ 6 ชั่วโมง ผลการทดลองแสดงว่าคุณภาพของอสุจิม้า รวมถึงการเคลื่อนที่ การอยู่รอด และการทำงานของเยื่อหุ้มเซลล์ ดีขึ้นในสารละลายน้ำเชื้อที่มีทรีฮาโลส มากกว่ากลุ่มที่ไม่มีการเติมทรีฮาโลส ($P<0.05$) จนถึง 6 ชั่วโมงหลังการละลาย โดยสรุปทรีฮาโลสช่วยเพิ่มความทนต่อแรงดันออสโมติกของอสุจิม้า และพัฒนาคุณภาพของอสุจิม้าหลังจากการแช่แข็ง

สาขาวิชา สรีรวิทยา ลายมือชื่อนิสิต .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5887142020 : MAJOR PHYSIOLOGY
KEYWORDS: COOLING, CRYOPRESERVATION, EQUINE, OSMOTIC TOLERANCE, SPERM, TREHALOSE

DISSAYA SRINUTIYAKORN: THE EFFECTS OF TREHALOSE ON OSMOTIC TOLERANCE, MEMBRANE INTEGRITY AND QUALITY OF EQUINE SPERM DURING COLD STORAGE AND CRYOPRESERVATION. ADVISOR: ASSOC. PROF. SUTTHASINEE POONYACHOTI, D.V.M., Ph.D., CO-ADVISOR: ASST. PROF. THEERAWAT THARASANIT, D.V.M., Ph.D., 58 pp.

This study aimed to investigate the protective effects of trehalose on osmotic tolerance and the quality of equine sperm before and after freezing and thawing. Equine ejaculated semen was collected from six stallions (3 ejaculates per stallion; >50% motility and >70% normal morphology). In the present study, the osmotic tolerance limit of equine sperm to osmotic changes was firstly verified, and followed with the protective effects of trehalose on osmotic tolerance of equine sperm. In the study, sperm was exposed to Tyrode's albumin lactate pyruvate (TALP) medium at different osmolalities: isosmolality (300 mOsm/kg; control) and anisosmolality (150, 450, 600 and 750 mOsm/kg) and was incubated at 37°C for 10 min. Sperm quality in terms of motility, viability and membrane functionality were evaluated. Results demonstrated that the average of motility was significantly lower in hypo- and hyperosmolality ($P<0.05$) when compared to isosmolality. The average of viability and membrane functionality were lower in hyperosmolality; however, these were not significantly different in hyposmolality ($P>0.05$). Moreover, these parameters worsen when the osmolality increased. This data indicated that equine sperm responded to osmotic changes and had limited osmotic tolerance. Co-treatment with 100 mM trehalose revealed that sperm motility, viability and membrane functionality significantly increased when compared to those in the absence of trehalose ($P<0.05$); except motility in 650 and 750 mOsm/kg ($P>0.05$). In addition, pre-treatment with 100 mM trehalose, where sperm was centrifuged to remove trehalose and challenged with different osmolalities, increased the sperm quality in term of motility, viability and membrane functionality ($P<0.05$). Therefore, these results demonstrated that trehalose enhanced osmotic tolerance of equine sperm. In the present study, the protective effects of trehalose on equine sperm before and after cryopreservation were further confirmed. In the first part of the cooling process, equine sperm was pre-equilibrated with isosmotic TALP in the absence (control) and the presence of 100 mM trehalose and incubated at 37°C for 10 min, cooled down to 4°C and maintained for 10 min and 60 min. Compared to the control (no trehalose), trehalose increased sperm quality when maintained in 4°C for 10 min ($P<0.05$). However, the sperm quality (i.e motility, viability and membrane functionality) were not significantly changed when maintained for 60 min ($P>0.05$). The second part, during the cryopreservation process, the standard extender were used to cryopreserve sperm in the absence (control) or the presence of 100 mM trehalose. Frozen sperm was thawed at 37°C for 30 second and post-thawed sperm quality was evaluated at 10 min, 2, 4 and 6 h. Results represented that equine sperm quality including motility, viability and membrane functionality had better improved in extender containing trehalose than those without trehalose supplementation ($P<0.05$) up to 6 hours post-thawing. In conclusion, trehalose enhanced osmotic tolerance of equine sperm and improved equine sperm quality after cryopreservation.

Field of Study: Physiology
Academic Year: 2017

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

This study was achieved at the Department of Obstetrics, Gynaecology and Reproduction (OGR), Faculty of Veterinary Science Chulalongkorn University, Bangkok, Thailand and First livestock and agriculture division, veterinary and remount department, Thai army, Kanchanaburi province, Thailand.

I would like to express my sincere gratitude to my advisor, Associate Prof. Dr. Sutthasinee Poonyachoti, who always supports me. She has been a tremendous mentor for me. I would like to thank you for encouraging my research and always being kind to me. I am truly thankful to my thesis co-advisor, Assistant Prof. Dr. Theerawat Tharasanit, who always has been supportive since the beginning to the end of my study. First of all, I would like to thank you for give me an opportunity to be your advisee. Your plentiful advice and dedication on both research as well as on my Master's degree life have been invaluable.

In addition, my appreciation goes to all of the students and staffs at OGR, Inter-Department of Physiology and Department of Physiology, Faculty of Medicine (CU, Thailand) for helping me though my Master's degree. Staff at First livestock and agriculture division, veterinary and remount department, Thai army, Kanchanaburi province, Thailand which provide equine semen sample. I wish to thank Ms. Junpen Suwimonteerabut for the technical support in chemical and equipment preparation. I would especially like to thank to TT's lab members: Dr. Ornwanya Nuchanart, Dr. Piyathip Setthawong, Dr. Apichaya Sudsukh, Dr. Saengtawan Arayatham and Dr. Praopilas Phakdeedindan for support my work, friendship, and share the best memorial time together.

Finally, special thanks to my family, Boat and my beloved friend for always support and stand by my side. Thank you for letting me through all the pressure and difficulties. They are the most important part of my life and my accomplishment.

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LIST OF ABBREVIATIONS

°C	degree Celsius
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar
AI	artificial insemination
ANOVA	Analysis of Variance
AO	Acridine orange
AV	artificial vagina
cm	centimeter
CPA	cryoprotective agent
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EthD-1	Ethidium homodimer-1
FITC-PNA	Fluorescein isothiocyanated peanut agglutinin
g	gram
h	hour
HOST	hypo-osmotic swelling test
kg	kilogram
LN_2	liquid nitrogen
M	molar

mg	milligram
min	minute
mL	milliliter
mM	millimolar
mOsm	milliosmole
PBS	phosphate buffered saline
rpm	round per minute
SD	standard deviation
SE	standard error of mean
TALP	Tyrode's albumin lactate pyruvate
V	volume
W	weight

CHAPTER I
INTRODUCTION

1.1 Background and rationale

Subfertility of mare after insemination with frozen-thawed semen is a crucial problem in equine reproduction. One of the best alternative solutions for treating subfertility is artificial insemination (AI) (Govaere et al., 2014). Recently, AI has been well-known and accepted by many veterinarians. The principle of the AI is to achieve the fertilization and pregnancy by direct deposition of semen into the female reproductive tract, without sexual intercourse. Fresh, chilled and frozen-thawed semen have been successfully used in the equine industry (Brinsko, 2006). Fresh semen provides highest sperm quality but sperm viability rapidly decreases within 2-3 hours. For chilled semen, temperature is gradually decreased to 4°C and sperm can be preserved for 2 – 3 days; this is called short-term storage. Frozen semen or cryopreserved semen is kept in liquid nitrogen (-196°C) and provides longest lifespan; this is called long-term storage. Longevity of chilled and frozen sperm can be extended by supplement with a chilled and freezing extender, respectively. However, semen cryopreservation is the best way to preserve semen for an indefinite period. This cryopreserved semen can be easily transported and thereby improving remotely genetic distribution (Loomis and Squires, 2005; Loomis and Graham, 2008) . Unfortunately, sperm is exposed to extreme changes in temperature and extracellular fluid compositions during the cryopreservation, which potentially cause the osmotic stress and membrane instability leading to the death of sperm (Pommer et al., 2002). Therefore, the response of sperm to osmotic stress need to be studied in order to improve the freezing ability of equine sperm.

Although there are several cryopreservation protocols and various extenders routinely used, previous reports indicated that cryodamage limited sperm survival and dramatically decreased sperm motility after freezing and thawing (Yeste, 2016). During cooling, plasma membrane undergoes a series of changes such as the rearrangement of membrane dynamic that frequently result in rigidity of cell membranes. Therefore, cryoprotective agents (CPA) that protect sperm from cryodamage has become a major attention.

Trehalose is a natural disaccharide sugar containing two **α**-glucose molecules. Trehalose, a non-permeable sugar, can be found naturally in bacteria, fungi, algae, plants and invertebrates; shrimp and insects (Ahmad and Aksoy, 2012) but not in mammals. Moreover, trehalose acts as an extracellular sugar resource and it is also used as a sweetener in commercial foods. There are several reports that trehalose has beneficial effects to protect cell against cryodamage during freezing and dehydration (Richards et al., 2002; Patist and Zoerb, 2005). It also has the protective effects on cellular protein and membrane stabilization. A previous study demonstrated that trehalose helps to protect the plasma membrane during dehydration (Patist and Zoerb, 2005). The mechanism of action of trehalose is a direct interaction with plasma membrane by forming hydrogen bond between the hydroxyl groups of trehalose and phospholipids polar head of membrane phospholipid bilayer. Subsequently trehalose can insert itself into the plasma membrane, thereby substituting water molecules (Patist and Zoerb, 2005). Therefore, trehalose protects the plasma membrane from ice crystal formation during cryopreservation, support membrane to be more stable and may help to maintain membrane integrity during dehydration and cryopreservation. Several reports revealed that trehalose has an outstanding cryoprotective capability and could improve sperm qualities during freezing and thawing of boar (Hu et al., 2009), ram (Aisen et al., 2002), mouflon ram (Berlinguer et al., 2007), goat (Aboagla and

Terada, 2003) sperm. These reports suggested that trehalose could be beneficial for equine semen cryopreservation.

However, it is worth to note that mammalian sperm cannot synthesize trehalose. Therefore, supplementation of trehalose in order to improve the efficiency of sperm to protect against cryodamage causing by excessive osmotic stress during freezing and thawing is needed. Moreover, individual variation of equine sperm also leads to different post-thaw semen quality. We propose that each horse may have different osmotic tolerance and the response of sperm to osmotic stress needs to be studied in order to improve the cryopreservation methods. Therefore, this study aims at investigating the response and osmotic tolerance limits of equine sperm to osmotic changes and examining the protective effects of trehalose on osmotic tolerance of equine sperm before and after freezing and thawing.

1.2 Research Objectives

1. To investigate the osmotic tolerance limit of equine sperm quality to osmotic changes
2. To investigate the protective effects of trehalose on osmotic tolerance of equine sperm
3. To investigate the protective effects of trehalose on quality of equine sperm before and after freezing and thawing

1.3 Research Hypothesis

1. Equine sperm respond to the osmotic change and have a limited osmotic tolerance.
2. Trehalose increases osmotic and cold tolerance of equine sperm.
3. Trehalose improves sperm post-thawed quality.

1.4 Expected Benefits and Application

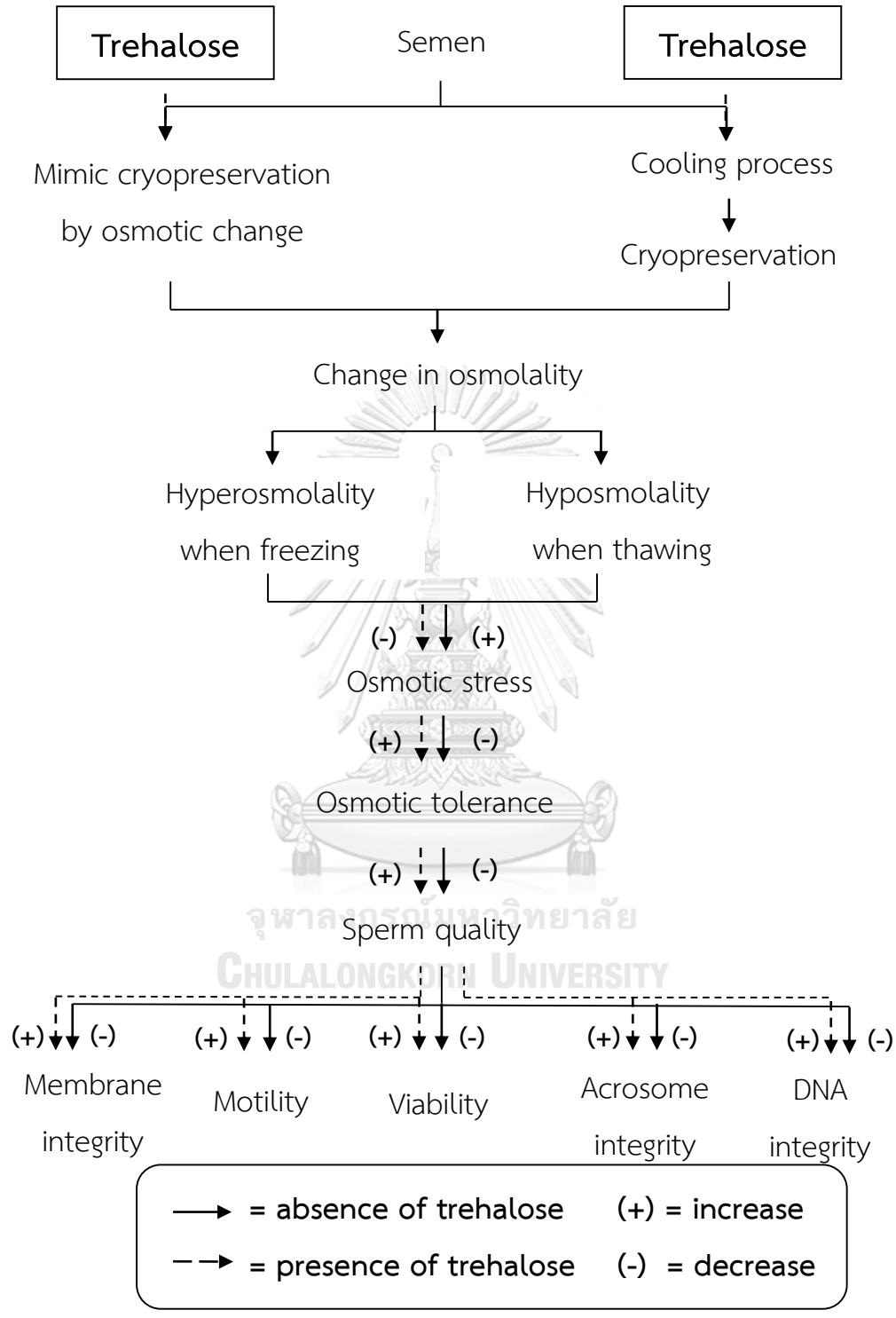
1. This study provides knowledge on osmotic tolerance limit of equine sperm.

2. This study aims to improve osmotic tolerance of equine sperm by trehalose.
3. This study aims to improve sperm quality during cold storage by trehalose.
4. This study will lead to an understanding the effects of trehalose on cryopreservation of equine sperm.

1.5 Places of study

Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn university, Bangkok, Thailand and First livestock and agriculture division, veterinary and remount department, Thai army, Kanchanaburi province, Thailand

1.6 Conceptual Framework



1.7 Keywords

Cooling, Cryopreservation, Equine, Osmotic tolerance, Sperm, Trehalose

CHAPTER II
LITERATURE REVIEWS

2.1 Male reproductive system and sperm structure

The male reproductive system composes of testes, scrotal sac, epididymis, vas deferens, accessory glands (seminal vesicles, prostate gland and Cowper's glands or bulbourethral glands), urethra, penis and prepuce. These reproductive organs work together to produce and deliver sperm in term of semen out of the body. Testes are the male gonad which are responsible for the production of sperm by spermatogenesis and the production of male sex hormone, testosterone. Spermatogenesis occurs in seminiferous tubules which are located inside of the testes. These tubules contain many stem cells that can divide and differentiate into the sperm cells. Before puberty, there is no spermatogenesis because of the lack of hormonal stimulation (Pesch and Bergmann, 2006). At puberty, aged between 18 – 24 months in horse, spermatogenesis begins when follicle stimulating hormone (FSH) and luteinizing hormone (LH) is produced from the anterior pituitary. LH stimulates Leydig cell to secrete testosterone while FSH triggers the maturation of gametes (Roser, 2001). Testosterone triggers spermatogonial stem cells in the seminiferous tubule to develop into spermatocytes. Then, spermatocyte undergoes meiosis I and II to produce 4 spermatid cells. The spermatid cells then differentiate to be a sperm cell, or spermatozoa through the process called spermiogenesis where a sperm flagellum and head develop. Then, sperm are mobilized within the seminiferous tubule, and they are released in a series of ducts to the epididymis, which the sperm complete the maturation process and are able to move on their own. In the stallion, the spermatogenic cycle happens as a wave and takes around 57 days (Johnson et al., 1997). This process starts at puberty and usually continues uninterrupted until death.

The sperm structure is made up of three parts which is entirely covered by the plasma membrane: the first part is a head containing a condensed haploid nucleus

which DNA is tightly packed and the anterior end of the nuclear envelope is an acrosome which contains hydrolytic enzyme to penetrate the egg's outer coat during fertilization. The second part is a midpiece consisting of many mitochondria in order to produce the energy for movement and the last part is a flagellum tail that provides propulsion (Amann and Pickett, 1987). The length of the equine sperm is approximately 60 μm (Pesch and Bergmann, 2006). The good sperm quality shows high motility, viability, plasma membrane integrity and also normal morphology. Previous report demonstrated that the good quality of fresh semen should have 70 – 80 % of total sperm motility (Yeste, 2016). The quality of sperm can be decreased by many causations which depend on variation of each stallion and post-thawed quality of sperm that will be discussed further.

2.2 Artificial insemination and semen cryopreservation

Artificial insemination (AI) is the process of collecting semen from a male animal and directly inserts the semen into the female reproductive tract instead of natural mating in order to achieve the fertilization and pregnancy (Samper and Plough, 2010). Nowadays, artificial insemination is commonly used, well-known and accepted by many veterinarians because there are many advantages (Govaere et al., 2014). These benefits include safety of the animals due to the risks during sexual intercourse. Particularly in equine, stallion is very large and often aggressive; therefore, AI can decrease a stress and also injuries for both mare and stallion during natural breeding. Moreover, AI can improve genetic values since we can select the semen from the desirable male breeder in order to produce the good genetic offspring. In each ejaculate, the male usually produces enough sperm to be diluted to make abundant semen doses and can breed with numerous females. AI is beneficial to transport genetics without shipping the animals. In addition, AI can limit the transmitting diseases since all equipment used for AI are sterilized and free of any chemical contamination; therefore, the process of AI is cleaner compared to natural breeding (Aurich and Aurich, 2006).

The most common technique to collect equine semen is using an artificial vagina (AV). Fresh, chilled and frozen semen collected from equine have been successfully used (Brinsko, 2006). Fresh semen provides highest sperm quality but sperm rapidly dies within a few hours. As for chilled semen, temperature is gradually decreased to 5 – 8°C and sperm can be preserved for a few days (24 – 72 hours); this is termed short-term storage. However, the limit of fresh and chilled semen is the long term preservation, it can be solved by using frozen semen. While frozen semen or semen cryopreservation is typically stored in liquid nitrogen (-196°C) and provides the longest lifespan; this is called long-term storage. Both chilled and frozen semen need to be extended the shelf life by supplementing with chilled and freezing extender respectively. However, the limitation of AI involves with the short lifespan of ejaculated sperm (Prien and Iacovides, 2016). Therefore, semen cryopreservation is the best choice and benefits to preserve semen at a sub-zero temperature for an undefined period, easy to transport to the remote area and to increase the genetic value (Loomis and Squires, 2005). For some animal species, including equine, diluted semen is packaged into straws and then frozen. These straws of frozen semen are typically stored in a liquid nitrogen tank where they can be preserved for years and can be used when needed. The semen can also be shipped to various livestock producers around the world, allowing them to be used for artificial insemination with the mare. This is beneficial to avoid transporting the mare to stallion or vice versa, which potentially decrease the cost (Amann and Pickett, 1987; Vidament et al., 1997).

Unfortunately, a particular limitation when using cryopreserved semen is the post-thawed quality of sperm is lower; motility, viability and also plasma membrane integrity are worsened when compared to both fresh and chilled semen (Aurich, 2012). Moreover, the insemination dose must be increased when cryopreservation is performed. The fact that sperm is exposed to a varied extreme temperature and osmolality change during cryopreservation, which may cause the osmotic stress leading to the damage of sperm, dramatic decrease of sperm motility and low cryosurvival rate. Since during semen freezing and thawing, the changes in osmolality occur (Prien and Iacovides, 2016). During freezing, water outside the sperm will be changed to ice

formation before water inside the sperm, therefore; sperm faces with hyperosmotic solution and dehydration occurs. By the way, during thawing, ice formation outside the sperm will be changed to water before ice formation inside the sperm, then sperm faces with hyposmotic solution and rehydration occurs. When sperm exposes to hyperosmotic solution, water inside the cell will move out and then the cell will be shrunk. On the contrary, when sperm exposes to hypo-osmotic solution, water will move into the cell and cause cells to be swollen. If the sperm swell or shrink excess their osmotic tolerance limits, these can lead to lethal of the cells (Benson et al., 2012). Therefore, during semen cryopreservation, sperm exposes to osmotic change which leads to osmotic stress and cryodamage. Since cryoinjury limits sperm survival and dramatically decreases sperm motility after semen cryopreservation. Cryopreservation requires protection of intracellular and extracellular structures and biomolecules, and thereby requires protective agents (Yeste, 2016). Therefore, cryoprotective agents which protect sperm from freezing damages have become a major attention.

2.3 Cryoprotective agent (CPA)

Cryoprotective agent is a substance used to protect cell, including sperm from cryopreservative injury and to minimize cellular damage induced by freezing. In addition, cryoprotective agent is used to stabilize the structure and biomolecules of cell and should support cell viability during freezing and thawing (Ball and Vo, 2001; Yeste, 2016). In general, the typical cryoprotective agent would be non-toxic to cells and reduce osmotic stress; however, at higher concentration, it could be toxic to cells (Prien and Iacovides, 2016). Cryoprotective agent can be divided into two types: penetrating cryoprotective agent and non-penetrating cryoprotective agent. The combination of these two types of cryoprotective agent should increase the beneficial effects to prevent against cyrodamage and promote cell survival (Wu et al., 2015).

Penetrating cryoprotective agent (e.g., glycerol, DMSO, ethylene glycol, propylene glycol) should be small and non-ionic molecules which is able to pass

cellular membrane and permeate into the cell replacing water molecules and thus prevents ice crystal formation inside the cell. The most typically membrane permeable cryoprotective agents used are glycerol and dimethyl sulfoxide (DMSO). Glycerol is the major cryoprotective agent for equine sperm. Previous study involving equine sperm showed that glycerol had the beneficial effect over DMSO in the aspect of improving sperm motility and viability (Wu et al., 2015). Nevertheless, some research reviewed that glycerol permeates slowly and induce osmotic stress, thereby leads to its toxicity. Therefore, this leads to the study of other effective cryoprotective agents (Prien and Iacovides, 2016). However, in cases glycerol and DMSO are toxic to some type of cells, ethylene glycol, propylene glycol, methylformamide, or dimethylformamide can also be used (Sieme et al., 2016). Penetrating cryoprotective agents are dispersed in both intracellular and extracellular sides equally, thereby they are osmotically inactive. Nevertheless, the initial addition of cryoprotective agent causes osmotic changes to the cells due to water moves quickly across the plasma membrane than the penetrating cryoprotective agents. Therefore, this results in an initial osmotic stress and shrinkage of the cells followed by an influx of water and cryoprotective agents until reaching the equilibrium; an equal distribution of cryoprotective agent on both sides of the cell (Sieme et al., 2016).

Non-penetrating cryoprotective agent is large or macromolecule which is not capable of penetrating across the plasma membrane and acts in the extracellular region. An example of non-penetrating cryoprotective agent are disaccharide sugars, polysaccharide, hydroxyethyl starch, egg yolk and milk protein. Non-penetrating CPA stabilize plasma membrane and prevent ice crystal formation since it can form hydrogen bonds with water molecules extracellularly (Yeste, 2016). Moreover, non-penetrating cryoprotective agent can osmotically extract water from cells.

Plasma membrane permeability to water indicates the ability of sperm to change their volume in order to reduce osmotic stress induced by freezing. The rate of water transport into and out of the cells can be decreased when temperature decreases, and this can be changed by cryoprotective agents. Previous reports indicated that cryoprotective agents increase membrane permeability to water, and alter cooling rate to be at optimum level in order to increase survival rate (Akhoondi et al., 2011). Currently, there is no standard cryoprotective agent or protocol to cryopreserve equine sperm since the undesirability of sperm post-thaw quality (Heape, 1897). In the horse, there is the specificity of species and high individual variation in equine sperm. Previous study reported that no two stallions have the same chemical composition and biomolecule, and thereby this can be frozen differently (Prien and Iacovides, 2016). Therefore, researchers still try to modify and improve the components of cryoprotective agents which minimizes sperm damages induced by cryopreservation.

2.4 Trehalose

Trehalose is a non-permeable disaccharide sugar composed of two glucose units linked by an alpha-1, 1 glucosidic linkage (Ahmad and Aksoy, 2012) in figure 1. Trehalose has a minor difference from maltose despite both containing two glucose molecules, as trehalose possesses a different bond and comprises of alpha-glucose molecules. Trehalose can be found naturally as a component of some bacteria, fungi, algae, plants and invertebrates; obtained from the cocoon and nest of some insects (Luyckx and Baudouin, 2011). In bacteria and mushrooms, the main biological function of trehalose is water regulation because trehalose can form as a gel to protect cell during dehydration (Furuki et al., 2009). However, trehalose does not exist naturally in mammals or mammalian cell.

Trehalose can act as an extracellular sugar resource and it is also used as a sweetener in commercial diet which is sweet as sucrose. In addition, trehalose is

accepted by the Food and Drug Administration (FDA) and the fact that it is nontoxic to be a food additive for the consumer. Trehalose appears to have a variety of therapeutic purposes due to its ability to protect the denaturation of protein, stabilize membrane and ability to tolerance against various types of stress including radiation, dehydration and freezing stresses (Crowe et al., 2005). Trehalose is a noticeable protective agent in cryopreservation since it has shown to protect mammalian cells against freezing-induced damage (Beattie et al., 1997). A previous report from indicated that trehalose protects plasma membrane during dehydration (Patist and Zoerb, 2005). The mechanism of action of trehalose shows its ability to substitute for water molecule in the membrane by forming a direct interaction with plasma membrane by making hydrogen bonds between sugar hydroxyl groups and the phospholipids polar head groups (Patist and Zoerb, 2005) in Figure 2. Hence, trehalose might protect the plasma membrane from ice crystallization during freezing, stabilize membrane and help to maintain membrane integrity during dehydration and cryopreservation (Woelders et al., 1997). Moreover, there are reports stating that trehalose supplementation provides hyperosmotic condition which causes cell to lose water and reduces intracellular injury from ice crystal formation during freezing (Storey et al., 1998). In addition, several studies indicated the trehalose added into semen freezing extenders improve the post-thawed semen qualities in boar (Hu et al., 2009), bovine (Woelders et al., 1997; Hu et al., 2010), stallion (Squires et al., 2004), dog (Yamashiro et al., 2007), ram (Aisen et al., 2002), mouflon ram (Berlinguer et al., 2007), buck (Aboagla and Terada, 2003). Therefore, recent reports suggested that trehalose exhibited the remarkably cryoprotective capability and could be used as cryoprotectant for cryopreservation of sperm cells. Nevertheless, mammalian including equine sperm cannot synthesize trehalose; therefore, the trehalose supplementation in order to protect the sperm from osmotic stress and cryoinjury during cryopreservation is needed.

Therefore, the supplementation of trehalose in order to improve the efficiency of sperm to protect against cryodamage causing by osmotic stress during freezing and thawing is needed to be studied. This study aims to investigate the response of sperm to osmotic stress and to investigate the protective effects of trehalose on osmotic

tolerance limits of equine sperm before and after freezing and thawing in order to improve the cryopreservation methods.

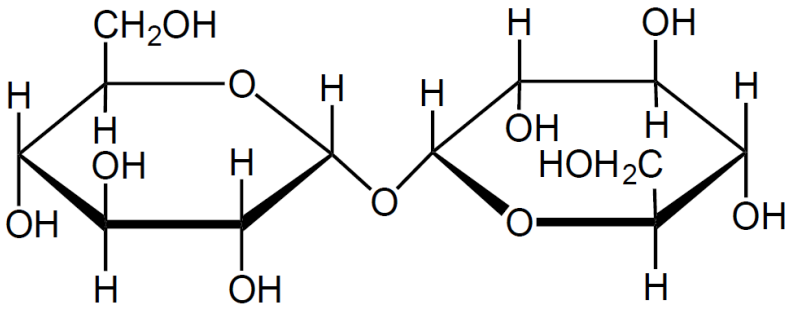


Figure 1 Structure of trehalose composed of two glucose units linked by an alpha-1, 1 glucosidic linkage.

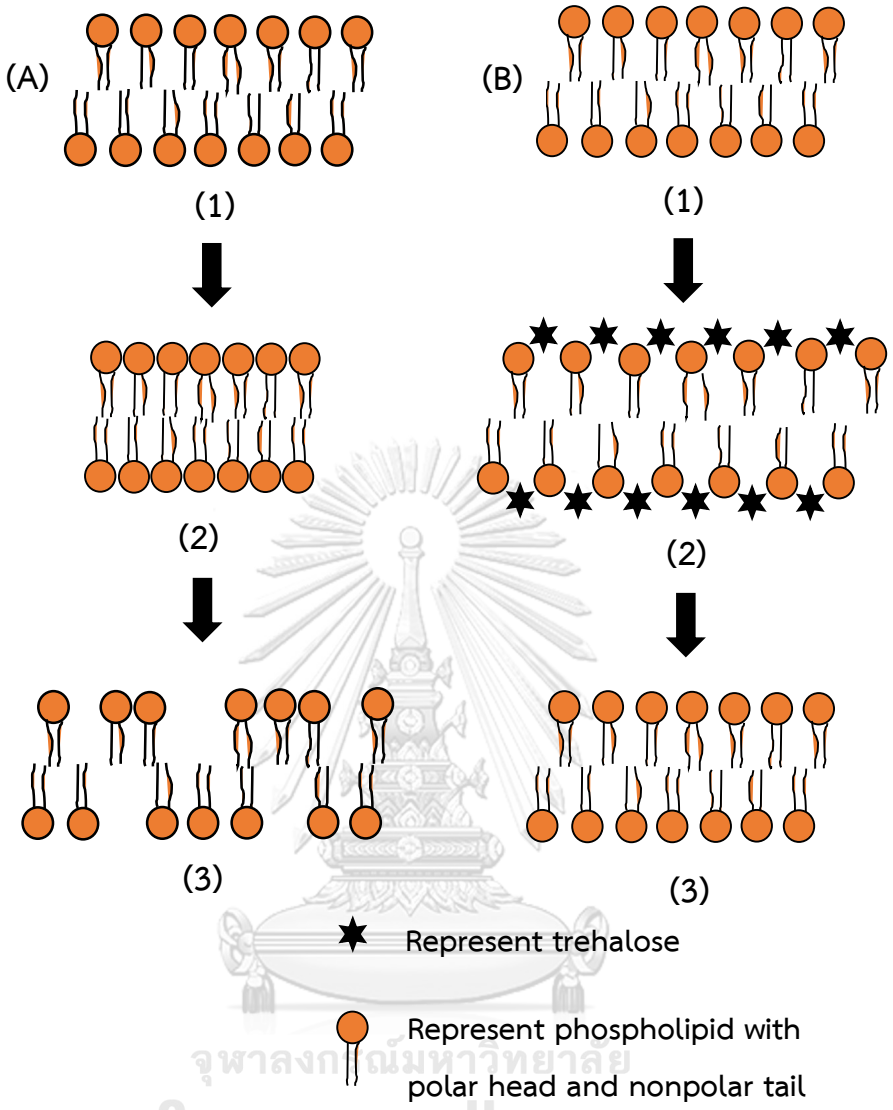


Figure 2 Mechanism of action of trehalose on the phospholipid bilayer of plasma membrane

(A) Phospholipid bilayer of the plasma membrane in the absence of trehalose (1) demonstrating conversion to gel phase during drying (2) and dehydration (3) making the leakage of the plasma membrane. (B) Plasma membrane in the presence of trehalose which showing the mechanism of action of trehalose in order to preserve plasma membrane. Trehalose binds directly to the hydrophilic head of phospholipid bilayer during drying (1 ⟶ 2). Subsequently, plasma membrane integrity stays intact during rehydration (3). Modified from Patist and Zoerb (2005).

CHAPTER III
MATERIALS AND METHODS

3.1 Experimental design

Experiment 1: The protective effects of trehalose on osmotic tolerance of equine sperm

This experiment was divided into three parts:

The first part (experiment 1.1) – the osmotic tolerance of equine sperm to osmotic changes. Equine sperm from ejaculation was exposed to modified Tyrode's albumin lactate pyruvate (TALP) medium (120 mM NaCl, 20 mM Hepes, 5 mM glucose, 3.1 mM KCl, 0.4 mM $MgSO_4 \cdot 7H_2O$, 21.7 mM Lactate, 0.3 mM $NaH_2PO_4 \cdot H_2O$, 1 mM Pyruvate, 1 mg/ml polyvinylpyrrolidone, 100 IU/ml penicillin and 100 µg/ml streptomycin) at different osmolalities: isosmotic TALP (300 mOsm/kg; control) and anisosmotic TALP (150, 450, 600 and 750 mOsm/kg) and was incubated at 37°C for 10 min (Ball and Vo, 2001; Pommer et al., 2002). The osmolalities were increased by adding NaCl and were decreased by adding distilled water and pH was adjusted to 7.3-7.4. The osmolality was measured by micro-osmometer (The FISKE® Micro-Osmometer model 210, USA) and pH was measured by pH meter (EUTECH Instruments pH 510, Singapore). Then, sperm quality in terms of sperm motility, viability and plasma membrane functionality were evaluated after 10 min of incubation.

The second part (experiment 1.2) – co-treatment, equine sperm was challenged with TALP medium (150, 300, 450, 600 and 750 mOsm/kg) supplemented with 50 and 100 mM trehalose and incubated at 37°C for 10 min. Sperm parameters were evaluated.

The third part (experiment 1.3) – pre-treatment, equine sperm was exposed to isosmotic TALP (300 mOsm/kg) supplemented with 100 mM trehalose at 37°C for 10 min. Sperm was centrifuged at 1,000 rpm at room temperature for 5 min in order to

remove trehalose. Then, sperm was challenged with different osmolalities: 150, 300, 450, 600 and 750 mOsm/ kg at 37°C for 10 min and sperm parameters were subsequently evaluated.

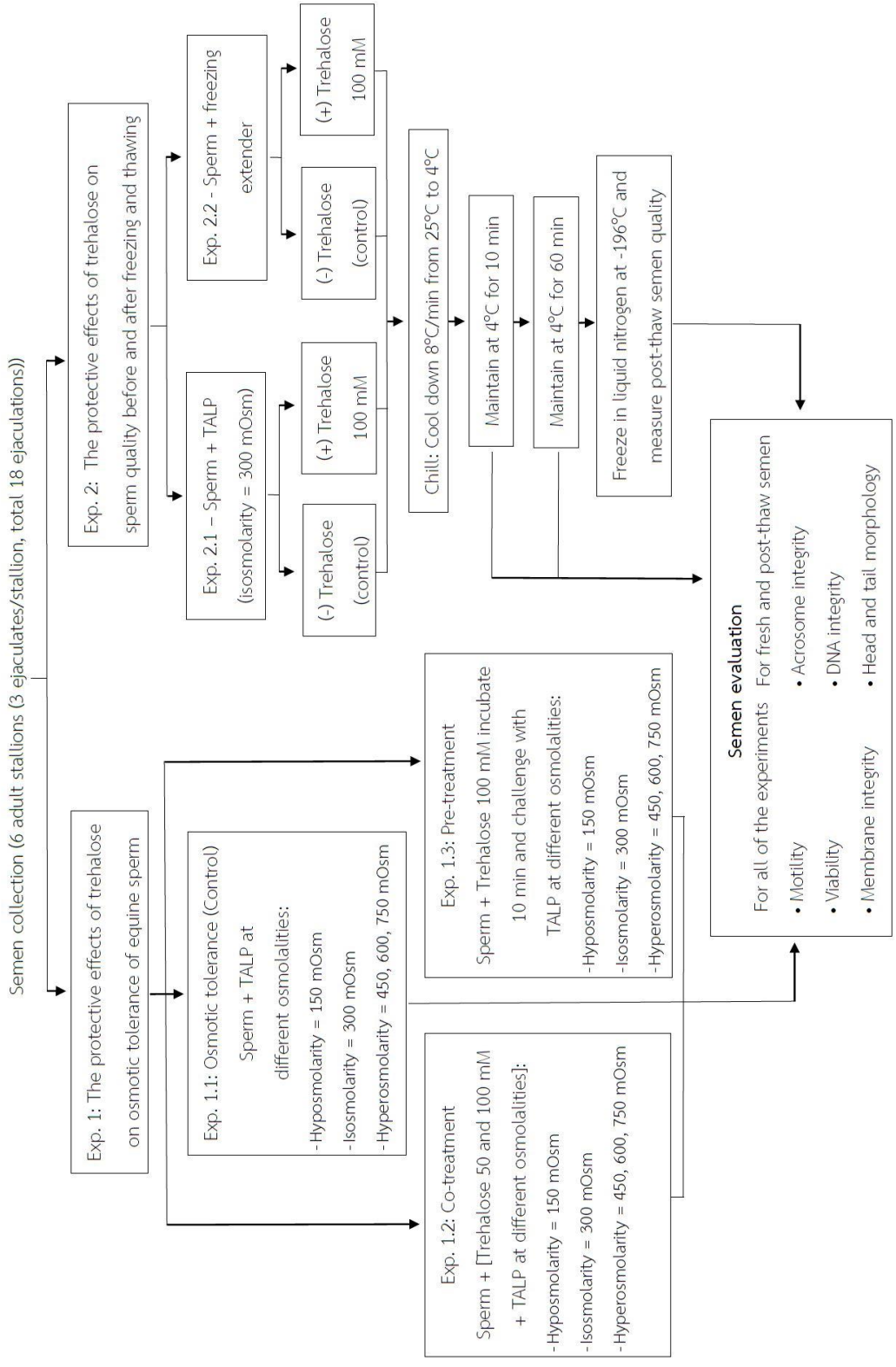
Experiment 2: The protective effects of trehalose on equine sperm before and after freezing and thawing

This experiment was divided into two parts:

The first part (experiment 2.1) - equine sperm was pre-equilibrated with isosmotic TALP (300 mOsm/kg) in the absence (control) and the presence of 100 mM trehalose and incubated at 37°C for 10 min. Sperm was cooled down with cooling rate 8°C/ min from 25°C to 4°C using freeze control cryopreservation systems (FREEZE CONTROL® CL-8800i Cryologic, Australia) and maintained at 4°C for 10 min and 60 min for semen evaluation. Then, sperm was frozen in liquid nitrogen at -196°C at least 24 hours before thawing.

The second part (experiment 2.2) – the freezing extender in the absence (control) and the presence of 100 mM trehalose were slowly added into sperm and sperm was loaded into 0.5 ml straws. Then, sperm was cooled down with cooling rate 8°C/ min from 25°C to 4°C and was equilibrated at 4°C for 10 and 60 min for semen evaluation same as experiment 2.1. The straw containing sperm was placed 4 cm above liquid nitrogen for 10 min, and then the straw was plunged into liquid nitrogen in order to be frozen. Equine sperm was frozen at least 24 hours in liquid nitrogen before thawing. Frozen sperm was thawed at 37°C for 30 second in water bath and post-thawed sperm quality was evaluated.

3.2 Research design



3.3 Chemicals

All chemicals used in this study were obtained from Sigma-Aldrich (USA), unless generally indicated.

3.4 Animals

Six adult stallions (Warmblood, Holsteiner, Irish Sport Horses and Oldenburg) aged between 5 – 12 years old maintained at First livestock and agriculture division, veterinary and remount department, Thai army, Kanchanaburi province were used in this study. Each stallion was access to grass and water ad libitum. The study was performed with an approval of Institutional Animal Care and Use Committee (IACUC) and Animal Use Protocol No. 1731056.

3.5 Semen collection

Equine semen samples were collected from six adult stallions (3 ejaculates per stallion) using artificial vagina (Missouri model, United States) with plastic liner and filter to separate the gel fraction at 45°C– 50°C and were collected above a phantom mare. Ejaculates (n = 18) with more than 70% motility and normal morphology were only used in this study. The collected semen was evaluated for volume, color and was immediately extended 1: 3 with skim milk glucose based semen extender. The skim milk extender consisted of skim milk (Himedia, India) 2.4 %, glucose-monohydrate (Merck, Germany) 4.9 %, sodium bicarbonate 0.15%, 100IU/ml penicillin and 100 µg/ml streptomycin. The pH was adjusted to 7.3 – 7.4 and the osmolality was adjusted to 350 – 360 mOsm/kg. The semen was transported to the laboratory at 4°C in an Equitainer (Hamilton Research, United States) within 4 – 5 hours for processing.

3.6 Semen freezing and thawing

Sperm, which was extended in the skim milk extender, was centrifuged at 2,000 rpm for 5 min at room temperature in order to remove skim milk extender and seminal plasma. The semen was divided into two parts and separately cryopreserved with different freezing extender (conventional freezing extender and freezing extender supplemented with 100 mM trehalose) as described in table 1. Sperm was slowly added with freezing extender to a final concentration of $100x10^6$ sperm/ml. Then, sperm was loaded into 0.5 ml straw and was cooled down with cooling rate 8°C/min from 25°C to 4°C and equilibrated at 4°C for 60 min. The straw containing sperm was placed 4 cm above liquid nitrogen for 10 min to receive the vapor of liquid nitrogen and then, the straw was plunged into liquid nitrogen. Equine sperm was frozen at least 24 hours in liquid nitrogen before thawing. Frozen sperm was thawed at 37°C for 30 second in the water bath. Subsequently, longevity after freezing and thawing was performed. Sperm was incubated at room temperature and sperm quality was evaluated at 10 min, 2 h, 4 h and 6 h.

Table 1 Compositions of conventional freezing extender (control) and freezing extender + 100 mM trehalose used in this experiment

Ingredients	Conventional freezing extender (control)	Freezing extender+ 100 mM trehalose
Solution I		
Sorbital (%)	6	6
Tri-sodium citrate (%)	0.37	0.37
Di-sodium EDTA (%)	0.37	0.37
Sodium bicarbonate (%)	0.12	0.12
Antibiotics	100 IU/ml penicillin and 100 µg/ml streptomycin	
Milli-Q water (ml)	100	100
Freezing extender (solution II)		
Lactose 11% (w/v) (ml)	50	16.7
Trehalose 100 mM (ml)	-	33.3
Solution I	26	26
Egg yolk (ml)	20	20
Equex STM (ml)	0.5	0.5
Glycerol (ml)	4	4
Osmolality (mOsm/kg)	1,085	970

3.7 Semen evaluation

3.7.1 Motility

Percentage of total sperm motility was determined by pipetting 10 µl of sperm suspension onto a clean, prewarmed microscope slide at 37°C and cover by cover slip. Then, total motility in five fields was examined subjectively under a phase-contrast microscopy (Olympus, Japan). Motility was expressed by the percentage of total motile sperm.

3.7.2 Viability

Sperm viability was evaluated by Calcein AM and Ethidium Homodimer (EthD-1) fluorescent probes labeling. Sperm viability via esterase enzyme activity was determined by a permeable green-fluorescent calcein AM staining (Molecular Probes™, Invitrogen, USA) and a non-permeable red-fluorescent DNA staining Ethidium homodimer-1 (EthD-1, Molecular Probes™, Invitrogen, USA). Sperm was stained with a mixture of EthD-1 and Calcein AM at a final concentration of 4 µM and incubated for 10 min in the dark. A total of 200 sperm per sample was evaluated under fluorescence microscope by DP2-BSW program (Olympus, Japan). Sperm was classified into 2 groups: viable sperm (positive to Calcein AM and negative to EthD-1) and dead sperm (negative to Calcein AM and positive to EthD-1). Then, the number of sperm in green and red fluorescent was expressed as the percentage of sperm viability. Photomicrograph of equine sperm stained with Calcein AM and Ethidium Homodimer (EthD-1) was demonstrated in Figure 3.

3.7.3 Plasma membrane functionality

Hypo-osmotic swelling test (HOST) was used to evaluate plasma membrane functionality of sperm. Briefly, a 25 µl volume of semen sample was added to 250 µl of hypo-osmotic sucrose solution (100 mOsm/kg) incubated at 37°C for 30 min. The

solution consisted of 100 mM sucrose in milli-q water. After 30 min incubation, semen-hypo-osmotic solution was fixed in hypo-osmotic solution supplemented with 5% (v/v) formaldehyde (BDH, UK). For evaluation, ten microliters of the sample were placed onto a pre-warm microscope slide and were covered by cover slip, then 200 sperm per sample were observed under a phase-contrast microscope at 200x magnification. Sperm with intact plasma membranes (HOST positive sperm) exhibited swollen or coiled tail as water enters the sperm cell. Sperm with non-intact plasma membrane (HOST negative sperm) did not exhibit swollen or coiled tail since water cannot restrict within the sperm cell as shown in figure 4. Therefore, the percentage of plasma membrane-intact sperm was expressed as the percentage of sperm with swollen or coiled tails.

3.7.4 Acrosome Integrity

A fluorescein isothiocyanated peanut agglutinin (FITC-PNA) staining was used to evaluate acrosome integrity and counter stained with EthD-1 (Cheng et al., 1996). Briefly, five µl of sperm suspension was smeared onto a microscope slide and air dried. Then, the sperm membrane was permeabilized with 95% ethanol for 30 second and air dried. FITC-PNA (10 µl of 100 µM) was mixed with EthD-1 (90 µl of 4 µM) diluted EthD-1 with PBS 1:1000. The mixture was spread over the slide and covered by parafilm and then, incubated in a dark moist chamber at 4°C for 60 min. The sample was rinsed with cold 1x PBS and air dried. A total of 200 sperm per slide was evaluated under a fluorescence microscope at 1,000x magnification. The sperm was classified as either intact acrosome (continuous bright fluorescent at acrosomal cap), partially damaged (discontinuous fluorescent at acrosomal cap) or completely damaged acrosome (no fluorescent at acrosomal cap). Photomicrograph of equine sperm stained with FITC-PNA and EthD-1 was exhibited in Figure 5.

3.7.5 DNA integrity

Sperm DNA integrity was performed by Acridine Orange (AO) fluorescent staining. In brief, semen was centrifuged at 2,000 rpm for 5 min and the pellet was resuspended with 1X PBS. Five microliters of sperm pellet were smeared onto a microscope slide and air dried. The slide containing sperm was fixed with freshly prepare Carnoy's solution (3 parts of methanol and 1 part of glacial acetic acid) for at least 2 hours or overnight and air dried. Then, the slide was soaked into the mixture of 10 ml of 1% (w/v) AO solution, 40 ml of 0.1 M citric acid and 2.5 ml of 0.3 M $Na_2HPO_4 \cdot 7H_2O$. The slide was stained for 5-10 min and then rinsed with distilled water for 2 times. A total of 200 sperm was examined with a fluorescence microscope at 1,000x magnification. Sperm was classified into 2 classes: normal DNA was demonstrated in green color and fragmented DNA was shown in red color as in Figure 6.

3.7.6 Morphology

Head morphology of 200 sperm was evaluated after William's staining under a light microscope at 1,000x magnification. Tail morphology of 200 sperm was examined by fixing sperm in formal saline solution in 1:20 dilution ratio and then 10 µl of diluted semen was placed onto microscope slide and determining under a phase-contrast microscope at 400x magnification. Sperm head morphology including narrow head, pear shape, variable size, acrosome defect and loose head was classified as abnormal head morphology. Sperm tail morphology including proximal and distal cytoplasmic droplets, abnormal mid-piece, bent tail, coiled tail and loose tail was determined as abnormal tail morphology.

3.7.7 Concentration

The sperm concentration was evaluated by a hemocytometer after dilution with formal saline solution 1:20 (v/v). Diluted semen (10 µl) was loaded into a chamber of hemocytometer and the number of sperm was counted under light microscope at

400x magnification. Then, sperm concentration was demonstrated as the number of sperm $x10^6$/ml.

3.8 Statistical analysis

All data was demonstrated as mean ± standard error of mean (SEM). The motility of fresh semen after adding skim milk extender of six stallions were performed by one-way analysis of variance (ANOVA) to test there was no significant different between the motility of semen of all six stallions used in his experiment. Paired t-test was performed to analyze between the motility of fresh semen before and after adding skim milk.

Experiment 1 (1.1, 1.2 and 1.3) was statistically analyzed by ANOVA followed by Duncan post – hoc test. Experiment 1.1 analyzed between isosmolality (control; 300 mOsm/kg) and anisosmolality (150, 450, 600 and 750 mOsm/kg). There were analyzed between the absence of trehalose (experiment 1.1), co-treatment of trehalose (experiment 1.2) and pre-treatment with trehalose (experiment 1.3). Experiment 2 (2.1 and 2.2) was tested by unpaired student's t-test in order to statistically analyze between no trehalose (control) and trehalose group. Data was analyzed by SPSS version 22.0 (SPSS Inc., USA) and significant difference was set at $p < 0.05$.

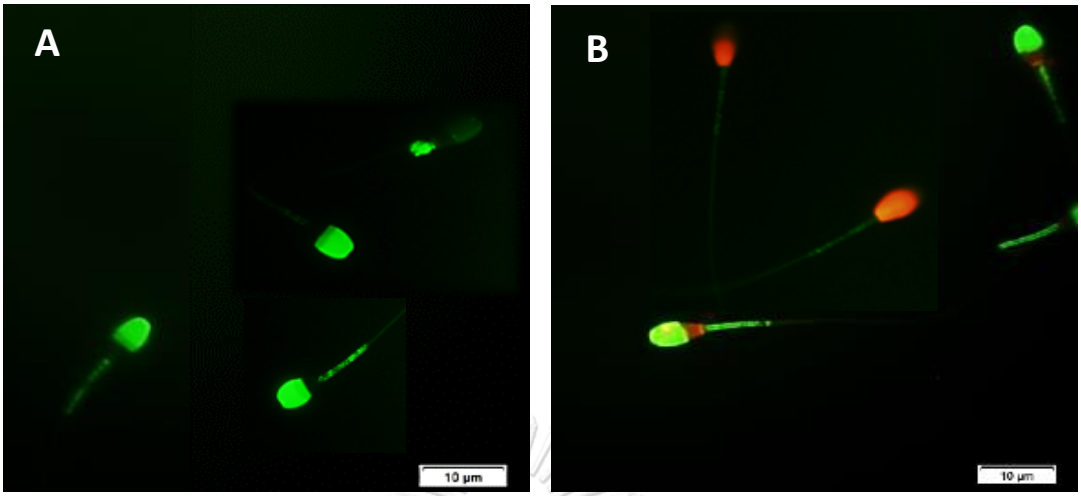


Figure 3 Photomicrograph of equine sperm stained with Calcein AM and Ethidium Homodimer (EthD-1) fluorescent probes labeling. The viable sperm (positive to Calcein AM and negative to EthD-1) were stained green (A; live). The dead sperm (positive to EthD-1) were stained red (B; dead).

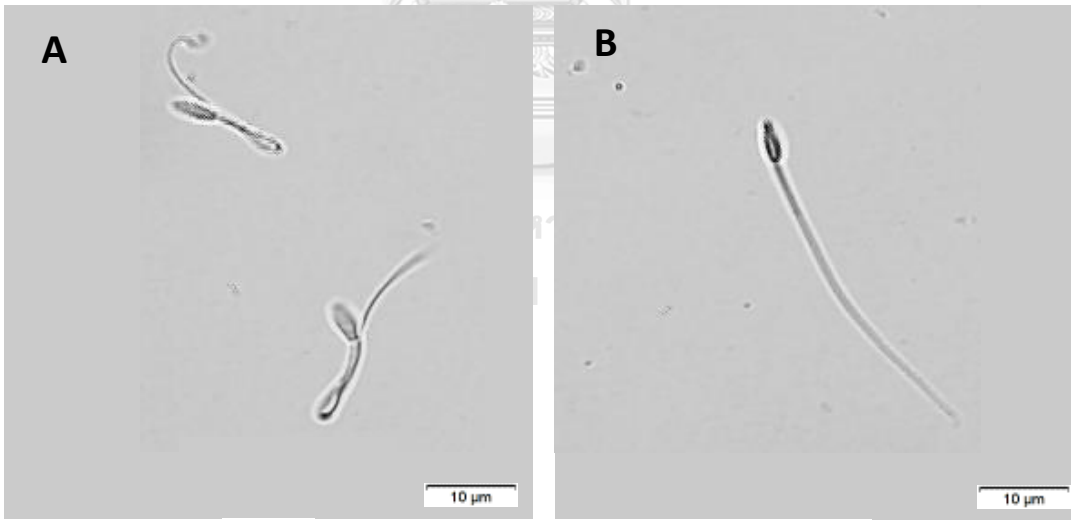


Figure 4 Photomicrograph of equine sperm evaluated with hypo-osmotic swelling test (HOST). Sperm with intact plasma membranes (HOST positive sperm) exhibited swollen or coiled tail (A). Sperm with non-intact plasma membrane (HOST negative sperm) did not exhibit swollen or coiled tail (B).

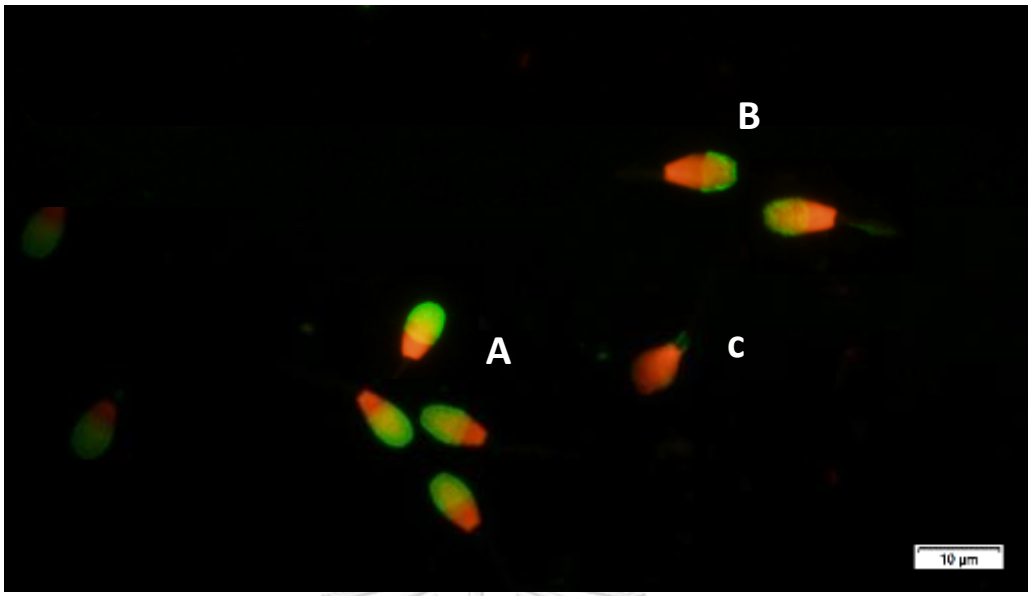


Figure 5 Photomicrograph of equine sperm stained with FITC-PNA and counter stained with EthD-1. The sperm was evaluated with intact acrosome (continuous bright green at acrosomal cap; A), partially damaged (discontinuous green at acrosomal cap; B) and completely damaged acrosome (no fluorescent at acrosomal cap; C).

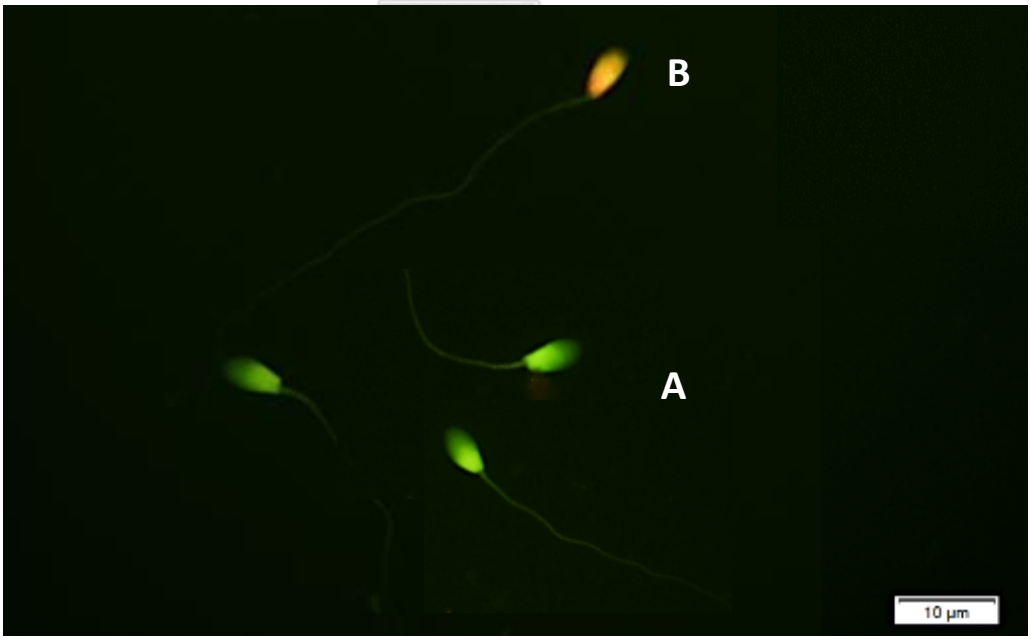


Figure 6 Photomicrograph of equine sperm stained with Acridine Orange (AO) fluorescent. The normal DNA was demonstrated in green fluorescent (A) and fragmented DNA was demonstrated in red fluorescent (B).

CHAPTER IV
RESULTS

A total of 18 ejaculates collected from six proven-fertility stallions (3 ejaculates per stallion) were used in this study for both experiment 1 and experiment 2. Descriptive data (mean ± standard error of mean) of the sperm parameters of fresh semen which were measured immediately after semen collection at First livestock and agriculture division, veterinary and remount department, Thai army, Kanchanaburi province, Thailand were presented in Table 2. Descriptive values of sperm motility after adding skim milk was not significantly different between six stallions (P>0.05). Therefore, the semen from six stallions could be combined altogether since there was no individual variation between the six stallions. The sperm parameters of chilled semen which were evaluated after transport to the laboratory within 4-5 h were presented in Table 3. The results revealed that the quality in term of motility of chilled semen after transport to the laboratory did not significantly changed compared with before transportation (P>0.05). Consequently, transportation within 4-5 h in equitainer had no affect to overall semen quality.

Table 2 Data represented in Mean ± SEM of sperm parameters in term of motility (before, after add skim milk and after transportation), volume, concentration and total sperm per ejaculation of fresh semen of six stallion (n = 3 ejaculates per stallion)

Stallion	Motility (%)			Volume (ml)	Concentration ($x10^6$/ml)	Total sperm per ejaculation ($x10^9$/ml)
	Before add skim milk	After add skim milk	After transportation			
No.1	60.0±5.8	76.7±3.3	75.0±5.0	27.8±12.3	304.0 ± 34.0	8.4 ± 3.4
No.2	80.0±0.0	80.0±0.0	83.3±3.3	25.0±10.0	386.0 ± 104.0	8.6 ± 1.3
No.3	80.0±3.2	84.0±4.0	82.0±3.7	22.4±3.0	411.6 ± 57.8	8.8 ± 1.0
No.4	78.8±3.1	80.0±0.0	77.5±2.5	33.0±7.6	210.4 ± 37.3	7.3 ± 2.2
No.5	76.7±3.3	70.0±5.8	71.7±4.4	20.7±6.4	246.0 ± 42.0	4.6 ± 0.8
No.6	53.3±3.3	70.0±5.8	70.0±5.8	38.3±1.7	349.5 ± 50.6	13.2 ± 1.3

Table 3 Data represented in Mean ± SEM of sperm parameters in term of motility, viability, membrane functionality, acrosome integrity, DNA integrity, head and tail morphology of chilled semen of six stallion after 4 - 5 h transportation (n = 3 ejaculates per stallion)

Stallion	Motility (%)	Viability (%)	Membrane functionality (%)	Acrosome integrity (%)	DNA integrity (%)	Morphology (%)	
						Normal head	Normal tail
No.1	75.0±5.0	78.3±1.9	67.8±1.3	83.0±2.4	90.9±4.0	92.3±1.7	71.8±0.3
No.2	83.3±3.3	79.8±6.1	74.7±1.2	81.8±1.9	87.3±4.8	82.8±3.3	78.8±6.3
No.3	82.0±3.7	79.7±3.6	73.1±1.2	82.9±3.0	92.2±1.2	91.2±1.8	81.4±2.5
No.4	77.5±2.5	83.6±2.0	69.9±1.9	79.0±4.4	96.3±0.9	90.8±1.5	82.0±4.1
No.5	71.7±4.4	79.3±1.6	56.8±2.8	80.0±0.0	94.2±1.6	89.5±0.3	77.3±0.3
No.6	70.0±5.8	72.8±0.7	67.8±1.2	72.2±4.7	90.5±3.8	80.2±0.2	84.5±1.5

Experiment 1: The protective effects of trehalose on osmotic tolerance of equine sperm

This experiment was divided into three parts:

The first part (experiment 1.1) – the osmotic tolerance limit of equine sperm to osmotic changes. A total of 18 ejaculates were used in experiment 1.1. The semen parameters in terms of motility, viability and plasma membrane functionality were presented in Table 4. The average of motility of equine sperm was significantly lower in hypo- (150 mOsm/kg) and hyperosmolality (450, 600 and 750 mOsm/kg) ($P<0.05$) when compared to isosmolality (300 mOsm/kg). The average of viability and plasma membrane functionality were lower in hyperosmolality; however, these were not significantly different in hyposmolality when compared to isosmolality ($P>0.05$) as shown in Table 4. Moreover, these parameters worsened when the osmolalities were increased. This data indicated that equine sperm responded to osmotic change and had limited osmotic tolerance.

From preliminary study, co-treatment with 50 mM trehalose was performed and result demonstrated that sperm motility, viability and plasma membrane functionality were not significantly different from control (no trehalose) ($P>0.05$) (see in appendix). Therefore, treatment with 50 mM trehalose had little effect to protect sperm when sperm exposed to osmotic changes. Moreover, sperm viability at 150 and 300 mOsm/kg and membrane functionality were increased in trehalose 100 mM when compared with trehalose 50 mM as also demonstrated in appendix. Therefore, trehalose at a concentration of 100 mM was selected in the following studies.

The second part (experiment 1.2) – co-treatment with 100 mM trehalose. A total of 18 ejaculates were used in this experiment. Results revealed that sperm motility, viability and also membrane functionality were significantly improved from control (without trehalose) (experiment 1.1) ($P<0.05$); except, motility at 600 and 750 mOsm/kg as presented in Table 5. Therefore, these results demonstrated that trehalose protected osmotic stress and enhanced osmotic tolerance of equine sperm.

The third part (experiment 1.3) – pre-treatment with 100 mM trehalose. A total of 18 ejaculates were used in this experiment. The results from Table 5 demonstrated that pre-treatment with 100 mM trehalose (Experiment 1.3), sperm motility, viability and membrane functionality significantly increased when compared to no trehalose (Experiment 1.1) ($P<0.05$). Therefore, these results demonstrated that trehalose protected osmotic stress and enhanced osmotic tolerance of equine sperm even though trehalose was removed.

Table 4 Data represented in Mean ± SEM of equine sperm parameters in term of motility, viability and membrane functionality after exposure to TALP at different osmolalities (n = 18 ejaculates per group)

Sperm parameter (%)	TALP at different osmolalities (mOsm/kg)				
	150	300	450	600	750
Motility	40.9 ± 2.7[b]	52.1 ± 2.4[a]	30.6 ± 2.9[c]	7.4 ± 1.7[d]	1.8 ± 0.6[d]
Viability	58.2 ± 2.1[a]	63.0 ± 2.4[a]	49.5 ± 1.7[b]	46.1 ± 1.3[b]	44.3 ± 1.5[b]
Membrane functionality	44.3 ± 2.3[a]	47.2 ± 1.8[a]	30.4 ± 1.6[b]	24.9 ± 1.6[c]	22.4 ± 1.1[c]

($P<0.05$ was significant difference from isosmolarity (300 mOsm/kg), marked by different superscript letters; a, b, c and d within row using ANOVA followed by Duncan test)

Table 5 Data represented in Mean ± SEM of equine sperm parameters in term of motility, viability and membrane functionality after exposed to osmotic challenge at different osmolalities in the absence of trehalose (Experiment 1.1) and co-treatment (Experiment 1.2) and pre-treatment with 100 mM trehalose (Experiment 1.3) (n = 18 ejaculates per group)

Sperm parameter (%)	Osmolality (mOsm/kg)	Absence of trehalose	Co-treatment with 100 mM trehalose	Pre-treatment with 100 mM trehalose
Motility				
	150	40.9 ± 2.7^{a}	53.1 ± 3.3^{b}	53.5 ± 3.3^{b}
	300	52.1 ± 2.4^{a}	63.6 ± 2.7^{b}	65.6 ± 2.6^{b}
	450	30.6 ± 2.9^{a}	46.4 ± 2.8^{b}	45.6 ± 3.7^{b}
	600	7.4 ± 1.7^{a}	13.6 ± 2.7^{a}	22.9 ± 3.1^{b}
	750	1.8 ± 0.6^{a}	2.5 ± 0.7^{a}	6.5 ± 1.3^{b}
Viability				
	150	58.2 ± 2.1^{a}	65.8 ± 1.7^{b}	66.0 ± 2.1^{b}
	300	63.0 ± 2.4^{a}	72.4 ± 2.0^{b}	69.8 ± 2.7^{b}
	450	49.5 ± 1.7^{a}	65.4 ± 2.4^{b}	67.2 ± 2.5^{b}
	600	46.1 ± 1.3^{a}	63.8 ± 2.0^{b}	60.2 ± 2.6^{b}
	750	44.3 ± 1.5^{a}	61.8 ± 2.2^{b}	58.9 ± 3.1^{b}
Membrane functionality				
	150	44.3 ± 2.3^{a}	53.8 ± 2.0^{b}	50.1 ± 1.8^{b}
	300	47.2 ± 1.8^{a}	53.2 ± 1.4^{b}	53.3 ± 1.5^{b}
	450	30.4 ± 1.6^{a}	42.8 ± 1.2^{b}	46.6 ± 1.7^{b}
	600	24.9 ± 1.6^{a}	36.9 ± 1.6^{b}	38.3 ± 1.6^{b}
	750	22.4 ± 1.1^{a}	29.2 ± 1.4^{b}	31.7 ± 1.5^{b}

($P<0.05$ was significant difference from absence of trehalose, marked by different superscript letters; a and b within row using ANOVA followed by Duncan test)

Experiment 2: The protective effects of trehalose on equine sperm before and after freezing and thawing

This experiment was divided into two parts:

The first part (experiment 2.1) – the protective effects of trehalose were examined during cooling process. A total of 18 ejaculates were used in this experiment. When sperm was cooled down to 4°C and maintained for 10 min, trehalose increased sperm quality in terms of motility, viability and plasma membrane functionality when compared with control (no trehalose) ($P<0.05$) as shown in Table 6. Therefore, trehalose decreased cool stress and increased sperm quality during cooling process for 10 min. However, sperm qualities including motility, viability and membrane functionality of trehalose group were not significantly different from control (no trehalose) ($P>0.05$) when maintained at 4°C for 60 min. It indicated that trehalose had limited ability to protect sperm against cold stress only for a short period of time (i.e. short-term, 10 min). In control group, sperm motility was decreased when maintained at 4°C for 60 min when compared to 10 min. In trehalose group, sperm motility and viability was diminished when maintained at 4°C for 60 min compared to 10 min as presented in Table 6. Therefore, when sperm was cooling down to 4°C and maintained for 60 min, the qualities of sperm were decreased in both control and trehalose group. Therefore, this experiment concluded that sperm cannot withstand for long-term period at 4°C without extender.

The second part (experiment 2.2) – the protective effects of trehalose were eventually tested for the cryopreservation process. A total of 16 ejaculates were used in this experiment. Before cryopreservation and after post-equilibration at 4°C, sperm qualities including motility, viability and membrane functionality of extender trehalose were not significantly different from control (extender without trehalose) ($P>0.05$) for both 10 and 60 min post-equilibration as shown in Table 7. In control group, sperm membrane functionality was decreased in 60 min post-equilibration when compared to 10 min post-equilibration; however, sperm motility and viability of 60 min post-equilibration were not significantly different from 10 min post-equilibration in Table 7. Comparison between experiment 2.1 in table 6 and experiment 2.2 in table 7

demonstrated that trehalose improved sperm quality in the absence of extender; however, trehalose had no effect on sperm quality in the presence of extender during cooling process before cryopreservation. During cooling process, trehalose did not increased sperm quality in the presence of extender. Therefore, other biological components of freezing extender such as egg yolk likely played more important role than trehalose to protect sperm against cold stress.

After cryopreservation, equine sperm motility, viability and membrane functionality improved in extender containing trehalose ($P<0.05$) when compared to control (extender without trehalose) up to 6 hours post-thawing as shown in Table 8. Therefore, we concluded that trehalose protected sperm from damages and improved sperm quality after cryopreservation. Descriptive statistic (Mean ± SE) of acrosome integrity (%) and DNA integrity (%) of equine sperm after cryopreservation were demonstrated in Table 9. Results revealed that there was no significant difference of acrosome and DNA integrity between trehalose and control group ($P>0.05$). Therefore, trehalose had no effect on acrosome and DNA integrity of equine sperm after cryopreservation.

Table 6 Data represented in Mean ± SEM of equine sperm parameters in term of motility, viability and membrane functionality of TALP (control) and TALP + 100 mM Trehalose at 300 mOsm/kg after cooling down to 4°C and maintain at 4°C for 10 and 60 min (n = 18 ejaculates per group)

		After cooling at 4°C	
	Sperm parameter (%)	TALP at 300 mOsm/kg (control)	TALP + 100 mM Trehalose at 300 mOsm/kg
Maintain at 4°C for 10 min	Motility	35.3 ± 2.9^{a,A}	49.7 ± 2.8^{b,A}
	Viability	50.4 ± 2.0^{a,A}	63.7 ± 2.1^{b,A}
	Membrane functionality	38.1 ± 2.1^{a,A}	49.3 ± 1.9^{b,A}
Maintain at 4°C for 60 min	Motility	13.8 ± 3.8^{a,B}	18.8 ± 6.6^{a,B}
	Viability	42.1 ± 9.1^{a,A}	51.9 ± 4.5^{a,B}
	Membrane functionality	36.9 ± 2.6^{a,A}	43.0 ± 2.5^{a,A}

($P<0.05$ was significant difference between TALP (control) and TALP + Trehalose, marked by different lowercase letters; a and b within row using unpaired t-test)

($P<0.05$ was significant difference between maintain for 10 and 60 min, marked by different uppercase letters; A and B within column in the same parameter using unpaired t-test)

Table 7 Data represented in Mean ± SEM of equine sperm parameters in term of motility, viability and membrane functionality of extender without trehalose group (control) and extender with 100 mM trehalose group after equilibration at 4°C for 10 and 60 min before cryopreservation (n = 16 ejaculates per group)

	Sperm parameter (%)	Extender	
		Control (without trehalose)	Treatment (100 mM trehalose)
Post-equilibration 10 min	Motility	65.0 ± 8.7^{a,A}	65.0 ± 8.7^{a,A}
	Viability	68.9 ± 2.3^{a,A}	73.5 ± 2.5^{a,A}
	Membrane functionality	64.1 ± 1.7^{a,A}	61.8 ± 1.6^{a,A}
Post-equilibration 60 min	Motility	63.4 ± 2.5^{a,A}	65.3 ± 2.6^{a,A}
	Viability	65.5 ± 2.0^{a,A}	71.4 ± 2.5^{a,A}
	Membrane functionality	52.4 ± 3.6^{a,B}	58.5 ± 2.5^{a,A}

(P<0.05 was significant difference between control and treatment, marked by different lowercase letters; a and b within row using unpaired t-test)

(P<0.05 was significant difference between post-equilibration 10 and 60 min, marked by different uppercase letters; A and B within column in the same parameter using unpaired t-test)

Table 8 Data represented in Mean ± SEM of equine sperm parameters in term of motility, viability and membrane functionality of extender without trehalose group (control) and extender with 100 mM trehalose group after cryopreservation and evaluate at 10 min, 2 h, 4 h and 6 h post-thawing (n = 16 ejaculates per group)

Time	Sperm parameter (%)	Extender	
		Control (without trehalose)	Treatment (100 mM trehalose)
10 min post-thawing	Motility	41.6 ± 3.2[a]	50.6 ± 2.8[b]
	Viability	50.3 ± 1.8[a]	60.1 ± 1.5[b]
	Membrane functionality	39.2 ± 2.8[a]	48.3 ± 2.6[b]
2 h post-thawing	Motility	50.6 ± 3.7[a]	61.9 ± 3.0[b]
	Viability	46.6 ± 2.2[a]	55.7 ± 2.0[b]
	Membrane functionality	35.3 ± 2.1[a]	45.2 ± 2.0[b]
4 h post-thawing	Motility	42.8 ± 3.2[a]	57.2 ± 2.8[b]
	Viability	42.7 ± 2.1[a]	51.9 ± 2.3[b]
	Membrane functionality	32.3 ± 2.2[a]	42.1 ± 2.0[b]
6 h post-thawing	Motility	41.6 ± 3.8[a]	51.9 ± 2.9[b]
	Viability	37.6 ± 1.9[a]	47.2 ± 1.9[b]
	Membrane functionality	32.7 ± 2.1[a]	40.9 ± 1.9[b]

($P<0.05$ was significant difference between control (without trehalose) and treatment (100 mM trehalose), marked by different superscript letters; a and b within row using unpaired t-test)

Table 9 Data represented in Mean ± SEM of equine sperm parameters in term of acrosome and DNA integrity of extender without trehalose group (control) and extender with 100 mM trehalose group after cryopreservation (n = 12 ejaculates per group)

Sperm parameter (%)	Extender	
	Control (without trehalose)	Treatment (100 mM trehalose)
Acrosome integrity	60.0 ± 4.2	63.9 ± 2.6
DNA integrity	90.4 ± 2.7	91.0 ± 2.2

CHAPTER VI
DISCUSSION

This study demonstrated that temperature of transported semen using Equitainer® was decreased to 10-15°C within 4 -5 h during semen transportation, while sperm quality in terms of viability and motility did not significantly change compared to before transportation. Previous study reported that equine semen could be slowly cooled down to about 5°C and maintained at this temperature for at least 24 h which semen quality still remained the same as initial (McKinnon, 2010).

During cryopreservation, sperm exposes to osmotic changes in both hypo- and hyperosmolality which induce osmotic stress (Kunkitti et al., 2017). When freezing, sperm inevitably face with hyperosmotic condition essentially when extracellular ice crystal is formed and water moves out of the sperm causing cell dehydration. Likewise, sperm face with hypo-osmotic condition when the extracellular ice is thawed. At this stage, the water rapidly moves into the sperm (Mazur, 1984) causes swelling of sperm. Due to the fact that sperm has small amount of cytoplasm compared to other types of cells; therefore, sperm is able to rapidly shrink and swell when cryopreservation is performed (Sieme et al., 2008). For this reason, osmotic tolerance of equine sperm to hypo- and hyperosmolality need to be studied in order to improve sperm quality during cryopreservation process.

In this study, the osmotic challenge (150 – 750 mOsm/kg) was used to imitate the cryopreservation process. The study from experiment 1.1 indicated that the quality of equine sperm declined when sperm was exposed to osmotic stress and motility, viability and also membrane functionality worsen when osmolality increased. Therefore, equine sperm responded to osmotic changes and had limited osmotic tolerance. This present study assists the earlier research that equine sperm has limited osmotic tolerance (Ball and Vo, 2001; Pommer et al., 2002). Our findings also supported previous study that equine sperm had a limited osmotic tolerance similar to boar sperm (Gilmore et al., 1998) which less tolerance than mouse (Willoughby et al., 1996)

and human sperm (Gao et al., 1995). Moreover, this study revealed that hyperosmolality was more detrimental to equine sperm than hyposmolality. This was because sperm viability and membrane functionality at 450 mOsm/kg (hyperosmolality) were decreased, while 150 mOsm/kg (hyposmolality) did not significantly change from 300 mOsm/kg (isomolarity). However, previous studies informed that equine sperm were more sensitive to hyposmolality than hyperosmolality (Burnaugh et al., 2010; Garcia et al., 2012). Nevertheless, hyperosmotic stress was re-examined which was the major source of damages to sperm (Morris et al., 2007) and our study supported this data.

Previous studies insisted on the beneficial effects of trehalose on frozen-thawed sperm quality in various types of animals: boar (Hu et al., 2009), bovine (Woelders et al., 1997; Hu et al., 2010), buffalo (Shiva Shankar Reddy et al., 2010), ram (Jafaroghli et al., 2011), goat (Khalili et al., 2009), stallion (Squires et al., 2004), rabbit (Dalimata and Graham, 1997; Zhu et al., 2017) and deer (Wang and Dong, 2017). Due to mammals or mammalian cell including equine sperm cannot synthesis trehalose (Richards et al., 2002), supplementation of trehalose in order to protect sperm during cooling and cryopreservation process is needed. The effective dosages of trehalose in order to improve sperm quality are 50 and 100 mM (Bucak et al., 2007; Hu et al., 2009; Shiva Shankar Reddy et al., 2010; Ahmad et al., 2015). In the present study, trehalose at the dose of 50 mM had little effect on sperm quality in terms of sperm motility, viability and membrane functionality when sperm were exposed to osmotic stress. Therefore, this result indicated that 50 mM trehalose was not effective to protect osmotic stress, while trehalose at the dose of 100 mM was able to improve osmotic tolerance of equine sperm. Furthermore, the previous study of rabbit sperm indicated that trehalose 100 mM was the most effective dose among 50, 75, 100, 150, 200 mM in order to enhance sperm parameters including motility, membrane functionality, mitochondrial membrane potential and increased antioxidant enzyme level during cryopreservation (Zhu et al., 2017). There was the study of ram sperm demonstrated that trehalose prevented osmotic stress when sperm faced with anisosmolality. As a result of sperm cryopreservation, trehalose at 50 and 100 mM also improves

cryosurvival of sperm and decreased acrosome reaction induced by LPC (Ahmad et al., 2015). In the same way as previous study, this current research also proved that trehalose enhances osmotic tolerance of equine sperm by sustaining plasma membrane, resulting in the reason for improving sperm quality in the aspect of sperm motility, viability and plasma membrane functionality during cryopreservation process.

The study from experiment 1.2 supports the hypothesis that the presence of extracellular trehalose heightened osmotic tolerance of equine sperm which is likewise the previous study in ram sperm (Ahmad et al., 2015). When trehalose presented and washed out as in experiment 1.3, trehalose showed the beneficial effects which was effective to enhance osmotic tolerance. This result might indirectly indicate that after removing trehalose, it might integrate to plasma membrane and maintained membrane integrity during osmotic challenge. There is the proposed mechanism which infer that since trehalose has hydrogen bond, it can bind directly to hydrophilic head of plasma membrane (Patist and Zoerb, 2005) and diminish the van der Waals force and consequently sustain membrane fluidity (Ahmad and Aksoy, 2012).

The result from experiment 2.1 when sperm were cooling down to 4°C with the rate 8°C/min and maintained at 10 and 60 min for short and long term incubation respectively, trehalose showed slightly protective effect against cool stress only in short-term incubation. In this experiment, supplementation of trehalose with TALP medium instead of extender was used to evaluate the effect of trehalose on cooling stress avoiding confounding factor from other cryoprotective agent in extender, since TALP medium has no other cryoprotective agent except trehalose. Furthermore, comparison between experiment 2.1 (trehalose in TALP medium without extender) and experiment 2.2 (trehalose with extender) revealed that trehalose improved sperm quality only in the absence of extender in short-term period. Extender contained cryoprotective agents which are glycerol and egg yolk. This result insisted that there should not have other cryoprotective agent in order to examine only the effect of trehalose on cool stress because the combination of cryoprotective agent might demonstrated the beneficial effect over trehalose. During cooling and cryopreservation

process, several cryoprotective agents are routinely used to protects sperm against cryodamages (Squires et al., 2004; Fayrer-Hosken et al., 2008). Egg yolk contains low density lipoprotein (LDL) which could attach to cell membrane and thus preserve sperm plasma membrane (Moussa et al., 2002) and increase membrane fluidity during cryopreservation process. Glycerol penetrate within the cell, and stabilizing intracellular components of sperm cell (Najafi et al., 2013) and then reduce osmotic stress. The combination of these two cryoprotective agents could have beneficial effect to diminish damages from cold stress. Therefore, during cooling process in the presence of cryoprotective agents, there is less stress causing damages to sperm. In the aspect of cooling rate, equine sperm could be cooled rapidly from 37 to 20°C, however; sperm should be slowly cooled from 20 to 5°C to maximize sperm survival rate (Kayser et al., 1992). Earlier study reported that the cryosurvival of equine sperm is maintained when cooling rates are between 5°C/min to 45°C/min (Moore et al., 2006).

In experiment 2.2, trehalose clearly improved sperm quality after cryopreservation, since cryopreservation extremely induces osmotic changes and trehalose enlarges osmotic tolerance of equine sperm. The possible assumption is trehalose protects extracellular ice crystal formation during freezing process. Moreover, trehalose is able to bind to plasma membrane replacing water molecule nearby plasma membrane; thus, reduce damages from ice crystal around membrane. During thawing process, trehalose reduces osmotic stress since when thawing water moves into cell rapidly, while cryoprotective agent (glycerol) move out from cell slower than water; therefore, water tends to enter the cell enormously and causes osmotic stress. Trehalose reduces extracellular water and thus diminishes the amount water entering the cell and consequently decreases osmotic stress. This present study provides the better understanding about the beneficial effect of trehalose to protect sperm against osmotic stress as previously describe in ram sperm (Ahmad et al., 2015). Extender with trehalose did not improve acrosome and DNA integrity more than conventional extender. Trehalose which is disaccharide sugar cannot penetrate though plasma membrane of sperm (Richards et al., 2002); therefore, trehalose acts outside of the cell. Reasonably, trehalose had no significantly positive affect to neither acrosome integrity nor DNA integrity of sperm since acrosome and DNA located inside the sperm

cell. Further study, artificial insemination with froze-thawed semen of extender containing trehalose might be processed in order to examine the effect of trehalose on fertility rate of the mare.

In conclusion, the present study demonstrated that equine sperm responded to osmotic change and had limited osmotic tolerance. Trehalose protected osmotic stress and enhanced osmotic tolerance of equine sperm when sperm exposed to osmotic changes. Furthermore, optimum concentration of trehalose in order to protect sperm against osmotic stress was 100 mM. Trehalose had limited ability to protect sperm against cold stress especially only in short-term. During cooling process, trehalose did not increased sperm quality in the presence of extender. Therefore, extender plays more important role than trehalose to protect sperm against cold stress. Finally, trehalose improved sperm quality and protected sperm from damages after cryopreservation.

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APPENDIX

Descriptive data (Mean ± SE) of equine sperm after exposed to osmotic challenge in the absence of trehalose (Experiment 1.1) and co-treatment (Experiment 1.2) with 50 mM trehalose (n = 3 ejaculates per group) and 100 mM trehalose (n = 18 ejaculates per group)

Sperm parameter (%)	Osmolality (mOsm/kg)	Trehalose 50 mM		Trehalose 100 mM	
		Experiment 1.1	Experiment 1.2	Experiment 1.1	Experiment 1.2
Motility					
	150	43.3 ± 3.3^{a}	50.0 ± 7.6^{a,A}	40.9 ± 2.7^{a}	53.1 ± 3.3^{b,A}
	300	70.0 ± 0.0^{a}	70.0 ± 0.0^{a,A}	52.1 ± 2.4^{a}	63.6 ± 2.7^{b,A}
	450	46.7 ± 8.8^{a}	50.0 ± 5.8^{a,A}	30.6 ± 2.9^{a}	46.4 ± 2.8^{b,A}
	600	8.3 ± 1.7^{a}	10.0 ± 2.9^{a,A}	7.4 ± 1.7^{a}	13.6 ± 2.7^{a,A}
	750	1.7 ± 1.7^{a}	1.7 ± 1.7^{a,A}	1.8 ± 0.6^{a}	2.5 ± 0.7^{a,A}
Viability					
	150	54.2 ± 4.9^{a}	51.8 ± 2.5^{a,A}	58.2 ± 2.1^{a}	65.8 ± 1.7^{b,B}
	300	62.8 ± 2.2^{a}	60.3 ± 4.0^{a,A}	63.0 ± 2.4^{a}	72.4 ± 2.0^{b,B}
	450	53.3 ± 2.8^{a}	60.7 ± 1.9^{a,A}	49.5 ± 1.7^{a}	65.4 ± 2.4^{b,A}
	600	54.5 ± 3.3^{a}	55.5 ± 4.0^{a,A}	46.1 ± 1.3^{a}	63.8 ± 2.0^{b,A}
	750	52.3 ± 5.1^{a}	51.7 ± 4.1^{a,A}	44.3 ± 1.5^{a}	61.8 ± 2.2^{b,A}
Membrane functionality					
	150	39.0 ± 3.9^{a}	40.7 ± 2.7^{a,A}	44.3 ± 2.3^{a}	53.8 ± 2.0^{b,B}
	300	41.7 ± 2.7^{a}	35.8 ± 2.6^{a,A}	47.2 ± 1.8^{a}	53.2 ± 1.4^{b,B}
	450	25.2 ± 1.9^{a}	23.8 ± 0.4^{a,A}	30.4 ± 1.6^{a}	42.8 ± 1.2^{b,B}
	600	23.3 ± 2.2^{a}	20.8 ± 2.3^{a,A}	24.9 ± 1.6^{a}	36.9 ± 1.6^{b,B}
	750	20.2 ± 0.8^{a}	19.5 ± 1.9^{a,A}	22.4 ± 1.1^{a}	29.2 ± 1.4^{b,B}

($P<0.05$, marked by different lowercase letters (a, b) compared between experiment 1.1 and 1.2 and uppercase letters (A, B) compared between trehalose 50 and 100 mM in experiment 1.2)

VITA

Dissaya Srinutiyakorn was born on April 13th 1993 in Bangkok, Thailand. She graduated with Degree of Bachelor of Science (B.Sc.) in Biology with First-Class Honors with Gold Medal Award from Faculty of Science, Chulalongkorn University, Bangkok, Thailand in 2015. She got a scholarship from the Development and Promotion of Science and Technology Talents (DPST) Project jointly administered by the Ministry of Science and Technology, the Ministry of Education since 2008. She received Young Scientist Award from the 8th FAOPS Congress and she went to Tokyo University of Agriculture and Technology to conduct the research in 2014. Dissaya is currently pursuing a Master's degree in the field of animal reproduction at Inter-Department of Physiology, Faculty of Graduate School, Chulalongkorn University, Bangkok, Thailand. She is interested in equine reproduction with the aim of improving equine sperm quality during cryopreservation.