ผลของตัวยับยั้งโรห์แอสโซซิเอสโปรตีนไคเนสต่อการเจริญพัฒนาไมโอสิสและตัวอ่อนของโอโอไซต์แมวภายหลังการแช่แข็งแบบวิทริฟิเคชั่น

นางสาวแสงตะวัน อารยธรรม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาการสืบพันธุ์สัตว์ ภาควิชาสูติศาสตร์-เธนุเวชวิทยาและวิทยาการสืบพันธุ์
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2558

Effects of Rho-associated protein kinase inhibitor on meiosis and embryo development of feline oocytes after vitrification

Miss Saengtawan Arayatham

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Theriogenology
Department of Obstetrics Gynaecology and Reproduction
Faculty of Veterinary Science
Chulalongkorn University
Academic Year 2015

Thesis Title	Effects of Rho-associated protein kinase inhibitor on meiosis and embryo development of feline oocytes after vitrification
By	Miss Saengtawan Arayatham
Field of Study	Theriogenology
Thesis Advisor	Assistant Professor Theerawat Tharasanit, D.V.M., Ph.D.

Accepted by the Faculty of Veterinary Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Veterinary Science

(Professor Roongroje Thanawongnuwech, D.V.M., M.Sc., Ph.D.)

THESIS COMMITTEE

.....Chairman

(Associate Professor Padet Tummaruk, D.V.M., M.Sc., Ph.D.)

.....Thesis Advisor

(Assistant Professor Theerawat Tharasanit, D.V.M., Ph.D.)

.....Examiner

(Associate Professor Kaywalee Chatdarong, D.V.M., M.Sc., Ph.D.)

.....External Examiner

(Assistant Professor Sukanya Manee-in, D.V.M., M.Sc., Ph.D.)

แสงตะวัน อารยธรรม : ผลของตัวยับยั้งโรห์แอสโซซิเอสโปรตีนไคเนสต่อการเจริญพัฒนาไมโอสิสและตัวอ่อนของโอโอไซต์แมวภายหลังการแช่แข็งแบบวิทริฟิเคชั่น (Effects of Rho-associated protein kinase inhibitor on meiosis and embryo development of feline oocytes after vitrification) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. น.สพ. ดร. ธีรวัฒน์ ธาราศานิต, 53 หน้า.

ในช่วงหลายทศวรรษที่ผ่านมาการเก็บรักษาเซลล์สืบพันธุ์ด้วยการแช่แข็งเป็นวิธีที่ได้รับความสนใจอย่างยิ่ง เทคโนโลยีการแช่แข็งนี้ช่วยเพิ่มประสิทธิภาพในการสืบพันธุ์ในมนุษย์และสัตว์หลายชนิดเช่น แมว สุนัข กระบือ และแพะ การแช่แข็งแบบวิทริฟิเคชั่นเป็นหนึ่งในวิธีการแช่แข็ง ช่วยให้สามารถเก็บรักษาเนื้อเยื่อและเซลล์ได้ระยะยาวนานขึ้น อย่างไรก็ตามกระบวนการเก็บรักษาเซลล์แช่แข็งมีข้อเสียหลายประการ ทำให้เกิดความเสียหายแก่เซลล์หรือเนื้อเยื่อและทำให้เกิดการตายของเซลล์ในที่สุดโรห์แอสโซซิเอสคอยล์คอยล์โปรตีนไคเนส (Rho-associated coil-coiled protein kinase, ROCK) มีหน้าที่ควบคุมการทำงานของเซลล์และมีบทบาทสำคัญในการเกิดการเสื่อมสลายและการตายของเซลล์ การศึกษาครั้งนี้ตรวจผลของตัวยับยั้ง ROCK ต่อความสามารถในการเจริญและพัฒนาของโอโอไซต์แมวที่ผ่านขั้นตอนการแช่แข็งแบบวิทริฟิเคชั่นและการทำละลาย ยีน LIMK-1 และ LIMK-2 จะถูกตรวจสอบในเซลล์คิวมูลัสและโอโอไซต์ (การทดลองที่ 1) และความเป็นพิษของตัวยับยั้ง ROCK ที่ความเข้มข้นต่างๆ (0, 10, 20 และ 40 ไมโครโมลาร์) จะถูกทดสอบในการทดลองที่ 2 การทดลองที่ 3 ทำการตรวจสอบผลของวิทริฟิเคชั่นต่อการแสดงออกของยีน การทดลองที่ 4 ศึกษาผลของการยับยั้ง ROCK ต่อการพัฒนาของโอโอไซต์ที่ผ่านการแช่แข็งแบบวิทริฟิเคชั่นภายหลังการทำละลาย การศึกษาครั้งนี้พบว่าโอโอไซต์ และเซลล์ คิวมูลัส แสดงออกยีน LIMK1 และ LIMK2 เมื่อทำการเลี้ยงโอโอไซต์ให้พร้อมปฏิสนธิใน ตัวยับยั้ง ROCK ความเข้มข้น10 μM พบว่าได้อัตราการพัฒนาของโอโอไซต์พร้อมปฏิสนธิในระยะเมตาเฟสที่ 2 ในระดับที่สูงอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) เมื่อเทียบกับกลุ่มควบคุม อย่างไรก็ตาม การเพิ่มความเข้มข้นของ ROCK inhibitor ในระดับสูง (40 μM) มีผลในทางลบต่ออัตราความสำเร็จในการเลี้ยงโอโอไซต์ให้พร้อมปฏิสนธิ แต่ไม่มีผลต่อประสิทธิภาพของการพัฒนาเป็นตัวอ่อน ยีน LIMK1 มีการแสดงออกในระดับสูงที่สุด เมื่อทำการเลี้ยงโอโอไซต์ที่ไม่ผ่านการแช่แข็งใน ROCK inhibitor ที่ระดับความเข้มข้น 10 μM นาน 12 ชั่วโมง ($p<0.05$) การใส่ ROCK inhibitor ที่ระดับความเข้มข้น10 μM ให้ผลในการเพิ่มจำนวนตัวอ่อนระยะคลีเวจ (36.13±3.76%) อย่างมีนัยสำคัญ และมีแนวโน้มเพิ่มร้อยละตัวอ่อนระยะโมรูล่าและบลาสโตซีส เมื่อทำการเปรียบเทียบกับกลุ่มควบคุม (27.40±2.54%) การศึกษาครั้งนี้สรุปว่ามีการแสดงออกของกลไก ROCK ในโอโอไซต์และเซลล์คิวมูลัสของแมวบ้าน ตัวยับยั้ง ROCK ความเข้มข้น10 μM เป็นระดับที่เหมาะสมในการนำไปใช้เพื่อเพิ่มอัตราการเลี้ยงโอโอไซต์ให้พร้อมปฏิสนธิ และเจริญเป็นตัวอ่อนโดยเฉพาะเมื่อโอโอไซต์ผ่านการแช่แข็งและทำละลาย แต่ตัวยับยั้ง ROCK ที่ความเข้มข้นสูงเกินมีผลในทางลบต่อโอโอไซต์

ภาควิชา สูติศาสตร์-เธนุเวชวิทยาและวิทยาการสืบพันธุ์ ลายมือชื่อนิสิต .....

สาขาวิชา วิทยาการสืบพันธุ์สัตว์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2558

5775313931 : MAJOR THERIOGENOLOGY
KEYWORDS: CAT / EMBRYO DEVELOPMENT / MEIOTIC RESUMPTION / OOCYTE / ROCK INHIBITOR / VITRIFICATION

SAENGTAWAN ARAYATHAM: Effects of Rho-associated protein kinase inhibitor on meiosis and embryo development of feline oocytes after vitrification. ADVISOR: ASST. PROF. THEERAWAT THARASANIT, D.V.M., Ph.D., 53 pp.

For decades, gamete cryopreservation is markedly interesting method for assisted reproductive technologies (ARTs) in several species such as human, cat, dog, buffalo and goat. Vitrification is a denoting applied procedure for tissues and cells for long term storage. However, there are several disadvantages of all types of cryopreservation that potentially damages to cell so-called cryoinjuries. Cryoinjuries occur during cryopreservation procedure that damages cells or tissues and finally lead to cell death. Indeed, rho-associated coiled-coil protein kinase (ROCK) manipulates the cellular function and plays a pivotal role in cell apoptosis and death. In the present study, we examined the effects of ROCK inhibitor on oocyte maturation and developmental competence of vitrified-warmed feline oocytes. LIMK-1 and LIMK-2 were determined in cumulus cells and oocytes (experiment 1). The toxicity of the ROCK inhibitors at various concentrations ROCK inhibitor (0, 10, 20 and 40 μM) was tested in experiment 2. The experiment 3 examined the effect of vitrification on gene expression. The effects of ROCK inhibitor treatment on post-warming development of vitrified oocytes were examined in experiment 4. Our results showed the expressions of LIMK1 and LIMK2 in fresh oocytes and cumulus cells. Cumulus oocyte complexes (COCs) cultured with ROCK 10 μM had the statistical highest metaphase II (MII) rate (p<0.05) when compared to controls. However, high concentration of ROCK inhibitor (40 μM) negatively affected on meiotic competence and but not for developmental competence. The gene expression of LIMK1 after *in vitro* maturation of non-cryopreserved oocytes supplemented with 10 μM ROCK inhibitor for 12 hours significantly up regulated to higher levels than other groups (p<0.05). Incubation of vitrified-warmed COCs with 10 μM ROCK inhibitor significantly increased cleavage rate (36.13±3.76%) and tended to increase morula and blastocyst rates when compared to non-treated group (27.40±2.54%) (p<0.05). In conclusion, this study demonstrated that ROCK cascade presences in feline oocytes and cumulus cells. A chosen dose of 10 μM ROCK inhibitor is an appropriate dose for improving feline meiotic resumption rate, especially after cryopreservation. However, high dose of ROCK inhibitor adversely affected to meiotic resumption.

Department:	Obstetrics Gynaecology and Reproduction	Student's Signature	----------
		Advisor's Signature	----------
Field of Study:	Theriogenology		
Academic Year:	2015		

ACKNOWLEDGEMENTS

This study was taken place at the Department of Obstetrics, Gynecology and Reproduction, Faculty of veterinary Science, Chulalongkorn University, Bangkok, Thailand.

I would like to express my appreciation to my advisor, Asst. Dr. Theerawat Tharasanit, for his support, encouragement and providing me a great chance to learn lots of techniques of Assisted Reproductive Technologies (ARTs) that could be advantage for studying the advance wildlife reproductive and conservative research.

To my colleagues, Dr. Narong Tiptanavattana, Dr. Ajjima Chansanroj, Dr. Piyathip Setthawong, Dr. Praopilas Phakdeedindan and OGR postgraduate students for our cordial friendship and encouragement.

Finally, I would like to thanks my family for supporting and beside me all times. They are the most important persons in my life, ever. My accomplishment could not be happened whether without their support. Especially, Rapin and Nga-Sai, my beloved cats, for your loveliness and make me happy. I'll never regret to have you.

CONTENTS

Page

Page

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	percent
°C	degree celsius
µg	microgramme
ANOVA	Analysis of Variance
ATPase	adenosine triphosphate enzyme
BAX	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
bp	basepair
BSA	bovine serum albumin
Ca^{2+}	calcium ion
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
COCs	cumulus-oocyte complexes
CPA	cryoprotectant agent
DAPI	4,6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
EG	ethylene glycol
EGF	epidermal growth factor
GAGs	glycoaminoglycan
GSH	intracellular glutathione
GTPase	guanosine triphosphate enzyme
GV	germinal vesicle
HM	holding medium
IU	international unit
IVC	in vitro culture
IVF	in vitro fertilization
IVM	in vitro maturation

LH	luteinizing hormone
LIM kinase/LIMK	lipase modulator kinase
M	molar
m	milli
MI	metaphase I
MII	metaphase II
MLC	myosin light chain
MPT	mitochondrial permeability transition pore
mRNA	messenger ribonucleic acid
n, No	number
$NaHCO_3$	sodium bicarbonate
NEAA	non-essential amino acid
OPS	open pulled straw
PH	Plecktrin homology
RBD	Rho-binding domain
rhFSH	recombinant human follicle stimulating hormone
RhoA	Rho protein type A
RhoB	Rho protein type B
RhoC	Rho protein type C
RhoD	Rho protein type D
RNA	ribonucleic acid
ROCK	Rho associated coiled-coil protein kinase
RT	reverse transcription
RT-PCR	reverse transcription polymerase chain reaction
SD	standard deviation
SOF	synthetic oviductal fluid
TNF	tumor necrosis factor

v	volume
VS	vitrification solution
w	weight
YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta

CHAPTER I

INTRODUCTION

Importance and rationale

A great of progress has been recently made towards the development of assisted reproductive technologies (ARTs) for conservation of several species. These technologies play important roles, not only for basic research but also for preserving genetic biodiversity in animal and wildlife conservation program. ARTs such as artificial insemination (AI), *in vitro* fertilization (IVF), embryo transfer (ET) and gamete cryopreservation have been used to support prepubertuation of genetically valuable endangered felids. Owing to the limitation of specimens from those valuable animals, domestic cat (*Felis catus*) is therefore logically used as a model for development of these techniques.

One of the promising technologies for genetic banking in ART program is gamete cryopreservation. This technique has been routinely used in humans and animals, although some techniques have still met a limited success in terms of cryosurvival and fertility after cryopreservation. In general, there are two methods of cryopreservation including slow-freezing and vitrification methods. The slow freezing technique has traditionally been used for several decades, while a new trend of "ice-free cryopreservation (vitrification)" has become attractive to cryobiologists. This is due to the fact that extensive intracellular ice formation during slow freezing is detrimental to cell viability. Vitrification, on the other hand, involves the conversion of a liquid substance into a glass-like, non-crystallization structure by ultrarapid freezing rate. Although live kittens have successfully been produced after vitrification of immature stage of cat oocytes, the meiotic and developmental competence of vitrified-warmed oocytes were markedly compromised. For instance, less than 1% of these vitrified-warmed oocytes could develop to term. Indeed, the cryopreservation causes irreversible and reversible damage at cellular and subcellular levels depending on the

degree and type of cryoinjury. Therefore, apoptosis and senescence of the oocytes (both surrounding cells and oocyte *per se*) would take part as a potential cause of poor oocyte viability and functionality. It has recently reported that physical stress and cryopreservation can trigger Rho-associated coil-coiled protein kinase (ROCK) cascades that, in turn, stimulate apoptotic pathways. It is hypothesized that this ROCK cascade is present in the feline oocytes and is also stimulated during vitrification. Furthermore, it is interesting to examine whether or not the addition of a selective ROCK inhibitor would really improve viability and fertilizing ability of vitrified-warmed oocyte.

CHAPTER II

LIERATURE REVIEW

Meiotic resumption

Somatic cells proliferate via mitosis, while the germ cells produce gamete (haploid= n) by meiosis. The new offspring is originated after fertilization by means of both male and female gametes fusion. The new progeny would contain a half genetic material from parents. To produce haploid cells, 2 sequential round of cellular and nuclear division would be carried out. These refer to Meiosis I and II. Parental chromosomes would be separated into sister chromatids during meiosis I after the end of S phase. Meiosis I, chromosome from one another would be homologously paired and secluded into various daughter cells. The end of meiosis I, the parental cells proliferate daughter cells which compose of both half parental chromosome. Meiosis II is a next step following the meiosis I which is similar to mitosis. Sister chromatid exclude to different daughter cells. Additionally, meiosis II finally produces four haploid cells containing one set of each parental chromosome. At metaphase I, the bivalent chromosomes align on the meiotic spindle. Sister chromatids would be attached homologously by microtubules at pole and anaphase I would be initiated. The chiasma of homologous chromosome would be disrupted and the chromosomes are finally segregated. Sister chromatids continue connected to one another at their centromere after homologous chromosomes have been separated. When meiosis I completes, sister chromatid which consists of one copy of each parental chromosome, homologously pair to another and meiosis II suddenly originates before decondensation of chromosomes. However, meiosis II undergoes as mitosis. At metaphase II, chromosomes rearrange on spindle fiber and sister chromatids attached by microtubules from opposite pole. Then, the sister chromatids separate by breaking at centromere and form new daughter cells. This gives rise to haploid daughter cells. Cell proliferation in meiosis I is asymmetric, as a result of a tiny polar body and oocyte formation. The oocyte undergoes forward to

meiosis II without neither re-formed a nucleus nor decondensed its chromosome. Oocytes are arrested at metaphase II before fertilization and continue further development (Levesque and Sirard, 1996; Tsafriri et al., 2005).

Cumulus function during oocyte maturation

Communication between cell to cell is important for oocyte and surrounding cells. The first is to arrest oocyte at prophase stage of meiosis I and then stimulate the oocyte to repossess meiosis after ovulation. Surrounding follicle cells or cumulus cells communicate with other cumulus cells and oocyte via gap junction. More specifically, gap junctions are located at the projections. The communication between oocyte and surrounding cells occurs through transversing the zona pellucida or transzonal process, and terminates on the oocyte plasma membrane (Tanghe et al., 2002; Van Soom et al., 2002; Yokoo and Sato, 2004).

Cumulus cells are necessary for *in vitro* maturation. It has been reported that denudation of cumulus cells was harmful for oocyte maturation in cattle. Cumulus cells are therefore an important effector during maturation of oocyte by keeping the oocyte arrest and on the other hand, resuming to meiosis. Moreover, cumulus cells play a role for enhancing cytoplasmic maturation. These key functions of the cumulus cells during oocyte maturation are attributed to their embellish gap junction and to their specific metabolizing capacity (Tanghe et al., 2002). Yokoo and Sato (2004) asserted that these communications are prominent, first to arrest the oocyte at prophase of meiosis and to cause repossess of oocyte to meiosis at ovulation. The oocyte continues arrested until the ovulatory gonadotropins surge and then stimulates the resumption of meiosis in the preovulatory follicle.

Cumulus cells associated with meiotic resumption

It has been affirmed that oocytes have no receptors of luteinizing hormone (LH), thus the initiation of oocyte maturation has to be interposed by the follicle. It has been reported that identification of receptors specifically bind to LH appeared on cumulus-

oocyte complex (COC) in bovine and murine species. This predicts the possibility of that the direct responses of gonadotropins upon the COCs.

Cumulus oophorus functionally communicate between oocyte and extrafollicular or culture surroundings. There have sufficient LH receptor only in preovulatory follicles in order to transmit the signal to the oocyte. Different hypotheses of resumption of meiosis that could stimulate the follicles by gonadotropins exposure are as the following: 1) by reducing the substances of oocyte maturation- inhibitor of intrafollicular substances; 2) by signal triggering by the granulosa cells to the oocyte which exceed the inhibitory environment of the follicle; or 3) by interfering the gap junction between cumulus cells and the oocyte (Tanghe et al., 2002; Yokoo and Sato, 2004). As recently proposed, the somatic cells receive the signals of LH that trigger and then produce two mechanisms of the second messenger to the oocyte. LH directly and indirectly acts on the follicle to induce theca and granulosa cells to provide a mediator through the follicular fluid or cumulus cells. There signals then transfer to the oocyte via cumulus-oocyte gap junctions. Cumulus cells respond to the signal according to LH with an intracellular calcium ion (Ca^{2+}) increase throughout all gap junctions into the oocyte (Tanghe et al., 2002; Van Soom et al., 2002; Yokoo and Sato, 2004). After LH surge, a temporal rise of cAMP takes place intracellularly. After exposed to LH in a few hours, cumulus cells endure plasma membrane depolarization and then lead to intercellular Ca^{2+} rise (Tanghe et al., 2002). Cumulus-oocyte coupling dynamically changes during maturation and the loss of the communication disturbs meiotic resumption by blocking the signal of meiosis inhibition from cumulus cells. After these phenomena, the oocyte resumes meiosis, continues through meiosis I, enters the second meiotic division and becomes arrest at meiosis II. This step is called oocyte maturation (Yokoo and Sato, 2004). The second half of maturation, somatic cells and oocyte are cooperated and confined to the corona radiata while the other layers of the cumulus cells are disconnected. According to this, down regulation of communication junctions is required for expansion of cumulus cells. A marked cumulus expansion is produced by large amount of secreted glycosaminoglycan (GAGs) (Tanghe et al., 2002; Van Soom et

al., 2002; Yokoo and Sato, 2004). Alternately, these GAGs interact with specific matrix substances and forming a network of viscoelastic and hydrate matrix in the extracellular part. This phenomenon facilitates detachment of the cumulus-oocyte complex from the follicle wall and then extrusion at ovulation (Yokoo and Sato, 2004).

Cumulus cells enhance cytoplasmic maturation of the oocyte

The cumulus cells are very essential for the oocyte to accomplish cytoplasmic maturation. It is important for the capacity to support male pronuclease formation, fertilization and embryonic development. In bovine, cumulus cells are beneficial for oocyte maturation either by secreting vital factors which induce developmental competence, or by against an embryo development repressive substance from the medium. Oocyte should be grown and successfully cultured when the communication junctions are still remained (Tanghe et al., 2002; Van Soom et al., 2002; Yokoo and Sato, 2004) . The cumulus cells play a role of protection and metabolism for oocyte cytoplasmic maturation. In cattle, cumulus cells reduce cysteine to cysteine and enhance cysteine uptake during maturation, as a result, bovine oocyte reaches higher content of intracellular glutathione (GSH). Stable levels of GSH in mature bovine oocyte lead to increase number of blastocysts that are normally fertilized.

Apoptosis

Programmed cell death process or apoptosis, is identified by detached appearances in morphology and energy-dependent mechanisms. Apoptosis is a crucial element of various processed containing cell turnover, normal immune function and development, hormone-induced atrophy, embryonic development as well as chemical-induced cell death. These cellular events moderate the life or death of cellular recognition for its immense therapeutic potential.

Apoptosis occurs generally during development and deterioration as well as a homeostatic mechanism to uphold their normal cell population in tissue. It also appears as a defense mechanism such as immunoreactions or when cells are damaged by harmful stimuli such as disease or lethal agent. Furthermore, irradiation or medication

also affects cells to apoptosis such as chemotherapy, used for cancer therapy, results in DNA damages and leads to apoptosis. Some hormones have been reported to cause apoptosis. Though, there are a variety of stimuli and conditions that motivate apoptosis. However, it does not mean that all cells would decide to dead when expose to similar stimuli. In conclusion, apoptosis is a correlated process to other cells and further related to energy-dependent process. Apoptosis undergoes the activation via a group of cysteine proteases called "caspases" and complex events that conjoin the originating incentive to final departure of the cell. During the early step of apoptosis, cell shrinkage and nuclear pyknosis are detected. The cells seem smaller, the cytoplasm appears round or oval shape, dark, dense nuclear formation and organelles appear to be a more strictly pack. These are the result in condensation of chromatin and appeared common feature form of apoptosis. Expanded cell membrane blebbing (also called budding) occurs following by karryorrhexis and disjoining of cellular fragments to form apoptotic bodies, strictly packed organelles with or without nuclear fragment. Theses buddings are subsequently eradicated by phagocytosis. The apoptotic mechanisms seem highly complicated, concerning an energy dependent cascade of molecular events (Elmore, 2007; Gozuacik and Kimchi, 2007; Ouyang et al., 2012; Chaabane et al., 2013). At the present, apoptosis is categorized into 2 major pathways. The first is the extrinsic pathway or death receptor pathway and another is the intrinsic pathway or mitochondrial pathway. The evidence shows that the two pathways are conjoined and molecules in one another pathway that can affect to the other. Both extrinsic and intrinsic pathways join on the same terminal. It is execution pathway. This pathway is originated by the motivation of caspase-3 and finally results in DNA damage, cytoskeletal degradation and nuclear proteins, cross-linking protein, formation of apoptotic bodies, expression of ligands receptors. Apoptotic cells exhibit several biochemical modification such as protein splitting, protein cross-linking, DNA fragmentation that cause the remarkable structural pathology. Caspases are protein that widely expressed in the most cells as a proenzyme form. These proteolytic cascades, in

which one caspase can activate the others, can amplify the apoptotic signaling pathway and lead to cell death rapidly (Elmore, 2007).

The extrinsic pathways that originate apoptosis by receptor-mediated interaction. " Death domain" composes of cysteine-rich extracellular domains and cytoplasmic domain. These domains play a significant role in conducting the signal of death that is sent from cellular surface through intracellular pathways. There are many typical feature mediators and analogous death receptor such as FasL/ FasR, TNF-α/TNFR1, APO3L/DR3. The sequences that define the extrinsic apoptotic pathway are best classified by the FasL/FasR and TNF-α/TNFR1 (Elmore, 2007; Chaabane et al., 2013). There are different of receptors that can be bound with the similar ligand. The adapter protein, FADD, associates with procaspases-8 via dimeriazation of the death domain. Accordingly, a death signal complex is formed resulting in the auto-catalytic motivation of procaspase-8. When caspase-8 is motivated, the apoptotic execution phase is activated.

The intrinsic signaling pathway that initiates apoptosis including a several arrangement of non-receptor-mediated inducement and mitochondrial-initial events (Elmore, 2007; Ouyang et al., 2012). The intrinsic pathway produces intracellular signals that respond in positive or negative manner. Other stimuli act in a positive manner, e.g. radiation, toxin, hypoxia, infection, free radical and others. These stimuli change the inner mitochondrial membrane to open the mitochondrial permeability transition (MPT) pore, transmembrane potential loss and then lead to the release of two groups of pro-apoptotic proteins from the intermembrane space into cytoplasm such as cytochrome C and serine protease.

The proteins mentioned above, trigger the caspase dependent intrinsic pathway. Cytochrome C binds activated proteins as well as procaspase-9 to form "apoptosome". The second group is proteins; AIF, endonuclease G and CAD, which release from mitochondria during apoptosis. AIF and endonuclease G function in a caspase-independent manner. In addition, CAD released from mitochondria plays a role via caspase-3 (Elmore, 2007). The mitochondrial related apoptosis is controlled and

regulated by the BCL-2 family. The BCL-2 family controls mitochondrial membrane permeability and can be the anti-apoptosis. There are 25 genes of BCL-2 family. Some of them play as anti-apoptotic proteins include Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w and BAG, while Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik and Blk are pro-apoptotic proteins. These proteins have a special significant since they can determine to die or to abort (Elmore, 2007; Gozuacik and Kimchi, 2007; Chaabane et al., 2013).

Execution pathway is the end of both extrinsic and intrinsic pathway. Execution apoptosis begins after activate of the execution caspase (caspase-8, caspase-9 and caspase-10). It has been reported that caspase-3 can cause cytoskeletal degradation as well as cell disintegration and finally induce apoptosis. Caspase-3 would cleave gelsolin and the gelsolin fragments lead to actin filaments fragmentation in a calcium independent manner, resulting in cytoskeletal, intracellular transport, cell division and signal disruption (Elmore, 2007).

Rho kinase mediated apoptosis

The recently research has enclosed that Rho family GTPase controlling pathway related to prominence morphologically change according to apoptotic processes. The Rho GTPases are the member of protein family such as RhoA, RhoB, RhoC and RhoD. These proteins switch molecular intracellular transuction signal. One functional member of Rho proteins family are RhoA that relate to actin fiber and actin-myosin II contractile force formation. There are numerous lines of evidence revealed that the presence of filamentous actin, increase myosin light chain (MLC) phosphorylation and myosin ATPase activity dealed with a dynamic of cellular contraction and membrane blebbing at the first phase. A second step of actin filament dispersal occurs via depolymerization and caspase-mediated break down of monomers of actin. Initially, contraction and blebbing effect of RhoA on enhancing actin bunding and actin-myosin II contractile force formation, it was purposed that the activated RhoA is coved with contraction during apoptosis. Cellular contraction and blebbing of cell membrane are sufficient supported by activated RhoA. Rho signal pharmacologically plays essential role as

hindrance of Rho effector kinase ROCK that prevent budding of cell membrane. Active ROCK1 was reported act on cellular contraction and blebbing of membrane in mouse. These are increase actin-myosin II contractile force being driven by Rho-dependent ROCK activity that finally lead to blebbing of cell membrane in apoptosis process. ROCK conduces to agonist-induced modification to actin structure without generating contraction and blebbing. The improved kinase activity is adequate to motivate caspase-independent cell contraction and membrane blebbing, consist with direct effect of ROCK on the morphologically apoptotic development. ROCK supports actin-myosin force after protein phosphorylation. ROCK-dependent activation of LIM kinase-1 and -2 results in phosphorylation and inactivation of actin serving protein, cofilin, thereby stabilizing filamentous actin. Inhibition of ATPase activity increase actin-myosin contractile force. Cell membrane degradation appears when interaction strength, is too high. This results in shift of intracellular hydrostatic force. Thus, cell membrane appears blebbing easier when this strength reduced. In apoptotic process, caspase–mediated proteolytic structure decreases the interaction between cell membrane to cytoskeletal structure and allows an increase of hydrodynamic force that originated by ROCK-induced cell contraction to push bleb protrusion at the weakest point. Next, depolymerization of actin filaments is a second step. Signaling of Rho GTPase related to actin reorganization on both steps. Apoptosis occurs when a C-terminal fragment of Rho kinase released by caspase-mediated proteolysis which leads to apoptosis by catalytic activity inhibition, especially antiapoptotic protein kinase and lessens phosphorylation inhibition of proapoptotic protein in BCL-2 family. These incline that cytoskeletal structure may be required for the final process of the apoptotic cell.

As mentioned above, four proteins related to Rho GTPase signaling and they classified as caspase-cleaved protein in apoptotic cells. Rho GTPase family is important in actin cytoskeleton regulation, when they change their signaling activity that result in apoptotic morphological change. The evidences shown that FAS ligands induce apoptosis undergo by stimulation of caspase-3 and -7 in several cell type. Apoptosis begins when N-terminal of these proteins has been cleaved. This terminal is an effector

domain that responsible for localization of the protein to the configuration of cell membrane (Coleman and Olson, 2002; Wettschureck and Offermanns, 2002; Street and Bryan, 2011).

ROCK inhibitor

ROCKs are the small GTP-binding proteins that classified as a member of the Rho family that regulates cell shape, motility, cell division and proliferation, migration, metabolism and apoptosis though manipulation of actin dynamics and contraction of cell. Rho kinases (ROCKs) are found to mediate RhoA-induces actin cytoskeletal shifts and additionally effects on phosphorylation of myosin light chain. ROCKs are classified as proteins in the serine/ threonine kinases family. ROCK structures compose of an amino kinase attached to a coiled-coil region and Rho-binding domain (RBD) . Furthermore, carboxyl terminal cysteine-rich domain (CRD) labeled with plecktrin homology (PH) domain. Mammalian ROCKs have been identified into two isoforms. ROCK1 or ROKβ or p160ROCK consists of 1354 amino acids and located on chromosome 18. ROCK2 or ROKα or Rho kinase consist of 1388 amino acid that are located on chromosome 12. The collaboration of the active GTP binding form to transform Rho to ROCK's RBD extends ROCK activity by the depression of the carboxyl-terminal RBD-PH domain. This results in an active domain or "open" kinase structure. The open structure is caused by arachidonic acid that binds to the PH domain of the carboxyl-terminus in ROCK1 breakdown by caspase-3 and the ROCK2 by caspase-2.

The Rho activator, such as lysophophatidic acid (LPA) or sphingosine-1 phosphate (S1P) that activate function of Rho guanine nucleotide exchange factor (GEF) and result in actin GTP-bound Rho formation, ROCK related to many cellular response including the actin cytoskeleton. To illustrate, ROCK regulates phosphorylating protein such as myosin light chain (MLC) phosphatase, LIM kinase, ezrin-radixin-moesin (ERM) and adductin. As a result, this manipulate cytoskeletal actin assembly and contractility. By the way, the actin proteins are also controlled by phosphorylation of other protein in serine-threonine kinase family such as protein kinase

A, C and G kinase. Additionally, ROCKs indirectly control MLC phosphorylation by inhibit MLC phosphatase (MLCP) activity. The phosphorylation of ROCK causes the degradation of ERM and actin cytoskeletal reconstruction controversy. ROCK1 transform LIM kinase-1 and LIM kinase-2 by phosphorylation, that support the phosphorylation of cofilin as well. Since cofilin is an actin-binding domain and and actin disassembly depolymerizing protein that systematizes actin filament turnover, the phosphorylation of LIM kinase by ROCKs inhibits cofilin-mediated actin filament disruption and bring on an upsurge in the number of actin filaments.

ROCKs are effectors of Rho that play a crucial role of controlling actin function. Inhibitions of ROCKs, such as fasudil or Y-27632 or overexpression of domain-negative mutant of ROCKs cause the loss of stress fibers and focal adhesion complexes. ROCKs rearrange polarity of cell, enhancing contraction of cytoskeletal organelles and focal adhesion. These lead to increase cellular contraction and migration (Wettschureck and Offermanns, 2002; Liao et al., 2007; Street and Bryan, 2011).

Oocyte cryopreservation

In the past decades, denoting overtures have been made in properous cryopreservation of mammalian oocytes. Beginning of oocyte cryopreservation, researchers pioneered the process of cryopreservation with versions of prolonged hoarding of cell and tissues that could be revived following suspended vivacity in cryostorage. They speculated that life could be revived after freezing of the entire human body. During cryopreservation, cell are exposed to numerous stressors like mechanical, chemical and extremely low temperature, which lead to disrupt cell function and cell death (Smith et al., 2011; Arav and Natan, 2013; Cil and Seli, 2013). Cil and Seli (2013) asserted that there are many factors affecting the achievement of oocyte cryopreservation including factors related to host (age or infertility factor), protocol and cryopreservation methods (slow freezing and vitrification). Disturbance of cellular or subcellular processes by mean of interfering cellular homeostasis leads to partial or completely alteration celled cellular stress. There are many studies affirmed that oocytes

are susceptible to cryodamage when compared to the embryo. However, all stress scarcely induce a cellular damage. In case of oocyte cryodamage, the most considerable damage is structural shift. These structures have been diminished by extremely low temperature, the function of cell would be compromised (Dinnyes et al., 2000; Chen and Yang, 2009; Smith et al., 2011; Chian et al., 2014; Simopoulou et al., 2014). There are a few reports revealed that mature oocyte cryopreservation is still interesting without expectation on nuclear structure or cryodamage (Chen and Yang, 2009; Smith et al., 2011). Theoretically, ultrastructures of oocyte are modified during meiotic division. In that events, stages of cell division differently response to cellular stress. Freezing-thaw process can cause depolymerization and spindle fiber dislocation. Surprisingly, there were a few reports asserted that spindle fiber depolymerization caused by freezing-thaw steps could be repaired themselves. Besides of spindle fiber, there are a few organelles that may damage by cryoinjuries like ribosome, golgi apparatus and mitochondria, all of which impair meiotic resumption, fertilizability and embryo development (Smith et al., 2011; Simopoulou et al., 2014).

Successful cryopreservation of oocytes

In the past few decades, there were many studies reporting that vitrified oocytes of mouse, bovine, equine and human, could survive and were able to develop to embryonic stage (Chaabane et al., 2013; Chian et al., 2014). Previous studies reported that human vitrified oocytes showed higher survival rate, higher embryo development than slow-freezing oocytes. Furthermore, the human vitrified oocytes showed no obvious difference compared to fresh control oocytes (Cobo and Diaz, 2011; Simopoulou et al., 2014). In other species, there was report claimed that both slow-freezing and vitrified mouse oocytes showed evidences of apoptosis. However, vitrification induced more apoptotic level than that of slow-freezing method (Kader et al., 2010). There was further study reported that there was no difference in meiotic resumption and embryo development between high cryoprotectant (CPA) and low CPA in vitrified mouse oocyte (Choi et al., 2015). In bovine species, Dinnyes et al. (2000) found that embryonic rates

(cleavage and blastocyst) of vitrified bovine oocyte were lower than fresh oocyte. However, these oocytes could still supported embryonic development after nuclear transfer. In canine, Turathum et al. (2010) succeeded in vitrified metaphase II oocyte but several organelles (smooth endoplasmic reticulum, lipid droplet, cortical granule and mitochondria) were affected. They emphasized the high expression of BCL-2 but other genes (BAX, SOD1, HSP70 and Dnmt1) did not differ when compared to control. Additionally, Tharasanit et al. (2011) and Galiguis et al. (2014) have successfully produced kittens from *in vitro* vitrified-warmed oocytes. The previous studies suggested that intracellular lipid levels in cat oocytes could improve their functional survival after vitrification (Gomez et al., 2003; Galiguis et al., 2014). Altogether, it is clearly evident that cryopreservation of oocytes using either slow freezing or vitrification has gained significant success in several species, though overall success is still markedly limited. However, it is also worth noting that cryopreservability of the oocytes can be variable among species. Several factors involving the success of oocyte cryopreservation remain to be studied in order to increase the viability and fertilizing ability of cryopreserved oocytes.

Objectives

1. To determine the presence of ROCK cascade in domestic cat oocytes
2. To determine the effects of ROCK inhibitor on oocyte quality in terms of nuclear and cytoplasmic maturation after vitrification
3. To examine the effect of vitrification on apoptosis of oocytes and cumulus cells

Hypothesis

1. ROCK cascade is present in cat oocytes.
2. Vitrification induces cumulus and oocyte apoptosis in the domestic cat.

3. ROCK inhibitor inhibits apoptosis in cumulus cells and oocytes in the domestic cat after vitrification.
4. ROCK inhibitor improves oocyte quality after vitrification in terms of meiotic and cytoplasmic competence.

Expected outcome

1. ROCK cascade would be presented in domestic cat oocytes
2. ROCK inhibitor would be improved quality in terms of nuclear and cytoplasmic maturation after vitrification.
3. The effects of apoptosis on oocytes and cumulus cells due to vitrification procedure would be examined.

CHAPTER III

MATERIALS AND METHODS

The thesis is divided into 4 experiments as follows

Experiment 1: Identification of ROCK cascade in feline cumulus oocyte complexes

Experiment 2: Determination of toxicity of ROCK inhibitor on meiotic and embryo competence

Experiment 3: Effect of vitrification on gene expression of oocytes and cumulus cells

Experiment 4: Effect of different concentrations of ROCK inhibitor on development of oocytes and embryo following vitrification and warming

Experimental design

Experiment 1: Identification of ROCK cascade in feline cumulus oocyte complexes

Cumulus oocyte complexes (COCs) were collected from feline ovaries. The oocytes (n=75, 3 replicates) were separated from cumulus cells. They were stored and analyzed for LIMK-1 (LIM kinase-1) and LIMK-2 (LIM kinase-2) gene expression using RT-PCR.

Experiment 2: Determination of toxicity of ROCK inhibitor on meiotic and embryo competence

Exp. 2.1 The COCs were matured in maturation medium supplemented with different concentrations of ROCK inhibitor (10, 20 and 40 μM; n=90 each group). *In vitro* maturation without ROCK inhibitor (0 μM) were as a control group. After 24 hours of *in vitro* maturation, the oocytes were denuded and fixed in 4% paraformaldehyde. The oocytes were then be stained with DNA staining (4,6-diamidino-2-phenylindole, DAPI) to assess the stage of nuclear maturation. The stage of nuclear maturation of cat oocytes were classified as germinal vesicle stage (GV), metaphase I (MI), metaphase II (MII) and degenerate oocytes.

Exp. 2.2 This experiment was performed as similar as Exp. 2.1 except that the mature oocytes (n=90 each group) were further fertilized *in vitro*. After *in vitro* fertilization (IVF, day 0), the embryo development was examined and classified as cleaved, morula and blastocyst stage embryos on day 2, 5 and 7 of development respectively. In addition, blastocysts were fixed and examined for embryo quality by mean of cell numbers.

Experiment 3: Effect of vitrification on quality of oocytes and cumulus cells

The COCs (n=90) were vitrified and warmed. These COCs were subjected to *in vitro* maturation and *in vitro* fertilization as described in Exp. 2. For quality of cumulus cells after vitrification, the cumulus cells were collected from vitrified-warmed COCs (n=75). They were examined for apoptotic gene expression (LIMK1 and LIMK2) using quantitative RT-PCR. YWHAZ was used as internal gene control. Non-cryopreserved and vitrified-warmed oocytes and cumulus cells cultured without and 10 μM ROCK inhibitor for 0 and 12 hours and were collected for quantitative RT-PCR.

Experiment 4: Effect of different concentrations of ROCK inhibitor on quality of oocytes and cumulus cells following vitrification and warming

The experiment were performed as similar to experiment 3 but three different concentrations of ROCK inhibitor were tested (10, 20 and 40 μM; n=90 each group). 0 μM were served as a control group. The effects of ROCK inhibitor on the quality of oocyte and cumulus cells were assessed. For oocyte quality, the vitrified-warmed oocytes were subjected to *in vitro* maturation and fertilization. Meiotic resumption and embryo development were determined as previously described. The expression levels of apoptotic genes were used to investigate the effect of ROCK inhibitor on cumulus cell quality after vitrification.

All chemicals used in this study would be purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

Source of ovaries

All experiment procedure involved animal was approved by the Research ethics committee of Chulalongkorn University (1531082) and licensed by the Institute of Animal for Scientific Proposes Development (U1-00064-2558)

Domestic cats' ovaries (*Felis catus)* were collected after routine ovariohysterectomy from the Veterinary Public Health Division of the Bangkok Metropolitan Administration. The ovaries were collected in 0.9% (w/v) normal saline solution with 100 IU/ml penicillin and 100 µg/ml streptomycin. Then, the ovaries were transported at environmental temperature (~26-30°C) to the laboratory within 2 hours.

After receiving the ovaries, they were washed twice in normal saline solution supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin. Then, ovaries were placed in holding medium (HM) (HEPES-buffered M199 containing 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin, and 4 mg/ml bovine serum albumin (BSA; embryo tested)). The ovaries were minced in HM to release cumulus-oocyte complexes (COCs), after that they were morphologically classified under 40X magnified stereomicroscope (SMZ645; Nikon, Tokyo, Japan). Grade A and B oocytes were used in this study. Grade A oocyte is fully surrounded by more than 5 layers of compacted cumulus cells and cytoplasm seems homogenously dark. Grade B oocyte is also fully surrounded by more than 5 compacted cumulus cells layers, though, cytoplasm seems homogenously slightly dark.

***In vitro* maturation and fertilization**

In vitro oocyte maturation (IVM) and fertilization (IVF) were performed as previous described (Sananmuang et al., 2010; Thongkittidilok et al., 2014). For IVM, COCs were divided into 4 groups, depending on Rho-associated, coiled-coil containing protein kinases inhibitor concentrations (0, 10, 20 and 40 µM) in 500 µl of IVM medium

($NaHCO_3$ buffered M199 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). Each group contained a total of 90 COCs. After 24 hours of *in vitro* maturation, 10 COCs were transported to 50 µl droplets of IVF medium (Tyrode's balanced salt solution containing 1% (v/v) non-essential amino acids (NEAA), 6 mg/ml BSA, 100 IU/ml penicillin and 50 µg/ml gentamicin). Conventional frozen semen used in this study was collected from a fertility-proven tom cat. Frozen semen was thawed at 37ºC for 30 seconds and evaluated subjectively. Sperm with more than 50% progressive motile were used in this study. A total of $5x10^5$ sperm/ml were added to each IVF droplet. In all steps, the culture would was performed at 38.5ºC in a humidified atmosphere of 5% CO_2 in air.

In vitro culture

After 24 hours of IVF, all COCs were denuded by mouth controlled glass pipette, washed twice and cultured for 24 hours in 50 µl droplets of synthetic oviductal fluid (SOF) containing 4 mg/ml BSA and antibiotic (IVC-1 medium). Next 24 hours, cleaved embryos without cumulus cell were collected, wash twice and then cultured in IVC-2 medium (SOF supplemented with 10% (v/v) fetal calf serum). The culture medium droplets were changed every other day. These culture systems were performed at 38.5ºC in humidified atmosphere of 5% CO_2 in air.

Assessment of embryo development

All cleaved embryos were morphologically observed under an inverted 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 4ºC overnight before staining with 4, 6-diamidino-2-phenylindole (DAPI, 0.1 µg/ml) in phosphate-buffered saline solution (PBS) for 10 min for nuclei count. The fluorescently labeled embryos were then determined using an epifluorescence microscope. The percentages of morula (>16 cells without blastocoels),

blastocysts (>50 cells with blastocoel formation) and hatching blastocysts were respectively examined on day 5, 6 and 7.

Stepwise vitrification

Open pulled straw (OPS) vitrification of COCs was modified by using 0.25 ml polyvinyl chloride straws and by heating over the hot plate and stretching them manually until the outer diameter was approximately half of the original size. Ethylene glycol (EG) was used as cryoprotectant in this study. Preparation of vitrification solution (VS) was as follows: VS0.5, 50% (v/v) HM base medium (20% (v/v) FBS in 80% (v/v) HM) mixed with 50% (v/v) VS1. For VS1, 80% (v/v) of HM base supplemented with 20% (v/v) EG. VS1.5 composed of 50% (v/v) of VS1 supplemented with 50% (v/v) of VS2. To prepare VS2, 60% (v/v) trehalose solution (0.83 M of trehalose in HM base) was mixed to make 40% EG. Four steps CPA exposure techniques was performed by incubating the COCs in VS0.5, VS1, VS1.5 and VS2 for 60, 30, 30 and 20 seconds, respectively. After that COCs were incubated in VS2, they were loaded into the OPS by capillary action and immediately submerged into liquid nitrogen. All vitrification procedures were performed at 37°C on a warm plate.

Warming of vitrified COCs was carried out by immersing the OPS in warm thawing medium at 37°C which composed of HM base and 0.25 M of sucrose. The COCs were expelled into the solution for 5 min. Following by maintaining the COCs in HM supplemented with 0.125 M and 0.0625 M of sucrose. The vitrified-warmed COCs were then gentle washed and held in HM at 37°C until the next procedure.

RNA extraction

Cumulus cells surrounded COCs were denuded by mouth controlled glass pipette. They were collected from fresh COCs before culture (fT0), 12 hours (fT12) and 12 hours of 10 μM ROCK inhibitor supplemented (f10T12) after culture. Moreover, vitrified COCs were also collected at immediately (vT0), 12 hours (vT12) and 12 hours of 10 μM ROCK inhibitor supplemented (v10T12) after thawing and culture. In one

replication, total RNA was extracted from cumulus cells (pools of denuded cumulus from 20 COCs) using the Absolutely RNA Nanoprep Kit following the manufacturer's instructions at room temperature. The extracted RNA was assessed for quality and quality using a spectrophotometer and immediately stored at -80ºC until use.

Qualitative and quantitative PCR

Reverse transcription (RT) was performed using a First-Strand cDNA Synthesis Kit (Superscript III Kit, Carlsbad, California, USA) following the manufacturer's instructions. The products were store at -20ºC for further in qPCR. The relative expression levels of LIMK1 and LIMK2 were normalized to the endogenous control gene (YWHAZ). The primers used in this study are listed in **Table 1**.

The PCR reaction performed as follows: 2 min at 95 °C for initial denaturation, followed by 30 cycles of 30 seconds at 95 °C, 30 seconds at different annealing temperatures for each primer and 30 seconds at 72 °C. Incubation at 2 min at 72 °C was used for the final extension. The PCR products were electrophoresed in 2% (w/v) agarose gel (Bio-Rad, CA, USA) in TBE buffer containing 0.4 mg/ml ethidium bromide (Promega, WI, USA). The amplified products were examined under UV light using a Gel Documentation system (Syngene, CB, UK). The specific primers used in qualitative PCR were LIMK-1 and LIMK-2.

Table 1 Description of forward and reverse primers that were used in this study

Gene	Forward primer	Reverse primer	Base pair (kb)	Assession No.
LIMK1	CTGGTCCGAGAGAACAAGAA	ATCTCACACAGGACGATTCC	144	XM_003998592.3
LIMK2	GTTCAAGTACCACCCAGAGT	CACTTCCCACAGTAAAGGGT	156	XM_003994813.3
YWHAZ	GAAGAGTCCTACAAAGACAGCACGC	AATTTTCCCCTCCTTCTCCTGC	115	HQ185236.1

Statistical analysis

Data were tested for normality and equal variance using the median test and be expressed as a mean±standard deviation (SD) in maturation and developmental experiment. The percentage of cleaved embryos was defined as the number of cleavage embryo relative to total number of oocytes in each group. The proportion of each embryo stage was relative to the total number of cleaved embryos. Logistic regression was used to test the differences of stage of oocytes and developmental competence (cleavage, morula and blastocyst formation rates), and analysis of variance (ANOVA) statistical test and LSD post-hoc were used for statistical comparison of cell numbers within blastocyst. Differences in quantitative mRNA expression were assessed by multiple ANOVA. *P* value less than 0.05 was considered significant.

CHAPTER IV

RESULTS

Experiment 1: Identification of ROCK cascade in feline cumulus oocyte complexes

RT-PCR products expression in Figure 1 displayed the presences of LIMK1 and LIMK2 in fresh oocytes and cumulus cells. Besides, our results enclosed that LIMK2 expressed more intensely than that LIMK1 both in feline oocytes and cumulus cells. This current study firstly confirmed the presence of ROCK cascade in feline oocytes and cumulus cells.

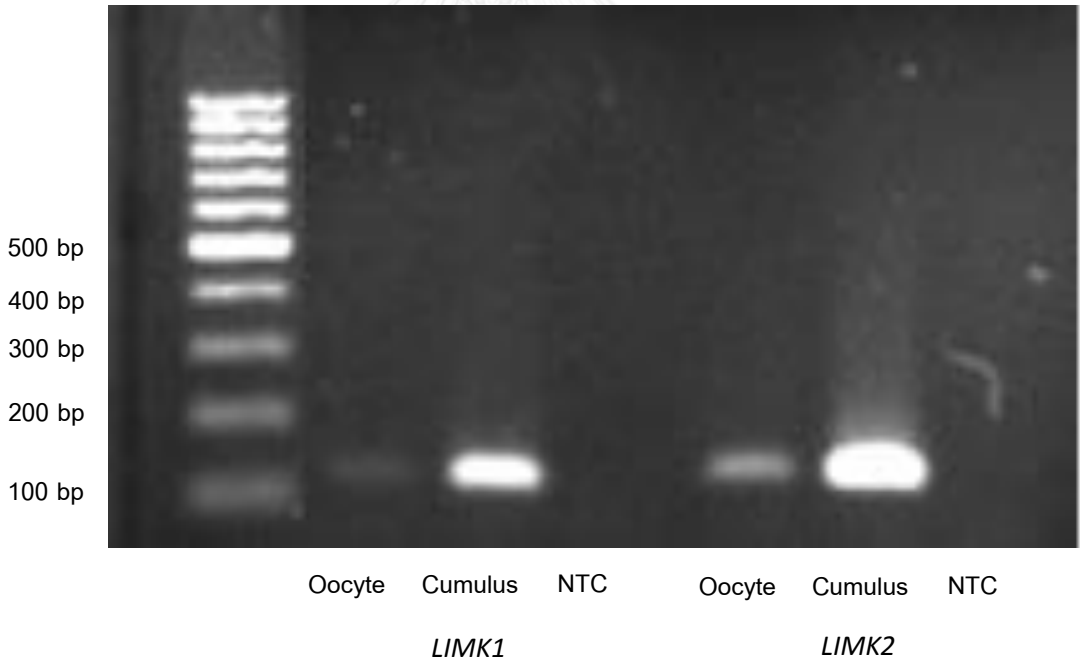


Figure 1 The RT-PCR expression of LIMK1 and LIMK2 in feline oocytes, cumulus cells and no template control (NTC).

Experiment 2: Determination of toxicity of ROCK inhibitor on meiotic and embryo competence

The results of toxicity of ROCK inhibitor on meiotic and embryo competence showed in Table 1. The percentage of metaphase II oocytes showed no statistical difference among 10 μM (71.01±3.40%), 20 μM (52.86±2.87%) of ROCK inhibitor treated and control (64.71±2.52%). Cumulus oocyte complexes cultured with ROCK 10 μM had the statistical highest metaphase II (MII) rate ($p<0.05$) and significantly differed to 20 μM and 40 μM (33.33±2.87%) of ROCK inhibitor treatment. In controversy, 40 μM of ROCK inhibitor treated oocytes had significant lowest MII rate among the experimental groups and also showed statistically highest rate of meiotic arrest at metaphase I rate (50.79±2.70%) when compared to control, 10 μM and 20 μM ROCK inhibitor (22.54±2.71%, 18.84±1.73%, 30.00±2.50%, respectively). ROCK inhibitor treatment during *in vitro* maturation did not affect on embryo developmental competence, in terms of cleavage, morula and blastocyst formation rates.

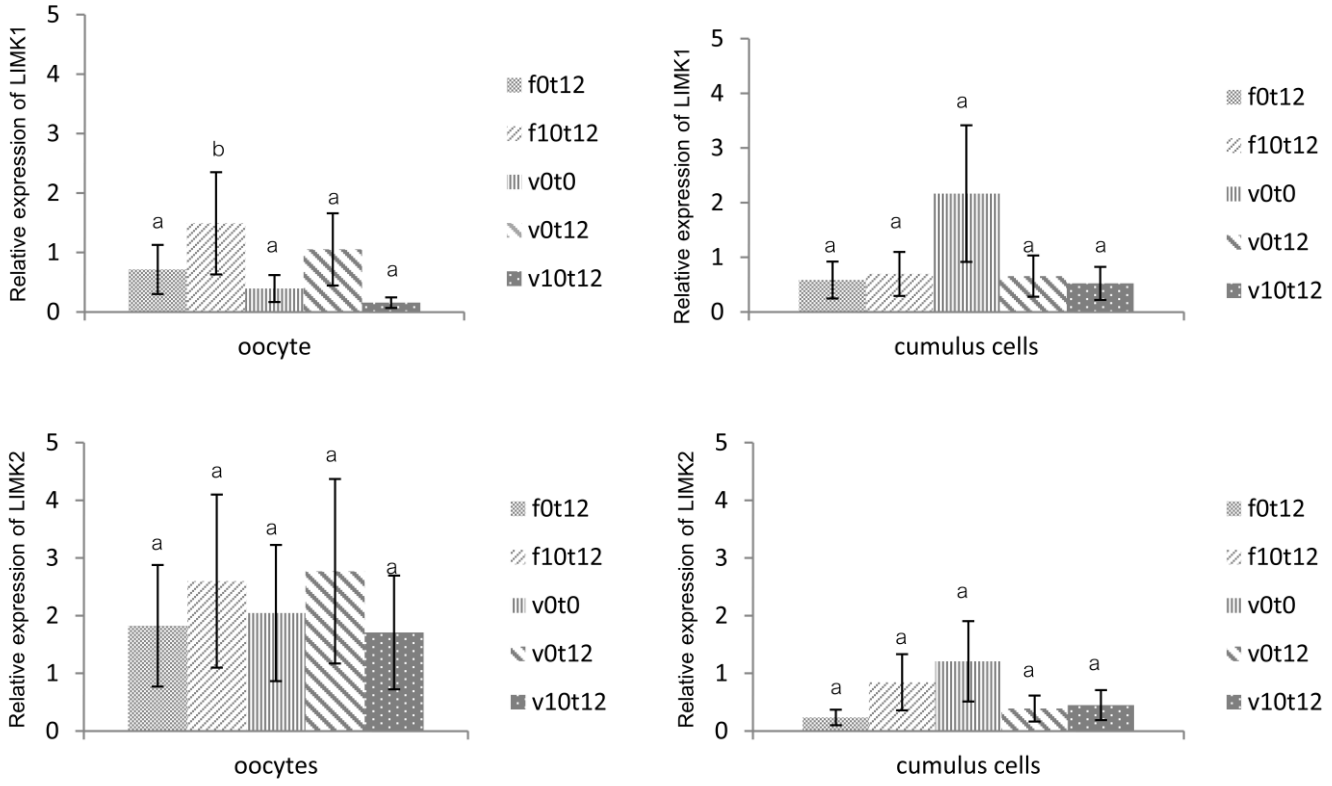
Table 2 *in vitro* maturation and embryo development of non-cryopreserved feline cumulus-oocyte complexes (mean±SD)

ROCK inhibitor concentration	Stage of nuclear maturation (%Mean±SD)			Stage of embryo development (%Mean±SD)			Blastocyst cell numbers
	GV (%)	MI (%)	MII (%)	Cleavage (% relative to total oocytes)	Morula (% relative to cleavage)	Blastocytst (% relative to cleavage)	
control	11.98±2.03	22.54±2.71[a]	64.71±2.52[a]	55.74±2.89	58.82±3.09	52.94±2.65	147.22±11.00
10 μM	6.87±1.92	18.84±1.73[a]	71.01±3.40[a,b]	51.72±3.00	60.00±1.73	53.33±1.53	176.26±17.59
20 μM	9.47±2.44	30.00±2.50[a]	52.86±2.87[a,c]	54.80±3.00	52.38±1.52	35.71±1.73	135.35±12.84
40 μM	7.44±2.02	50.79±2.70[b]	33.33±2.87[d]	45.59±2.52	41.94±1.52	32.86±0.58	143.80±14.83

[a,b,c,d] within a column, different superscripts denote values that differ significantly ($p<0.05$).

Experiment 3: Effect of vitrification on gene expression of oocytes and cumulus cells

The quantitative RT-PCR expression of LIMK1 and LIMK2 derived from vitrified-warmed oocytes and cumulus cells were displayed in Figure 2A-2D. Figure 2A enclosed that the expressions of LIMK1 after *in vitro* maturation of non-cryopreserved oocytes supplemented with ROCK inhibitor 10 μM for 12 hours showed significantly up regulation than other oocyte groups ($p<0.05$). Nonetheless, Figure 2B-2D showed that LIMK1 expression in any cumulus cell groups and LIMK2 expression levels of any oocyte or cumulus cell showed no statistical difference ($p\geq0.05$).



Different superscripts (a,b) denote values that differ significantly ($p<0.05$).

Figure 2 Quantitative RT-PCR relative expression of LIMK1 and LIMK2 in feline oocyte and cumulus cells after in vitro maturation 12 hours (f0t12), 12 hours after in vitro maturation supplemented with ROCK inhibitor 10 μM (f10t12), vitrified-warmed oocyte before in vitro maturation (v0t0), vitrified-warmed oocyte after in vitro maturation 12 hours (v0t12), and vitrified-warmed oocyte supplemented with ROCK inhibitor 10 μM and in vitro maturation 12 hours (v10t12). (A) Quantitative RT-PCR relative expression of LIMK1 in oocyte (B) Quantitative RT-PCR relative expression of LIMK1 in cumulus cells (C) Quantitative RT-PCR relative expression of LIMK2 in oocyte (D) Quantitative RT-PCR relative expression of LIMK2 in cumulus cells.

Experiment 4: Effect of different concentration of ROCK inhibitor on development of oocytes and embryo following vitrification and warming

Vitrified COCs maturation showed similar tendency as non-cryopreserved experiments. High concentration of ROCK inhibitor (40 μM) treated adversely affected in oocyte maturation, by mean of high MI rate and statistical lowest MII rate (**Table 3**). Treated vitrified COCs with 10 μM of ROCK inhibitor significantly enhanced developmental competence in cleavage rate (36.13±3.76%) compared to non-treated group (27.40±2.54%) ($p<0.05$). However, increase ROCK inhibitor to 20 μM did not further improve cleavage rate.

Incubation of 10 μM ROCK inhibitor after vitrification and warming tended to increase morula and blastocyst rate compared to other experimented group. There was no effect of ROCK inhibitor on blastocyst quality by means of cell number.

Table 3 *in vitro* maturation and fertilization of vitrified-warmed feline cumulus-oocyte complexes (mean±SD)

ROCK inhibitor concentration	Stage of nuclear maturation (%Mean±SD)			Stage of embryo development (%Mean±SD)			Blastocyst cells number
	GV (%)	MI (%)	MII (%)	Cleavage (% relative to total oocytes)	Morula (% relative to cleavage)	Blastocyst (% relative to cleavage)	
control	14.43±2.52	18.56±2.00	55.67±8.54[a]	27.40±2.54[a]	18.61±1.23	6.95±6.16	66.00±18.38
10 µM	10.10±1.15	28.28±5.13	48.48±8.54[a]	36.13±3.76[b]	29.05±0.82	16.20±7.87	68.40±12.22
20 µM	17.04±2.00	20.45±6.1	48.86x±6.11[a]	33.18±9.22[a,b]	23.48±3.29	14.24±6.16	96.25±35.80
40 µM	26.92±5.20	34.61±7.24	17.95±10.01[b]	24.46±7.80[a]	20.12±9.01	5.59±4.90	81.00±36.76

[a,b,c,d] within the same meiotic stage, different superscripts denote values that differ significantly ($p<0.05$).

CHAPTER V

DISCUSSION

Oocytes are the largest cells in our body, and the ooplasm contains complex structures that are prone to damage during cryopreservation. Cryopreservation of the oocytes are therefore the most challenging cell type for cryobiologists. Furthermore, the dynamic changes of oocytes during meiosis and fertilization also play an important role in determining the cryoinjuries that frequently occur during freezing and thawing processes. These changes are also associated with bidirectional communication between cumulus cells and the oocyte, essentially via gap junctions that transport necessary factors during oocyte maturation. This junctions have been reported to be damaged after cryopreservation (Hochi et al., 1998; Cocchia et al., 2010). As previously reported, cumulus-oocyte interconnection is necessary for oocyte maturation (Wood and Wildt, 1997; Van Soom et al., 2002; Yokoo and Sato, 2004). Thus, severe disruption of the communication junctions between the oocyte and cumulus cells would be inferiorly affected to their developmental competence. Additionally, oocyte cytoskeleton such as actin microfilaments and microtubules play an essential role during oocyte maturation (Duan et al., 2014; Zhang et al., 2014; Li et al., 2016). However, these structures are very sensitive to cold stress (Comizzoli et al., 2009; Cocchia et al., 2010; Saragusty and Arav, 2011). After oocyte vitrification, the cold sensitizing functional structures, that are important for meiotic development would be damaged and resulted in poor meiotic resumption rate. Few reports acclaimed that lipid droplets in oocyte were the main factor that impaired oocyte maturation, decreased cryosurvival rate and changed membrane integrity after cryopreservation (Liang et al., 2012; Dunning et al., 2014; Prates et al., 2014). In this study we emphasized on the use of selective ROCK inhibitor during *in vitro* culture of oocytes in order to improve cryosurvival of oocytes in terms of maturation and fertilization and embryo development. ROCK or rho-associated coiled-coil kinase plays a key role of cellular motility, cytoskeletal dynamic, especially actin and microtubule

functions (Coleman and Olson, 2002; Chan et al., 2005; Liao et al., 2007; Duan et al., 2014; Zhang et al., 2014; Lee et al., 2015). ROCK further involves in caspase dependent of apoptotic pathways. Thus, ROCK inhibitor (Y-27632) plays a pivotal role on cytoskeletal functions especially actin modification, microtubule stabilization and spindle organization (Duan et al., 2014; Zhang et al., 2014; Li et al., 2016). In the present study we demonstrated that ROCK inhibitor improved oocyte quality after vitrification but the effect was dependent on the concentration used. ROCK inhibitor at 10 to 20 μM did not affect maturation rate of non-cryopreserved oocytes (Table 1). This might be the fact that several factors have been shown to involve this process (Tanghe et al., 2002), and ROCK pathway is not an obligatory signal for meiosis resumption. As described above, oocytes are very sensitive and frequently damage by hypothermal changes, resulting in malfunctioned organelles (Arav and Natan, 2013; Chian et al., 2014). Cumulus cells are also affected by cold stress, and intercellular connecting junctions are mostly disrupted and intercellular transportation would be poor (Luvoni et al., 1997; Mikolajewska et al., 2012; Apparicio et al., 2013). High concentration of Y-27632 adversely affected to oocyte maturation owing to actin disruption (Duan et al., 2014; Zhang et al., 2014) and microtubule disorganization (Li et al., 2016). Microtubule disorganization resulted in the failure of polar bodies extrusion and caused oocyte meiotic arrested (Li et al., 2016). This study revealed that 40 μM of ROCK inhibitor (Y-27632) inhibited feline oocyte maturation. High concentration of Y-27632 may result in the decrease of the phosphorylation level of ROCK and cofilin (Chan et al., 2005). As a reason of previously described, we agreed that ROCK inhibitor played a chief part of oocyte meiotic resumption but only in a manner of dose dependence (Chan et al., 2005; Duan et al., 2014; Zhang et al., 2014). Inhibition of ROCK by Y27632 could not reduce either LIMK1 or LIMK2 in this study because there are many factors that influence on oocyte apoptosis (Chan et al., 2005; Elmore, 2007; Turathum et al., 2010; Street and Bryan, 2011) and other signaling pathway of ROCK may be alternatively activated. For example, cryoinjuries occurred due to vitrification lead to membrane damage, cytoskeleton and DNA fragmentation, mitochondrial malfunction and malorganization.

These are factors that affect on cell function and finally can lead to cellular apoptosis (Mikolajewska et al., 2012). There were a recently report asserted that LIM kinases (LIMK1 and LIMK2) activities are prerequisite for oocyte maturation during meiosis. LIMK expression levels have been demonstrated to be expressed since germinal vesicle stage, gradually increased and peaked at metaphase I and metaphase II (Li et al., 2016). During oocyte maturation of cat, the meiotic stage develop from germinal vesicle to metaphase I spend for 12-18 hours, and totally spend 24 hours for develop from germinal vesicle to metaphase II (Tharasanit, 2008). However, it is still unclear whether pattern of these gene expression in domestic cat oocytes would be similar to other species. Furthermore it is important to further investigate the exact role of these gene on oocyte maturation and apoptosis. In the current study, we found a positive effect of ROCK inhibitor when ROCK inhibitor was included in the oocyte maturation medium only if the oocytes were vitrified-warmed (experiment 4). However, the positive effect of ROCK inhibitor was found only when 10 μM ROCK inhibitor was used when compared to a control group. This is likely that the ROCK pathway is activated somehow and it negatively affected to oocyte meiotic and developmental competence. The selective ROCK inhibitor is therefore preferable, at least to reduce detrimental changes of the oocytes following vitrification and warming. Although it is clearly demonstrated in this study that although 10 μM ROCK inhibitor could improve cryosurvival of vitrified-warmed oocytes, the cellular responses of ROCK inhibitor on cytoskeleton changes during maturation still need to be investigated. In addition, our study also indicated that cryoinjuries occurs at several cellular levels. Therefore, inhibition a cellular cascade, such as ROCK pathway in this study, seemed insufficient to fully protect the oocytes or to recover the oocytes from cryoinjuries. Further study to determine the types of cryoinjuries at subcellular levels together with novel cryopreservation technology will improve success of oocyte cryopreservation.

Conclusions

This study found that ROCK cascade presences in feline oocytes and cumulus cells by mean of LIMK expression. We suggest that 10 μM of ROCK inhibitor treated is the appropriate dose that is useful for improving feline meiotic and developmental competence of feline oocytes after vitrification and warming. However, high dose of ROCK inhibitor addition adversely affected to meiotic resumption.

REFERENCES

Apparicio M, Ruggeri E and Luvoni GC. 2013. Vitrification of immature feline oocytes with a commercial kit for bovine embryo vitrification. Reprod Domest Anim. 48(2): 240-244.

Arav A and Natan Y. 2013. Vitrification of oocytes: from basic science to clinical application. Adv Exp Med Biol. 761: 69-83.

Chaabane W, User SD, El-Gazzah M, Jaksik R, Sajjadi E, Rzeszowska-Wolny J and Los MJ. 2013. Autophagy, apoptosis, mitoptosis and necrosis: interdependence between those pathways and effects on cancer. Arch Immunol Ther Exp (Warsz). 61(1): 43-58.

Chan CC, Khodarahmi K, Liu J, Sutherland D, Oschipok LW, Steeves JD and Tetzlaff W. 2005. Dose-dependent beneficial and detrimental effects of ROCK inhibitor Y27632 on axonal sprouting and functional recovery after rat spinal cord injury. Exp Neurol. 196(2): 352-364.

Chen SU and Yang YS. 2009. Slow freezing or vitrification of oocytes: their effects on survival and meiotic spindles, and the time schedule for clinical practice. Taiwan J Obstet Gynecol. 48(1): 15-22.

Chian RC, Wang Y and Li YR. 2014. Oocyte vitrification: advances, progress and future goals. J Assist Reprod Genet. 31(4): 411-420.

Choi JK, Huang H and He X. 2015. Improved low-CPA vitrification of mouse oocytes using quartz microcapillary. Cryobiology. 70(3): 269-272.

Cil AP and Seli E. 2013. Current trends and progress in clinical applications of oocyte cryopreservation. Curr Opin Obstet Gynecol. 25(3): 247-254.

Cobo A and Diaz C. 2011. Clinical application of oocyte vitrification: a systematic review and meta-analysis of randomized controlled trials. Fertil Steril. 96(2): 277-285.

Cocchia N, Ciani F, Russo M, El Rass R, Rosapane I, Avallone L, Tortora G and Lorizio R. 2010. Immature cat oocyte vitrification in open pulled straws (OPSs) using a cryoprotectant mixture. Cryobiology. 60(2): 229-234.

Coleman ML and Olson MF. 2002. Rho GTPase signalling pathways in the morphological changes associated with apoptosis. Cell Death Differ. 9(5): 493-504.

Comizzoli P, Wildt DE and Pukazhenthi BS. 2009. In vitro compaction of germinal vesicle chromatin is beneficial to survival of vitrified cat oocytes. Reprod Domest Anim. 44 Suppl 2: 269-274.

Dinnyes A, Dai Y, Jiang S and Yang X. 2000. High developmental rates of vitrified bovine oocytes following parthenogenetic activation, in vitro fertilization, and somatic cell nuclear transfer. Biol Reprod. 63(2): 513-518.

Duan X, Liu J, Dai XX, Liu HL, Cui XS, Kim NH, Wang ZB, Wang Q and Sun SC. 2014. Rho-GTPase effector ROCK phosphorylates cofilin in actin-meditated cytokinesis during mouse oocyte meiosis. Biol Reprod. 90(2): 37.

Dunning KR, Russell DL and Robker RL. 2014. Lipids and oocyte developmental competence: the role of fatty acids and beta-oxidation. Reproduction. 148(1): R15-27.

Elmore S. 2007. Apoptosis: a review of programmed cell death. Toxicol Pathol. 35(4): 495-516.

Galiguis J, Gomez MC, Leibo SP and Pope CE. 2014. Birth of a domestic cat kitten produced by vitrification of lipid polarized in vitro matured oocytes. Cryobiology. 68(3): 459-466.

Gomez MC, Pope E, Harris R, Mikota S and Dresser BL. 2003. Development of in vitro matured, in vitro fertilized domestic cat embryos following cryopreservation, culture and transfer. Theriogenology. 60(2): 239-251.

Gozuacik D and Kimchi A. 2007. Autophagy and cell death. Curr Top Dev Biol. 78: 217-245.

Hochi S, Ito K, Hirabayashi M, Ueda M, Kimura K and Hanada A. 1998. Effect of nuclear stages during IVM on the survival of vitrified-warmed bovine oocytes. Theriogenology. 49(4): 787-796.

Kader A, Falcone T, Sharma RK, Mangrola D and Agarwal A. 2010. Slow and ultrarapid cryopreservation of biopsied mouse blastocysts and its effect on DNA integrity index. J Assist Reprod Genet. 27(8): 509-515.

Lee SR, Xu YN, Jo YJ, Namgoong S and Kim NH. 2015. The Rho-GTPase effector ROCK regulates meiotic maturation of the bovine oocyte via myosin light chain phosphorylation and cofilin phosphorylation. Mol Reprod Dev. 82(11): 849-858.

Levesque JT and Sirard MA. 1996. Resumption of meiosis is initiated by the accumulation of cyclin B in bovine oocytes. Biol Reprod. 55(6): 1427-1436.

Li X, Zhu Y, Cao Y, Wang Q, Du J, Tian J, Liang Y and Ma W. 2016. LIM kinase activity is required for microtubule organising centre positioning in mouse oocyte meiosis. Reprod Fertil Dev.

Liang Y, Rakwongrit D, Phermthai T, Somfai T, Nagai T and Parnpai R. 2012. Cryopreservation of immature buffalo oocytes: effects of cytochalasin B pretreatment on the efficiency of cryotop and solid surface vitrification methods. Anim Sci J. 83(9): 630-638.

Liao JK, Seto M and Noma K. 2007. Rho kinase (ROCK) inhibitors. J Cardiovasc Pharmacol. 50(1): 17-24.

Luvoni GC, Pellizzari P and Battocchio M. 1997. Effects of slow and ultrarapid freezing on morphology and resumption of meiosis in immature cat oocytes. J Reprod Fertil Suppl. 51: 93-98.

Mikolajewska N, Muller K, Nizanski W and Jewgenow K. 2012. Vitrification of domestic cat oocytes--effect on viability and integrity of subcellular structures. Reprod Domest Anim. 47 Suppl 6: 295-299.

Ouyang L, Shi Z, Zhao S, Wang FT, Zhou TT, Liu B and Bao JK. 2012. Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. Cell Prolif. 45(6): 487-498.

Prates EG, Nunes JT and Pereira RM. 2014. A role of lipid metabolism during cumulus-oocyte complex maturation: impact of lipid modulators to improve embryo production. Mediators Inflamm. 2014: 692067.

Sananmuang T, Techakumphu M and Tharasanit T. 2010. The effects of roscovitine on cumulus cell apoptosis and the developmental competence of domestic cat oocytes. Theriogenology. 73(2): 199-207.

Saragusty J and Arav A. 2011. Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. Reproduction. 141(1): 1-19.

Simopoulou M, Asimakopoulos B, Bakas P, Boyadjiev N, Tzanakaki D and Creatsas G. 2014. Oocyte and embryo vitrification in the IVF laboratory: a comprehensive review. Folia Med (Plovdiv). 56(3): 161-169.

Smith GD, Motta EE and Serafini P. 2011. Theoretical and experimental basis of oocyte vitrification. Reprod Biomed Online. 23(3): 298-306.

Street CA and Bryan BA. 2011. Rho kinase proteins--pleiotropic modulators of cell survival and apoptosis. Anticancer Res. 31(11): 3645-3657.

Tanghe S, Van Soom A, Nauwynck H, Coryn M and de Kruif A. 2002. Minireview: Functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. Mol Reprod Dev. 61(3): 414-424.

Tharasanit T, Techakumphu M., Lohachit, C. 2008. Reorganisation of cell cytoskeleton of cat oocytes matured in vitro. Thai Journal of Veterinary Medicine. 38(1): 7.

Thongkittidilok C, Tharasanit T, Sananmuang T, Buarpung S and Techakumphu M. 2014. Insulin-like growth factor-1 (IGF-1) enhances developmental competence of cat embryos cultured singly by modulating the expression of its receptor (IGF-1R) and reducing developmental block. Growth Horm IGF Res. 24(2-3): 76-82.

Tsafriri A, Cao X, Ashkenazi H, Motola S, Popliker M and Pomerantz SH. 2005. Resumption of oocyte meiosis in mammals: on models, meiosis activating sterols, steroids and EGF-like factors. Mol Cell Endocrinol. 234(1-2): 37-45.

Turathum B, Saikhun K, Sangsuwan P and Kitiyanant Y. 2010. Effects of vitrification on nuclear maturation, ultrastructural changes and gene expression of canine oocytes. Reprod Biol Endocrinol. 8: 70.

Van Soom A, Tanghe S, De Pauw I, Maes D and de Kruif A. 2002. Function of the cumulus oophorus before and during mammalian fertilization. Reprod Domest Anim. 37(3): 144-151.

Wettschureck N and Offermanns S. 2002. Rho/Rho-kinase mediated signaling in physiology and pathophysiology. J Mol Med (Berl). 80(10): 629-638.

Wood TC and Wildt DE. 1997. Effect of the quality of the cumulus-oocyte complex in the domestic cat on the ability of oocytes to mature, fertilize and develop into blastocysts in vitro. J Reprod Fertil. 110(2): 355-360.

Yokoo M and Sato E. 2004. Cumulus-oocyte complex interactions during oocyte maturation. Int Rev Cytol. 235: 251-291.

Zhang Y, Duan X, Xiong B, Cui XS, Kim NH, Rui R and Sun SC. 2014. ROCK inhibitor Y-27632 prevents porcine oocyte maturation. Theriogenology. 82(1): 49-56.

VITA

Miss Saengtawan Arayatham was born on the 14th of March 1987 in Nakhon sawan province, Thailand. She was graduated high school from Nakhon sawan school in 2005, graduated with her bachelor degree of Doctor of Veterinary Medicaine (D.V.M.) with 2nd degree honour in 2014. She received scholarship from the 90th anniversary of Chulalongkorn University fund (Ratchadaphiseksomphot Endowment Fund) for her M.Sc. programme of Theriogenology at the Department of Obstetrics Gynaecology and reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. Her field of interest was about cryopreservation and biotechnology. Her study was focused on the effect of ROCK inhibitor on vitrified-warmed cumulus-oocyte complexes in cat.

Publication

1. Arayatham, S., Tharasanit, T. and Baurpung, S. 2015. Effects of Rho-associated Coiled-Coil (ROCK) inhibitor on meiotic resumption and development competence of domestic cat oocytes. Proc. 40th International Conference on Veterinary Science 2015 DLD, FAO & APHIS Joint Symposium. 11th-13th November 2015, Impact Forum, Muang Thong Thani, Thailand. P.55.

2. Arayatham, S., Tiptanavattana, N., Setthawong, P. and Tharasanit, T. 2016. Effects of Rho-associated Coiled-Coil Kinase (ROCK) inhibitor on meiotic resumption and development competence of domestic cat oocyte after vitrification and warming. Proc. 3rd of the Joint Symposium of Thai and Japanese Societies for Animal Reproduction. 13th-14th January 2016, Faculty of Veterinary Medicine, Kasetsart University. P. 13-14.

3. Arayatham, S., Tiptanavattana, N. and Tharasanit, T. 2016. Effects of Rho-associated protein kinase inhibitor on meiosis and embryo development of feline oocytes after vitrification. (In preparation)