EFFECTS OF SUPPLEMENTED INTRA- AND EXTRA-CELLULAR TREHALOSE ON CRYOPRESERVATION OF FELINE OOCYTES

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Theriogenology
Department of Obstetrics Gynaecology and Reproduction
FACULTY OF VETERINARY SCIENCE
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ผลของการเสริมทรีฮาโลสแบบภายในและภายนอกเซลล์ต่อการแช่แข็งโอโอไซต์แมว

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การศึกษาที่ 1 ศึกษาผลของระยะการเจริญของโอโอไซต์และความเข้มข้นของทรีฮาโลสต่อความสามารถในการพัฒนาของตัวอ่อนของโอโอไซต์แมวภายหลังการแช่แข็ง โดยทำการแช่แข็งโอโอไซต์ระยะไม่พร้อมปฏิสนธิและระยะพร้อมปฏิสนธิ ใช้โอโอไซต์ที่ไม่ได้รับการแช่แข็งเป็นกลุ่มควบคุม ทำการศึกษาความเข้มข้นของทรีฮาโลสที่แตกต่างกัน (0, 0125, 0.25, 0.5, 1 โมลาร์) ต่อความสามารถในการแช่แข็งและการพัฒนาของตัวอ่อน ผลการทดลองพบว่าการแช่แข็งส่งผลให้ตัวอ่อนพัฒนาได้น้อยลง และโอโอไซต์ระยะไม่พร้อมปฏิสนธิมีความไวรับและเสียหายได้มากกว่าโอโอไซต์ระยะพร้อมปฏิสนธิ ($P<0.05$) การบ่มโอโอไซต์ด้วยทรีฮาโลสความเข้มข้นสูง (0.5, 1 โมลาร์) ลดการพัฒนาของตัวอ่อนอย่างมีนัยสำคัญทางสถิติ ความเข้มข้นของทรีฮาโลสที่เหมาสมระหว่างการแช่แข็งโอโอไซต์ระยะไม่พร้อมปฏิสนธิและระยะพร้อมปฏิสนธิคือ 0.125, 0.25 โมลาร์ ตามลำดับ สรุปได้ว่าระยะการเจริญของโอโอไซต์แมวมีผลต่อความสามารถในการแช่แข็ง และความเข้มข้นของทรีฮาโลสที่เหมาะสมมีความแตกต่างกันสำหรับระยะการเจริญของโอโอไซต์

การศึกษาที่ 2 เพื่อศึกษาผลของทรีฮาโลสที่สามารถผ่านเยื่อหุ้มเซลล์ได้ต่อตวามสามารถในการแช่แข็งโอโอไซต์แมว ทรีฮาโลสภายในเซลล์ (ทรีฮาโลสเฮกซะอะซีเตต; Tre-(OAc)$_6$) ได้ถูกสังเคราะห์ขึ้นจากสารตั้งต้นทรีฮาโลสและตรวจสอบด้วยแมส สเปคโตรมีทรี ทำการทดสอบความสามารถในการผ่านเยื่อหุ้มเซลล์ของ Tre-(OAc)$_6$ ที่ความเข้มข้นต่างๆกัน (3, 15,30 มิลลิโมลาร์) ทำการประเมินหาความเข้มข้นที่เหมาะสมและความเข้มข้นเป็นพิษของ Tre-(OAc)$_6$ ทำการศึกษาผลของ Tre-(OAc)$_6$ ต่อความสามารถในการแช่แข็งโอโอไซต์แมว โดยตรวจจากการแสดงออกของยีนที่เกี่ยวข้องกับอะโพโตซิสและการพัฒนาของตัวอ่อน พบว่า Tre-(OAc)$_6$ สามารถผ่านเข้าสู่ไซโตพลาสซึมของโอโอไซต์แมวโดยปริมาณทรีฮาโลสจะมีความสัมพันธ์กับความเข้มข้นและระยะเวลาในการบ่มโอโอไซต์ โดยระดับทรีฮาโลสภายในเซลล์ที่มีความเข้มข้นสูงที่สุดเมื่อทำการเสริม Tre-(OAc)$_6$ ที่ 30 มิลลิโมลาร์ เป็นระยะเวลา 24 ชั่วโมง สำหรับการทดสอบระดับความเป็นพิษพบว่าการเสริม Tre-(OAc)$_6$ 3 มิลลิโมลาร์ เป็นระยะเวลา 24 ชั่วโมง ไม่ส่งผลกระทบต่อการเจริญของโอโอไซต์และการพัฒนาของตัวอ่อน อย่างไรก็ตาม Tre-(OAc)$_6$ ที่ระดับความเข้มข้นสูง (15, 30 มิลลิโมลาร์) ลดอัตราการเจริญของโอโอไซต์และอัตราการปฏิสนธิอย่างมีนัยสำคัญทางสถิติ ($P<0.05$) นอกจากนั้นยังพบว่า การเสริม Tre-(OAc)$_6$ 3 มิลลิโมลาร์เพิ่มการแสดงออกของยีนยับยั้งอะพอพโตซิส (*BCL-2*) เมื่อเปรียบเทียบกับกลุ่มควบคุมและที่ระดับความเข้มข้นอื่นๆ (15, 30 มิลลิโมลาร์) การเสริม Tre-(OAc)$_6$ ก่อนกระบวนการแช่แข็งให้ผลอัตราความสามารถในการพัฒนาของตัวอ่อนในระยะคลีเวจและบลาสโตซิสต์ดีขึ้นเมื่อเปรียบเทียบกับกลุ่มควบคุม ($P<0.05$) การศึกษาครั้งนี้สรุปได้ว่าได้ว่าความเข้มข้นของทรีฮาโลสภายในเซลล์ที่เพิ่มขึ้นโดยการเสริม Tre-(OAc)$_6$ ทำให้ความสามารถในการแช่แข็งดีขึ้นแต่จำเป็นต้องใช้ความเข้มข้นจำเพาะ

การศึกษาที่ 3 เพื่อศึกษาผลของการใช้ร่วมกันของทรีฮาโลสแบบผ่าน (Tre-(OAc)$_6$) และไม่ผ่านเยื่อหุ้มเซลล์ (α,α-trehalose) ต่อความสามารถในการแช่แข็งโอโอไซต์แมว ทำการทดสอบความเข้มข้นที่เหมาะสมของ Tre-(OAc)$_6$ ที่ความเข้มข้น 0, 1.5, 3 มิลลิโมลาร์ และทรีฮาโลส ที่ความเข้มข้น 0, 0.125, 0.25, 0.5 โมลาร์ทำการศึกษาผลของการใช้ทรีฮาโลสทั้งสองชนิดร่วมกันร่วมกันต่อประสิทธิภาพของการแช่แข็งโอโอไซต์ ผลการศึกษาพบว่า การเสริม Tre-(OAc)$_6$ ที่ความเข้มข้น 3 มิลลิโมลาร์ทำให้การพัฒนาของตัวอ่อนหลังผ่านการแช่แข็งดีขึ้นในขณะที่ทรีฮาโลสแบบภายนอกเซลล์ที่ 0.25 โมลาร์เป็นระดับความเข้มข้นที่เหมาะสมในการช่วยเพิ่มความสามารถในการแช่แข็ง ($P<0.05$)

สาขาวิชา	วิทยาการสืบพันธุ์สัตว์	ลายมือชื่อนิสิต ....
ปีการศึกษา	2565	ลายมือชื่อ อ.ที่ปรึกษาหลัก ....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม ....

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Saengtawan Arayatham : EFFECTS OF SUPPLEMENTED INTRA- AND EXTRA-CELLULAR TREHALOSE ON CRYOPRESERVATION OF FELINE OOCYTES. Advisor: Assoc. Prof. Dr. THEERAWAT THARASANIT, D.V.M., Ph.D. Co-advisor: Asst. Prof. Dr. Panuwat Padungros, B.Sc., Ph.D.

Study 1 aimed at examining the effects of different stages of oocyte maturation and trehalose concentrations on freezing ability and developmental capability of feline oocytes. The immature and matured feline oocytes were cryopreserved and thawed. They were assessed for developmental competence. The effects of different concentrations of trehalose (0, 0.125, 0.25, 0.5, 1 M) on the freezing ability of feline oocytes were tested. The cryopreservation generally induced poor embryo development, while immature oocytes were more susceptible to cryodamage compared with the matured oocytes ($P<0.05$). Incubation of oocytes with high concentrations of trehalose (0.5, 1 M) significantly reduced the developmental competence. Optimal concentrations of trehalose during cryopreservation of immature and matured oocytes were different (0.125 M and 0.25 M, respectively). It is concluded that stages of meiotic maturation affected the freezing ability of feline oocytes. Optimal concentration of trehalose was differently required for immature and matured feline oocytes.

Study 2 aimed at examining the effect of membrane-permeable trehalose on the freezing ability of feline oocytes matured in vitro. intracellular trehalose (trehalose hexaacetate; Tre-$(OAc)_6$) was synthesized from trehalose precursor and subjected to spectroscopic characterization. The membrane permeability of the Tre-$(OAc)_6$ was investigated by incubating oocytes with different concentrations of Tre-$(OAc)_6$ (3, 15, and 30 mM) and optimum concentration and the toxicity of Tre-$(OAc)_6$ were subsequently assessed. The effects of Tre-$(OAc)_6$ on freezing ability in terms of apoptotic gene expression and developmental competence of in-vitro matured oocytes were examined, respectively. The Tre-$(OAc)_6$ permeated into the ooplasm of cat oocytes in a dose- and time-dependent manner. The highest concentration of intracellular trehalose was detected when the oocytes were incubated for 24 h with 30 mM Tre-$(OAc)_6$. For the toxicity test, incubation of oocytes with 3 mM Tre-$(OAc)_6$ for 24 h did not affect maturation rate and embryo development. However, high doses of Tre-$(OAc)_6$ (15 and 30 mM) significantly reduced maturation and fertilization rates ($P < 0.05$). In addition, frozen-thawed oocytes treated with 3 mM Tre-$(OAc)_6$ significantly upregulated anti-apoptotic (*BCL-2*) gene expression compared with the control (0 mM) and other Tre-$(OAc)_6$ concentrations (15 and 30 mM). Oocyte maturation in the presence of 3 mM Tre-$(OAc)_6$ prior to cryopreservation significantly improved oocyte developmental competence in terms of cleavage and blastocyst rates when compared with the control group ($P < 0.05$). Our results lead us to infer that increasing the levels of intracellular trehalose by Tre-$(OAc)_6$ during oocyte maturation improves the freezing ability of feline oocytes, albeit at specific concentrations.

Study 3 aimed at examining the effects of combination of intra-cellular (Tre-$(OAc)_6$) and extra-cellular (α,α-trehalose) trehalose on freezing ability of feline oocytes. Optimal concentrations of Tre-$(OAc)_6$ (0, 1.5, and 3 mM) and trehalose (0, 0.125, 0.25, and 0.5 M) were investigated for cryopreservation of matured feline oocytes. The combination of the two optimal concentrations were subsequently examined. The results showed that 3 mM of Tre-$(OAc)_6$ improved post-thawed developmental competence, while extra-cellular trehalose at 0.25 M significant enhanced freezing ability of feline oocytes ($P<0.05$). The combination of intra- and extra-cellular trehalose significantly improved developmental capability of frozen-thawed matured feline oocytes compared to non-trehalose control.

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Field of Study:	Theriogenology	Student's Signature ....
Academic Year:	2022	Advisor's Signature ....
		Co-advisor's Signature ....

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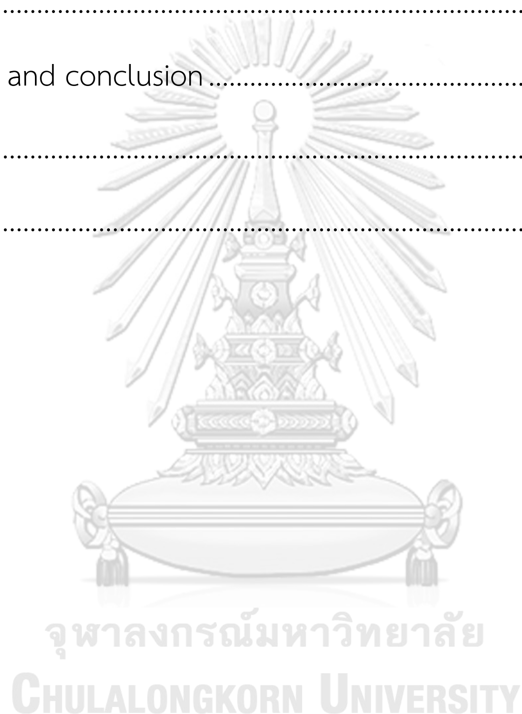

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List of Abbreviation

%	Percent
°C	Degree Celsius
α	alpha
β	beta
μ	micro
g	gram
H	Hour
IU	International unit
l	liter
m	milli
M	Molarity
MHz	Megahertz
vol/vol	Volume by volume
wt/vol	Weight by volume
ANOVA	Analysis of Variance
ART	Assisted reproductive technology
BAX	BCL-2 associated X protein
BCL-2	B-cell chronic lymphoma leukemia 2
BD	2,3-butanediol
BH3	BCL-2 homology domain 3
Bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
$CDCl_3$	Deuterated chloroform
CPA	Cryoprotective agent/ Cryoprotectant
CO_2	Carbon dioxide
COCs	Cumulus oocyte complexes
D	Day
DAPI	4,6-diamidino-2-phenylindole

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List of Abbreviation

DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra-acetic acid
EG	Ethylene glycol
EGF	Epidermal growth factor
ESI-QTOF	Electrospray-ionisation quardrupole time-of-flight
FBS	Fetal bovine serum
$FeCl_3$	Iron (III) chloride
FMD	Formaldehyde
FP	Forward primer
FSH	Follicle stimulating hormone
GLY	Glycerol
GV	Germinal vesicle
^{1}H	proton
HB	Hydrogen bond
HM	Holding medium
HRMS	High-resolution mass spectrometry
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
LH	Luteinizing hormone
METH	Methanol
MI	Metaphase I
MII	Metaphase II
Min	Minute
mRNA	Messenger ribonucleic acid
$NaHCO_3$	Sodium bicarbonate
NMR	Nuclear magnetic resonance

List of Abbreviation

O	Oxygen
OVH	Ovariohysterectomy
Pbs	Phosphate-buffered saline
PCR	Polymerase chain reaction
PF	Paraformaldehyde
PG	1,2-propanediol/propylene glycol
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
rhFSH	Recombinant human follicle stimulating hormone
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RP	Reverse primer
SDS	Sequence Detection System
sec	second
SEM	Standard error of the mean
SOF	Synthetic oviductal fluid
$T_g^{trehalose}$	Glass transition temperature/crystalline temperature of trehalose
$T_g^{sucrose}$	Glass transition temperature/crystalline temperature of sucrose
TLC	Thin-layer chromatography
TM	Thawing medium
Tre-$(OAc)_6$	Membrane-permeable trehalose/trehalose hexaacetate
Tris	2-Amino-2-(hydroxymethyl)-propane-1,3-diol/trimethyloaminomethane
YWHAZ	Tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein zeta

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CHAPTER I
Introduction

1.1 Importance and rationale

Assisted reproductive technologies (ARTs) including cryopreservation have been established and utilized to savage the genetic potentials of desired animals. These techniques have also been used as an excellent tool for safeguarding the valuable/endangered species. Domestic cats are being employed as developing models for wildlife conservation and also for biomedical proposes due primarily to the available samples and its similarity of reproductive physiology. Cryopreservation or subzero temperature storage is an interesting method for genetic banking. This technique allows long-term storage of cells, while the cells remain alive with preserved cellular functions. However, cryopreservation *per se* also inevitably induces cell damage such as disruption of cell membrane and organelles. There are many factors relating to cryoinjuries including freezing procedure, cooling rate, type of cell, stage of cell, cryoprotective agents (CPA). It is worth nothing that these cryoinjuries involve the formation of lethally intra-cellular ice formation occurred during cryopreservation and thawing process. Therefore, it has been hypothesized, in principle, that reduction of intra-cellular ice formation will increase the success rate of cryopreservation especially for oocytes. The cryoprotectant agents (CPA) that are normally used in the cryopreservation medium can be generally divided into two categories including penetrating and non-penetrating CPA. The non-penetrating CPA acts as a cellular buffer against osmotic pressure and also to protect the cell membrane. More importantly, penetrating CPA penetrate into the cell cytoplasm and replace the cellular water, thereby reducing intra-cellular fluid and subsequently the intra-cellular ice formation. However, high concentration of the intra-cellular CPA potentially leads to cellular damage and death. To overcome this problem, novel strategy to reduce intra-cellular ice formation using natural ice-inhibiting substance especially the trehalose would be interesting. However, it is worth noting that the trehalose can only be intra-cellular synthesized by particular plants and invertebrate

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organisms but not by the most of mammalian cells. This thesis, therefore, aims at synthesizing and examining the roles of a novel intra-cellular trehalose (trehalose hexa-acetate, trehalose-6-acetate) during cryopreservation process. It is hypothesized that this trehalose-6-acetate would permeate into the oocyte cytoplasm and protect the oocyte against cryoinjuries. This novel cryopreservation technology will indeed improve success rate of oocyte cryopreservation in terms of oocyte cryosurvival and developmental competence following cryopreservation.

1.2 Keywords

Cat, cryopreservation, oocyte, trehalose

1.3 Objectives of this study:

To determine the effects of stages of maturation stage on cryopreservability of feline oocytes

To synthesize and study the effectiveness of intra-cellular trehalose (trehalose hexa-acetate) as cryoprotective agent for oocyte cryopreservation

To determine the effectiveness of intra-cellular trehalose and extra-cellular trehalose on freezing ability of feline oocytes

1.4 Hypothesis of this study

Different stages of feline oocyte maturation are different by means of their freezing ability.

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Trehalose hexa-acetate can be synthesized and passively permeate through the ooplasm.

A combination of intra-cellular trehalose and extra-cellular non-reducing disaccharide can improve freezing ability of feline oocytes.

1.5 Literature review

1.5.1 Oocyte maturation

Mammals are born with full storehouse of oocytes (immature oocytes) that are provisional held in meiotic arrest until puberty. The pubertal hormonal changes in female stimulate the oocytes resumed meiotic division as known as oocyte maturation. There are three parts of oocyte maturation: nuclear, epigenetic and

cytoplasmic maturation. Oocyte maturation depended on several factors such as stage of estrous cycle, the oocyte quality, time of culture and hormonal supplementation (Merlo et al., 2005). According to the nuclear maturation of oocytes, the oocytes are arrested at prophase I of meiosis until the gonadotropin hormone, follicle stimulating hormone (FSH) and luteinizing hormone (LH), secreted to stimulate follicular growth and development. During the FSH and LH increasing, the oocytes are re-entry meiotic division II and oocytes are then arrested at metaphase II (MII) until fertilization (Eppig et al., 2004; Jamnongjit and Hammes, 2005). Epigenetic maturation refers to genomic modification that regulate gene expression during the fertilization and embryo development (Eppig et al., 2004). As nuclear maturation, cytoplasmic substances are simultaneously signaled and changed to promote for fertilization, cytoplasmic maturation, such as organelle re-organization, synthesis and accumulation of nutrient, protein, and genetic materials (Eppig et al., 2004; Watson, 2007).

To knowledge, oocyte developmental rate depended on oocyte maturation. The favorable both of nuclear and cytoplasmic of oocyte maturation provided superior oocyte developmental competence which revealed advanced embryo developmental rate as cleavage, morula, blastocyst, and blastocyst cell number (Merlo et al., 2005).

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1.5.2 Cryopreservation

Cryopreservation is an alternative and reliable method for long-term conservation of genetic resources. All metabolic cycles and physiological functions are discontinued at cryogenic stage (-196°C). This technique has been modified and used for gamete cryopreservation in order to improve genetic preservation of several domestic animal species. This will be beneficial for safeguarding the valuable gametes, embryos and cells derived from endangered species. In the future, cryopreservation with ultra-low temperature of living cells and organs would be increasingly more important method for maintaining biodiversity and species for preserving genetics and medical proposes (Luvoni and Colombo, 2020).

1.5.3 Oocyte cryopreservation

Cryopreservation plays an important role in assisted reproductive technologies (ARTs). For gamete cryopreservation, semen cryopreservation is the most promising technology for preserving male gametes as the quality and fertility of frozen-thawed sperm can be successfully high in terms of post-thaw sperm viability and pregnancy rate after artificial insemination. Controversy, oocytes are larger cells compared with the sperm and compose of complex organelles. Therefore, the oocytes are easy to fragile and sensitive to in subzero temperature (Tharasanit and Thuwanut, 2021). In addition, the use of CPA also leads to osmotic stress and organelle malfunction (Best, 2015), such as zonal hardening, premature cortical granule exocytosis and meiotic spindle disruption. As a result, frozen-thawed oocytes are more difficult to be fertilized when compared with fresh/non-frozen oocytes (Best, 2015; Tharasanit and Thuwanut, 2021). It is also well accepted that different approaching methods for cryopreservation of the oocytes revealed different results by means of survival rate developmental competence (Luvoni and Pellizzari, 2000; Luciano et al., 2009). In general, there are two major methods which are routinely applied for oocyte preservation including controlled-slow freezing and vitrification. These techniques have been used to successfully cryopreserve the oocytes both at immature and matured stage of the oocytes. An immature stage of the oocyte arrests at prophase I (germinal vesicle stage; GV) where the nuclear membrane appears to protect the tightly packed chromosomes. Notably, the thermosensitive meiotic spindle has not yet form at this stage. However, other structures such as cell organelles and gap junction’s communication between the oocytes and surrounding cumulus cells can be other factors contributing to the freezing susceptibility of the immature oocytes (Luciano et al., 2009). Similarly, matured (metaphase II, MII) oocytes containing meiotic spindle are susceptible to cryodamage in terms of chromosome arrangement and disorganization of microtubular structure (Luciano et al., 2009). It has been suggested that the programmable freezing protocol is suitable for matured oocytes compared to vitrification method.

Programmable controlled-rate freezing

Programmable freezing process is a slow and controllable reduction of temperature to an adequate stage of cellular dehydration which is preferred for minimization the intra-cellular ice crystal formation (Chen et al., 2017). This technique is required freezing machine by which the temperature can be controlled by the software and freezing apparatus. The cooling rate of this technique is fast in the initial time and then very slow once the ice formation has been initiated (FAO, 2012; Chen et al., 2017). In the past few years, programmable freezing is a technique of choice for cryopreservation of mammalian oocyte. Oocytes expose to low concentration of cryoprotective agent (CPA), and the slow cooling is performed (Luciano et al., 2009). According to the low CPA concentration used for slow freezing, the oocytes are less likely expose to osmotic shock and CPA's toxicity. However, this technique requires an expensive freezing apparatus, and the procedure is time consuming. In addition, different types of cells will also require an optimal freezing rate in order to minimize an excessively intra-cellular ice formation that is formed during slow freezing and thawing process (Luciano et al., 2009).

Vitrification

Vitrification is an alternative ultra-rapid method of cryopreservation which could be used to preserve living cells in an absent of ice crystal. This technique depends on the solidification of the liquid into a glass matter or no crystalline matter. Vitrification requires an extra-high concentration of CPA (20%-50% vol/vol) and high cooling rate during freezing (10ºC/min-100ºC/min) and warming (10^2-10^3ºC/min-10^5-10^7ºC/min) steps. This technique enhances and improves cell survival because it serves diminutive ice formation which results in less cellular damages. However, the use of vitrification has been hampered by toxicity and the excessive osmotic stress generated by the extra-high concentration of CPA. It therefore requires a skillful person to perform the vitrification process (Fahy and Wowk, 2015).

Different meiotic stages of oocytes require different freezing method. In feline oocytes, immature oocytes had high survival rate after warming step when compared with programmable freezing methods (Luciano et al., 2009). Vitrification is therefore suggested for immature feline oocytes, although the survival results has yet to be

satisfied owing to the low developmental competence (Luvoni and Pellizzari, 2000; Colombo et al., 2019).

1.5.4 Cryoprotective agents

Cryoprotective agent is a substance that is used for protecting tissues or cells from cryodamage during freezing process due principle to overgrowth of intra-cellular and extra-cellular ice crystal formation that subsequently cause cell disruption, malfunction and death. The freezing cells in subzero temperature are prone to death due to the ice formation and dehydration situation (Wright et al., 2004; FAO, 2012; Lin and Tsai, 2012; Russ et al., 2014; Best, 2015; Bozkurt, 2018). To preserve and minimize the cell death, the penetrating and non-penetrating CPA and also optimal freezing rate are necessary for determining the amount of intra- and extra-cellular ice formation.

1.5.5 Mechanism of action of CPA

There are many types of CPA such as glycerol, ethylene glycol, dimethyl sulfoxide (DMSO) and carbohydrate polymers. After dissolving in freezing solutions, the CPA act by reducing the melting point of surrounding solution. Thus, cells can be frozen within the surrounding solution without excessive ice crystal. Basically, sub-zero temperature has been observed to associate with the problems such as freezing injuries, ice crystal formation all of which can damage the cell functions. During freezing process, the CPA initially causes cell shrinkage due to the change of solution’s osmolarity. Later, the cells will be swollen after lipophilic CPA molecules penetrate into the cells. This “shrinkage-swelling” will be discontinued when the osmolarity between the intra-cellular and extra-cellular fluid is equal (Bhattacharya, 2018). In addition, some CPA also have special properties such as polyol CPA (glycerol and several sugars) can stabilize lipophilic part of the cell membrane by hydrogen bonding with the polar hear of phospholipid which is beneficial for dehydration stage (FAO, 2012). The prevention of ice crystal formation happens according to the properties of CPA which is interfering with hydrogen bonding between water molecules (Best, 2015). These problems could be restricted by the use of CPA. This CPA having hydrophilic property and can pass through the cell membrane are classified as penetrating CPA, such as glycerol, ethylene glycol, DMSO.

Besides, the remaining CPA, which rarely enter into the cell cytoplasm and usually have hydrophilicity with large molecules, are classified as non-penetrating CPA (Best, 2015; Pegg, 2015). When the penetrating CPA are used at a concentration of 5-15%, the unfrozen pockets could be largely observed and helped to prevent excessive formation of intra-cellular ice formation (Best, 2015). In contrast, vitrification requires high number of CPA. However, the levels of CPA exceeding 50% must be avoid because of cytotoxicity and also extreme osmotic stress (Bhattacharya, 2018).

1.5.6 CPA toxicity

At subzero temperature, intra-cellular fluid is estimated to replace by, at least, fifty percent of CPA molecules. The penetrating CPA having lipophilic molecules are able to self-permeate into cytoplasm. This CPA will substitute intra-cellular water and act as ice formation preventive agent. Unfortunately, extra high concentration of intra-cellular CPA causes distinctive disadvantages of cell survival, frequently causing cell death as a result of CPA toxicity. According to desiccation stage, CPA also induces osmotic shock, oxidative stress, chilling damages and other causes of cellular damage (FAO, 2012; Best, 2015; Pegg, 2015).

1.5.7 Types of cryoprotective agent

Cryoprotective agents are classified as penetrating CPA and non-penetrating CPA according to their ability to permeate through the plasma membrane into the intra-cellular fluid.

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Penetrating CPA

Penetrating CPA are lipophilic substances that passively permeate cell membrane, such as ethylene glycol (EG), propylene glycol (PG; 1,2-propanediol), dimethyl sulfoxide (DMSO), glycerol (GLY), formaldehyde (FMD), methanol (METH), and butanediol (BD; 2,3-butanediol). High level of penetrating CPA can reduce ice formation during freezing processes of cells, tissues and organs in supercooling temperature. In new age penetrating CPA, which are used during cryopreservation and pharmaceutical industry for preserving moisture during freezing process, a molecular mass less than 100 Dalton has been suggested. However, penetrating CPA become toxicity due to very high concentration. Thus, many resolutions have been

approached in order to solve the problems of ice formation with less cell toxicity (FAO, 2012; Pegg, 2015; Bozkurt, 2018).

Non-penetrating CPA

Non-penetrating CPA is a type of CPA that have been used to optimize unfrozen water during cryopreservation processes. Moreover, these CPA also play an effective role by reducing toxicity of penetrating CPA during the processes. Non-penetrating CPA are used in a combination of penetrating CPA (Wright et al., 2004; FAO, 2012; Waterstone et al., 2018).

Sugar

During cooling, concentration of extra-cellular solution plays a key role to determine the effectiveness of the cooling rate. As previous description, there is direct variation between cooling rate and salt concentration in freezing solution. Electrolytic salts and non-electrolytic salts affect to the cooling rate. These electrolytes increase constantly within the cytoplasm when temperature is gradually reduced (Mazur and Rigopoulos, 1983). The sugar has been used as non-electrolytic substance and also plays a role as extra-cellular salt concentration. In the same way, sugar in the freezing medium also affects the osmotic stress of cell during freezing process. The sugar acts as non-penetrating CPA and helps to move water from intra-cellular fluid to extra-cellular fluid due principle to the high osmolarity of the medium containing sugar. In practice, a combination of penetrating CPA and non-penetrating CPA or sugar in the medium is preferable. The advantages of this combination involving several reasons such as serving optimal cell volume and homeostatic ratio of intra-cellular water and electrolytes. As mentioned, sugar containing medium will lead to an efflux of intra-cellular water, while penetrating CPA can be efficiently influx into the cells. The further advantages of sugar containing in the freezing medium is to decrease the toxicity of penetrating CPA during the process. This combination of the two types of CPA resulted in a small amount of ice formation and limited cell shrinkage (Mazur and Rigopoulos, 1983; Wright et al., 2004; FAO, 2012). Sugar has evidently been reported to stabilize the lipid membrane during dehydration and to serve as water replacement from water-bound membrane,

thereby reducing cell membrane injuries during dehydration stress (Wright et al., 2004).

In freezing protocol, sugar containing medium is routinely used in humans and animals. Several types of sugars have been added into the freezing medium, such as sucrose, trehalose, lactose, fructose, raffinose, mannitol, sorbitol and hydroxyethyl starch (Eroglu et al., 2002; Wright et al., 2004; Eroglu et al., 2009; Eroglu, 2010; FAO, 2012; Abazari et al., 2015; Fahy and Wowk, 2015; Pegg, 2015; Waterstone et al., 2018). Of these sugars, trehalose is the most popular sugar that has been applied and used in cryopreservation of many cell types due to its excellent properties in protecting ice crystal formation.

Trehalose

Trehalose (α-d-glucopyranosyl-α-d-glucopyranoside) is a non-reducing disaccharide sugar which is composed of two molecules of glucose and formed by α,α-1,1 glycosidic linkage (figure 1). It presents and produces in mushrooms, yeast, fungi, insects, plant and prokaryotes (Eroglu et al., 2002; Richards et al., 2002; Kikawada et al., 2007);). In natural environment, trehalose is mostly formed in α,α-1,1 structure, and two others isoform of trehalose are neotrehalose (α,β-1,1 glycosidic linkage) and isotrehalose (β,β-1,1 glycosidic linkage) (Richards et al., 2002). Trehalose is bodily digested by trehalase and hydrolyzed only in small intestine (Montalto et al., 2013). High concentration of trehalose enhances desiccation and rehydration without injuries of cryptobiotic cells (Montalto et al., 2013; Martinetti et al., 2017). This is inspired cryobiologist to apply trehalose as protective substance during cryopreservation.

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Trehalose has been applied in cell cryopreservation for a long period of time due to its excellent cryoprotective properties. However, the beneficial effects of extra-cellular trehalose has been observed as the trehalose only acts as extra-cellular CPA. It would be interesting if trehalose can be delivered into the cytoplasm especially for the oocytes that have a large amount of cytoplasm. Several approaches have been used to introduce the trehalose through the cell membrane, such as membrane poration, microinjection, bacterial trehalose gene insertion. The

previous data suggested that only 0.2 M of intra- and extra-cellular of trehalose was adequate to prevent ice crystal induced cell damage (Wright et al., 2004). As recent report, microinjection of 0.15 M trehalose into human ooplasm resulted in high oocyte survival rate (60%) (Eroglu, 2010; Uchida et al., 2017). Furthermore, a previous report also revealed that different isoforms of trehalose appeared to have different cryoprotective properties. The natural trehalose likely performed best for its benefits during cryopreservation (Seo et al., 2006).

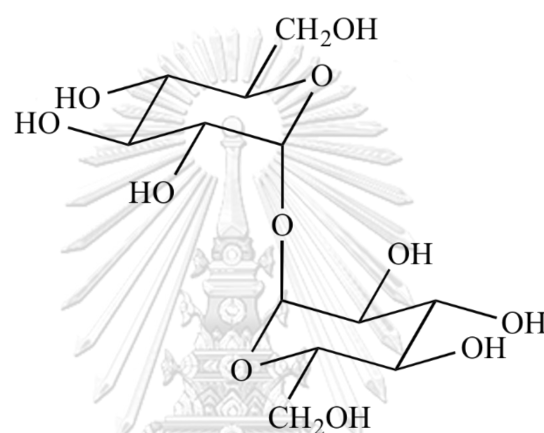

Figure 1 structure of trehalose

Trehalose is non-reducing sugar, forms by α,α,1,1 glycosidic bond between two molecules of glucose.

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Biological protection of trehalose during cryopreservation

According to structure, trehalose forms two to three hydrogen-bonded (HB) water molecules per one intra-structure of trehalose. This HB is likely folded with internal water molecules, and this is counteracted against water lacking by trapping these internal water molecules. Moreover, trehalose molecule presents two tipping oxygen (O) sites and six internal O sites. There have been reports indicating that one water molecule could bind at tipping O site and two water molecules bond at one internal O sites. The large number of HB and dramatically modification of water molecules has been thought to explain trehalose ability to biological protection from extremely thermal change and desiccation. This also resulted in an increase of crystalline temperature/ glass temperature ($T_g^{trehalose}$368 K) to be higher than other

sugar solution ($T_g^{glucose}$295 K, $T_g^{sucrose}$319 K) (Seo et al., 2006; Simperler et al., 2006; Pagnotta et al., 2010; Chiu et al., 2011; Zhang et al., 2016). In addition, the trehalose protects the cryopreserved cell during the process by prolonging time for cell dehydration and stronger membrane phase transition (Younis et al., 2009; Zhang et al., 2016).

1.5.8 Oocyte developmental competence

Developmental competence of mammalian oocytes is considered as capabilities of maturation, fertilization, and zygote formation. The oocyte developmental competence is controlled by intrinsic and extrinsic factors covered by gene and epigenetic regulation. For extrinsic factors, follicular size and heat shock are the significant influence factors related to the oocyte quality and their ability to develop during oogenesis and folliculogenesis. Oocyte developmental competence generally defines as the development capability of the oocytes throughout the process of gamete maturation, fertilization and embryo development which involves the biochemical and molecular changes (Kempisty et al., 2015).

1.5.9 Apoptosis in oocytes

Programmed cell death or apoptosis is a natural self-decided to destroy of living cells. In oocytes, apoptosis also occurs during oocyte development and folliculogensis. Moreover, the ovulated oocytes can also undergo several changes that lead to cell apoptosis (Tiwari et al., 2015). Spectacularly, cryopreservation process frequently induces apoptosis cascade and also cell senescence or cell death. By the low temperature process, the oocytes exposed to oxidative stresses activate the apoptotic signals both extrinsic and intrinsic pathways (Tiwari et al., 2015). The extrinsic apoptotic pathway of oocytes is originated by activation of the ligands of tumor necrosis factor family. Signals activate procaspase-8 to transform into an active form. Active caspase-8 motivates apoptosis by activating caspase-3. The ooplasm becomes fragment and oocyte’s structures become disruptive (Tiwari et al., 2015). The extrinsic apoptotic pathway depends on oolemma disturbance and disruption. The intrinsic pathway, however, presents principally by alteration of mitochondrial functions. There are several factors that motivate the intrinsic pathway of oocyte including reactive oxygen species (ROS), osmotic stress led organelle

malfunction, biochemical and ion imbalance. In cryopreservation, intrinsic pathway of oocytes can be affected by osmotic stress, intra-cellular ice formation, DNA fragmentation and chemical toxicity. According to mitochondrial change, cytochrome c, a small protein which is found in an inner mitochondrial membrane, leaks into ooplasm and activates caspase cascade in the oocyte (Tiwari et al., 2015). After cytochrome c leaking, proapoptotic proteins that regulate apoptosis in oocyte are triggered. BH3 protein family is the main apoptotic regulating protein of the oocyte (Felici et al., 1999; De Bem et al., 2014; Hutt, 2015; Majidi Gharenaz et al., 2016). High cytochrome c upregulates the Bcl-2 associated X protein (BAX), a member of BH3 family, resulted in deadly caspase-9 and caspase-3 activation. The caspase-3 adversely acts to cause structural, chemical and morphological change during oocyte apoptosis (Filali et al., 2009; De Bem et al., 2014; Tiwari et al., 2015; Majidi Gharenaz et al., 2016). In order to overcome apoptosis, the B-cell CLL/lymphoma protein 2 (Bcl-2) is synthesized and acts as anti- apoptotic mediated protein. The Bcl-2 prevents apoptosis via restoring the lipid transition and preventing cytochrome C release from mitochondria (Felici et al., 1999; Filali et al., 2009; Hutt, 2015; Kempisty et al., 2015). During oocyte maturation and early embryo development, the Bcl-2 is the mainly protein that prevents apoptosis. The previous studies suggested that the Bcl-2 is a good representative protein which indicate good quality of oocytes. Furthermore, *Bcl-2* expression level is antagonized with *BAX* expression level. This is inferred that *Bcl-2/BAX* ratio can be served as a good biomarker of oocyte developmental competence (Felici et al., 1999; Filali et al., 2009; Boumela et al., 2011; De Bem et al., 2014; Hutt, 2015; Majidi Gharenaz et al., 2016). The activation of intrinsic apoptotic pathway in the oocyte can therefore be detected by the high ratio of *BAX/Bcl2* gene in relation to the loss of mitochondrial membrane potential.

1.5.10 Novel approaches for improving quality of cryopreserved oocytes

Recently, gamete cryopreservation has increasingly been used in clinical practice because the technique is being developed to possess the higher survival rate with improved outcome (Wright et al., 2004; Younis et al., 2009; Abazari et al., 2015; Uchida et al., 2017). However, it is very challenging to cryopreserve the oocytes as the oocytes have complex structures and contains with many cryo-

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susceptible organelles. The cryo-susceptible organelles are less tolerant and easy to damage by intra-cellular ice crystal formation. As a resulted, low survival rate and poor post-thawed developmental competence are frequently observed (Eroglu et al., 2002; Wright et al., 2004; Eroglu et al., 2009; Younis et al., 2009; Eroglu, 2010; Monzo et al., 2012; Abazari et al., 2015; Zhang et al., 2016; Zhang et al., 2017). To improve the cryosurvival, several approaches have been tested. Of the strategies, addition of trehalose as extra-cellularly non-penetrating CPA also improved cryosurvival rates of several cell types such as sperm, oocytes and tissues (Younis et al., 2009; Eroglu, 2010; Abazari et al., 2015; Zhang et al., 2016; Zhang et al., 2017). Interestingly, trehalose could be modified to have more hydrophobic property. This allowed trehalose to permeate into the cytoplasm as intra-cellular trehalose. There have been several reports revealed that intra-cellular trehalose improved post-thawed survival quality of many cell types such as hepatocytes, fibroblast and oocytes. Trehalose was initially introduced into cytoplasm of murine cells before subzero preservation. This resulted in a high survival rate of frozen-thawed fibroblasts (Zhang et al., 2016) and hepatocytes (Abazari et al., 2015). The latter report suggested that trehalose hexaacetate could penetrate, and the optimal concentration of trehalose hexaacetate was found to be 30 mM for 12 hours (Abazari et al., 2015). For gamete cryopreservation, vitrification of human oocytes with freezing medium containing trehalose appeared to increase embryo development when compared with control without trehalose supplementation (Zhang et al., 2017). Also, loading trehalose into murine oocytes via microinjection significantly improved survival and blastocyst formation rates (Eroglu et al., 2009). The introduction of intra- and extra-cellular trehalose with DMSO in slow freezing of human oocytes obtained the highest survival rate (88.5%) of human cryopreserved oocyte compared to control group (52.2%) (Younis et al., 2009). Although intra-cellular trehalose cryopreserved oocyte gained high developmental competence in human and murine, the positive effects in freezing oocytes from other species have yet to be determined.

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Chapter II

Optimal concentration of trehalose solution on freezing ability of feline oocytes at different stages of maturation

2.1 Abstract

Oocytes cryopreservation is a valuable method for long-term storage of female genetics. However, the developmental competence of frozen-thawed oocytes is frequently poor. Here, we examined the effects of trehalose supplementation during cryopreservation of feline oocytes at different stages of maturation on developmental competence. In experiment 1, the feline oocyte at different stages of maturation were controlled-rate slow frozen without trehalose supplement. The probable cytotoxic effects of different concentrations (0, 0.125, 0.25, 0.5 and 1M) of trehalose was tested in experiment 2. In experiment 3, the oocytes at both maturation stages were frozen-thawed using medium with different concentrations of trehalose. The freezing ability of feline oocytes in terms of maturation and embryo developmental rates was subsequently assessed. Cryopreservation generally affected to the developmental competence of feline oocytes irrespective the stages of maturation. However, the immature oocytes were more susceptible to cryodamage when compared with matured oocytes ($P<0.05$). Incubation of oocytes with freezing medium containing trehalose significantly reduced the developmental competence especially when high concentrations (0.5 and 1 M) of trehalose was used. Optimal concentrations of trehalose for protecting cryoinjury during cryopreservation of immature and matured oocytes was different (0.125 M and 0.25 M, respectively). It is concluded that stages of meiotic maturation affected the freezing ability of feline oocytes. High concentrations of trehalose negatively affected the oocyte developmental competence. Supplementation of trehalose in freezing medium improved the freezing ability of feline oocytes. However, optimal concentration of trehalose is differently required for immature and matured feline oocytes.

Keywords: cat, cryopreservation, stage of oocytes maturation, trehalose

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2.2 Introduction

Cryopreservation or subzero-temperature preservation is one of the assisted reproductive technologies (ARTs) that is commonly used to preserve gametes for further use in genome banking and fertility treatment (Cocchia et al., 2010; Colombo et al., 2021; Comizzoli et al., 2004; Elliott et al., 2015; Luvoni and Colombo, 2020; Tharasanit and Thuwanut, 2021). However, cryoinjury occurred during cryopreservation frequently induces several physical and biological changes that render cell apoptosis and cell death (Arayatham et al., 2017; Vining et al., 2021). It is therefore still challenging for cryobiologist to minimize the cryoinjury especially the tissues or cells that have complex structures such as ovary and oocyte (Songsasen et al., 2002; Rajaei et al., 2013; Arayatham et al., 2017; Vining et al., 2021). The oocyte is the largest cell that have a low membrane permeability to cryoprotectants. It also contains a large amount of lipid content (Apparicio et al., 2012; Mokrousova et al., 2020; Okotrub et al., 2018) and thermo-sensitive organelles such as microtubules, actins and chromatins (Arayatham et al., 2017; Cocchia et al., 2010; Comizzoli et al., 2004, 2009; Luciano et al., 2009; Okotrub et al., 2018). These dynamical organelles and cellular cascades are important during oocyte development and meiotic resumption in order to support cytoplasmic maturation and their developmental competence (Tharasanit and Thuwanut, 2021; Vining et al., 2021). For cryopreservation, several factors have been demonstrated to affect the survival of cryopreserved oocytes such as maturation stage (Luvoni et al., 2000; Comizzoli et al., 2008), freezing procedures (Luvoni et al., 2020; Tharasanit and Thuwanut, 2021), and types and concentration of cryoprotective agent (CPA) (Best, 2015; Jang et al., 2017). There have been reported that stages of maturation and freezing techniques played a key role in determining the freezing ability of feline oocytes in terms of oocyte survival rates and their developmental capability (Luvoni and Colombo, 2020; Colombo et al., 2021, Tharasanit and Thuwanut, 2021). Both immature and matured stages of feline oocytes have been successfully cryopreserved (Luvoni and Colombo, 2020; Jewgenow and Zahmel, 2020; Arayatham et al., 2022; Tharasanit et al., 2011). However, it is still difficult to compare the results among laboratory since the freezing ability was predominantly dependent upon freezing techniques (Comizzoli et

al., 2009; Luvoni and Pellizzari, 2000 Arayatham et al., 2017; Luvoni et al., 1997; Snoeck et al., 2018) and types of CPA (Comizzoli et al., 2004; Tharasanit et al., 2011). Two types of CPAs including membrane permeable and non-permeable CPAs are generally used to mitigate the cryo-injuries. The penetrating CPAs act to reduce intra-cellular ice formation by free water substitution, while the non-penetrating CPAs such as sugar (trehalose, sucrose, and maltose) theoretically promote osmotic balance between intra-cellular and extra-cellular fluid. Among the non-penetrating CPAs, trehalose demonstrated to have an excellent property for reduction of cryoinjury (Best, 2015; Fahy, 1986; Fahy et al., 1990), extra-cellular osmotic protection and stabilization of cell membrane and protein structures during the freezing (Elbein et al., 2003; Jain and Roy, 2009; Schlichter et al., 2001). During cryopreservation, optimal concentration of CPA is required for different stages of oocyte maturation (Best, 2015; Fahy et al., 1990). In cat, the responses of extra-cellular trehalose and its properties to reduce cryoinjury during freezing and thawing of oocytes at different stages of maturations has yet to be determined. This study aimed at examining the effects of different stages of oocyte maturation and trehalose concentrations on viability and development capability following CPA incubation or after cryopreservation.

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2.3 Materials and methods

2.3.1 Experimental design

Experiment 1: Effect of maturation stages on cryopreservability of feline oocytes

Cumulus oocyte complexes (COCs) were collected either at immature or after *in vitro* maturation. The COCs were then subjected to controlled-rate slow freezing without trehalose supplementation. Three replicates (20-40 COCs per replicate) were performed. The frozen-thawed immature COCs were further matured *in vitro*. The developmental competence of the oocytes was tested by *in vitro* fertilization and embryo culture. The embryo development was examined on day 2, 5 and 7 for cleavage, morula, and blastocyst rates, respectively. Non-cryopreserved oocytes were used as a control group.

Experiment 2: Effects of different stages of oocyte maturation and trehalose concentrations on viability and development capability

The immature and matured oocytes were incubated for 15 min in freezing medium supplemented with different concentrations of trehalose (0.125 M, 0.25 M, 0.5 M, 1 M trehalose). The COCs exposed to freezing medium without trehalose (0 mM) served as a control group. The COCs were then washed and maintained in a holding medium. The COCs were matured and fertilized as described in experiment 1. A total of 30-40 oocytes were used in each experiment group and replicate. Three replicates were performed in this experiment.

Experiment 3: Effect of different concentrations of trehalose on freezing ability of feline oocytes

The COCs at different stages of maturation (immature versus mature stage) were incubated with freezing medium containing different concentrations of extra-cellular trehalose as described in experiment 2. After freezing and thawing, immature oocytes were matured *in vitro*. The maturation rate of oocytes after cryopreservation were examined after maturation for 24 hours. The maturation rates were compared among experimental groups. The matured oocytes derived either from frozen-immature oocytes or from frozen- matured oocytes were fertilized and cultured to determine their fertilizing ability and the developmental competence. A total of 30-40 oocytes were used in each experiment group and replicate. Experiment was performed triplicate. Frozen-thawed oocytes without trehalose served as a control group.

2.3.2 Reagents

All chemical reagents used in this study were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) unless otherwise specified

2.3.3 Ethics

The animal use protocol was followed and approved by the Chulalongkorn University of Animal Care and Use (Accession No. 2031002). All chemical reagents used in this study were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) unless otherwise specified.

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2.3.4 Source of ovary and sperm for IVF

The feline ovaries were obtained after a routine ovariohysterectomy which were collected from the Veterinary Public Health Division of the Bangkok Metropolitan Administration. The samples were transported in 0.9% (wt/vol) irrigation saline supplemented with 100 IU/ml penicillin and 100 IU/ml streptomycin. The samples were transported at approximately 30 °C to the laboratory within 1 hour.

The frozen-thawed semen used for IVF was collected from a proven-fertility tom cat. The semen was collected by electroejaculation under general anesthesia. The cat was premedicated by atropine sulfate (0.04 mg/kg) and xylazine (2 mg/kg) and then anesthetized with ketamine (10 mg/kg) intramuscularly. Three electroejaculation series was performed with 3 min interval (Howard et al., 1990; Comercio et al., 2013). The collected semen was examined and then frozen with egg yolk-Tris based freezing medium containing 5 % (vol/vol) glycerol.

2.3.5 *In vitro* maturation (IVM)

The feline ovaries were washed and maintained in a holding medium (HM: HEPES-buffered M199 supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 4 mg/ml bovine serum albumin, and 100 IU/ml penicillin). They were repeatedly sliced to obtain the cumulus oocyte complexes (COCs) from the ovarian follicles. The COCs were classified for their quality. The only A grading (dark cytoplasm and more than 5 layers of cumulus cell surrounding) and B grading (dark cytoplasm and 3-5 layers of cumulus cell surrounding) were only used in this study. The selected COCs were randomly allocated into experimental groups. The IVM medium consists of $NaHCO_3$ buffered M199 supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin, 50 μg/ml gentamicin, 4 mg/ml BSA and 0.05 IU/ml recombinant human follicle-stimulating hormone (rhFSH; Organon, Bangkok, Thailand) and 25 ng/ml EGF). The oocytes were matured for 24 hours at 38.5 °C in a humidified condition and 5% carbon dioxide (Sananmuang et al., 2010).

2.3.6 Oocyte cryopreservation and thawing

The oocytes subjected to cryopreservation were maintained at 24 °C in a freezing medium containing 10% (vol/vol) ethylene glycol. The freezing temperature

was maintained and controlled by a programable freeze-control system (CryoLogic CL-8800i, Australia). The freezing protocol was set as the followings: holding at 24 °C for 5 min; cooling down to -6 °C at a rate of 1 °C/min; holding for 5 min and seeding; holding for 5 min; cooling down to -33 °C at a freezing rate of 0.3 °C/min. The straws containing the oocytes were immediately plunged and stored in liquid nitrogen for at least 1 week. For thawing, the oocytes were thawed in air for 10 seconds and subsequently submerged into 37°C water bath for 30 seconds. The thawed oocytes were pooled and serially diluted in thawing medium (TM; 0.25 M sucrose in HM based medium) followed by 0.125TM and HM, respectively. Finally, frozen-thawed oocytes were maintained in HM at 37°C for further used.

2.3.7 *In vitro* fertilization and *in vitro* culture

For *in vitro* fertilization, ten *in vitro* matured COCs were cultured in a 50 µl droplet of *in vitro* culture medium (synthetic oviductal fluid: SOF) containing 4 mg/ml BSA and antibiotic (IVC-1 medium). Frozen semen used in this study was collected from a fertility-proven tom cat. The semen was thawed at 37°C for 30 sec and subjectively evaluated before co-culture with the matured COCs. Sperm with more than 50% progressive motility were acceptable for IVF. Total concentration of sperm was set at $5x10^5$ spermatozoa/ml. All culture steps were performed at 38.5°C in a humidified atmosphere of 5% CO_2 in air. After 24 hours of sperm/oocyte co-culture, all COCs were denuded by mouth-controlled glass pasture pipette and then cultured for additional 24 hours in synthetic oviductal fluid containing 4% (wt/vol) BSA (IVC-1). On day 2 after fertilization, cleaved embryos were selected and cultured in IVC-2 medium (SOF supplemented with 10% vol/vol fetal calf serum). The culture medium droplets were changed every other day. All culture steps were performed at 38.5°C in a humidified atmosphere of 5% CO_2 in air.

2.3.8 Assessment of embryo development

The embryo development was determined under an inverted light microscope (40x magnification) on day 2, 5 and 7 for cleavage, morular and blastocyst rates, respectively (day of IVF = day 0). All day-7 embryos assessed for their morphology and then fixed in 4% paraformaldehyde. The embryos were then

stained with 4’,6’-diamidino-2-phenylindole (DAPI, 0.1 ug/ml). The fluorescent-labeled embryos were then determined using an epifluorescence microscope. The embryos contained with more than 50 cells and blastocoel formation were considered as blastocysts.

2.3.9 Statistical analysis

Data were tested for normality and equal variance using the proportional data and were reported in the percentages ± SEM. The percentage of cleaved embryos, morula and blastocyst were defined as the number of cleavage embryo relative to total number of oocytes in each group. The difference of stages of oocytes and developmental competence (cleavage, morula, and blastocyst formation rates) were tested by *Chi*-square. Comparison of cell numbers within blastocyst among groups were compared by one-way ANOVA and post-hoc Bonferroni correction. P value less than 0.05 was considered as significance. All statistical analysis were analyzed using SPSS ver. 22.0.0 software (IBM, Armonk, NY, USA).

2.4 Results

Experiment 1: Effect of maturation stages on cryopreservability of feline oocytes

The developmental competence of frozen-thawed feline oocytes at different stages of maturation (immature and matured oocytes) are presented in table 1. The results revealed that the freezing and thawing, irrespective the stages of meiotic maturation, significantly reduced the developmental competence of feline oocytes when compared with non-frozen controls (P<0.05, table 1). However, the proportions of frozen-thawed matured oocytes developed to cleavage (31.5±1.7 versus 21.0±1.8%), morula (9.8±0.4 versus 5.9±2.3%) and blastocyst (4.4±2.0% versus 0%) stages were significantly greater than those observed from oocytes frozen at immature stage (P<0.05). In addition, cryopreservation also negatively affected to blastocyst quality by mean of blastocyst cell number (P<0.05).

Table 1 The effect of different stages of maturation on developmental competence of frozen-thawed feline oocytes

Group	N	Cleavage* (%)	Morula* (%)	Blastocyst* (%)	Blastocyst cell no.
Control	131	$63.2±2.8^{a}$	$42.9±4.0^{a}$	$39.0±3.4^{a}$	$136.3±8.2^{a}$
Immature	86	$21.0±1.8^{b}$	$5.9±2.3^{b}$	0^{b}	0^{b}
Mature	92	$31.5±1.7^{c}$	$9.8±0.4^{c}$	$4.4±2.0^{c}$	$74.4±7.6^{c}$

Data represent mean±SEM. Different superscripts (^{a,b,c}) within the column denote values that differ significantly (P<0.05). * in relation to total number of oocytes.

Experiment 2: Effects of different stages of oocyte maturation and trehalose concentrations on viability and development capability

The effects of trehalose on meiotic and developmental competence are shown in Table 2. The results showed that different trehalose concentrations affected the meiotic resumption and developmental competence of feline oocytes. The immature oocytes incubated in freezing medium supplemented with 0.125 or 0.25 M trehalose had similar MII rates when compared with control oocytes (P>0.05, Table 2). However, these trehalose concentrations significantly reduced further embryonic development in terms of cleavage, morula and blastocyst formation rates (P<0.05). The quality of blastocyst by mean of total cell number was also affected. Higher concentrations of trehalose (0.5 and 1 M) worsened the effects of trehalose on both meiotic and developmental competence, especially arrested the oocyte maturation at metaphase I stage (Table 2). In contrast to immature oocytes, incubation of trehalose at 0.125 and 0.25 M after oocyte maturation did not affect cleavage rates as compared to controls (P>0.05). However, the further embryo development (morula and blastocyst rates) and blastocyst quality were still significantly affected (P<0.05). Furthermore, high concentrations of trehalose (0.5 M and 1 M) were negatively affected to all stages of embryo development.

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Table 2 The effects of different stages of maturation on developmental competence of feline oocytes incubated with different concentrations of trehalose

Stage of oocyte maturation	Trehalose concentration (M)	Stage of nuclear maturation				Stage of embryo development				Blastocyst cell No.
		Total oocyte	GV (%)	MI (%)	MII (%)	Total oocyte	Cleavage* (%)	Morula* (%)	Blastocyst* (%)	
	0 (control)	86	0	$18.0±4.8^{a}$	$82.0±4.8^{a}$	104	$60.7±4.4^{a}$	$57.7±6.0^{a}$	$49.1±3.4^{a}$	$160.4±8.1^{a}$
Immature	0.125	88	0	$13.5±7.6^{a}$	$86.5±7.6^{a}$	88	$53.0±1.3^{b}$	$42.9±3.7^{b}$	$40.9±5.5^{b}$	$126.4±8.7^{b}$
	0.25	92	0	$15.5±6.3^{a}$	$84.5±6.3^{a}$	98	$51.9±1.8^{b,c}$	$43.1±6.0^{b}$	$40.4±1.41^{b}$	$125.5±9.0^{b}$
	0.5	48	0	$31.8±7.6^{b}$	$68.2±6.7^{b}$	48	$50.0±2.4^{b,c}$	$25.1±1.6^{c}$	$20.9±3.8^{c}$	$128.8±11.0^{b}$
	1	54	0	$30.1±6.8^{b}$	$69.9±12.8^{b}$	54	$29.0±3.7^{d}$	$16.8±1.5^{d}$	$12.8±1.9^{d}$	$107.5±10.6^{c}$
Matured	0.125	-	-	-	-	80	$55.9±3.2^{a,b}$	$45.7±6.2^{b}$	$43.0±4.1^{b}$	$129.3±8.7^{b}$
	0.25	-	-	-	-	139	$56.9±3.0^{a,b}$	$47.8±5.4^{b}$	$45.5±4.9^{a,b}$	$128.0±8.6^{b}$
	0.5	-	-	-	-	102	$51.8±2.1^{b,c}$	$32.2±4.8^{c}$	$30.8±2.0^{e}$	$130.5±4.9^{b}$
	1	-	-	-	-	54	$41.6±1.3^{e}$	$18.9±4.2^{d}$	$12.0±2.2^{d}$	$98.8±12.6^{c}$

Data represent mean ± SEM, ^{a,b,c,d,e} different superscripts in the same column denoted differ significant value ($P<0.05$). * in relation to total number of oocytes.

Experiment 3: Effect of different concentrations of trehalose on freezing ability of feline oocytes

Cryopreservation significantly impaired developmental competence of feline oocytes for both immature and matured stages compared to fresh oocytes (Table 3). Moreover, trehalose solution was effective for feline oocyte cryopreservation as indicated by developmental competence. Supplementation of different concentrations of trehalose in freezing medium improved the developmental competence when compared to non-trehalose supplemented group. For immature oocytes, trehalose at 0.125 M significantly improved developmental competence of frozen-thawed oocytes compared with control ($P<0.05$). However, there were no difference in terms of morula and blastocyst rates, and blastocyst cell number between 0.125 M and 0.25 M groups. In addition, trehalose at 0.5 M negatively affected to embryo development. This study also demonstrated that cryopreservation of feline oocytes at mature stage generally yielded higher embryo development when compared with oocytes frozen at immature stage ($P<0.05$). The concentrations of trehalose also affected to the cryopreservability of mature oocytes. However, 0.25 M trehalose was the best to protect the oocytes against cryoinjury, resulting the highest embryo development post-thawing.

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Table 3 The effect of different stages of maturation on cryopreserved feline oocytes with different concentrations of trehalose

Stage of oocyte	Trehalose concentration (M)	Stage of embryo development				
		Total oocyte	Cleavage (%)	Morula* (%)	Blastocyst* (%)	Blastocyst cell No.
Non frozen-thawed	0	104	63.2 ± 2.8^{a}	42.9 ± 4.0^{a}	39.0 ± 3.4^{a}	136.3 ± 8.2^{a}
Frozen-thawed immature	0	86	23.1 ± 3.0^{b}	3.8 ± 0.5^{b}	0^{b}	0^{b}
	0.125	101	29.5 ± 2.9^{c}	10.9 ± 0.9^{c}	4.0 ± 1.7^{c}	91.8 ± 4.1^{c}
	0.25	106	25.5 ± 1.2^{b}	$9.8 \pm 4.2^{c,d}$	3.8 ± 1.5^{c}	90.7 ± 6.0^{c}
	0.5	81	25.9 ± 1.6^{b}	7.8 ± 2.4^{d}	3.9 ± 1.0^{c}	75.3 ± 7.2^{d}
Frozen-thawed matured	0	90	32.2 ± 1.3^{c}	10.0 ± 0.8^{c}	4.5 ± 1.9^{c}	81.7 ± 8.1^{d}
	0.125	74	48.8 ± 1.1^{d}	18.5 ± 2.1^{f}	16.1 ± 1.6^{e}	108.2 ± 5.7^{e}
	0.25	117	59.4 ± 0.9^{d}	27.5 ± 4.2^{e}	26.0 ± 5.1^{d}	112.5 ± 9.8^{e}
	0.5	66	39.1 ± 3.2^{e}	16.1 ± 2.0^{f}	14.1 ± 1.8^{e}	107.4 ± 5.1^{e}

Data represent mean ± SEM, ^{a,b,c,d,e} different superscripts in the same column denoted differ significant value ($P<0.05$). * in relation to total number of oocytes

2.5 Discussion

Cryopreservation is the remarkable useful method for long-term genetic storage. Unfortunately, cryopreservation protocol induced severe damages to intra- and extra-cellular cellular compartments such as membrane damage (Luvoni and Pellizari, 2000), cytoskeletal disruption (Comizzoli et al., 2004; Luciano et al., 2009), protein denaturation (Clark and Swain, 2013), mitochondrial membrane disruption and finally cellular apoptosis (Clark and Swain, 2013; Arayatham et al., 2017). This study further demonstrated that stage of maturation affected to cryo-survival of frozen-thawed feline oocytes. According to the result (experiment 1), the frozen-thawed immature oocytes without trehalose poorly developed to blastocyst stage. No blastocyst was found for frozen-thawed immature oocytes. However, cryopreservation at matured stage significantly improved oocyte's developmental capability compared to frozen-thawed immature and non-frozen-thawed oocytes (3.3±0.1% vs. 0%, and 47.9±4.9%, respectively). It has been suggested that the immature oocytes demonstrate higher cryosensitivity when compared to mature oocytes. Although the exact mechanism is still unclear but poor cryo-survival of immature oocytes may cause by the irreversible damage of intra-cellular structures (Luvoni and Pellizari, 2000; Comizzoli et al., 2004; Luciano et al., 2009; Clark and Swain, 2013), which was induced by the formation of intra-cellular ice crystal during the subzero situation. This event interrupted the meiotic competence during the oocyte maturation (Luvoni and Pellizari, 2000; Cocchia et al., 2010; Clark and Swain, 2013). The cryoinjuries that are responsible for poor embryo development of frozen-thawed oocytes at both meiotic stages appear to involve several factors such as freezing technique, freezing medium and cell types (Luvoni, 2000; Ledda et al., 2007; Clark and Swain, 2013).

In experiment 2, cryoinjuries could be mitigated by adding or combinidng different types of CPAs in the freezing medium. Previous study suggested that ethylene glycol and DMSO could be used as penetrating CPA for feline oocyte cryopreservation (Cocchia et al., 2010; Tharasanit et al., 2011). It has become clear that supplement of non-penetrating CPA would help to reduce cryoinjury at freezing and thawing

processes. Among non-penetrating CPAs used to date, trehalose has been demonstrated to have an excellent property to reduce cryoinjuries and improve developmental competence of frozen-thawed oocytes (Arayatham et al., 2022; Elbein et al., 2003; Rayos et al., 1994). Different concentrations of trehalose have been used for oocyte cryopreservation. However, optimal concentration for each meiotic stage of feline oocytes has not previously been reported. As a non-penetrating CPA, trehalose plays as osmotic controller. In addition, trehalose plays an important role during cryopreservation by strong membrane phase transition and protein stabilization according to strong hydrogen bonding (Younis et al., 2009; Zhang et al., 2016). Trehalose is essential reduced ice crystal formation owing to high glass temperature ($T_g^{trehalose}$ 368 K) compared to other solution (T_g^{water} 140 K, $T_g^{glucose}$295 K, $T_g^{sucrose}$319 K) (Katkov and Levine, 2004; Seo et al., 2006; Simperler et al., 2006; Pagnotta et al., 2010). Excessive concentration of trehalose can adversely affect to oocyte's quality by dehydration effect. In this study, we found that too high concentration of trehalose (1 M, experiment 2) negatively affected to both immature and matured oocytes. This probably involves the fact that adding trehalose will increase solution osmolality. Concentration of trehalose (0.125 M, 0.25 M, 0.5 M, and 1 M) had the osmolarities 329±2.6, 368.8±7.6, 875±7.1 and 1557.75±8.1 mOsm, respectively. It therefore suggests that the feline oocytes have limited osmotic tolerant. Too high osmotic solution can unfortunately affect to water efflux and leads to over dehydration, and disturbing the homeostasis of oocyte (Best, 2015; Tharasanit and Thuwanut, 2021). Moreover, the different stages of oocyte maturation contain the different components that may be differently affected by the CPA, water permeability and osmolarlity (Agca et al., 1998; Agca et al., 2000). Experiment 3 found that the frozen-thawed immature oocytes had high developmental competence when frozen with to 0.125 M of trehalose concentration, while frozen-thawed matured oocytes required the different concentration (0.25 M trehalose). This suggests that different meiotic stages of oocytes require different condition for oocyte cryopreservation. This probably involves the membrane permeability to CPA and also osmotic tolerance of the oocytes at different stages of maturation.

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2.6 Conclusion

This study emphasized that cryopreservation interrupted oocyte meiotic resumption and developmental competence. Furthermore, immature feline oocytes are more susceptible to cryoinjuries compared to mature oocytes. This study also demonstrated the importance of trehalose for mitigate the cryoinjuries and for improvement of embryo development of frozen-thawed oocytes. The feline oocytes require an optimal concentration of trehalose, and this concentration was different for freezing oocyte at immature versus matured stage.

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CHAPTER III
Membrane-permeable trehalose improves the freezing ability and developmental competence of *in-vitro* matured feline oocytes

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3.1 Abstract

Oocytes are highly sensitive to cryopreservation, which frequently results in an irreversible loss of developmental competence. We examined the effect of membrane-permeable trehalose on the freezing ability of feline oocytes matured in vitro. In Experiment 1, intra-cellular trehalose (trehalose hexaacetate; Tre-$(OAc)_6$) was synthesized from trehalose precursor and subjected to spectroscopic characterization. The membrane permeability of the Tre-$(OAc)_6$ was investigated by incubating oocytes with different concentrations of Tre-$(OAc)_6$ (3, 15, and 30 mM). Optimum concentration and the toxicity of Tre-$(OAc)_6$ were assessed in Experiment 2. The effects of Tre-$(OAc)_6$ on freezing ability in terms of apoptotic gene expression and developmental competence of in-vitro matured oocytes were examined in Experiments 3 and 4, respectively. The Tre-$(OAc)_6$ permeated into the ooplasm of cat oocytes in a dose- and time-dependent manner. The highest concentration of intra-cellular trehalose was detected when the oocytes were incubated for 24 hours with 30 mM Tre-$(OAc)_6$. For the toxicity test, incubation of oocytes with 3 mM Tre-$(OAc)_6$ for 24 hours did not affect maturation rate and embryo development. However, high doses of Tre-$(OAc)_6$ (15 and 30 mM) significantly reduced maturation and fertilization rates ($P<0.05$). In addition, frozen-thawed oocytes treated with 3 mM Tre-$(OAc)_6$ significantly upregulated anti-apoptotic (*BCL-2*) gene expression compared with the control (0 mM) and other Tre-$(OAc)_6$ concentrations (15 and 30 mM). Oocyte maturation in the presence of 3 mM Tre-$(OAc)_6$ prior to cryopreservation significantly improved oocyte developmental competence in terms of cleavage and blastocyst

rates when compared with the control group ($P<0.05$). Our results lead us to infer that increasing the levels of intra-cellular trehalose by Tre-$(OAc)_6$ during oocyte maturation improves the freezing ability of feline oocytes, albeit at specific concentrations.

Keywords: cat, cryopreservation, oocyte, trehalose hexaacetate

3.2 Introduction

Assisted reproductive technologies (ARTs), including cryopreservation, have been established and used to conserve the genetics of desired animals, especially for endangered and valuable wild species (Pope et al., 2006; Luvoni and Colombo, 2020). Domestic cats have been intensively used as an animal model for genetic conservation due to their relatively similar physiological and biological functions of the reproductive system when compared to other wild felid species (Jewgenow and Zahmel, 2020). Cryopreservation is the method of choice for the long-term preservation of biological materials (Luvoni, 2000, 2006; Comizzoli et al., 2009; Eroglu et al., 2009; Luciano et al., 2009; Eroglu, 2010). Compared with different types of gametes, oocytes are the most difficult cells to be cryopreserved due to their structural complexity and molecular networks during oocyte maturation and subsequent embryo development (Arav and Natan, 2013; Rienzi et al., 2017). The oocyte is the largest cell that contains large amounts of lipids and cryosensitive organelles such as microtubules and chromatins (Comizzoli et al., 2004, 2009; Tharasanit et al., 2006; Luciano et al., 2009; Apparicio et al., 2012; Mokrousova et al., 2020). Several factors can affect the viability and developmental capacity of cryopreserved oocytes, such as maturation stage (Luvoni and Pellizzari, 2000; Commizoli et al., 2009), cryoprotectant type (Cocchia et al. 2010; Tharasanit et al., 2011), and freezing technique (Luvoni et al., 1997; Merlo et al., 2008; Luciano et al., 2009; Arayatham et al., 2017). The success rate of freezing oocytes is also dependent upon the species studied (Leibo and Songsasen, 2002; Smith and Silva, 2004; Amstistavsky et al., 2019). In domestic cats, immature and matured oocytes have

been successfully cryopreserved using both conventional slow freezing (Luvoni et al., 1997; Mokrousova et al., 2020) and vitrification (Comizzoli et al., 2009; Cocchia et al., 2010; Tharasanit et al., 2011; Galigius et al., 2014). However, the developmental competence in terms of blastocyst formation and pregnancy has been poor (Murakami et al., 2004; Tharasanit et al., 2011; Galigius et al., 2014), and therefore, the application of novel technologies for oocyte freezing, aimed at minimizing cryoinjury, is essential. For conventional slow freezing, intra-cellular and extra-cellular ice crystal formation increases simultaneously with temperature reduction. This excessive formation, especially in the case of intra-cellular ice formation, causes cryodamage and disrupts cellular structures and functions (Mazur et al., 2005, 2016; Eroglu, 2009). To minimize this problem, the combination of penetrating and non-penetrating cryoprotective agents (CPAs) is generally required (Wright et al., 2004; Cocchia et al., 2010, Tharasanit et al., 2011; Best, 2015; Zhang et al., 2016).

Hydrophilic sugars are commonly supplemented into freezing media for oocyte cryopreservation as membrane non-permeable CPAs. They mainly minimize the excessive osmotic pressure and extra-cellular ice formation during freezing and thawing (Wright et al., 2004; Younis et al., 2009; Eroglu, 2010). Of several types of sugars used for cryopreservation, trehalose is most suitable to stabilize proteins and membranes during cryopreservation (Crowe et al., 1984; Lins et al., 2004; Corradini et al., 2013; Camisasca et al., 2020; Starciuc et al., 2020). Trehalose (α-D-glucopyranosyl-(1,1)-α-D-glucopyranoside) is a non-reducing disaccharide sugar composed of two glucose molecules and formed by α,α-(1,1) glycosidic linkage (Elbein et al., 2003; Jain and Roy; 2009). High concentrations of naturally produced intra-cellular trehalose enhance cryosurvival *at subzero temperatures for some* plants, insects, and microorganisms (Elbein; 1974; Nwaka and Holzer; 1998; Muller et al., 2001; Khan et al., 2012). Most mammalian cells cannot produce trehalose, and therefore, extra-cellular trehalose is generally added to the freezing medium. Although several techniques, such as the addition of molecule-engineered trehalose (Abazari et al., 2015), thermal induction (Zhang et al., 2016), electrical stimulation (Dovgan et al., 2017), and the addition of nanoparticles (Zhang et al., 2009), have

been successfully used to load the trehalose into the cell cytoplasm, such technologies have not been tested for oocytes. Trehalose hexaacetate (Tre-$(OAc)_6$) is a novel interesting trehalose that passively permeate through the cell membrane. It is modified increasing the hydrophobicity by multi-molecules of acetylation. After passthrough, the acetyl groups of Tre-$(OAc)_6$ are cleaved by intra-cellular esterase enzyme and finally transformed into natural hydrophilic trehalose in the cytoplasm and lack of passive permeability function (Abizari et al., 2015). This study hypothesized that intra-cellular trehalose plays a role as a cryoprotection as in extra-cellular freezing medium. In this sense, this study aims to synthesize Tre-$(OAc)_6$ that possesses higher hydrophobicity and membrane permeability. Using the cat model, the effects of different concentrations of Tre-$(OAc)_6$ during *in-vitro* maturation on intracytoplasmic trehalose levels, apoptotic gene expression, oocyte developmental capability, and oocyte freezing ability were examined.

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3.3 Materials and methods

3.3.1 Experimental design

Experiment 1: Synthesis of Tre-$(OAc)_6$ and its membrane permeability

Membrane-permeable Tre-$(OAc)_6$ was synthesized by using a commercially available α,α-D-trehalose dihydrate as a precursor according to Abazari et al. (2015), with minor modifications from the original methods. The Tre-$(OAc)_6$ was subsequently characterized by nuclear magnetic resonance (NMR) spectroscopy and high-resolution mass spectrometry (HRMS). To determine its membrane permeability, the cumulus oocyte complexes (COCs) were incubated with different concentrations of Tre-$(OAc)_6$ and α,α-D-trehalose (0, 3, 15, and 30 mM) in *in-vitro* maturation (IVM medium). At 12 and 24 hours of incubation, the oocytes were denuded and lysed to detect intra-cellular trehalose.

Experiment 2: Optimum concentration and toxicity of Tre-(OAc)6

The COCs were matured *in-vitro* for 24 hours in the presence of different concentrations of Tre-$(OAc)_6$ (3, 15, and 30 mM). The IVM without Tre-$(OAc)_6$ (0 mM) served as a control group. At least three replicates were performed, with a total of 30–50 COCs per experimental group. After IVM, the oocytes were either assessed for stages of nuclear maturation or fertilized *in-vitro*.

Experiment 3: Effect of Tre-$(OAc)_6$ on apoptotic gene expression of frozen-thawed oocytes

The COCs were matured *in-vitro* with different concentrations of Tre-$(OAc)_6$ (3, 15, and 30 mM). Maturation without trehalose served as a control group. Following IVM, they were subjected to conventional slow freezing. After thawing, the oocytes were incubated in IVM medium for 3 H for recovery (Eroglu et al., 1998; Chen et al., 2003, 2009; Gao et al., 2009) and assessed for apoptotic gene expression (*BCL*-2, *BAX*) using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). The relative expression levels of these apoptotic genes were normalized to endogenous gene, *YWHAZ* (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta gene).

Experiment 4: Effect of Tre-$(OAc)_6$ on developmental competence of frozen-thawed oocytes

The COCs were matured *in-vitro* with different concentrations of Tre-$(OAc)_6$ and subjected to conventional slow freezing and thawing. The frozen-thawed oocytes were incubated in IVM medium for 3 h for intracytoplasmic organelles restoration and reorganization (Eroglu et al., 1998; Chen et al., 2003, 2009; Gao et al., 2009), before subsequently fertilized and cultured to access the fertilizing ability (cleavage rate) and developmental competence (morula and blastocyst rates).

3.3.2 Animal ethics and chemicals

This study was performed according to the approval of animal ethics by the Animal Care and Use Committee of Chulalongkorn University (Accession No. 2031002). All chemical reagents used in this study were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) unless otherwise specified.

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3.3.3 Synthesis of trehalose hexaacetate (2,2′,3,3′,4,4′-hexa-*O*-acetyl-α,α-D-trehalose; Tre-$(OAc)_6$)

The Tre-$(OAc)_6$ was synthesized from a commercially available α,α-D-trehalose dihydrate precursor. Firstly, the α,α-D-trehalose was subjected to tritylation in the presence of pyridine to yield 6,6′-di-*O*-trityl-α,α-D-trehalose intermediate (1, Figure 2). Progress of the reaction was monitored by thin-layer chromatography (TLC). Once the α,α-D-trehalose was totally consumed, acetic anhydride was added to furnished 2,2′,3,3′,4,4′-hexa-*O*-acetyl-6,6′-di-*O*-trityl-α,α-D-trehalose intermediate (2, Figure 2) as a yellow liquid after column chromatography. Removal of the trityl groups was accomplished using iron (III) chloride ($FeCl_3$) as Lewis acid to yield the 2,2′,3,3′,4,4′-hexa-*O*-acetyl-α,α-D-trehalose (Tre-$(OAc)_6$) as a white solid after chromatographic purification (Figure 2). To ensure the high purity of the product, the white solid product was subjected to recrystallization with ethanol and hexanes to provide the desired Tre-$(OAc)_6$ as a white crystal in 27% yield over three steps. nuclear magnetic resonance (NMR) spectroscopy and high-resolution mass spectrometry (HRMS) analyses confirmed the chemical structure of Tre-$(OAc)_6$.

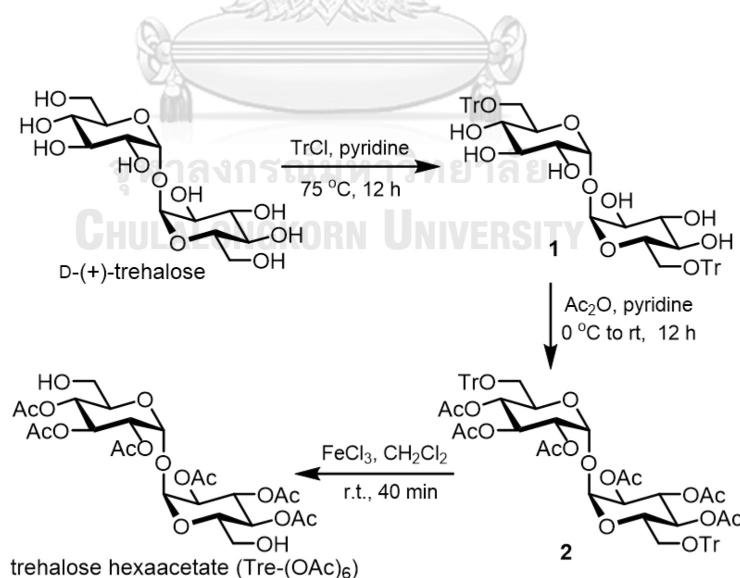

Figure 2 Synthesis of hydrophobic trehalose (trehalose hexaacetate).

3.3.4 Oocytes and *in-vitro* maturation

The feline ovaries were donated after routine ovariohysterectomy (OVH) from the Veterinary Public Health Division of the Bangkok Metropolitan Administration, Bangkok, Thailand. The ovaries were transported in a thermal protective container containing 0.9% (wt/vol) normal saline supplemented with 100 IU/mL penicillin and 100 IU/mL streptomycin. Within 2 h after OVH, the ovaries were separated from the surrounding tissues and then maintained in a holding medium (HM: HEPES-buffered M199 supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 4 mg/mL bovine serum albumin (BSA), and 100 IU/mL penicillin). The COCs were released from the ovaries by repeating slices in HM under a stereomicroscope (SMZ645; Nikon, Tokyo, Japan) and examined and classified according to the appearance of ooplasm and cumulus cells (Chatdarong et al., 2021). Only grade A and B COCs were used in this study.

In brief, the COCs were matured at 38.5 °C, 5% CO_2, for 24 hours in IVM medium (NaHCO_3-buffered M199 with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/mL penicillin, 50 µg/mL gentamicin, 4 mg/mL BSA, 0.05 IU/mL recombinant human follicle-stimulating hormone (rhFSH; Organon, Bangkok, Thailand), and 25 ng/mL epidermal growth factor.

3.3.5 *In-vitro* fertilization and embryo culture

After IVM, the matured oocytes were transferred to 50-µL droplets of CO_2-pre-equilibrated IVF medium (SOF, synthetic oviductal fluid supplemented with 4 mg/mL BSA). The frozen semen used in this study was obtained from a fertility-proven tomcat; semen collection and freezing were performed as previously described (Tharasanit et al., 2020). The frozen semen was thawed at 37 °C for 30 sec and then examined for progressive motility. Only sperm with progressive motility greater than 50% was used for IVF. The IVF was performed with a final concentration of 5×10^5 sperm/mL in a culture condition of 38.5 °C and a humidified atmosphere of 5% CO_2 in the air. After 24 hours of IVF (day 0), the inseminated oocytes were carefully denuded by a glass-mouth pipette and then further cultured for an additional 24 hours, after which the cleaved embryos were counted and selected. The selected embryos were cultured in SOF medium supplemented with 10%

(vol/vol) fetal bovine serum (FBS); the culture medium was changed every other day. The embryo culture system was set at 38.5 °C in a humidified atmosphere of 5% CO_2 in the air.

3.3.6 Assessment of meiotic resumption and embryo development

Oocytes subjected for examination of meiotic stages were mechanically denuded to remove the surrounding cumulus cells and then fixed in 4% (wt/vol) paraformaldehyde (PF) overnight. The oocytes were stained with 0.1 µg/mL DAPI (diamidino-2-phenylindole) and examined using an epifluorescence microscope (200X, Olympus, Shinjuku, Japan). Subsequently, they were classified as germinal vesicle stage (GV), metaphase I (MI) and metaphase II (MII), and degenerated oocytes. For embryo development, the morphology was examined with an inverted microscope (40–1000X, Olympus, Shinjuku, Japan). The cleaved embryos contained at least 2–4 cells on day 2 after IVF, whereas compacted blastomeres with more than 16 cells were classified as morula on day 5 of development. On day 7 post-IVF, the blastocoelic-stage embryos were fixed in PF and stained with DAPI. Total cell count per embryo was performed under an epifluorescence microscope (200X).

3.3.7 Control-rated freezing and thawing

After IVM, the COCs were maintained in basal medium for cryopreservation, containing HM and 20% (vol/vol) FBS. Two-step CPA exposure was performed by incubating the COCs in a cryopreservation medium in the presence of 5% (vol/vol) and 10% (vol/vol) ethylene glycol (EG) for 5 minutes, each. The COCs (10 COCs per straw) were then loaded into a 0.25-mL min-straw prior to cryopreservation and, controlled-rate cryopreservation was performed using a programable freeze control system (CryoLogic CL-8800i, Australia). The freezing protocol was set as follows: holding at 24 °C for 5 min, cooling down to -6 °C at a rate of 1 °C/min, holding for 5 min and seeding, holding for 5 min, cooling down to -33 °C at a freezing rate of 0.3 °C/min. Subsequently, the frozen oocytes were plunged and stored in liquid nitrogen. For thawing, the straws containing frozen oocytes were held in the air for 10 sec and then rapidly submerged in a 37 °C water bath for 30 sec. The oocytes were then released into HM-based thawing medium containing 0.5 M sucrose for 3 min and serially diluted within 0.25 M, 0.125 M and finally submerged into HM and 20%

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(vol/vol) FBS for 3 min, each. The thawed oocytes were further washed with HM twice and maintained at 37 °C in HM for analysis.

3.3.8 Measurement of intra-cellular trehalose

The COCs matured *in-vitro* with and without Tre-$(OAc)_6$ were mechanically denuded by mouth pipetting. The pooled denuded oocytes (n = 20) were washed twice in phosphate-buffered saline (PBS) and kept at -80 °C until used. The intra-cellular trehalose was measured in three replicates using the trehalose assay kit (Megazyme™, Wicklow, Ireland) following the manufacturer's protocol. In short, the oocytes were lysed by a lysis buffer (Agilent Technologies, USA), and the initial and final glucose concentrations were detected *via* spectrophotometry before and after enzymatic digestion. This procedure was run simultaneously with a standard trehalose reagent.

3.3.9 Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

The oocytes were first denuded free from the cumulus cells, washed twice in PBS, and kept in a microcentrifuge tube with minimum volume of liquid at -80 °C. In one replication contained 25 oocytes and three replicates were performed in this experiment. The RNA was extracted using the Absolutely NanoPrep Kit (Stratagene™, Agilent Technologies, CA, USA) as described by the manufacturer. Total RNA was measured for quality and concentration by a spectrophotometer (Nanodrop™ 2000, USA). The complementary DNA was synthesized by the First-Strand cDNA Synthesis Kit (Superscript III Kit, Invitrogen, USA) following the manufacturer's instructions. The qRT-PCR was performed to detect the relative expression levels of antiapoptotic *BCL-2* and apoptotic *BAX* mRNA, followed by normalization with a housekeeping gene (*YWHAZ*). The primers and amplicon sizes are shown in Table 4. The qRT-PCR was performed using the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Bedford, USA) with the KAPA SYBR® FAST qPCR Kit (Merck, Boston, MA, USA) as previously described (Arayatham et al., 2017). The relative expressions were analyzed by a Sequence Detection System (SDS) software (Applied Biosystem). The PCR products were verified by gel electrophoresis following staining with 5% (vol/vol) RedSafe nucleic acid staining solution (iNtRON Biotechnology, Hyeonggi-do, Korea) in

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a Tris borate EDTA buffer. The amplicon sizes were examined with the Gel documentation system (Syngenic, Cambridge, UK) under ultraviolet excitation.

Table 4 Forward (FP) and reverse primers (RP) and amplicon sizes used for qRT-PCR.

Gene	Primer (5'-3' orientation)	Product size (bp)	Reference
YWHAZ	FP: GAAGAGTCCTACAAAGACAGCACGC RP: AATTTTCCCCTCCTTCTCCTGC	115	HQ185236.1
BAX	FP: GTCAGCACTCCCGCCACAAA RP: CCGATGGCAACTTCAACTGG	244	NM_001009282
BCL-2	FP: GGATGCCTTTGTGGAACTGT RP: CGTTTCATGGGACATCACTG	223	AB096611

3.3.10 Statistical analysis

The experiments were performed with three to four replicates. Data were expressed as mean ± standard error of the mean (SEM) and tested for normality and equal variance using the median tested. Oocyte maturation rates were calculated by the percentage of oocytes that reached GV, MI, MII, and degenerated stages in relation to the total oocytes. Fertilizing ability was defined as the percentage of IVF oocytes that developed to cleaved embryos on day 2 of development (IVF oocytes= total cultivated oocytes on D0). The embryo developmental competence in terms of percentages of morula and blastocyst formation was expressed as a percentage in relation to the total number of cleaved embryos. The *Chi*-square test was used to test the differences in oocyte maturation, fertilizing ability, and embryo development. One-way analysis of variance (ANOVA) and Bonferroni post-hoc test was used to determine the differences in blastocyst cell numbers and the relative expression of *BCL-2* and *BAX*. Multivariate analysis of variance and Bonferroni correction were used for intra-cellular trehalose detection. All statistical evaluations were processed using SPSS version 22 (IBM Corp., USA); a p value less than 0.05 (95% confidence) was considered significant.

3.4 Results

Experiment 1: Synthesis of Tre-$(OAc)_6$ and its membrane permeability

Trehalose was converted into the trehalose hexaacetate to increase hydrophobicity. The final product appeared as a white crystalline powder after recrystallization. The desired trehalose hexaacetate product was obtained in 0.71 g (27% yield over three steps) from 2.08 g of the initial trehalose starting material. The spectroscopic data of trehalose hexaacetate was characterized as ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.52 (t, J = 9.9 Hz, 2H), 5.29 (d, J = 4.0 Hz, 2H), 5.05–4.94 (m, 4H), 3.94 (ddd, J = 10.3, 4.6, 2.6 Hz, 2H), 3.64–3.53 (m, 4H), 2.19 (bs, 2H), 2.08 (s, 6H), 2.07 (s, 6H), 2.03 (s, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.5, 170.4, 170.0, 93.7, 70.5, 70.3, 69.8, 68.9, 61.1, 20.8. HRMS (ESI-QTOF): *m/z* calcd for $C_{24}H_{34}O_{17}Na$ $[M+Na^+]$ 617.1699, found 617.1698.

Intra-cellular trehalose detection was verified by the trehalose assay. The results revealed that hydrophilic trehalose (α,α-D-trehalose dihydrate) had poor oocyte membrane permeability as the level of detected trehalose was extremely low. Increase concentrations and incubation times did not significantly increase the levels of trehalose inside the ooplasm ($P>0.05$; Figure 3). However, engineering the molecular structure of trehalose to Tre-$(OAc)_6$ significantly increased the intra-cellular levels of trehalose when compared with the trehalose dihydrate ($P<0.05$). In general, the levels of intra-cellular trehalose in oocytes increased in response to time and concentrations of Tre-$(OAc)_6$ added to the maturation medium (Figure 3). The concentrations of intra-cellular trehalose did not differ between incubation periods of 12 and 24 hours when 3 mM Tre-$(OAc)_6$ was supplemented ($P>0.05$). However, the levels of trehalose in the oocytes were significantly different between 12- and 24-hours treatment periods for high-Tre-$(OAc)_6$ groups (15 and 30 mM, $P<0.05$). Following oocyte maturation for 24 hours, the concentration of trehalose within the matured oocytes was significantly dependent on the concentration of Tre-$(OAc)_6$ added (Figure 3).

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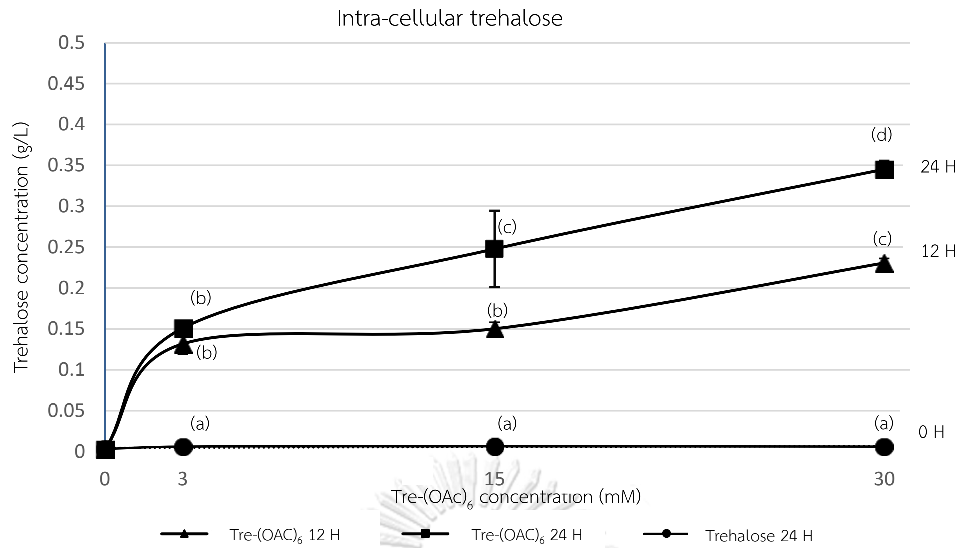

Figure 3 Intra-cellular trehalose detection

Accumulated levels (mean ± SEM) of intra-cellular trehalose in feline oocytes supplemented with trehalose hexaacetate (Tre-(OAc)$_6$) and D-(+)-trehalose (control) during *in-vitro* maturation. Different letters indicate significant differences ($p < 0.05$).

Experiment 2 Optimal concentration and toxicity of Tre-(OAc)$_6$

The effects of trehalose hexaacetate on meiotic resumption and embryo development are presented in Table 4. Supplementation with Tre-(OAc)$_6$ affected meiotic resumption and embryo developmental competence of feline oocytes in a dose-dependent manner. Low concentration of Tre-(OAc)$_6$ (3 mM) supplemented during *in-vitro* maturation had no adverse effects on oocyte maturation rate and developmental competence in terms of cleavage, morula, and blastocyst formation ($P>0.05$) when compared with the control (Table 4, $P>0.05$). However, high concentrations of Tre-(OAc)$_6$ (15 and 30 mM) significantly induced meiotic arrest at MI and resulted in a decreased number of oocytes reaching the MII stage (Table 4, $P>0.05$). In addition, these high concentrations of Tre-(OAc)$_6$ also significantly reduced the fertilization rate *via* the cleavage rate ($P<0.05$). In all cases, Tre-(OAc)$_6$, irrespective of the concentrations used, did not significantly affect the development of cleaved embryos to morulae and blastocysts ($P>0.05$).

Table 4 Effects of trehalose hexaacetate (Tre-$(OAc)_6$) supplementation during IVM on meiotic and developmental competence of cat oocytes.

Tre-$(OAc)_6$ Conc. (mM)	N	Stage of nuclear maturation				Stage of embryo development			Blastocyst cell numbers
		Degenerate (%)	GV (%)	MI (%)	MII (%)	Cleavage (%) *	Morula (%) **	Blastocyst (%) **	
0	101	2.6 ± 0.5	2.6 ± 0.5	2.6 ± 0.6^{a}	92.3 ± 1.9^{a}	47.8 ± 6.8^{a}	57.0 ± 22.4	17.8 ± 5.9	72.0 ± 38.6^{a}
3	96	3.7 ± 0.5	3.3 ± 0.6	3.0 ± 0.5^{a}	89.9 ± 0.8^{a}	47.9 ± 6.6^{a}	53.8 ± 9.3	21.3 ± 9.7	111.3 ± 21.8^{a}
15	93	2.8 ± 0.6	2.8 ± 0.6	12.5 ± 0.5^{b}	84.5 ± 1.3^{b}	44.1 ± 1.4^{b}	52.2 ± 2.3	15.6 ± 5.7	92.3 ± 33.2^{a}
30	95	0	9.44 ± 1.3	10.1 ± 0.5^{b}	82.2 ± 0.6^{c}	42.0 ± 5.9^{b}	50.6 ± 6.7	16.3 ± 6.1	105.7 ± 39.6^{a}

Data represent mean±SEM. ^{a,b,c} different superscripts in the same column indicate significant differences ($p < 0.05$). *, ** development in relation to the total COCs and cleaved embryos, respectively

Experiment 3 Effect of Tre-(OAc)$_6$ on apoptotic gene expression of frozen-thawed oocytes

Gel electrophoresis verified the expression of the antiapoptotic gene (*BCL-2*) and apoptotic gene (*BAX*), irrespective of the Tre-(OAc)$_6$ treatments (Figure 4A). *In-vitro* oocyte maturation with Tre-(OAc)$_6$ for 24 hours prior to cryopreservation generally increased the levels of *BCL-2* when compared to non-treated control oocytes. The results also indicated that the 3-mM Tre-(OAc)$_6$ group was most effective in maintaining high levels of *BCL-2* gene expression when compared to the control and other Tre-(OAc)$_6$ concentrations (P<0.05, Figure 4B). In addition, the levels of *BAX* mRNA expression of 3-mM Tre-(OAc)$_6$ and control groups were not significantly different (P>0.05). High doses of Tre-(OAc)$_6$ (15 and 30 mM) significantly downregulated *BAX* gene expression when compared to the control and 3-mM groups.

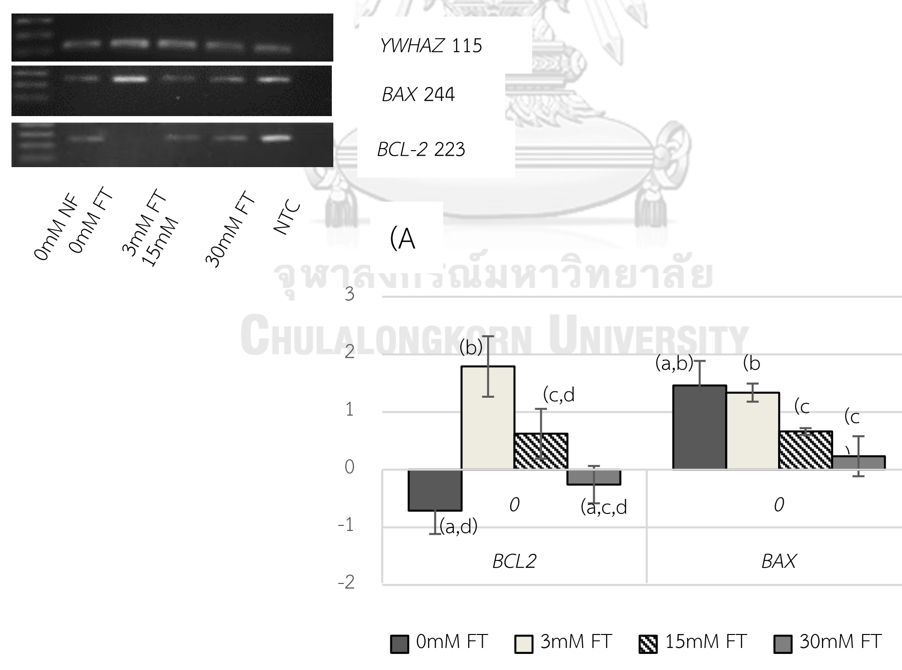

Figure 4 Relative gene expression

A: RT-PCR products showing the expression of *BAX* and *BCL-2* gene in feline oocytes following Tre-(OAc)$_6$ treatment and cryopreservation (NF: non-frozen, FT: frozen-thawed, NTC: no template control). B: quantitative RT-qPCR expression of *BAX*

and *BCL-2* indicating the effects of Tre-(OAc)$_6$ and cryopreservation at the level of gene expression.

Experiment 4 Effect of Tre-(OAc)$_6$ on the developmental competence of frozen-thawed oocytes

Table 6 shows that supplementation with Tre-(OAc)$_6$ (3 and 15 mM) during *in-vitro* maturation significantly improved the freezing ability of the oocytes, as indicated by the fertilization rate (cleavage rates: 33.2 ± 7.5% and 36.1 ± 3.1%, respectively), compared to the control group (27.4 ± 2.1%). However, oocytes treated with only 3 mM Tre-(OAc)$_6$ had greater blastocyst rates ($P<0.05$) and blastocyte cell numbers ($P>0.05$) than those of the control and those treated with other Tre-(OAc)$_6$ concentrations.

Table 6 Effects of Tre-(OAc)$_6$ supplementation during IVM on developmental competence of frozen-thawed cat oocytes.

Tre-(OAc)$_6$ Conc. (mM)	N	Stage of embryo development			Blastocyst cell numbers
		Cleavage (%) *	Morula (%) **	Blastocyst (%) **	
0	96	27.4 ± 2.1^{a}	16.1 ± 4.3^{a}	6.4 ± 4.5^{a}	66.0 ± 18.4^{a}
3	93	33.2 ± 7.5^{b}	22.3 ± 3.8^{a}	16.2 ± 5.2^{b}	96.3 ± 35.8^{a}
15	104	36.1 ± 3.1^{b}	24.2 ± 6.2^{a}	12.6 ± 6.6^{a}	68.4 ± 12.2^{a}
30	98	25.6 ± 5.1^{a}	12.4 ± 11.3^{a}	6.7 ± 4.6^{a}	81.0 ± 36.8^{a}

Data represent mean±SEM. ^{a,b,c} different superscripts within the same column indicate significant differences ($P<0.05$). *, ** embryo development in relation to total COCs and cleaved embryos, respectively.

3.5 Discussion

We synthesized and applied the Tre-(OAc)$_6$ to substantially increase the levels of intra-cellular trehalose. To our knowledge, this is the first report

demonstrating that modifications of the trehalose molecular structure to gain more hydrophobic properties could improve membrane permeability and the freezing ability of feline matured oocytes. Several studies have reported that trehalose protects the cell membrane and cellular proteins by chemical bonding and structure stabilization (Crowe et al., 1996; Eroglu et al., 2000; Chen et al., 2001; Elliott et al., 2006; Jain et al., 2009; Zhang et al., 2016; Bosch et al., 2016). According to its physiochemical properties, trehalose efficiently decreases lethal ice formation due to its high glass temperature (Tg) compared to other sugar types (Miller et al., 1997; Chen et al., 2000; Simperler et al., 2006; Olgenblum et al., 2020). A high concentration of trehalose is therefore required for the survival of several plants and microorganisms at subzero temperatures (Elbein et al., 1974; Nwaka et al., 1998; Muller et al., 2001; Khan et al., 2012). However, most mammalian cells do not produce this trehalose. As trehalose cannot permeate the cell membrane due to its high hydrophilic property, it mainly functions as an extra-cellular cryoprotectant (Kikawada et al., 2007; Eroglu et al., 2009; Tharasanit et al., 2011; Cai et al., 2020; Zhang et al., 2020). Several attempts have been made to artificially increase the levels of intra-cellular trehalose of mammalian cells, such as microinjection (Eroglu et al., 2003, 2017), specific channel induction (Elliott et al., 2006), gene editing (Uchida et al., 2017), freezing-induced osmotic stress (Zhang et al., 2016), and modification of trehalose solubility (Abazari et al., 2015). Of these technologies, only engineering trehalose molecules to gain more hydrophobic properties is a promising approach since it does not result in genomic changes and altered cell membrane functions.

In Experiment 1, we synthesized trehalose hexaacetate to increase its hydrophobicity compared to the trehalose. Our results demonstrate, for the first time, that this technique is applicable for oocytes since the synthesized Tre-$(OAc)_6$ permeated through the oolemma into the ooplasm. Once accumulated, the Tre-$(OAc)_6$ will be cleaved by intra-oocyte biological esterase enzyme, resulting in trehalose and acetic acid as final products (Abazari et al., 2015). Clearly, the levels of intra-cellular trehalose were dependent upon the initial Tre-$(OAc)_6$ loading concentration and on the incubation time (Figure 3). However, it should be noted

that the levels of intra-cellular trehalose observed in the current study were lower compared to those of hepatocytes (Abazari et al., 2015). It is possible that oocytes may have a limited Tre-$(OAc)_6$ transmembrane permeation and require longer incubation periods when compared with somatic cells. In addition, tightly packed cumulus cells surrounding the oocytes may alter membrane permeability to the CPA (Fabbri et al., 2001). Although incubation of the oocytes with 30 mM Tre-$(OAc)_6$ for 24 hours resulted in a greater level of trehalose when compared to 3 and 15 mM, this adversely affects meiosis resumption and, subsequently, embryo development (Table 2). It is hypothesized that extremely high concentrations of intra-cellular trehalose may contribute to an increase in excessive osmotic stress, which may disrupt cellular structures and functions (Bhowmick et al., 2002; Comizzoli et al., 2008; Abazari et al., 2015; Pollock et al., 2016). In addition, the decrease in intra-cellular pH as the acetic acid is accumulated as a side-product from the hydrolysis of Tre-$(OAc)_6$ by esterase is a matter of concern (Abazari et al., 2015). Although oocytes have been reported to tolerate acidity (Dale et al., 1998), the relationship between poor meiotic competence and intraoocyte pH after Tre-$(OAc)_6$ loading should be further examined. In Experiments 3 and 4, cryopreservation and thawing adversely affected the developmental competence of feline oocytes; this is in agreement with the activation of the apoptotic cascade by cryopreservation (Dai et al., 2015; Niu et al., 2016). Our results revealed that cryopreservation and concentrations of Tre-$(OAc)_6$ significantly affected *BAX* and *BCL-2* gene expression. Both *BAX* and *BCL-2* are pro-apoptotic and anti-apoptotic proteins associated with mitochondrial-dependent apoptosis pathways (Gomez-Crisostomo et al., 2013). Our results indicate that loading trehalose into ooplasm prior to cryopreservation reduced *BAX* expression, whereas anti-apoptotic *BCL-2* mRNA was well preserved (Figure 4). The patterning of gene expression corresponds to the findings that treating oocytes with 3 mM Tre-$(OAc)_6$ for 24 hours was superior in reducing oocyte apoptosis compared to other concentrations, whereas fertilization and embryo development were significantly improved (Table 6). These results were in agreement with previous reports indicating that oocytes require a small amount of intra-cellular trehalose to protect them against cryoinjury (Bhowmick et al., 2002; Eroglu et al., 2003, 2009). Thus, the

optimum concentration of intra-cellular trehalose is likely the most important factor that determines the freezing ability of feline matured oocytes in terms of meiotic competence, fertilizing ability, and embryo development.

3.6. Conclusions

In this study, Tre-$(OAc)_6$ was successfully synthesized and could be used to deliver trehalose into the feline oocytes. Cryopreservation of matured oocytes previously treated with various concentrations of Tre-$(OAc)_6$ differently affected meiosis resumption, apoptotic gene expression, and embryo development. Incubation of oocytes with 3-mM Tre-(OAc)6 during *in-vitro* maturation for 24 hours was optimal to improve meiotic competence and freezing ability in terms of cleavage rates and subsequent blastocyst development.

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Chapter IV
The combination of intra- and extra-cellular trehalose for cryopreservation of feline oocytes

4.1 Abstract

Cryopreserved oocytes have been used for genetic preservation. Domestic cat is a useful biological model for the endangered felid wildlife. However, the developmental competence of cryopreserved feline oocytes must be improved prior to realistically clinical use. Cryoinjuries cause poor post-thawed developmental competence and cellular damages. Cryoprotective agents (CPAs) are effective substances that help to reduce cryoinjuries during the cryopreserved process. Penetrating CPA reduces intra-cellular damages and non-penetrating CPA acts similar as extra-cellular part. Trehalose is a hydrophilic sugar that enhances and widely applies as a CPA. Unfortunately, mammalian cells *perse* cannot produce trehalose. This study examined the effect of trehalose using a combination of trehalose hexaacetate (Tre-$(OAc)_6$) as a penetrating CPA and alpha D-trehalose as a non-penetrating CPA (extra-cellular trehalose) on freezing ability of feline matured oocytes. In Experiment 1, Tre-$(OAc)_6$ was investigated to find out the optimal concentration (0, 1.5, and 3 mM). The optimal concentration of extra-cellular trehalose (0, 0.125, 0.25, and 0.5 M) was examined in Experiment 2. A combination of the two optimal concentrations were tested in Experiment 3. The results showed that 3 mM of Tre-$(OAc)_6$ improved post-thawed oocyte's developmental competence in terms of cleavage, morula, blastocyst rates and blastocyst cell numbers. It was also revealed that extra-cellular trehalose at 0.25 M was significant enhanced freezing ability of feline oocytes ($P<0.05$). Additionally, the combination of intra- and extra-cellular trehalose was significantly supported the frozen-thawed matured feline oocyte compared to non-trehalose supplementation. Our results highlighted that trehalose significantly improved freezing ability of matured feline oocytes.

Keywords: cryoprotective agents, domestic cats, frozen oocyte, trehalose, trehalose hexaacetrate

4.2 Introduction

Assisted reproductive technologies (ARTs) are technological processes which conserve genetic biodiversity for endangered species. There are various methods of ARTs that can be applied for animals and those are especially beneficial for valuable animal and endangered species. Domestic cat has been applied as a model for other felid species as they have similar physiological and biological reproductive functions compared to the wild felids. Cryopreservation is a method of choice for biological long-term preservation. Among the biological cryopreserved cells, oocytes are the most challenging cell types due to the fact that they contain very complex structures with cryosensitive organelles and dynamically molecular changes during oocyte maturation and embryo development. In cryopreserved feline oocytes, both immature and mature oocytes have been achieved to cryopreserved by conventional controlled rate freezing and vitrification. However, the post-thawed viability rates and developmental rates remains to be improved. Thus, optimization of freezing techniques aimed at minimizing the cryodamages is required. During controlled rate freezing, ice crystal formation at intra- and extra-cellular compartment is formed along with gradual decrease of temperature. The excessively increase of ice crystals destroys the normal cellular function such as cytoskeletal disruption, cell membrane imperforation, cellular malfunctions, and leads to cellular dead. To reduce the ice crystal formation, cryoprotective agents (CPAs) is normally supplemented into freezing medium to avoid excessive water cellular content during the freezing process. CPAs are divided into penetrating molecules and non-penetrating molecules. Penetrating CPAs are lipophilic molecules that play as intra-cellular free water replacement such as dimethysulfoxided (DMSO), propanediol $(PrOH)_2$, ethylene glycol (EG). These substances are chemicals that have been widely used for avoiding ice crystal formation. The unfavorable effects of penetrating CPAs were high toxicity to cellular functions. Non-penetrating CPAs such as yolk, protein, and sugar are lipophobic molecules, act as osmotic controller to prevent excessive extra-cellular ice crystal formation. They usually have large molecules certainly do not permeate through the plasma membrane. A combination

of penetrating and non-penetrating CPAs consequently mitigates the ice crystal formation during the freezing process.

Trehalose is biological disaccharide sugar, composed of two molecules of glucose at α,α-(1,1) glycosidic bond. Most mammals cannot produce it but it is naturally found in yeast, fungi, plants, and insects. The molecule of trehalose has lipophobicity and chemical inert. Thus, trehalose is low cellular toxicity and rarely permeate through cell membrane. During freezing stage, high concentration of trehalose prevents cellular damages as protein and cell membrane stabilizing agent and also reduces ice crystal formation.

Recently, membrane-permeable trehalose hexaacetate (Tre-$(OAc)_6$) as a penetrating trehalose has been engineered and used as penetrating CPA. Tre-$(OAc)_6$ self-permeates through cytoplasm and cleaves by intra-cellular esterase enzyme to natural trehalose form. Intra-cellular trehalose remains the natural trehalose properties; lipophobicity, subcellular stabilization and minimizing ice formation. However, the totally replacement of chemical penetrating CPA by Tre-$(OAc)_6$, combine with natural trehalose as a non-penetrating CPA, has yet to be studied. This study therefore aimed to evaluate the effects of a combination of penetrating- and non-penetrating trehalose on freezing ability and developmental competence of frozen-thawed feline oocytes.

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4.3 Materials and methods

4.3.1 Experimental design

Experiment 1: Effect of intra-cellular trehalose on cryopreservability of feline matured oocytes

The COCs were matured *in vitro* for 24 hours. The oocytes then subjected to cryopreservation medium containing with different concentrations of trehalose hexaacetate (Tre-$(OAc)_6$) (3 mM and 15 mM). The oocytes were then conventionally cryopreserved and then stored in the liquid nitrogen until further steps. For examination the efficiency of the intra-cellular trehalose, the frozen oocytes were

thawed and evaluated for oocyte quality and its developmental competence. The developmental competence was assessed following *in vitro* fertilization and *in vitro* culture. The percentages of cleavage, morular and blastocysts were examined. The quality of blastocysts was further determined by mean of cell number and its morphology.

Experiment 2: Effects of extra-cellularly trehalose solution on frozen-thawed feline oocyte developmental competence

The feline COCs were collected and matured *in vitro*. The matured oocytes were cryopreserved with freezing medium containing different concentrations of trehalose at 0.125 M, 0.25 M, 0.5 M. After thawing, the oocytes were subsequently *in vitro* fertilized. The developmental competence (cleavage rate, morula rate, blastocyst rate and blastocyst cell number) of treated oocytes was compared to non-trehalose supplementation as a control group (0 M). A total of 40 oocytes were used per treatment (tree replicates).

Experiment 3: Effects of a combination of intra- and extra-cellular trehalose on cryopreservability of feline oocytes

This study was performed as similar as experiment 1 and experiment 2. However, the COCs were matured in maturation medium containing Tre-$(OAc)_6$. After 24 hours of maturation, the oocytes were subjected to cryopreservation. The optimal concentration of extra- and intra-cellular trehalose were determined according to the experiment 1 and experiment 2. The oocyte quality following cryopreservation was examined by mean of post-thawing developmental competence.

4.3.2 *In vitro* maturation

The IVM medium consists of $NaHCO_3$ buffered M199 supplemented with 1.0 mM sodium pyruvate, 2.0 mM L-glutamine, 100 IU/ml penicillin, 50 µg/ml gentamicin, 4 mg/ml BSA and 0.05 IU/ml recombinant human follicle-stimulating hormone (rhFSH; Organon, Bangkok, Thailand) and 25 ng/ml EGF). To assess the developmental competence of the oocytes, the COCs were cultured as mentioned. However, the *in vitro* matured oocytes were fertilized *in vitro* with frozen semen. The embryo development was determined on day 2, 5 and 7 for cleavage, morular and blastocyst

rates, respectively. In all cases, the processes for *in vitro* oocyte maturation (IVM) and fertilization (IVF) were performed as previous described by Sananmuang *et al.* (2010) and Thongkittidilok *et al.* (2014).

4.3.3 Oocyte cryopreservation and thawing

The matured oocytes subjected to cryopreservation were maintained at 24 °C in a freezing medium containing 10% (vol/vol) ethylene glycol. The freezing temperature was maintained and controlled by a programable freeze control systems (CryoLogic CL-8800i, Australia). The freezing protocol was set as the followings: holding at 24 °C for 5 min; cooling down to -6 °C at a rate of 1 °C/min; holding for 5 min and seeding; holding for 5 min; cooling down to -33 °C at a freezing rate of 0.3 °C/min. The straws containing oocytes were immediately plunged and stored into liquid nitrogen. For thawing, the oocytes were thawed in air for 10 seconds and rapidly submerged into 37°C water bath for 30 seconds. The thawed oocytes were pooled and serially diluted in thawing medium (TM; 0.25 M sucrose in HM base medium) followed by ½TM, ¼TM and HM, respectively. Finally, frozen-thawed COCs were pooled in HM at 37°C before next step.

4.3.4 *In vitro* fertilization and *in vitro* culture

For IVF, ten *in vitro* matured COCs were cultured in a 50 µl droplets of *in vitro* culture 1 medium (synthetic oviductal fluid (SOF) containing 4 mg/ml BSA and antibiotic (IVC-1 medium). Frozen semen used in this study was collected from a fertility-proven tom cat. The semen was thawed at 37°C for 30 sec and subjective evaluation before co-culture with COCs. More than 50% of sperm progressive motility were considered to use for IVF. Total concentration of sperm that was co-cultured in each COCs droplets will be $5x10^5$ spermatozoa/ml. All culture steps were performed at 38.5°C in humidified atmosphere of 5% CO_2 in air. After 24 hours of sperm/oocyte co-culture, all COCs were denuded by mouth-controlled glass pasture pipette. Then, oocytes were washed twice and further cultured for 24 hours in 50 µl droplets of IVC-1. Next 24 hours, cleaved embryos were collected, washed twice and then cultured in IVC-2 medium (SOF supplemented with 10% vol/vol fetal calf serum).

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The culture medium droplets were completely changed every other day. All culture steps were performed at 38.5°C in humidified atmosphere of 5% CO_2 in air.

4.3.5 Assessment of embryo development

All cleaved embryos were observed under an invert microscope (40x magnification) to evaluate morula and blastocyst on day 5 and day 7, respectively (day of IVF = day 0). On day 7, all embryos were fixed in 4% (wt/vol) paraformaldehyde and kept at 4°C overnight before staining with 4',6-diamidino-2-phenylindole (DAPI, 0.1 µg/ml) in phosphate-buffered saline solution (PBS) for 10 minutes for nuclei count. The fluorescently labeled embryo were then determined using an epifluorescence microscope. The percentage of morula (>16 cells without blastocoels), blastocysts (>50 cells with blastocoel formation) were respectively examined on day 5, 6 and 7.

4.3.6 Statistical analysis

Data were tested for normality and equal variance using the proportion and reported in the percentage. The percentage of cleaved embryos were defined as the number of cleavage embryo relative to total number of oocytes in each group. The proportion of each embryo stage wase relative to the total number of cleaved embryos. The differences of stage of oocytes and developmental competence (cleavage, morula and blastocyst formation rates) were tested by *Chi*-square and statistical comparison of cell numbers within blastocyst were tested by one-way ANOVA. P value less than 0.05 considered as significance.

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4.4 Results

Experiment 1: Effects of intra-cellular trehalose on cryopreservability of feline matured oocytes

The effects of trehalose hexaacetate (Tre-$(OAc)_6$) supplementation on developmental competence are shown in Table 7. Freezing and thawing process negatively affected to feline developmental competence in terms of cleavage rate, morula rate, blastocyst rates and average cell numbers compared to non-frozen-thawed feline oocytes ($P<0.05$). Tre-$(OAc)_6$ supplementation during freezing process

improved cleavage, morula and blastocyst rates and also average cell numbers compared to none of Tre-$(OAc)_6$ group. After IVF, cleavage rates observed in 1.5 and 3 mM of Tre-$(OAc)_6$ supplementation groups did not significantly different (30.0±1.9% and 30.7±1.3%, respectively). However, the morula and blastocyst rates developed from oocytes frozen with 3 mM Tre-$(OAc)_6$ were significantly greater than 0 mM and 1.5 mM of Tre-$(OAc)_6$ supplementation.

Table 7 Effects of intra-cellular trehalose (Tre-$(OAc)_6$) on developmental competence of frozen-thawed feline oocytes

Oocytes	Tre-$(OAc)_6$ (mM)	N	%Cleavage*	%Morula*	%Blastocyst*	Average cell no.
Non-frozen	0	92	59.9±1.0[a]	41.6±1.8[a]	39.1±0.9[a]	140.4±4.8[a]
Frozen-thawed	0	146	27.4±1.3[b]	4.8±1.2[b]	2.7±1.7[b]	79.2±8.6[b]
	1.5	137	30.0±1.9[c]	8.0±0.7[c]	4.4±0.4[c]	88.7±6.5[c]
	3	153	30.7±1.3[c]	9.2±1.4[d]	7.2±1.3[d]	96.7±1.8[d]

Data represent mean±SEM. [a,b,c,d,e] different superscripts in the same column denoted significant differences ($p < 0.05$). * development in relation to the total COCs.

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Experiment 2: Effects of extra-cellular trehalose on developmental competence of frozen-thawed feline oocytes

The results showed that cryopreservation significantly reduced developmental competence of frozen-thawed oocytes, compared to non-frozen controls. The supplementation of extra-cellular trehalose during the freezing process of feline oocytes significantly improved developmental competence of post-thawed oocytes compared to no supplementation, as indicated by higher cleavage, morula, blastocyst rates, and average cell numbers. Additionally, the results showed that 0.25 M of trehalose significantly improved the embryo development in terms of cleavage, morula and blastocyst rates (58.3±1.4%, 26.0±2.3% and 24.4±2.5, respectively) compared to other trehalose supplementation groups. However, there were no

difference in average cell numbers among the trehalose supplementation groups where non-trehalose supplementation group was the least (Table 8).

Table 8 Effects extra-cellular trehalose on developmental competence of frozen-thawed feline matured oocytes

Oocytes	Trehalose Conc. (M)	N	%Cleavage*	%Morula*	%Blastocyst*	Average cell no.
Non-frozen control	0	92	59.9±1.0^{a}	41.6±1.8^{a}	39.1±0.9^{a}	140.4±4.8^{a}
Frozen-thawed matured	0	92	31.6±4.5^{b}	10.4±1.2^{b}	10.4±1.2^{b}	74.7±7.7^{b}
	0.125	93	50.7±1.9^{c}	19.6±4.7^{c}	18.7±2.7^{c}	110.8±6.8^{c}
	0.25	74	58.3±1.4^{d}	26.0±2.3^{d}	24.4±2.5^{d}	109.2±4.7^{c}
	0.5	79	49.2±2.3^{c}	19.3±1.6^{c}	14.2±2.0^{e}	103.8±7.8^{d}

Data represent mean±SEM. ^{a,b,c,d,e} different superscripts in the same column denote significant differences ($p < 0.05$). * development in relation to the total COCs.

Experiment 3 Effects of a combination of intra- and extra-cellular trehalose on cryopreservability of feline oocytes

Table 9 presents the effects of the combination of intra- and extra-cellular trehalose supplementation. The combination of two concentrations of Tre-$(OAc)_6$ as intra-cellular trehalose (1.5 mM and 3 mM) and three different extra-cellular trehalose concentration (0 M, 0.125 M, and 0.25 M) was tested. The results showed that 3 mM of Tre-$(OAc)_6$ combined with 0.25 M of trehalose was significantly effective for cryopreservation of feline oocytes as indicated by cleavage, morula and blastocyst rates, and blastocyst cell numbers (45.2±6.0%, 17.0±1.4%, 13.8±1.9%, and 96.08±6.4 cells, respectively) compared to no trehalose supplementation (32.2±1.0%, 10.0±0.7%, 4.5±1.9%, and 81.7±8.1 cells, respectively).

Table 9 The combination effects of intra- and extra-cellular trehalose on developmental competence of frozen-thawed feline oocytes

	$Tre\text{-}(oAc)_6$ conc. (mM)	trehalose conc. (M)	N	%Cleavage*	%Morula**	%Blastocyst**	Average cell No.
Non frozen-thawed	0	0	92	59.9 ± 1.0^{a}	41.6 ± 1.8^{a}	39.1 ± 0.9^{a}	140.4 ± 4.8^{a}
Frozen-thawed	1.5	0	73	29.4 ± 2.1^{a}	19.8 ± 5.4^{a}	16.4 ± 7.7^{a}	73.7 ± 4.6^{a}
		0.125	79	33.61 ± 3.76^{b}	34.3 ± 1.8^{b}	15.3 ± 3.8^{a}	94.67 ± 5.1^{b}
		0.25	54	36.88 ± 1.19^{c}	44.3 ± 5.1^{c}	24.5 ± 4.3^{b}	96.5 ± 7.4^{b}
	3	0	57	33.4 ± 1.9^{b}	26.2 ± 8.6^{d}	15.8 ± 1.4^{a}	99.7 ± 11.5^{b}
		0.125	91	32.98 ± 3.19^{b}	30.2 ± 3.0^{b}	16.4 ± 4.7^{c}	89.0 ± 11.5^{b}
		0.25	58	45.17 ± 6.0^{d}	37.7 ± 2.2^{e}	31.2 ± 7.9^{d}	96.08 ± 6.4^{c}

[a,b,c,d,e] different superscripts in the same column denoted significant differences ($p < 0.05$). * development in relation to the total COCs.

4.5 Discussion

In this study was examined the effects of intra- and extra-cellular trehalose as cryoprotective agents on developmental competence of frozen-thawed feline oocytes. Cryopreservation is a useful method for long-term preservation but successful result is still restricted due to cellular damage induced by ice formation during the freezing (Luvoni et al., 1997; Comizzoli et al., 2004, 2009, 2004; Jain and Roy, 2009; Jewgenow and Zahmel, 2020; Luvoni and Colombo, 2020; Mokrousova et al., 2020a,b). The injuries therefore induce cellular damages at several levels such as cytoskeletal disruption (Comizzoli et al., 2004, 2009, Arayatham et al., 2017), lipid inhomogeneity (Okotrub et al., 2018; Amstislavsky et al., 2019; Mokrousova et al., 2020), protein denaturation (Eroglu et al., 2020; Tharasanit and Thuwanut, 2021), intra-cellular organelle, and membrane perforation, and terminated cellular apoptosis (Comizzoli et al., 2004, 2009; Chen et al., 2009; Arayatham et al., 2017, 2022). Our findings agree with several reports that cryopreservation induced irreversible cellular damages, resulting poor embryo development (Luvoni, 2012, Arayatham et al., 2017; Ochota and Nizanski, 2018; Luvoni et al., 2020).

To reduce cryoinjuries, cryoprotective agents are generally used. The penetrating CPAs is often hydrophobic chemical that permeate and accumulate in intra-cellular fluid. However, suboptimal concentration may lead to subcellular toxicity. On the other hand, non-membrane permeable trehalose was reported as the most effective osmotic controller as non-penetrating CPAs. However, trehalose could only be synthesized in some prokaryote, plants, and insects, while most of mammalian cells cannot synthesize it. Tre-$(OAc)_6$ is a novel synthetic self-permeable trehalose that has been demonstrated as an effective penetrating CPA during cryopreservation. As our knowledge, Tre-$(OAc)_6$ is deacetylating hydrolyzed by cellular esterase enzyme into natural trehalose molecules. The property of natural trehalose, the hydrolyzed trehalose, played an effective role for reducing ice formation. Our result (experiment 1) showed that Tre-$(OAc)_6$ reduced intra-cellular ice formation injury as indicated by improved developmental rates of frozen-thawed feline oocytes. Likewise, extra-cellular trehalose played role as osmotic controller

and also reduced extra-cellular ice formation injury (Eroglu et al., 2000; Eroglu, 2010, Abazari et al., 2015). According to previous report, Tre-$(OAc)_6$ self-permeated and accumulated in feline ooplasm into natural trehalose form at approximately 0.15 g/l (0.0004 M), where other mammalian somatic cells preserved normal cellular function at least up to 0.2 M (Eroglu et al., 2000). Therefore, the particular types and species of cryopreserved cells required different types and concentrations of cryoprotective agent (Best, 2015; Tharasanit and Thuwanut, 2021). Trehalose exchanges molecular hydrogen bonding with water and biological molecules: lipid bilayers of cell membrane, cellular protein, and many cellular substances. The strong hydrogen bonding networks induced biological stabilization, especially unfolding protein (Olgenblum et al., 2020). This study supported that extra-cellular trehalose enhanced quality of frozen-thawed feline oocytes by mean of embryo developmental rate compared to no trehalose supplementation. However, different trehalose concentrations (0.125, 0.25 and 0.5 M) potentially induce different osmotic pressures (329±2.6, 368.8±7.6, 875±7.1 mOsm, respectively). In agreement with previously report, feline oocytes could be tolerated to the osmotic pressure in range 250-750 mOsm (Comizzoli et al., 2008), whereas 0.5 M trehalose resulted in extremely high osmolality. High osmotic pressure could result in the over-dehydration and ended up with poor freezing ability and developmental competence of feline oocytes (Comizzoli et al., 2008).

The combination of intra- and extra-cellular trehalose (Experiment 3) was chosen by the highest embryo developmental rate of intra- and extra-cellular trehalose in Experiment 1 and 2. The results emphasized that trehalose was necessary for oocyte cryopreservation. However, the optimal concentration of a combination of intra- and extra-cellular trehalose should be further examined as the developmental competence was still poor compared to only extra-cellular trehalose supplementation (Luvoni and Luvoni et al., 1997; Colombo, 2020; Jewgenow and Zahmel, 2020; Mokrousova et al., 2020b). It has been suggested that penetrating CPA induced higher intra-cellular dehydration by water replacement, and high extra-

cellular osmolarity could diminish cellular function by high osmotic stress (Eroglu et al., 2005; Fahy, 2010; Best, 2015; Tharasanit and Thuwanut, 2021).

4.6 Conclusion

This study highlighted that trehalose was necessary for feline oocyte cryopreservation. Cryopreservation of mature feline oocyte supplemented with Tre-$(OAc)_6$ improved post-thawed developmental competence. Incubation of oocytes with 3 mM during *in vitro* maturation for 24 hours was the highest effective. The 0.25 M trehalose was an optimal concentration as an extra-cellular cryoprotective agent. Furthermore, a combination of 3 mM of Tre-$(OAc)_6$ and 0.25 M of trehalose was effective for oocyte cryopreservation.

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Chapter V
General discussion and conclusion

5.1 Cryopreservation of feline oocytes and cryoinjuries

To date, cryopreservation of feline oocytes has been successful for long-term preservation. The frozen-thawed feline oocytes can develop post-thawing up to blastocyst stage, and pregnancy has been ontained (Tharasanit et al., 2011; Galigius et al., 2014). However, the rates of embryo development of post-thawed oocytes has still poor (Galigius et al., 2014; Arayatham et al., 2017, 2022; Mokrousova et al., 2020). Since then, there have been several studies aimed at improving the feline oocyte freezing ability by challenging the different freezing procedures (Luvoni et al., 1997; Mokrousova et al., 2020), stage of oocyte maturation (Luvoni and Pellizari, 2000), and types of penetrating and non-penetrating cryoprotective agents (CPAs) (Mokrousova et al., 2020; Tharasanit and Thuwanut, 2021). Indeed, freezing ability of feline oocytes has been variable depending on freezing procedures and stages of maturation of oocytes (Luvoni and Pellizari, 2000, Comizzoli et al., 2004; Tharasanit and Thuwanut, 2021) which were ranging from 20 to 48 % (Luvoni and Pellizari, 2000; Tharasanit et al., 2011; Arayatham et al., 2017; Mokrousova et al., 2020; Fernandez-Gonzalez et al., 2021) compared unfavorable to non-cryopreservation (Herrick et al., 2007; Cocchia et al., 2010; Tharasanit et al., 2014, Thongkittidilok et al., 2014). Although progress have been made, the pregnancy and live kitten born have been difficult (Tharasanit et al., 2011; Galigius et al., 2014). During the transition temperature, intra- and extra-cellular water molecules transform to consolidated structures which could be damage and imperforated intra- and extra-cellular organelles so-called "cryoinjuries/cryodamages" (Bhowmick et al., 2002; Eroglu et al., 2003, 2009; Best, 2015). Cryoinjuries further affect by suboptimal osmotic control (Eroglu et al., 2010; Camisasca et al., 2020). Osmotic changes cause hyper osmolarity induce cellular dehydration. In contrast, hyo-osmolarity induces intra-cellular water influx and disturbs cellular functions (Comizzoli et al. 2008; Tharasanit and Thuwanut, 2021). Suboptimal osmolarity can disrupt cellular functions (Comizzoli et al. 2008; Arayatham et al., 2017; Tharasanit and Thuwanut, 2021) at several levels such as

cytoskeleton disruption (Luciano et al., 2009; Arayatham et al., 2017), protein denaturation (Comizzoli et al., 2009), lipid phase transition (Mokrousova et al., 2020), and subsequently program cell death (apoptosis) (Arayatham et al., 2017, 2022). Oocytes per se has a low freezing ability due to the complexity of cellular (Okotrub et al., 2018; Mokrousova et al., 2020; Tharasanit and Thuwanut, 2021) and high cellular barrier such as cumulus surrounding cells (Okotrub et al., 2018). As a result, cryoinjured oocytes frequently show poorly meiotic resumption and embryo development (Arayatham et al., 2017). However, the effects of cryoinjuries could also activate genes (upregulation of *BAX* and downregulation of *BCL-2* expression) that are responsible for apoptosis.

Cryoprotective agents (CPAs): Trehalose and synthesized membrane-permeable trehalose

Cryoprotective agents (CPAs) are used to reduce an excessive water and to minimize the cryoinjuries. Currently, many studies reported that trehalose helped to reduce cryoinjuries during freezing procedure. However, trehalose acts only as non-penetrating CPA because of its hydrophilicity (Best, 2015, Tharasanit and Thuwanit, 2021). The previous studies reported intra-cellular trehalose loaded into frozen cells could improve viability of cells post-thawing (Eroglu et al. 2009, 2020, Abazari et al., 2015, Zhang et al., 2016). This study applied trehalose hexaacetate (Tre-$(OAc)_6$) as self-permeable trehalose, as penetrating CPAs similar to previously report (Abazari el al., 2015). We found that the Tre-$(OAc)_6$ permeated into oocyte cytoplasm but the intra-cellular levels of trehalose are dependent on used concentration and incubation time. According to previous report, Tre-$(OAc)_6$ self-permeated and accumulated in feline ooplasm into natural trehalose form at approximately 0.15 g/l (0.0004 M), where other mammalian somatic cells preserved normal cellular function at least up to 0.2 M (Eroglu et al., 2000). Moreover, Tre-$(OAc)_6$ could reduce cryoinjuries by enzymatic transformation into natural trehalose, and by lowering apoptotic-related gene expression. Of trehalose property as cryoprotective agent, there were several studies reported that trehalose exchanged molecular hydrogen bonding with water and biological molecules: lipid bilayers of cell membrane, cellular protein, and many cellular substances (Eroglu et al., 2000, Eroglu, 2010,

Bosch et al., 2016; Camisasca et al., 2020). The strong hydrogen bonding networks induces biological materials stabilization, especially unfolding protein (Olgenblum et al., 2020). This study further emphasized that trehalose plays a crucial role in preventing the cryoinjuries. However, trehalose prevents cryoinjuries in a manner of concentration dependence as similar as Tre-$(OAc)_6$.

5.2 Cryopreservation at different stages of feline oocyte maturation

Of the stage of maturation, the previous reports indicated that different stages of feline oocytes required different freezing procedure. Immature oocytes have a low tolerance as they contain a highly sensitive cytoskeleton such as cell cytoskeletons (Cocchia et al., 2010; Comizzoli et al., 2004; Galiguis et al., 2014; Tharasanit et al., 2006). It has become clear in the chapter II that different stages of oocyte maturation require different freezing techniques/conditions. When use the slow freezing, cryopreservation of oocytes at matured stages demonstrated higher embryo development compared with the oocytes frozen at immature stage. It is possible that slow freezing with trehalose supplementation is a technique of choice if the mature oocytes are to be cryopreserved. However, different result may be obtained if the vitrification is applied.

5.3 Combination of intra-cellular and extra-cellular CPAs: Tre-$(OAc)_6$and natural trehalose

This study (Chapter IV) is the first report to use the combination of intra- and extra-cellular trehalose as penetrating and non-penetrating CPAs, respectively. Intra-cellular trehalose, Tre-$(OAc)_6$ is self-permeated as the levels of trehalose is concentration and time dependence (Abazari et al., 2015). Although Tre-$(OAc)_6$ can be enzymatic cleaved and transformed into natural form of trehalose, too high concentration of intra-cellular trehalose may become toxic. Therefore, an optimal incubation time and concentration need to be empirically tested if this Tre-$(OAc)_6$ will be used in other species. Efficacy of different types of intra-cellular sugar in protecting oocytes against cryoinjury should be further investigated.

5.4 Suggestion for future investigations

Cryopreservation of oocytes is widely applied for genetic banking and cell reservation in many species. As aforementioned, trehalose prevents intra- and extra-cellular cryoinjuries in a manner of concentration and time dependence. The optimal concentration of the combination of intra- and extra-cellular trehalose must be further investigated for vitrification. Other types of sugar may be potentially used to mitigate cryoinjury that inevitably occur during freezing and thawing processes.

5.5 Conclusion

This dissertation demonstrates the effects of different stages of feline oocyte maturation and types of trehalose on developmental competence of feline frozen-thawed oocytes. Stages of maturation significantly affected to the freezing ability of feline oocytes, owing that the mature oocytes were superior to freezing compared with immature stage. It has become clear that freezing medium played a crucial role to determine the viability and developmental competence of frozen-thawed oocytes. The Tre-$(OAc)_6$ was engineered to permeate the cell membrane. This novel form of trehalose could increase intra-cellular levels of trehalose, while improved freezing ability of the mature oocytes. Natural trehalose are also important as non-permeating CPAs. In all cases, the optimal concentration and exposure time are critical.

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