การศึกษาการพัฒนาและอณูชีววิทยาของตัวอ่อนแมวที่ผลิตภายนอกร่างกายในระบบการเลี้ยงที่แตกต่างกัน

นางสาว ธนิดา สนั่นเมือง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ปีการศึกษา 2553

DEVELOPMENTAL AND MOLECULAR BIOLOGICAL STUDIES OF *IN VITRO* PRODUCED CAT EMBRYOS IN DIFFERENT CULTURE SYSTEMS

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A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy Program in Theriogenology
Department of Obstetrics, Gynaecology and Reproduction
Faculty of Veterinary Science
Chulalongkorn University
Academic Year 2010

Thesis Title	DEVELOPMENTAL AND MOLECULAR BIOLOGICAL STUDIES OF *IN VITRO* PRODUCED CAT EMBRYOS IN DIFFERENT CULTURE SYSTEMS
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ธนิดา สนั่นเมือง : การศึกษาการพัฒนาและอณูชีววิทยาของตัวอ่อนแมวที่ผลิตภายนอกร่างกายในระบบการเลี้ยงที่แตกต่างกัน (DEVELOPMENTAL AND MOLECULAR BIOLOGICAL STUDIES OF *IN VITRO* PRODUCED CAT EMBRYOS IN DIFFERENT CULTURE SYSTEMS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.น.สพ.ดร. มงคล เตชะกำพุ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ.น.สพ.ดร. ธีรวัฒน์ ธาราศานิต และ ดร. แดทเทอรีน นูเยน, 100 หน้า

การทดลองที่ 1 ศึกษาผลของสารโรสโควิตินต่อความสามารถในการพัฒนาของโอโอไซต์แมวบ้านภายนอกร่างกาย โอโอไซต์แมวบ้านถูกนำมาเลี้ยงในสารโรสโควิตินที่ความเข้มข้นต่าง ๆ กัน (0, 12.5, 25, 50, 100 และ 200 µM) นาน 24 ชั่วโมง จากนั้นนำมาเลี้ยงต่อในน้ำยาเลี้ยงโอโอไซต์นาน 24 ชั่วโมงเพื่อให้พร้อมปฏิสนธิ นำโอโอไซต์มาประเมินระยะของการพัฒนาแบบไมโอซิสทั้งก่อนและหลังทำการเลี้ยงรวมถึงการเปลี่ยนแปลงที่เกิดขึ้นกับเซลล์คิวมูลัสภายใต้กล้องฟลูออเรสเซนต์ ผลการศึกษาพบว่าโรสโควิตินมีผลต่อการยับยั้งการเจริญของโอโอไซต์ขึ้นกับปริมาณของโรสโควิตินที่ใช้ อย่างไรก็ตามพบว่าการเลี้ยงในสารดังกล่าวที่ความเข้มข้นสูง (200 µM) มีผลเพิ่มอัตราการตายที่สูงขึ้นของเซลล์คิวมูลัสและโอโอไซต์ ($P<0.05$) ดังนั้นโรสโควิตินที่ความเข้มข้น 12.5 และ 25 µM ซึ่งเป็นความเข้มข้นที่โอโอไซต์สามารถพัฒนาถึงระยะพร้อมปฏิสนธิ (เมตาเฟส II) มากที่สุด($P<0.05$) ได้ถูกนำมาประเมินผลของโรสโควิตินต่อความสามารถในการพัฒนาของโอโอไซต์ภายหลังการปฏิสนธิภายนอกร่างกาย ผลการศึกษาพบว่าโอโอไซต์ที่เลี้ยงในโรสโควิตินสามารถเจริญเป็นตัวอ่อนได้ต่ำกว่าโอโอไซต์ที่ไม่ได้ผ่านการเลี้ยงในโรสโควิติน ($P<0.05$) ดังนั้นจึงสรุปได้ว่าสารโรสโควิตินสามารถยับยั้งการเจริญของโอโอไซต์แมวได้โดยไม่มีผลต่อความสามารถในการพัฒนาของโอโอไซต์ แต่พบว่ามีผลในเชิงลบต่อเซลล์คิวมูลัสและการพัฒนาเป็นตัวอ่อนภายหลังการปฏิสนธิภายนอกร่างกาย

การทดลองที่ 2 ศึกษาผลของชนิดและปริมาตรน้ำยาเลี้ยง ตัวอ่อนจำนวน 8-10 ตัว ถูกนำมาเลี้ยงในน้ำยาเลี้ยงชนิด SOF, modified Tyrode's solution และ MK-1 ที่ปริมาตรต่าง ๆ คือ 20, 50 และ 100 ไมโครลิตร จากนั้นทำการเลี้ยงตัวอ่อนในน้ำยาเลี้ยงชนิด SOF ที่มีน้ำตาลกลูโคสความเข้มข้น 1.5, 3.0 หรือ 6.0 mM เพื่อศึกษาผลของความเข้มข้นน้ำตาลกลูโคสในน้ำยาเลี้ยง โดยตัวอ่อนระยะบลาสโตซิสถูกนำมาศึกษาการเปลี่ยนแปลงของยีน BAX, BCL-2 และ GLUT-1 ร่วมกับการประเมินความสามารถในการพัฒนาของตัวอ่อน ผลการศึกษาพบว่าตัวอ่อนที่เลี้ยงในน้ำยาชนิด SOF และ MK-1 สามารถพัฒนาได้ดีกว่าตัวอ่อนที่เลี้ยงใน modified Tyrode's solution ($P<0.05$) โดยน้ำยาเลี้ยงปริมาตร 20 ไมโครลิตร ส่งผลให้ความสามารถในการเจริญของตัวอ่อนลดลง ($P<0.05$) นอกจากนี้พบว่ากลูโคสที่ความเข้มข้น 6.0 mM ซึ่งใกล้เคียงกับความเข้มข้นในน้ำยาเลี้ยง modified Tyrode's solution (5.6 mM) ส่งผลให้ตัวอ่อนโตได้น้อยลง ($P<0.05$) โดยพบการแสดงออกของยีน BCL-2 สูงขึ้นในตัวอ่อนกลุ่มนี้ กล่าวโดยสรุปได้ว่าการเลี้ยงตัวอ่อนในปริมาตรต่ำ (20 ไมโครลิตร)และน้ำยาเลี้ยงที่มีกลูโคสความเข้มข้นสูง (6 mM)นั้นลดความสามารถในการเจริญของตัวอ่อน ความเข้มข้นของน้ำตาลกลูโคสในน้ำยาเลี้ยง modified Tyrode's solution น่าจะเป็นปัจจัยสำคัญที่ทำให้ตัวอ่อนพัฒนาน้อยลงเมื่อเทียบกับการเลี้ยงในน้ำยา SOF และ MK-1 ทั้งนี้การเพิ่มสูงขึ้นของ BCL-2 บ่งชี้ถึงกลไกสำคัญที่ทำให้ตัวอ่อนสามารถมีชีวิตอยู่รอดได้ในภาวะดังกล่าว

การทดลองที่ 3 ศึกษาผลของความหนาแน่นและจำนวนตัวอ่อนที่เลี้ยงร่วมกันต่อการพัฒนาของตัวอ่อนแมว ตัวอ่อนถูกนำมาเลี้ยงเป็นกลุ่มๆ (10 และ 5 ตัว)และเลี้ยงเดี่ยวในปริมาตรน้ำยาเลี้ยง12.5, 25, 50, 100 และ 200 ไมโครลิตร ตัวอ่อนระยะบลาสโตซิสถูกนำมาศึกษาการแตกหักของสายโครโมโซมและรูปแบบการแสดงออกของยีน BAX, BCL-2 และ HSP70 ร่วมกับความสามารถในการพัฒนาของตัวอ่อน ผลการศึกษาพบว่าตัวอ่อนที่เลี้ยงเป็นกลุ่มมีแนวโน้มที่สามารถพัฒนาได้ดีกว่าตัวอ่อนที่เลี้ยงเดี่ยว โดยตัวอ่อนที่เลี้ยงเป็นกลุ่มละ 10 ตัวที่ความหนาแน่น 1:5, 1:10 และ1:20 พัฒนาได้ดีกว่าที่เลี้ยงความหนาแน่นสูง 1:1.25 and 1:2.5 ($P<0.05$) นอกจากนี้ตัวอ่อนระยะบลาสโตซิสที่เลี้ยงในความหนาแน่นที่สูง (1:1.25) ยังพบการแตกหักของสายโครโมโซมจำนวนมากและมีการแสดงออกของยีน BAX and HSP70 สูงขึ้นเมื่อเทียบกับตัวอ่อนที่เลี้ยงที่ความหนาแน่นต่ำ (1:5 and 1:20) ($P<0.05$) อย่างไรก็ตามไม่พบความแตกต่างของการพัฒนาในตัวอ่อนที่เลี้ยงเดี่ยวที่ความหนาแน่นต่างกันรวมถึงรูปแบบยีนในตัวอ่อนที่เลี้ยงจำนวนต่างกัน (10, 5 และ 1 ตัว) ในปริมาตรที่เท่ากัน (200 ไมโครลิตร) การศึกษานี้สรุปได้ว่าการเลี้ยงตัวอ่อนที่ความหนาแน่นสูงส่งผลลดความสามารถในการพัฒนาของตัวอ่อน ทั้งนี้การเพิ่มสูงขึ้นของยีน BAX และ HSP70 ในตัวอ่อนกลุ่มนี้บ่งชี้ให้เห็นถึงความเครียดที่เกิดขึ้นอย่างมากในสภาวะการเลี้ยงที่ความหนาแน่นสูง

ภาควิชาสูติศาสตร์ เธนุเวชวิทยาและวิทยาการสืบพันธุ์
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ลายมือชื่อนิสิต.....
ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....
ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

4975583931: MAJOR THERIOGENOLOGY
KEYWORDS: *IN VITRO* FERTILIZATION/ROSCOVITINE/CULTURE MEDIUM/EMBRYO DENSITY/GENE EXPRESSION/FELINE

THANIDA SANANMUANG: DEVELOPMENTAL AND MOLECULAR BIOLOGICAL STUDIES OF *IN VITRO* PRODUCED CAT EMBRYOS IN DIFFERENT CULTURE SYSTEMS. THESIS ADVISOR: PROF. MONGKOL TECHAKUMPHU, D.V.M., DOCTORAT 3e CYCLE THESIS CO-ADVISOR: AISST. PROF. THEERAWAT THARASANIT, D.V.M., PH.D., CATHERINE NGUYEN, PH.D., 100 pp.

EXP. 1 aimed to investigate the effect of roscovitine (ROS) on the developmental competence of cat oocytes matured *in vitro*. Groups of COCs were cultured in 0, 12.5, 25, 50, 100, and 200 μM ROS for 24 h and were either fixed to assess the stages of nuclear maturation or additionally matured *in vitro* for 24 h before fixation. Cumulus cells from the COCs treated with ROS were examined for late apoptosis. The developmental competence of cat oocytes after ROS treatment and *in vitro* fertilization was determined. ROS reversibly arrested cat oocytes at an immature stage in a dose-dependent manner. ROS at 12.5 and 25 μM demonstrated less efficiency to arrest the oocytes compared with other doses. However, higher doses of ROS induced cumulus cell apoptosis and resulted in a high number of degenerated oocytes after *in vitro* maturation ($P<0.05$). ROS at 12.5 and 25 μM, which gave rise to the highest rate of mature stage (MII) ($P<0.05$), were therefore used to evaluate their effect on embryo development. Pretreatment with 12.5 and 25 μM ROS prior to *in vitro* maturation decreased the developmental competence of cat oocytes compared with non–ROS-treated controls ($P<0.05$). In conclusion, ROS reversibly maintained cat oocytes at the germinal vesicle stage without detrimental effect on nuclear maturation. However, it negatively affected cumulus cell viability and developmental competence.

EXP. 2 aimed to define the effects of culture media and culture volume in cat embryos. Groups of 8 to 10 embryos were cultured in SOF, modified Tyrode's solution and MK-1 medium in different volumes (20-, 50- and 100-μl drops). SOF supplemented with different concentrations of glucose (1.5, 3.0 and 6.0 mM) was used to examine the effect of glucose in culture medium on embryo development. Quantitative polymerase chain reaction was used to determine the relative transcripts of BAX, BCL-2 and GLUT-1 genes in blastocysts derived from various concentrations of glucose. SOF and MK-1 supported feline embryo development better than modified Tyrode's solution ($P<0.05$). Embryos cultured in 20-μl droplets showed decreased development in all three media ($P<0.05$). Increasing the glucose concentration in SOF to 6.0 mM adversely affected embryo development and tended to increase the BCL-2 transcript in blastocysts ($P<0.05$). In conclusion, type of culture medium, culture volume and glucose concentration affected the development of domestic cat embryos. Decreased culture volume (20 μl) and high glucose concentration (6 mM) negatively affected embryo development. The increase of anti-apoptotic BCL-2 expression found in blastocysts cultured in 6.0 mM glucose may prevent an increase of apoptosis. In the present study, it was clearly demonstrated that differential gene expression occurred in embryos with similar morphology.

EXP. 3 aimed to determine the effects of embryo density and number on feline embryo development. Embryos were randomly cultured in group (n=10 and 5) or singly in different medium volume (12.5, 25, 50, 100 and 200 μl). They were examined for their developmental competence and fragmentation of blastocyst cell nuclei using DNA labeling. Only expanded blastocysts acquired from different density and numbers were collected to examine their mRNA transcripts of BAX, BCL-2 and HSP70 genes using quantitative polymerase chain reaction. Embryos cultured in groups tended to develope better than those cultured singly. For group cultured embryos (n=10), embryos acquired from low culture density (1:5, 1:10 and 1:20) could develop better than those acquired from high density (1:1.25 and 1:2.5) ($P<0.05$). Moreover, fragmentation of the blastocyst cell nuclei tended to increase in high culture density. On the other hand, there was no significant difference of developmental competence among embryos cultured singly in varied densities. Blastocysts derived from high culture density (1:1.25) also significantly up-regulated BAX and HSP70 transcripts comparing with those of low culture densities (1:5 and 1:20) ($P<0.05$). However, there was no significant difference in relative transcripts among varied embryo numbers (n=10, 5, 1) cultured in fixed 200 μl culture medium. In conclusion, high density negatively affected the developmental competence and up-regulated pro-apoptotic (BAX) and stress response (HSP70) transcripts in embryos. This highlighted the mechanisms used to protect the embryo against suboptimal culture condition.

Department: Obstetrics Gynaecology and Reproduction Student's signature.....
Field of study: Theriogenology Advisor's signature.....
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ACKNOWLEDGEMENTS

This study was carried out at the Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. The study was financially supported by Royal Golden Jubilee PhD program of Thailand Research Fund, French Embassy, CHE-TRF Senior Research Fund (RTA 5080010), The National Research University Project of CHE, Rajadaphiseksomphot Endowment Fund (HR1166I) and Chulalongkorn University Centenary Academic Development Project.

I would like to express my sincere gratitude to my advisor, Prof. Dr. Mongkol Techakumphu, for his support and giving me the opportunity to learn new knowlegde in Assisted Reproductive Technologies (ARTs). This makes me realize the importance of this technology for wild life conservation. I'm really appreciated for his advocate during my PhD study including teaching me to become a good scientist. I would like to deeply thank my main co-advisor, Assist. Prof. Dr. Theerawat Tharasanit, for his accompany and valuable suggestions on my work. I'm really grateful for Dr. Nawapen Phutikanit for teaching and helping me setting up the molecular laboratory, including her encouragement. I would like to thank Dr. Catherine Nguyen, my beloved co-advisor, for giving me the great opportunity to learn advance technologies in molecular biology and always taking care of me during practicing in France. Many thanks to Myriam Yammine, Sarwat Afridi, Aurélie Bergon, Geneviève Victorero and other staff from TAGC, INSERM, Marseille for their kindness and friendship. I also wish to express my appreciation for all the members of committee for valuable comments and advices. I also would like to thank Assoc. Prof. Dr. Daungsmorn Suwattana and Ms. Jutarat Jirasupphachok for allowing me to perform my experiments in their laboratory and also giving me the suggestions.

I would like to thank Dr. Suparlark Nuntawan na Ayudhya from the Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University for helping me setting up the technique of gene expression in embryos and also instructing me the basic knowledge in molecular biology. Many thanks for Dr. Sukanya Manee-in for aiding me in *in vivo* embryo production and also advised me about the reproductive physiology in cat. Thanks also to Ms. Junpen Suwimonteerabutr for technical support and chemical/equipment preparation. I also would like to thank Ms. Pranee Numchaisrika for guiding me the intracytoplasmic sperm injection (ICSI) technique during my first to second year of PhD. To my lovely colleagues, Dr. Panida Chanapiwat, Dr. Sirirak Buapeung, Dr. Vibuntita Chankitisakul and Dr. Nutthee Am-In, their encouragement and warm friendship always make me optimistic and pass through every obstacle. I'm really happy having them as my friends. Special thanks for other staff and students in the department for helping me in many aspects including the management of documents and cat's herd.

Finally, I would like to thank my family and Dr. Kaj Chockchai-usaha for their support and love. They are the most important part of my life and my accomplishment. Without their encouragement and support, this achievement would have not been happened.

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AI	artificial insemination
Akt	protein kinase B
Ala-Gln	alanyl L-glutamine
ARTs	assisted reproductive technologies
BAX	Bcl-2-associated X
BCL-2	B-cell lymphoma 2
Bid	BH3 interacting domain death agonist
BLI	butyrolactone I
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
cDNA	complementary DNA
CDKI	cyclin-dependent kinase inhibitor
CL	corpus luteum
cm	centimeter
CO_2	carbon dioxide
COCs	cumulus oocyte complexes
DAPI	4',6-diamidino- 2-phenylindole
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	the mixture of four deoxyribonucleotides: dATP (deoxy adenine triphosphate), dCTP (deoxy cytosine triphosphate), dGTP (deoxy guanine triphosphate), dTTP (deoxy thymine triphosphate)
DTT	dithiothreitol
eCG	equine chorionic gonadotropin
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ET	embryo transfer
FCS	fetal calf serum
FP	forward primer
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GLUT	glucose transport
GV	germinal vesicle
GVBD	germinal vesicle breakdown
h	hour
hCG	human chorionic gonadotropin
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HM	holding medium
HSPs	heat shock proteins
HSP70s	70 kilodalton heat shock proteins
H_2O_2	hydrogen peroxide
ICSI	intracytoplasmic sperm injection
i.m.	intramuscular
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
IVP	*in vitro* production
IVV	*in vivo* production
JNKs	c-Jun N-terminal kinases
KCl	potassium chloride
Kg	kilogram
KH_2PO_4	monopotassium phosphate
l	liter
LOS	large offspring syndrome
M199	Medium 199
MAPK	mitogen activated protein kinases
MEM	Minimum Essential Media
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
MI	metaphase I
MII	metaphase II
MK-1	modified Earle's balanced salt solution
mOsm	milliosmole
MPF	M-phase promoting factor/ mitosis promoting factor
mg	milligram
min	minute
ml	milliliter
mM	millimole
mRNA	messenger ribonucleic acid
N	normal solution
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NaH_2PO_4	monosodium phosphate
NaOH	Sodium hydroxide
NEAA	nonessential amino acids
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NT	nuclear transfer
OH^-	hydroxide

OVH	ovariohysterectomy
O_2^-	superoxide anion
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pHe	environmental pH
pHi	intracellular pH
Pi	inorganic phosphate
qPCR	quantitative polymerase chain reaction
rhFSH	recombinant human follicle-stimulating hormone
RNase	ribonuclease
RNA	ribonucleic acid
ROS	roscovitine (Chapter II) / reactive oxygen species (Chapter IV)
RP	reverse primer
RQ	relative quantitation
RT	reverse transcriptase
sec	second
SEM	standard error of the mean
SOF	synthetic oviductal fluid
TBE	Tris, Borate, EDTA
TdT	terminal deoxynucleotidyl transferase
TUNEL	terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling
Tyrode	tyrode's balanced salt solution
UV	ultraviolet
wt	weight
vol	volume
µg	microgram
µl	microlitre
µm	micrometer
µM	micromole
β-ME	β-Mercaptoethanol

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

During the past few decades, assisted reproductive technologies (ARTs) such as artificial insemination (AI), embryo transfer (ET), gamete cryopreservation, *in vitro* embryo production (IVP) and nuclear transfer (NT) have been used to produce and to propagate genetically superior livestock. In companion animals, these technologies have been also applied for the international trade of frozen semen or live breeding animals and biomedical research (Farstad, 2000). In domestic cat, IVP techniques such as *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI) and nuclear transfer (NT) have been continually developed. Owing to the similarity of reproductive physiology and easily obtainable gametes, domestic cat has been used as a model for application of ARTs in other felid species especially for wild cat conservation.

Figure 1. Most of the 36 wild felid species (A) are threatened or endangered due to rapidly decreasing of habitat area and hunting. Domestic cat (B) has been currently used as a model for developing ARTs for wild cat conservation owing to the similarity of reproductive physiology and easily obtainable gametes (www.hdw-inc.com/lgmarbledcatlg.htm; www.yenra.com/pet-cloning/).

Although IVP technique has been remarkably progressed in domestic cat, success rate of embryo production is still lower than that of *in vivo* (Farstad, 2000). The problems usually occured are the inability of immature oocytes to undergo meiosis resumption after performing *in vitro* oocyte maturation, as well as the failure of *in vitro* fertilized oocytes to develop to blastocyst stage when being cultured *in vitro*. Many studies revealed that only 40-60% of immature cat oocytes resumes and reaches metaphase II (MII), and blastocyst formation rate ranges from only 30-40% of immature oocytes (Farstad, 2000). Fundamental study of

contributing factors is required for the improvement of culture system and also for the implement of the IVP technique in other felid species in the future.

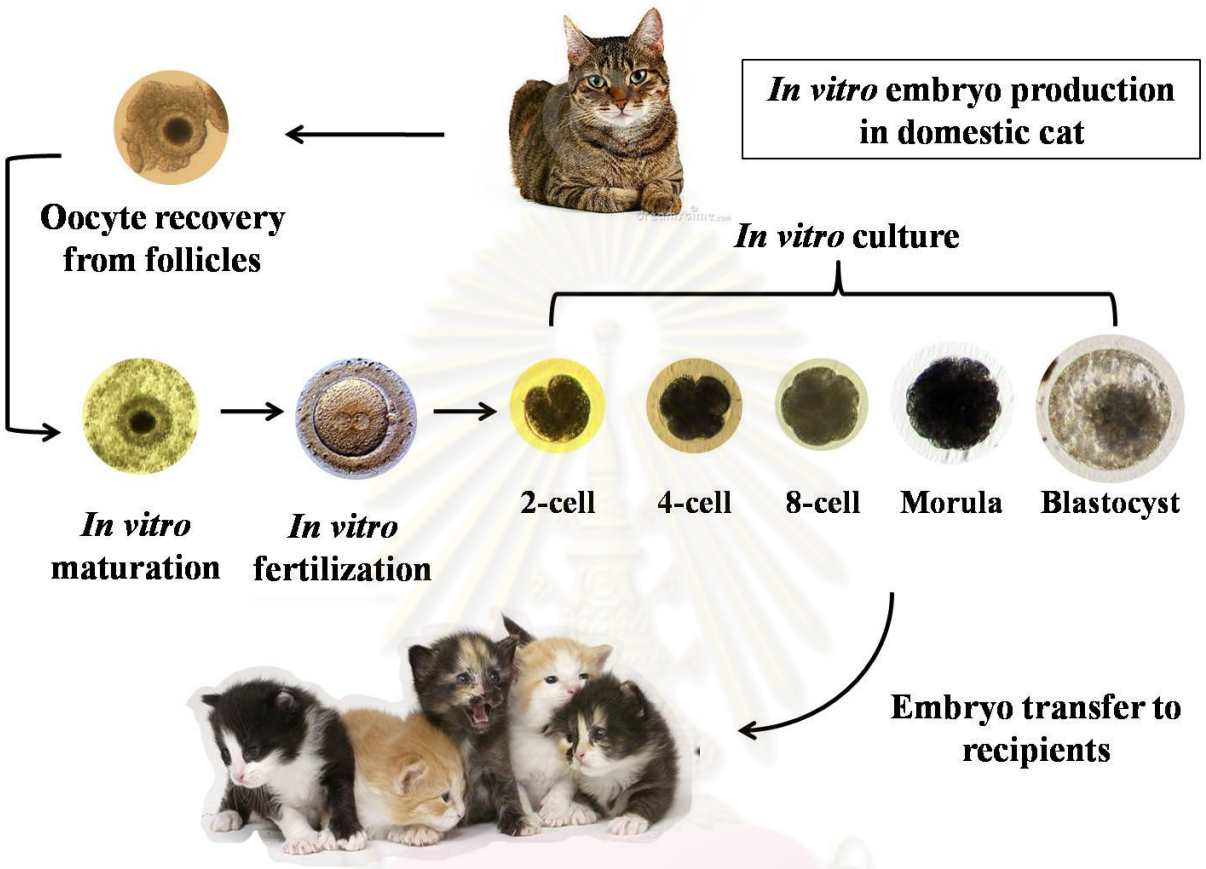

Figure 2. Schematic figure of *in vitro* embryo production (IVP) system. The system involves four main steps: oocyte recovery from the follicles; *in vitro* maturation (IVM) of the oocytes; *in vitro* fertilization (IVF) of the matured oocytes and *in vitro* culture (IVC) of the embryos (modified from Freitas and Melo, 2010).

Before fertilization, optimization of *in vitro* maturation (IVM) technique plays a central role in an improvement of embryo production. Quality of the oocyte *per se* is also recognized as a potential factor affecting development competence of the cumulus oocyte complexes (COCs) in several species. Although a large number of Grade II-III cat COCs are normally obtained during isolation of the COCs, they are usually discarded since they attain lower developmental capacity than Grade I COCs (Wood and Wildt, 1997). In addition, functions of cumulus cells also play a pivotal role during meiotic progression (Zhuo and Kimata, 2001; Yokoo and Sato, 2004). These cumulus cells regulate oocyte's cytoplasmic maturation by providing metabolic substances such as sugar, amino acids, and nucleotides (Farin et al., 2007). Normally, oocyte maturation consists of nuclear and cytoplasmic maturation. The delay of cytoplasmic maturation

during *in vitro* oocyte maturation has been introduced as the cause of developmental incompetence of oocyte. This cause is known to impair not only the ability of oocyte to perform meiosis resumption but also the ability of fertilized oocyte to develop to blastocyst (Ajduk et al., 2008). Of many strategies to improve cytoplasmic maturation of the oocyte, roscovitine (ROS), a potent cyclin-dependent inhibitor of the MPF (M-phase promoting factor) activity that reversibly inhibits meiotic progression, has been examined in many species (Mermillod et al., 2000). Although ROS potentially inhibits meiotic progression of the immature oocytes, subsequent embryo development of ROS-treated oocytes has been variable among species studied (Hinrichs et al., 2002; Han et al., 2006). It is therefore hypothesized that ROS may affect the developmental competence of the oocytes in a species-specific manner. In this regard, using of reversible meiotic inhibitor (roscovitine) might be useful means of improving developmental competence of cat oocytes; particularly for poor quality COCs (Grade II-III).

After fertilization, embryo culture system is also considered critically for the success of IVP. Many factors such as culture medium (Johnston et al., 1993; Herrick et al., 2007), group culture (Spindler and Wildt, 2002), gas atmosphere (Johnston et al., 1991) and culture temperature (Johnston et al., 1991) affect the developmental competence of embryos. There are many types of culture media developed for IVP in feline species (Kanda et al., 1998; Herrick et al., 2007). However, the most suitable media is variable among the lab. Furthermore, embryo density (embryo number: medium volume ratio) and the embryo number (single or group) are also known as the factors affecting the developmental competence of IVP in many species (Paria and Dey, 1990; Gardner et al., 1994; O'Doherty et al., 1997; de Oliveira et al., 2005). Due to the difficulty in obtaining wild cat oocytes, beneficial culture system requiring least number of embryos and suitable culture condition for feline embryo should be examined in domestic cat.

Indeed, success of IVP can be justified from embryo quality. Normally, the quality of embryos is determined from their morphology and the success of ET. However, embryos exhibiting the similar features might have different developmental competence. Moreover, success of ET depends on many factors and a large number of embryos are required in order to produce acceptable result. Therefore, the conventional methods might not be sufficient for evaluation of embryo quality (Lane and Gardner, 1996). Nowadays, molecular techniques have been used for embryo evaluation. The techniques can clarify the abnormal gene expression and protein products of *in vitro* culture embryos and provide more clues to the effect of *in vitro* culture on the embryos at the molecular level.

The objective of this study was to determine the effect of various culture systems on the efficiency of *in vitro* embryo production. Molecular techniques will also be applied to determine gene expression patterns of embryos developed in varied culture systems. The knowledges acquired from this study would provide the information leading to generate the optimal culture condition to improve both phenotype and molecular characteristics of cat embryos. It would contribute to the improvement of developmental competence of cat oocytes, define culture media of choice, proper embryo density and the number of cultured embryos for the optimal feline embryo culture condition. The examination of gene expression profile during embryo

development might help us understand the mechanisms that control blastocyst formation, assess the normality of *in vitro* produced embryos and optimize the *in vitro* culture condition.

1.2 Literature Review

1.2.1. *In vivo* oocyte maturation

The oocyte maturation is the lengthen process which provides the oocytes with the competence to be fertilized and undergo embryogenesis. Most of oocytes in the ovary are small and immature (arrested at diplotene stage of the first meiotic prophase: germinal vesicle (GV) stage). In response to hormonal stimulus, some of them start to grow and mature, while the rest become atresia (Hardy et al., 2000). The maturing follicles provide a special micro-environment to oocytes, so they acquire the "prematuration" or "capacitation" (ultrastructural modification that takes place in the oocytes of dominant follicles) enabling them to attain full developmental competence. This process consists of nuclear and cytoplasmic maturation which occur simultaneously. Nuclear maturation is the acquisition of competence to complete the meiosis (meiotic competence). The oocytes would undergo germinal vesicle breakdown (GVBD), metaphase I, anaphase I, telophase I and arrest again at metaphase II (MII). Oocytes at MII stage called secondary oocytes would be ovulated and be stimulated to complete their meiosis by fertilization (Figure 3). Cytoplasmic maturation is the process that allows the oocytes to undergo normal fertilization and embryo development. The morphological and ultrastructural changes taken place during cytoplasmic maturation consist of changes of oocytes and cumulus cells interaction and also changes of organelle redistribution such as increasing level of kinase activity, peripheral migration of mitochondria, increasing number of golgi apparatus, peripheral migration and attachment to the plasma membrane of cortical granule (Gosden et al., 1997).

The major changes occurring during oocyte maturation are mainly related to activity of two major kinases: M-phase promoting factor (MPF) and mitogen-activated protein kinase (MAPK) (Figure 4). In domestic cat, MPF comprises of two subunits, $p34^{cdc2}$kinase and cyclin B1. It is responsible for inducing spindle assembly, chromatin condensation and nuclear envelop break down (Murray, 1994). MAPK activity is required for maintaining chromatin condensation during meiosis I - II transition and prevents nuclear envelope formation (Murray, 1994). Increased levels of these kinases trigger nuclear maturation by inducing the geminal vesicle breakdown and chromosomal condensation before the onset of metaphase I (Bogliolo et al., 2004).

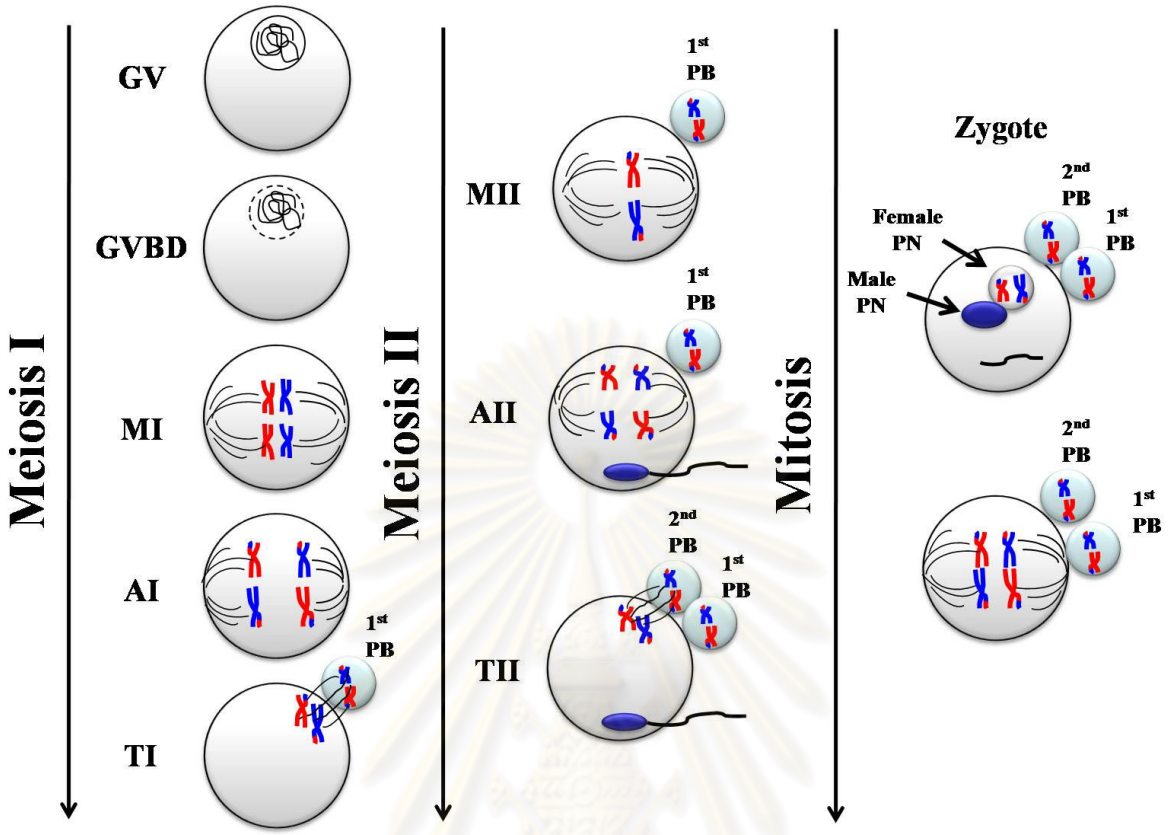

Figure 3. Meiosis and meiotic spindle formation in oocytes. The oocytes are normally arrested at the last stage of prophase before entering the puberty. In this stage, the oocytes have a large nucleus called the germinal vesicle (GV). After hormonal stimulation, resumption of meiosis I is occured by GV breakdown followed by metaphase I (MI), in which a spindle forms and homologous chromosomes align at the spindle's equator. After all chromosomes align at the spindle equator, the oocyte enters anaphase I (AI) and chromosomes start to separate, followed by telophase I (TI) in which a polar body (1st PB) containing half of chromosomes is extruded from the oocyte. The complete of meiosis I is indicated by the extrusion of the first polar body. Then, the oocyte re-organizes a second meiotic spindle and chromosomes re-align at the equator of the spindle, and the oocyte is arrested at metaphase II (MII). Meiosis II is initiated by fertilization or parthenogentic activation, which causes segregation of chromatids through anaphase II (AII) and telophase II (TII) stages, and extrusion of the second polar body (2nd PB). After fertilization, a female pronucleus (female PN) and a male pronucleus (male PN) form in the oocyte, followed by formation of a mitotic spindle, allowing the oocyte (embryo) to enter mitosis (modified from Wang and Sun, 2006).

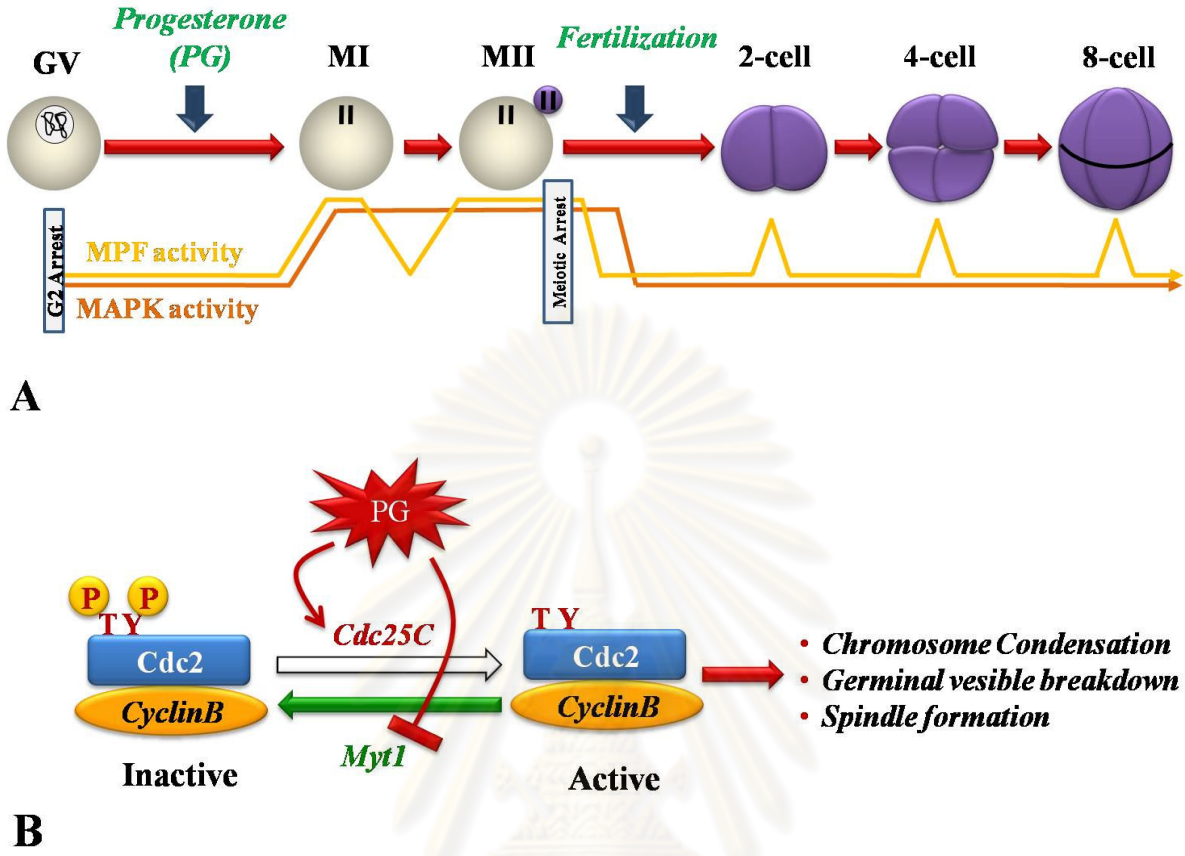

Figure 4. Pathways involved in oocyte maturation. (A) Fully grown immature oocytes are arrested at the G2/M border of the meiotic cell cycle. After receiving the progesterone (PG), this arrest is overcomed and oocyte maturation is initiated by entering meiosis I (MI). This is followed by a transient decline in MPF activity and entry into meiosis II (MII), culminating with arrest at this stage. Fertilization overcomes this arrest, followed by exit from meiosis II and entry into the embryonic cell cycles. Two important signal transduction pathways crucial for the process of oocyte maturation are MPF (yellow) and MAPK (orange). (B) Pathway of MPF activation. M-phase promoting factor (MPF) is a protein complex composed of a Cdc2 protein kinase subunit and a cyclin B regulatory subunit. MPF is found in an inactive form in immature oocytes due to phosphorylation on Thr 14 (T) and Tyr 15 (Y) on Cdc2 by the inhibitory kinase Myt1. Progesterone stimulation of the immature oocytes brings about the activation of a protein phosphatase, Cdc25C that directly counteracts the Myt1 phosphorylation of MPF, leading to its activation. Progesterone (PG) also activates pathways that inhibit the Myt1 kinase. MPF activity is crucial for the process of oocyte maturation by inducing chromosome condensation, germinal vesicle breakdown (GVBD), and formation of the meiotic spindle, thus driving entry into M phase. Progesterone stimulation also leads to the activation of MAPK which facilitates the MPF-driven process of oocyte maturation by contributing to the inhibition of Myt1 during meiosis I (modified from Tunquist and Maller, 2003).

1.2.2. *In vitro* oocyte maturation

Although most *in vitro* matured oocytes are able to complete nuclear maturation, only a few reach blastocyst stage. Since *in vitro* matured oocytes normally resume their meiosis spontaneously after removal from their follicles despite the cytoplasmic maturation, incomplete of cytoplasmic maturation is known as the cause of developmental incompetence of *in vitro* matured oocytes. Bogliolo et al. (2004) also demonstrated that MPF and MPK activity is higher in *in vivo* matured oocytes than in *in vitro* matured oocytes, suggesting a possible incomplete cytoplasmic maturation after culture. Poor cytoplasmic maturation is known to impair not only the ability of oocyte to perform meiosis resumption but also the ability of fertilized oocyte to develop to blastocyst (Ajduk et al., 2008).

Of many strategies to improve cytoplasmic maturation of the oocyte, roscovitine (ROS), a potent cyclin-dependent inhibitor of the MPF activity that reversibly inhibits meiotic progression, has been examined in many species (Mermillod et al., 2000). Most of studies hypothesized that treatment of the oocytes with kinase inhibitor might allow them to undergo cytoplasmic maturation during meiotic arrest and give rise to the synchronization between nuclear and cytoplasmic maturation. Although ROS potentially inhibits meiotic progression of the immature oocytes, subsequent embryo development of ROS-treated oocytes has been variable among species studied (Hinrichs et al., 2002; Han et al., 2006). For example, ROS did not affect the embryo development in bovine (Mermillod et al., 2000) but improve developmental competence of equine oocytes (Hinrichs et al., 2002). In contrast, ROS failed or adversely affected the blastocyst formation rate in goat (Han et al., 2006). It is therefore hypothesized that ROS may affect the developmental competence of the oocytes in a species-specific manner. The effect of ROS has never been studied in domestic cat, so treatment of cat oocytes in this kinase inhibitor might improve their cytoplasmic maturation and give rise to the improvement of their developmental competence after *in vitro* maturation, fertilization and culture.

Quality of the oocyte *per se* is also recognized as a potential factor affecting development competence of the COCs in several species. Although a large number of Grade II-III cat COCs are normally obtained during isolation of the COCs, they are usually discarded since they attain lower developmental capacity than Grade I COCs (Figure 5) (Wood and Wildt, 1997). In this regard, using of reversible meiotic inhibitor (roscovitine) might be useful means of improving developmental competence of cat oocytes; particularly for poor quality COCs (Grade II-III).

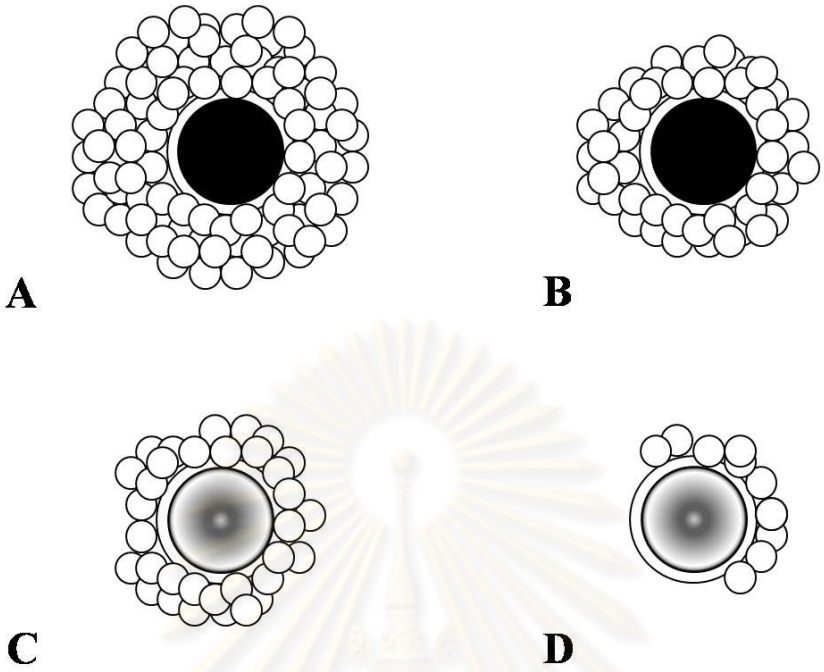

Figure 5. Grades of cumulus oocyte complexes (COCs) derived from domestic cat ovaries, (a) Grade I, excellent: oocytes contain a uniform, dark cytoplasm with spherical eccentric nuclei surrounded with full complement of five or more layers of compacted cumulus cells, (b) Grade II, good: oocytes contain a uniform, dark cytoplasm with complete complements of corona radiata cells, but fewer than five layers of cumulus cells, (c) Grade III, fair: oocytes lack uniformity which is expressed as mosaic transparency of the cytoplasm; oocytes have nearly a full complement of corona radiata and some cumulus cells, but are not as compacted as higher Grades, (d) Grade IV, poor: oocytes with severe mosaic transparency or fragmentation of the cytoplasm and sparse complements of corona radiate and cumulus oophorus cells, some nearly denuded (modified from Wood and Wildt, 1997).

1.2.3. *In vitro* embryo culture (IVC)

The success of IVF in terms of embryo and offspring production has been reported in domestic cat (Goodrowe et al., 1989; Gomez et al., 2000). Although the IVF system has been remarkably improved, the blastocyst rate from oocytes matured and fertilized *in vitro* is still lower than those matured and fertilized *in vivo* (30-40% vs 50-70%, respectively) (Farstad, 2000). *In vitro* fertilization (IVF) is the technique developed to mimic normal fertilization process outside the body. It is the procedure that sperm are co-incubated with unfertilized oocytes in proper condition to achieve fertilization. Fertilization rate of *in vivo* matured oocytes is normally ranging from 60-80% and 40-60% of *in vitro* matured oocytes (Farstad, 2000). Cleavage rate after fertilization of oocytes matured *in vivo* is ranging from 50-80% and 40-60% of *in vitro* matured oocytes (Gomez et al., 2003; Pope et al., 2006a).

The developmental competence of embryos depends on the culture system such as culture medium (Johnston et al., 1993; Herrick et al., 2007), group culture (Spindler and Wildt, 2002), gas atmosphere and culture temperature (Johnston et al., 1991). There are many kinds of cultured media such as Ham F-10 (Roth et al., 1994), Synthetic oviductal fluid (SOF) (Freistedt et al., 2001), modified Tyrode's balanced salt solution (modified Tyrode's solution) (Gomez et al., 2003) and modified Earle's balanced salt solution (MK-1) (Kanda et al., 1998) known to support feline embryo development. The component of these culture media can differently affect the embryonic morphology, metabolism, and gene expression (Lane and Gardner, 1998; Niemann and Wrenzycki, 2000; Crosier et al., 2001; Holm et al., 2002; Niemann et al., 2002; Rinaudo and Schultz, 2004; de Oliveira et al., 2005; Gardner and Lane, 2005; Lane and Gardner, 2005). However, *in vitro* feline embryo development following IVF differs greatly among laboratories using a variety of culture media (Kanda et al., 1998; Freistedt et al., 2001; Gomez et al., 2003). For example, blastocyst rate acquired from embryo cultured in Ham F-10 medium is ranging from 0-50% of cleaved embryos (Roth et al., 1994; Spindler and Wildt, 2002).

A sequential culture system using Tyrode's balanced salt solution as the basal medium (contains 137.0 mM NaCl, 2.7 mM KCl, 5.6 mM glucose, 2.2 mM lactate, 0.36 pyruvate, and 1.0 mM glutamine) has been developed for feline species (Gomez et al., 2000). Supplementation of this medium with 10% FCS from day 4 to 7 after IVF could produce 50% blastocyst formation rate from cleaved embryos (Gomez et al., 2000). The percentage of blastocyst ranges from 30 to 40% of embryos cultured in SOF, while the blastocyst yield was increased (~60% of embryos) after supplementation of growth factor in maturation medium (Merlo et al., 2005). Using of modified Earle balanced salt solution, designated MK-1 (contains 116.4 mM NaCl, 5.4 mM KCl, 1.5 mM glucose, 1.8 mM lactate, 0.36 pyruvate, and 1.0 mM glutamine) supplemented with 10% human serum yielded ~70% of cleaved embryos developing into blastocysts on day 6 after IVF (Kanda et al., 1998).

Besides suboptimal embryo culture media, embryo density (embryo number: medium volume ratio) and number of cultured embryos (single or group) also affect developmental competence and transcription of important related genes of *in vitro* produced embryos (Spindler and Wildt, 2002; de Oliveira et al., 2005). In mouse (Paria and Dey, 1990), sheep (Gardner et al., 1994) and bovine (Ferry et al., 1994; O'Doherty et al., 1997), embryos cultured in groups and/or reduced incubation volumes showed superior developmental competence than those cultured singly (Paria and Dey, 1990; Lane and Gardner, 1992; Keefer et al., 1994; Donnay et al., 1997; O'Doherty et al., 1997). The advantages of group embryo culture on suboptimal culture condition are contributed to autocrine and paracrine factors (O'Neill, 1997). However, the excessive increase of embryo density might have negative consequence on embryo development due to improper pattern of gene expression reported in bovine species (de Oliveira et al., 2005).

In domestic cat, no blastocysts were produced after culturing in Ham F-10 medium (contains 126.7 mM NaCl, 3.8 mM KCl, 6.1 mM glucose, 0.11 mM lactate, 1.1 mM pyruvate, and 2.0 mM glutamine) supplemented with 5% FCS (Roth et al., 1994). However, blastocyst (50% of embryos derived from *in vitro* matured oocytes) could be produced from this medium

when culture volume was reduced from 100 to 20 µl per 10 embryos along with changing the culture medium every two days (Spindler and Wildt, 2002). Up to now, the comparative effects of culture medium types and embryo density on developmental competence of cat embryos have never been seriously studied.

1.2.4. Gene expression during embryo development

Normally, the quality of embryos is determined from their morphology and the success of ET. However, embryos exhibiting the similar features might have different developmental competence. This statement was supported from the failure of implantation after ET of most transferred embryos (Farstad, 2000). Nowadays, molecular techniques have been applied for embryo evaluation. The techniques could clarify the abnormal gene expression and protein products of *in vitro* culture embryos and provide more clues to optimize *in vitro* culture condition.

The survival of the pre-implantation embryos depends not only on proper conditions for normal development but also on mechanisms by which embryos normally use to cope with the stress. The ability of embryos to resist the stress is regulated by the balance between programmed cell death (apoptosis) and protective mechanism of the cell. It has been demonstrated that *in vitro* environments alter the expression patterns of a number of genes involving stress regulation in early mouse and bovine embryos compared to *in vivo* controls (Ho et al., 1995; Wrenzycki et al., 1998). Normally, cells can response to the stress via enzymatic and non-enzymatic defense mechanism. According to the previous studies, embryos could synthesize anti-stress protein so-called heat shock proteins (HSPs) to resolve the effect of stress and allow cells to survive in suboptimal conditions. Stress-induced expression of HSPs, particularly the HSP70s, occurs when cells are exposed to heat, amino acid analogues, heavy metals, metabolic poisons and oxidative stress.

Heat shock 70 kDa proteins (HSP70s) are molecular chaperones that participate in many biological processes, such as modulating polypeptide folding, degradation and translocation across membranes, and protein-protein interactions (Figure 6). In addition to these roles under optimal condition, stress can cause protein conformational problems (for example, heat shock causes protein unfolding). Althouh chaperones can facilitate folding or refolding, often is not possible. In such cases, chaperones can facilitate degradation, either by preventing aggregation and keeping them susceptible to proteolysis or by facilitating their transfers to proteolytic systems. This indicates that HSP70s plays the essential roles in both normal cell development and protection against damage from stressors.

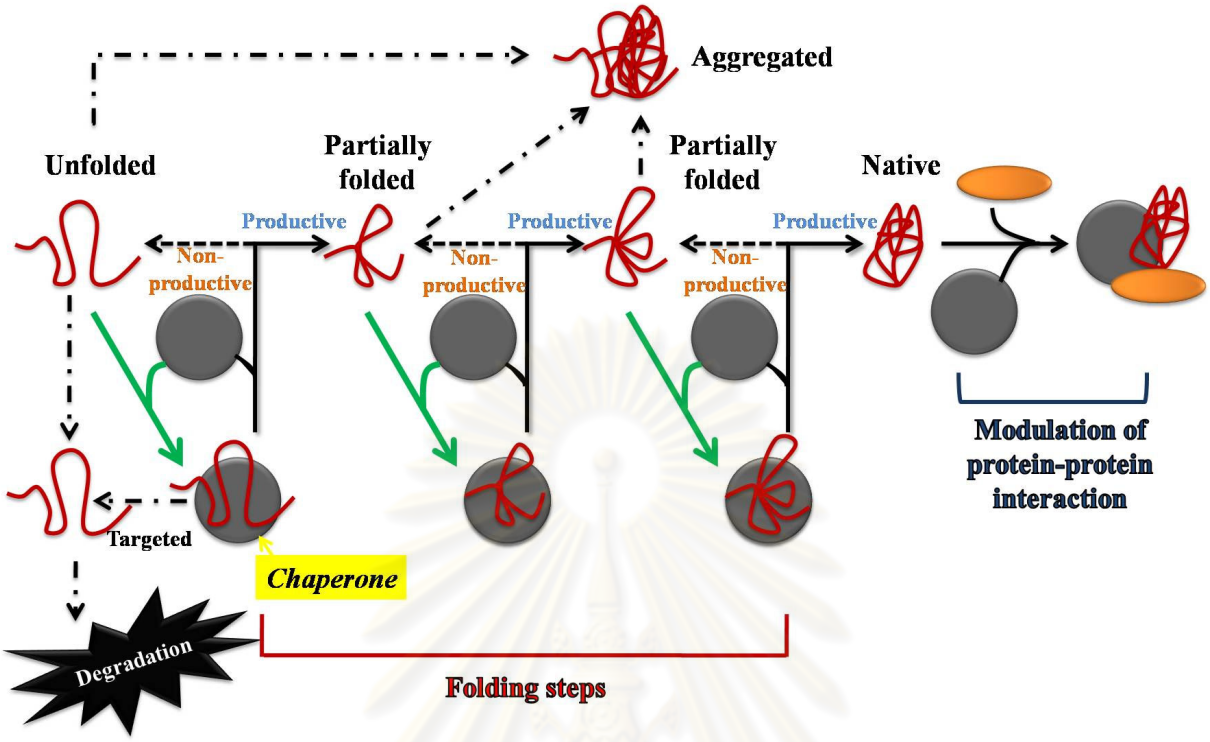

Figure 6. Chaperones are a group of proteins that interact with various non-native polypeptides, facilitating the acquisition of their native conformation when their natively folded and functional structure (centre). Chaperone functions are not restricted to assisting protein folding and assembly, but also to facilitate protein degradation through both proteasomal and autophagasomal pathways (left), as well as to stabilize or destabilize interactions between mature, folded proteins (right). Productive folding occurs through a series of steps, and chaperones are recycled for polypeptide binding. If folding fails or a non-foldable polypeptide re-binds to the chaperone, the protein is degraded by the proteasome in a stochastic (passive) manner (left). Some chaperones can also actively direct clients towards degradation (targeted degradation). In addition, chaperones can bind folded proteins and induce conformational changes (right), thereby regulating protein–protein interactions and the functionality of protein complexes (modified from Kampinga and Craig, 2010).

Inability to maintain the balance between the survival and death factors could initiate the cascade of apoptosis signaling pathway leading to developmental blockage and cell death (Figure 7) (Betts and King, 2001). Apoptosis is an essential feature of many normal processes and pathological conditions, and serves a variety of purposes, including tissue homeostasis and remodelling, and the removal of unwanted cells (Wyllie et al., 1980). It may result from activation of an endogenous programme or be induced by stimuli (Hardy, 1999). Since cell death occurred in the embryo could be a means to eliminate abnormal cells and cells with inappropriate developmental potential (Hardy, 1999), the regulation of cell death during pre-implantation

embryo development is considered the critical point for the development of conceptus (Betts and King, 2001). Two members of BCL-2 family, BAX and BCL-2, play the important role in embryo apoptosis. BAX promotes apoptosis, while BCL-2 promotes survival of the cells. For this reason, the expression ratio of BCL-2/BAX can effectively indicate cellular sensitivity to apoptosis (Wyllie, 1995).

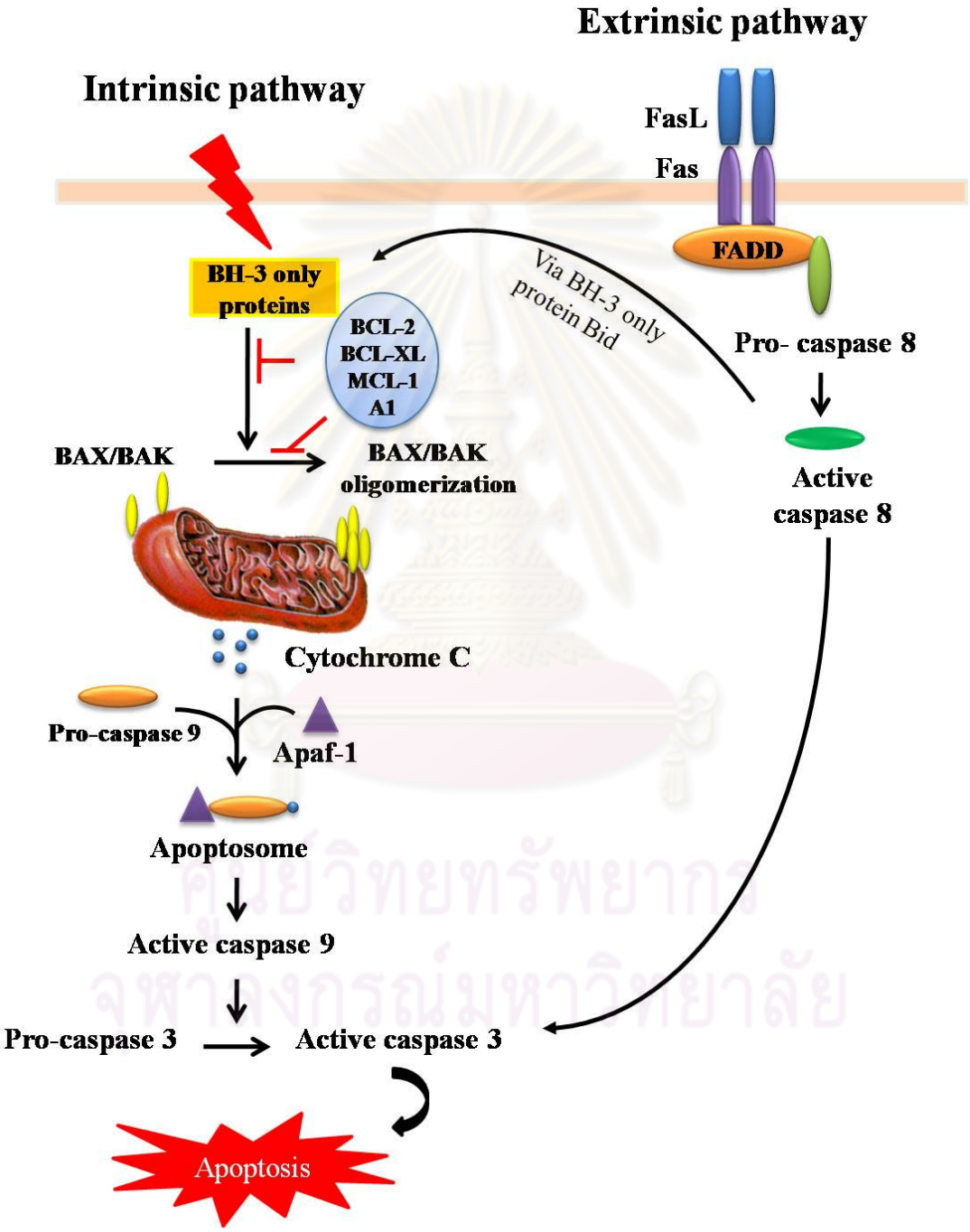

Figure 7. Apoptotic pathways. Two major pathways lead to apoptosis: the intrinsic cell death pathway controlled by BCL-2 family members and the extrinsic cell death pathway controlled by death receptor signaling. During embryo development, apoptosis are known to be occurred via the intrinsic pathway. In this pathway, BCL-2 family proteins are main regulators and are grouped into three subfamilies based on the number of BH (BCL-2 Homology) domains they share. The first subfamily includes the anti-apoptotic proteins BCL-2, BCL-XL, BCL-W, MCL-1 and A1/BFL-1which possess four BH domains—BH1–4. The next two groups are pro-apoptotic proteins possessing three BH (BH1–3) domains represented by BAX, BAK and BOK and BH3-only proteins characterized by the presence of only the BH3 domain. Normally, various cell death stimuli activate one or more of BH3-only effectors that integrate and transmit the death signal through the multi-domain BH1–3 pro-apoptotic proteins, BAX and BAK. These proteins will undergo conformational activation leading to oligomerization at the outer mitochondrial membrane resulting in subsequent release of pro-apoptotic factors. Normally, BAX protein is directly responsible for the damage to the mitochondria, but pro-survival family members, especially BCL-2 (B-cell lymphoma protein 2) protein prevent the cell from this damage. The activated BAX molecule could be captured by free pro-survival protein allowing cell to survive. In contrast, apoptotic initiation might be occurred if the amount of activated BAX molecules is greater than the amount of pro-survival proteins (modified from Zhang et al., 2005).

According to the previous statement, assessment of stress-induced gene (HSP70), pro-apoptotic (BAX) and anti-apoptotic (BCL-2) gene products using quantitative RT-PCR might provide further understanding of the molecular regulation involved in preventing improper embryonic cell apoptosis during embryo development *in vitro*. The data acquired from the study would determine embryo quality and help finding out the optimal *in vitro* culture conditions.

1.3 Objectives of the thesis

1. To evaluate the effect of specific cyclin-dependent kinase inhibitor (roscovitine: ROS) on developmental competence of cat oocytes
2. To evaluate the developmental competence and gene expression of cat embryos cultured in different types of culture media and culture volumes
3. To compare developmental competence and gene expression of cat embryos cultured in different density and numbers

1.4 Hypothesis

1. Inhibition of meiotic resumption with specific cyclin-dependent kinase inhibitor could improve developmental competence of cat oocytes
2. Cat embryos acquire developmental competence and pattern of gene expression differently after culture in different media types and volumes
3. Embryos cultured in different density and numbers demonstrate the different developmental competence and pattern of gene expression

1.5 Keywords: *in vitro* fertilization, roscovitine, culture medium, culture volume, embryo density, embryo number, developmental competence, gene expression, feline

1.6 Research merits:

1. The appropriate cultured condition for *in vitro* embryo production in feline specie
2. The knowledge about the alteration of gene expression pattern in embryos cultured in specific conditions
3. Application of molecular techniques as a tool for the embryo quality evaluation

CHAPTER II

THE EFFECT OF SPECIFIC CYCLIN-DEPENDENT KINASE INHIBITOR (ROSCOVITINE: ROS) ON DEVELOPMENTAL COMPETENCE OF CAT OOCYTES

2.1 Abstract

The developmental competence of cat oocytes matured *in vitro* is relatively poor when compared with that of *in vivo* matured oocytes. The study aimed to investigate the effect of roscovitine on the developmental competence of cat oocytes matured *in vitro*. Cumulus-oocyte complexes (COCs) were classified as Grade I and II to III. Groups of COCs were cultured in 0, 12.5, 25, 50, 100, and 200 μM roscovitine for 24 h and were either fixed to assess the stages of nuclear maturation (Experiment I) or additionally matured *in vitro* for 24 h before fixation (Experiment II). In Experiment III, cumulus cells from the COCs treated with roscovitine were examined for apoptosis. Experiment IV examined the developmental competence of cat oocytes after roscovitine treatment and *in vitro* fertilization in terms of cleavage and morula and blastocyst formation rates. Roscovitine reversibly arrested cat oocytes at an immature stage in a dose-dependent manner. Roscovitine at 12.5 and 25 μM demonstrated less efficiency compared with that of other doses. However, higher doses of roscovitine induced cumulus cell apoptosis and resulted in a high number of degenerated oocytes after *in vitro* maturation. Roscovitine at 12.5 and 25 μM were therefore used to evaluate their effect on embryo development. Pretreatment with 12.5 and 25 μM roscovitine prior to *in vitro* maturation decreased the developmental competence of cat oocytes compared with that of non–roscovitine-treated controls. In conclusion, roscovitine reversibly maintained cat oocytes at the germinal vesicle stage without detrimental effect on nuclear maturation. However, it negatively affected cumulus cell viability and developmental competence.

2.2 Introduction

Domestic cat is considered a valuable model for assisted reproductive biotechnology in non-domestic felids. However, the overall success of *in vitro* embryo production related to *in vitro* maturation in this species remains inconsistent. *In vivo*, mammalian oocytes acquire their nuclear and cytoplasmic maturation during follicle growth (Fortune, 1994). The highly competent dominant follicles are selected and will release oocytes at ovulation, whereas the remaining follicles undergo atresia (Hagemann et al., 1999). In contrast with the *in vivo* situation, oocytes collected for *in vitro* maturation originate from follicles at various stages of their growth. In parallel, they demonstrate relatively poor developmental potential compared with that of *in vivo* matured oocytes (Farstad, 2000). The quality of the oocyte and cumulus cells is recognized as a potential factor associated with its developmental competence, in terms of the capability of oocytes to resume meiosis, to mature, and to be fertilized and develop up to blastocyst stage *in vitro*. Cumulus cells surrounding the oocytes also play a critical role in their growth and

maturation by providing nutrients and several signals into the oocytes (Zhuo and Kimata, 2001; Yokoo and Sato, 2004; Farin et al., 2007).

In domestic cats, only 40% to 60% of immature oocytes reach metaphase II (MII) *in vitro*, while blastocyst formation rates of 30% to 40% from *in vitro* matured oocytes can be achieved (Farstad, 2000). Understanding the mechanism that regulates oocyte maturation thus plays a central role in the optimization of *in vitro* maturation technique and in the improvement of embryo production. Until recently, although the exact pathway that regulates the developmental competence of cat oocytes is unclear, the M-phase promoting factor (MPF) and mitogen activated protein kinases (MAPK) have been shown to actively induce meiotic progression (Bogliolo et al., 2004). Of the many strategies to improve the cytoplasmic maturation of the oocyte, two-step culture using MPF inhibitors, such as butyrolactone and roscovitine, has been developed to increase the developmental competence of immature oocytes from immature follicles. The first step involves the inhibition of the germinal vesicle breakdown, and the second step aims to stimulate the completion of nuclear and cytoplasmic maturation of the oocyte. This technique increased significantly the meiotic competence of cattle oocytes isolated from small antral follicles (Pavlok et al., 2000). Roscovitine, a potent cyclin-dependent inhibitor of the MPF activity that reversibly inhibits meiotic progression, has been examined in many species such as bovine (Mermillod et al., 2000; Ponderato et al., 2002), goat (Han et al., 2006), and pig (Krischek and Meinecke, 2001; Schoevers et al., 2005). Although this effect does not compromise the establishment of pregnancy or fetal development during organogenesis (Ponderato et al., 2002), *in vitro* embryo development of roscovitine-treated oocytes has been variable among species studied (Mermillod et al., 2000; Hinrichs et al., 2002; Han et al., 2006). It is therefore hypothesized that roscovitine may affect the *in vitro* developmental competence of the oocytes in a species-specific manner.

In this regard, prematuration with roscovitine has to be evaluated as a means of improving the developmental competence of cat oocytes, particularly for poor quality cumulus-oocyte complexes (Grade II to III). This study aimed to examine the effect of roscovitine on cat oocytes, specifically on meiosis inhibition, cumulus cell apoptosis, and developmental competence after *in vitro* fertilization.

2.3 Materials and methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

2.3.1 Oocyte recovery

Ovaries were collected from domestic cats after ovariohysterectomy and transported to the laboratory in NaCl 0.9% in deionized water supplemented with 100 IU/ml penicillin and 100 mg/ml streptomycin. Within 2 h, the ovaries (Figure 8) were washed and placed in holding medium (HM) consisting of HEPES buffered M199, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 4 mg/ml bovine serum albumin (BSA;

embryo tested). Cumulus-oocyte complexes (Figure 9A) were recovered by ovarian mincing in HM and then morphologically classified at x 40 magnification using a stereomicroscope (SMZ645; Nikon, Tokyo, Japan) as described (Wood and Wildt, 1997). In brief, Grade I COCs were typified by the oocytes being completely surrounded with more than five layers of compacted cumulus cells and containing a homogeneous-darken ooplasm (Figure 9B), while Grade II to III COCs had irregular pale ooplasm surrounded with fewer layers of compacted cumulus cells (Figure 9C).

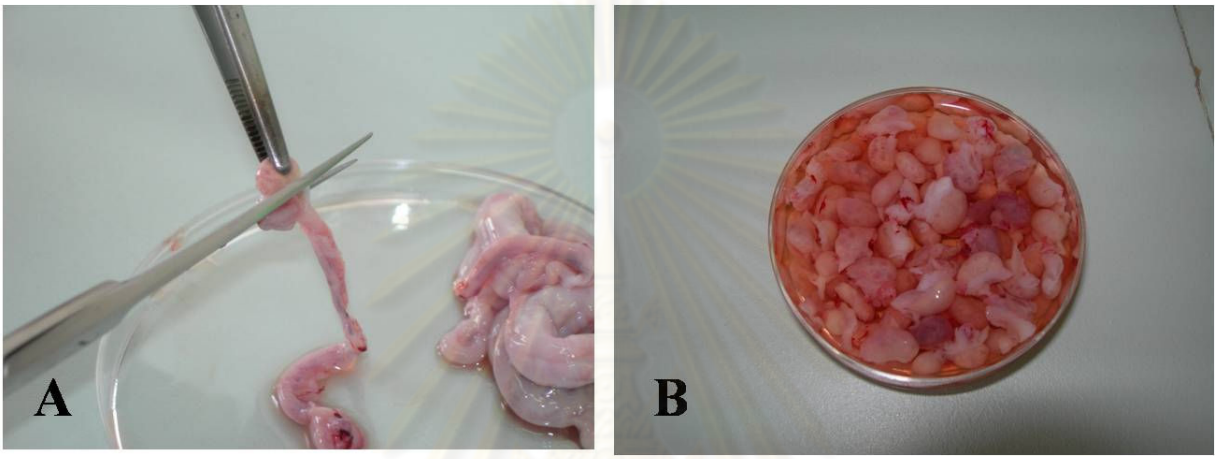

Figure 8. Cat ovaries derived from ovariohysterectomized domestic cats were collected in HM (holding medium) before ovarian mincing (A-B).

2.3.2 Oocyte culture

Groups of 20 to 30 COCs were cultured in 500 µl of a basic *in vitro* maturation medium ($NaHCO_3$-M199 supplemented with 1.0 mM sodium pyruvate, 2.0 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 4 mg/ml BSA) containing roscovitine at different concentrations (0, 12.5, 25, 50, 100, and 200 µM) in a 4-well plate (Nunc, Roskilde, Denmark). After this prematuration in roscovitine, *in vitro* maturation was performed at 38.5˚C in a humidified atmosphere with 5% CO_2 in air for 24 h in an IVM medium (basic *in vitro* maturation medium supplemented with 0.05 IU/ml recombinant human follicle stimulating hormone (rhFSH; Organon, Bangkok, Thailand).

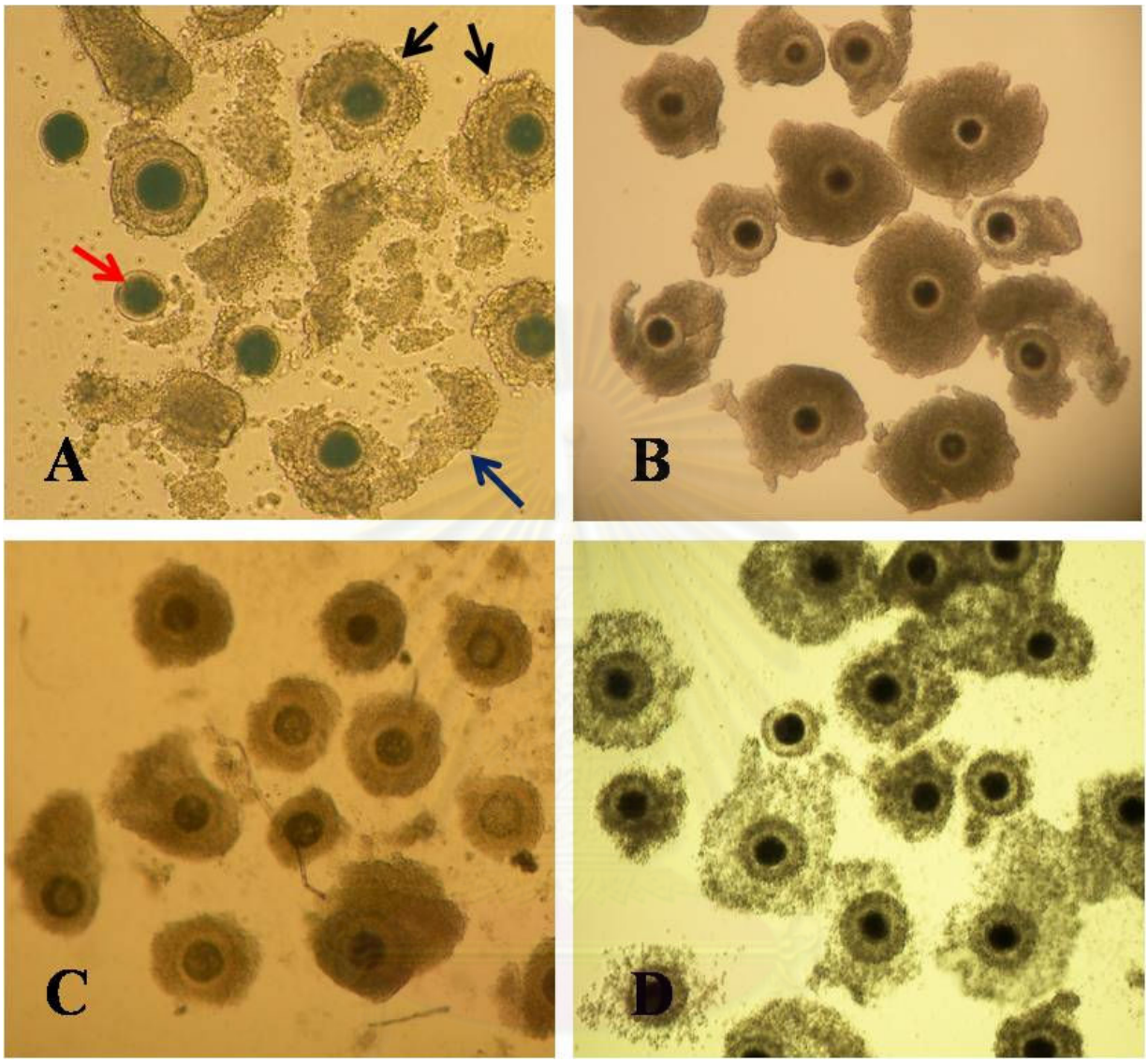

Figure 9. Cumulus oocyte complexs (COCs) (black arrow) are characterized by the oocyte (red arrow) surrounded with cumulus cells (blue arrow) (A). Grade I immature COCs (B) and Grade II-III immature COCs (C) were selected for *in vitro* maturation. After culturing for 24 h, the matured oocytes (D) demonstrated the expanded cumulus cells surrounding the oocytes.

2.3.3 *In vitro* fertilization

After maturation (Figure 9D), the cumulus cells were partially removed by gentle pipetting. *In vitro* fertilization was performed essentially as described (Pope, 2004) with minor modifications. In brief, groups of 5 to 10 oocytes were transferred to 50-µl droplets of IVF medium (Tyrode's balanced salt solution containing 1% (v/v) MEM nonessential amino acids (NEAA), 6 mg/ml BSA, 100 IU/ml penicillin, 100 µg/ml streptomycin, 30 mg/ml heparin, 1 mM L-glutamine, 0.36 mM sodium pyruvate, and 0.11 mM calcium lactate) (Pope, 2004). The semen

used in this study was collected from two fertility-proven tom cats and then frozen according to Rota et al. (1997) with minor modifications. In brief, cats were anesthetized with 0.04 mg/kg atropine sulfate (A.N.B. Laboratories, Bangkok, Thailand), 3 mg/kg xylazine (Laboratorioscalier, Barcelona, Spain), and 10 mg/kg ketamine hydrochloride (Gedeon Richter, Budapest, Hungary). The semen was collected by electroejaculation (Zambelli and Cunto, 2006). The semen was cryopreserved by placing the straws horizontally 4 cm above liquid nitrogen vapors for 10 min and then plunging into liquid nitrogen. Before *in vitro* fertilization (IVF), the semen was thawed at 70˚C for 6 sec. After thawing, the sperm was subjectively evaluated, and only sperm that had more than 50% progressive motility were used for the *in vitro* fertilization. The oocytes were co-cultured with the sperm at a final concentration of 0.5 x 10^6 sperm/ml for approximately 18 h.

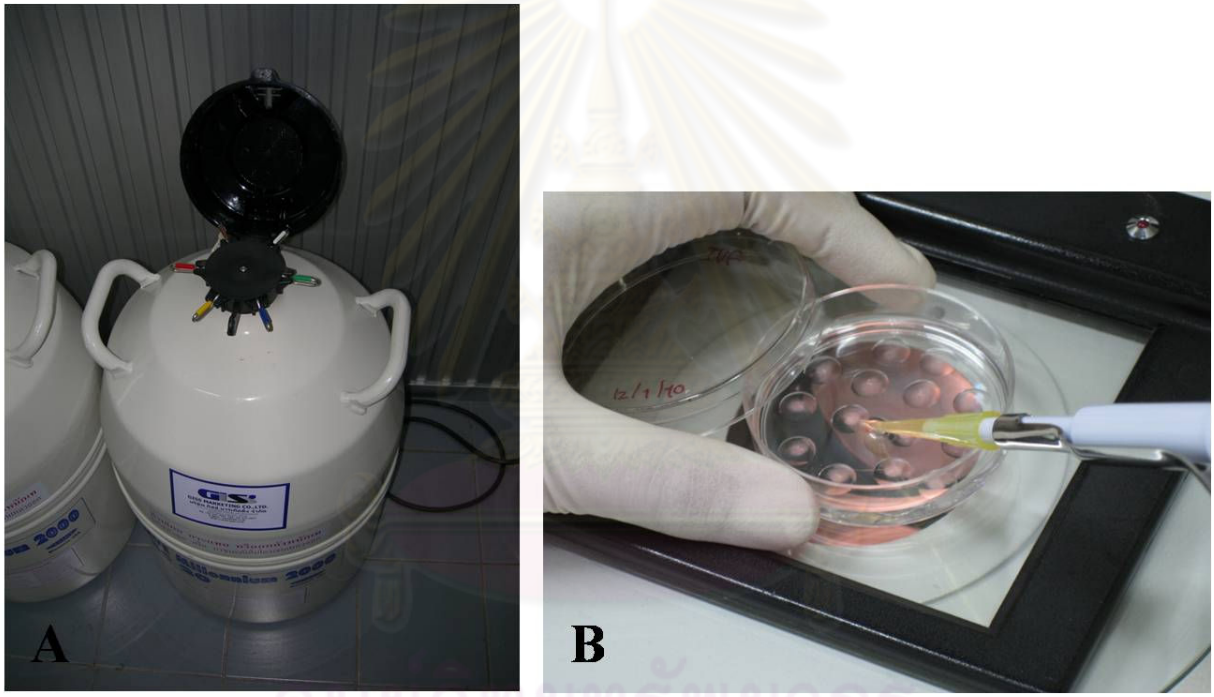

Figure 10. Fresh semen collected from proven tom was collected in liquid nitrogen until use (A). The frozen-thawed semen was washed with IVF medium twice before incubation with matured oocytes (B).

2.3.4 Embryo culture

After the co-incubation of oocytes and sperm, presumptive zygotes were denuded by pipetting, washed, and cultured in 50-µl droplets of synthetic oviductal fluid (SOF) containing 4 mg/ml BSA, 100 mg/ml streptomycin, and 100 IU/ml penicillin (IVC-1 medium). After incubation for 24 h in the IVC-1 medium, the embryos were washed and cultured in IVC-2 medium (SOF containing 10% (vol/vol) Fetal calf serum (FCS) (Gibco, Invitrogen, CA, USA)). In all cases, *in vitro* culture was performed at 38.5˚C in a humidified atmosphere with 5% CO_2.

2.3.5 Assessment of nuclear status and embryo development

To evaluate nuclear status after pretreatment with roscovitine and *in vitro* maturation, the COCs were denuded, fixed in 4% (wt/vol) paraformaldehyde, and stained with fluorescent DNA labeling (4',6-diamidino- 2-phenylindole; DAPI). Cumulus-oocyte complexes were fixed in 4% paraformaldehyde and kept at 4˚C for 2 days before staining procedure. They were stained with 1 μg/ml DAPI in PBS at 37˚C for 10 min. The oocytes were then examined for their nuclear status using an epifluorescent microscope (BX51; Olympus, Shinjuku, Japan). The nuclear status of the oocytes was classified as the germinal vesicle (GV), metaphase I (MI), and metaphase II (MII) stages. The GV oocytes were classified by the oocytes that had a single mass of diffuse or condensed chromatin configurations confined within a specified area (germinal vesicle) (Figure 11A). Metaphase I (MI) was classified as one set of chromosome aligned at the metaphase plate in the ooplasm (Figure 11B), while metaphase II (MII) was classified as the ooplasm contained one set of aligned chromosome at metaphase plate with an extrusion of the first polar body (Figure 11C). The oocytes displayed with the scattered chromatins or containing with no chromatin materials in ooplasm were classified as degenerated (Figure 11D) (Han et al., 2006). Oocytes exhibiting a germinal vesicle, germinal vesicle break down or metaphase I were termed immature, while oocytes in metaphase II were considered mature (Johnston et al., 1989). The percentage of cleaved embryos (2 to 16 cells), morula (16 or more cells without blastocoele), and blastocysts (>50 cells with blastocoele formation) were evaluated on day 2, 5, and 7 of *in vitro* culture (IVC), respectively. Numbers of embryonic nuclei in embryos were evaluated using DAPI staining.

2.3.6 Assessment of apoptosis in cumulus cells and oocytes

After *in vitro* culture for 24 h in roscovitine, COCs were washed in phosphate-buffered saline (PBS), and cumulus cells were then separated from the oocytes. Triple staining technique was used to determine viable, dead, and apoptotic cells. Pools of cumulus cells from 5 COCs were incubated with 4 mM ethidium-homodimer-1 (Molecular Probe, Invitrogen, Oregon, CA, USA) for 5 min at 37˚C to detect any cell death. After washing and centrifugation, cumulus cells were smeared on silane-coated slides and then fixed overnight in 4% paraformaldehyde. Detection of DNA fragmentation (late apoptosis) was performed by Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay (In Situ Cell Death Detection Kit; Roche, Mannheim, Germany) following the manufacturer's instructions. Briefly, the slides containing cumulus cells were washed twice in PBS and incubated with a mixture of TUNEL reaction (TdT enzyme and nucleotide mix) for 1 h at 37˚C in a humidified chamber. The cumulus cells were counterstained with 1 μg/ml DAPI. Cells positive to only TUNEL were classified as apoptotic, and cells positive to ethidium-homodimer-1 or both ethidium-homodimer-1 and TUNEL were classified as dead cells. A total of 3,000 to 5,000 cumulus cells/slide (three replicates per experimental group) was randomly counted.

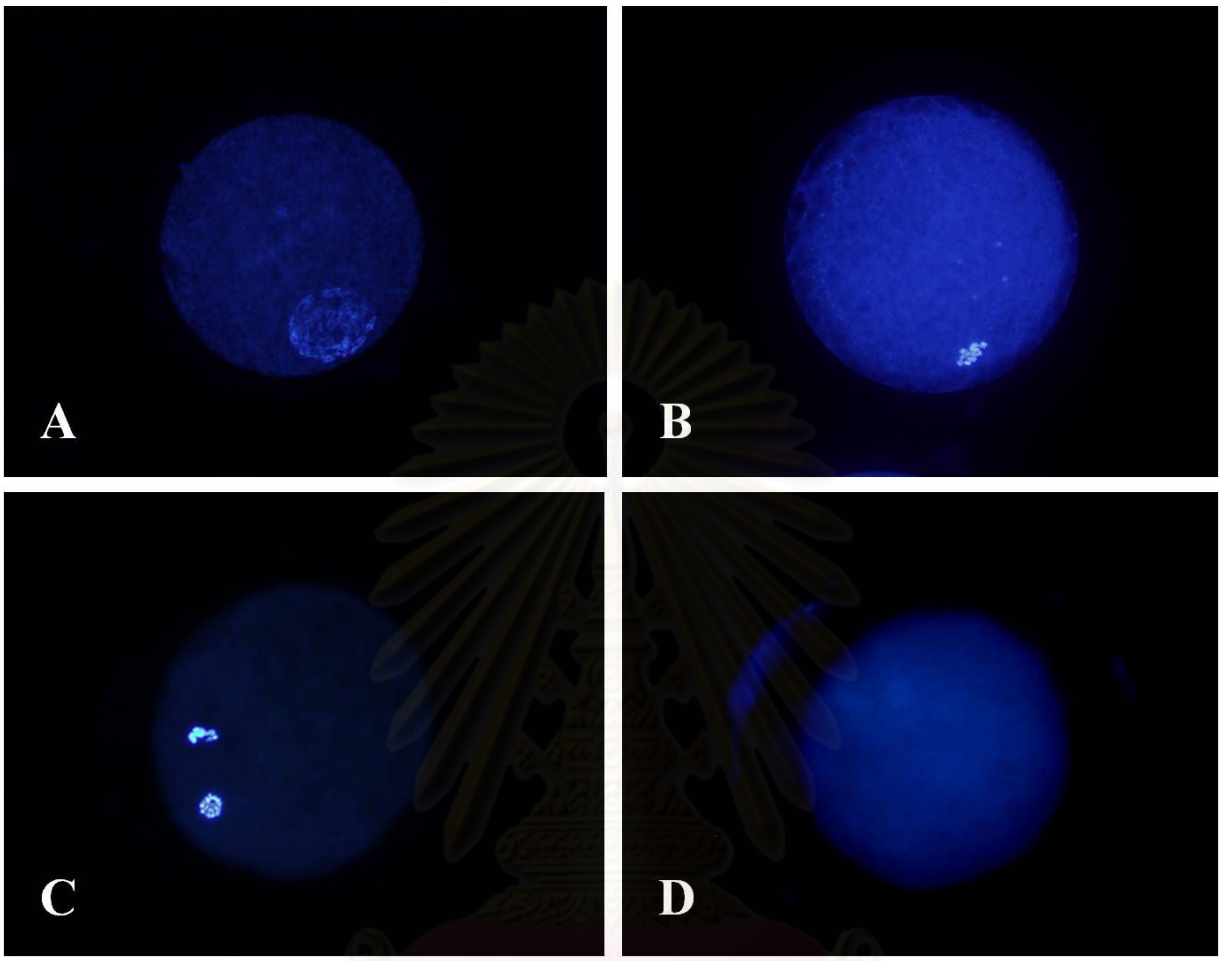

Figure 11. Nuclear statuses of oocytes after DAPI labeling under epifluorescence microscope (BX51; Olympus, Shinjuku, Japan) at a magnification x 400. They were classified as germinal vesicle (GV), metaphase I (MI), and metaphase II (MII) stages. (A) GV stage was classified by a single mass of diffuse or condensed chromatin configurations confined within a specified area (germinal vesicle), (B) MI stage was classified as one set of chromosome aligned at the metaphase plate in the ooplasm, (C) MII stage was classified as the ooplasm contained one set of aligned chromosome at metaphase plate with an extrusion of the first polar body. Oocytes displayed with scattered chromatins or containing with no chromatin materials in ooplasm were classified as degenerated (D).

2.3.7 Statistical analysis

Three to four replicates were performed in each experiment. Results were expressed as mean ± standard error of the mean (SEM). Differences among groups were assessed by the one-way ANOVA statistical test and Duncan analysis. Differences with $P<0.05$ were considered statistically significant.

2.3.8 Experimental design

Experiment I: Effect of roscovitine on the meiotic arrest of cat oocytes

Groups of 20 to 30 Grade I and II to III COCs were cultured for 24 h in a basic maturation medium containing different concentrations of roscovitine (0, 12.5, 25, 50, 100, and 200 μM).

Experiment II: Effect of roscovitine on the nuclear maturation of cat oocytes

This experiment was performed similarly to Experiment I but, after washing three to four times in roscovitine-free medium, the COCs (Grade I and II to III) were matured *in vitro* in IVM medium for 24 h. The reversibility of meiotic inhibition was analyzed.

Experiment III: Effect of roscovitine on cumulus cell apoptosis

To determine the effect of roscovitine on the viability and apoptosis of oocytes and cumulus cells, Grade I COCs were incubated with different concentrations of roscovitine as in Experiment I. Oocyte/cumulus cells were subsequently stained with ethidium-homodimer-1 and TUNEL.

Experiment IV: Developmental competence of roscovitine-treated cat oocytes

After 24 h of roscovitine incubation and subsequent washing, the COCs were matured for 24 h and *in vitro* fertilized. Cleaved embryos were recorded at 48 h after fertilization, and noncleaved embryos were removed from the culture. On day 5 of IVC, the number of embryos at morula stage was recorded. Blastocysts at day 7 were fixed and stained with DNA labeling to count the total cell numbers. For the controls, oocytes were matured in IVM medium supplemented either with or without 25 ng/ml epidermal growth factor (EGF) (Merlo et al., 2005). Epidermal growth factor, known to enhance cytoplasmic maturation of oocytes, was additionally used as positive control.

2.4 Results

2.4.1 Experiment I: Roscovitine-induced meiotic arrest of cat oocytes

A total of 404 Grade I and 415 Grade II to III cat COCs were used in this study. All roscovitine concentrations significantly arrested cat oocytes at the GV stage in a dose-dependent manner, which was significantly greater than that in the controls (0 μM roscovitine). For Grade I COCs, a large number of oocytes cultured in the presence of roscovitine (50, 100, and 200 μM) arrested at the GV stage (%GV: 85.7 ± 4.4, 87.1 ± 3.8, and 75.9 ± 4.7, respectively). These were significantly greater than those obtained with roscovitine at 12.5 and 25 μM (%GV: 42.6 ± 6.7 and 67.6 ± 5.8) (Figure 12A) ($P<0.05$). Similarly, all concentrations of roscovitine were capable of arresting Grade II to III COCs at the GV stage (Figure 12B). The degeneration of Grade I COCs was significantly decreased in a dose-dependent manner ($P<0.05$), whereas the

degeneration rate of Grade II to III COCs did not significantly differ among roscovitine concentrations (12.5, 25, 50, and 100 μM). In both cases, the highest concentration of roscovitine (200 μM) contributed to the increasing rate of oocytes degeneration (P<0.05).

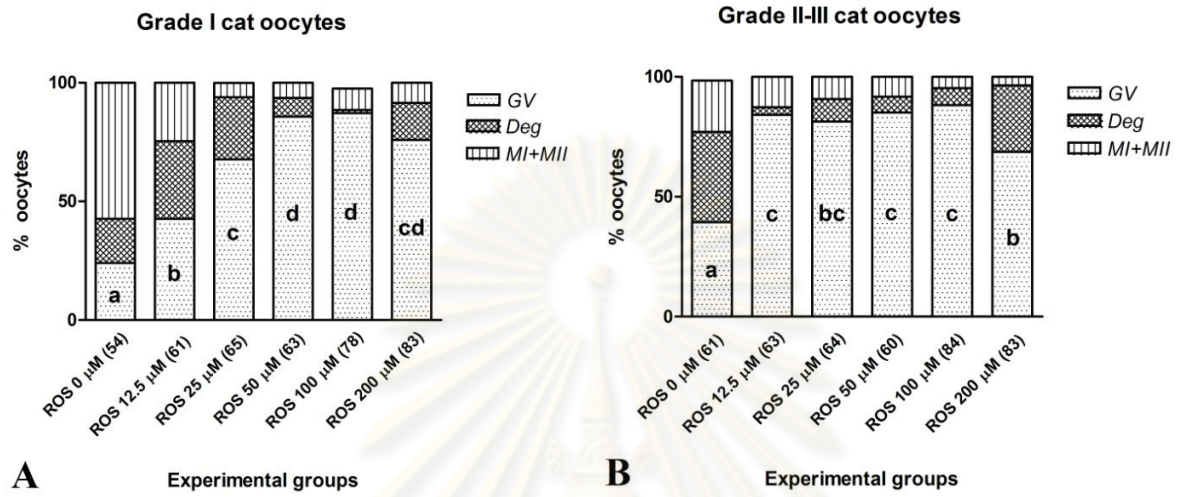

Figure 12. Percentages at the GV stage (GV), metaphase I stage (MI), metaphase II stage (MII), and degenerated oocytes (Deg) after culture for 24 h in basic maturation medium containing varied concentrations of roscovitine. (A) Percentage of Grade I cat oocytes obtained from different roscovitine concentrations; (B) percentage of Grade II to III cat oocytes obtained from different roscovitine concentrations. [a,b,c,d] Different superscripts within the graph denote values of %GV among the experimental groups that differ significantly. ROS= roscovitine

2.4.2 Experiment II: Reversibility of roscovitine-induced meiotic arrest

A total of 365 Grade I and 387 Grade II to III cat COCs were used in this study. Generally, COCs treated with roscovitine for 24 h were able to resume meiosis and reach MII. Grade I COCs matured in IVM medium resumed and reached the MII stage in greater proportions than Grade II to III COCs (%MII: 74.1 ± 6.0 and 47.1 ± 6.0, respectively). The COCs treated with 12.5 and 25 μM roscovitine gave significantly higher rates of MII stage oocytes (%MII: 63.3 ± 6.2 and 50.9 ± 6.8 for Grade I COCs (Figure 13A); 47.1 ± 6.1 and 33.8 ± 5.9 for Grade II to III COCs (Figure 13B), respectively) than those treated with other roscovitine concentrations (P<0.05). MII rates were significantly reduced in 100 and 200 μM roscovitine groups compared with those of other roscovitine concentrations. MII rates obtained with 12.5 and 25 μM roscovitine treatments were comparable with those obtained from non-treated controls (P>0.05).

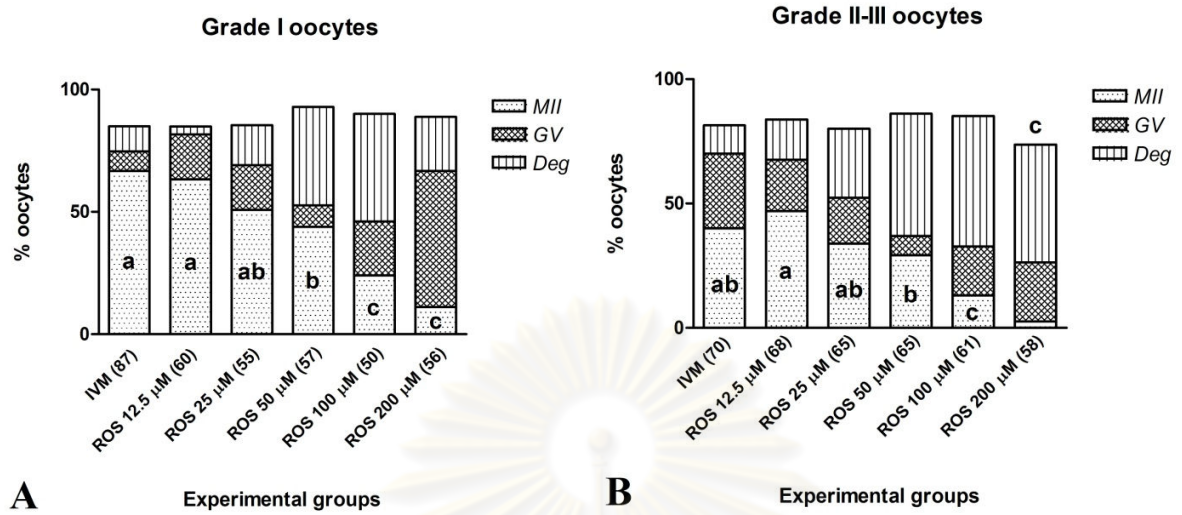

Figure 13. Percentages at the GV stage (GV), MII stage (MII), and degenerate oocytes (Deg) after roscovitine removal and culture in IVM medium for 24 h. Oocytes matured in IVM medium without roscovitine preincubation served as controls. (A) Percentage of Grade I cat oocytes obtained from different roscovitine concentrations; (B) percentage of Grade II to III cat oocytes obtained from different roscovitine concentrations. Percentages of MI stage oocytes were excluded in this figure. [a,b,c] Different superscripts within the graph denote values of %MII among the experimental groups that differ significantly. ROS= roscovitine

2.4.3 Experiment III: Roscovitine-induced cumulus cell apoptosis

Prior to oocyte culture, DNA fragmentation (late apoptosis) of the oocyte and cumulus cells was scarcely detected (% apoptosis: 4.7 ± 1.9). In contrast, a dose-dependent occurrence of apoptosis in cumulus cells was shown after roscovitine treatment (% apoptosis: 8.4 ± 2.3, 10.0 ± 5.0, 11.7 ± 6.2, 28.9 ± 27.3, 58.4 ± 18.8, and 65.9 ± 31.35 for 0, 12.5, 25, 50, 100, and 200 μM roscovitine; respectively) (Figure 14-15). Roscovitine, however, did not affect oocyte viability and apoptosis (0% dead and apoptotic oocytes).

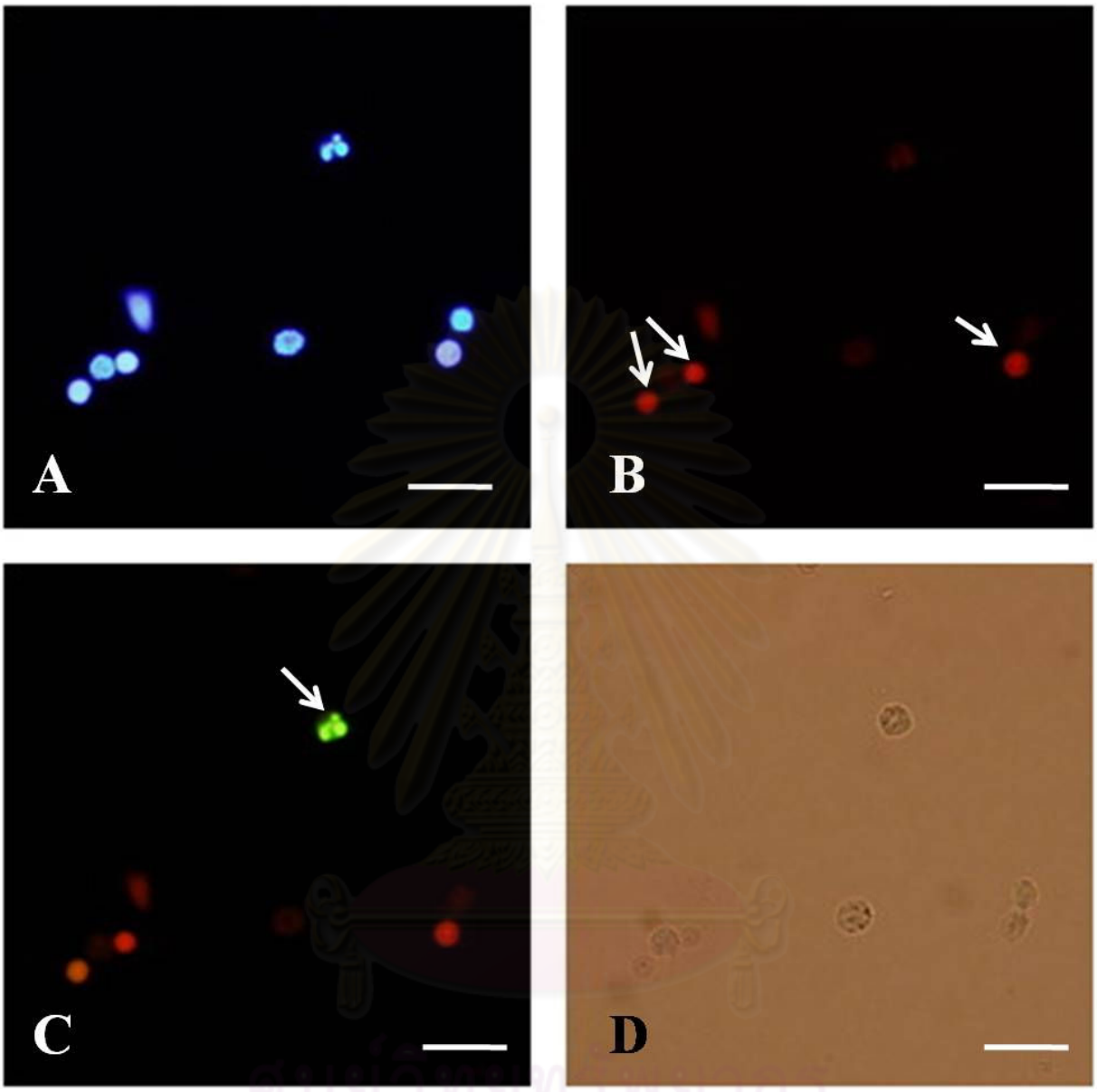

Figure 14. Cumulus cell assessment using DAPI, ethidium-homodimer and TUNEL staining. (A) Total cumulus cell counter stained with DAPI, (B) dead cumulus cells, positive only to ethidium-homodimer (arrowheads), (C) apoptotic cumulus cells positive only to TUNEL (arrowhead), (D) the same microscopic view of cumulus cells under bright-field visualization via light microscope. Scale bars = 20 μm.

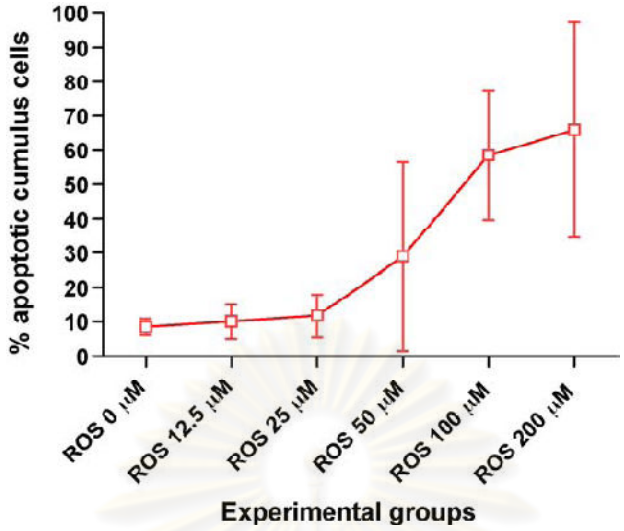

Figure 15. Percentage of cumulus cell (Grade I COCs) apoptosis after roscovitine treatment for 24 h. Cumulus oocyte complexes cultured without roscovitine treatment served as controls. ROS = roscovitine

2.4.4 Experiment IV: Developmental competence of roscovitine-pretreated oocytes

Due to the highest MII rate obtained with 12.5 and 25 µM roscovitine, these concentrations were selected to assess the effect of roscovitine on the developmental competence of cat oocytes (Grade I and II to III). In this study, supplementation of the IVM medium with 25 ng/ml EGF, known to enhance cytoplasmic maturation thereby increasing the developmental competence of oocytes (Merlo et al., 2005), gave the highest developmental rates (% cleavage: 65.3 ± 5.5 and 42.5 ± 5.5; % blastocyst: 36.0 ± 5.5 and 15.0 ± 4.0 for Grade I and II to III, respectively) compared with that of non-EGF-treated and roscovitine-treated groups ($P<0.05$; Figure 16; Table 1-2). Prematuration of the oocytes with roscovitine did not improve cytoplasmic maturation of the oocytes because developmental rates gained from Grade I and II to III oocytes exposed to 12.5 and 25 µM roscovitine were significantly lower than those of non–roscovitine-treated group (% blastocyst: 4.1 ± 2.3 and 7.5 ± 2.9 for Grade I and 1.4 ± 1.3 and 0 for Grade II to III COCs treated with 12.5 and 25 µM roscovitine; respectively) ($P<0.05$).

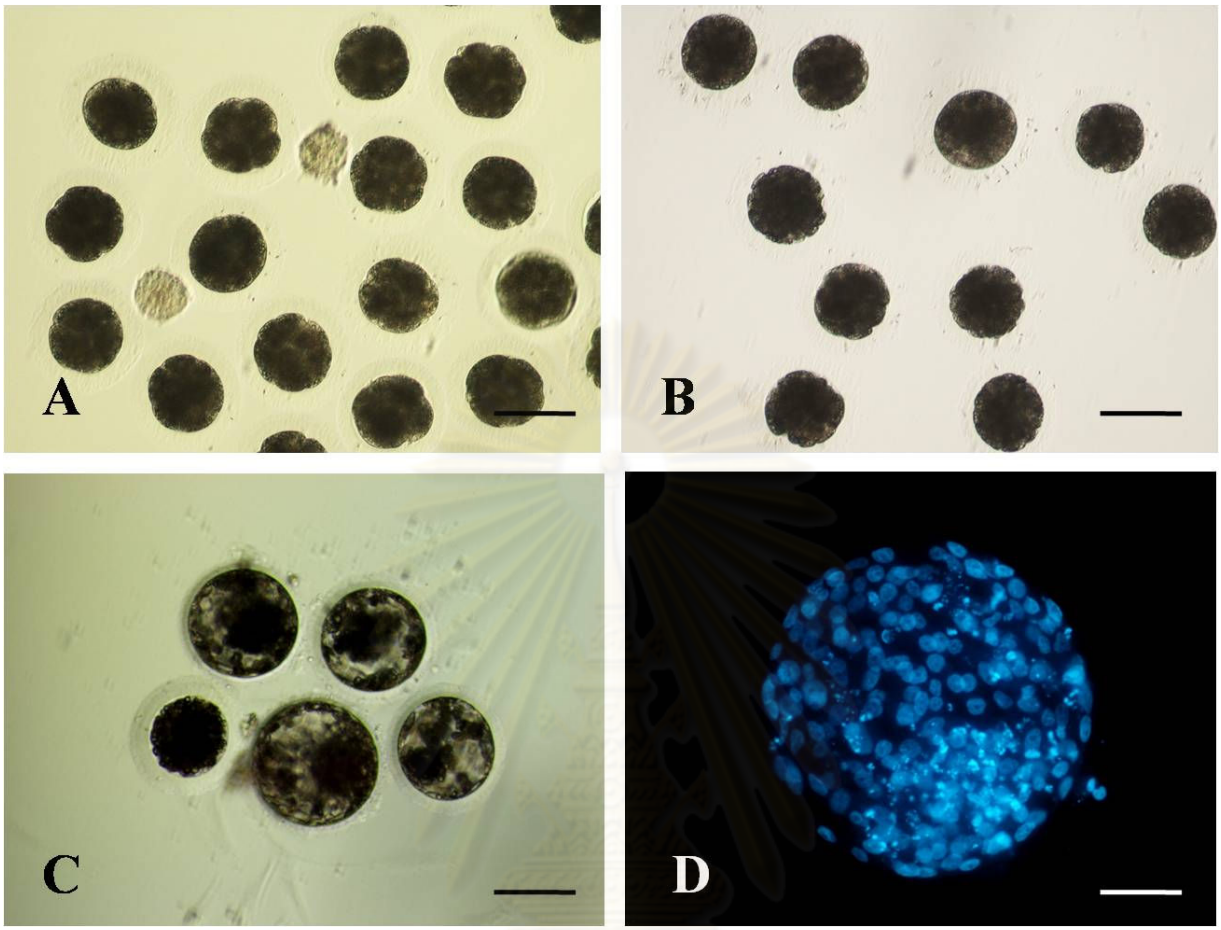

Figure 16. Light microscope appearance of cat embryos at cleavage (A), morula (B) and blastocyst (C) stage on day 2, 5 and 7 respectively derived from oocytes cultured in IVM medium supplemented with 25 ng/ml EGF (magnification x 100); (D) cat embryo after DAPI labeling under epifluorescence (magnification x 400). Scale bars: (A-C) 100 µm; (D) 50 µm.

Table 1. Developmental competence of cat oocytes (Grade I COCs) after *in vitro* fertilization and embryo culture

Groups		N	Cleavage (%)	Percentage (%) total oocytes developed to:		Total cell number
Prematuration	*In vitro* maturation			Morula	Blastocyst	
None	rhFSH	79	37.9±5.5[a]	31.6±5.2[b]	20.2±4.5[b]	260±57.5
ROS 12.5 µM	rhFSH	73	28.7±5.3[a]	15.0±4.2[a]	4.1±2.3[a]	73.33±11.5
ROS 25 µM	rhFSH	80	30.0±5.1[a]	20.0±4.5[ab]	7.5±2.9[a]	126.67±47.6
None	rhFSH+EGF	75	65.3±5.5[b]	61.3±5.6[c]	36.0±5.5[c]	148±76.9

rhFSH= recombinant human follicle-stimulating hormone; EGF= epidermal growth factor
[a,b,c] Values within columns with different superscripts were significantly different

Table 2. Developmental competence of cat oocytes (Grade II to III COCs) after *in vitro* fertilization and embryo culture

Groups		N	Cleavage (%)	Percentage (%) total oocytes developed to:		Total cell number
Prematuration	*In vitro* maturation			Morula	Blastocyst	
None	rhFSH	85	35.2±5.2[b]	22.3±4.5[b]	8.2±3.0[bc]	220±64.3
ROS12.5 μM	rhFSH	74	17.5±4.4[a]	8.1±3.2[a]	1.4±1.3[ab]	80
ROS 25 μM	rhFSH	76	15.7±4.2[a]	7.9±3.1[a]	0[a]	0
None	rhFSH+EGF	80	42.5±5.5[b]	35.0±5.3[c]	15.0±4.0[c]	142.5±117.5

rhFSH= recombinant human follicle-stimulating hormone; EGF= epidermal growth factor
[a,b,c] Values within columns with different superscripts were significantly different.

2.5 Discussion

This study examined the effect of roscovitine on meiotic and developmental competence of cat oocytes. The results demonstrated that roscovitine (cyclin-dependent kinase inhibitor; CDKI) efficiently arrested feline oocytes at the GV stage and this effect was reversible after culturing roscovitine-treated oocytes in ‘‘roscovitine-free’’ IVM medium. Similar to other domestic species such as cow, pig, and sheep, roscovitine and other CDKIs have successfully been used to prevent meiotic resumption aiming ultimately to improve the oocyte’s developmental competence (Mermillod et al., 2000; Hashimoto et al., 2002; Schoevers et al., 2005). The results indicated convincingly as in other mammals that the meiotic resumption of cat oocytes is controlled by a cyclin-related pathway. In cats, the activation of maturation promoting factors (MPFs), a protein complex composed of two subunits of $P34^{cdc2}$ and cyclin B, and mitogen activated protein (MAP) kinase are considered as essential pathways during *in vitro* resumption of meiosis (Bogliolo et al., 2004), although several other signaling pathways have also been demonstrated to be involved in meiosis resumption. In Experiments I and II, the ability of roscovitine to arrest GV-stage oocytes was dose-dependent and depended also on the COCs quality. The percentage of Grade II to III COCs that were arrested at GV stage after roscovitine (12.5 and 25 μM) treatment was higher compared with that of Grade I COCs. This could be due to the fact that Grade II to III COCs have poorer meiotic competence than Grade I COCs (Wood and Wildt, 1997). This study also demonstrated that roscovitine did not improve meiotic competence of Grade II to III cat COCs, although it has been shown to accelerate meiosis progression in other species (Schoevers et al., 2005). The suitable concentrations of roscovitine used to arrest immature oocytes have been variable among species studied (pig, 25 to 80 μM (Krischek and Meinecke, 2001; Ju et al., 2003; Le Beux et al., 2003; Schoevers et al., 2005); cow and calf, 25 μM (Mermillod et al., 2000; Albarracin et al., 2005); horse, 66 μM (Franz et al., 2003)). Our results demonstrated that roscovitine at concentrations of 12.5 and 25 μM efficiently arrested the meiotic resumption of cat oocytes, in terms of the number of MII and degenerated oocytes. This was similar to that of non-roscovitine-treated controls. Given that the interaction between an oocyte and the surrounding cumulus cells is essential to successful nuclear and

cytoplasmic maturation during *in vitro* maturation, and high dose of roscovitine (50, 100, and 200 μM) significantly increased the numbers of apoptotic cumulus cells, we therefore selected roscovitine at 12.5 and 25 μM for subsequent study. Several previous reports have revealed that meiotic inhibitors seem to accelerate nuclear maturation after removal and maturation *in vitro* (Mermillod et al., 2000; Ponderato et al., 2001; Lagutina et al., 2002; Adona et al., 2008). This phenomenon has also been observed in pig (Hirao et al., 2003) and goat (Han et al., 2006). The reason for this acceleration is unclear, but it has been suggested that some factors related to cell-cycle progression control might accumulate during the block of meiosis (Vigneron et al., 2004a; Vigneron et al., 2004b). These oocytes reached the MII stage faster and remained for too long a time at this stage, leading to chromatin and spindle abnormalities (Ponderato et al., 2001; Han et al., 2006). Because a large proportion of oocytes arrested at MI and MII during 24 h incubation with 12.5 and 25 μM roscovitine and the effect of roscovitine was completely reversible, we speculated that these oocytes would resume and arrest at MII stage after IVM for a longer period of time compared with the roscovitine-treated oocytes that had been arrested at GV stage. Prolonging culture of these ‘‘aging’’ MII oocytes appeared to cause the poor developmental capability in this study.

Furthermore, the effects of roscovitine on oocyte developmental competence depended also on the incubation time used. For example, goat oocytes pretreated with 200 μM roscovitine for 24 h were able to resume meiosis at high rates after *in vitro* maturation, but these oocytes supported poor embryo development. However, the percentage of oocytes developing to blastocysts increased to the level of the control when the roscovitine treatment time was reduced to 8 h (Han et al., 2006). Adona et al. (2008) revealed that butyrolactone I (BLI) and roscovitine added in DMEM resulted in decreased blastocyst rates, whereas BLI and roscovitine added into TCM-199 resulted in similar developmental rates as those of the controls. This observation demonstrated that the culture conditions in which the inhibitor is applied may affect the outcome of the treatment. However, most studies have shown that prematuration in the presence of BLI or roscovitine maintained blastocyst development and cell numbers similar to those of the controls (Lonergan et al., 2000; Mermillod et al., 2000; Hashimoto et al., 2002). Moreover, Hashimoto et al. (2002) obtained a high blastocyst rate after using prematuration of bovine oocytes with BLI only but using different culture conditions, including low oxygen tension (5% oxygen) and the presence of fetal calf serum.

In contrast with the reports that roscovitine did not impair subsequent development of goat and bovine oocytes (Mermillod et al., 2000; Han et al., 2006), we found that feline oocytes pretreated with roscovitine for 24 h prior to IVM demonstrated poor developmental competence by means of cleavage and blastocyst formation rates when compared with that of non–roscovitine-treated controls. To date, the mechanism underlying roscovitine-induced poor embryo development is still unclear. In the current study, roscovitine incubation for 24 h at high doses (50, 100, and 200 μM) markedly affected cumulus cell viability and DNA fragmentation in a dose-dependent manner. DNA fragmentation (TUNEL-positive nuclei) known as the late stage of apoptosis demonstrated the negative effect of roscovitine on cumulus cells viability. Given

that bidirectional communication between cumulus cells and oocytes via gap junctions determines the cytoplasmic maturation of oocytes (Eppig, 1991; Matzuk et al., 2002) and high degree of cumulus cell apoptosis/cell death contributes to poor embryo development (Host et al., 2002), insufficiently viable cumulus cells or the disruption of gap junctions between cumulus cells and oocytes after roscovitine treatment could have been responsible for the poor developmental competence of cat oocytes in this study. Roscovitine at 12.5 and 25 μM exhibited the lowest apoptotic rate of cumulus cells, and therefore these doses were chosen as candidate doses for subsequent IVF and IVC study. Lonergan et al. (2003a) reported that roscovitine disrupted the integrity and expansion ability of cumulus cells, which is in accordance with our observations of cat oocytes. In addition, they also found that the incubation of roscovitine for 24 h led to the swelling of mitochondria cristae, degeneration of cortical granules especially during IVM, and a convolution of the nuclear membrane.

In conclusion, roscovitine was able to block the meiosis of immature cat oocytes without any remarkable effect on nuclear maturation. However, the experiments demonstrated the dramatically negative effect on embryo development possibly via cumulus cell apoptosis.

CHAPTER III

THE EFFECT OF CULTURE MEDIUM TYPES AND CULTURE VOLUMES ON DEVELOPMENTAL COMPETENCE AND GENE EXPRESSION OF CAT EMBRYOS

3.1 Abstract

Morphology and gene expression are currently used methods for assessing the effect of culture medium and culture volume on embryos of several species. To define their effects on domestic cat embryos, groups of 8 to 10 embryos were cultured in SOF, modified Tyrode's solution or MK-1 medium in a fixed volume (50 µl) and in different volumes (20, 50 and 100 µl). SOF supplemented with different concentrations of glucose (1.5, 3.0 and 6.0 mM) was used to examine the effect of glucose level in culture media on embryo development. Real-time reverse transcriptase polymerase chain reaction was used to determine the relative transcripts of BAX, BCL-2 and GLUT-1 genes in blastocysts derived from various concentrations of glucose. SOF and MK-1 supported feline embryo development better than modified Tyrode's solution. Embryos cultured in 20-µl droplets showed decreased development in all three media ($P<0.05$). Increasing the glucose concentration in SOF to 6.0 mM adversely affected embryo development and tended to increase the BCL-2 transcript in blastocysts. In conclusion, type of culture medium, culture volume and glucose concentration affected the development of domestic cat embryos. Decreased culture volume and high glucose concentration negatively affected embryo development. The increase of anti-apoptotic BCL-2 expression found in blastocysts cultured in 6.0 mM glucose may prevented an increase in the incidence of apoptosis. In the present study, it was clearly demonstrated that differential gene expression occurred in embryos with similar morphology.

3.2 Introduction

Over the past few decades, success of *in vitro* fertilization (IVF) in domestic cats has been improved. However, blastocyst rates from oocytes matured and fertilized *in vitro* are still lower than those matured and fertilized *in vivo* (30 to 40% vs. 50 to 70%, respectively) (Farstad, 2000; Gomez et al., 2003; Pope et al., 2006a; Yin et al., 2007). The restricted development of IVM/IVF derived embryos is due to a poor understand of culture requirement less than ideal *in vivo* environment and also how oocytes/embryos response to the specific condition. This includes both *in vitro* maturation and embryo culture environment (Herrick et al., 2007). Although determination of phenotypic changes in IVF embryos, such as incidence of blastocyst development and occurrence of cell fragmentation, are commonly used tools to access quality of embryo development *in vitro*, phenotype alone does not provide sufficient information on the detrimental factors presented in by *in vitro* culture. Lately, determination of gene expression has become an increasingly powerful tool to access embryo quality. Analysis of phenotypic changes,

along with analysis of gene expression can provide useful insight into mechanisms regulating embryo development under different culture conditions.

Type of culture medium (Johnston et al., 1993; Herrick et al., 2007), culture volume (Spindler and Wildt, 2002), gas atmosphere (Johnston et al., 1991) and culture temperature (Johnston et al., 1991) are among the main factors that determine the success of *in vitro* embryo production (IVP). Composition of the culture medium markedly affects embryonic morphology, metabolism, gene expression and epigenetic modification (Lane and Gardner, 1998; Doherty et al., 2000; Niemann and Wrenzycki, 2000; Holm et al., 2002; Niemann et al., 2002; Rinaudo and Schultz, 2004; de Oliveira et al., 2005; Gardner and Lane, 2005; Lane and Gardner, 2005). It is therefore not surprising that the rate of feline blastocyst development has shown considerable variability (ranging from 30 to 70% of cleaved embryos) (Kanda et al., 1998; Gomez et al., 2000; Spindler and Wildt, 2002; Merlo et al., 2005) in different culture media. Globally, various types of culture media have been used to support feline embryo development *in vitro*, including Ham's F-10, (Roth et al., 1994), Synthetic oviductal fluid (SOF) (Freistedt et al., 2001), modified Tyrode's balanced salt solution (modified Tyrode's solution) (Gomez et al., 2003) and modified Earle's balanced salt solution (MK-1) (Kanda et al., 1998). Although the culture system used for *in vitro* maturation and embryo culture markedly influences developmental competence (Kanda et al., 1998; Freistedt et al., 2001; Gomez et al., 2003), a simultaneous comparison of these culture media, in terms of their ability to support embryo development has not been done. In addition, culture volume has been demonstrated to affect *in vitro* development, including the quality of embryos (Lane and Gardner, 1992; Vutyavanich et al., 2010). Culture of mouse embryos in reduced incubation volume, from 320 to 20 µl, significantly increased blastocyst cell number and embryo development after transfer (Lane and Gardner, 1992). Moreover, mouse embryos cultured singly in 0.5 µl had fewer trophectoderm cells than those in 10 µl (Vutyavanich et al., 2010).

Indeed, several components in culture media have been shown either to support or to adversely affect embryo development *in vitro*. Although embryos require a specific energy substrate such as glucose and pyruvate to promote development (Gardner, 1998; Gardner et al., 2000; Doblado and Moley, 2007), the rate of glucose utilization as a sole energy substrate differs both among species and developmental stages. Excessive glucose levels impair embryo development by the induction of oxidative stress and an increased incidence of apoptosis and cell death (Moley et al., 1991; Moley et al., 1994; Moley et al., 1998b; Leunda-Casi et al., 2002). Furthermore, early fetal loss and congenital malformation frequently occurs during pregnancy in diabetic women (Kalter, 1987). The poor development of human and mouse embryos in a high glucose condition has been postulated to be the result of altered expression of genes regulating glucose transport (Hahn et al., 2000). Previously, glucose transport (GLUT) in preimplantation murine embryos was known as facilitative glucose transporters: GLUT-1, GLUT-2, and GLUT-3 (Hogan et al., 1991; Aghayan et al., 1992). Although there are many other types of GLUTs that have been detected in preimplantation embryos, their function is largely unknown. GLUT-1 mRNA is expressed throughout preimplantation development in the mouse (Hogan et al., 1991;

Aghayan et al., 1992; Morita et al., 1992), rabbit (Robinson et al., 1990), cow (Lequarre et al., 1997; Augustin et al., 2001), and human (Dan-Goor et al., 1997). It has been hypothesized that the function of GLUT-1 in the developing embryo is to transport glucose from the embryonic extracellular space into the inner cell mass (Morita et al., 1992). A relationship between the decrease in GLUT-1 expression and the increase of apoptosis in murine blastocysts has been found (Chi et al., 2000). The decrease of GLUT-1 elevated the cell death signal that triggers a BAX - dependent apoptotic cascade (Moley et al., 1998b; Chi et al., 2000). Indeed, alterations of glucose uptake via the glucose transport gene have not been previously examined in cat embryos. However, an *in vitro* high glucose condition increases the incidence of nuclear fragmentation and apoptosis in murine blastocysts (Pampfer et al., 1997). Apoptosis in embryos has been known to occur via an intrinsic pathway (Hardy, 1999). BCL-2 (B-cell lymphoma 2) family, a family protein consisting of pro-survival and pro-apoptotic members primarily controls the process. Pro-apoptotic BH3-only proteins are responsible for the first step in the apoptosis signaling cascade and function upstream pro-apoptotic BAX (Bcl-2-associated X) protein. Normally, BAX protein is directly responsible for the damage to the mitochondria but pro-survival family members, especially BCL-2 (B-cell lymphoma 2) protein, protect the cell from this damage (Giam et al., 2008). The activated BAX molecule can be captured by free pro-survival protein, thus allowing the cell to survive. In contrast, initiation of apoptosis may occur if the number of activated BAX molecules is greater than the number of pro-survival proteins (Leber et al., 2007; Giam et al., 2008).

The objectives of the present study were to examine the effect of culture medium, culture volume and glucose concentration on the *in vitro* developmental competence of cat embryos. Additionally, expression levels of BAX, BCL-2 and GLUT-1 genes were examined in blastocysts produced by culture in different concentrations of glucose.

3.3 Materials and methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, USA), unless otherwise indicated.

3.3.1 Oocyte recovery

Ovaries were collected from domestic cats subjected to ovariohysterectomy and were transported to the laboratory in a 0.9% (v/w) saline solution supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin. Within 2 h, the ovaries were washed and placed in a holding medium (HM) consisting of Hepes-buffered M199, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 4 mg/ml bovine serum albumin (BSA, embryo tested). Cumulus oocyte complexes (COCs) were recovered by ovarian mincing in HM and then morphologically classified at a magnification x 40 using a stereomicroscope (SMZ645 Nikon, Tokyo, Japan). Only COCs surrounded with more than five layers of compacted cumulus cells and containing homogeneous-dark ooplasm were used.

3.3.2 Oocytes culture

In vitro oocyte maturation was essentially performed as previously described (Sananmuang et al., 2010). Groups of 20 to 30 COCs were cultured for 24 h in 500 µl of *in vitro* maturation medium ($NaHCO_3$-M199 supplemented with 1.0 mM sodium pyruvate, 2.0 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 4 mg/ml BSA) containing 0.05 IU/ml recombinant human follicle stimulating hormone; rhFSH, (rhFSH; Organon, Bangkok, Thailand) and 25 ng/ml epidermal growth factor (EGF).

3.3.3 *In vitro* fertilization

For IVF, groups of 8 to 10 oocytes were transferred to 50-µl drops of IVF medium (Pope, 2004) with minor modification (Tyrode's balanced salt solution containing with 11.9 mM $NaHCO_3$, 1% (v/v) MEM non-essential amino acids (NEAA), 6 mg/ml BSA, 100 IU/ml penicillin, 100 µg/ml streptomycin, 30 µg/ml heparin, 1 mM L-glutamine, 0.36 mM sodium pyruvate and 0.11 mM calcium lactate). The semen was collected by electro-ejaculation (Zambelli and Cunto, 2006) from two fertile cats during anesthesia with 0.04 mg/kg atropine sulphate (A.N.B. Laboratories, Bangkok, Thailand), 3 mg/kg xylazine hydrochloride (Laboratorios calier, Barcelona, Spain) and 10 mg/kg ketamine hydrochloride (Gedeon Richter, Budapest, Hungary). Semen samples were cryopreserved according to Rota et al., (1997) with minor modifications. Briefly, straws were placed horizontally 4 cm above liquid nitrogen vapors for 10 min and then plunged into liquid nitrogen. After thawing at 70˚C for 6 sec, sperm were subjectively evaluated, and only samples that had more than 50% progressive motility were used. Oocytes were co-cultured with sperm at a concentration of 0.5 x 10^6 sperm/ml at 38.5°C in a humidified atmosphere of 5% CO_2 in air.

3.3.4 Embryo culture

After 18 h of co-incubation with sperm, cumulus cells were removed by mechanical pipetting. Presumptive zygotes were then washed and cultured in IVC-1 media for 24 h. Cleaved embryos (day 2 post-IVF) were subsequently cultured in IVC-2 media. Embryo culture was performed at 38.5°C in a humidified atmosphere of 5% CO_2 in air. Composition of the three culture media (MK-1, SOF and modified Tyrode's solution) are shown in Table 3. MK-1 was slightly modified by addition of sodium pyruvate and alanyl L-glutamine (Ala-Gln). Modified Tyrode's solution was also modified by supplementation with 11.9 mM $NaHCO_3$ instead of 25 mM $NaHCO_3$. The media were equally adjusted the pH before gas incubation and the pH of modified Tyrode's was tested and ranged between 7.2-7.4 after 1 to 2 h of CO_2 equilibration (Figure 17). The media were also overlaid with mineral oil and changed every 2 day to minimize the possible pHe change during culture.

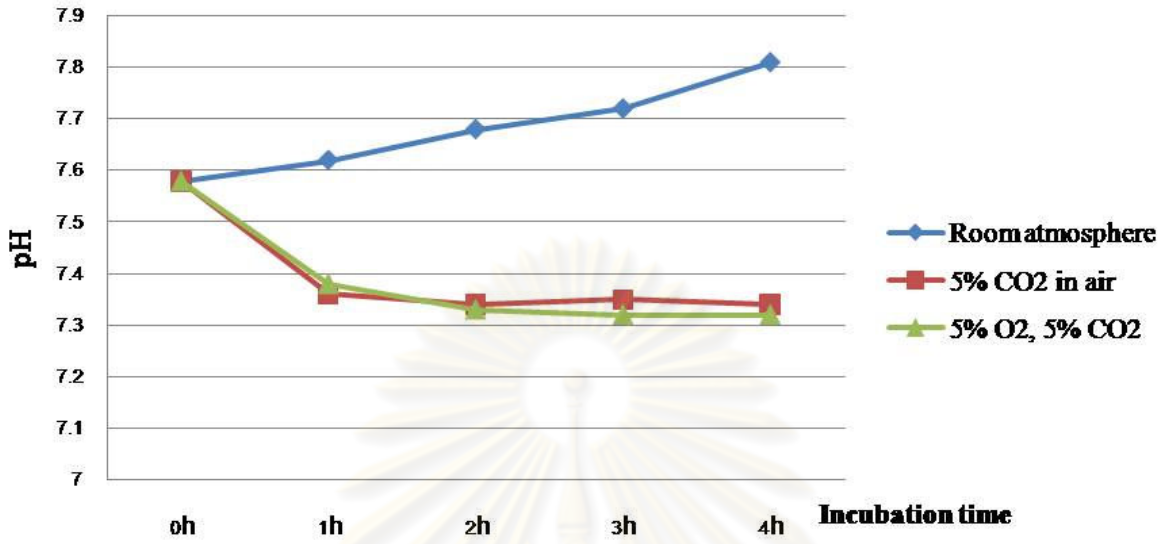

Figure 17. The effect of culture atmospheres on pH of modified Tyrode's medium (IVC). Modified Tyrode's medium was prepared according to Table 3 and then split to 3 incubation conditions: 1) room atmosphere 2) 5% CO_2 in air and 3) 5% CO_2, 5% CO_2 and 90% N_2. In all cases, the medium was maintained at 38.5°C. The pH was measured using a pH meter (EUTECH Instruments Pte Ltd., pH 510, Ayer Rajah Crescent, Singapore). The pH of this medium was in an optimal range for embryos (between 7.2-7.4) after approximately 1 h of incubation (for both 5% CO_2, 20% O_2 and 5% CO_2, 5% O_2, 90% N_2).

3.3.5 Assessment of embryo development

The percentage of cleaved embryos (2 to 16 cells), morula (≥16 cells without blastocoel) and blastocysts (>50 cells with blastocoel formation) were evaluated on day 2, 5 and 7 of IVC, respectively. The percentage of hatched blastocysts was calculated relative to the total number of blastocysts. To count the number of nuclei, blastocysts were fixed in 4% (w/v) paraformaldehyde and kept at 4˚C for 2 days before staining with 0.1 µg/ml 4',6-Diamidino-2-phenylindole (DAPI) in PBS at 37˚C for 10 min. The fluorescently labeled embryos were then examined using an epifluorescent microscope (BX51 Olympus, Shinjuku, Japan).

Table 3. Chemical components of three different culture media (SOF, modified Tyrode's solution and MK-1) used for culturing feline embryos. IVC-1 and IVC-2 were used for day 1 to 2, and day 2 to day 7 of embryo development, respectively (day 0 = day of IVF). m-Tyrode = modified Tyrode's solution

Media	IVC-I			IVC-II		
	SOF	m-Tyrode	MK-1	SOF	m-Tyrode	MK-1
NaCl	107.6 mM	136.9 mM	116.4 mM	107.6 mM	136. 9 mM	116.4 mM
KCl	7.1 mM	2.7 mM	5.4 mM	7.1 mM	2.7 mM	5.4 mM
$NaHCO_3$	25.0 mM	11.9 mM	25.0 mM	25.0 mM	11.9 mM	25.0 mM
Na-Lactate (60% w/w)	5.3 mM	-	-	5.3 mM	-	-
$L(+)Ca(lactate)_2$	-	0.1 mM	1.8 mM	-	0.1 mM	1.8 mM
$MgSO_4.7H_2O$	-	-	0.8 mM	-	-	0.8 mM
$MgCl_2.6H_2O$	0.5 mM	1.1 mM	-	0.5 mM	1.1 mM	-
$CaCl_2.2H_2O$	1.7 mM	1.8 mM	-	1.7 mM	1.80 mM	-
KH_2PO_4	1.2 mM	-	-	1.2 mM	-	-
$NaH_2PO_4.H_2O$	-	-	1.0 mM	-	-	1.03 mM
NaH_2PO_4 (anhyd)	-	0.4 mM	-	-	0.36 mM	-
Phenol red	←		500 µl		→	
MEM NEAA	←		1%		→	
BME EAA	2%	-	2%	←	2%	→
Glucose	1.50 mM	5.6 mM	1.5 mM	1.5 mM	5.6 mM	1.50 mM
Pen/strep	←	100 IU penicillin G and 100 µg/mL streptomycin				→
Sodium Pyruvate	0.3 mM	0.4 mM	0.4mM	0.3 mM	0.4 mM	0.4 mM
Ala-Gln	2.1 mM	1.0 mM	1.0 mM	2.1 mM	1.0mM	1.0 mM
BSA	4	3	3	-	-	-
FCS	-	-	-	←	10% (v/v)	→

SOF, modified Tyode's solution and MK-1 media were prepared according to (Freistedt et al., 2001), (Pope, 2004) and (Kanda et al., 1998) respectively. Modified Tyrode's solution was slightly modified by supplementation with 11.9 mM $NaHCO_3$.

3.3.6 RNA extraction

Total RNA was extracted from five blastocysts in one replicate using the Absolutely RNA Nanoprep Kit (Stratagene, San Diego, CA, USA) following the manufacturer's instruction at room temperature. Briefly, blastocysts were lysed using a lysis buffer containing 0.7% β-Mercaptoethanol (β-ME). An equal volume of 80% sulfolane was added to the cell lysate. The mixture was then transferred to an RNA-binding nano-spin cup and centrifuged at ≥12,000 x g

for 60 sec. The filtrate was discarded and a low-salt wash buffer was added to the re-seated spin cup. After centrifugation, the filtrate was discarded and the cup was re-seated and dried by centrifuging at ≥12,000 x g for 60 sec. DNase I (Stratagene, San Diego, CA, USA) was added to the fiber matrix inside the cup and incubated at 37˚C for 15 min. After incubation, the fiber matrix was washed with high-salt wash buffer once and low-salt wash buffer twice. The RNA was eluted by centrifuging (≥12,000 x g for 5 min) with 12 µl RNase-free water. The extracted RNA was assessed for quality and quantity using a spectrophotometer (Nanodrop ND-2000, Wilmington, Delaware, USA) and immediately stored at -80˚C until further processing.

3.3.7 Reverse Transcription (RT)

RT was performed using a First-Strand cDNA Synthesis Kit (SuperScript III Kit, Invitrogen, Carlsbad, CA, USA). The RT reactions (20 µl) were performed according to the kit manufacturer's protocol. Eight µl of extracted RNA was incubated with 1 µl of random hexamers (50 ng/µl) and 1 µl of 10 mM dNTP mix at 65˚C for 5 min, then immediately placed on ice for at least 1 min. After chilling on ice, the mastermix containing 2 µl of 10xRT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT, 1 µl of RNaseOut (40U/µl) and 1 µl of SuperScript III RT (200 U/µl) was then added to the reaction. The reaction was further incubated at 50 ˚C for 50 min followed by 5 min incubation at 85˚C. RNA template from the cDNA:RNA hybrid molecule was removed by digestion with 1 µl of RNase H (2U/µl) after first-strand synthesis at 37˚C for 20 min. The product was stored at -20˚C for further use in PCR.

3.3.8 Quantitative Real-Time PCR

The relative expression levels of individual target genes (GLUT-1, BAX and BCL-2) were normalized to the endogenous normalizer (GAPDH: glyceraldehyde 3-phosphatedehydrogenase), and were run in separate wells. The PCR was performed using the ABI PRISM 7300 Real-time cycler (Applied Biosystems, Foster City, CA, USA) with Power SYBR Green PCR Master Mix (Applied Biosystems, WA, UK). Each PCR reaction (total volume of 20 µl) consisted of 2 µl of reverse transcription product (equivalent to 0.5 blastocyst) and 18 µl of reaction mixture which contained 10 µl of SYBR Green Master Mix, 1 µl of both 5 µM forward and reverse primer (Table 4) and 6 µl of nuclease free water. The thermal cycling conditions were as follows: 10 min at 95˚C to activate tag DNA polymerase, 40 cycles of 15 sec at 95˚C for denaturing, 30 sec at 55˚C for annealing and 60 sec at 72˚C for extension. The Sequence Detection System (SDS) Software Version 1.4 (Applied Biosystems, USA) was used to quantify and analyze the relative quantitation (RQ). Calculations of relative quantitation were performed by the comparative Ct method, using blastocysts cultured in 1.5 mM glucose as a control group. Data was reported as relative n-times difference, in relation to the control sample. At the end of program, PCR products were confirmed by melting curve analysis and run in gel electrophoresis. The amplified products were run in 2% agarose gel (Bio-Rad, CA, USA) prepared in 1 x TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH8)

containing 0.4 mg/ml ethidium bromide (Promega, WI, USA). The separated products in agarose gel were visualized under UV light (Syngene, CB, UK).

Table 4. Description of forward (FP) and reverse (RP) primers used to assess the expression of target genes in blastocysts obtained from different glucose culture conditions.

Genes	Sequence (5′-3′ orientation)	Fragment length (bp)	Reference
GLUT-1	FP: ATTGTGGCTGAACTCTTCAG RP: CCAGGAGTACGGTGAAGATG	157	(Zini et al., 2009)
BAX	FP: CCGATGGCAACTTCAACTGGG RP: GTCAGCACTCCCGCCACAAAG	244	(Zhao et al., 2008)
BCL-2	FP: GGAGGATTGTGGCCTTCT RP: GTTATCCTGGATCCAGGTGT	143	(Yamazaki et al., 2004)
GAPDH	FP: GGAGAAAGCTGCCAAATATG RP: AGGAAATGAGCTTGACAAAGTGG	191	(Sano et al., 2005)

3.3.9 Statistical analysis

Three to four independent replicates were performed in each experiment. Data was expressed as a mean ± standard error of the mean (SEM). Differences among groups were assessed by one-way ANOVA statistical test and DUNCAN analysis. Differences with a $P<0.05$ were considered to be statistical significance.

3.3.10 Experimental design

Experiment I: Effect of embryo culture media on embryo development

A total of 175 cat COCs was used. Following IVM/IVF, groups of 8 to 10 cat embryos were randomly cultured in 50-µl droplets of SOF, modified Tyrode's solution or MK-1 for 7 days. The incidence of cleavage and number of embryos at the morula stage were recorded on day 2 and 5, respectively. Blastocysts at day 7 of development were fixed and stained with DNA labeling to count the total cell number/blastocyst.

Experiment II: Effect of culture volume on embryo development

A total of 778 cat COCs was used. Following IV/IVF, groups of 8 to 10 cat embryos were randomly cultured in different volumes (20-, 50- and 100-µl drops) of SOF, modified Tyrode's solution or MK-1 for 7 days. Embryo examination was performed as described in experiment I.

Experiment III: Effect of different glucose concentrations on cat embryo development

A total of 370 cat COCs was used. Following IVM/IVF, groups of 8 to 10 cat embryos were randomly cultured in 50-µl droplets of SOF containing different concentrations of glucose

(1.5, 3.0 or 6.0 mM). Embryo development and quality were assessed as previously described in experiment I. Additionally, the percentages of hatched blastocysts and fragmented embryonic nuclei were examined.

Experiment IV: Effect of glucose on the gene expression of cat embryos

Cat blastocysts at day 7 of development cultured in different concentrations of glucose (as in experiment III) were used. Five expanded blastocysts showing the same morphology from each replicate were collected in a minimum volume (less than 2 µl) of PBS containing 0.1% BSA and were immediately stored at -80˚C until use. They were analyzed for expression levels of GLUT-1, BAX and BCL-2 genes relative to the house keeping gene (GAPDH). The relative expression levels assessed by real-time PCR were shown as relative quantitation (RQ) and the size of products were confirmed by gel electrophoresis.

3.4 Results

3.4.1 Experiment I: Effect of embryo culture media on cat embryo development

Frequencies of cleavage and development to the morula stage were not different among the three culture media. However, the percentages of embryos that developed to the blastocyst stage in SOF (61.0±7.7) and MK-1 (62.5±8.7) were higher than that of modified Tyrode's solution (33.3±9.8) ($P<0.05$). The overall average cell number/blastocyst was similar among the experimental groups (123.1±5.8 cells/blastocyst) (Table 5).

Table 5. Mean percent ±SE of developmental competence and embryo quality of cat embryos cultured in different culture medium types

Cultured media	N	Cleavage	Cleaved embryo developed to		Cell number
			Morula	Blastocyst	
SOF	63	55.6±6.3 [a]	90.2±4.7 [a]	61.0±7.7 [a]	121.6±12.2 [a]
m-TYRODE	53	45.3±6.9 [a]	83.3±7.8 [a]	33.3±9.8 [b]	113.8±20.5 [a]
MK-1	59	54.2±6.5 [a]	87.5±5.9 [a]	62.5±8.7 [a]	134.0±24.9 [a]

[a,b] Within a column, different superscripts denote values that differ significantly ($P<0.05$). m-Tyrode = modified Tyrode's solution

3.4.2 Experiment II: Effect of culture volume on embryo development

Frequency of cleavage was not affected by the volume of the culture droplet (~50 to 60%). The incidence of development to the morula stage of embryos cultured in SOF was higher ($P<0.05$) in 50-µl and 100-µl droplets (96.3±3.7 and 93.9±3.6, respectively) than in 20-µl droplets (80.3±4.0). Blastocyst formation rates of embryos cultured in 50- and 100-µl droplets of SOF (62.5±7.8 and 56.7±6.5, respectively) and MK-1 (60.9±7.7 and 53.3±7.5, respectively) were similar, and both were higher than blastocyst development of embryos in modified Tyrode's solution (45.2±7.8 and 30.2±7.1, respectively). Culture of embryos in 20 µl of SOF, MK-1 or

modified Tyrode's solution decreased blastocyst formation rates (20.0±5.4, 39.6±6.8 and 13.6±5.2, respectively). Cell numbers of blastocysts were not affected by the type or volume of culture medium (Table 6-8). Overall, frequency of blastocyst formation in embryos cultured in modified Tyrode's solution tended to be lower than that of embryos in SOF and MK-1 regardless of culture volume.

Table 6. Mean percent ± SE of developmental competence and embryo quality of cat embryos cultured in SOF with different culture volumes

Volume(µl): 5-10 embryos	N	Cleavage	Cleaved embryo developed to		Cell number
			Morula	Blastocyst	
20	92	59.9±3.4 [a]	80.3±4.0 [a]	20.0±5.4 [a]	94.5±6.2 [a]
50	73	54.5±8.7 [a]	96.3±3.7 [b]	62.5±7.8 [b]	119.2±11.7 [a]
100	92	64.6±8.6 [a]	93.9±3.6 [b]	56.7±6.5 [b]	99.9±7.8 [a]

[a,b] Within a column, different superscripts denote values that differ significantly (P<0.05).

Table 7. Mean percent ± SE of developmental competence and embryo quality of cat embryos cultured in modifiedTyrode's solution with different culture volumes

Volume(µl): 5-10 embryos	N	Cleavage	Cleaved embryo developed to		Cell number
			Morula	Blastocyst	
20	87	51.9±9.7 [a]	79.7±2.4 [a]	13.6±5.2 [a]	119.2±27.8 [a]
50	78	54.8±8.9 [a]	94.4±5.6 [a]	45.2±7.8 [b]	123.7±10.2 [a]
100	90	48.0±3.2 [a]	89.9±6.8 [a]	30.2±7.1 [ab]	95.5±10.6 [a]

[a,b] Within a column, different superscripts denote values that differ significantly (P<0.05).

Table 8. Mean percent ± SE of developmental competence and embryo quality of cat embryos cultured in MK-1 with different culture volumes

Volume(µl): 5-10 embryos	N	Cleavage	Cleaved embryo developed to		Cell number
			Morula	Blastocyst	
20	94	56.9±6.4 [a]	79.4±4.4 [a]	39.6±6.8 [a]	101.4±7.7 [a]
50	79	52.7±9.7 [a]	83.6±5.4 [a]	60.9±7.7 [a]	137.0±21.4 [a]
100	93	48.6±2.7 [a]	91.4±4.3 [a]	53.3±7.5 [a]	136.1±16.3 [a]

[a,b] Within a column, different superscripts denote values that differ significantly (P<0.05).

3.4.3 Experiment III: Effect of different glucose concentrations on cat embryo development

Frequency of cleavage in all concentrations of glucose were similar (50-60%) (P>0.05). However, the frequencies of embryo development to morula and blastocyst stages of embryos cultured in SOF containing 1.5 and 3.0 mM glucose were higher than that obtained from embryos cultured in SOF containing 6.0 mM glucose (P<0.05). Cell numbers of blastocysts were

also higher in embryos cultured in 1.5 and 3.0 mM glucose as compared to 6.0 mM glucose ($P<0.05$). Although hatching and cell fragmentation rates were not significantly different among glucose concentrations, the results indicated that increasing glucose levels of up to 6 mM in SOF tended to negatively affect blastocyst quality, in terms of cell nuclear fragmentation and the number of hatched blastocysts (Table 9 and Figure 18).

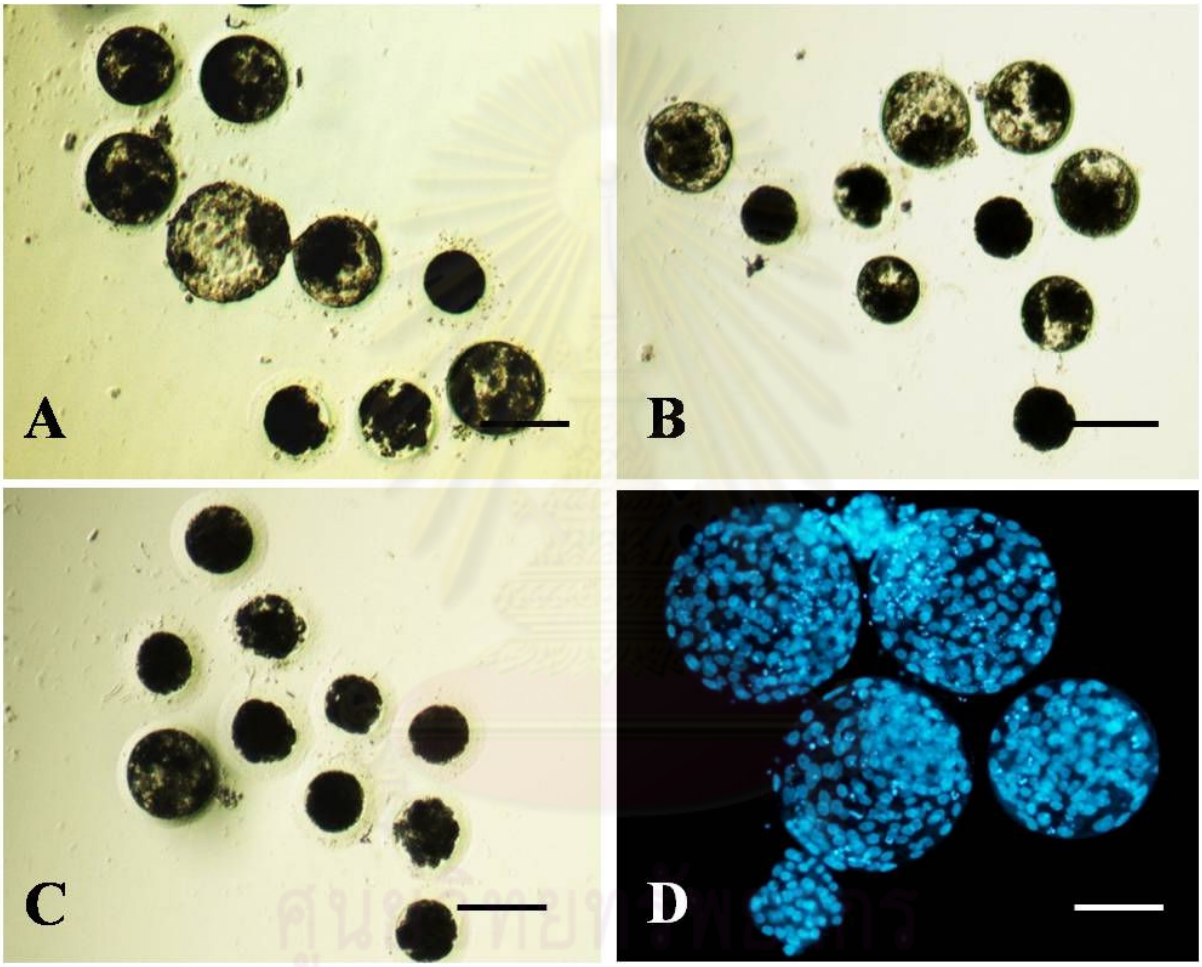

Figure 18. Day 7 cat embryos derived from *in vitro* culture. They were assessed under bright field visualization via a light microscope at a magnification x 10 (A-C). (A) Blastocysts derived from embryos cultured in SOF containing 1.5 mM glucose, (B) Blastocysts derived from embryos cultured in SOF containing 3.0 mM glucose, (C) Blastocysts derived from embryos cultured in SOF containing 6.0 mM glucose, D) Blastocsyts derived from SOF containing 1.5 mM glucose stained with DAPI and evaluated using an epifluorescent microscope at a magnification x 200. Scale bars represent 200 (A-C) and 100 (D) µm.

Table 9. Mean percent ± SE of developmental competence and embryo quality of cat embryos cultured in SOF containing with different glucose concentrations

Glucose conc. (mM)	N	Cleavage	Cleaved embryo developed to		Hatched blastocyst	Cell number	Fragmented cells
			Morula	Blastocyst			
1.5	124	58.5±1.5 [a]	95.0±1.7 [a]	55.6±7.8 [a]	29.1±10.7 [a]	170.9±22.3 [a]	12.8±3.0 [a]
3.0	124	53.8±1.9 [a]	88.8±4.8 [a]	41.2±6.8 [a,b]	25.6±4.8 [a]	167.1±8.3 [a]	16.3±2.5 [a]
6.0	122	51.9±5.2 [a]	76.2±3.1 [b]	24.8±5.8 [b]	12.5±7.2 [a]	103.1±4.6 [b]	19.3±1.1 [a]

[a,b] Within a column, different superscripts denote values that differ significantly (P<0.05).

3.4.4 Experiment IV: Effect of glucose on gene expression of cat embryos

The melting curve analysis showed no primer-dimers or nonspecific products in all assays (Figure 19-22) and PCR products were confirmed correct amplicon sizes by running in gel electrophoresis (Figure 23). The expression levels of GLUT-1, BCL-2 and BAX were not significantly different among the blastocysts cultured in various concentrations of glucose (P>0.05). However, the expression levels of BCL-2 gene tended to increase in blastocysts cultured in 6.0 mM glucose as compared to that of blastocysts cultured in 1.5 and 3.0 mM glucose (Figure 24).

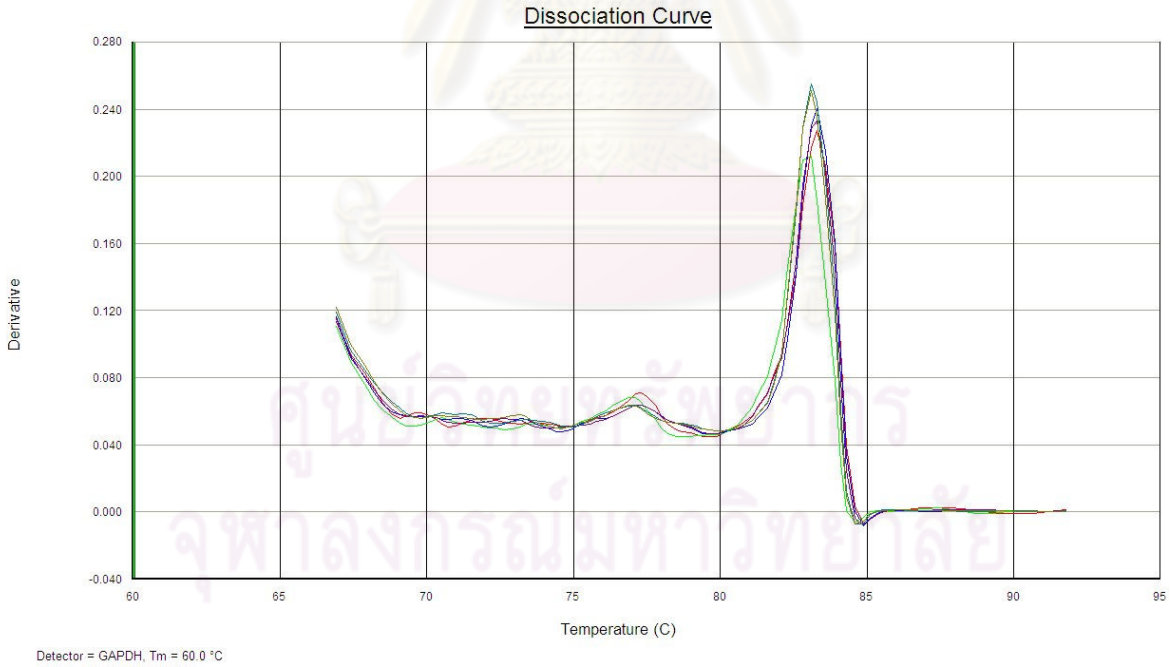

Figure 19. Melting curve analysis of GAPDH performed at the end of the real-time PCR. There were no significant primer-dimers or nonspecific products present on the curve.

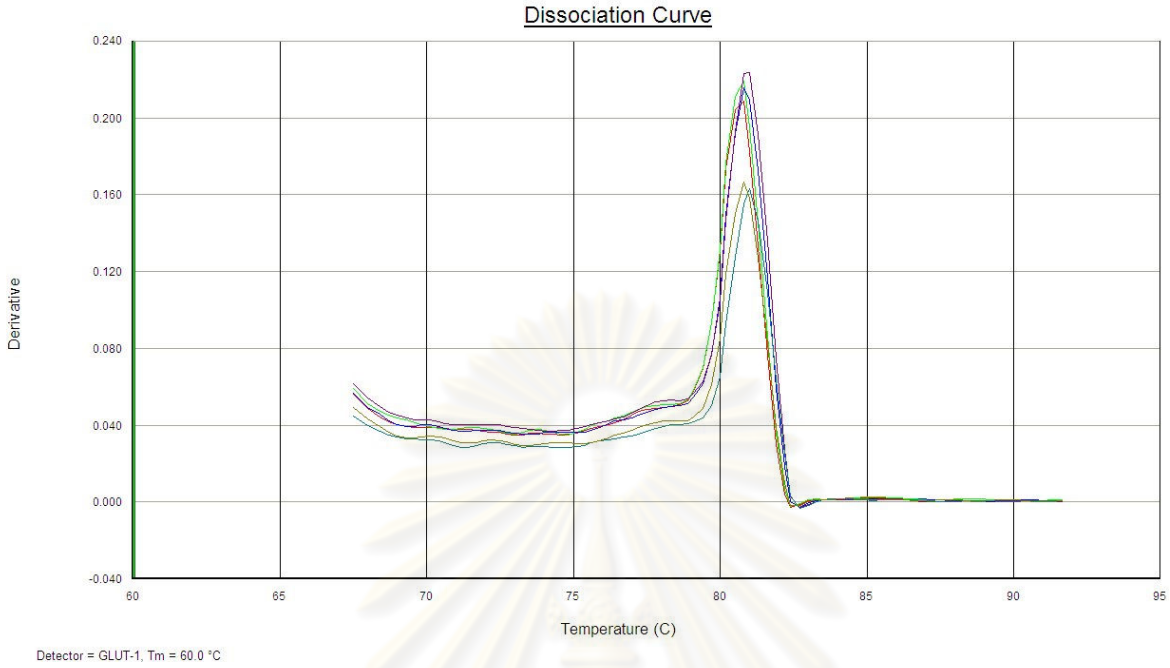

Figure 20. Melting curve analysis of GLUT-1 performed at the end of the real-time PCR. There were no significant primer-dimers or nonspecific products present on the curve.

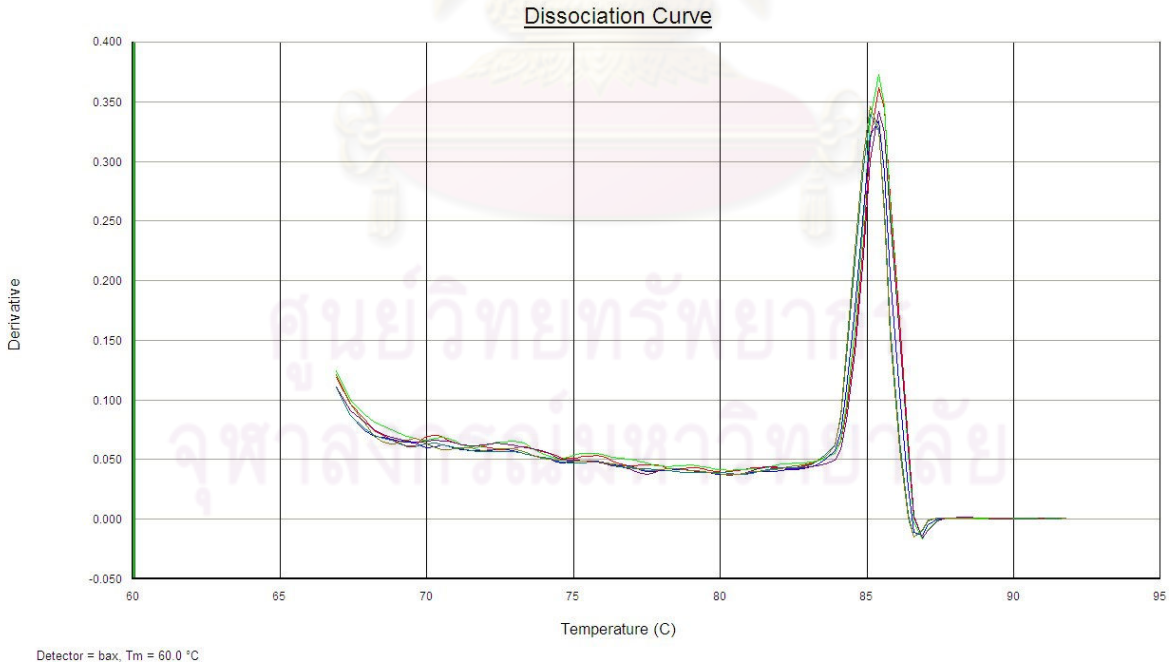

Figure 21. Melting curve analysis of BAX performed at the end of the real-time PCR. There were no significant primer-dimers or nonspecific products present on the curve.

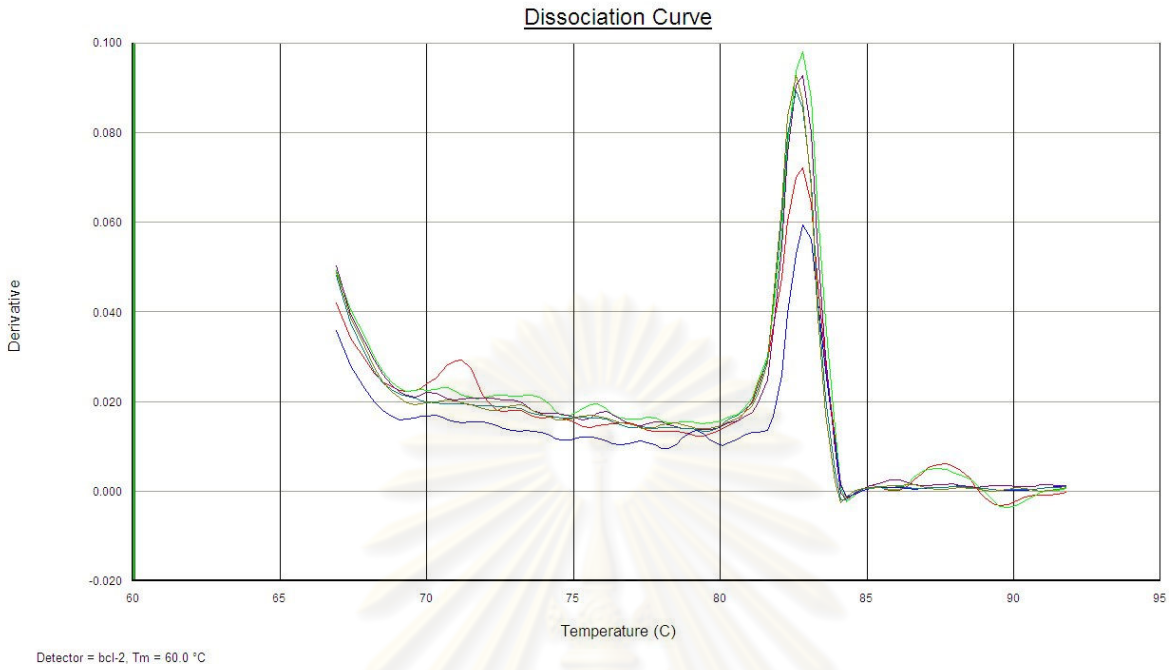

Figure 22. Melting curve analysis of BCL-2 performed at the end of the real-time PCR. There were no significant primer-dimers or nonspecific products present on the curve.

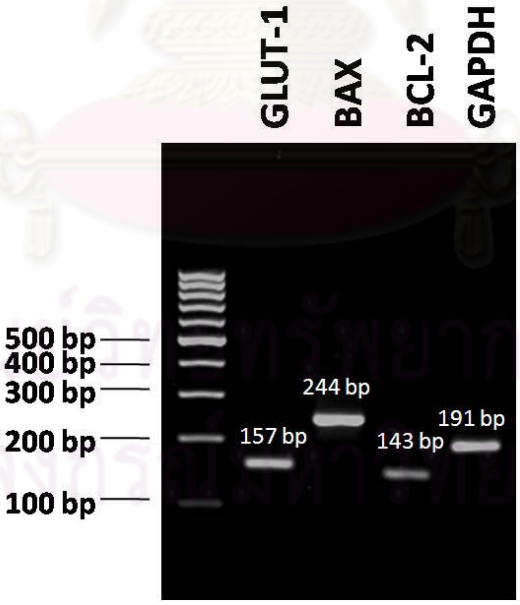

Figure 23. The amplicon sizes of PCR products (GLUT-1, BAX, BCL-2 and GAPDH) acquired from real-time PCR were confirmed their correct sizes by gel electrophoresis. They were run on 2% agarose gel contained with ethidium bromide in 1 x TBE buffer and were visualized under UV light.

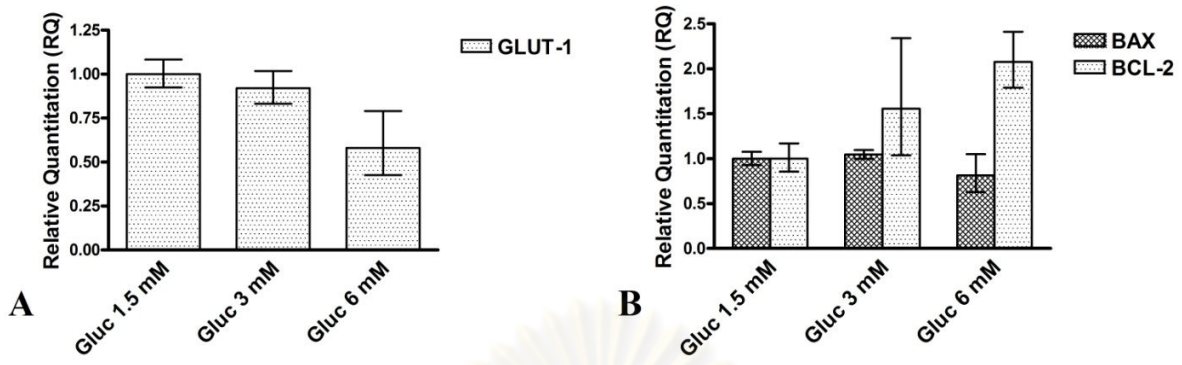

Figure 24. Relative expression of GLUT-1 (A), BAX and BCL-2 (B) transcripts in blastocysts acquired from embryos cultured in SOF containing 1.5 mM (Gluc 1.5 mM), 3 mM (Gluc 3 mM) and 6 mM (Gluc 6 mM) glucose. The expression of each gene showing as relative quantitation (RQ) was analyzed using real-time PCR. GAPDH was used to normalize each gene, and embryos cultured in glucose 1.5 mM were used as calibrators.

3.5 Discussion

Synthetic oviductal fluid (SOF) (Freistedt et al., 2001), modified Tyrode's balanced salt solution (modified Tyrode's solution) (Gomez et al., 2003; Pope, 2004) and modified Earle's balanced salt solution (MK-1) (Kanda et al., 1998) are generally known to support feline embryo development *in vitro*. However, the efficacy of these media has been variable among laboratories (Kanda et al., 1998; Freistedt et al., 2001). Culture volume also affected cat embryo development in the present study. A decreasing culture volume to 20-µl drop/8 to 10 embryos negatively affected the developmental competence of embryos in all culture media as compared to those of 50- and 100-µl drops/8 to 10 embryos. These were likely due to the accumulation of toxic substances in a small volume of culture medium, resulting in cell stress and poor embryo development (Fujitani et al., 1997; O'Doherty et al., 1997; de Oliveira et al., 2005).

Our results indicated that SOF and MK-1 supported feline embryo development better than modified Tyrode's solution in all culture volumes (Table 5-9) when cultured in 5% CO_2 condition, although Tyrode's solution has been widely used to culture feline embryos with a high rate of embryo development (Gomez et al., 2003; Pope, 2004; Pope et al., 2006a). It is worth noting that the concentration of sodium bicarbonate (11.9 mM) in modified Tyrode's solution used in this study was lower than SOF and MK-1 (25 mM). However, this Tyrode's balance salt solution was able to maintain pH in an optimal range for embryo culture (pH 7.2 to 7.4) (Figure 17). Because the maintenance of intracellular pH (pHi) is vital for normal regulation of several intracellular metabolisms and development of embryos (Swain, 2010), it therefore seems likely that other factors in Tyrode's solution contributed to the poor embryo development. In fact, we cultured feline embryos with Tyrode's solution in the presence of 20% O_2 in contrast to the previous studies that cultured cat embryos with 5% O_2 (Gomez et al., 2003; Pope, 2004; Pope et al., 2006a). Since the oxygen tension in culture environment is a major contributor to the reactive oxygen species production, reduction of O_2 level in culture environment from 20% to 5% results

in the lower ROS production (e.g. superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-)), thereby improving the embryo development (Fujitani et al., 1997; Kwon et al., 1999; Karja et al., 2006). Furthermore, amino acids in SOF and MK-1 may also act as osmoregulators, osmoprotectants, pH regulators and energy sources for pre-implantation embryos. These properties alleviate the stress at early cleavage stages which in turn, improve the quality of developing embryos (Swain, 2010).

In addition to the above mentioned factors, high concentration of glucose (5.6 mM) in Tyrode's solution appeared to play a key role since suboptimal glucose concentration in culture medium potentially induces poor embryo development (Swanson et al., 1996). *In vivo*-derived feline embryos metabolized glucose approximately twice as much as pyruvate on day 3 and approximately five times as much on day 6. An increased utilization of glucose through glycolysis during compaction and blastocyst formation is common among embryos of several species (Gardner, 1998; Thompson, 2000). On the other hand, an increasing glucose concentration lowered glycolytic activity during blastocyst formation (Gardner, 1998; Gardner et al., 2000). Because SOF medium tended to provide the best feline embryos' development in the present study, we selected SOF as the model to study the effects of glucose concentrations on feline embryo development. Increasing the glucose concentration in SOF (6.0 mM) comparable to that of modified Tyrode's solution (5.6 mM) negatively affected feline embryo development by reducing the blastocyst rate and cell number of blastocysts. Moreover, it also tended to reduce hatching blastocyst rate and increased the fragmented nuclei of blastocysts. These results correspond to previous reports demonstrating the detrimental effects of a high glucose condition on embryo development (Moley et al., 1991; Moley et al., 1994; Leunda-Casi et al., 2002; Doblado and Moley, 2007). Although the mechanism underlying poor embryo development under high glucose conditions is still unclear, it has been postulated that high glucose concentrations may induce oxidative stress or down regulate the glucose uptake into cells resulting in insufficient metabolic requirements (Doblado and Moley, 2007). A high concentration of glucose has been shown to increase cell death and oxidative stress, and a decrease of glucose transporter and metabolism in mouse blastocysts (Leunda-Casi et al., 2002). In addition, elevated glucose concentration in SOF may also contribute to the imbalance of inorganic phosphate which potentially affects the development of feline embryos cultured *in vitro* (Herrick et al., 2007). While the elevated phosphate concentration inhibits early cleavage stage (Quinn, 1995; Lane and Gardner, 2000), increase of KH_2PO_4 concentration in medium from 0.25 mM to 1.0 mM with constant 3 mM glucose improved cleavage rates of feline embryos. It is therefore implied that feline embryos need a specific requirement for inorganic phosphate, and the management of glucose and inorganic phosphate (Glu/Pi) balance in feline embryo appear to be different when compared to other species.

For quantitative PCR, blastocysts with the same morphology were selected in order to minimize the variation of embryo quality acquired from each culture medium. These blastocysts represented the produced feline embryos that could tolerate and survive in their culture systems. This study only determined BCL-2 and BAX expressions as representatives of anti-apoptotic and

pro-apoptosis gene expression, respectively. Though the expression of BCL-2 in blastocysts produced in high glucose concentration (6 mM) tended to be higher than those of other groups (1.5 and 3 mM), expressions of BAX gene were similar among the culture groups. Over-expression of BCL-2 can enhance cell survival by suppressing apoptosis in cells subjected to apoptosis-inducing stimuli. Moreover, increasing BCL-2 is the mechanism by which cells become resistant to apoptosis in the presence of glucose (Abu-El-Asrar et al., 2004). Because of these, the results acquired from the study supported the protective role of BCL-2 in protecting feline embryos against suboptimal culture condition (high glucose in this study). However, our results contradicted to a previous study in which a high glucose condition up-regulated BAX in human blastocysts (Moley et al., 1998a). It is possible that high glucose concentration (6 mM) used in this study was far less than those demonstrated in the previous study (30 mM glucose) (Moley et al., 1998a), and the 6 mM glucose may not efficiently stimulate BAX expression in feline embryos. Another explanation was the nature of BAX response which appeared to be tissue-, cell- and stage-specific (Evan and Littlewood, 1998; Kockx and Knaapen, 2000; Giam et al., 2008). Since the study about BAX expression in feline embryos is still limited, further study on other networks of BAX, such as BH3-only protein that have been reported to be involved in the modulation of cellular function and the localization of BAX (Eskes et al., 2000) may also help to elucidate the changed patterns of BAX/BCL-2, thereby substantially providing the clues as to how feline embryos respond to the high glucose culture condition.

Initiation of an apoptotic cascade in embryos during hyperglycemic culture conditions by a decrease of glucose transport has been previously demonstrated (Chi et al., 2000). Such extensive apoptosis has long-lasting detrimental effects on embryo development and pregnancy outcomes, including fetal malformation or increased occurrence of pregnancy reabsorption (Heilig et al., 2003). In order to avoid the effects of a hyperglycemic condition and subsequent induction of cell death, embryonic cells develop mechanisms to promote survival. Cells regulate glucose uptake by the glucose transporter protein (GLUT) on the cell plasma membrane (Pantaleon and Kaye, 1998) and also by modifying glycolytic enzyme activity (Riley and Moley, 2006). Though high glucose concentration used in the current study was not completely representative of hyperglycemic condition, we still considered GLUT gene to imply embryo's glucose regulation. According to our study, GLUT-1 gene expressions were not significantly different among the blastocysts cultured in various concentrations of glucose. There were some explainable causes of insignificant difference of GLUT-1 expression among embryos cultured in various glucose concentrations. Firstly, high glucose concentration (6 mM) used in this study may not efficiently altered GLUT-1 expression. Another explanation was poorly explored role of GLUT-1 in feline embryos. There was an evidence in preimplantation mouse embryos indicated the unequal response of GLUT 1-3 protein to maternal diabetic state (Moley et al., 1998b). In addition, different patterns and roles of GLUTs expression during preimplantation period have been found among species (Augustin et al., 2001). This may also imply the variable sensitivity of each GLUT members in feline embryos. Because only blastocysts were selected for gene expression study, it is also possible that 'survival' blastocysts can find their ways to maintain the

levels of GLUT-1 expression and also to balance glucose metabolism even in the suboptimal glucose condition.

In conclusion, type of culture medium, culture volume and glucose concentration affected the development of domestic cat embryos. SOF and MK-1 supported feline embryo development better than modified Tyrode's solution at least under culture system used in the current study. Reduced culture volume and high glucose concentration negatively affected embryo development. The increase of anti-apoptotic BCL-2 expression found in blastocysts cultured in 6.0 mM glucose appeared to be one of the mechanisms to protect feline embryos against high-glucose induced apoptosis. In the present study, it was clearly demonstrated that similar embryo morphology may have a different pattern of gene expression which was influenced by culture environment. Although this study examined only the effect of glucose concentration and embryo density on embryo development, it is still not possible to rule out other confounding factors such as oxygen tension and buffer system that are also crucial for feline embryo development.

CHAPTER IV

THE EFFECT OF EMBRYO DENSITY AND NUMBER OF CULTURED EMBRYOS ON DEVELOPMENTAL COMPETENCE AND GENE EXPRESSION OF CAT EMBRYOS

4.1 Abstract

Embryo density affects the developmental competence of cultured embryos in several species. Gene expression is currently used for assessing the embryo quality along with the morphology. To determine the effects of this factor on feline embryo development, embryos were randomly cultured in group (n=10 and 5) or singly in different media volumes (12.5, 25, 50, 100 and 200 µl). They were examined their developmental competence and fragmentation of blastocyst cell nuclei using DNA labeling. Only expanded blastocysts acquired from different densities and numbers were collected to examine their mRNA transcripts of survival related genes (BAX, BCL-2 and HSP70) using real-time RT-PCR. Transcripts of *in vivo* produced blastocysts were used as the control. The results showed that embryos cultured in groups tended to develop better than those cultured singly. For group cultured embryos (n=10), embryos acquired from low culture density (1:5, 1:10 and 1:20) could develop better than those acquired from high density (1:1.25 and 1:2.5) ($P<0.05$). Moreover, fragmentation of the blastocyst cell nuclei tended to increase in high culture density. On the other hand, there was no significant difference of developmental competence among embryos cultured singly in varied densities. Blastocysts derived from high culture density (1:1.25) also significantly up-regulated BAX and HSP70 transcripts comparing with those of low culture densities (1:5 and 1:20) ($P<0.05$). However, there was no significant difference in relative transcripts between group and single embryos cultured in fixed 200-µl culture medium. In conclusion, high density negatively affected the developmental competence of feline embryos cultured in groups. The increase of BAX and HSP70 transcripts in these embryos suggested the stress in this group highlighted the mechanisms used to protect the embryo against suboptimal culture condition. The study clearly indicated that determination of mRNA transcripts could describe the metabolic changes that could not be observed by morphology and could be used as a tool for optimization of *in vitro* culture condition in feline species.

4.2 Introduction

Domestic cat has been considered as a potential model for conservation of wild felids essentially via *in vitro* embryo production (IVP) technique. However, realistic application of the technique for endangered wild cat species has met only a limited success due to a number of critical limitations, such as inadequate numbers and low quality of gametes acquired from wild cat samples. Although culture system for single embryos has been developed, developmental

capability of these embryos has been restricted when compared to group culture (Spindler et al., 2006).

Embryo number (single or group) and embryo density (embryo number: medium volume ratio) have been demonstrated to affect the developmental competence of embryos in several species (Paria and Dey, 1990; Gardner et al., 1994; O'Doherty et al., 1997; de Oliveira et al., 2005). Embryos cultured in group and/or in reduced incubation volume had often superior developmental competence than those cultured singly (Paria and Dey, 1990; Lane and Gardner, 1992; Ferry et al., 1994; Keefer et al., 1994; Donnay et al., 1997; O'Doherty et al., 1997; Spindler and Wildt, 2002) suggesting the importance of autocrine and paracrine factors on embryo development (O'Neill, 1997). Previous studies demonstrated that the developmental competence of embryos was enhanced by co-culturing with other cells or tissues (Ellington et al., 1990; Goto et al., 1994) and supplementation of growth factor in culture medium (Larson et al., 1992a; b). In addition, blastocyst formation rate of single culture embryo was increased when cultured in a conditioned medium derived from group-embryo culture in bovine (Fujita et al., 2006). By contrast, excessive embryo number/density negatively affected the embryo development and quality possibly due to the accumulated toxic substances and the aberrant pattern of gene expression (de Oliveira et al., 2005).

In domestic cat species, only few studies demonstrate the beneficial effects of increasing embryo density on the embryo development. *In vitro* development of solitary cat embryos could be improved by culturing with better or equal-quality conspecific companion embryos, particularly companions of an advanced age (Spindler and Wildt, 2002). Besides the increasing of developmental competence, single embryos co-cultured with the heterospecific companion were also demonstrated better quality in terms of increasing total cells per embryos (Spindler et al., 2006). Nevertheless, the study about relationship between embryo numbers and density is still not thoroughly determined in this species. Moreover, the insight mechanisms underlying the developmental outcome are still not clearly clarified.

Recently, examination of gene expression has become as a potential tool for assessing the embryo quality. It provides valuable data of how the embryos manage to survive in the given culture condition by altering the production rate of particular proteins (Liu et al., 1997). Heat shock proteins (HSPs) are one of essential factors allowing the cells to adapt and survive in sub-optimized condition. The proteins have two major functions namely as molecular chaperone. The first one involved in protein folding, assembly, and translocation. The other is to stabilize the damaged protein allowing them promptly to be repaired or degraded in the cell exposed to stress (Santoro, 2000). Normally, most of cellular stress activates similar endpoints – free radical formation, membrane destabilization, protein denaturation, DNA damage, and apoptosis. Cytoprotective mechanisms including the heat-shock protein response, DNA repair cycle checkpoint regulation, and antioxidant system are commonly engaged to protect cells from a variety of adverse environments (Hansen, 2007). HSP70 protects the cell by facilitate folding or refolding of the proteins (Kampinga and Craig). Since the increasing level of HSP70 expression indicate the struggle of the embryos to survive in stress condition, this protein becomes as an

indicator for embryo quality assessment. Previous study in bovine embryos indicated the correlation between HSP70 expression and the sup-optimized culture condition in IVP system. For example, increase of HSP70 expression in bovine blastocysts cultured in high numbers (20 embryos/drop) correlated with the decrease of the developmental competence comparing with those of low numbers (5 embryos/drop) (de Oliveira et al., 2005).

HSP70 also plays the role in anti-apoptotic function in embryonic cells (Paula-Lopes and Hansen, 2002). It interacts with intrinsic and extrinsic pathways of apoptosis at a number of steps. These include the inhibition of translocation of BAX into mitochondria, release of cytochrome c from mitochondria, formation of apoptosome and inhibition of activation of initiator caspases. It also modulates JNK, NK-κB and Akt signaling pathways in the apoptotic cascade (Arya et al., 2007). Apoptosis determined as programmed cell death is a common mechanism used among organisms to eliminate mutated, damaged or even healthy unwanted cells (Hardy, 1997). The evidences of apoptosis in blastomeres of preimplantation embryos have been demonstrated in several species such as mouse, sheep, horse, cattle, pig and human (Hardy, 1997; 1999; Pomar et al., 2005) and this phenomenon occurrs in both *in vivo* and *in vitro* produced blastocysts (Pomar et al., 2005). Basically, increasing incidence of apoptosis in cultured embryonic cells indicates the sub-optimized culture condition (Fabian et al., 2005). To balance between live and death of cells, two well-known pro-apoptotic BAX (Bcl-2 associated X) and anti-apoptotic BCL-2 (B-cell lymphoma protein 2) proteins play an important role in balancing the homeostasis. BAX protein directly responses to the damage of mitochondrial membrane contributing to the further steps of apoptotic process, while BCL-2 protein can prevent the cell from this damage by capturing with BAX protein. Apoptotic process could be occurred if the amount of pro-apoptotic protein is higher than anti-apoptotic protein (Giam et al., 2008). So, the ratio between BAX and BCL-2 (BAX/BCL-2 ratio) is commonly used to predict the cell fate (Perlman et al., 1999).

Measurement of developmental competence combined with the expression of survival related genes, apoptotic (BAX and BCL-2) and stress response genes (HSP70) therefore may provide the mechanism how the embryos respond to the specific culture condition. This study aimed to define the effect of embryo density on the developmental competence of feline embryos cultured in group or singly by morphology and gene expression.

4.3 Materials and methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, USA), otherwise indicated.

4.3.1 Oocyte recovery

The ovaries from ovariohysterectomized domestic cats were collected in a 0.9% (v/w) saline solution supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin. Within 2 h, they were washed and minced in holding medium (HM) consisting of Hepes-buffered M199, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin, 100µg/ml streptomycin and 4 mg/ml bovine serum albumin (BSA, embryo tested) to recover the cumulus oocyte complexes

(COCs) under a stereomicroscope (SMZ645 Nikon, Tokyo, Japan) at a magnification x 40. Only COCs surrounded with more than five layers of compacted cumulus cells and containing with a homogeneous-darken ooplasm were used in this study (Wood and Wildt, 1997).

4.3.2 Oocytes culture

In vitro oocyte maturation was essentially performed as previously described (Sananmuang et al., 2010). Groups of 20 to 30 COCs were cultured in 500 µl of IVM media ($NaHCO_3$-M199 supplemented with 1.0 mM sodium pyruvate, 2.0 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 4 mg/ml BSA and 25 ng/ml EGF) containing with 0.05 IU/ml recombinant human follicle stimulating hormone; rhFSH, (Organon, The Netherlands) for 24 h.

4.3.3 *In vitro* fertilization

In vitro fertilization was essentially performed as previously described (Sananmuang et al., 2010). After oocyte culture, the cumulus were partially removed by gently pipetting, and a group of 8 to 10 oocytes were cultured in 50-µl drops of IVF media (Tyrode's balanced salt solution containing 1% MEM non-essential amino acid (NEAA), 6 mg/ml BSA, 100 IU/ml penicillin, 30 µg/ml heparin and IVFx100) (Pope, 2004). The semen was collected from two fertile cats, and then frozen (Rota et al., 1997) with minor modifications. In brief, the cats were anesthetized with 0.04 mg/kg atropine sulphate (A.N.B. Laboratories, Bangkok, Thailand), 3 mg/kg xylazine hydrochloride (Laboratorios calier, Barcelona, Spain) and 10 mg/kg ketamine hydrochloride (Gedeon Richter, Budapest, Hungary). The semen was collected by electro-ejaculation (Zambelli and Cunto, 2006). The semen were cryopreserved by placing the straws horizontally 4 cm above liquid nitrogen vapors for 10 min and then plunged into liquid nitrogen. After thawing at 70˚C for 6 sec, the sperm were subjectively evaluated, and only sperm that had more than 50% progressive motility were used for the *in vitro* fertilization. The frozen-thawed sperm from 2 proven toms were washed twice. The sperm with a final concentration of 0.5 x 10^5 sperm/µl were co-incubated with matured oocytes in 50-µl drops for 18 h.

4.3.4 Embryo culture

After co-incubation with spermatozoa, cumulus cells were removed by gently pipetting. Presumptive zygotes were then washed and cultured in synthetic oviductal fluid (SOF) containing 4 mg/ml BSA, 100 µg/ml streptomycin and 100 IU/ml penicillin (IVC-1 media). After culture for 24 h in IVC-1 media, only cleaved embryos were then washed and cultured in IVC-2 media (SOF containing 10% (v/v) FCS (Gibco®, Invitrogen, CA, USA)). Media were overlaid with mineral oil and changed every two days. In all cases, *in vitro* culture was performed at 38.5°C in a humidified condition of 5% CO_2 in air.

4.3.5 *In vivo* produced embryos

Surgical collection of feline embryos was performed according to the Ethical committees of animal ethic, Faculty of Veterinary Science, Chulalongkorn University (accession no. 0831078). Four queens were induced their estorus cycles by intramuscular injection (i.m.) with 150 IU equine chorionic gonadotropin (eCG, Intervet-Schering Plough, Boxmeer, The Netherlands) (Figure 25). The response of eCG injection was checked by observing changes in estrus behavior, characterized by the continuous rubbing of head and neck against the objects, contant vocalizing, lordosis posturing and rolling (Figure 26A). After eCG injection for 96 h, the queens were received 150 IU human chorionic gonadotropin (hCG, Intervet-Schering Plough, Boxmeer, The Netherlands), i.m. to ensure the ovulation and simultaneously allowed to mate with a tom cat (Figure 26B). The natural mating was performed three times a day at 3-h intervals on the first and second days (day 0) after hCG injection (Figure 25). Ovulation was expected to occur 24 to 28 h after mating/hCG injection (Sojka et al., 1970). On day 7 post hCG injection, embryos were recovered from the oviduct and uterine horn by flushing with HM (Figure 27-28). The embryos classified as blastocysts (Figure 29A) were washed twice in PBS containing with 0.1% BSA and immediately stored at -80˚C in a minimum volume (less than 2 µl) into a 0.6 ml sterile eppendorf tubes for further analysis. Those not classified as blastocysts (Figure 29B) or undeveloped/fragmented embryos (Figure 29C) were discarded.

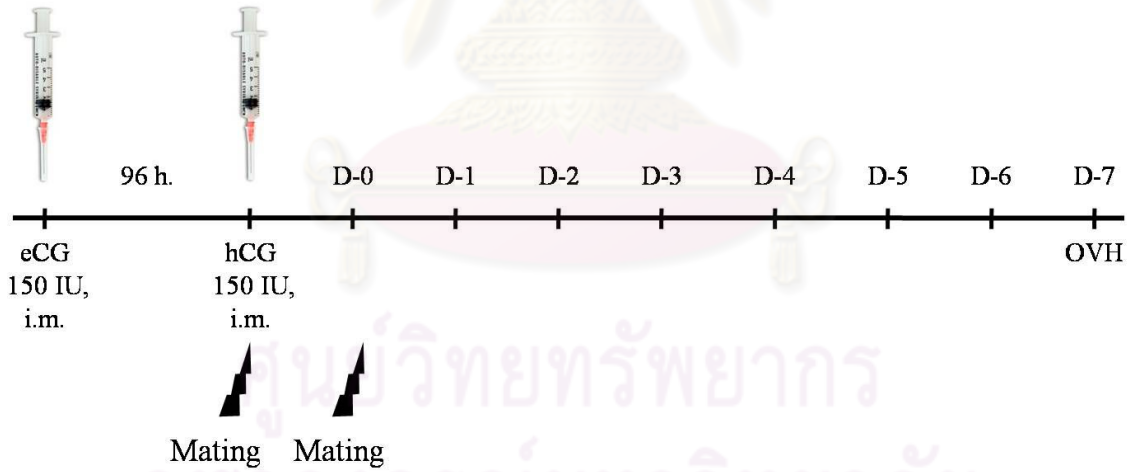

Figure 25. Schematic figure of treatment used for *in vivo* embryo production in domestic cats. The queens were administred with 150 IU eCG for estrus induction. Approximately 96 h after eCG injection, the ovulation was induced by 150 IU hCG injection and mating with cat. The embryos were recovered from the queens by ovariohysterectomy on day 7 after hCG injection.

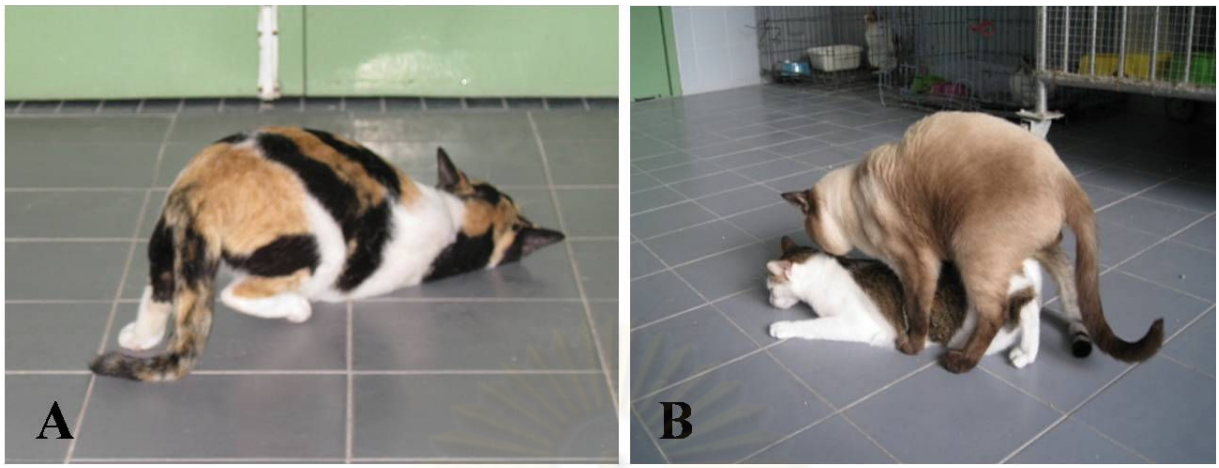

Figure 26. The response of eCG injection was checked by observing changes in estrus behavior, characterized by the continuous rubbing of the head and neck against the objects, constant vocalizing, lordosis posturing and rolling (A). At 96 h later, ovulation was induced by hCG injectin and mating with tom (B). Estrual queens were mated with tom three times per day on the first and (day 0) second days after hCG injection.

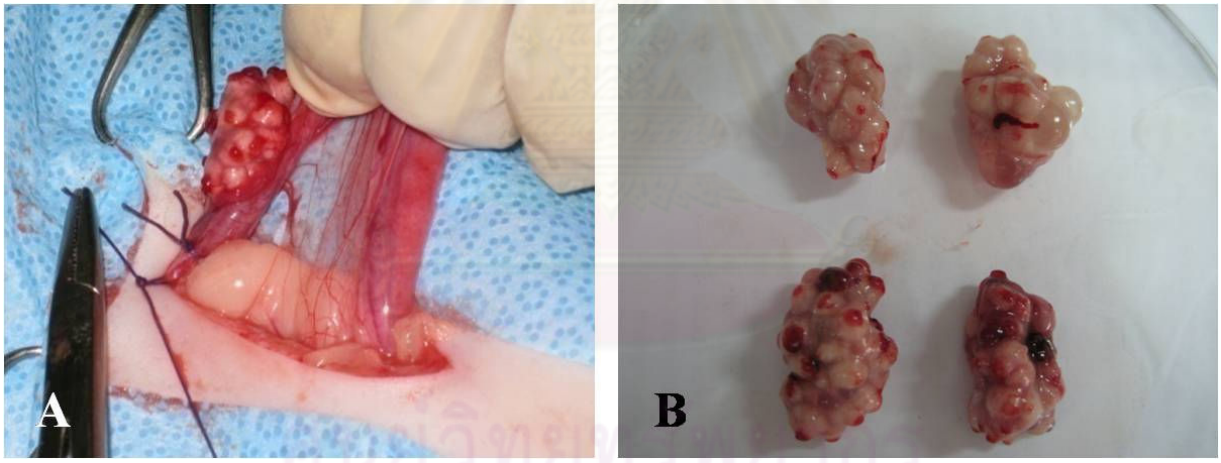

Figure 27. Ovaries and uterine horns were recovered from the queens by ovariohysterectomy (A). The ovaries were counted for the number of corpus luteum (CL) to estimate the number of ovulated oocytes (B).

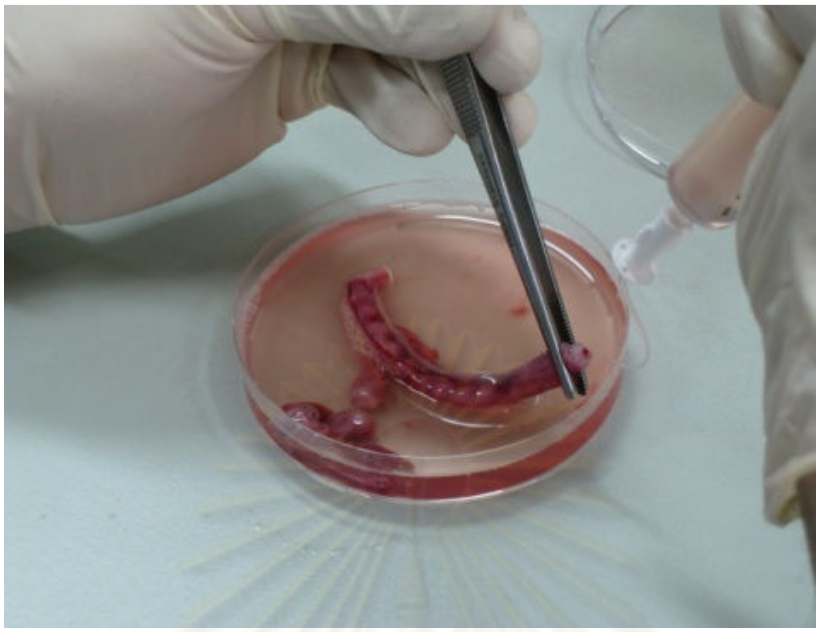

Figure 28. The embryos were recovered from the oviducts and uterine horns by flushing with HM. The embryos in the flushing medium were then searched under a light microscope.

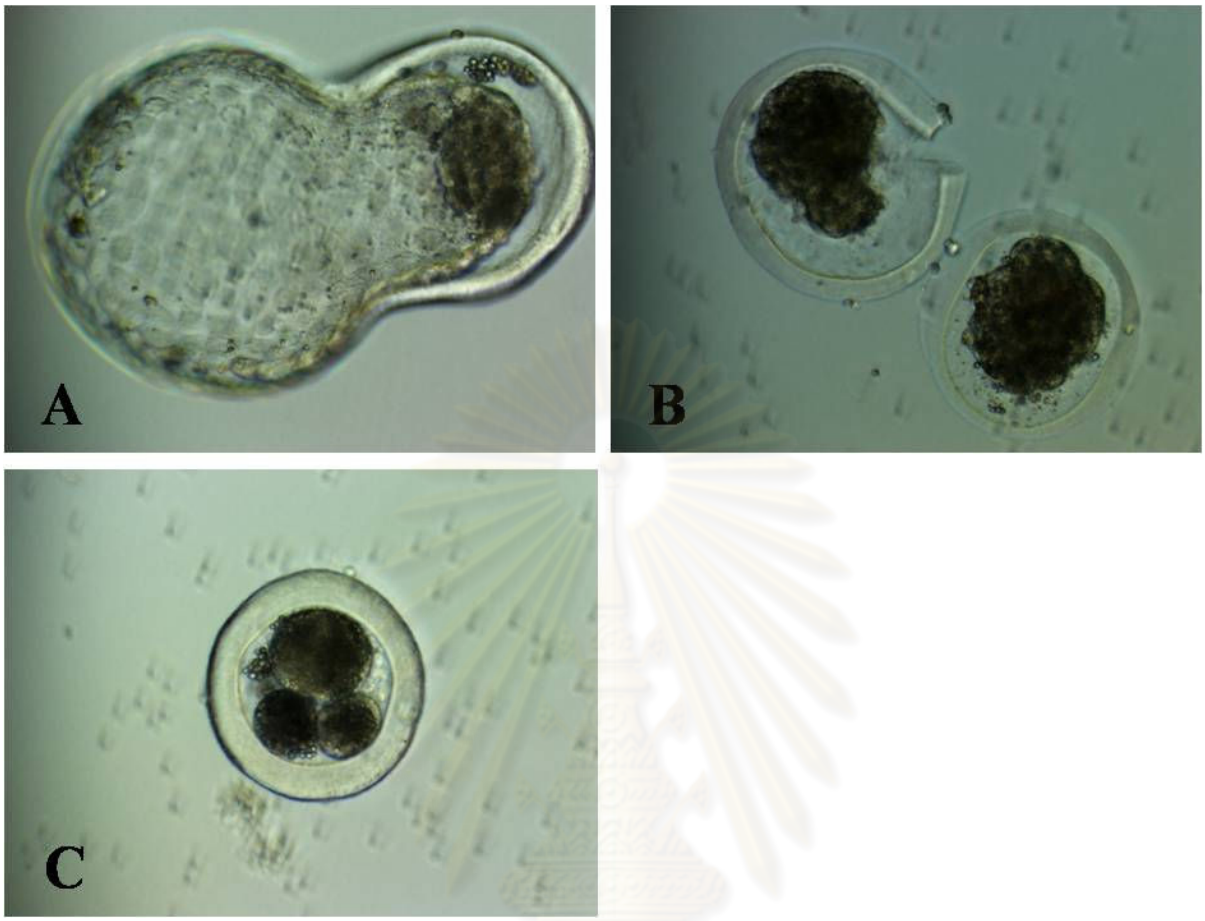

Figure 29. Day 7 cat embryos acquired from uterine flushing after performimg *in vivo* embryo production. The embryos classified as blastocysts on day 7 (A) were washed twice in PBS containing with 0.1% BSA and immediately stored at -80˚C in a minimum volume (less than 2 µl) into 0.6 ml eppendorf tubes. Those not classified as blastocysts on day 7 (B) or undeveloped or fragmented embryos (C) were discarded.

4.3.6 Assessment of embryo development

The percentage of cleaved embryos (2 to 16 cells), morula (≥16 cells without blastocele) and blastocysts (≥50 cells with blastocele formation) were evaluated on day 2, 5 and 7 of IVC, respectively. Morula and blastocyst rates were calculated relatively to the cleaved embryos, while hatching rates were calculated relatively to the number of blastocyst. To count the number of nuclei, blastocysts were fixed in 4% (w/v) paraformaldehyde and kept at 4˚C for 2 days before staining procedure. They were stained with 1 µg/ml 4',6-Diamidino-2-phenylindole (DAPI) in PBS at 37˚C for 10 min. Fragmentation was calculated relatively to the total embryonic cell nuclei under an epifluorescent microscope (BX51 Olympus, Shinjuku, Japan).

4.3.7 RNA extraction

Absolutely RNA Nanoprep Kit (Stratagene, San Diego, CA, USA) was used to extract total RNA from five blastocysts acquired from the same replication following the manufacturer's instruction at the room temperature. Briefly, blastocysts were lysed using lysis buffer containing with 0.7% (v/v) β-Mercaptoethanol (β-ME). Cell lysate was added with equal volume of 80% sulfolane. The mixture was then transferred to RNA-binding nano-spin cup and was centrifuged at ≥12,000 x g for 60 sec. The filtrate was discarded and re-seated spin cup was added with low-salt wash buffer. After centrifugation, the filtrate was discarded and the cup was re-seated and dried by centrifuging at ≥12,000 x g for 60 sec. DNase I (Stratagene, San Diego, CA, USA) was added onto the fiber matrix inside the cup and was incubated at 37˚C for 15 min. After incubation, the fiber matrix was washed once with high-salt washing buffer and twice with low-salt washing buffer. RNA was eluted with 12 µl RNase-free water by centrifugation (≥12,000 x g for 5 min). Extracted RNA was assessed the quality and quantity using spectrophotometer (Nanodrop ND-2000, Wilmington, Delaware, USA) and was immediately stored at -80˚C.

4.3.8 Reverse Transcription (RT)

RT reaction (20 µl) was performed using First-Strand cDNA Synthesis Kit (SuperScript III Kit, Invitrogen, Carlsbad, CA, USA) following the kit manufacturer's protocol. Initially, 8 µl of extracted RNA was incubated with 1 µl of random hexamers (50 ng/µl) and 1 µl of 10 mM dNTP mix at 65˚C for 5 min, then immediately placed on ice for at least 1 min. After chilling on ice, the reaction was then added with the mastermix containing 2 µl of 10xRT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT, 1 µl of RNaseOut (40U/µl) and 1 µl of SuperScript III RT (200 U/µl). The reaction was further incubated at 50˚C for 50 min followed by 85˚C for 5 min. RNA template from the cDNA:RNA hybrid molecule was removed by digestion with 1 µl of RNase H (2U/µl) after first-strand synthesis at 37˚C for 20 min. The product was stored at -20˚C for further use in PCR.

4.3.9 Quantitative Real-Time PCR

The mRNA transcript levels of individual target genes (HSP70, BAX and BCL-2) were normalized to the endogenous normalizer (GAPDH: Glyceraldehyde 3-phosphate dehydrogenase), and were run in separate wells. The PCR was performed using the ABI PRISM 7300 Real-time cycler (Applied Biosystems, Foster City, CA, USA) with Power SYBR Green PCR Master Mix (Applied Biosystems, WA, UK). Each PCR reaction (total volume of 20 µl) consisted of 2 µl of reverse transcription product (equivalent to 0.5 blastocyst) and 18 µl of reaction mixture which consisted of 10 µl of SYBR Green Master Mix, 1 µl of both 5 µM forward and reverse primer (Table 10) and 6 µl of nuclease free water. The primers were used according to the previous reference except for HSP70 primer. It was designed from heat shock protein 70 mRNA, complete cDNA of rat (Accession number: L16764) using the LightCycler Probe Design Software Version 2.0. HSP70 amplicon were purified with NucleoSpin® Extract II kits (Macherey-Nagel, Germany) and both strands were sequenced using the same primers. The

sequences derived from both strands were assembled using BioEdit Version7.0.8.0. Assembled sequences were then blasted in the NCBI gene bank to determine the similarity to other species. Thermal cycling conditions were as follows: 10 min at 95˚C to activate Tag DNA polymerase, 45 cycles of 15 sec at 95˚C for denaturing, 30 sec at 55˚C for annealing and 60 sec at 72 ˚C for extension. PCR products were confirmed by melting curve analysis and running in gel electrophoresis. The amplified products were run in 2% agarose gel (Bio-Rad, CA, USA) prepared in 1 x TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH8) containing 0.4 mg/ml ethidium bromide (Promega, WI, USA). The separated products in agarose gel were visualized under UV light (Syngene, CB, UK). Three sets of embryos were analyzed for each gene evaluation. All PCRs were duplicated for every transcripts of interest.

The Sequence Detection System (SDS) Software Version 1.4 (Applied Biosystems, USA) was used to analyze the relative quantitation (RQ) by comparative C_T method using the blastocysts derived from *in vivo* (IVV) as a control group. RQ was reported as relative n-times difference, in relation to the control sample. In brief, the ΔC_T was calculated from the target gene C_T (HSP70, BAX and BCL-2) subtracted with endogenous normalizer (GAPDH: Glyceraldehyde 3-phosphate dehydrogenase) C_T. $\Delta\Delta$Ct was formulated from $\Delta Ct_{\text{IVP embryo}} - \Delta Ct_{\text{IVV embryo}}$ and the RQ was calculated from $2^{-\Delta\Delta Ct}$.

Table 10. Description of forward (FP) and reverse (RP) primers used to assess expression of target genes in blastocysts acquired from varied culture conditions.

Genes	Sequence (5′-3′ orientation)	Fragment length (bp)	Genebank accession number or Reference
HSP70	FP: ATCCAGGTGTACGAGGG RP: TGGTGATCTTGTTGGCCT	190	L16764
BAX	FP: CCGATGGCAACTTCAACTGGG RP: GTCAGCACTCCCGCCACAAAG	244	(Zhao et al., 2008)
BCL-2	FP: GGAGGATTGTGGCCTTCT RP: GTTATCCTGGATCCAGGTGT	143	(Yamazaki et al., 2004)
GAPDH	FP: GGAGAAAGCTGCCAAATATG RP: CAGGAAATGAGCTTGACAAAGTGG	191	(Sano et al., 2005)

4.3.10 Statistical analysis

Three to four independent replicates were performed in each experiment. The data were expressed as a mean ± standard error (SE). Values were analyzed using one-way ANOVA statistical test and DUNCAN analysis for statistical difference among groups. Differences with a $P<0.05$ were considered statistical significance.

4.3.11 Experimental design

Experiment I: Effect of embryo density on developmental competence of cat embryos cultured in group or singly

Cat COCs were matured and fertilized *in vitro*. To evaluate the effect of embryo density, in Trial 1, 10 cleaved embryos were cultured in different culture volumes (12.5, 25, 50, 100 and 200 µl per drop) to acquire density of 1:1.25, 1:2.5, 1:5, 1:10 and 1:20. The developmental competence was determined by morula and blastocyst rates in relation to the number of cleaved embryos. Blastocysts at day 7 were fixed and stained with DNA labeling to count total cell numbers and fragmentation. In Trial 2, COCs were similarly matured and fertilized *in vitro* as described above; however, 1, 5 and 10 cleaved embryos were cultured in same culture volumes as Trial 1 to evaluate effect of single culture in comparison to group culture systems on embryo development.

Experiment II: Effect of embryo density on the transcripts of BAX, BCL-2 and HSP70 genes of cat blastocysts cultured in group or singly

In vivo produced blastocysts were used as control group for relative transcript analysis. To evaluate culture density's effect on gene expression, expanded blastocysts on day 7 derived from embryos cultured in the density of 1:1.25, 1:5 and 1:20 from Trial 1 were selected as the representative culture densities for relative transcript analysis. Effect of embryo number (single and group culture) on gene expression was determined, as well. Expanded blastocysts on day 7 derived from 1, 5 and 10 embryos cultured in 200 µl from Trial 2 were selected as the representative culture number for relative transcript analysis. The produced blastcysts were washed twice in PBS containing with 0.1% BSA. The pooled embryos (n=5) were then collected in a minimum volume (less than 2 µl) of washing into a 0.6 ml eppendorf tube. The samples were immediately stored at -80˚C until further use.

4.4 Results

4.4.1 Experiment I: Effect of embryo density on developmental competence of cat embryos cultured in group or singly

In Trial 1, cleavage rates of embryos used in the current experiment was approximately 50 to 70%. Morula rates were not different among culture densities (Table 11). Blastocyst rates acquired from culture density of 1:1.25 was significantly lower than those 1:5, 1:10 and 1:20 ($P<0.05$), while blastocyt rate of 1:2.5 was also significantly lower than those 1:10 and 1:20 ($P<0.05$). Hatching rates, blastocyst cell numbers and cell fragmentation were tended to improve when the culture density was less than 1:1.25 (Table 11 and Figure 30).

Table 11. Mean percent ± SE of developmental competence of cat embryos cultured in a group of ten in different medium volume / density

Culture volume (µl)	Culture density	N	Cleaved embryo developed to		Hatching	Cell number	Fragmented cells
			Morula	Blastocyst			
12.5	1:1.25	50	81.7±7.3[a]	15.0±10.4[a]	0.0±0.0[a]	69.1±42.7[a]	19.5±12.1[a]
25	1:2.5	50	80.0±7.6[a]	20.0±5.0[ab]	13.3±13.3[a]	180.1±41.8[b]	22.5±6.6[a]
50	1:5	60	97.2±1.5[a]	39.4±7.5[bc]	21.0±1.0[a]	140.5±15.4[ab]	14.3±1.4[a]
100	1:10	50	86.7±3.3[a]	48.3±1.7[c]	14.1±7.1[a]	149.2±14.9[ab]	14.4±1.5[a]
200	1:20	60	90.6±4.7[a]	43.3±3.3[c]	19.4±10.0[a]	152.2±28.0[ab]	14.3±1.2[a]

[a, b, c] Values within columns with different superscripts were significantly different ($P<0.05$). N = Total number of cleaved embryos. Culture density means 1 embryo: media volume (µl)

In Trial 2, cleavage rate of embryos used was approximately 50 to 70%. Single, five and ten cleaved embryos were cultured in various culture volumes according to the experimental design. Morula rate was similar among culture conditions (approximately 80 to 95%). Blastocyst rates of embryos cultured in a group of ten in the volumes of 50, 100 and 200 µl was significantly higher than those of 12.5 µl ($P<0.05$). No significant difference of blastocyst rate was observed in five and single embryo cultured among various culture volumes ($P>0.05$) (Figure 31A). Hatching rates, blastocyst cell numbers and cell fragmentation (data not shown) (Figure 31B) of 1, 5 and 10 embryos acquired among culture volumes was not significantly different ($P>0.05$). However cell fragmentation tended to increase in 5 and 10 embryos cultured in decreasing volume (Figure 31B). In comparison between group and single culture, embryos cultured in group tended to develop better than single cultured, especially in high culture volumes ($P>0.05$). At 200 µl, blastocysts derived from embryos cultured singly demonstrated significant high fragmentation rate compared with those cultured in group ($P<0.05$) (Figure 31B).

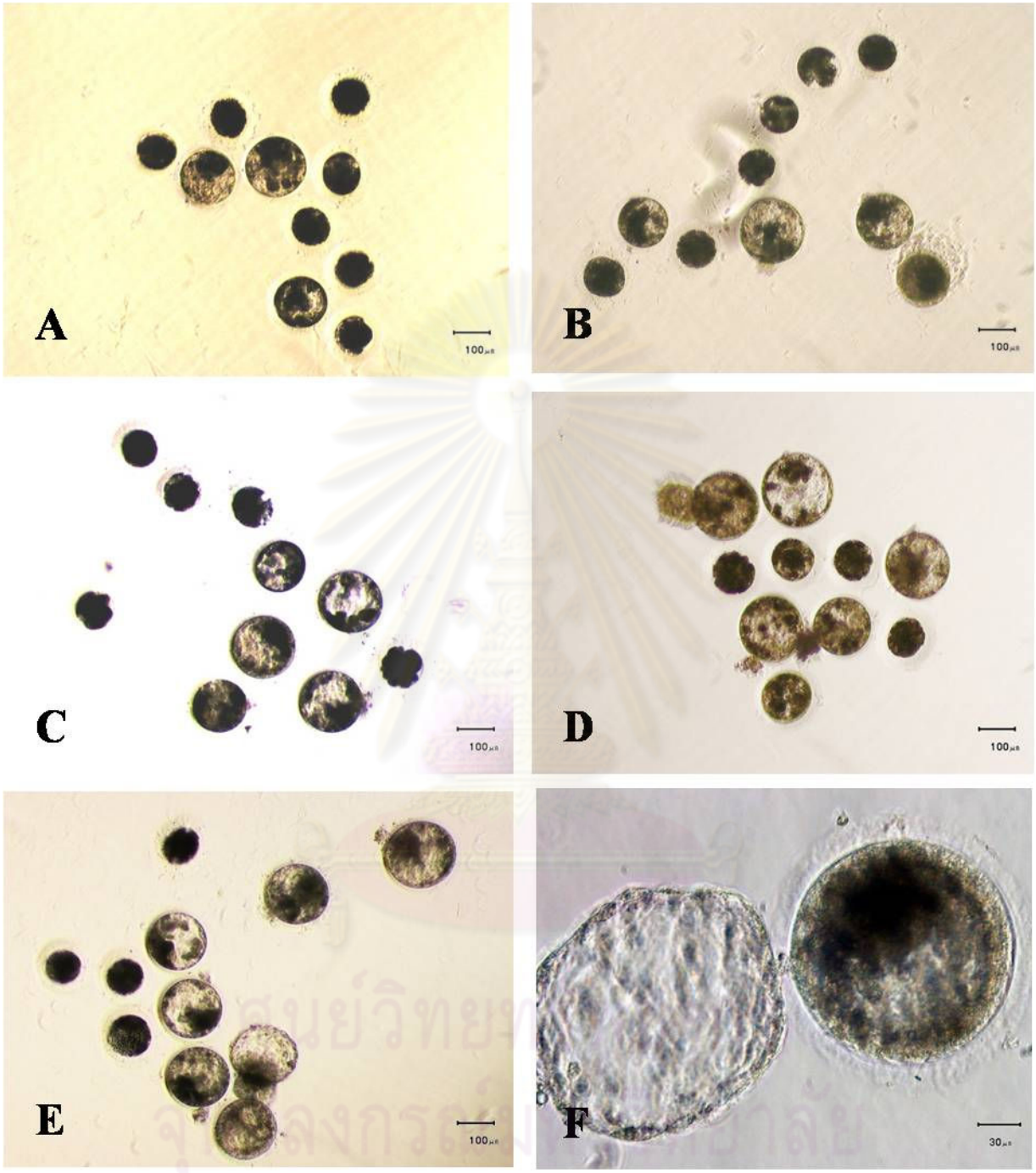

Figure 30. Day 7 cat embryos cultured in group of ten in different densities (10 embryos: medium volume). They were cultured in the density of 1:1.25 (A), 1:2.5 (B), 1:5 (C), 1:10 (D) and 1:20 (E). (F) Hatching blastocyst. Their developments were assessed under bright field visualization via light microscope (SMZ645 Nikon, Tokyo, Japan).

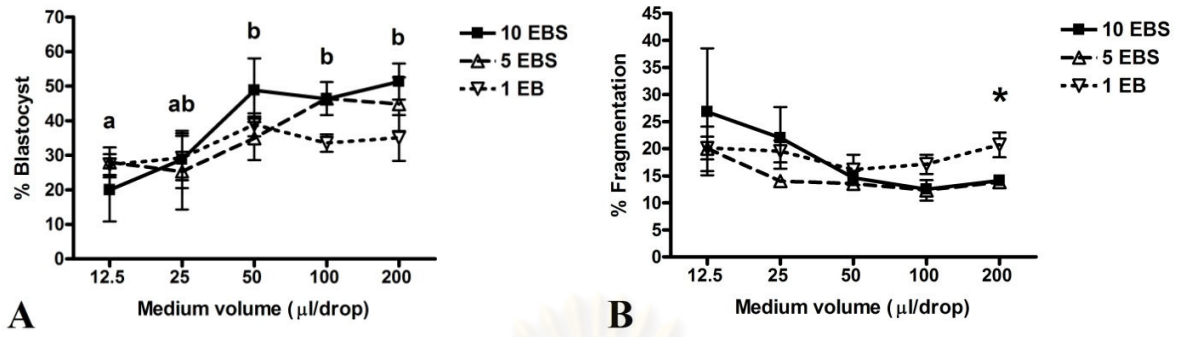

Figure 31. Developmental competence and morphology of cat embryos cultured in different numbers (1, 5 and 10 embryos) in different medium volumes (12.5, 25, 50, 100 and 200 µl). Percentage of blastocyst (A) and fragmentation of blastocyst cell nuclei (B) were compared among those cultured in different medium volumes. [a,b] Values with different superscripts were significantly different ($P<0.05$) within the different medium volumes of group culture embryos (10 EBS). An asterisk indicated the significant difference of fragmentation rate between group and single cultured embryos at 200 µl ($P<0.05$).

4.4.2 Effect of embryo density on the transcripts of BAX, BCL-2 and HSP70 genes of cat blastocysts cultured in group or singly

By applying a PCR-based approach, we partially determined the feline-specific mRNA sequences of HSP70 (Figure 32). High similarities (93 to 96% nucleotide identity) of HSP70 mRNA sequence were found between feline and other species (Table 12). This HSP70 designed primers were then used for quantification of expression levels of HSP70 genes feline blastocysts by real-time PCR. The melting curve analysis showed no primer-dimers or nonspecific products in all assays (Figure 33 to 36). The PCR product sizes of BAX, BCL-2 and HSP70 transcripts derived from *in vivo* produced blastocysts were confirmed correct amplicon sizes by running in the gel electrophoresis (Figure 37). As differences in development were observed after culturing the embryos in the density of 1:1.25, 1:5 and 1:20 (Experiment I, Trial 1), we selected only blastocysts derived from these densities for transcripts analysis of HSP70, BAX and BCL-2. The HSP70 (Figure 38A) and BAX (Figure 38B) transcripts of blastocysts cultured in the density of 1:1.25 were significantly higher than the others ($P<0.05$). Although BCL-2 transcripts (Figure 38B) did not significantly differ among all experimental groups ($P>0.05$), the BAX/BCL-2 ratio of blastocysts in 1:1.25 density was higher than the others (Figure 38C).

```
Query  1     TGGTGATCTTGTTGGCCTTGCCCGTGCTCTTGTCCGTGGCCGTGACGTTCAGGATGCCAT  60
             ||||||||||||||||||||| ||||||||||||||||||||||||||||||||||| |
Sbjct  1507  TGGTGATCTTGTTGGCCTTGCCGGTGCTCTTGTCCGTGGCCGTGACGTTCAGGATGCCGT  1448

Query  61    TGGCATCGATGTCGAAGGTCACCTCGATCTGGGGCACTCCCCGTGGG-GCCGGGGGGATG  119
             ||||  ||||||||||||||||||||||||||||||  ||||  ||||  |||||  ||||||
Sbjct  1447  TGGCGTCGATGTCGAAGGTCACCTCGATCTGGGGCACGCCCC-TGGGAGCCGGCGGGATG  1389

Query  120   CCGCTCAGCTCGAAGCGCCCCAGCAGGTTATTGTCCCGCGTCATGGCCCTCTCACCCTCG  179
             |||||||  |||||||||||||||||||  |||||  ||||||||||||||||  ||||||
Sbjct  1388  CCGCTCAACTCGAAGCGCCCCAGCAGGTTGTTGTCGCGCGTCATGGCCCTCTCGCCCTCG  1329

Query  180   TACACCTGGAT  190
             ||||||||||
Sbjct  1328  TACACCTGGAT  1318
```

Figure 32. The alignment between feline heat shock protein 70 (HSP70) mRNA, partial coding sequence (Query) and rat heat shock protein 70 (HSP70) mRNA, complete coding sequence (L16764.1) (Subject). The size of feline mRNA product was 190 bp and the position of the product on rat mRNA was from 1318 to 1507 bp.

Table 12. GenBank accession number and mRNA homology of HSP70 gene between cat and other species

Species	Gene Bank Accession Number	mRNA identity (%)
Rat (*Rattus norvegicus*)	L16764.1	94
Mouse (*Mus Musculus*)	M35021.1	93
Human (*Homo sapiens*)	DQ451402.1	93
Canine (*Canis lupus familiaris*)	AB114672.1	95
Bovine (*Bos Taurus*)	AY662497.1	96
Swine (*Sus scrofa*)	M69100.1	95
Buffalo (*Bubalus bubalis*)	EU099315.1	95

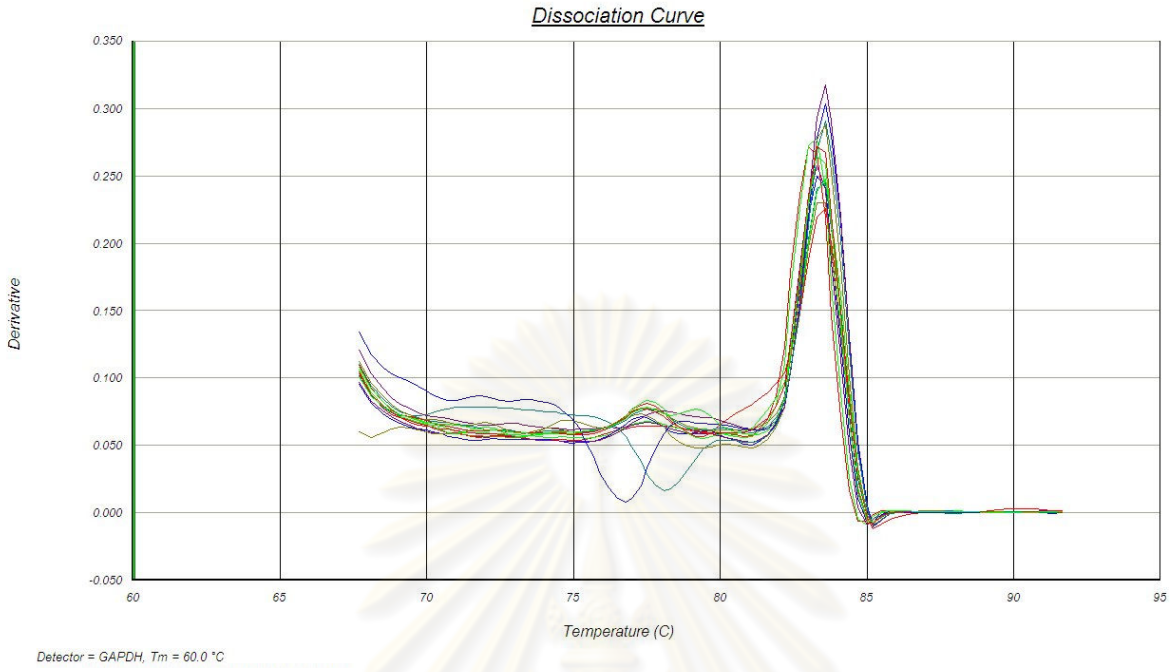

Figure 33. Melting curve analysis of GAPDH performed at the end of the real-time PCR. There were no significant primer-dimers or nonspecific products present on the curve.

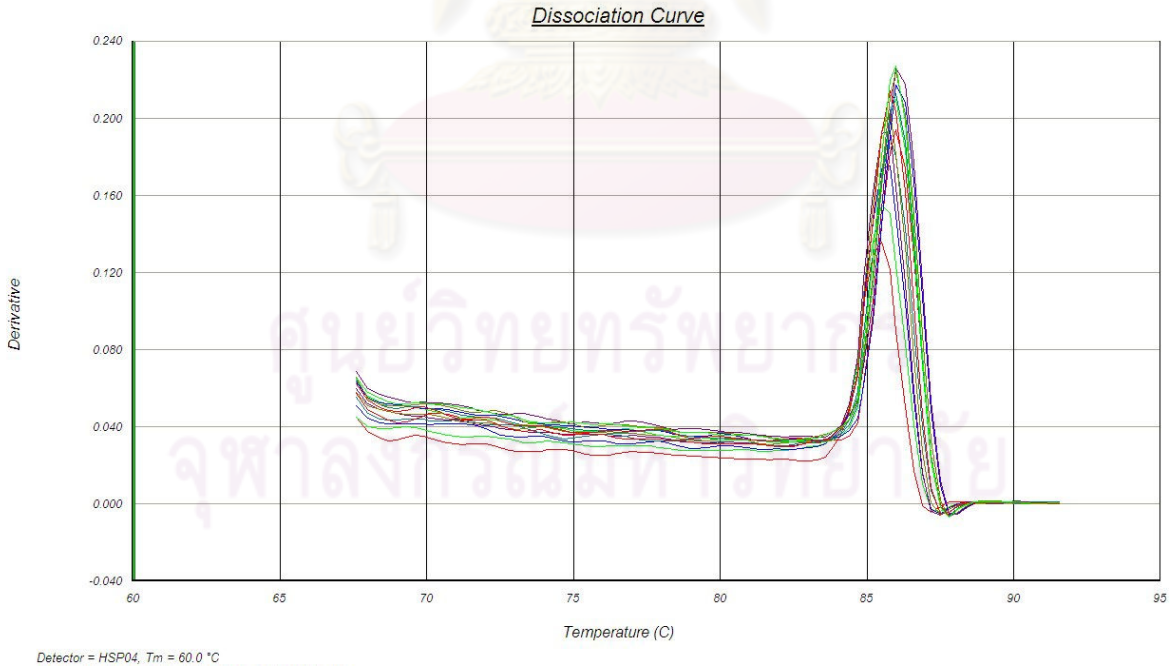

Figure 34. Melting curve analysis of HSP70 performed at the end of the real-time PCR. There were no significant primer-dimers or nonspecific products present on the curve.

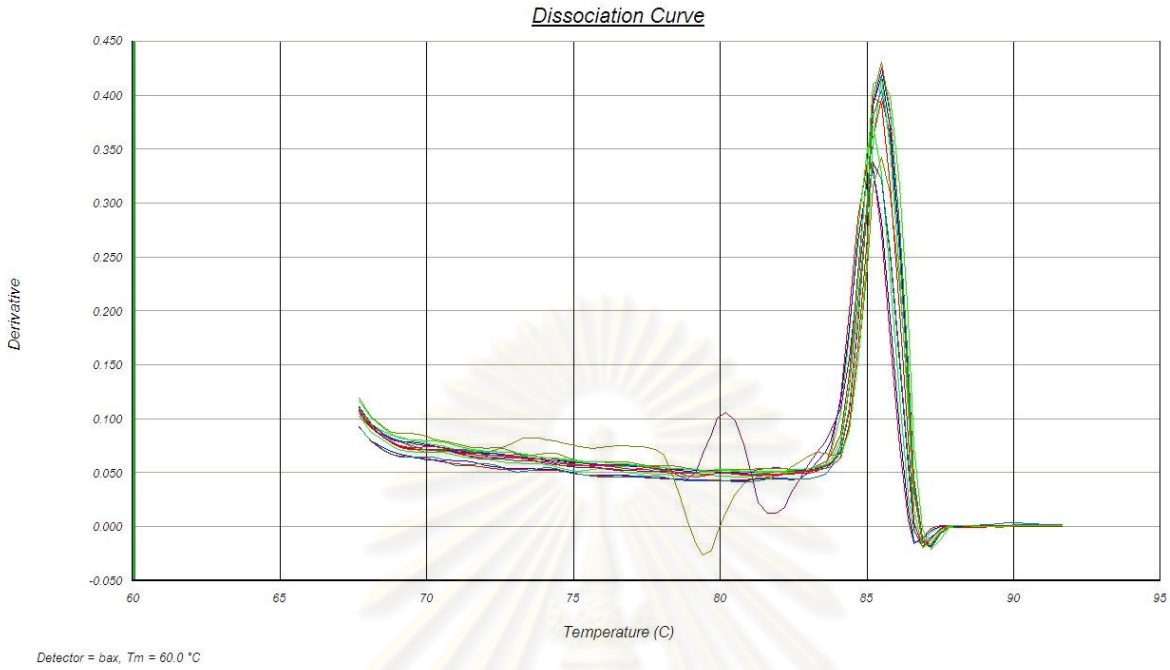

Figure 35. Melting curve analysis of BAX performed at the end of the real-time PCR. There were no significant primer-dimers or nonspecific products present on the curve.

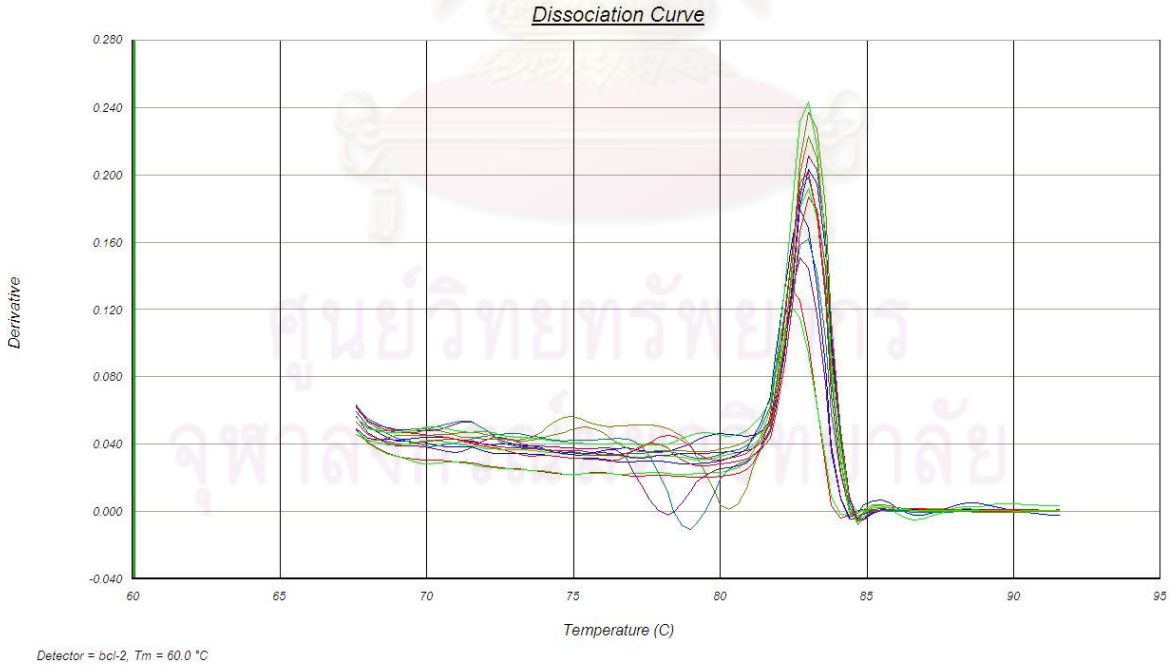

Figure 36. Melting curve analysis of BCL-2 performed at the end of the real-time PCR. There were no significant primer-dimers or nonspecific products present on the curve.

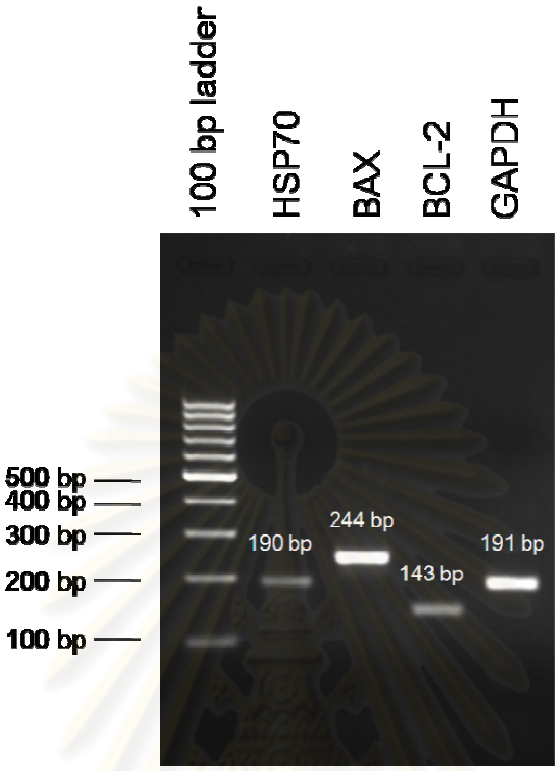

Figure 37. The amplicon sizes of PCR products (HSP70, BAX, BCL-2 and GAPDH) acquired from real-time PCR were confirmed their correct sizes by gel electrophoresis. They were run on 2% agarose gel contained with ethidium bromide in 1 x TBE buffer and were visualized under UV light.

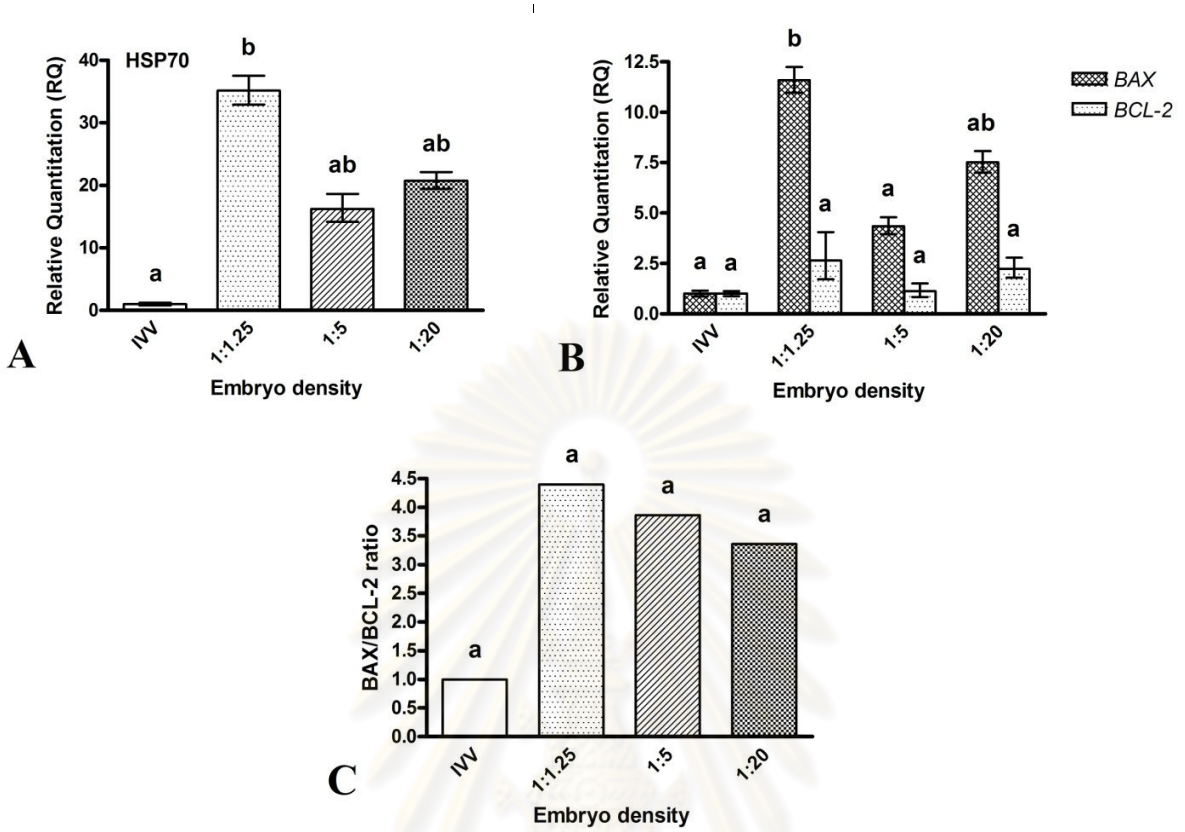

Figure 38. The comparison of specific transcripts of HSP70 (A), BAX and BCL-2 (B) and BAX/BCL-2 ratio (C) analyzed by real-time PCR in feline blastocysts produced *in vivo* (IVV) and *in vitro*. The *in vitro* derived blastocysts were obtained from groups of ten embryos cultured in the density of 1:1.25, 1:5 and 1:20. Mean values were obtained from three independent replicates. Bars indicated upper and lower range of values. ^{a,b} different superscripts denoted values that differ significantly ($P<0.05$).

Due to the significant difference of fragmentation of blastocyst cell nuclei after culturing the embryos with different numbers (1, 5 and 10 embryos) in 200 µl (Experiment I, Trial 2), we selected only those blastocysts derived from these groups for transcript analysis. HSP70 and BAX transcripts of *in vitro* produced blastocysts were significantly higher than those of *in vivo* ($P<0.05$) (Figure 39A-39B). However, they were not significantly different among *in vitro* blastocysts cultured in different numbers ($P>0.05$). On the other hand, no significant difference of BCL-2 transcripts (Figure 39B) and BAX:BCL-2 ratio (Figure 39C) was observed among the experimental groups ($P>0.05$).

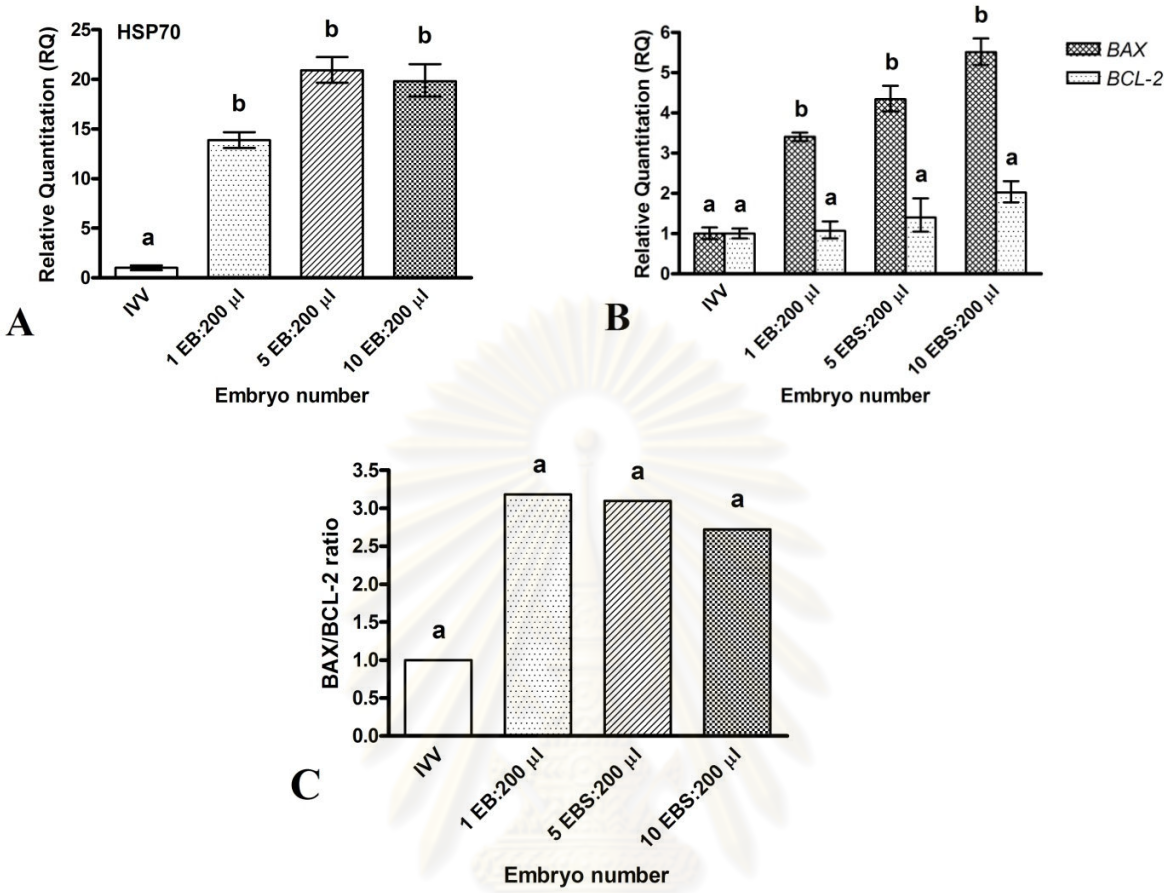

Figure 39. The comparison of specific transcripts analyzed by real-time PCR in feline blastocysts produced *in vivo* (IVV) and *in vitro* at different numbers (1, 5 and 10 embryos) in fixed volume (200 µl). Mean values were obtained from three independent replicates. Bars indicated upper and lower range of values. [a,b] different superscripts denoted values that differ significantly ($P<0.05$). EB = embryo

4.5 Discussion

In this study, embryo density and single culture condition affected developmental competence and gene expression of *in vitro* produced feline embryos. The effect of embryo density was mainly observed only in embryos cultured in group of ten. Effect of same densities in different embryo numbers was not determined in the study and should be regarded as limitation for our result's implication. Effect of single embryo culture condition was evaluated by culturing 1, 5 and 10 embryos in the different media volumes. Five and ten embryos were regarded as the control groups. Since the culture volumes were commonly used for feline embryo culture, only culture volumes but not densities were controlled in our culture condition and should be noted for our result's interpretation. High culture density reduced developmental competence and quality of feline embryos cultured in group of ten by decreasing blastocyst rate

but increasing cell nuclei fragmentation of blastocysts. On the other hand, various culture volumes in this study did not affect developmental competence in single cultured embryos. However its developmental competence was tended to be lower than that of group culture control (5 and 10 embryos cultured in groups). The relative transcript analysis indicated the dramatically increase of stress-response (HSP70) and pro-apoptotic (BAX) transcripts in blastocysts derived from ten embryos cultured in high density, while no difference was observed among those derived from embryos cultured individually or in groups at 200 ml culture volume.

The effect of embryo density on the embryo development was previously determined in other species. It could provide both positive and negative aspects on embryo development. The positive aspect related to embryo-derived factor stimulating the embryo development, while the negative one related to the accumulation of embryotoxic elements normally secreted from cell metabolism (Gardner et al., 1994; Bavister, 1995). In bovine, high embryos density negatively affected the embryo development and the expression of cellular marker (HSP70.1) (de Oliveira et al., 2005). In this study, high culture density (1:1.25) detrimentally affected developmental competences of group cultured feline embryos (ten embryos per drop) by reducing their blastocyst rate, but increasing their fragmentation (Table 11 and Figure 30-31). On the other hand, the effect of embryo density was not obviously different after culturing the embryos in group of 5 or 1 embryo (Figure 31). It had to be noted that culture volumes used for single culture were 10 times lesser in densities in comparison to ten embryo cultured in the same volumes. These tremendous differences possibly masked density's effects in single culture. However, embryos cultured in group of 5 tended to developed better in terms of blastocyst rate and the fragmentation of blastocyst cell nuclei after culturing in higher culture volumes, similar to the group of 10 embryos (Figure 31).

Besides the embryo density, embryo number (group and single culture) also affected the success of IVP in several species (Spindler et al., 2006). Previous studies in mice (Lane and Gardner, 1992), sheep (Gardner et al., 1994) and bovine (de Oliveira et al., 2005; Fujita et al., 2006) demonstrated developmental improvement of embryos cultured in group comparing with those cultured singly. Results from the study supported beneficial effects of group cultured embryo comparing to single cultured since embryos cultured in group tended to develop better than those cultured singly in high culture volume (Figure 31A).

The developmental incompetence of *in vitro* cultured embryos could be resulted from many factors. Poor blastocyst rate may not always correlate with the embryo' poor quality since there was no difference among the embryo morphology (Figure 30). Several methods have been developed to appraise embryo quality including gene expression analysis. Study of transcriptions can beneficially imply the metabolic quality of embryos with indiscriminate morphology and the response of the cell under the given condition (Wrenzycki et al., 2005). Good quality embryos usually express high level of survival promoting genes, but low level of apoptotic promoting genes (Lonergan et al., 2003b; Lonergan et al., 2003c). Alterations of these gene expressions could affect embryo viability and further gestation (Niemann and Wrenzycki, 2000; Niemann et al., 2002). As previously described, *in vitro* culture embryos usually faced

with the stress caused by improper culture environment including high culture density. Since data about transcriptional change in feline embryos cultured in high density is still limited, HSP70, BAX and BCL-2 genes were used as potential markers for assessing feline embryos quality correlating with their morphology. They also help determining the response of the cell to the culture condition which probably relating to the factor of developmental incompetency. As previously described, increase of HSP70 expression could protect cells from stress-induced molecular damage by facilitating cellular recovery (Santoro, 2000) and protecting them from BAX induced apoptosis (Arya et al., 2007). In comparison of HSP70 expressions acquired among different *in vitro* culture densities, the results suggested detrimental effect of high culture density (1:1.25) on embryos' stress (Figure 38A). Interestingly, this was co-related with poor morphology of these embryos, as well. Correlation between increase of DNA fragmentation and up-regulation of BAX and BAX/BCL-2 ratio of embryos cultured at this density (1:1.25) was also demonstrated in this study. Since the function of HSP70 on anti-apoptosis does not affect BAX expression (Figure 40) (Arya et al., 2007), embryos expressed high level of both HSP70 and BAX could indicate the sup-optimize cultured in high density. The result, thus suggested embryonic cell stress (Luft and Dix, 1999; Neuer et al., 1999) inducing apoptosis (Betts and King, 2001) due to high culture density. The stress in high culture density could be due to the oxidative stress which was one of many causes of stress triggering the expression of HSP70 (Arya et al., 2007). This stress could be caused from the excessive accumulation of embryotoxic elements such as radical oxygen species (Fujitani et al., 1997) and ammonium (Gardner and Lane, 1993) secreted from crowded embryos (O'Doherty et al., 1997; de Oliveira et al., 2005) or even an inadequacy of nutritional requirement and pH imbalance (Swain, 2010). Recently, there are the evidences suggesting the role of reactive oxygen species (ROS) generated from the cells in the initiation of apoptotic cell death (Kane et al., 1993; Guerin et al., 2001) which is responsible for embryo fragmentation and related embryonic loss (Yang and Rajamahendran, 2002). Due to these reasons, morphological and molecular evidences acquired from this study partially supported the contribution of high culture density to stress induced apoptosis of *in vitro* cultured feline embryos. However other techniques to determine embryo quality and apoptosis should be included to further confirm this implication.

To determine the mechanisms underlying the developmental incompetence of single culture embryos, transcripts of stress and apoptosis genes were evaluated among different numbers of cultured embryos in fixed highest media volume (200 μl) to minimize the effects of density. The acquired results revealed no difference in transcripts of stress and apoptotic-related genes (Figure 39A-C). The data implied that developmental incompetence of embryos cultured singly was not due to stress like those cultured at high density. One possible cause of their developmental incompetence was the lack of paracrine factors related to growth (Kaye and Harvey, 1995; Matsui et al., 1995; Herrler et al., 1998). To prove this hypothesis, further study related to the effect of growth factor supplementation on single embryo culture should be performed.

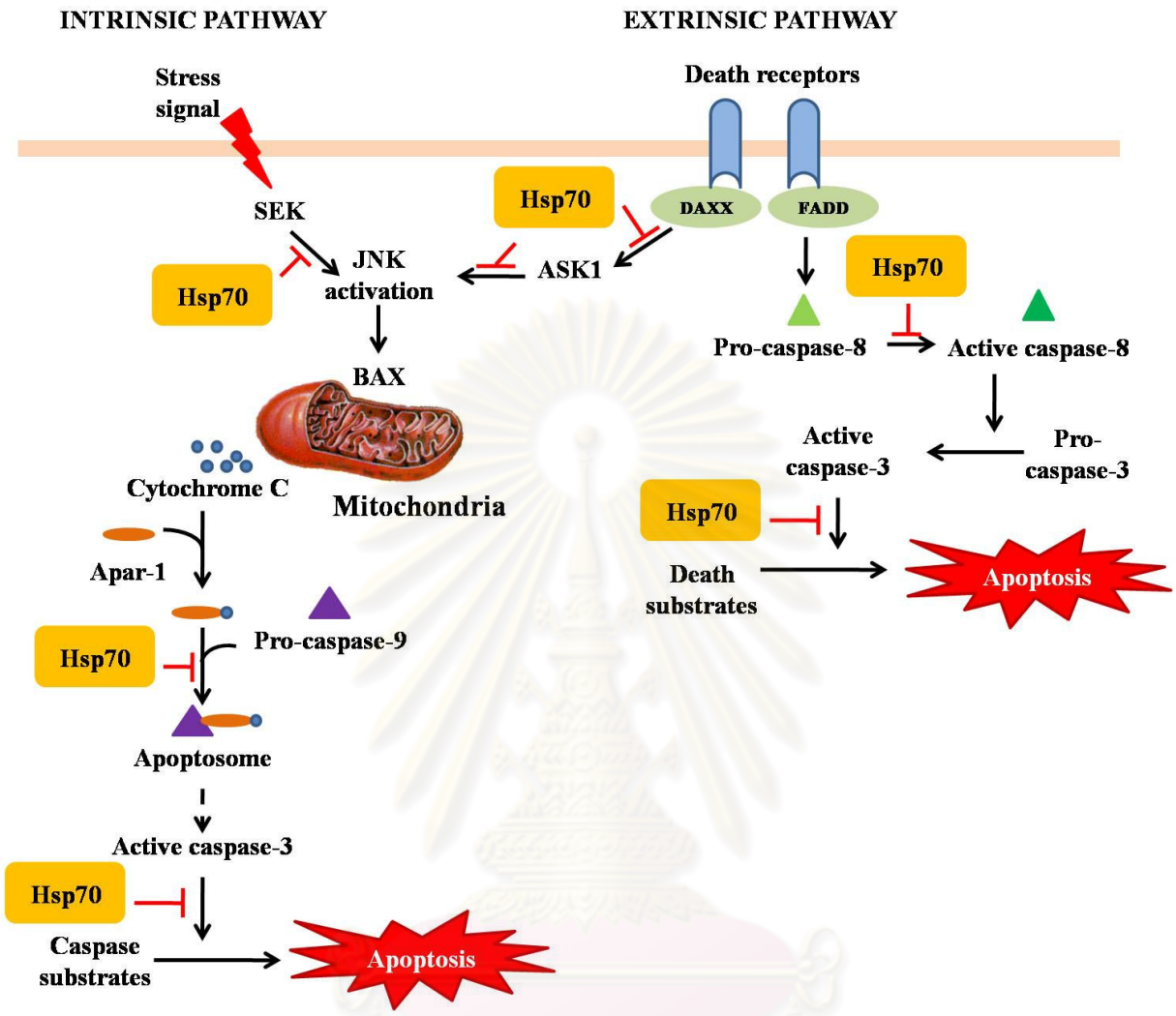

Figure 40. HSP70 (yellow boxes) is generally anti-apoptotic since it inhibits (red lines) many steps in the apoptotic pathways. These include inhibition of translocation of BAX into mitochondria, release of Cytochrome C from mitochondria, formation of apoptosome and inhibition of activation of initiator caspases. HSP70 also modulates other signaling pathways in the apoptotic cascade (modified from Arya et al., 2007).

Unequal quality between *in vivo* and *in vitro* derived feline embryos was demonstrated in this study (Yang and Rajamahendran, 2002). *In vitro* culture embryos express more HSP70 than those acquired from *in vivo* control. Moreover, BAX up-regulation related with increasing DNA fragmentation was also demonstrated (Table 11 and Figure 38B-38C). Increase of BAX expression related with high DNA fragmentation rate of *in vitro* derived embryonic cells comparing with those of *in vivo* were demonstrated in several species (Long et al., 1998; Melka et al., 2009). These evidences supported the idea that BAX up-regulation was a consequence of inappropriate *in vitro* culture condition and at least partially responsible for the death of embryos

(Lonergan et al., 2003b; Melka et al., 2009). According to the study, this suggested the sup-optimize culture condition, irrespective the culture density.

In conclusion, effects of density and number of embryos in culture condition on their developmental competence were clearly demonstrated by both morphology and gene expressions. Embryos' stress inducing apoptosis appeared to be responsible for incompetency at high culture density. Accumulation of toxic substances might be the cause of the stress. Incompatible transcripts of *in vivo* and *in vitro* produced embryos also implied us several other factors affecting development of embryos. Even though the low culture density could improve the developmental competence of cultured embryos, the expressions of these stress and apoptotic related genes were still higher than those produced *in vivo*. This seems to be the important factor caused the limited success of IVP system. Of note, this study demonstrated embryos with same morphology but different qualities due to their gene expressions. Therefore, observation of both morphological and molecular changes should be considered together for potential optimization of culture condition in feline species.

CHAPTER V

GENERAL DISCUSSION AND CONCLUSION

Current strategies: Optimization of *in vitro* embryo production in domestic cat

Nowadays, *in vitro* embryo production (IVP) has become an important technology for wild life conservation. The technique is commonly applied for animal propagation and numerous researches about animal reproduction and embryology. In felidae family, most of 36 wild species are threatened and nearly extincted (Farstad, 2000). The development of IVP in these species is then remarkably progressed. Due to the limited availability and accessibility of gametes acquired from wild cats, domestic cat is commomly used as a model for wild cats study (Goodrowe et al., 2000).

Even though the success of this technology has been reported in domestic cat by giving birth of kittens (Gomez et al., 2000), its efficiency is still low by which only 30 to 40% of oocytes can develop to blastocysts (Farstad, 2000). There are several factors contributing to the differences beween *in vivo* and *in vitro* produced embryos and thus influencing on IVP efficiency and success. These differences involve both morphological and molecular aspects which can influence on not only embryo survival rate but also phenotypic disorder of fetuses and offspring (Wrenzycki et al., 2007). Initial oocyte quality, follicular environment and culture environment are considered as main factors responsible for these aberrant outcomes (Pope et al., 2006b). Although mammalian embryos have a great plasticity allowing them to survive *in vitro*, they usually have low quality and viability in *in vitro* environment (Lane, 2001). These are due to incompatibility between *in vitro* and *in vivo* environment resulting in the altered morphology and gene expression of the produced embryos (Wrenzycki et al., 1996; Wrenzycki et al., 2005). In persuing of these unrelenting researches, our study continued to optimize *in vitro* environment for feline IVP. The study demonstrated the effect of culture environment on the success of IVP in domestic cat in terms of developmental competence and quality of embryos evaluated from phenotypic and molecular changes. We expected that the knowledges acquired from this study could provide the valuable data on how the embryo response to the provided culture condition, and thus help optimizing suitable culture condition for wild feline embryos in the future.

Decreasing of developmental competence of *in vitro* matured oocytes compared with *in vivo* matured oocytes has been known as one of the important factors resulting in the failure of IVP in feline specie (Farstad, 2000; Roth et al., 1994). During oocyte growth *in vivo*, follicular environment offers the suitable condition to complete the maturation process. In contrast, incomplete of cytoplasmic maturation or asynchronization between nuclear and cytoplasmic maturation usully occur in oocytes matured *in vitro* (Gomez et al., 2000; Bogliolo et al., 2004). These events impair not only ability of oocyte to undergo meiosis resumption but also ability to complete the fertilization process and development to blastocyst stage (Ajduk et al., 2008). Several methods including addition of particular chemical substances in culture condition are

invented to improve developmental capacity of *in vitro* matured oocytes. Roscovitine, a potent cyclin-dependent inhibitor of the MPF activity has been applied for this purpose in several species (Mermillod et al., 2000; Hashimoto et al., 2002; Schoevers et al., 2005). The effect of roscovitine was previously demonstrated as species-specific; therefore, this study aimed to evaluate roscovitine's effect on *in vitro* feline oocyte maturation. Since oocyte quality is a crucial factor for the success of fertilization and embryo development (Wood and Wildt, 1997), we examined effects of roscovitine on both good (Grade I) and poor quality oocytes (Grade II to III). The study exhibited that the ability of roscovitine to arrest meiotic resumption at GV-stage oocytes was dose-dependent manner without detrimental effect on meiotic resumption after culturing in IVM medium. However, it did not improve meiotic competence of poor quality COCs. Moreover, both good and poor quality of feline oocytes pretreated with roscovitine for 24 h prior to IVM demonstrated poorer developmental competence by means of cleavage, morula and blastocyst formation than non–roscovitine-treated control groups. These results contrasted with some previous reports that roscovitine did not impair subsequent development of goat and bovine oocytes (Mermillod et al., 2000; Han et al., 2006). The result thus supported the species specific effect of roscovitine. Detrimental effect of roscovitine was linked to its negative effect on cumulus cells viability. Since high concentration of roscovitine contributed to the high incidence of cumulus cell apoptosis, it was a most likely cause of poor embryo development after roscovitine treatment observed in this study. However, there are other factors influencing on the potency of roscovitine on the developmental capacity of oocytes such as incubation time and culture conditions (Hashimoto et al., 2002; Han et al., 2006).

Commonly, proportion of embryos developing to the blastocyst stage can range from 40% to ≥ 60% of *in vitro* cultured embryos (Freistedt et al., 2001; Karja et al., 2002; Gomez et al., 2003). Several types of culture media have been developed for feline embryo culture. These media are usually formulated and contained with nutrients to meet embryo requirements. However, they are still not precisely optimized and can cause molecular and phenotypic alterations in embryos, fetus and neonates (Farin et al., 2001; Lonergan et al., 2006). Common media used in feline embryo culture are synthetic oviduct fluid (SOF) (Freistedt et al., 2001), modified Earle's balanced salt solution supplemented (MK-1) (Kanda et al., 1998; Karja et al., 2002) and modified Tyrode's balanced salt solution (Gomez et al., 2003). Since the culture system and media components are usually varied among laboratory, it is difficult to compare each medium's efficiency to support feline embryo development. To establish effective IVP system for feline in our lab, we determined efficiency of each medium type using identical culture environment. The study demonstrated that SOF and MK-1 supported feline embryo development better than modified Tyrode's solution. Difference of medium compositions should be one of important factors contributed to the difference of their efficacy. High glucose concentration in modified Tyrode's solution seemed to be the cause of detrimental effect on feline embryo development. This was partially supported by negative effect of increasing glucose concentration in SOF (6.0 mM) which was comparable to glucose concentration in modified Tyrode's solution (5.5 mM) on blastocyst rate and fragmented nuclei of blastocysts.

Although the mechanism underlying poor embryo development under the high glucose condition is still unclear, it has been postulated that this condition may induce oxidative stress or down regulate the glucose uptake into cells resulting in insufficient metabolic requirements (Doblado and Moley, 2007). Besides the factors mentioned above, low concentration of sodium bicarbonate in modified Tyrode's solution (11.9 mM) as compared to SOF and MK-1 (25 mM) might contribute to its poor nourishment for embryos. Bicarbonate plays an important role in balancing of buffer system. Imbalance of bicarbonate/CO_2 buffer system might occur in low bicarbonate culture condition and result in aberrant embryo's metabolisms and development (Swain, 2010). Lacking of several amino acids in IVC-1 medium of modified Tyrode's solution in comparison to SOF and MK-1 might be the other factor contributing to less protective ability of produced embryos from occurring oxidative stress (Swain, 2010).

Not only culture media, embryo density but numbers also affect developmental competence of *in vitro* produced embryos (de Oliveira et al., 2005; Gardner et al., 1994; O'Doherty et al., 1997; Paria and Dey, 1990). Generally, wild cat's gametes obtained from alive or unexpected die animals have low numbers and quality (Pope et al., 2006a). Therefore, the limited number of gametes is considered as crucial obstacle for IVP application in real wild cat. The effect of embryo density was previously reported in several species (de Oliveira et al., 2005; Gardner et al., 1994; O'Doherty et al., 1997; Paria and Dey, 1990). Most of studies indicated that embryos cultured in group could develop better than those cultured individually (Paria and Dey, 1990; Lane and Gardner, 1992; Ferry et al., 1994; Keefer et al., 1994; Donnay et al., 1997; O'Doherty et al., 1997; Spindler and Wildt, 2002). Moreover, high number of cultured embryos could develop better than low number in bovine species (de Oliveira et al., 2005). In domestic cat, the effect of embryo density and number on IVP success was also implied in heterospecific companion (Spindler et al., 2006) and conspecific companions embryos's co-culture (Spindler and Wildt, 2002). However, the direct study about these factors in feline species is still limited making the difficulty for optimizing the culture system for small number embryo's culture. According to this, we then examined the effect of embryo density (culture volume: embryo numbers) on developmental competence of embryos cultured in group and singly.

Our results revealed that high culture densities (1:1.25 and 1:2.5) detrimentally affected developmental competence and quality of group cultured feline embryos. Decrease of blastocyst rates, but increase of fragmentation were presented in these groups. This may probably due to the accumulation of toxic substances normally secreted from the metobolism of the cells. The excessive level of these toxic substances has been postulated as the cause of embryonic stress and induces apoptosis in the cell (O'Doherty et al., 1997; Lonergan et al., 2003b; de Oliveira et al., 2005; Melka et al., 2009). Supporting previous studies (Spindler et al., 2006), culturing embryos in group tended to improve the developmental competence than single culture in this study. This suggested the beneficial effect of group culture system. A possible cause of developmental incompetence in single cultured embryos was insufficiency of paracrine factors related to growth (Kaye and Harvey, 1995; Matsui et al., 1995; Herrler et al., 1998). To find out

the mechanisms underlying these morphological results, we then further study them at molecular levels.

Future direction: Application of gene expression as a tool for culture optimization

Healthy embryo is a crucial factor for successful establishment and maintenance of pregnancy following embryo transfer. Although the ultimate test of embryo quality is establishment of pregnancy after embryo transfer, this procedure is time-consuming and depends on several factors (Wrenzycki et al., 2007). Though embryo quality is primarily implied by morphology, only this criterion is insufficient. Since the incidence of "large offspring syndrome (LOS)" has been occured after transfer the *in vitro* produced embryos (Walker et al., 1996; Kruip et al., 1997), detection of gene expression pattern has been introduced as additional criterions for embryo quality's determination along with the morphology (Rizos et al., 2002; Wrenzycki et al., 2007). Culture media, culture conditions as well as the production procedure can affect many steps during embryo's development such as maturation, fertilization, timing of first cleavage, activation of the embryonic genome, compaction, blastocyst formation and also the genetic program of produced embryos (Sagirkaya et al., 2006; Niemann and Wrenzycki, 2000; Niemann et al., 2002). These perturbations can possibly result in decrease of blastocyst quality, and even viability of offspring born after transfer (Wrenzycki et al., 2007). Several gene transcripts associated with embryo quality are used to evaluate the culture conditions (Wrenzycki et al., 2005). However, most of these data in feline are still lacking. In this study, gene expression was applied as a tool for feline embryo evaluation cultured in the specific provided conditions. Hereby, we aimed to apply it as a criterion for feline embryo quality assessment and also to understand the response of embryo to given culture condition

Gene expression was applied to evaluate the quality of blastocysts derived from embryos cultured in different glucose concentrations (Chapter III). Since each glucose concentration in the culture media resulted in varied blastocyst rates and percentages of fragmented cells; changes of gene expression associated with cell apoptosis were expected. In this study, blastocysts with the same morphology exhibited different gene expression patterns. The expression of BCL-2 in survived blastocysts cultured in highly concentrated glucose (6 mM) culture condition tended to be higher than those of lower concentrated groups (1.5 and 3 mM glucose). Increase of BCL-2 expression could partially indicate the mechanism by which the cells become resistant to apoptosis (Abu-El-Asrar et al., 2004) in high glucose condition. However, there was no difference of BAX and GLUT-1 expressions among the blastocysts cultured in varied concentrations of the glucose. The result, thus contradicted to previous studies in which high glucose condition up-regulated BAX in human blastocysts (Moley et al., 1998a) and down-regulated glucose transport in murine blastocysts (Chi et al., 2000). A possible explanation is the concentration of glucose used in this study was far less than many previous studies (Moley et al., 1998a), and thus may not efficiently stimulate BAX and GLUT-1 expressions. Another explanation was the nature of BAX response appeared to be tissue-, cell-, stage- and species-

specific (Evan and Littlewood, 1998; Kockx and Knaapen, 2000; Giam et al., 2008). Unequal response and expressions of GLUTs family's members in various stages of embryos were also demonstrated in other species (Moley et al., 1998b; Augustin et al., 2001; Lequarre et al., 1997; Wrenzycki et al., 1998; Augustin et al., 2001). Since the study about BAX and GLUT1 expressions in feline embryos was still limited, final conclusion for this is still unknown. According to the morphological and molecular changes in embryos cultured in difference glucose concentrations, high glucose concentration in culture media seemed to be one of many factors detrimentally affected on embryo development. The evidence, thus partially supported the detrimental effect of high glucose concentration in modified Tyrode's solution on feline embryo development.

The effects of density and number of feline embryos in culture condition on their developmental competence were clearly demonstrated by both morphology and gene expression in this study (Chapter IV). Since the up-regulation of HSP70, BAX and BAX/BCL-2 ratio in high density (1:1.25) implied embryos' stress inducing apoptosis in this culture condition, accumulation of embryotoxic substances such as radical oxygen species (Fujitani et al., 1997) and ammonium (Gardner and Lane, 1993) secreted from crowded embryos (O'Doherty et al., 1997; de Oliveira et al., 2005) may be the cause of the stress. Correlation between BAX up-regulation relating with increasing DNA fragmentation of feline embryos was clearly demonstrated in the study.

Apart from the density, embryo number's effect was also determined in this study. Interestingly, the acquired result implied that developmental incompetence of embryos cultured singly seemed not due to the same stress presented in group culture at high density. Since there was no difference of HSP70, BCL-2 and BAX expressions among the blastocysts derived from embryos cultured in 1, 5 and 10 in fixed volume (200 µl) of SOF. Supporting previous studies in other species, insufficiency of paracrine secretion was likely to be major cause of developmental incompetence of single embryo culture (Kaye and Harvey, 1995; Matsui et al., 1995; Herrler et al., 1998). To prove this hypothesis, further study related to the effect of growth factor supplementation on single embryo culture should be performed.

Incompatible transcripts of *in vivo* and *in vitro* produced embryos also implied us unequal quality between *in vivo* and *in vitro* derived feline embryos. Even though *in vitro* low culture density could improve the embryo's developmental competence, the expressions of stress and apoptotic related genes were still much higher than those of *in vivo*. This incompatibility may be a cause of limited success of feline IVP. Of note, this study demonstrated embryos with same morphology but different qualities due to their gene expressions. Therefore, observation of both morphological and molecular changes should be considered together for potential optimization of culture condition in feline species.

Conclusion

In vitro embryo production of feline has been greatly progressed during the past few decades. Although the culture system has been markedly improved, low embryo survival rate after embryo transfer in terms of low nomal pregnancy rates of recipients and in fewer kittens born is still occured. This strongly reflects the incompatibility between *in vivo* and *in vitro* culture environment. To improve in *vitro* embryo's quality, new techniques and knowledges are continuously introduced in feline IVP system. In this study, effects of roscovitine (cyclin-dependent inhibitor of the MPF activity); culture medium types/components; culture density and number were observed and studied. The acquired results introduced us a numbers of factors that could affect developmental competence of produced embryos during IVP. These, therefore confirmed the need of further optimization of feline IVP. Beside morphological evaluation, this study also included gene expression analysis of cultured feline embryos. Interestingly, the study demonstrated the benefit of the assay to imply the quality and its underlying intracellular metabolism of the produced embryos. According to all mentioned above, we introduced a combination of morphological and molecular evaluation as a method of choice for optimization of feline IVP system in the future.

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APPENDICES

APPENDIX A

All media were prepared using MiliQ water, otherwise indicated. The pH of all media was adjusted to 7.2-7.4 for holding medium and 7.6-7.8 for culture media; the osmolarity was 285-295 mOsm/kg for culture medium. All media were sterilized by filtration immediately after preparation then aliquoted and stored at 4 ˚C or -20 ˚C

Stock solution

FSH	100 IU/ml diluent
EGF	25 ng/µl PBS+0.1%BSA
hCG	300 IU/ml diluent
eCG	300 IU/ml diluent
DAPI	0.1 mg/ml PBS

Oocyte holding medium

Hepes	5.985 g/l
Medium M-199 (M3769: Sigma)	1 bottle/l
BSA	4 mg/ml
L-glutamine	0.292 g/l
1 M Sodium pyruvate	1 ml/l
Penicillin	100 IU/ml
Streptomycin	100 µg/ml
Phenol red	500 µl/l

Oocyte maturation medium (prepared in HCO_3 buffered M-199)

1 M Sodiun pyruvate	1 µl/ml
BSA	4 mg/ml
L-glutamine	0.000292 g/ml
Penicillin	100 IU/ml
Streptomycin	100 µg/ml
rhFSH	0.05 IU/ml
EGF	25 ng/ml

***In vitro* fertilization medium (prepared in Tyrode's balanced salt solution)**

BSA	6 mg/l
NEAA	1% v/v
IVF X 100 stock	1% v/v
Penicillin	100 IU/ml
Streptomycin	100 µg/ml
Heparin	30 µg/ml

IVFx100 stock: 100 mM L-glutamine, 36.3 mM Sodium Pyruvate and 110.89 mM Calcium Lactate were solubilized in 10 ml Tyrode's balanced salt solution.

Synthetic oviductal fluid medium (SOF) – IVC I

NaCl	6.29 g/l
KCl	0.53 g/l
$NaHCO_3$	2.1 g/l
Na-Lactate (60% w/w)	0.5969 g/l
$MgCl_2.6H_2O$	0.10 g/l
$CaCl_2.2H_2O$	0.25 g/l
KH_2PO_4	0.16 g/l
MEM NEAA	10 ml/l
BME EAA	20 ml/l
Glucose	0.27 g/l
Pen/strep	10^4 IU penicillin + 0.1 g streptomycin
Sodium Pyruvate	0.0363 g/l
Ala-Gln	0.3 g/l
BSA	4 g/l
phenol red	500 µl/l

Synthetic oviductal fluid medium (SOF) – IVC II

NaCl	6.29 g/l
KCl	0.53 g/l
$NaHCO_3$	2.1 g/l
Na-Lactate (60% w/w)	0.5969 g/l
$MgCl_2.6H_2O$	0.10 g/l
$CaCl_2.2H_2O$	0.25 g/l
KH_2PO_4	0.16 g/l
MEM NEAA	10 ml/l
BME EAA	20 ml/l
Glucose	0.27 g/l
Pen/strep	10^4 IU penicillin + 0.1 g streptomycin
Sodium Pyruvate	0.0363 g/l
Ala-Gln	0.3 g/l
FCS	10% v/v
phenol red	500 µl/l

Modified Tyrode's balanced salt solution (modified Tyrode's solution) – IVC I

NaCl	8 g/l
KCl	0.2 g/l
$NaHCO_3$	1 g/l
NaH_2PO_4 (anhyd)	0.05 g/l
$MgCl_2.6H_2O$	0.214 g/l
$CaCl_2.2H_2O$	0.265 g/l
Cal Lactate	0.024 g/l
MEM NEAA	10 ml/l
BME EAA	10 ml/l
Glucose	1 g/l
Pen/strep	10^4 IU penicillin + 0.1 g streptomycin
Sodium Pyruvate	0.0396 g/l
Ala-Gln	0.2172 g/l
BSA	3 g/l
phenol red	500 µl/l

Modified Tyrode's balanced salt solution (modified Tyrode's solution) – IVC II

NaCl	8 g/l
KCl	0.2 g/l
$NaHCO_3$	1 g/l
NaH_2PO_4 (anhyd)	0.05 g/l
$MgCl_2.6H_2O$	0.214 g/l
$CaCl_2.2H_2O$	0.265 g/l
Cal Lactate	0.024 g/l
MEM NEAA	10 ml/l
BME EAA	10 ml/l
Glucose	1 g/l
Pen/strep	10^4 IU penicillin + 0.1 g streptomycin
Sodium Pyruvate	0.0396 g/l
Ala-Gln	0.2172 g/l
FCS	10% v/v
phenol red	500 µl/l

Modified Earle's balanced salt solution (MK-1) – IVC I

NaCl	6.8 g/l
KCl	0.4 g/l
$NaHCO_3$	2.1 g/l
$NaH_2PO_4.H_2O$	0.16 g/l
L(+)Ca(lactate)2	0.39 g/l
$MgSO_4.7H_2O$	0.2 g/l
MEM NEAA	10 ml/l
BME EAA	20 ml/l
Glucose	0.27 g/l
Pen/strep	10^4 IU penicillin + 0.1 g streptomycin
Sodium Pyruvate	0.04 g/l
Ala-Gln	0.22 g/l
BSA	3 g/l
phenol red	500 µl/l

Modified Earle's balanced salt solution (MK-1) – IVC II

NaCl	6.8 g/l
KCl	0.4 g/l
$NaHCO_3$	2.1 g/l
$NaH_2PO_4.H_2O$	0.16 g/l
L(+)Ca(lactate)2	0.39 g/l
$MgSO_4.7H_2O$	0.2 g/l
MEM NEAA	10 ml/l
BME EAA	20 ml/l
Glucose	0.27 g/l
Pen/strep	10^4 IU penicillin + 0.1 g streptomycin
Sodium Pyruvate	0.04 g/l
Ala-Gln	0.22 g/l
FCS	10% v/v
phenol red	500 µl/l

0.5 M EDTA (pH 8)

Na_2EDTA	186 g/l

Adjust pH = 8 by 10 N NaOH, then autoclave and store at 4°C

10 x TBE buffer

Tris	108 g/l
Boric acid	55 g/l
0.5 M EDTA (pH 8)	40 ml/l

Adjust water up to 1 litr, then autoclave and store at 25°C (no longer than 1 month)

APPENDIX B

List of publications and conferences

1. Sananmuang, T., Tharasanit, T., Nguyen, C., Phutikanit, N. and Techakumphu, M. 2011. Culture medium and embryo density influence on developmental competence and gene expression of cat embryos. **Theriogenology**: In Press.
2. 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: 199-207.
3. Sananmuang, T., Tharasanit, T., Nguyen, C., Phutikanit, N., Manee-in, S. and Techakumphu, M. 2010. The effects of embryo density on developmental competence in relation to gene expression of cat embryos.**13th Association of Institutions for Tropical Veterinary Medicine (AITVM) Conference**, Sofitel Centara Grand Hotel, Bangkok, Thailand, 23 - 26 August 2010, p194.
4. Sananmuang, T., Tharasanit, T. and Techakumphu, M. 2010. The effects of culture media types and embryo density on development of cat embryo. **The 9th Chulalongkorn University Veterinary Annual Conference**, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand, 1 April 2010, p115.
5. Sananmuang, T., Tharasanit, T., Nguyen, C., Phutikanit, N. and Techakumphu, M. 2010. Developmental competence of cat embryos cultured in different densities and numbers. **The 9th Chulalongkorn University Veterinary Annual Conference**, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand, 1 April 2010, p114.
6. Sananmuang, T., Tharasanit, T., Nguyen, C., Phutikanit, N. and Techakumphu, M. 2010. Developmental competence and GLUT-1/BAX gene expressions of cat embryos cultured in different concentrations of glucose. **The RGJ Seminar Series LXXI "Perspectives and Innovation in Veterinary Biosciences"**, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand, 24 February 2010, p40.
7. Sananmuang, T.**,** Panasopolkul, S., Tharasanit, T. and Techakumphu, M. 2009. The effects of culture media and embryo density on developmental competence of cat embryos. **RGJ-Ph.D. Congress X**, Jomtean Plam Beach Resort Hotel, Pattaya, Chonburi, Thailand, 3-5 April 2009, p159.
8. Tharasanit, T., Sanuanmuang, T. and Techakumphu, M. 2008. *In vitro* embryo production in cat: State of the art. **Proc. of CU VET Graduate Seminar**, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand, 8-9 April 2008, p21.
9. Sananmuang, T., Buarpung, S., Techakumphu, M. and Tharasanit, T. 2008. Embryo production in cat by intracytoplasmic sperm injection. **Proc. of CU VET Graduate Seminar**, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand, 8-9 April 2008, p22-23.
10. Sananmuang, T., Suwimonteerabutr, J., Techakumphu, M. and Tharasanit, T. 2007. Effect of roscovitine on meiotic competence of cat oocytes. **The 4th Annual Conference of Asian Reproductive Biotechnology Society "Reproductive Biotechnology, Stem cells and Regenerative Medicine"**, Sigapore, 24-28 November, 2007, p105.
11. Sananmuang, T., Techakumphu, M. and Tharasanit, T. 2007. Effects of roscovitine on meiotic arrest and resumption of cat oocytes. **Proc. of the 33th Veterinary Medical**

Association, Sofitel Centara Grand Hotel, Bangkok, Thailand, 31 October-2 November 2007, p63-65.

12. Tharasanit, T., Manee-In, S., Rungarunlert, S., Sananmuang, T., Chatdarong, K., Techakumphu, M. and Lohachit, C. 2007. Recombinant human follicle stimulating hormone exerts nuclear and cytoplasmic maturation of cat oocytes. **Proc. of the 33th Veterinary Medical Association**, Sofitel Centara Grand Hotel, Bangkok, Thailand, 31 October-2 November 2007, p59-61.
13. Sananmuang, T., Suwimonteerabutr, J., Rungarunlert, S., Techakamphu, M. and Tharasanit, T. 2007. Maintaining the meiotic arrest of cat oocytes by a specific cyclin-dependent kinase inhibitor. **The 6th Chulalongkorn University Veterinary Annual Conference**, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand, 26-27 April 2007, p112.
14. Tharasanit, T., Manee-In, S., Rungarunlert, S., Sananmuang, T., Chatdarong, K., Techakamphu, M. and Lohachit, C. 2006. The effect of recombinant human follicle stimulating hormone on developmental competence of cat oocytes. **Proc. of the 3rd Annual Conference of Asian Reproductive Biotechnology Society "Innovation for future life"**, Hanoi, Vietnam, 29 November - 3 December 2006, p143.

VITAE

Miss Thanida Sananmuang was born on March 19th 1982 in Bangkok province, Thailand. She graduated with Degree of Doctor of Veterinary Medicine (DVM) with the 1st honour from Faculty of Veterinary Science, Chulalongkorn University, in 2006. In the same year, 2006, she received a scholarship from the Royal Golden Jubilee PhD program of Thailand Research Fund to perform a PhD program of Theriogenology at Department of Obstetrics Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. Her focus research is about *in vitro* embryo production in feline, which aims to find out the appropriate culture condition in feline species using domestic cat as a model. This research introduces gene expression analysis together with observed embryo morphology for culture system evaluation in feline.