Follicular responsiveness to equine chorionic gonadotropin in cat species: study in *in vitro* culture

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การตอบสนองของฟอลลิเคิลต่อฮอร์โมนอีไควน์คอริโอนิคโกนาโดโทรปินในสัตว์ตระกูลแมวโดยการศึกษาจากการเลี้ยงฟอลลิเคิลภายนอกร่างกาย

น.ส.อัจจิมา จันท์แสนโรจน์

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อัจจิมา จันท์แสนโรจน์ : การตอบสนองของฟอลลิเคิลต่อฮอร์โมนอีโควน์คอริโอนิคโกนาโดโทรปินในสัตว์ตระกูลแมวโดยการศึกษาจากการเลี้ยงฟอลลิเคิลภายนอกร่างกาย. (Follicular responsiveness to equine chorionic gonadotropin in cat species: study in *in vitro* culture) อ.ที่ปรึกษาหลัก: ศ. สพ.ญ. ดร.เกวลี ฉัตรดรงค์, อ.ที่ปรึกษาร่วม: ดร.นุชรินทร์ ศงสะเสน

อีโควน์คอริโอนิคโกนาโดโทรปินได้ถูกใช้ในการเหนี่ยวนำการเป็นสัดในสัตว์ป่าตระกูลแมวหลากหลายสปีชีส์ อย่างไรก็ตามกลไกของโกนาโดโทรปินที่ควบคุมกระบวนการสร้างฟอลลิเคิลในแมวนี้ยังคงไม่ชัดเจน ในการทดลองนี้ ทำการประเมิน 1) การตอบสนองของฟอลลิเคิลในรังไข่แมวที่ระยะการพัฒนาของฟอลลิเคิลที่ต่างกันต่ออีโควน์คอริโอนิคโกนาโดโทรปินด้วยการเลี้ยงฟอลลิเคิลภายนอกร่างกาย 2) การตอบสนองของฟอลลิเคิลเสือในระยะแอนทรัลต่ออีโควน์คอริโอนิคโกนาโดโทรปิน 3) อิทธิพลของอีโควน์คอริโอนิคโกนาโดโทรปินร่วมกับอินซูลินไลค์โกรทแฟคเตอร์, สเต็มเซลล์แฟคเตอร์ และการรวมกันของอินซูลินไลค์โกรทแฟคเตอร์และสเต็มเซลล์แฟคเตอร์ต่อฟอลลิเคิลในรังไข่แมวที่ระยะการพัฒนาของฟอลลิเคิลที่ต่างกัน

การทดลองที่ 1 ฟอลลิเคิลแยกเดี่ยวจากรังไขของแมว 22 ตัว แบ่งเป็น 3 ระยะการพัฒนา ตามลักษณะภายนอกและเส้นผ่านศูนย์กลางของฟอลลิเคิล: 1) ฟอลลิเคิลระยะที่สองที่มีสองชั้น ขนาด100-150 ไมโครเมตร (จำนวน 139 ฟอลลิเคิล) 2) ฟอลลิเคิลระยะที่สองที่มีหลายชั้น, 150-300 ไมโครเมตร (จำนวน 154 ฟอลลิเคิล) และ 3) แอนทรัลฟอลลิเคิลระยะเริ่มต้น ≥ 300-500 ไมโครเมตร (จำนวน 123 ฟอลลิเคิล) จากนั้นฟอลลิเคิลจะถูกห่อหุ้มใน 0.5 เปอร์เซ็นต์ (น้ำหนัก/ปริมาตร) โซเดียมอัลจิเนต และเลี้ยงเป็นเวลา 12 วันในน้ำยาเลี้ยงซึ่งเสริมด้วย 0, 0.05, 0.1, 0.5 ไอยู/มิลลิลิตร อีโควน์คอริโอนิคโกนาโดโทรปิน หลังจากทำการเลี้ยงไปแล้ว 12 วัน การเติบโตของฟอลลิเคิลและการแสดงออกของยีนของฟอลลิเคิลระยะที่สองที่มีสองชั้นไม่ได้รับอิทธิพลโดยอีโควน์คอริโอนิคโกนาโดโทรปินที่ทุกความเข้มข้น ($P > 0.05$) อย่างไรก็ตามความเข้มข้นของอีโควน์คอริโอนิคโกนาโดโทรปินที่ 0.05 ไอยู/มิลลิลิตร กระตุ้นการเติบโตของฟอลลิเคิลและการแสดงออกของยีนในฟอลลิเคิลระยะที่สองที่มีหลายชั้นและแอนทรัลฟอลลิเคิลระยะเริ่มต้น ($P < 0.05$) ความคล้ายคลึงกันที่พบคือเส้นผ่านศูนย์กลางของโอโอไซต์ในฟอลลิเคิลระยะที่สองที่มีหลายชั้นและแอนทรัลฟอลลิเคิลระยะเริ่มต้นที่ใส่ 0.05 ไอยู/มิลลิลิตร นั้นไม่มีการเปลี่ยนแปลง เมื่อพิจารณาจากการแสดงออกของยีน ระดับของ *STAR* เพิ่มขึ้นในแอนทรัลฟอลลิเคิลระยะเริ่มต้น ($P < 0.05$) และ มีแนวโน้มเพิ่มขึ้นในฟอลลิเคิลระยะที่สองที่มีหลายชั้น ($P = 0.08$) ที่เลี้ยงใน 0.05 ไอยู/มิลลิลิตร อีโควน์คอริโอนิคโกนาโดโทรปิน ในขณะที่ไม่มีผลต่อการแสดงออกของยีนอื่นๆ ดังนั้นการตอบสนองของฟอลลิเคิลแมวต่ออีโควน์คอริโอนิคโกนาโดโทรปินพบได้ตั้งแต่ฟอลลิเคิลระยะที่สองที่มีหลายชั้นเป็นต้นไป และอีโควน์คอริโอนิคโกนาโดโทรปินที่ 0.05 ไอยู/มิลลิลิตรนั้นเหมาะสมสำหรับการเลี้ยงฟอลลิเคิลในแมวบ้าน

การทดลองที่ 2 ความเข้มข้นของอีโควน์คอริโอนิคโกนาโดโทรปินที่เหมาะสมจากการทดลองที่ 1 ถูกเลือกมาเพื่อตรวจสอบการตอบสนองของฟอลลิเคิลเสือต่ออีโควน์คอริโอนิคโกนาโดโทรปิน เนื้อเยื่อรังไข่ที่ได้จากการแช่แข็งและทำละลาย 6 ชิ้นที่ได้มาภายหลังการตายของเสือหนึ่งตัวถูกนำมาประเมินฟอลลิเคิลแยกเดี่ยว 12 ฟอลลิเคิล ถูกแบ่งอย่างสุ่มเป็น 2 สภาวะการเลี้ยง (กลุ่มควบคุมและ 0.05 ไอยู/มิลลิลิตร อีโควน์คอริโอนิคโกนาโดโทรปิน) และเลี้ยงในอัลจิเนต ไฮโดรเจล 3 วัน เส้นผ่านศูนย์กลางของฟอลลิเคิลในกลุ่มควบคุมลดลงอย่างมีนัยสำคัญ ($P < 0.05$) หลังจากเลี้ยงไป 3 วัน ในขณะที่เส้นผ่านศูนย์กลางของฟอลลิเคิลในกลุ่มที่เสริมอีโควน์คอริโอนิคโกนาโดโทรปินยังคงมีขนาดเหมือนตอนเริ่มต้น ($P > 0.05$) การรอดชีวิตของฟอลลิเคิลคือ 100% ในทั้งสองกลุ่ม อย่างไรก็ตามโอโอไซต์ที่เก็บได้ในระหว่างกลุ่มควบคุมและกลุ่มเสริมอีโควน์คอริโอนิคโกนาโดโทรปินนั้นแตกต่างกันอย่างมีนัยสำคัญ (33%, จำนวน 6 ฟอลลิเคิล และ 67%, จำนวน 6 ฟอลลิเคิล ตามลำดับ) ($P < 0.05$) สถานะของนิวเคลียสในโอโอไซต์ที่ได้มาทั้งหมดยังคงอยู่ในระยะเจอร์มินอลเวสสิเคิล การทดลองนี้แสดงให้เห็นว่าแอนทรัลฟอลลิเคิลที่ได้จากเนื้อเยื่อที่แช่แข็งและทำละลายของเสือไม่สามารถรักษาลักษณะภายนอกและการทำงานของฟอลลิเคิลได้หากขาดการเสริมอีโควน์คอริโอนิคโกนาโดโทรปินในน้ำยาเลี้ยง เราสรุปว่าอีโควน์คอริโอนิคโกนาโดโทรปินมีบทบาทสำคัญกับการเติบโตของแอนทรัลฟอลลิเคิลและการรอดชีวิตของโอโอไซต์

การทดลองที่ 3 อิทธิพลของการเสริมโกรทแฟคเตอร์ (อินซูลินไลค์โกรทแฟคเตอร์, สเต็มเซลล์แฟคเตอร์และการรวมกันของแฟคเตอร์ทั้งสอง) ต่อฟอลลิเคิลในรังไข่แมวที่ระยะการพัฒนาของฟอลลิเคิลที่ต่างกันได้ถูกตรวจสอบ ฟอลลิเคิลได้รับจากแมว 12 ตัว ถูกห่อหุ้มในไฟบรินอัลจิเนต และเลี้ยงเป็นเวลา 18 วัน ในน้ำยาเลี้ยงซึ่งมี 0.05 ไอยู/มิลลิลิตร อีโควน์คอริโอนิคโกนาโดโทรปิน ที่ไม่มีการเสริมโกรทแฟคเตอร์ (กลุ่มควบคุม) หรือ เสริมด้วย 1 นาโนกรัม/มิลลิลิตร อินซูลินไลค์โกรทแฟคเตอร์ (IGF-I), 50 นาโนกรัม/มิลลิลิตร สเต็มเซลล์แฟคเตอร์ (SCF50), 100 นาโนกรัม/มิลลิลิตร สเต็มเซลล์แฟคเตอร์ (SCF100), 1 นาโนกรัม/มิลลิลิตร อินซูลินไลค์โกรทแฟคเตอร์ กับ 50 นาโนกรัม/มิลลิลิตร สเต็มเซลล์แฟคเตอร์ (IGF-SCF50) หรือ 1 นาโนกรัม/มิลลิลิตร อินซูลินไลค์โกรทแฟคเตอร์ กับ 100 นาโนกรัม/มิลลิลิตร สเต็มเซลล์แฟคเตอร์ (IGF-SCF100) การเสริมโกรทแฟคเตอร์ไม่มีผลต่อการเติบโตของฟอลลิเคิลระยะที่สองที่มีสองชั้นและฟอลลิเคิลระยะที่สองที่มีหลายชั้น ในขณะที่ SCF100 ส่งเสริมการเติบโตของแอนทรัลฟอลลิเคิลระยะเริ่มต้นตลอดระยะเวลาการศึกษา ($P<0.05$) อย่างไรก็ตามการเติบโตของโอโอไซต์แตกต่างกันไปตามระยะการพัฒนาของฟอลลิเคิล ในฟอลลิเคิลระยะที่สองที่มีสองชั้นนั้นอินซูลินไลค์โกรทแฟคเตอร์ไม่สามารถรักษาขนาดของโอโอไซต์ภายหลังการเลี้ยง 12 วัน ในทางกลับกันโอโอไซต์ของฟอลลิเคิลระยะที่สองที่มีหลายชั้นนั้นรักษาขนาดของโอโอไซต์ได้ในทุกกลุ่มที่ใส่โกรทแฟคเตอร์จนกระทั่งจบการทดลอง อย่างไรก็ตามการมีหรือไม่มีโกรทแฟคเตอร์ต่างไม่เป็นผลต่อโอโอไซต์ของแอนทรัลฟอลลิเคิลระยะเริ่มต้น SCF50กระตุ้นการสร้างช่องว่างในแอนทรัลฟอลลิเคิลและยีนที่ควบคุมการเติบโตของฟอลลิเคิลและโอโอไซต์และการสร้างสเตียรอยด์ฮอร์โมนในฟอลลิเคิลระยะที่สองที่มีหลายชั้น ในขณะที่ IGF-SCF50 ดีที่สุดในการเสริมให้กับแอนทรัลฟอลลิเคิลในระยะเริ่มต้นเพราะมันเพิ่มการแสดงออกของยีน *FSHR*, *GDF9* และยีนที่เกี่ยวกับการสร้างสเตียรอยด์ฮอร์โมน ดังนั้นสเต็มเซลล์แฟคเตอร์มีอิทธิพลต่อทุกระยะการพัฒนาของฟอลลิเคิลในแมว

โดยสรุป การตอบสนองของฟอลลิเคิลแมวต่ออีโควน์คอริโอนิคโกนาโดโทรปินพบได้ตั้งแต่ฟอลลิเคิลระยะที่สองที่มีหลายชั้นเป็นต้นไป และการเสริมอีโควน์คอริโอนิคโกนาโดโทรปินที่ 0.05 ไอยู/มิลลิลิตร เหมาะสมต่อการเลี้ยงฟอลลิเคิลภายนอกร่างกายในแมวบ้าน การเพิ่มสเต็มเซลล์แฟคเตอร์ลงในน้ำยาเลี้ยงช่วยส่งเสริมกระบวนการสร้างฟอลลิเคิลในแมวตั้งแต่ฟอลลิเคิลระยะที่สองที่มีหลายชั้นถึงแอนทรัลฟอลลิเคิลระยะเริ่มต้น ยิ่งไปกว่านั้นฟอลลิเคิลเสือก็ตอบสนองต่อการเสริมอีโควน์คอริโอนิคโกนาโดโทรปิน 0.05 ไอยู/มิลลิลิตร เช่นเดียวกับที่พบในแมวบ้าน

สาขาวิชา	วิทยาการสืบพันธุ์สัตว์	ลายมือชื่อนิสิต .....
ปีการศึกษา	2561	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORD: Follicle, Gonadotropin, Growth factor, Response, Tiger

Ajjima Chansaenroj: Follicular responsiveness to equine chorionic gonadotropin in cat species: study in *in vitro* culture. Advisor: Prof. KAYWALEE CHATDARONG, D.V.M., Ph.D. Co-advisor: Dr. Nucharin Songsasen, D.V.M., Ph.D.

Equine chorionic gonadotropin (eCG) has been commonly used to induce estrus in several felid species. However, the mechanisms by which this gonadotropin regulates cat folliculogenesis are still unclear. In this present study, we investigated 1) the in vitro responsiveness of cat ovarian follicles at different follicle developmental stages to various eCG concentration; 2) tiger antral follicle responsiveness to eCG; 3) the influence of eCG combination with insulin-like growth factor I (IGF-I) and/or stem cell factor (SCF) on cat ovarian follicles at different developmental stages.

Study 1, the isolated follicles from the ovaries of 22 cats were classified into three developmental stages based on their morphology and diameter: 1) two-layered secondary follicle (SF), 100-150 μm (n = 139); 2) multi-layered SF, 150-300 μm (n = 154); and 3) early antral follicle (AF), ≥ 300-500 μm (n = 123). The follicles were then encapsulated in 0.5 % (w/v) sodium alginate and cultured for 12 days in culture medium supplemented with 0, 0.05, 0.1 or 0.5 IU/mL eCG. After being cultured for 12 days, follicle growth and gene expression of two-layered SF were not influenced by eCG at all concentrations ($P > 0.05$). However, the concentration of eCG at 0.05 IU/mL stimulated follicular growth and gene expressions in the multi-layered SF and early AF ($P < 0.05$). Correspondingly, the diameter of oocytes in the multi-layered SF and early AF treated with 0.05 IU/mL eCG was unchanged. Considering the gene expression, the level of *STAR* was enhanced in the early AF ($P < 0.05$) and tended to increase in the multi-layered SF ($P = 0.08$) cultured in 0.05 IU/mL eCG, whereas the expression of other genes was not affected. Therefore, the responsiveness of cat follicles to eCG is apparent from the multi-layered SF stage onward and the eCG supplementation at 0.05 IU/mL appeared to be optimal for the follicle culture in the domestic cat.

Study 2, the optimal concentration of eCG supplementation from the experiment 1 was selected to investigate the responsiveness of the tiger follicle to eCG. Six frozen-thawed ovarian tissue from a tiger obtained post-mortem were evaluated. Twelve isolated antral follicles recovered were randomly allocated into two culture conditions (control and 0.05 IU/mL eCG supplementation) and cultured in an alginate hydrogel for 3 days. The follicle diameters in the control group significant decreased ($P < 0.05$) after 3 days of culture, while the size of those in eCG supplementation group were remained constant ($P > 0.05$). Follicle survival was 100% in both groups. However, the oocyte retrieval rate was significant different between the two treatments (control, 33%, n = 6; eCG, 67%, n = 6) ($P < 0.05$). The nuclear status of all recovered oocytes was remained in germinal vesicle phase. The present study showed that the tiger frozen-thawed antral follicles could not maintain their morphology and function without eCG supplementation in the culture medium. We concluded that eCG plays an important role on tiger antral follicle growth and the survival of the oocyte.

Study 3, the influence of growth factor supplementations (IGF-I, SCF and the combination of these two factors) on cat ovarian follicles at different follicle developmental stages were examined. The follicles obtained from twelve cats, encapsulated in a fibrin-alginate hydrogel and cultured for 18 days in the culture medium contained 0.05 IU/mL eCG without growth factor supplementation (control group) or supplemented with 1 ng/mL IGF-I (IGF-I), 50 ng/mL SCF (SCF50), 100 ng/mL SCF (SCF100), 1 ng/mL IGF-I + 50 ng/mL SCF (IGF-SCF50) or 1 ng/mL IGF-I + 100 ng/mL SCF (IGF-SCF100). The growth factors supplementations had no effect to two-layered SF and multi-layered SF growth, whereas SCF100 supported the growth of early AF throughout the culture period ($P<0.05$). However, the oocyte growth was varied among developmental stages. In the two-layered SF, IGF-I failed to maintain oocyte size after culture for 12 days. Oocytes of multi-layered SF were sustained in their initial size until the end of the study in all growth factor treated follicles. However, the present or absence of growth factor showed no effect to the oocyte of early AF. SCF50 stimulated antral cavity formation and gene regulating follicle and oocyte growth and steroidogenesis in multi-layered SF. While IGF-SCF50 was the best supplementation to early AF because it increased *FSHR*, *GDF9* and steroidogenic genes. Therefore, SCF influenced all stages of follicle development in cat.

In conclusions, the responsiveness of cat follicles to eCG was apparent from the multi-layered SF stage onward and the eCG supplementation at 0.05 IU/mL appeared to be optimal for the in vitro follicle culture in the domestic cats. The addition of SCF to culture medium supported cat folliculogenesis from multi-layered SF to early AF stage. Furthermore, similar to the domestic cat, tiger follicles also respond to 0.05 IU/mL eCG supplementation.

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ABBREVIATIONS

AF	Antral follicle
AI	Artificial insemination
AKT	Serine/threonine-specific protein kinase
ARTs:	Assisted reproductive technologies
BMP 15	Bone morphogenetic protein 15
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
cAMP	Cyclic adenosine 3′,5′-monophosphate
cAMP/PKA	Cyclic adenosine 3′,5′-monophosphate/ Protein kinase A
cDNA	Complementary deoxyribonucleic acid
cGMP	Cyclic guanosine monophpsphate
c-kit	Stem cell factor receptor
COCs	Cumulus oocyte complexes
CPAs	Cryoprotective agents
Ct	Cycle threshold
CYP17A1	Cytochrome P450 steroid 17 α-hydroxylase/17,20 lyase
CYP19A1	Cytochrome P450 aromatase
DNA	Deoxyribonucleic acid

eCG	equine chorionic gonadotropin
EGF	Epidermal growth factor
ET	Embryo transfer
FCS	Fetal calve serum
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone receptor
GDF-9	Growth differentiation factor-9
GnRH	Gonadotropin releasing hormone
hCG	Human chorionic gonadotropin
ICSI	Intracytoplasmic sperm injection
IGF-I	Insulin-like growth factor-I
IGFR-I	Insulin-like growth factor receptor-I
IVF	*In vitro* fertilization
LH	Luteinizing hormone
LHCGR	Luteinizing hormone/chorionic gonadotropin receptor
M199	Medium 199 with Earle's salt
MAPK	Mitogen-activated protein kinase
MEM	Minimum essential medium

mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NPPC	Natriuretic peptide precursor type C
NPR2	Natriuretic peptide precursor type C receptor
OSFs	Oocyte secreted factors
PDE3A	Phosphodiesterase 3A
PF	Primary follicle
pFSH	Porcine follicle stimulating hormone
PI3K/AKT	Phosphatidylinositol 3-kinase/serine/threonine-specific protein kinase
pLH	Porcine luteinizing hormone
POF	Preovulatory follicle
PrF	Primordial follicle
qRT-PCR	Quantitative real-time polymerase chain reaction
rhFSH	Recombinant human follicle stimulating hormone
RNA	Ribonucleic acid
SCF	Stem cell factor
SCF/c-kit	Stem cell factor/stem cell factor receptor
SDHA	Succinate dehydrogenase complex flavoprotein subunit A

SEM	Standard error of mean
SF	Secondary follicle
STAR	Steroidogenic acute regulatory protein
ΔΔCt	Delta delta Ct

Chapter I

Introduction

1.1 Importance and rational

During the past few decades, many wild felids have gradually decreased in numbers, and many of which are being classified as threatened, valuable, endangered or critically endangered largely due to extensive deforestation, habitat loss and illegal hunting. Of 38 wild felids worldwide nine species have been found in Thailand: tiger (*Panthera tigris*), leopard (*Panthera pardus*), clouded leopard (*Neofelis nebulosa*), Asian leopard cat (*Prionailurus bengalensis*), fishing cat (*Prionailurus viverrinus*), flat-headed cat (*Prionailurus planiceps*), Asian golden cat (*Catopuma temminckii*), jungle cat (*Felis chaus*) and marbled cat (*Pardofelis marmorata*) (Srisamoot et al., 2007). According to the International Union for Conservation of Nature, five of the nine species are at risk of losing their genetic diversity (The IUCN Red List of Threatened Species. Version 2018-1. <www.iucnredlist.org>. Download on 06 August 2018).

Assisted reproductive technologies (ARTs) such as artificial insemination (AI), *in vitro* fertilization (IVF), embryo transfer (ET) and gamete cryopreservation have been used to support perpetuation of genetically valuable endangered felids. Assisted breeding is a valuable tool for conservation of wild cat species, especially for developing successful *ex situ* breeding programs.

Induction of estrus cycles in felids can be performed by using equine chorionic gonadotropin (eCG), follicle stimulating hormone (FSH), gonadotropin releasing hormone (GnRH), GnRH analogues and opioid antagonists with varying results (Kutzler, 2007).

Porcine FSH (pFSH) has amino acids homology with tiger FSH and purified porcine FSH has a minimal LH activity (Crichton et al., 2003). Therefore, pFSH seemed to induce follicular growth in felids. However, FSH has a short half-life and need to be injected twice daily for 3-6 consecutive days which may cause stress associated with animal restraint. Apart from the above, because of its long half-life (120 h); eCG has been a hormone of choice to induce folliculogenesis in a single injection. Because of this advantage, eCG is widely used for ovarian stimulation in many felids including the domestic cat (Swanson et al., 1995; Roth et al., 1997b), cheetah (Howard et al., 1997), clouded leopard (Howard et al., 1997), tiger (Donoghue et al., 1990; Donoghue et al., 1996; Graham et al., 2006), puma (Moore et al., 1981; Miller et al., 1990), ocelot (Swanson et al., 1996), tigrina (Swanson and Brown, 2004) and snow leopard (Roth et al., 1997a). However, the disadvantage of eCG in felids is the production of anti-gonadotropin antibodies resulting in decreased ovarian responsiveness when the hormone is administered repeatedly in the same animal within 6 months (Swanson et al., 1995). The responsiveness of the ovary to exogenous gonadotropin has been shown to be species specific with the optimal eCG dosage not being associated with body weight (Howard et al., 1997). Moreover, the results of estrous induction are uncertain.

Several animals did not respond to the hormonal induction with unexplained causes (Pelican et al., 2006b). Furthermore, gonadotropin hyper-stimulation may be detrimental to animal health, reproductive organ or the quality of the follicles and oocytes that can subsequently cause the improper breeding and infertility problems (Goodrowe and Wildt, 1987).

Various protocols using eCG in combination with human chorionic gonadotropin (hCG) to induce estrus and ovulation prior to AI have been developed in the domestic cats (*Felis catus*) since the domestic cat is logically considered a suitable model for endangered felids. However, the modified AI protocols from the domestic cat provide uncertain outcomes in different felids. The AI success rate in the felids is low (Table 1) which may be due to the variation in ovarian responsiveness to exogenous gonadotropin treatment among different felid species.

Table 1. Pregnancy rate from artificial insemination by using fresh semen in felids.

Species	Gonadotropin dosage		Time of ovulation after hCG (h)	Pregnancy rate (%)	References
	eCG (IU)	hCG (IU)			
Tigrina *(Leopardus tigrinus)*	200	150	30-36	25	Swanson and Brown, 2004
Ocelot *(Leopardus pardalis)*	400	200	30-36	25	Moraes et al., 1997
Ocelot *(Leopardus pardalis)*	500	225	30-36	25	Swanson et al., 1996
Clouded leopard *(Neofelis Nebulosa)*	100	75	37-40	5	Howard et al., 1996
Snow leopard *(Panthera uncia)*	600	300	40	6.7	Roth et al., 1997
Cheetah *(Acinonyx jubatus)*	200	100	40-42	46.2	Howard et al., 1997
Puma *(Puma concolor)*	200	100	33-40	12.5	Barone et al., 1994
Tiger *(Panthera tigris)*	1000	750	39-46	10	Donoghue et al., 1996

The endangered felids in the wild and captivity are rare. Therefore, hormonal responsiveness tested in wild animals is limited. Recently, *in vitro* culture technology become more feasible using ovarian tissue. The overall aim of this thesis is to find the efficacy of estrous induction using eCG in terms of follicular growth and mRNA expressions in isolated follicles of the domestic cat. The intense growth and gene expressions are considered the indicators for eCG responsiveness. Moreover, growth factors that may enhance the effect of eCG on follicular growth were also assessed. The knowledge obtained in this study will be valuable to understand the follicle responsiveness to eCG in other wild felids species to improve ARTs.

1.2 Literature reviews

1.2.1 Reproductive biology in the domestic cat

The female cat is classified as seasonally polyestrous (Shille et al., 1979). Unlike other domesticated species, the cat is categorized as induced ovulation animal, although in some cases females ovulate spontaneously (Feldman and Nelson, 1996). The onset of puberty in female cats occurs at 4–12 months of age (Bristol-Gould and Woodruff, 2006). The exact time of entering to the puberty is depending on photoperiod (Hurni, 1981; Leyva et al., 1989; Michel, 1993), breed and body weight (Johnston et al., 2001) of the cat. The estrous cycle in the cat consists of five phases (proestrus, estrus, interestrus, diestrus and anestrus) (Feldman and Nelson, 1996). The proestrus phase consists of follicular growth and the synthesis of estrogen, often twice as high as the level observed during anestrus or interestrus (Shille et al., 1979). During the proestrus, cats start showing signs of estrus behavior such as rubbing and rolling,

but refuse mounting and copulation (da Silva et al., 2006). The end of proestrus is marked by females' acceptance to the male (Olson et al., 1984). Estrus period is a phase for mating that normally lasts for 2-19 days (Shille et al., 1979). Females exhibit similar behaviors to those observed during proestrus, but allow a male to mount and copulate. The typical signs of estrus are vocalization, lordosis, pelvis elevation, tail deviation, and the hind limbs treading, especially in the presence of a tom (da Silva et al., 2006). The estrus behavior is related to the follicular phase, which is defined as the period of time when the follicles produce and secrete high levels of estrogens (Verhage et al., 1976). The estrogens in blood stream continue to increase and reach a peak concentration on Day 3 of the follicular phase and rapidly decline (Banks and Stabenfeldt, 1982), although most queens continue so show estrus behavior for 1-4 days after the follicular phase is ended (Shille et al., 1979). Even if ovulation is induced and plasma progesterone starts to increase, the sexual receptivity behavior of the queen continues until the end of the estrus phase (Paape et al., 1975). The queen will enter the interestrus phase when the period of estrus is over. If ovulation does not occur during estrus, the queen will enter an anovulatory interestrus phase (da Silva et al., 2006), and proceed into proestrus again. In addition, the non-ovulatory cycles were characterized by a serum progesterone concentration on Days 11–15 that is below 5 nmol/L and a normal interestrus interval of 7–14 days (Chatdarong et al., 2006). If the ovulation occurs during estrus, the queen will become either pregnant or pseudopregnant (Feldman and Nelson, 1996). Ovulatory cycles were characterized by

a serum progesterone concentration on Days 11–15 that is above 5 nmol/L and the duration of interestrus phase was more than 30 days (Chatdarong et al., 2006). The average of interestrus period is 9 days (Kutzler, 2007). The female returns to normal behavior and does not attract to the tom cat (Feldman and Nelson, 1996). Diestrus is a period of progesterone-dominated luteal phase, which is the phase after estrus if ovulation occurs. The corpora lutea develop 24-48 hours after ovulation (Wildt et al., 1981) and start producing and secreting progesterone, which inhibits the secretion of gonadotropin-releasing hormone (GnRH), follicle stimulating hormone (FSH), luteinizing hormone (LH) from the pituitary gland. At the end of diestrus, the queen proceeds into either proestrus or anestrus, depending on the season (Feldman and Nelson, 1996). The anestrus is a phase of reproductive inactivity which estrogen and progesterone remain in their basal level (Brown, 2011). The queens are neither attract to the tom cats nor express any sexual behavior.

The queen is considered as an induced ovulator, with copulation triggering the release of luteinizing hormone (LH) from the pituitary gland leading to ovulation (Feldman and Nelson, 1996). Ovulation in the queen is dependent on several factors (the number of mating, mating intervals and individual differences in the LH peak) (Concannon et al., 1980). Multiple copulations are required for ovulation induction (Concannon et al., 1980). However, it has been described that many queens can spontaneously ovulate in the presence of a tomcat or estrous queens. It has been reported that spontaneous ovulation occurs up to 60% of the female cats (Kutzler,

2007). In several wild felids, including, clouded leopard, lion and fishing cat (Pelican et al., 2006b), the presence of estrous females often triggers ovulation (Pelican et al., 2006a).

1.2.2 Folliculogenesis in the domestic cats

Folliculogenesis is a complex process regulated by various autocrine, paracrine and endocrine factors (Demeestere et al., 2005). Folliculogenesis begins with the formation of the primordial follicles which initiates during the fetal life in the human, monkey, horse, cow and pig, but not until during neonatal period in the mouse and rat or later in the second or third weeks after birth in cats, dogs, ferrets, rabbits and minks (Peter and McNatty, 1980). Most primordial follicles (90%) leave the resting pool via apoptosis, while the remainders are activated by poorly understood mechanisms to enter the growing follicle pool and develop into the primary, secondary and antral stage (Picton, 2001; Gougeon, 2010). During the early stage of follicle development, the oocyte rapidly increases in size; however, as folliculogenesis proceeds, the growth rate significantly declines and the gamete reaches the maximum size shortly after antral formation (Griffin et al., 2006; Reynaud et al., 2009; Songsasen et al., 2009).

The developmental stage of ovarian follicles can be divided into primordial follicles, primary follicle, secondary follicle and antral follicle (Table 1). The oocytes are approximately 85–100 μm in size and usually positioned in the cortex of the ovary (Bristol-Gould and Woodruff, 2006).

In adult domestic cat ovaries, all types of follicle primordial, primary, secondary and antral are present. The average number of primordial follicles in feline ovaries was estimated as 74,520 (Gosden and Telfer, 1987). Microscopic evaluation of harvested follicles population from mechanical dissection by using a cell dissection sieve reveals that almost 90% are primordial and primary follicles (diameter = 40-50 μm), and 10% are secondary follicles (<60 μm), which slightly different from recent study using light and transmission electron microscopy to estimate the population of ovarian follicles and morphologically characterize preantral follicular stages in queens' ovary report that the queen ovaries present 37,853 ± 6,118 preantral follicles, with consist of 87% primordial (22-41 μm), 10.4% primary (25-64 μm) and 2.3% secondary follicles (43-110 μm) (Carrijo et al., 2010). Only about 2% of all follicles leave the resting pool of primordial follicles and start growing (Jewgenow and Paris, 2006). The follicle classification is divided into eight types as following in Table 2.

Table 2. Classification of follicles in the ovary (Pedersen and Peters, 1968).

Classification	Type of follicles	Morphology
Primodial follicle (PrF)	1	A small oocyte with no follicle cells attached to its surface
	2	A small oocyte has a few cells attached to its cell surface, but not a complete ring of cells
Primary follicle (PF)	3a	A complete ring of follicle cells surrounds the oocyte (≤20 cells)
	3b	A complete ring of follicle cells surrounds a growing oocyte (21-60 cells)
Secondary follicle (SF)	4	Two layers of follicle cells surround a growing oocyte (61-100 cells)
	5a	Three layers of follicle cells (101 to 200 cells) contain with the oocyte (≤70 μm)
	5b	Fully grown oocyte is surrounded by many layers of follicle cells (201-400 cells)
Antral follicle (AF)	6	A large oocyte with many layers of cells separated by scattered areas of fluid (401-600 cells)
	7	A follicle with a single cavity containing follicle fluid and form cumulus oophorus (≤600 cells)
Preovulatory follicle (POF)	8	A large follicle with a single cavity with follicle fluid and cumulus stalk

During folliculogenesis, ooplasm and granulosa cells connect via gap junctions (Russell et al., 2016). Granulosa cell plays an important role in oocyte growth and maturation by providing essential nutrients to oocyte (Coticchio et al., 2015). The gap junctions allow small molecules, such as calcium, pyruvate, inositol, amino acid and intracellular signaling molecules to pass from granulosa cells to the oocyte (Downs, 1995; Webb et al., 2002; Luvoni et al., 2006; Zheng et al., 2012; Liu et al., 2014). Oocytes metabolize pyruvate through oxidative phosphorylation to produce energy for growth and maturation (Roberts et al., 2004). The granulosa cells of preantral follicles sort into two differentiated populations at the time of follicular antrum formation: mural granulosa cells, which line the follicular wall, and cumulus granulosa cells, which are associated with the oocyte (Wigglesworth et al., 2015). While mural granulosa cells produce steroid hormones, cumulus cells play roles in supporting oocyte growth and maturation (Mattioli and Barboni, 2000; Gilchrist et al., 2004) by maintain meiotic arrest and the resumption of meiosis. Granulosa cells produce natriuretic peptide precursor type C (NPPC) which binds to NPPC receptors (NPR2) on cumulus cells resulting in cyclic guanosine monophosphate (cGMP) production. The cGMP transfers to the oocyte via gap junctions to inhibit phosphodiesterase 3A (PDE3A), Therefore, the hydrolysis of cAMP is inhibited resulting in maintenance oocyte meiotic arrest. After LH surge, oocyte cAMP falls because the gap junctions are closed. The granulosa cell derived epidermal growth factors increases and activate MAPK pathway for meiotic resumption, with cumulus cells expansion (Dumesic et al., 2015).

Gonadotropins and the paracrine factors (the oocytes secrete paracrine factors such as growth differentiation factor 9 (GDF9) (Gilchrist et al., 2004) and bone morphogenetic protein 15 (BMP15) (Persani et al., 2014) promote follicular growth and differentiation (Vanderhyden et al., 1990; Knight and Glister, 2003). In addition, FSH plays an important role in promoting granulosa cell proliferation and differentiation from preantral follicles stage (Eppig, 1991). FSHR expression is triggered by oocyte-derived GDF9 (Orisaka et al., 2006). FSH stimulation is also essential for LHCGR expression. LH also plays a major role in regulating follicle and oocyte maturation (De Los Reyes et al., 2017). LH exerts its effects through binding to its receptor (LHCGR) and enhances the final differentiation of the granulosa cells and the enzymes responsible for androgen production in the theca cells (Hattori et al., 2018). Besides the factors described above, various local factors such as insulin-like growth factor I (IGF-I) (Zhao et al., 2001), epidermal growth factor (EGF) (Fujihara et al., 2014; Alves et al., 2017), fibroblast growth factor (FGF) (Lu et al., 2015) have to work synergistically in an autocrine or paracrine manner to enhance granulosa cell proliferation, maintenance of follicular integrity, and the survival of oocytes *in vitro*.

1.2.3 Estrous induction and manipulation in domestic cats and wild felids

Estrous induction has been performed as one of the assisted breeding techniques for the animals that missed breeding opportunities, treatment of prolong anestrus, attempted to service in non-breeding seasonal, superovulation and synchronization of ovulation for embryo transfer programs (Kutzler, 2007). The most common hormones

used to stimulate folliculogenesis in felids are pFSH (Tsutsui et al., 1989; Verstegen et al., 1993) and eCG (Wildt et al., 1978). The recent availability of recombinant follicle stimulating hormone (rhFSH), with its high level of purity and batch-to-batch consistency appear to be an attractive alternative for ovarian stimulation in several mammalian species (Daya and Gunby, 2000). However, FSH has a short half-life and need to be used twice daily for 3-6 consecutive days that in turn, can cause stress to the animals (Kutzler, 2007). Thus, apart from FSH, other hormones have been used and are considered as potentially alternatives for estrous induction in wild felids. In contrast to pFSH, eCG has a long half-life. This gonadotropin can initiate folliculogenesis after a single injection (Tsutsui et al., 2000b). Due to this important reason, eCG is generally used in practice (Tsutsui et al., 2000b; Graham et al., 2006; Pelican et al., 2006a; Kutzler, 2007; Santymire et al., 2011). However, the disadvantage of eCG in felids are anti-gonadotropin antibodies (Swanson et al., 1995) and less precise information about an appropriate dose for either domestic cat or wild felids (Barone et al., 1994). Folliculogenic and luteotrophic activities of eCG have been well documented *in vivo* and *in vitro*. In the cats, eCG traditionally uses for stimulate follicular growth. High dosages or multiple injections of eCG are known to induce ovulation (Swanson et al., 1997). The gonadotropin responsiveness is influenced by the stage of estrous cycle, for an example the good ovarian response to gonadotropin treatment in Cheetah has been observed when they are under anestrous stage or inactive ovarian activity (Howard et al., 1997).

Several protocols using eCG to induce follicular development and human chorionic gonadotropin (hCG) to induce ovulation (Table 2) prior to artificial insemination (AI) have been developed in the domestic cat for application in wild felids

(Pelican et al., 2006b). Unfortunately, the success rate of AI remains inconsistent in many wild cat species including tiger (Donoghue et al., 1990; Pukazhenthi and Wildt, 2004) , clouded leopard (Howard et al., 1997), and puma (Barone et al., 1994). The pregnancy rates from AI after gonadotropin treatment are 12.5% in pumas (Barone et al., 1994), 10% in tigers (Donoghue et al., 1996) and 20% in clouded leopards (Barone et al., 1994). The reason for the low AI success rate in felids is likely multifactorial and varied among different species. The studies in the domestic cat suggested that the elimination rate of eCG in blood circulation are very slow. This phenomenon can cause ancillary follicle and secondary corpora luteal formation (Swanson et al., 1995) resulting in an abnormal endocrine environment (Graham et al., 2000). Another study comments that the low ovulation rate may associated with LH receptor on gonadotropin treated cats. The low eCG dosages may be insufficient to stimulate LH receptor expression in some wild felids. Earlier report of eCG/hCG regimens showed ovarian hyperstimulation in the puma (Miller et al., 1990), cheetah (Howard et al., 1992), clouded leopard (Howard et al., 1997) and fishing cat (Santymire et al., 2011). From the results of those relevant studies, it is appeared to be remarkable that there is species specific sensitivity to eCG among felids species and should be reminded that the chorionic gonadotropin dosage is not depend on body mass (Barone et al., 1994) (Table 3).

Table 3. Comparison of gonadotropins dosages in different felids species.

Species	Average body weight (kg)	Gonadotropins dosage		Outcomes	References
		eCG (IU)	hCG (IU)		
Domestic cat	2	100	75	95% exhibited behavioral estrus	Swanson et al., 1997
		100	75	100% fecal estradiol metabolite concentration increased	Graham et al., 2000
		150	100	83% proportion of recovered oocytes grade mature	Roth et al., 1997
Black-footed cat	2	100	75	100% Estradiol peak	Herrick et al., 2010
Tigrina	2	200	150	25% pregnancy	Swanson and Brown, 2004
Sand cat	2.5	150	100	100% estradiol peak	Herrick et al., 2010
Pallas' cat	3.5	100	75	100% estradiol peak	Brown et al., 2002
		200	150	100% estradiol peak	Brown et al., 2002
		300	150	66% estradiol peak	Brown et al., 2002
Fishing cat	6	150	100	100% fecal estradiol metabolite concentration hyper-elevated	Santymire et al., 2011
Ocelot	9	100	75	100% elevated serum estradiol-17β	Swanson et al., 1996
		200	150	100% elevated serum estradiol-17β	Swanson et al., 1996
		400	150	100% elevated serum estradiol-17β	Swanson et al., 1996
		500	225	100% elevated serum estradiol-17β	Swanson et al., 1996

Species	Average body weight (kg)	Gonadotropins dosage		Outcomes	References
		eCG (IU)	hCG (IU)		
Clouded leopard	15	25	50	100% peak preovulatory estradiol concentration	Brown et al., 1995
		50	75	100% peak preovulatory estradiol concentration	Brown et al., 1995
		75	50	100% peak preovulatory estradiol concentration	Brown et al., 1995
		100	75	100% peak preovulatory estradiol concentration	Brown et al., 1995
		50	75	100% elevated serum estradiol-17β	Howard et al., 1996
		100	75	100% elevated serum estradiol-17β	Howard et al., 1996
		200	100	100% elevated serum estradiol-17β	Howard et al., 1996
		25	75	25% ovulation	Howard et al., 1997
		50	75	100% ovulation	Howard et al., 1997
		75	75	80% ovulation	Howard et al., 1997
		100	75	80% ovulation	Howard et al., 1997
		200	140	100% ovulation	Howard et al., 1997
		400	280	100% ovulation	Howard et al., 1997
		100	75	42% ovulation	Pelican et al., 2006

Species	Average body weight (kg)	Gonadotropins dosage		Outcomes	References
		eCG (IU)	hCG (IU)		
Snow leopard	30	600	300	100% elevated serum estradiol-17β	Roth et al., 1997
		100	100	16% ovulation	Howard et al., 1997
Cheetah	35	200	100	70% Ovulation	Howard et al., 1997
		400	250	70% ovulation	Howard et al., 1997
		100	100	75% ovulation	Barone et al., 1994
		200	100	50% ovulation	Barone et al., 1994
Puma	35	1,000	800	80% follicular development	Miller et al., 1990
		1,250	1,000	100% ovulation, 33% pregnancy	Moore et al., 1981
		2,000	800	100% follicular development	Miller et al., 1990
Asiatic lion	120	750	750	76% overt signs of behavioral estrus	Umapathy et al., 2007
Siberian tiger	200	1,000	750	overt signs of behavioral estrus	Donoghue et al., 1993

1.2.4 Assisted Reproductive Technologies (ARTs) in the cats

In the last four decades, there has been extensive progress in feline assisted reproduction. Artificial insemination (AI) in domestic cats often used in research to serve as a model for wild felids species. The first successful pregnancy from AI was occurred in 1970 (Sojka et al., 1970). To date, AI technique using fresh, chilled and frozen semen has been developed. The sperm concentration and the insemination site are critical for successful fertilization (Table 4). In the absence of the mating stimuli, ovulation must be artificially induced before AI. Spermatozoa should be inseminated within 49 hours after ovulation induction because no fertilizations occurred after this time (Sojka et al., 1970; Howard et al., 1993). While the frozen-thawed spermatozoa, which affected sperm longevity, should inseminate close to ovulation. The insemination tools and techniques are modified in many studies. The AI techniques divide into two main methods. First, the surgical methods, including intrauterine AI (Swanson and Godke, 1994; Tsutsui et al., 2000a; Tsutsui et al., 2011), laparoscopic intrauterine AI (Roth et al., 1997b), intratubal AI (Tsutsui et al., 2001) and laparoscopic oviductal AI (Lambo et al., 2012; Swanson, 2012). Second, the non-surgical methods consist of intravaginal AI (Tanaka et al., 2000), Transcervical AI (intrauterine AI with tom cat catheter) (Zambelli and Cunto, 2005) and endoscopic intrauterine AI (Zambelli et al., 2015a; Zambelli et al., 2015b). However, to fertilization achievement, bearing in mind that AI success rate is influenced by type of spermatozoa, the number of total spermatozoa used, the site of sperm deposition (Tsutsui, 2006) and ovulation time (Howard et al., 1992).

The concepts of *in vitro* producing embryo from the oocyte has been developed in both domestic and wild cats in order to conserve the female gamete from the

ovariohysterectomy or accidentally died. The cumulus oocyte complexes (COCs) are rescued from the ovaries and cultured. The classification of COCs is divided into four grades as shown in Table 5.

Table 4. The summary of artificial insemination guideline in domestic cat.

Insemination site	Fresh semen (10^6 spermatozoa)	Pregnancy rate	Frozen semen (10^6 spermatozoa)	Pregnancy rate
Intravagina	5-50	54	50-100	11
	80	78	25	0
Intrauterine	2.4-19.2	14*	50	57
		50**	40***	28
	8	80	25	75
Intratubal	2	25	10***	80*
	4	43		20**

*AI before ovulation, **AI after ovulation, ***epididymal sperm

Table 5. Classification of COCs (Wood and Wildt, 1997).

Class	Morphology
I	Five or more layers of cumulus cells
II	Two to four layers of cumulus cells
III	Partial layers of cumulus cells with complete corona radiata
IV	Partially-to-completely denuded oocytes

The good quality COCs (class I and II) have ability to develop more than poor quality. Therefore, the COCs grading before *in vitro* maturation is one of the important steps of successful fertilization (Wood and Wildt, 1997). The *in vitro* maturation (IVM) is important process which is allowing the good quality COCs to develop into metaphase II stage and prompt for fertilization (Pope et al., 1997). After maturation process, there are two techniques for *in vitro* insemination. First, *in vitro* fertilization (IVF) (Pope et al., 1993), the viable spermatozoa penetrated to mature oocyte and fertilization occurred. Second, intracytoplasmic sperm injection (ICSI) (Pope et al., 2012), the sperm (from ejaculated sperm (Pope et al., 1998), epididymal sperm (Bogliolo et al., 2001) and testicular sperm (Comizzoli et al., 2006; Buarpung et al., 2013)) is injected into the oocyte by the glass capillary pipette. The embryo from both IVF and ICSI procedures can be transferred into egg donors or into gonadotropin treated recipients (Pope, 2000). The embryo transfer (ET) has been performed in both domestic (Dresser et al., 1988; Kanda et al., 1995) and wild cats using both fresh (Kanda et al., 1995) and cryopreserved embryos (Dresser et al., 1988). Transferring embryos to either the oviduct or the uterine horn has resulted in pregnancies (Pope et al., 1994; Pope et al., 2006).

During the last twenty years, *In vitro* follicle culture presents a possibility to grow early stage follicles to the advanced stage producing developmentally competence oocytes (Jewgenow, 1998; Fujihara et al., 2012; Gupta and Nandi, 2012; Songsasen et al., 2012). Such approach would benefit young cancer survivors who are facing infertility or premature menopause due to cancer treatments. Furthermore, this strategy also rescues genetics of endangered wildlife that die prematurely or unexpectedly before being able to reproduce.

For the cat, previous studies have shown that ovarian tissue culture of primordial and primary follicles sustained their viability up to 14 d if culture on an agarose gel since the tissue can expose to the oxygen in *in vitro* environment (Fujihara et al., 2012). Secondary and early antral follicles have been cultured directly in the culture plate (two-dimensional culture system). The most successful experiments were carried out in the mouse since it was possible to obtain normal offspring from preantral follicles culture (Motohashi et al., 2011). Although this two-dimensional culture system has been successful in the rodent model, it has not been shown to support follicular development in larger mammalian (West et al., 2007). It may be because the lack of proper development of their larger oocyte size and required longer culture period for larger animal species (West et al., 2007). After three to five days of culture, the follicles lose their structure by attachment on the culture plate surface (Desai et al., 2012). Follicles cultured in two-dimensional approach lose their normal morphology within 3 to 5 days, as the granulosa cells proliferate and spread across the surface of the plate (Desai et al., 2012). This results in follicle extrusion, basement membrane disruption and dysfunction of the gap junctions, which play a key role for the communication between the oocyte and cumulus cells (Gilchrist et al., 2008). Dysfunction of the gap junctions can cause arrest in follicular growth and impaired meiotic competence (Teng et al., 2016). Thus, the three-dimensional culture system imitates ovarian stromal structure was invented in order to sustain follicular architecture. Although, live mouse offspring were produced from preantral follicle cultured in alginate hydrogel (Xu et al., 2006a), this approach has not resulted in live birth in other species, including the cat. During the last decade, the biomedical engineering techniques became important tools for discover the novel materials to mimic ovarian cortex texture. The small size follicles

need more density surrounding tissue than the larger size counterparts (Kreeger et al., 2005). Therefore, the appropriate biomaterials for follicle culture must 1.) maintain their spherical architecture and contact with surrounding somatic cells (Green and Shikanov, 2016) 2.) absorb nutrients and exchange waste products between the follicle and culture medium (Li et al., 1996) 3.) non or less toxicity to the follicle (Vanacker and Amorim, 2017) and 4.) degradable material allowing follicle growth (Shikanov et al., 2011; Shea et al., 2014; Zhou and Shikanov, 2018). Various types of biomaterials such as collagen (Loret de Mola et al., 2004; Sharma et al., 2009), alginate (Xu et al., 2009a; Xu et al., 2009b; Xu et al., 2010; Brito et al., 2014; Filatov et al., 2015; Vanacker and Amorim, 2017), fibrin (Xu et al., 2013; Luyckx et al., 2014; Paulini et al., 2016), collagen-alginate (Kreeger et al., 2005) fibrin-alginate (Shikanov et al., 2009; Brito et al., 2016) and matrigel-alginate (Vanacker et al., 2012; Sadr et al., 2018) have been applied for these purposes.

1.2.5 Factors influence folliculogenesis

Folliculogenesis starts when the resting follicles leave their ovarian reserve pool to enter the growing phase. The understanding of molecular mechanisms control activation of resting follicles is important for clinical implications in mobilizing resting follicles in patients suffering from infertility due to either ovarian dysfunction or cancer treatments (Luyckx et al., 2013; Amorim and Shikanov, 2016; Chiti et al., 2016; Paulini et al., 2016; Chiti et al., 2017). Various mechanisms regulating follicular growth and atresia in mammalian ovaries have been clarified, not only their responses to gonadotropin hormone or endocrine stimulation but also their intraovarian regulation by growth factors, cytokines and intracellular proteins (Knight and Glister, 2003). Autocrines, paracrines and endocrines from follicular cell itself are the factors effecting

to follicular growth and development (Gilchrist et al., 2006). The oocyte and somatic cells are driving folliculogenesis from the primordial to the preovulatory follicle stage (Eppig et al., 1997; Eppig, 2001; Gilchrist et al., 2004). The secreting factors from each follicle cell type contribute their action either directly or indirectly by supporting other growth factors function (Fortune, 2003; Su et al., 2003). The growth and development of follicles are depended on effective two-way communication between oocyte and its surrounding follicular cells (Su et al., 2004; Hirao, 2012). Paracrine factors regulating folliculogenesis can divide into oocyte-secreted factors (OSFs) (Gilchrist et al., 2008; Emori and Sugiura, 2014), granulosa cell secreting factors (Monniaux, 2016) and theca cell secreting factor (Parrott and Skinner, 1998; Orisaka et al., 2009). However, the previous studies are widely discussed in mouse (Eppig et al., 1996; Eppig and O'Brien, 1996; Eppig et al., 1998) and human (Telfer and McLaughlin, 2011). In the cat, there are some publications concerning the impact of growth factors on follicular development (Alves et al., 2012; Fujihara et al., 2012; Fujihara et al., 2014; Alves et al., 2017; Thuwanut et al., 2017). However, the overall mechanisms effecting folliculogenesis in the cat still need further investigation.

One of the interesting factors is insulin-like growth factor I (IGF-I). Several studies found the stimulating effect of IGF-I on ovarian folliculogenesis by enhance both proliferation and differentiation of follicular cells by potentiating gonadotropin's actions (Monget et al., 1996; Mason and Franks, 1997). The interaction between IGF-I and FSH increases mRNA levels of IGF-I and FSHR, and FSH increases the expression of the IGFR-I mRNA in caprine secondary follicle culture (Brito et al., 2012). Moreover, IGF-I also stimulates steroidogenesis in thecal cells (Spicer and Stewart, 1996). In bovine, it was clearly established that, IGF-I stimulates proliferation of granulosa cells from

1–3 mm follicles (Monget et al., 2002) and stimulate secretion of progesterone by granulosa from the follicles which are larger than 5 mm (Monniaux and Pisselet, 1992). In the mouse, low doses of IGF-I (1 or 10 ng/mL) increases diameter of 140-160 μm preantral follicle, while 100 ng/mL exerts no effect (Zhao et al., 2001). From the previous studies, it can infer that the effect of IGF-I is depended on the stage of follicle development or different pathways of the intracellular signaling during follicle growth. In the cat, IGF-I exerts positive effects on follicles collected during the luteal phase (Alves et al., 2012). Moreover, the combination of IGF-I and epidermal growth factor (EGF) have been shown to promote preantral follicles growth, but not the enclosed oocyte (Alves et al., 2017). Therefore, IGF-I driving folliculogenesis from preantral to antral stage is also interesting to discover in the cat.

Another key factor is stem cell factor (SCF), which is a cytokine growth factor secreted by granulosa cells. SCF presents in all stages of follicle development in the mouse (Driancourt et al., 2000) and human (Tuck et al., 2015). SCF supports primordial follicle growth in the rat (Parrott and Skinner, 1999) and rhesus monkey (Lu et al., 2015). This paracrine signal binds to c-kit receptor which is expressed in theca cells and oocytes (Hutt et al., 2006a; Hutt et al., 2006b; Thomas and Vanderhyden, 2006). The SCF promotes the growth of primordial germ cells and regulate oogenesis and folliculogenesis (Parrott and Skinner, 1998) as well as support follicle survival. (Carlsson et al., 2006). In the cat, SCF promotes primordial to secondary follicles growth activation through the PI3K/AKT signaling pathway by upregulating mRNA expression of its receptor (Thuwanut et al., 2017). However, there have been no studies that recognize the effect of SCF in the preantral to antral follicles in this species. Therefore,

the information of the role of SCF on the middle to late stage folliculogenesis may improve the *in vitro* follicle culture quality in the cat.

1.3 Thesis Objective

1. To investigate responsiveness of domestic cat follicles to eCG at different developmental stages.

2. To determine the tiger follicle responsiveness to eCG.

3. To examine the effect of eCG in combination with IGF-I and/or SCF on various stages of folliculogenesis

1.4 Hypothesis

1. Follicles at different developmental stages show differ responses to eCG.

2. The responsiveness of tiger follicles to eCG is different from the domestic cat.

3. The influence of eCG in combination with IGF-I and/or SCF is different among developmental stages.

4. *In vitro* follicle culture with eCG and growth factors improve follicle development in domestic cat.

1.5 Keyword

Feline, folliculogenesis, growth factor, *in vitro* culture, preantral follicle

1.6 Research merits

1. Provide basic knowledge of feline folliculogenesis during secondary follicle to antral follicle stage.

2. Describe the responsiveness of the follicle after eCG stimulation in the domestic cat and the tiger.

3. Provide *in vitro* stimulation protocols of eCG alone or combination with growth factors for domestic cat follicle.

4. Potentially produce antral follicle from *in vitro* culture.

5. Develop the assisted reproductive technologies (ARTs) in order to apply in genetically valuable wild felids.

Chapter II

Equine chorionic gonadotropin induces *in vitro* follicular growth from the multi-layered secondary developmental stage in cats

2.1 Abstract

Equine chorionic gonadotropin (eCG) has been commonly used to induce estrus in several felid species. However, the mechanisms by which this gonadotropin regulates cat folliculogenesis are still unclear. We investigated the responsiveness of cat ovarian follicles at different developmental stages to various eCG concentrations supplemented *in vitro*. Follicles were mechanically isolated from the ovaries of 22 cats and categorized into three developmental stages based on their morphology and diameter: 1) two-layered secondary follicle (SF), 100-150 µm (n = 139); 2) multi-layered SF, 150-300 µm (n = 154); and 3) early antral follicle (AF), ≥ 300-500 µm (n = 123). The follicles were then encapsulated in 0.5 % (w/v) sodium alginate and cultured for 12 days in Minimum Essential Medium supplemented with 0, 0.05, 0.1 or 0.5 IU/mL eCG. Follicle and oocyte diameters were assessed every 3 days. On Day 12, mRNA expression levels of *FSHR, LHCGR, GDF9, BMP15, CYP17A1, CYP19A1* and *STAR* were analyzed using real-time PCR. After being cultured for 12 days, follicle growth and mRNA expression of two-layered SF were not influenced by eCG at all concentrations ($P > 0.05$). However, the concentration of eCG at 0.05 IU/mL stimulated follicular growth and gene expressions in the multi-layered SF and early AF ($P < 0.05$). Correspondingly, the diameter of oocytes in the multi-layered SF and early AF treated with 0.05 IU/mL eCG was unchanged. Considering the mRNA expression, the level of *STAR* was enhanced in the early AF ($P < 0.05$) and tended to increase in the multi-

layered SF (P = 0.08) cultured in 0.05 IU/mL eCG, whereas the expression of other genes was not affected. In sum, the responsiveness of cat follicles to eCG is apparent from the multi-layered SF stage onward. The eCG supplementation at 0.05 IU/mL appeared to be optimal for the follicle culture in the domestic cats.

2.2 Introduction

Reproductive outcomes of the zoo-housed felids are poor due to behavioral incompatibility (DeCaluwe et al., 2016) and poor semen quality (Axner and Linde Forsberg, 2007; Ruiz-Lopez et al., 2012). Therefore, assisted reproductive technologies (ARTs), including estrous synchronization and artificial insemination (AI) have been introduced to produce offspring from non-reproductive animals and help to maintain gene diversity *ex situ* (Brown, 2011). Exogenous gonadotropins, including equine chorionic gonadotropin (eCG), porcine follicle stimulating hormone (pFSH), gonadotropin releasing hormone agonist (GnRH agonist), human chorionic gonadotropin (hCG), human menopausal gonadotropin (hMG) and porcine luteinizing hormone (pLH) have been used to manipulate reproductive cycles in both domestic and wild cats, including tigers (Donoghue et al., 1996; Chagas e Silva et al., 2000; Gjorret et al., 2002; Graham et al., 2006), Asiatic lions (Umapathy et al., 2007), cheetahs (Brown et al., 1996), snow leopards (Brown et al., 1994), clouded leopards (Tipkantha et al., 2017), ocelots (Swanson et al., 1996), fishing cats (Santymire et al., 2011), Pallas's cats (Delaski and Gamble, 2015), sand cats (Herrick et al., 2010), tigrinas (Micheletti et al., 2015) and black-footed cats (Pope et al., 2012) with varying results. Although eCG has been frequently used for folliculogenesis purposes in felids, understanding of the associated molecular changes is limited.

Several genes are involved during folliculogenesis, and can therefore be used as indicators for ovarian follicle development, i.e., oocyte growth and granulosa cell proliferation. An *in vitro* bioassay study has demonstrated that eCG stimulate follicle-stimulating hormone receptor (*FSHR*) and luteinizing hormone/chorionic gonadotropin receptor (*LHCGR*) expression in the granulosa cells (Saint-Dizier et al., 2007). From preantral to early antral follicular stage, growth differentiation factor-9 (*GDF-9*) plays both an autocrine and a paracrine role in regulating oocyte growth and granulosa cell proliferation, respectively (Thomas et al., 2003).

Bone morphogenetic protein-15 (*BMP-15*) is also an oocyte derived factor that plays a key role in follicular growth after the primary follicular stage (Cadenas et al., 2017). In addition to the assessment of oocyte growth, granulosa cell proliferation and theca cell formation can be determined by the up-regulation of cytochrome P450 aromatase (*CYP19A1*) and cytochrome P450 steroid 17 α-hydroxylase/17,20 lyase (*CYP17A1*), respectively. These enzymes are involved in estrogen and androgen production, and thus, indicate the degree of steroidogenesis. Moreover, steroidogenesis representing the advanced stage of folliculogenesis could be determined by the expression of the steroidogenic acute regulatory protein (*STAR*) in the granulosa and theca cells (Murayama et al., 2012).

Previous studies in cats have demonstrated that FSH supplemented medium accelerated *in vitro* growth of isolated follicles (Songsasen et al., 2012). However, there was no report on eCG supplementation *in vitro* despite eCG being widely used to induce estrus *in vivo*. The aims of the present study were to investigate the responsiveness of different follicle developmental stages to eCG and the optimal concentration of eCG to support *in vitro* follicle growth in domestic cats.

2.3 Materials and Methods

The procedures used in this study was approved by Chulalongkorn University Animal Care and Use Committee.

2.3.1 Chemicals

All chemicals used in this study were purchased from Sigma Aldrich, St Louis, MO, USA, unless otherwise indicated.

2.3.2 Sample collection

Inactive ovaries without corpora lutea and containing < 2 mm follicles were collected from 22 domestic cats (aged between 5 months and 3 years) that underwent routine ovariohysterectomies at local veterinary clinics in Front Royal, Stephens City, and Harrisonburg, Virginia, USA. Ovaries were transported to the laboratory at 4°C in L-15 medium supplemented with antibiotics (3 mg/mL penicillin G sodium, and 3 mg/mL streptomycin sulfate) within 6 h after surgery.

2.3.3 Follicles isolation, encapsulation and culture

Upon arrival at the laboratory, two-layered secondary follicles (100-150 µm; n = 139) and multi-layered secondary follicles (150-300 µm; n = 154) were mechanically isolated from one ovary of each cat using a method described by Jewgenow and Goritz (Jewgenow and Göritz, 1995). The remaining ovary of each cat was processed for the isolation of early antral follicles (the presence of an antral cavity and surrounded by two to three layers of thecal cells, diameter less than 500 µm; n = 123) using a 25-gauge needle as described by Kreeger et al (Kreeger et al., 2005). Individual follicles were then encapsulated into a bead of alginate solution (slightly adapted from methods previously described) (Heise et al., 2005) with the first step being transferred into a 3 µL droplet of 0.5% alginate using a 10 µL pipette. Single droplets were

immersed into a solution containing 50 mM $CaCl_2$ and 140 mM NaCl and alginate beads were allowed to cross-link for 2 min, and then washed three times in a culture medium: MEM (supplemented with 3 mg/mL BSA, 2 mM L-glutamine, 10 ng/mL activin, 10 µg/mL insulin, 1.9 µg/mL transferrin, 5 µg/mL selenium, 10 IU/mL penicillin G sodium and 10 mg/mL streptomycin sulfate). The entire procedure was performed on a warm plate set at 38°C. Two to three follicles of the same developmental stage were cultured in 500 µL of culture medium supplemented with one of four eCG concentrations (0, 0.05, 0.1 and 0.5 IU/mL) at 38.5°C in 5% CO_2 in humidified air for 12 d. Half volume of the culture medium was changed every 3 d throughout the study period.

2.3.4 Assessment of follicle and oocyte growth

Follicle and oocyte diameters were assessed under an inverted microscope (Leica LEITZ DM IL; Leica Germany). Each follicle was sized from the outer layer of somatic cells, with the measurements including the widest diameter and perpendicular width in the initial assessment. The mean of these two metrics was calculated and reported in terms of diameter. Oocyte size, excluding the zona pellucida, was evaluated using the same method. The mean actual diameters of each follicle and oocyte on Days 0, 3, 6, 9 and 12 were recorded. The actual diameter of Day 3, 6, 9 and 12 divided by the actual diameter of Day 0 was calculated and reported as the relative diameter of the follicle. Follicles were considered to be degenerating if either the oocyte was no longer surrounded by a layer of granulosa cells; the oocyte was dark, the granulosa cells had become dark and fragmented, or the diameter of the follicle decreased (Xu et al., 2009b).

2.3.5 Quantitative RT-PCR (qRT-PCR)

To examine mRNA expression in follicles of different developmental stages cultured in different concentrations of eCG (total 12 treatment groups), follicles were frozen at -20°C. Total RNA was extracted from cat follicles using an absolutely RNA nanoprep kit (Agilent Technologies, Santa Clara, California, USA) following the manufacturer's protocol. DNA contamination was treated by Rapid Out DNA removal kit (Thermo-Scientific, Massachusetts, USA). The quantity of RNA was measured using a spectrophotometer (NanoDrop One, Thermo-Scientific, Massachusetts, USA). Complementary DNA was synthesized from mRNA using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Mannheim, Germany) with the same amount of mRNA (2 ng from each sample), and stored at -20°C until quantitative polymerase chain reaction (qPCR) analysis was performed. SyBr Green Mastermix (FastStart Essential DNA Green Master, Roche, Mannheim, Germany) was applied as a fluorescence probe for qPCR. All primer sequences used in this present study are shown in Table 6. Each PCR reaction consisted of 2 µL DNA and 18 µL of a mixture (10 µL of Fast Start Essential DNA green master (Roche, Basel, Switzerland), 1 µL each of 10 mM forward and reverse primers and 6 µL of nuclease-free water). qPCR reactions were performed in duplicate using Light Cycler® 96 (Roche, Basel, Switzerland). Each amplification reaction consisted of the pre-incubation period (95°C for 10 min), 45 cycles of three steps of amplification (95°C for 30 s, specific annealing temperature of primers of interest for 10 s and 72°C for 10 s) and a melting step (65°C for 30 s). The negative control was run together with samples from every amplification. Primer efficiency was performed in each gene by five serial dilutions of DNA. The Ct value of each gene was normalized against the Ct of beta-actin which were shown to be more stable than succinate

dehydrogenase complex flavoprotein subunit A (SDHA) using Best Keeper software evaluation (Pfaffl et al., 2004). The delta delta Ct (ΔΔCt), which included primer efficiency, was used to calculate fold changes of interesting genes in every group of cultures normalized to the control group, as described by Pfaffl (Pfaffl, 2001).

Fold change = $(1 + \text{primer efficiency})^{-(\Delta\Delta Ct)}$ (Rao et al., 2013)

Table 6. Primers sequence for qPCR.

Primer	Sequence 5'-3'	Accession number	Annealing temperature (°C)	Product size (bp)	Efficiency (%)	Reference
β-actin	F: ATCCACGAGACCACCTTC	AB051104	57	75	94	Songsasen et al., 2017
	R: CACCGTGTTAGCGTAGAG					
SDHA	F: GCCTTGGATCTCCTGATG	DQ402986	57	75	107	Songsasen et al., 2017
	R: ATGGATGGACCCGTCTTC					
FSHR	F: GCCTCACCTACCCTAGCCACTG	NM_001048014.1	64	163	84	Hobbs et al., 2012
	R: GTAACTGGATTCCTCGTCTTCTGC					
LHCGR	F: GCACAGCAAGGAGACCAAATA	XM_011281083.1	58	101	91	Veraguas et al., 2017
	R: CTTGGGTGAGCAGAAACCATAG					
GDF9	F: CATCCGTGGACCTGCTATTT	NM_001165900.1	58	129	94	Veraguas et al., 2017
	R: CCAGGTTGCACACACATTTC					
BMP15	F: AGGCTTTGGAATAAGAAGGG	NM_001165898.1	55	124	102	*
	R: ACAAAAATGCAGTGTCCAAG					
CYP17A1	F: CCGAGATGAGTTGCTGAG	NM_001009371.2	57	105	87	Songsasen et al., 2017
	R: GAGTTCATCCTGGCTTGG					
CYP19A1	F: CAATCCTGCTGCTCACTG	GU306147.1	57	84	92	Songsasen et al., 2017
	R: CCATGCAATAGCCAGGAC					
STAR	F: ATGGAAGCGATGGGAGAG	NM_001246196.1	57	90	107	Songsasen et al., 2017
	R: CAACTCGTGGGTGATGAC					

*BMP15 was designed by NCBI/ Primer-BLAST.

2.3.6 Statistical analysis

Data were analyzed using R, version 3.3.2 R, (http://www.r-project.org). Normal distribution and equal variances were tested by Shapiro test and Bartlett's test, consecutively. Follicle growth on Days 3, 6, 9 and 12 was presented as the relative diameter compared to the initial diameter (Day 0). The linear mixed model was followed by the least square mean, and a pairwise test (Tukey's HSD) was used to evaluate the differences in the mean relative diameter of the follicles in each developmental stage across 3, 6, 9 and 12 days. A paired t- test was used to compare the difference in growth data of the follicles between Day 0 and Day 12 of culture. The ΔΔCt of qPCR expression data from each treatment were analyzed by Kruskal-Wallis test followed by pairwise comparisons using Tukey and Kramer (Nemenyi) test. Differences were considered significant when $P < 0.05$.

2.3.7 Experimental design

Effects of eCG on growth of two-layered SF (100-150 μm), multi-layered SF (150-300 μm) and early AF (≥ 300-500 μm) were tested by culturing the follicles in MEM supplemented with eCG at 0 IU/mL (control), 0.05 IU/mL, 0.1 IU/mL and 0.5 IU/mL. Follicle and oocyte diameters were determined in terms of actual and relative diameter on Days 0, 3, 6, 9 and 12. On Day 12 of culture, all follicles were assessed for mRNA expression of gonadotropin receptor genes (*FSHR* and *LHCGR),* oocyte ligands (*GDF9* and *BMP15)* and steroidogenic enzyme activities (*CYP17A1, CYP19A1* and *STAR*) using qPCR (three replications).

2.4 Results

The follicle of each developmental stage responded differently to eCG supplementation. The actual and relative diameters of two-layered SF were not

different among eCG concentrations ($P > 0.05$) (Table 7) and among days of culture ($P > 0.05$) (Fig. 1A). However, the actual diameter of an oocyte of the two-layered SF in all culture treatments decreased on Day 12 of *in vitro* culture (Table 7). On Day 12 of culture, the actual diameter of multi-layered SF supplemented with 0.05 IU/mL eCG was higher than Day 0 ($P < 0.05$) (Table 7). Furthermore, on Day 12, the relative diameter of follicles incubated in 0.05 IU/mL eCG was higher than those exposed to 0 and 0.1 IU/mL eCG ($P < 0.05$) (Fig. 1B). The oocyte diameters of multi-layered SF cultured in 0.05 IU/mL and 0.5 IU/mL remained constant whereas the diameter of the gamete in 0 IU/mL and 0.1 IU/mL concentration groups decreased after *in vitro* culture ($P < 0.05$) (Table 7).

After 12 days of culture, the actual diameter of early AF cultured in all eCG concentrations increased ($P < 0.05$) (Table 7). However, the relative diameter of the follicles in this stage increased only when cultured in 0.05 IU/mL eCG in relation to Day 3 ($P < 0.05$) (Fig. 1C). The oocyte diameter of early AF supplemented with eCG at all concentrations was not different from the initial size ($P > 0.05$) (Table 7).

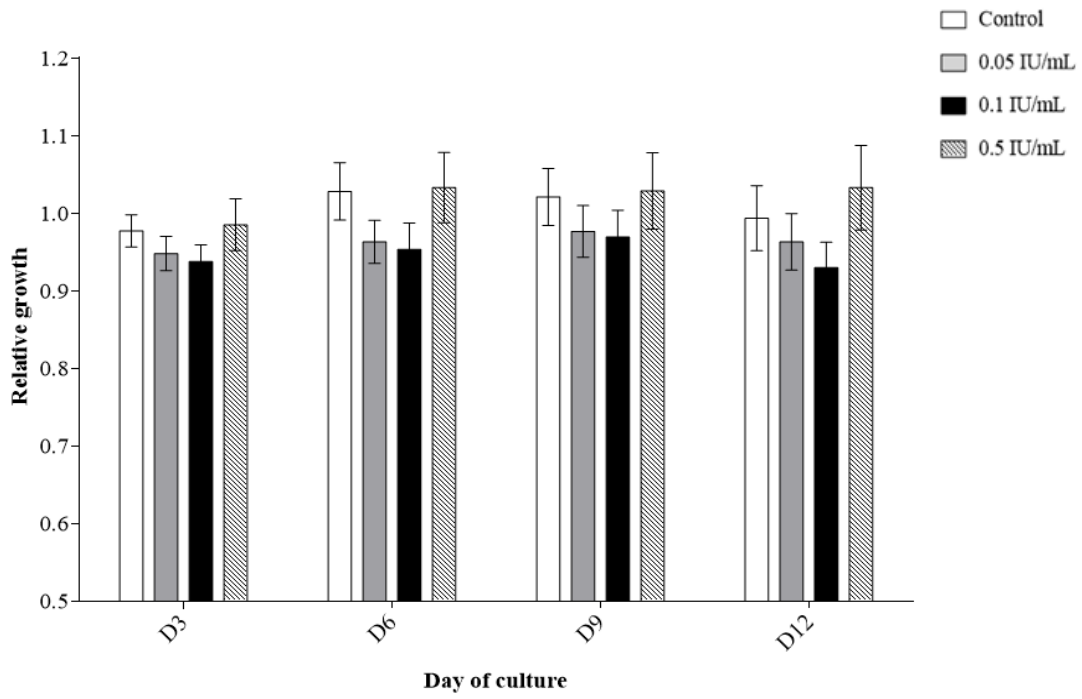
Table 7. Mean (± SEM) diameters of follicles and oocytes of various developmental stages on Days 0 and 12 of culture in the medium containing different concentrations of eCG.

Original stage (μm)	eCG (IU/mL)	n	Follicle (μm)		Oocyte (μm)	
			Day 0	Day 12	Day 0	Day 12
Two-layered secondary follicle	0	37	133.38±3.59	129.44±5.59	60.14±1.76[c]	51.53±2.91[d]
(100-150 μm)	0.05	40	128.88±4.56	122.03±6.79	56.63±2.26[c]	44.46±2.79[d]
	0.1	35	137.71±3.76	125.76±5.78	62.57±2.49[c]	49.70±3.03[d]
	0.5	27	141.30±7.05	139.00±9.51	60.74±3.28[c]	50.00±3.46[d]
Multi-layered secondary follicle	0	43	200.58±5.42	196.46±8.68	73.49±1.97[c]	64.15±2.73[d]
(150-300 μm)	0.05	41	194.76±6.96[a]	220.12±14.62[b]	70.00±1.61	68.66±2.85
	0.1	32	203.13±6.66	196.29±8.19	70.47±2.34[c]	58.55±2.85[d]
	0.5	39	207.50±4.78	214.87±9.69	72.89±1.56	66.45±3.40
Early antral follicle	0	30	366.67±10.99[a]	416.33±19.45[b]	88.50±3.87	85.50±3.94
(≥300-500 μm)	0.05	33	335.97±8.56[a]	396.13±13.13[b]	88.03±2.51	89.67±3.65
	0.1	32	347.83±11.19[a]	391.50±21.45[b]	95.63±3.21	89.33±3.69
	0.5	28	336.35±11.92[a]	372.88±20.08[b]	89.64±4.40	83.80±4.15

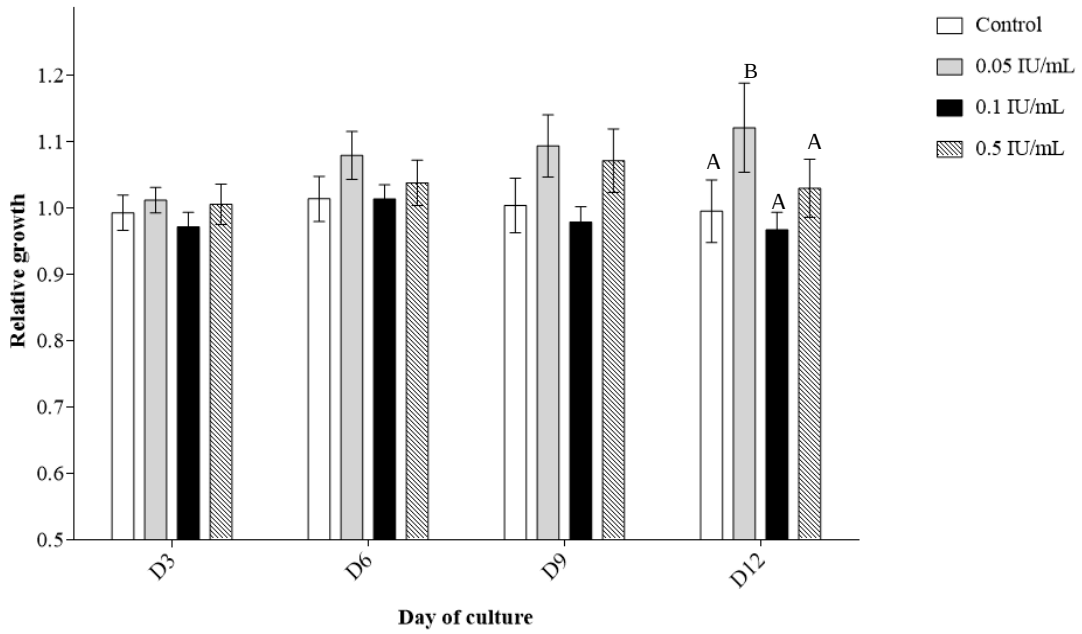
a, b are statistically significant differences ($P < 0.05$) in follicle diameter between Day 0 and Day 12 of culture in the same treatment

c, d are statistically significant differences ($P < 0.05$) in oocyte diameter between Day 0 and Day 12 of culture in the same treatment

A


Control
0.05 IU/mL
0.1 IU/mL
0.5 IU/mL
Relative growth
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
D3
D6
D9
D12
Day of culture

B


Control
0.05 IU/mL
0.1 IU/mL
0.5 IU/mL
Relative growth
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
A
B
A
A
D3
D6
D9
D12
Day of culture

C

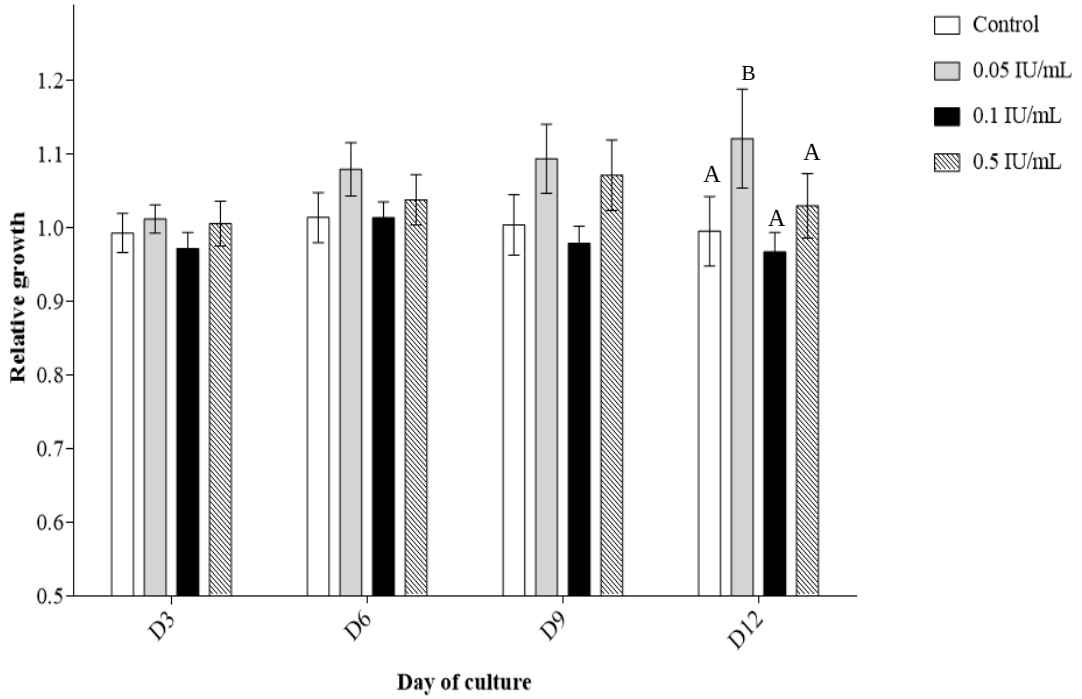
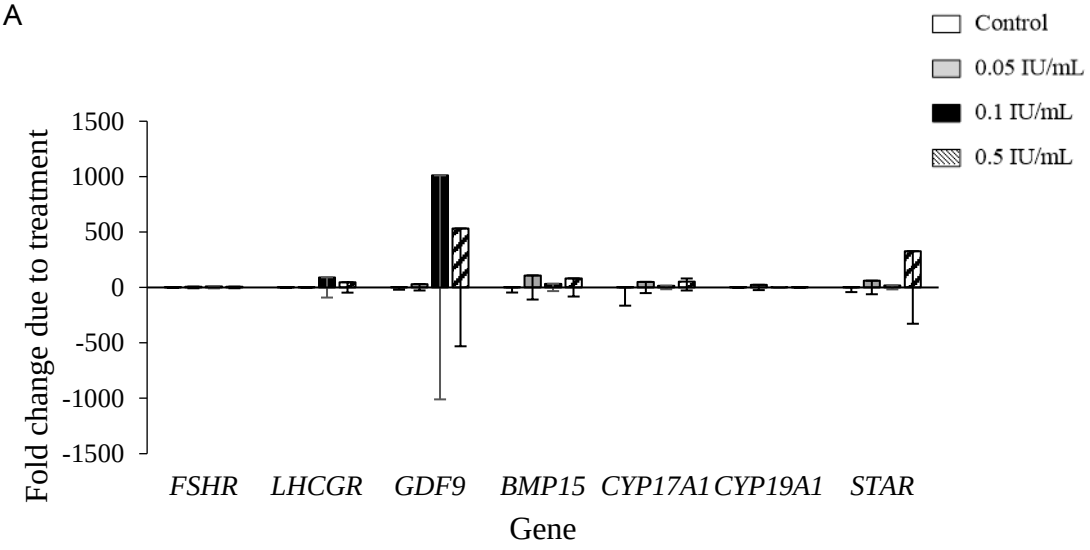
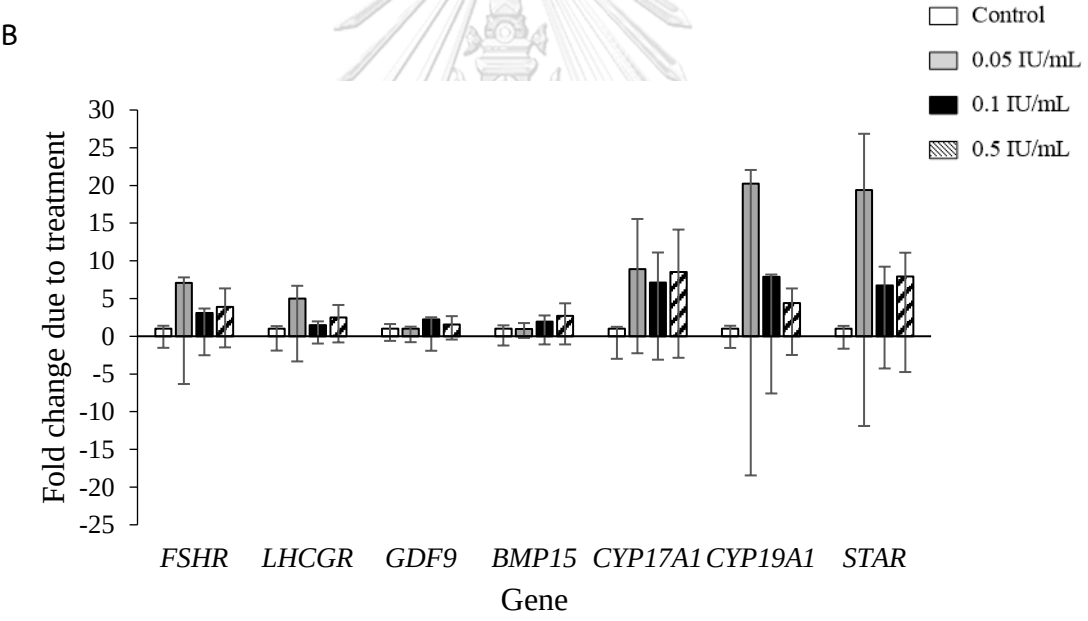


Figure 1. Relative growth of the follicles (compared to day 0). A) Relative growth of two- layered secondary follicles, B) multi-layered secondary follicles, and C) early antral follicles. Values with different superscripts (a, b) within the same treatment differ significantly ($P < 0.05$).

A


Control
0.05 IU/mL
0.1 IU/mL
0.5 IU/mL
Fold change due to treatment
1500
1000
500
0
-500
-1000
-1500
FSHR
LHCGR
GDF9
BMP15
CYP17A1
CYP19A1
STAR
Gene

B


Control
0.05 IU/mL
0.1 IU/mL
0.5 IU/mL
Fold change due to treatment
30
25
20
15
10
5
0
-5
-10
-15
-20
-25
FSHR
LHCGR
GDF9
BMP15
CYP17A1
CYP19A1
STAR
Gene

C

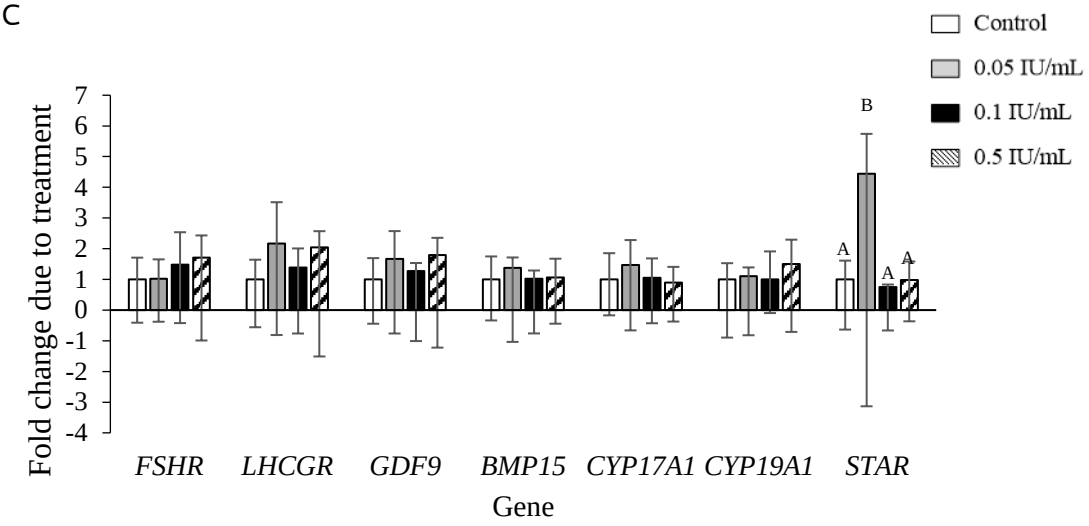


Figure 2. Fold differences in gene expression of the follicles. A) Fold change in expression of two-layered secondary follicles, B) multi-layered secondary follicles, and C) early antral follicles. Values with different superscripts (A, B) differ significantly ($P < 0.05$) among treatments.

Supplementation of eCG to the culture medium did not influence gene expression levels of the two-layered SF (Fig. 2A). However, eCG supplement at 0.05 IU/mL tended to increased *STAR* expression compared to the control group (P = 0.06) in the multi-layered SF (Fig. 2B). In the early AF, eCG supplementation at 0.05 IU/mL promoted *STAR* expression compared to the 0.1 IU/mL (P < 0.05) (Fig. 2C), while *FSHR, LHCGR, GDF9, BMP15, CYP17A1, CYP19A1 and STAR* expressions were not impacted by eCG.

2.5 Discussion

Follicular responses to FSH have been widely studied. Secondary stage follicles have proven to be a gonadotropin responsive stage in many species. However, the follicles were classified into eight types following the study of Pederson and Peter in 1968. The secondary follicles were subdivided into three groups: type 4 (early secondary: 2 layers of granulosa cells), type 5a (mid secondary) and type 5b (late secondary). In the current study, type 5a and type 5b were combined together and referred to as multi-layered SF. Here, the responsiveness of different follicle developmental stages to eCG and the optimal concentration of this gonadotropin to support *in vitro* growth of isolated cat ovarian follicles was evaluated in a three-dimensional culture system. The three different stages of follicles were encapsulated in alginate hydrogel and cultured in a medium supplemented with various doses of eCG for 12 days.

We discovered that eCG affected the development and gene expression of multi-layered SF while this gonadotropin exert no impact on two-layered SF. This assertion is supported by a previous study in cats that demonstrated the lack of responsiveness of the small-sized (60-100 μm) SF to FSH despite the presence of FSH

receptors in the granulosa cells (Jewgenow and Pitra, 1993). A previous study in the mouse has also shown that FSH stimulated *in vitro* growth of small SF (<100 μm) although the diameter change was less than that of larger follicles >130 μm (Hardy et al., 2017). Collectively, it appeared that the onset of responsiveness to gonadotropin stimulation may be slightly delayed in the cat compared to the mouse. The second reason for the lack of *in vitro* growth of the two-layered SF might be the different *in vitro* conditions in which eCG is required for the later developmental stages.

It was found that 0.05 IU/mL eCG supplementation supported the relative growth of multi-layered SF after culture for 12 days compared to control group. The signs of responsiveness were initiated by increasing follicle size and/or an increase in relative growth. Although gene expressions of multi-layered SF were not obviously changed, the levels of mRNA of the receptors (*FSHR* and *LHCGR*) and steroidogenic genes (*CYP17A1, CYP19A1* and *STAR*) increased, especially in the 0.05 IU/mL eCG-treated group. Our findings are consistent with that of a study in the mouse where it was demonstrated that high FSH dosages impaired multi-layered SF follicle development. It has been suggested that the detrimental effect of a high gonadotropin concentration is likely due to the disruption of bi-directional communication between oocytes and granulosa cells initiated by the rapid growth of the follicles (Russell et al., 2016). The response of the multi-layered SF to the eCG was different from FSH because the FSH supported follicle growth and steroidogenesis in a dose dependent manner (Songsasen et al., 2011). However, due to the growth and gene expressions of multi-layered SF, it may be stated that the proliferation and differentiation of multi-layered SF had occurred. Moreover, the granulosa and theca cells began functioning in terms of producing steroid hormone. However, the enclosed oocytes sustained their size

over the culture period and the oocyte specific genes were unchanged.

The beneficial influence of eCG was also found in early AF supplement with the 0.05 IU/mL eCG group. The responses of early AF were characterized by 1) the increase of actual and/or relative follicle diameter, 2) the steroidogenic capability, demonstrated by upregulation of *STAR* mRNA expression, and 3) the maintenance of the oocyte diameter during *in vitro* culture. Even though the absence of eCG in the culture system can conserve follicular structure in alginate hydrogel, proliferation of granulosa cells and ovarian follicle growth required gonadotropin stimulation suggesting that eCG becomes essential. We also found that increasing eCG concentration was not beneficial because *in vitro* follicle growth and expression of genes regulating folliculogenesis were not enhanced with higher gonadotropin dosages (0.1 and 0.5 IU/mL). This evidence may reinforce the fact that early AF in the cat has a limited response to eCG, as demonstrated in the multi-layered SF.

Based on the results of the current study, alginate hydrogel concentration might be one of the contributing factors for follicle development. The rigid hydrogel was required for supporting the growth of two-layered SF while the softer hydrogel allowed the expansion of the medium-size growing follicles, as demonstrated in the mouse (Xu et al., 2006b). A slow growth rate compared to previous reports was found in multi-layered SF and early AF, even if they had significantly grown in size relative to the retrieval follicles. A more easily degradable biomaterials may be applied to support follicular growth and expansion (Shikanov et al., 2009).

For the oocyte growth of the multi-layered SF, eCG sustained oocyte size compared to the control. Various growth factors including epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I) might be applied to regulate the oocytes

growth collateral with the growth of the follicles (Zhou and Zhang, 2005). The oocyte of early AF remained their initial size after cultured both in the presence or absence of eCG. This finding suggests that the growth of the oocytes after antral formation was not directly dependent on eCG. The complex mechanisms of the local factors in the oocyte itself, combined with cytokines and growth factors in the follicular fluid, may be associated with oocyte growth (Dumesic et al., 2015).

In terms of the effect of eCG on gonadotropin receptors, eCG did not impact *FSHR* and *LHCGR* expressions among treatment groups. In this study, two-layered SF appeared to be gonadotropin-independent. Thus, two-layered SF did not show the activation of *FSHR* and *LHCGR*, which was similar to the findings of the mouse study (Hardy et al., 2017). The lack of changes in *FSHR* and *LHCGR* expression in multi-layered SF and early AF in the presence of eCG was unexpected. The follicle growth should be accompanied by increasing expression of *FSHR* and *LHCGR*, which increase with exposure to FSH-like hormone (Saint-Dizier et al., 2007). The slow growth rate of the follicles in this culture system might contribute to the little change observed in mRNA of receptors.

The present study demonstrated that 0.05 IU/mL eCG had a stimulatory effect on multi-layered SF growth, but presented no effect on oogenesis, nor on oocyte-derived factor (*GDF9* and *BMP15*) expression. The lack of upregulation of oocyte ligands by eCG in our culture system showed that this culture system failed to support gamete's growth.

In this study, the level of *STAR* transcripts significantly increased in the early AF ($P < 0.05$), but did not affect the expression of *CYP17A1* and *CYP19A1*. The increase in *STAR* transcript suggested that eCG promotes steroid synthesis by supporting

cholesterol transportation (Manna et al., 2009). A previous study showed that gonadotropins (FSH and LH) activated the cAMP/PKA pathway, which in turn stimulated the production of a series of enzymes involved in steroidogenesis (Filatov et al., 2017). Accordingly, the two specific steroidogenic transcripts, *CYP17A1,* the gene encoding enzyme required for androgen synthesis (Murayama et al., 2012; Orisaka et al., 2013) and *CYP19A1*, the gene encoding P450 aromatase enzyme required for estrogen synthesis (Zhao et al., 2016), are produced by theca and granulosa cells, respectively. Non-appearance of *CYP17A1* and *CYP19A1,* especially in the early AF stage, showed that eCG has no stimulating effect on androgen and estrogen production. In contrast with the previous study in the mouse, FSH-dependent expression of genes encoding key steroidogenic enzymes (*STAR* and *CYP19A1*) were upregulated in the two-layered SF (Hardy et al., 2017). In conclusion, our findings elucidated that the responsiveness of domestic cat follicles to eCG supplementation depended on the developmental stage. Specifically, cat follicles acquire the responses to gonadotropin treatment when they reached the multi-layered SF stage. Furthermore, we also demonstrated that a low dosage of eCG (0.05 IU/mL) promoted the growth of multi-layered SF and sustained oocyte viability possibly through *STAR*. Although eCG exerted a modest effect on early AF growth, this gonadotropin upregulated *STAR* transcript, and thus, potentially influenced steroid production in this developmental stage. Finally, the study provides insight into gonadotropin regulations of cat ovarian follicles, information that is important to develop strategies to provoke *in vivo* and *in vitro* folliculogenesis.

Chapter III

Restoration of tiger (*Panthera tigris*) follicles from frozen-thawed ovarian tissues

3.1 Abstract

Cryopreservation of gametes is noteworthy as a potential tool to conserve valuable genetics of endangered wild species post-mortem. However, the quality of the follicle and oocyte within the frozen-thawed tissue is the most important part for further applications. Therefore, this study utilized frozen-thawed tiger ovarian tissue to evaluate follicular growth and oocyte nuclear configuration. Six frozen-thawed ovarian tissue from a tiger obtained post-mortem were evaluated (two tissues/replicate). Twelve isolated antral follicles obtained by mechanical isolation method were randomly allocated into two culture conditions (control and 0.05 IU/mL eCG supplementation) and cultured in an alginate hydrogel for three days. Follicle growth was evaluated on Day 0 and Day 3 by using image J program. At the end of culture, decapsulated follicles were stained with neutral red for follicle viability screening test, thereafter the oocytes were collected for nuclear status staining by using Hoechst 33258. The results showed that the follicle diameters in control group significant decreased ($P < 0.05$) after 3 days of culture. While the follicle diameters of eCG supplementation group were remained in their initial size ($P > 0.05$). The follicle survival was 100% in both groups. However, the oocyte retrieval was significant difference between the control and eCG groups (33%, n = 6 and 67%, n = 6, respectively) ($P < 0.05$). The nuclear status of all recovery oocytes was remained in germinal vesicle phase. The present study showed that tiger frozen-thawed antral follicles could not

maintain their morphology and function without eCG supplementation in the culture medium. In sum, eCG plays an important role in tiger antral follicle growth and the survival of the oocyte.

3.2 Introduction

Cryopreservation of genetic resources has proven to be a successful fertility preservation technique (Amorim et al., 2012; Dolmans et al., 2013). The ovarian tissue cryopreservation was developed for application in human cancer patients to preserve the fertility before chemotherapy or radiotherapy (Paulini et al., 2016; Dolmans, 2018). This approach has provided options for those who will lose their reproductive capacity. The biotechnology developed for human has been applied to endangered species for establishing the gamete cryopreservation banks (Jewgenow and Paris, 2006).

The tiger (*Panthera tigris*) is one of the world's most endangered felids species. Tiger population rapidly decreased because of loss of habitat, declined prey population and direct persecution (Goodrich, 2010). Assisted reproductive technology (ART) is considered as an important tool in the conservation of this endangered species, but the limiting factor of ART is the availability of mature oocytes. In cat species, several studies of frozen-thawed domestic cat (Luvoni et al., 2012; Mouttham and Comizzoli, 2017; Brito et al., 2018; Martins et al., 2018) and wild felids (Wiedemann et al., 2012; Wiedemann et al., 2013) ovarian tissue culture were demonstrated. Previous studies were focused on: 1) preantral follicle morphology/viability evaluation; 2) cumulus oocyte complexes culture; and 3.) transplantation of ovarian tissue. However, the information of the isolated follicle culture from the cryopreserved tissues especially in tiger is limited. The aims of the present study were to 1) evaluate the tiger follicle growth in an alginate hydrogel three-dimensional culture system supplemented

with/without eCG and 2) investigate the feasibility of female gametes restoration from the frozen thawed tiger ovaries.

3.3 Materials and Methods

3.3.1 Chemicals

All chemical used in this study were purchased from Sigma Aldrich, St Louis, MO, USA, unless otherwise indicated.

3.3.2 Samples

Two ovaries of post-mortem animal were obtained from The Zoological Park Organization under the Royal Patronage of His Majesty the King. Cryopreserved ovarian tissues were donated from Research unit of Obstetrics and Reproduction in Animals. The storage time in liquid nitrogen was 5 years.

3.3.3 Ovarian tissue thawing

The ovarian tissues were taken out of the cryotubes and transferred into the thawing solution, composed of 12 ml of medium 199 with Earle's salts (M 199), 20% (v/v) fetal calve serum (FCS) and 1 M sucrose at room temperature for 10 min and then transferred to the holding medium composed of M 199 containing 25 mM HEPES and 10% (v/v) FCS prior to follicle culture.

3.3.4 Follicle isolation, encapsulation and culture

Antral follicles (>500 µm; n = 12) were isolated from the cortical strips using 25G gauge needles. Individual follicles were then encapsulated into a bead of alginate solution with the first step being transferred into a 5 to 10 µL droplet (depended on follicular size) of 0.5% alginate using a 20 µL pipette. Single droplets were immersed into a solution containing 50 mM $CaCl_2$ and 140 mM NaCl and alginate beads were

allowed to cross-link for 2 min, and then washed three times in a culture medium: MEM (supplemented with 3 mg/mL BSA, 2 mM L-glutamine, 10 ng/mL activin, 10 µg/mL insulin, 1.9 µg/mL transferrin, 5 µg/mL selenium, 10 IU/mL penicillin G sodium and 10 mg/mL streptomycin sulfate). The entire procedure was performed on a warm plate set at 38°C. Two follicles were cultured in 500 µL of culture medium supplemented with 0 (control) and 0.05 IU/mL eCG at 38.5 °C in 5% CO_2 in humidified air for 3 d.

3.3.5 Assessment of follicle growth

Follicle diameters were assessed under an inverted microscope. Each follicle was sized from the outer layer of somatic cells, with the measurements including the widest diameter and perpendicular width in the initial assessment. The mean of these two metrics was calculated and reported in terms of diameter. The mean diameters of each follicle on Day 0 and Day 3 were recorded.

3.3.6 Follicle survival and oocyte nuclear configuration

Viability of isolated follicles were primary detected by neutral red. The oocytes of positive stained follicles were collected and transferred into a mixture of 2 µM Hoechst 33258 in 500 µL holding medium for 15 min in the dark. The nuclear configuration of stained oocytes was examined under a fluorescence microscope (BX51; Olympus) at X 2,000 magnification.

3.3.7 Statistical analysis

Data are analyzed using IBM SPSS Statistics for Windows, Version 23.0 (Armonk, NY: IBM Corp.). Normal distribution and equal variances were tested by Shapiro test and Bartlett's test, respectively. Differences in follicle diameters between Days 0 and 3 were determined by Wilcoxon Signed Rank test. Pearson Chi-Square test was used

to evaluate the differences in the number of oocytes recovery between control and eCG treated group at the end of culture. For all statistical analysis, differences were regarded to be significant if $P < 0.05$.

3.4 Results

The results of frozen thawed tiger ovarian tissue are presented in Table 8. At the end of culture, the follicle diameter significantly decreased in the control group ($P < 0.05$), whereas the follicle diameter of eCG treated group sustained their initial size ($P > 0.05$). However, there was no significant difference between control and eCG treated groups after 3 days of culture ($P > 0.05$). Although the neutral red staining of all follicles showed 100% follicle survival, the number of oocytes recovered from neutral red positve follicles were not equal to the number of viable follicles. The numbers of rescued oocytes of eCG treated group were not different between two groups ($P > 0.05$). Only two follicles were recovered from control group (33.33%). Whereas four follicles were retrieved from eCG treated group (66.67%). All oocytes' size was ~ 140 µm. The Hoechst 33258 staining elucidated that the nuclear configuration of all rescued oocytes was remained in germinal vesicle stage (Fig. 3).

Table 8. Mean ± SEM of follicle diameter on Day 0 and 3 of culture and the percentage of oocyte recovery at the end of culture.

Group	n	Follicle diameter (μm)		Oocyte recovery (%)
		Day 0	Day 3	
Control	6	1,553.10 ± 269.50[a]	1,514.58 ± 270.84[b]	33.33
eCG	6	1,541.45 ± 210.54[a]	1,644.73 ± 230.90[a]	66.67

Value with different superscripts (a, b) differ significantly (P<0.05) among culture days.

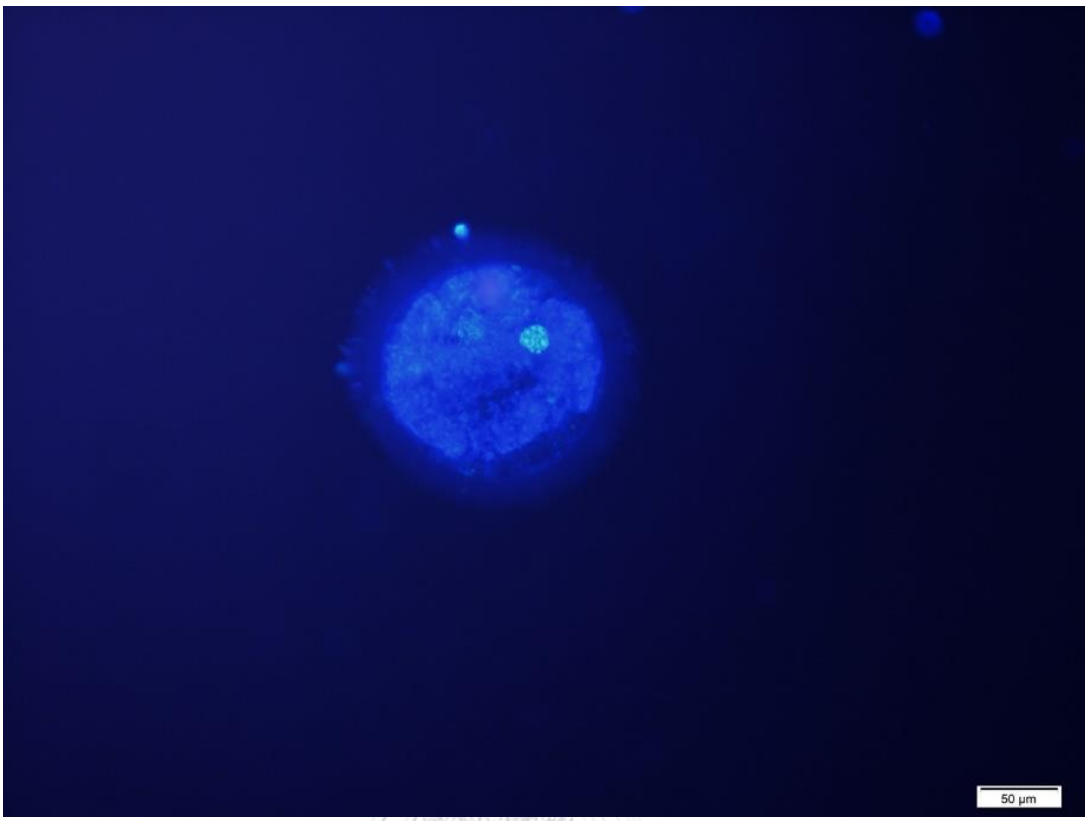


Figure 3. The germinal vesicle stage oocyte stained with Hoechst 33258 at the end of culture. Scale bar, 50 µm.

3.5 Discussion

In this study, post mortem tiger ovarian tissues were cryopreserved by vitrification method five years ago. Although the successful vitrification protocols for feline ovarian tissue cryopreservation has been established (Mouttham and Comizzoli, 2017; Martins et al., 2018), the frozen thawed tissues in our experiment showed signs of degeneration (dark and decompose). Our tissue's dimensions were 5 x 5 x 3 mm^3, approximately. The degeneration may occur because of the oversize tissues. In the baboon, the percentages of normal follicles in vitrified-warmed ovarian tissues slicing into 0.5 x 1.5 x 1 mm^3 were significantly higher than those slicing into 2 x 1 x 1 mm^3 (Lu et al., 2014). In the domestic cat, ovarian cortical tissues were sliced into 1 x 1 x 0.2 mm^3 before cryopreservation and obtained 47% of normal follicles after thawing (Mouttham and Comizzoli, 2017). Another factor of degeneration was our vitrification tissues used sucrose as a cryoprotective agent and the time of incubation was 10 min. The diffusion rates of CPAs in tissue depend on molecular weight (Sharma et al., 2007). Sucrose diffusion rate was reported that it predominantly found in the outer regions of the tissue and partially penetrated into the tissues volume 150 mm^3 within 30 min (Vasquez-Rivera et al., 2018). Thus, the degeneration of our vitrified-warmed tissues probably due to inappropriate size and CPA incubation time. The small amounts of normal follicles obtained in this study may associated with these two degeneration factors. The appropriate tissue size and time of CPA incubation should be concerned for improve the post-thawed quality.

To the best of our knowledge, there is no review of tiger isolated follicle culture. Our present study is the first report of tiger antral follicle culture from the vitrified-warmed ovarian tissue after long term storage. The retrieval of germinal vesicle

stage oocytes is the major finding of our experiment and is valuable because it confirmed that the tiger ovarian tissues are able to restore their viability after long-time storage in liquid nitrogen. The tiger antral follicles responded to eCG similar to the domestic cat (Chansaenroj et al., 2019). The follicle culture with 0.05 IU/mL eCG tended to stimulate antral follicle growth after culture for 3 days (P = 0.07). In this present study, the actual oocyte size could not measure because the large volume of antral cavity. Thus, the weak point of this study was unknown initial oocyte diameter. However, the oocytes were evaluated at the end of culture. The culture effect on the oocyte was more pronounced when eCG was treated in the culture medium compared to control. The culture medium without eCG had detrimental effect on oocyte recovery. Since the antral follicle was gonadotropin dependent stage (Craig et al., 2007), the folliculogenesis was stimulated by eCG. Previous study suggested that the presence of eCG suppressed granulosa cell apoptosis and follicular atresia of antral follicle (Li et al., 1998). Although the number of oocyte recovery was not different between two groups, the percentage of oocyte recovery in eCG group was higher than control group.

All recovery oocyte diameters were approximately 140 μm which were larger than normal size of the oocyte obtained from antral follicles (120 μm) of the domestic cat (Izumi et al., 2012). Our finding suggested that the fully-grown oocyte of the tiger was slightly larger than the domestic cat. In addition, these oocytes obtained from the cultured follicles can further developed if performed the *in vitro* maturation. Since the relation between oocyte diameter and meiotic competence of the domestic cat has been reported (Otoi et al., 2001). The final growth of the oocytes to their full size was

essential for their developmental competence after *in vitro* maturation and fertilization (Pavlok et al., 1992).

In conclusion, vitrification technique was suitable for ovarian cryopreservation in felids species. However, the tissue size and thickness including with the incubation time should be concerned because these factors influenced to the quality of thawing tissue. The number of normal follicles decreased in the damaged frozen thawed tissue. In this study, vitrified-warmed tiger ovarian tissues provided some antral follicles. The recovery antral follicles were able to grow and produce normal oocytes in a three-dimensional alginate hydrogel culture system supplementation with eCG in the culture medium. However, further study of meiotic resumption and *in vitro* fertilization should be performed.

Chapter IV

Requirements of insulin-like growth factor-I and stem cell factor for cat follicles cultured in a fibrin-alginate three-dimensional system

4.1 Abstract

Insulin-like growth factor-I (IGF-I) and stem cell factor (SCF) play roles in supporting proliferation, differentiation and survival of the ovarian follicle. Here, we examined the influence of these two factors on the development of varying stages cat follicle and expressions of genes regulating follicle and oocyte growth and steroidogenesis. Two-layered secondary follicles (two-layered SF), multi-layered secondary follicles (multi-layered SF) and early antral follicles (early AF) were mechanically isolated from twelve cats, encapsulated in a fibrin-alginate hydrogel. The encapsulated follicles were in Eagle's Minimum Essential Medium (MEM) without growth factor supplementation (Control) or with 1 ng/mL IGF-I (IGF-I), 50 ng/mL SCF (SCF50), 100 ng/mL SCF (SCF100), 1 ng/mL IGF-I + 50 ng/mL SCF (IGF-SCF50) or 1 ng/mL IGF-I + 100 ng/mL SCF (IGF-SCF100) for 18 d, Relative growth of follicles and oocytes were assessed every 3 days. At the end of the culture period, morphology of follicles in all developmental stages was evaluated and mRNA expression of *FSHR, LHCGR, GDF9, BMP15, CYP17A1, CYP19A1* and *STAR*) in multi-layered SF and early AF were determined by real-time PCR. The results showed that growth factors supplementations did not affect two-layered SF and multi-layered SF growth, whereas SCF100 supported ($P < 0.05$) growth of early AF throughout the culture period. However, oocyte growth was varied among stages of follicle development. In the two-layered SF, IGF-I failed to maintain oocyte size after culture for 12 days. Oocytes

enclosed within multi-layer SF cultured on the presence of growth factors were able to sustain their initial size whereas those in the Control group decreased the diameter as *in vitro* culture progressed. However, the present or absence of growth factor showed no effect to the oocyte of early AF. SCF50 stimulated antral cavity formation and genes regulating follicle and oocyte growth and steroidogenesis in multi-layered SF. IGF-SCF50 was the best supplementation for early AF because it upregulated *FSHR*, *GDF9* and steroidogenic genes. In conclusion, SCF is important for folliculogenesis in the multi-layered SF and early AF stages.

4.2 Introduction

Ovarian folliculogenesis is a dynamic biological process that involves the activation of primordial follicles and the transition of this primitive stage into primary, secondary and advanced antral follicles containing a developmentally competent oocyte (El-Hayek and Clarke, 2016; Shah et al., 2018). The ability to grow early stage follicles to the advanced antral stage would be valuable for preserving the fertility of women as well as rescuing genomes of genetically valuable animals. Live offspring have been produced from oocytes recovered from *in vitro* grown mouse follicles (Eppig and O'Brien, 1996; Xu et al., 2006a; Higuchi et al., 2015). However, such accomplishment has not been demonstrated in other large mammalian species including the human (Shea et al., 2014; Yin et al., 2016), sheep (Kamalamma et al., 2016; Lunardi et al., 2017), cow (Gutierrez et al., 2000; Bus et al., 2018), goat (Celestino et al., 2009; Brito et al., 2012; Silva et al., 2017; Ferreira et al., 2018), macaque (Xu et al., 2013; Lu et al., 2015), baboon (Fortune et al., 1998; Xu et al., 2011), dog (Songsasen et al., 2011; Fujihara et al., 2012; Nagashima et al., 2018) and cat (Fujihara et al., 2012; Alves et al., 2017; Songsasen et al., 2017; Thongkittidilok et al., 2018; Chansaenroj et al., 2019).

Several studies have shown that follicular growth and survival depend on the presence of gonadotropins and variety of factors that are locally produced in the ovary (Silva et al., 2004; Demeestere et al., 2005; Zhou and Zhang, 2005) . For the cat, studies have examined the impact of endocrine, including follicle stimulating hormone (FSH) (Jewgenow and Pitra, 1993), equine chorionic gonadotropin (Chansaenroj et al., 2019) and insulin (Thongkittidilok et al., 2018) as well as paracrine factors, such as insulin growth factor-I (IGF-I) and epidermal growth factor (Alves et al., 2017) on *in vitro* growth and survival of isolated follicles. Although these studies have shown that such treated follicles increase in size, resident oocytes have poor developmental competence (Songsasen et al., 2012; Alves et al., 2017; Chansaenroj et al., 2019). Therefore, there still are needs to establish an improved *in vitro* culture system that supports growth and maturation of immature cat follicles as a model for rescuing valuable genetic of wild felids.

Among paracrine factors studied to-date, IGF-I has been shown to regulate ovarian folliculogenesis by stimulating the proliferation and steroidogenesis of granulosa cells *in vitro* (Guthrie et al., 1998). In the mouse, IGF-I works synergistically with follicle stimulating hormone and stimulates *in vitro* growth of preantral follicles (Liu et al., 1998). IGF-I also increases bovine (Itoh et al., 2002) and caprine (Zhou and Zhang, 2005) preantral follicle and oocyte diameters during *in vitro* culture. Moreover, IGF-I increases granulosa cell proliferation, promotes follicular antrum formation and decreases apoptosis in porcine preantral follicles *in vitro* (Mao et al., 2004). In the cat, IGF-I supplementation *in vitro* increases preantral follicle diameter, granulosa cell viability and decreases degeneration rate (Alves et al., 2017). However, the effect of this growth factor on the development of antral follicles have not been demonstrated.

In addition to IGF-I, SCF has been shown to play important roles in mammalian

oogenesis and folliculogenesis (Parrott and Skinner, 1997; Reynaud et al., 2000; Ronnstrand, 2004). SCF regulates cell survival, proliferation, differentiation and migration (Ronnstrand, 2004). The signaling pathways activated by the SCF/c-KIT system involve phosphatidylinositol 3-kinase (PI3K)/AKT and mitogen-activated protein kinases (MAPK) pathways (Cardoso et al., 2014). Parrott and Skinner (1999) reported that the activation of primordial follicles is promoted by SCF during ovarian organ culture (Parrott and Skinner, 1999). For the preantral follicle, supplementation of SCF in combination with IGF-I promoted the production of mature oocytes in the mouse (Jee et al., 2012). In the early antral stage, SCF promotes theca cell recruitment, and thus, contributing to the regulation of androgen production in the mouse, pig and cow (Parrott and Skinner, 1997; Reynaud et al., 2000; Brankin et al., 2003). SCF also upregulates mRNA and protein expression of *STAR* and P450 aromatase in mice (Jin et al., 2005). In the cat, SCF promotes *in vitro* activation of primordial follicles enclosed within the ovarian cortex by upregulating c-kit mRNA expression and AKT phosphorylation (Thuwanut et al., 2017).

The objectives of the present study were to investigate (1) the influence of IGF-I, SCF and synergistic effects of IGF-I and SCF on cat secondary and early antral follicles development *in vitro*; (2) antrum formation rate of the multi-layered SF; (3) mRNA expression level of gonadotropin receptor genes (*FSHR* and *LHCGR*), oocyte ligands (*GDF9* and *BMP15*) and steroidogenic enzyme activity (*CYP17A1*, *CYP19A1* and *STAR*).

Because there has been evidence of differing responses to growth factor stimulation among varying stages of follicle development (Cook-Andersen et al., 2016; Cadenas et al., 2017), we also investigated the effect of developmental stages on the responses of follicle to growth factors supplementations. We hypothesized that IGF-I,

SCF and the combination of IGF-I and SCF (1) promote follicular growth and survival in cat follicles; (2) support antral cavity formation in the multi-layered secondary SF. (3) upregulate the folliculogenesis related genes.

4.3 Materials and Methods

4.3.1 Chemicals

Unless noted otherwise, the chemicals used in the present study were purchased from Sigma Aldrich, St. Louis, MO, USA.

4.3.2 Source of ovaries

Ovaries were collected from 12 cats (aged between 6 months to 3 years) underwent routine ovariohysterectomy at local veterinary clinics in Front Royal, Stephens City, and Harrisonburg, Virginia, USA. Ovaries were transported to the laboratory at 4°C in L-15 medium supplemented with 3 mg/mL penicillin G sodium, and 3 mg/mL streptomycin sulfate within 6 h after surgery.

4.3.3 Collection and *in vitro* culture of isolated follicles

At the laboratory, one ovary was dissected and pressed through a cell dissociation sieve-tissue grinder kit. The cell suspension obtained was passed through 100 µm nylon sieves (BD Biosciences, Bedford, MA, USA) which was then rinsed with 10 mL of Maintenance medium (Eagle's Minimum Essential Medium (MEM) containing 3 mg/mL BSA, 2 mM L-glutamine, 10 IU/mL penicillin G sodium and 10 mg/mL streptomycin sulfate and 20 mM Hepes) to recover two-layered SF (≤ 150 µm; n = 120) and multi-layered SF (150 to 300 µm; n = 120). The remaining ovary was carefully sliced into cortical strips using a scalpel blade and then kept in the maintenance medium. Early AF (> 300-500 µm; n = 120) were isolated from the cortical strips using 25G gauge needles. Isolated follicles were individually-encapsulated in a bead with

31.25 mg/mL fibrinogen and 0.5% (w/v) alginate (FMC BioPolymers, Philadelphia, PA) according to methods described by Shikanov (Shikanov et al., 2009). Briefly, follicles in fibrinogen-alginate (3 µL) were pipetted into 100 U/mL thrombin in Tris BuPH with 40 mM $CaCl_2$. Alginate crosslinks in the presence of a divalent cation, such as calcium (West et al., 2007), and fibrinogen polymerize into fibrin when thrombin activates factor XIIIa (Shikanov et al., 2009). Beads then were washed three times in Minimum Essential Medium containing 3 mg/mL BSA, 2 mM L-glutamine, 10 ng/mL activin, 10 µg/mL insulin, 1.9 µg/mL transferrin, 5 µg/mL selenium, 10 IU/mL penicillin G sodium and 10 mg/mL streptomycin sulfate and 0.05 IU/mL eCG, hereafter called “Control medium”. The encapsulated follicles were allocated into six groups: 1) Control (no growth factor supplementation), control medium supplemented with 2) 1 ng/mL IGF-I (IGF-I), 3) 50 ng/mL SCF (SCF50), 4) 100 ng/mL SCF (SCF100), 5) 1 ng/mL IGF-I + 50 ng/mL SCF (IGF-SCF50) and 6) 1 ng/mL IGF-I + 100 ng/mL SCF (IGF-SCF100). Two to three follicles of the same developmental stage were cultured in 500 µL of respective culture medium and incubated for 18 d at 38.5°C in 5% CO_2 in humidified air. Half volume of the culture medium was changed every 3 d throughout the study. This study consisted of eight replications.

4.3.4 Follicle and oocyte assessment

Follicle and oocyte diameter were assessed under an inverted microscope (Leica LEITZ DM IL; Leica Germany). Each follicle was sized from the outer layer of somatic cells, with the measurements including the widest diameter and perpendicular width to the initial assessment. The mean of these two metrics was calculated and reported as diameter (Thongkittidilok et al., 2018) The mean diameters of each follicle and oocyte on Days 0, 3, 6, 9, 12, 15 and 18 were recorded. The formation of antral cavity

in the multi-layered SF was examined every 3 d. Follicular atresia was recognized if a follicle decreased significantly in diameter over several culture days, contained a degenerate oocyte, or basement membrane disruption. Follicle survival was determined at the end of the culture period.

4.3.5 Quantitative RT-PCR (qRT-PCR)

To examine mRNA expression in multi-layered SF and early AF cultured for 18 days under different culture conditions, total RNA was extracted from cat follicles using absolutely RNA nanoprep kit (Agilent Technologies, Santa Clara, California, USA) following the manufacturer's protocol. DNA contamination was treated by Rapid Out DNA removal kit (Thermo-Scientific, Massachusetts, USA). The quantity of RNA was measured using a spectrophotometer (NanoDrop One, Thermo-Scientific, Massachusetts, USA). mRNA converted to complementary DNA (cDNA) by using a Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Mannheim, Germany) with the same amount of mRNA (2 ng from each sample), and stored at -20° C until quantitative polymerase chain reaction (qPCR) analysis. A fluorescence probe for qPCR was SyBr Green Mastermix (FastStart Essential DNA Green Master, Roche, Mannheim, Germany). All primer sequences used in the present study are shown in Table 6 (in Chapter II). Each PCR reaction consisted of 2 µL DNA and 18 µL of a mixture (10 µL of Fast Start Essential DNA green master (Roche, Basel, Switzerland), 1 µL each of 10 mM for forward and reverse primers and 6 µL of nuclease-free water). qPCR reactions were performed in duplicate using Light Cycler® 96 (Roche, Basel, Switzerland). Each amplification reaction consisted of the pre-incubation period (95 °C for 10 min), 45 cycles of three steps amplification (95 °C for 30 s, specific annealing temperature of primers of interest for 10 s and 72 °C for 10 s) and melting step (65 °C for 30 s). The negative control

without cDNA was run together with samples in every amplification. Primer efficiency of each gene calculated by using five serial dilutions of DNA. The Ct value of each gene was normalized against the Ct of beta-actin which was shown to be more stable than succinate dehydrogenase complex flavoprotein subunit A (*SDHA*) using Best Keeper software evaluation. The delta delta Ct (ΔΔCt) including primer efficiency was used to calculate fold changes of a given gene in experimental groups normalize to the control as described by Pfaffl (Pfaffl, 2001).

Fold change = $(1+ \text{primer efficiency})^{-(\Delta\Delta Ct)}$ (Rao et al., 2013)

4.3.6 Statistical analysis

Data were analyzed using SAS 9.0 to perform statistical analysis. Normal distribution and equal variances were tested by Shapiro test and Bartlett's test, respectively. Follicles and oocytes growth on Days 3, 6, 9, 12, 15 and 18 were presented as relative diameter to the initial diameter (Day 0). Kruskal-Wallis test following by Duncan's multiple range test were used to evaluate the differences of mean relative growth of the follicles and oocytes in each developmental stage among 3, 6, 9, 12, 15 and 18 days. The descriptive statistical analysis was used to calculate the percentage of antral formation and follicular survival on Day 18 of culture. Chi-square test with p value adjusted for multiple testing with Bonferroni test was used to analyze the differences of survival rate among treatments in each stage. The ΔΔCt of qPCR expression data from each treatment were analyzed by Kruskal-Wallis test following by pairwise comparisons using Tukey and Kramer (Nemenyi) test. Differences were considered significant when $P < 0.05$.

4.4 Results

4.4.1 Follicle and oocyte growth

Different developmental stages of the follicles showed varying growth rate and gene expressions responses to growth factor supplementation in fibrin-alginate three-dimensional culture system. With one exception, cat follicles exhibited modest growth (10%) during the 18 days culture period. However, early AF cultured with SCF100 displayed 20% growth after 18 days of *in vitro* culture.

When IGF, SCF or their combination was added to the culture medium, no differences were observed in the growth of the secondary follicle ($P > 0.05$) (Fig. 4A, 4B). A positive effect on follicle growth was observed when the early AF were culture in the presence of SCF. Specifically, SCF50 slightly increased follicle growth until Day 9 of culture, while SCF100 promoted follicle growth throughout the culture period ($P < 0.05$) (Fig. 4C) However, there were no significant differences in relative growth among treatments ($P > 0.05$).

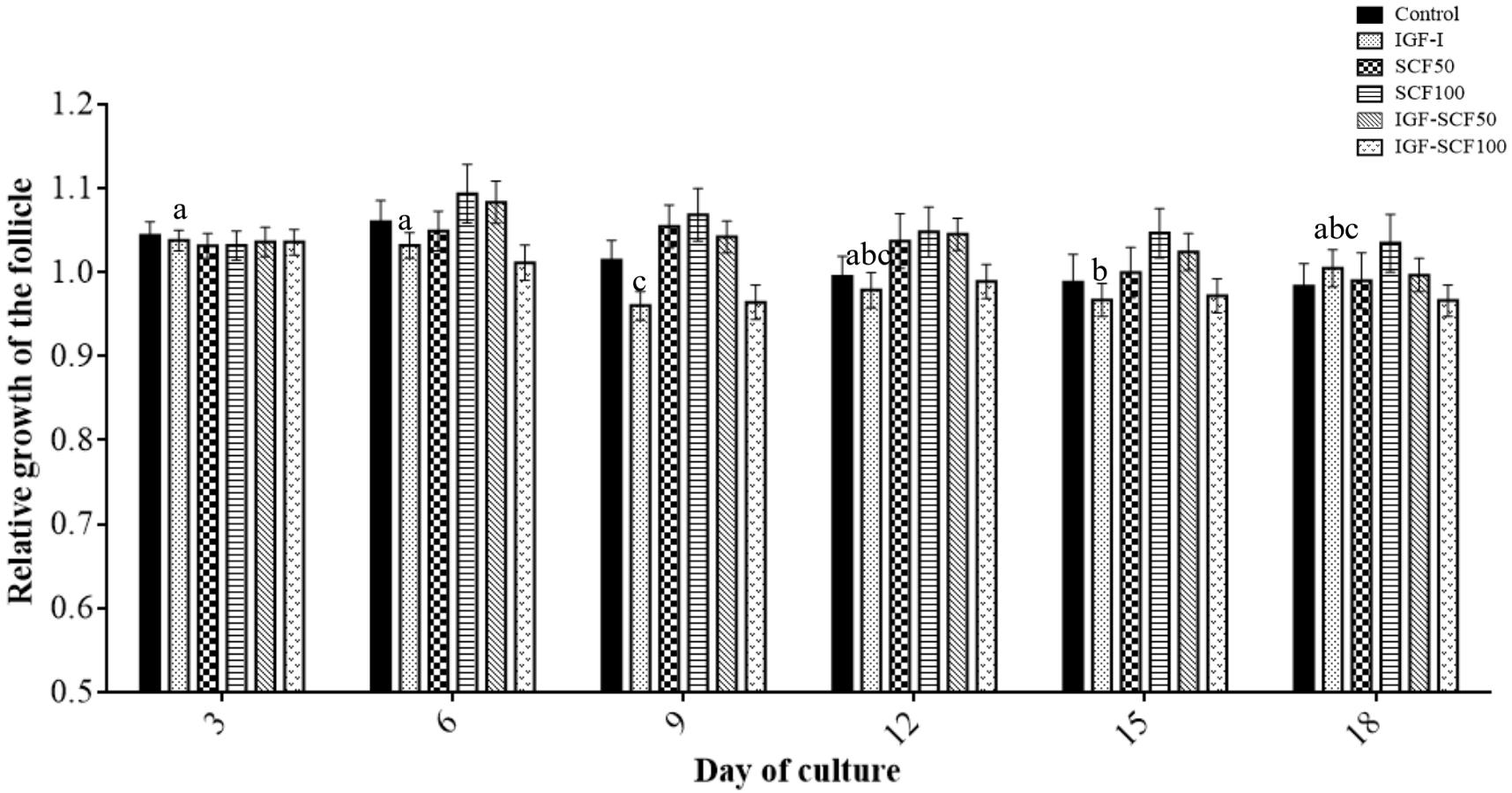
Supplementing the culture medium with IGF-I alone gradually decreased the relative growth of oocytes within two-layer SF during the first 15 days of *in vitro* culture ($P < 0.05$) (Fig. 5A). However, oocytes enclosed within multi-layer SF cultured on the presence of growth factors were able to sustain their initial size whereas those in the Control group decreased the diameter as *in vitro* culture progressed (Fig. 5B). Growth factors also had no effect on the oocyte of early AF ($P > 0.05$) (Fig. 5C).

4.4.2 Antral formation

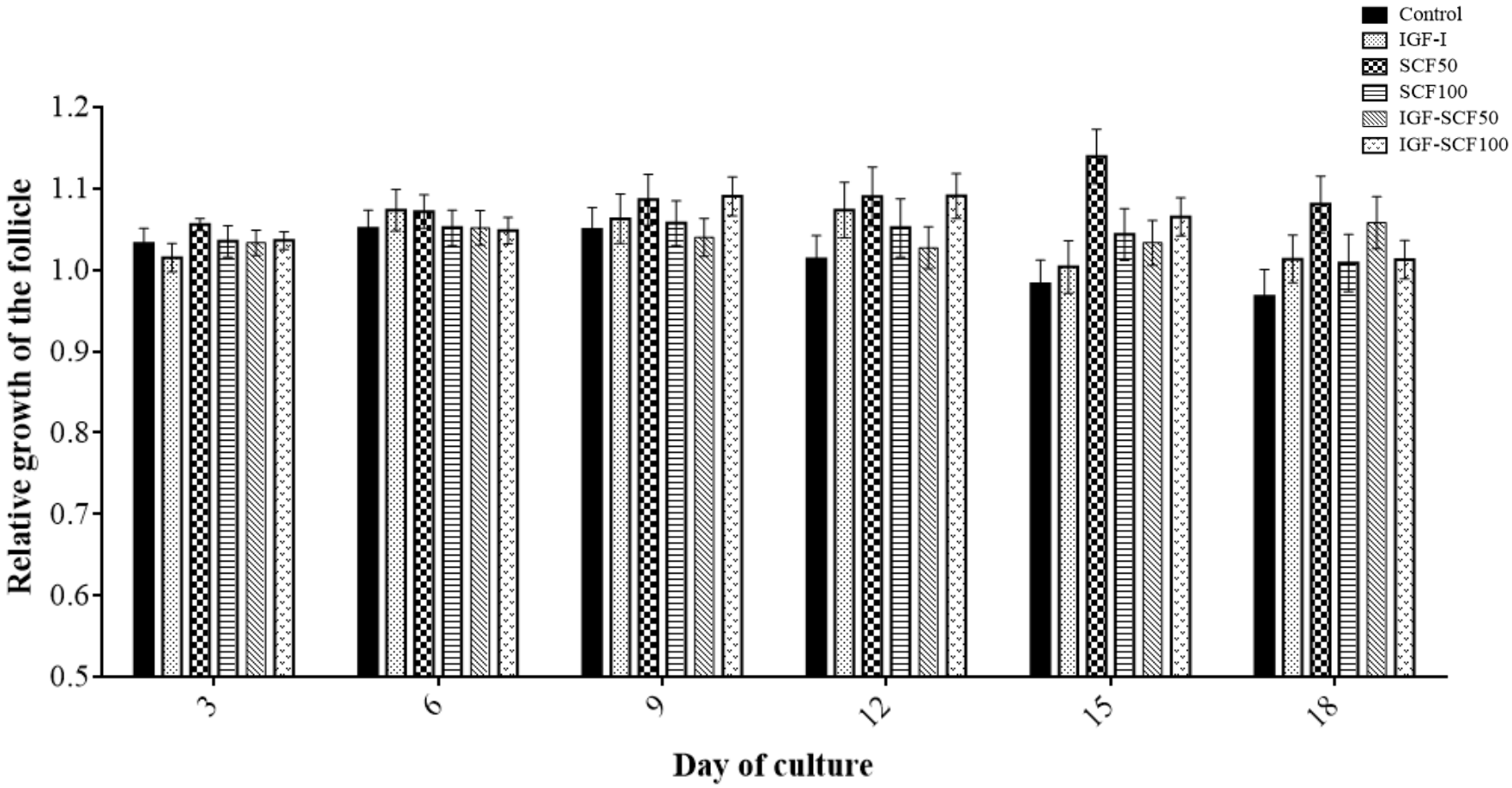
Antral cavity formation was observed in multi-layered SF. The first detection of antrum was found on Day 3 in the medium supplemented with SCF100 and IGF-SCF50. The antral cavity of multi-layered SF incubated with IGF-I, SCF50 and IGF-SCF100 groups

was found on Day 6. The last antral cavity appeared on Day 12 in control group. The highest rate of total antral formation was observed in SCF50 group (50%) (Fig. 6).

A


Control
IGF-I
SCF50
SCF100
IGF-SCF50
IGF-SCF100
Relative growth of the follicle
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
a
a
c
abc
b
abc
3
6
9
12
15
18
Day of culture

B


Control
IGF-I
SCF50
SCF100
IGF-SCF50
IGF-SCF100
Relative growth of the follicle
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
3
6
9
12
15
18
Day of culture

C

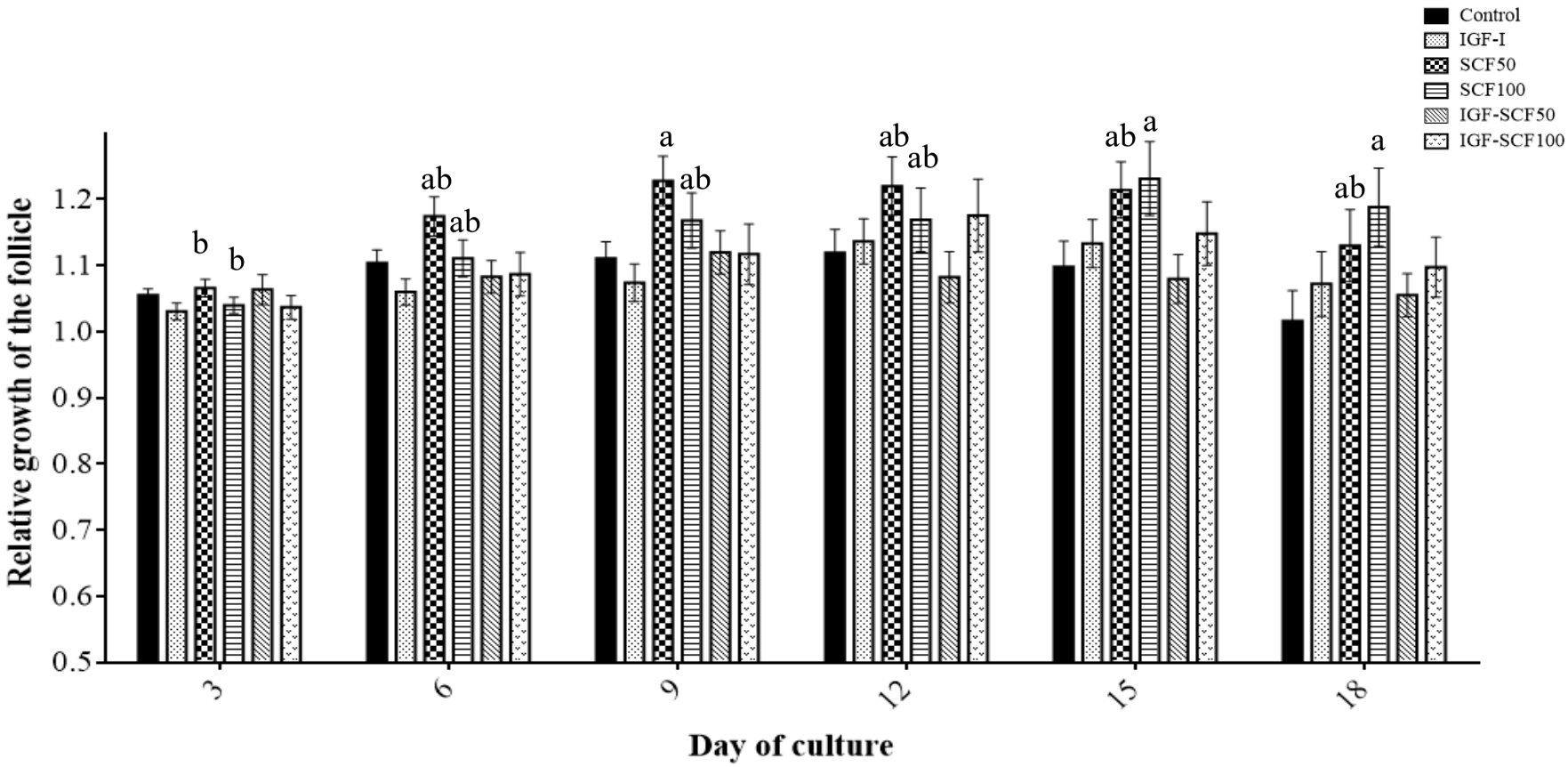
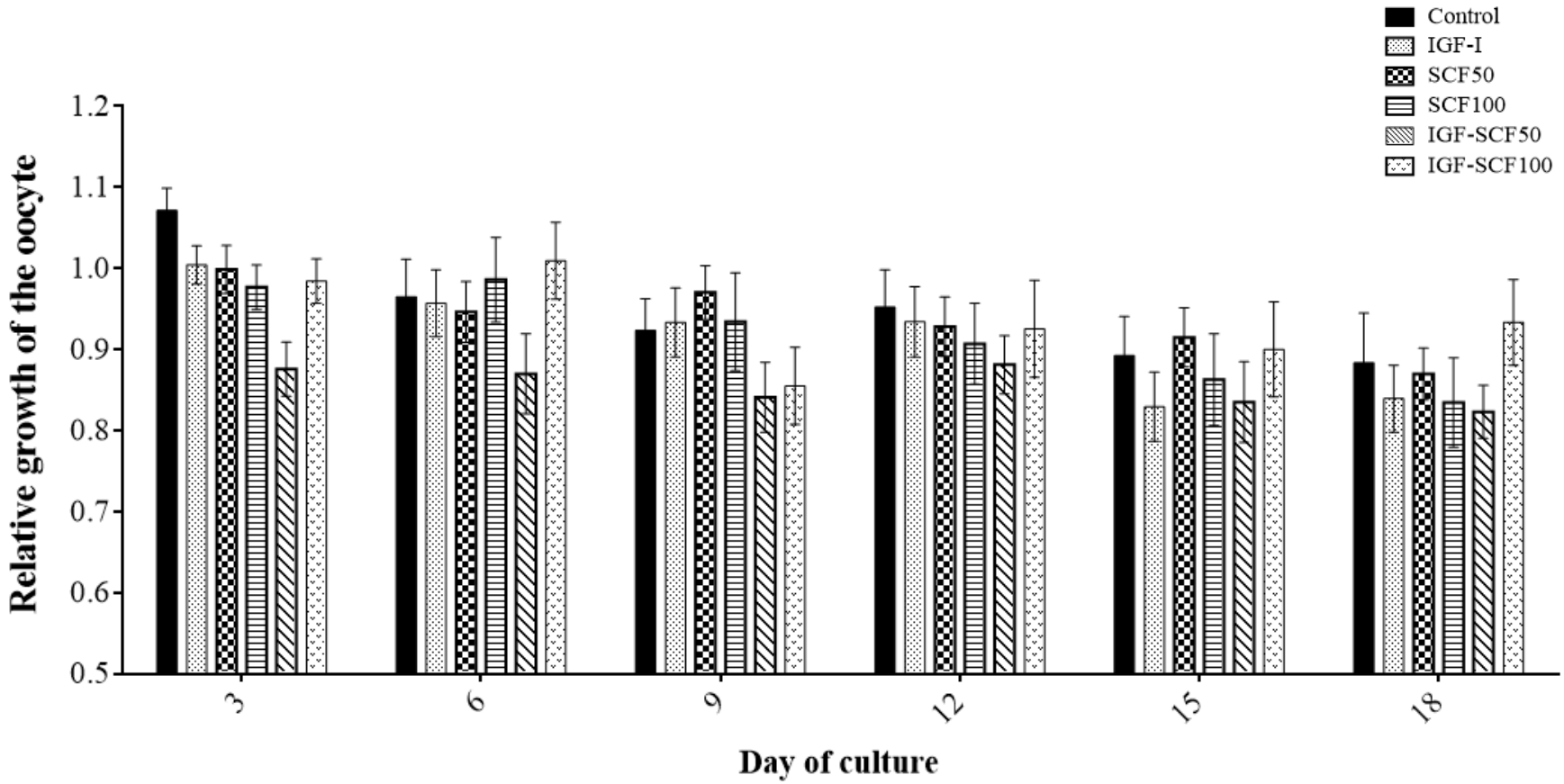
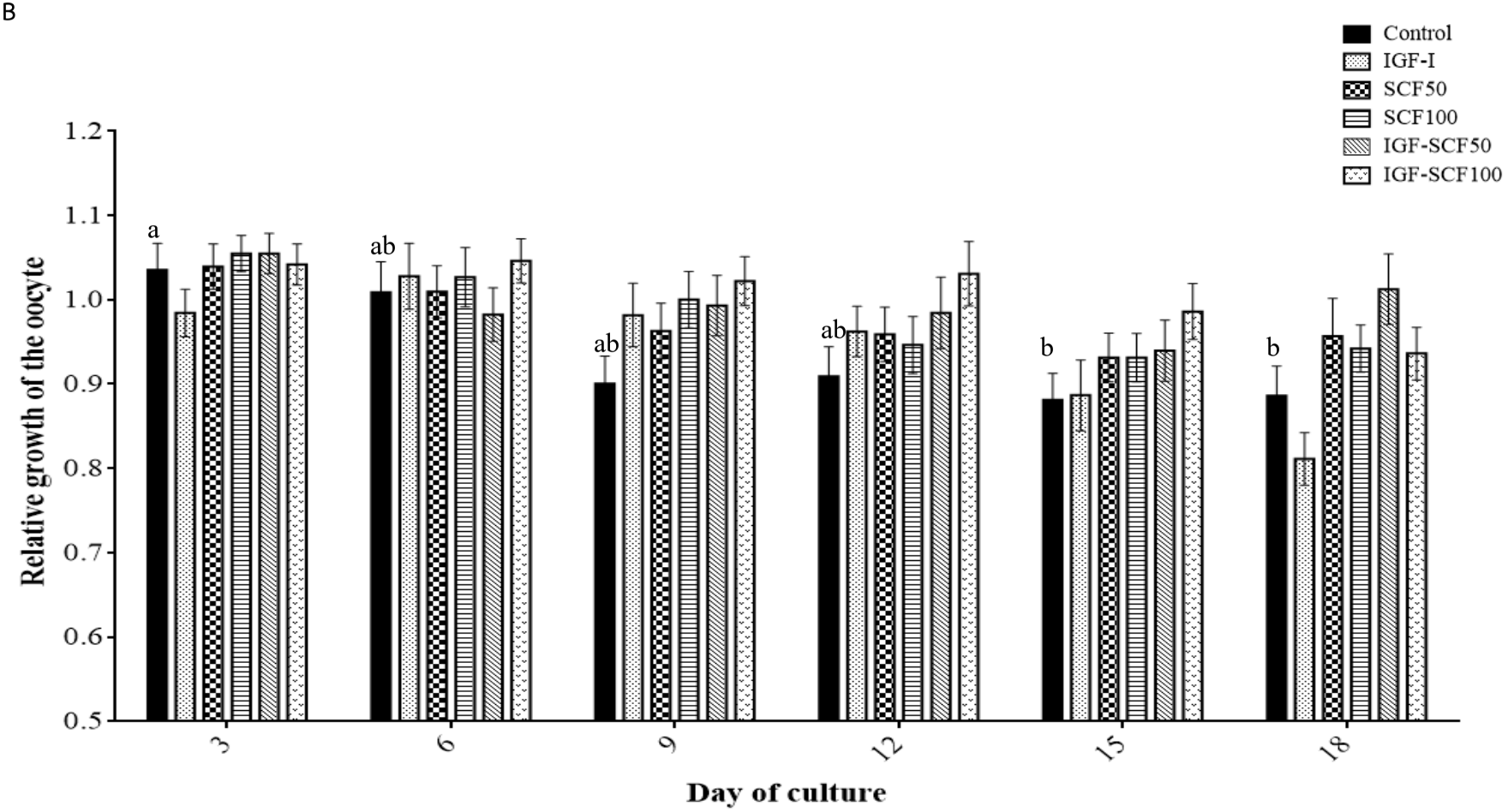


Figure 4. Relative growth of the follicles (compare to day 0) after 3, 6, 9, 12, 15 and 18 d of culture. A) Two-layered SF, B) Multi-layered SF and C) Early AF, respectively. Value with different superscripts (a, b, c) differ significantly ($P < 0.05$) among culture days within the same treatment.

A


Control
IGF-I
SCF50
SCF100
IGF-SCF50
IGF-SCF100
Relative growth of the oocyte
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
3
6
9
12
15
18
Day of culture

B


Control
IGF-I
SCF50
SCF100
IGF-SCF50
IGF-SCF100
Relative growth of the oocyte
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
a
ab
ab
ab
b
b
3
6
9
12
15
18
Day of culture

C

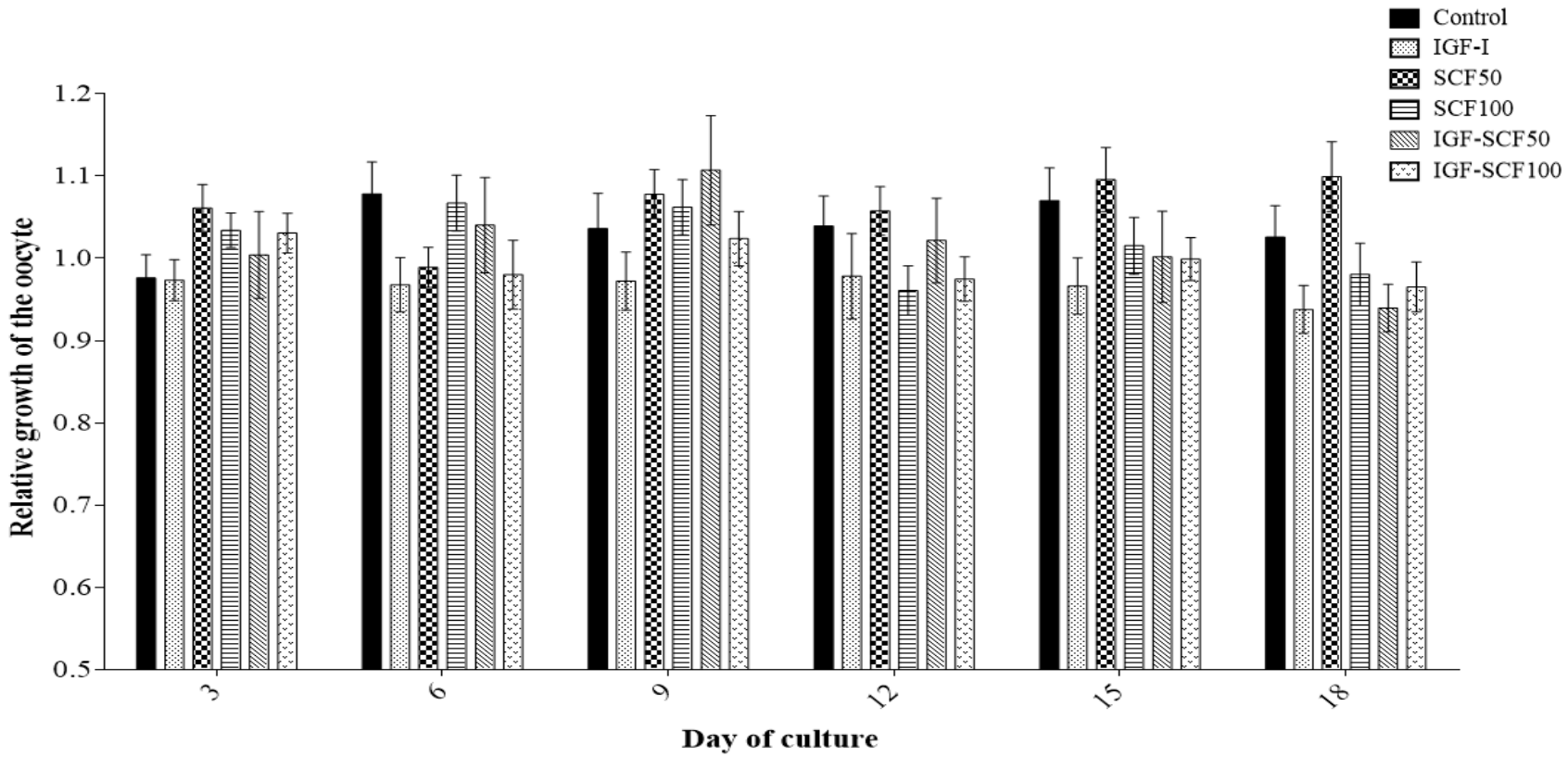


Figure 5. Relative growth of the oocytes (compare to day 0) after 3, 6, 9, 12, 15 and 18 d of culture. A) Two-layered SF stage, B) Multi-layered SF stage and C) Early AF stage, respectively. Value with different superscripts (a, b) differ significantly (P < 0.05) among culture days. Value with different superscripts (A, B) differ significantly (P < 0.05) among treatments in the same day of culture.

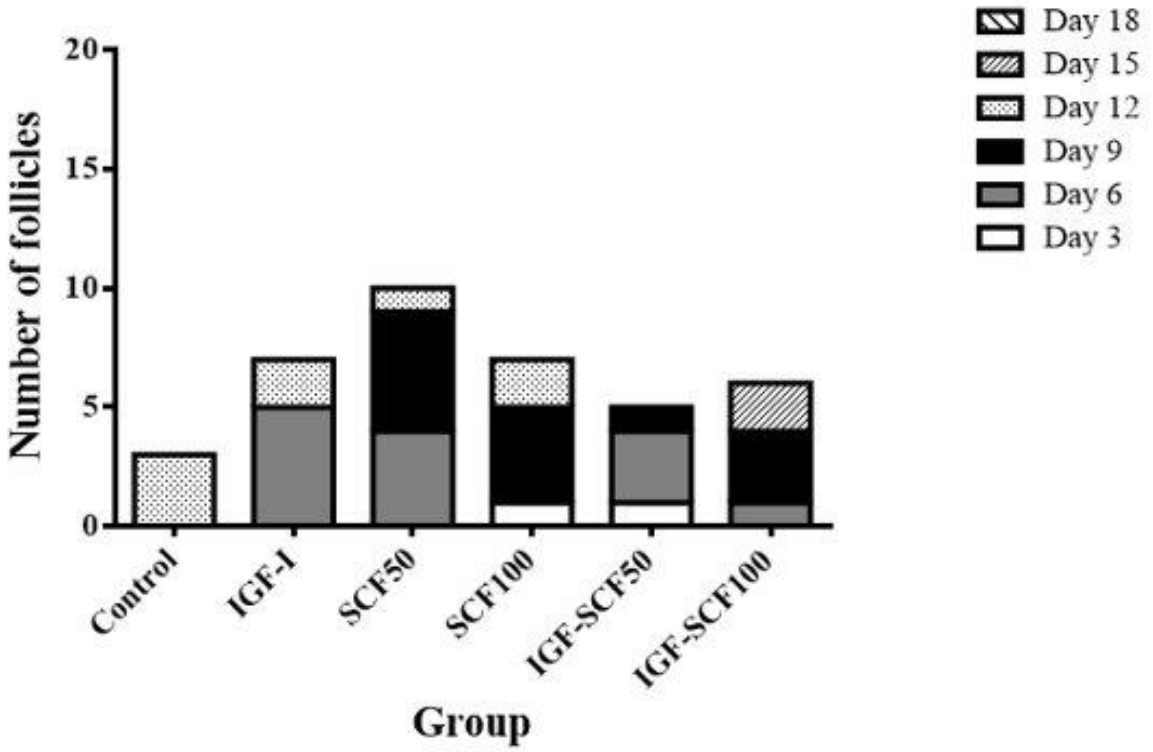


Figure 6. The percentage of multi-layered SF antral formation classify by treatment.

4.4.3 Survival rate

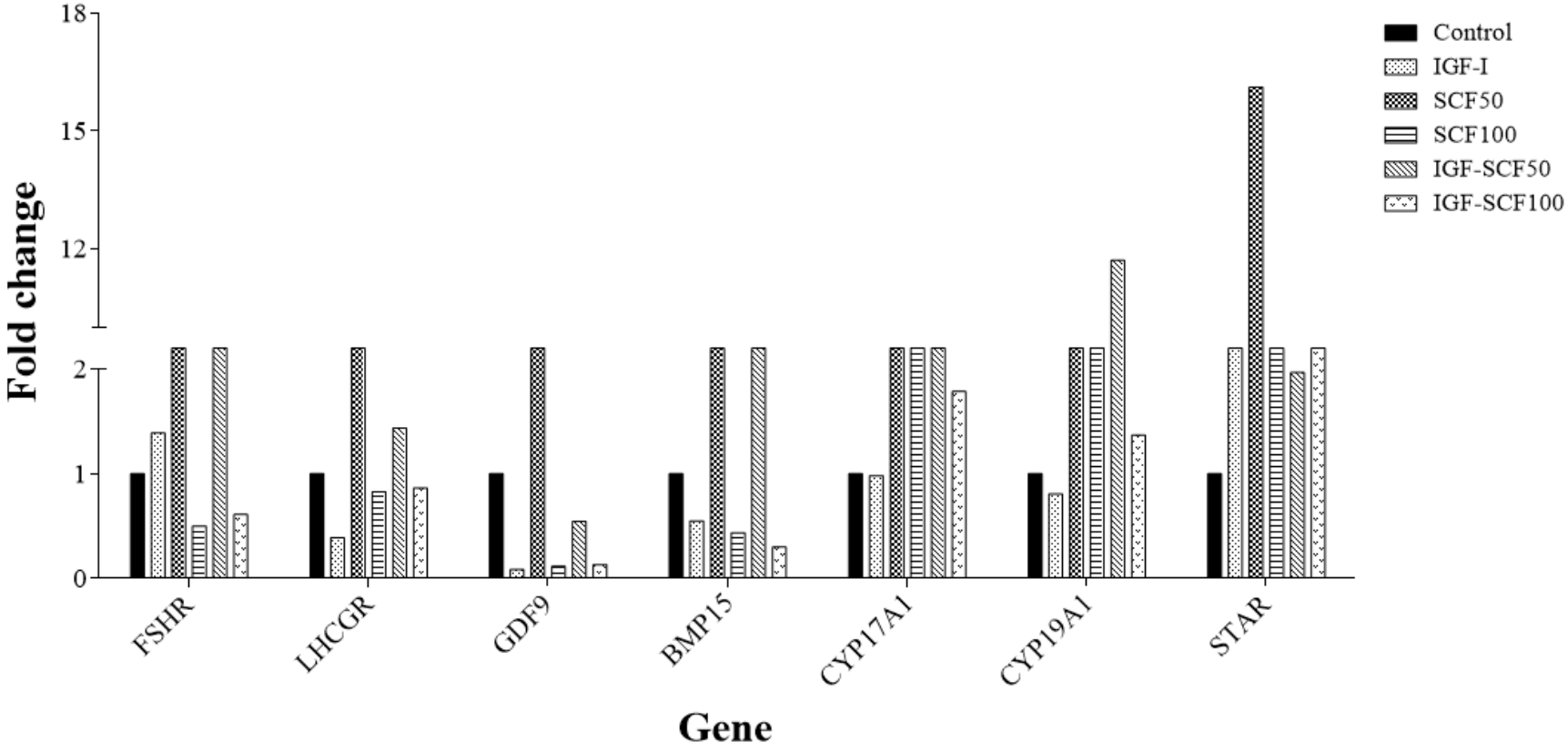
The follicle survival rate of two-layered SF stage decreased significantly ($P < 0.05$) in IGF-SCF100 treated group compared to the other groups. However, there were no significant differences in the survival rate (80%) among treatments in multi-layered SF and early AF stage. ($P > 0.05$).

4.4.4 Quantitative RT-PCR (qRT-PCR)

SCF50 upregulated gonadotropin receptors (*FSHR* and *LHCGR*), oocyte derived growth factors (*GDF9* and *BMP15*) and steroidogenic genes (*CYP17A1, CYP19A1* and *STAR*) in multi-layered SF (Fig 7A), whereas SCF100 stimulated steroidogenic gene expressions (Fig 7A). IGF-SCF50 upregulated *FSHR, BMP15, CYP17A1* and *CYP19A1* (Fig 7A).

Early AF cultured on the presence of growth factors upregulated *FSHR* expression (Fig 7B). The other genes upregulated by SCF50 and IGF-SCF50. SCF50 upregulated *CYP19A1* and *STAR* transcripts (Fig 7B). IGF-SCF50 supplementation to early AF increased *FSHR, GDF9, CYP17A1, CYP19A1* and *STAR* (Fig 7B).

A


Fold change
18
15
12
2
1
0
Control
IGF-I
SCF50
SCF100
IGF-SCF50
IGF-SCF100
FSHR
LHCGR
GDF9
BMP15
CYP17A1
CYP19A1
STAR
Gene

B

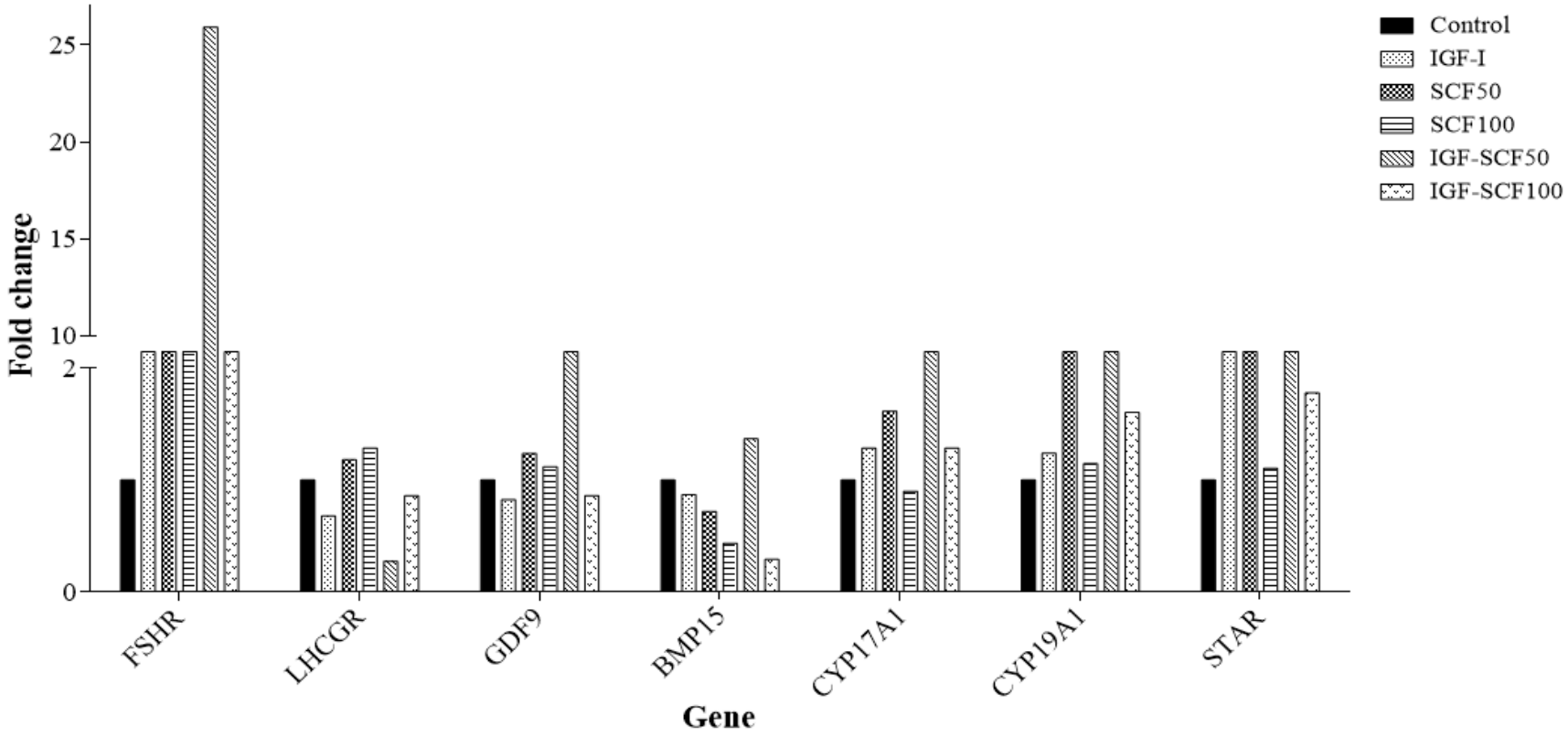


Figure 7. Gene expressions of A) Multi-layered SF and B) Early AF.

4.5 Discussion

In the present study, we examined the influence of two key growth factors, IGF-I and SCF, on *in vitro* development of isolated cat follicles. We discovered that SCF exerted beneficial effects on growth, antral cavity formation and mRNA expressions of the cat follicle.

SCF stimulated follicular growth of early AF and sustained oocyte size in all developmental stage. IGF-I did not exert beneficial effects on follicle and oocyte growth in all developmental stages. This growth factor also negatively influenced the viability (decreased diameter) of the enclosed oocyte, especially in the two-layered SF. Moreover, there was no synergistic effect between SCF and IGF-I in promoting *in vitro* development of isolated cat follicles.

IGF-I plays autocrine and paracrine roles in early follicular development and steroid production in many species (Sharma et al., 2010; Magalhaes-Padilha et al., 2012; Stubbs et al., 2013; Jimenez et al., 2016) including the cat (Alves et al., 2017). The effect of IGF-I in different species is still controversial. Some authors had reported no beneficial effects of IGF-I on *in vitro* follicle development (Brito et al., 2012). However, a previous report in the cat indicated that the presence of IGF-I and gonadotropin (FSH) in the culture medium stimulates secondary follicle growth (Jewgenow and Pitra, 1993). It has been demonstrated that the combination of IGF-I and EGF influences preantral follicle growth and granulosa cell proliferation. However, there was no effect on oocyte growth and antral cavity formation (Alves et al., 2017). In the present study, IGF-I supplementation did not enhance the growth of follicles at all developmental stages. Moreover, the diameter of oocytes recovered from IGF-I treatment decreased after 15 days of culture. In our preliminary study, we did not observe the different of

follicle diameter among 1, 10 and 100 ng/mL IGF-I after 18 day of culture. Thus, the 1 ng/mL IGF-I, the effective dose in human (Stubbs et al., 2013), was used in this present study. The unexpected outcomes of IGF-I may due to the ideal concentration of IGF-I was species-specific. The concentration of IGF-I (1 ng/mL) in our study may have been insufficient to promote cat two-layered SF growth. The effect of IGF-I on the two-layered SF in this present study is consistent to that reported in the goat. Adding IGF-I to the culture medium did not stimulate goat secondary follicle growth after culture for 6 days (Brito et al., 2012). The oocyte diameters in this present study were also sustained in their size until Day 12 of culture then gradually decrease in size. This evidence suggested that long term *in vitro* cat two-layered SF culture may associated with the progressive reduction of the oocytes. IGF-I alone was unable to maintain the oocyte size though 18 days. This may be due to IGF-I promoted follicle growth but may impaired the bi-directional communication between granulosa cells and oocyte (Russell et al., 2016).

The growth factors supplementation did not show satisfactory results in the present study. The multi-layered SF and the enclosed oocyte did not grow when supplement with IGF-I, SCF and their combination compared to previous studies (Lima et al., 2011; Magalhaes-Padilha et al., 2012). Although IGF-I, SCF and their combination do not promote the follicle and oocyte growth (Brito et al., 2012; Jee et al., 2012), they play important roles in maintaining the size of oocytes enclosed within multi-layered SF. In this study, growth factors supplementations had no effect on the survival rate. In contrast to our results, previous work showed positive effect of IGF-I and SCF on enhancing follicle survival (Sharma et al., 2009; Lima et al., 2011). The ideal concentration of IGF-I may be species-specific. The concentration of IGF-I (1 ng/mL) in

our study may have been insufficient to ensure the efficient removal of those undesirable factors that would maintain cat two-layered SF viability during *in vitro* culture. Indeed, some studies have suggested that increasing gonadotropin (FSH) dosage to the basic culture medium sequentially, i.e., in increasing concentrations, because as the follicles develop, the gonadotropin levels increase to meet the requirements of different follicular stages (Saraiva et al., 2011). Therefore, it is possible that optimal dosage of sequential eCG concentration presence in the basic medium may concern in order to promote follicle growth and survival *in vitro*. The antral cavity formation was observed in all groups. However, growth factors treated groups formed an antral cavity earlier than control group. Moreover, SCF50 group formed antral cavity at the highest rate (50%) compared to other treatments. This finding confirmed an earlier observation reported in the mouse which antrum formation was inhibited after received an antibody of SCF (Yoshida et al., 1997). Although IGF-SCF50 could stimulate *FSHR, BMP15, CYP17A1* and *CYP19A1*, SCF50 upregulate all folliculogenesis related genes. Therefore, SCF50 may be the best choice for multi-layered SF supplementation. This present observation emphasized that folliculogenesis and steroidogenesis in the multi-layered SF required growth factors supplementation.

For the early AF, it has been reported that SCF is detected in granulosa cells isolated from antral follicles and partially found in theca cells (Silva et al., 2006). SCF is considered regulatory factors that stimulate oocyte growth via the interaction between granulosa cells and the oocyte. (Park et al., 2013). The effect of SCF supported early AF growth was also seen in this study. The higher concentration of SCF stimulated the growth of the follicle better than the lower concentration. However, SCF did not stimulate oocyte growth. This finding indicated that early AF may

need more amount of SCF to promote follicle growth than secondary follicle because SCF has been shown to influence on the theca cell growth and differentiation (Parrott and Skinner, 1997). Nevertheless, the growth of the follicle may impair the communication between oocyte and granulosa cells. Thus, the oocyte growth was not occurred in this study. Although SCF50 upregulated *FSHR, CYP19A1* and *STAR*, the addition of IGF-SCF50 to a culture medium increased the transcript level of *FSHR, GDF9* and all steroidogenic genes (*CYP17A1, CYP19A1* and *STAR*). Therefore, IGF-SCF50 may be the best choice for early AF supplementation. These IGF-SCF50 stimulated mRNA expressions elucidated that granulosa cells, theca cells and the oocyte of early AF were proliferated, differentiated and still function. Although the mRNA expressions of the early AF were inconsistent with the relative growth, the mRNA expressions, which indicating proper development of the follicle and the oocyte, were more important than the increasing diameter. Therefore, we suggested that the IGF-SCF50 was appropriated for early AF supplementation in this study.

In conclusion, the growth factors supplementation has various beneficial effects to the follicles and oocytes. Thus, we proposed the applicable guideline of IGF-I and SCF supplementations for the multi-step follicle culture the in the domestic cat in Fig. 8.

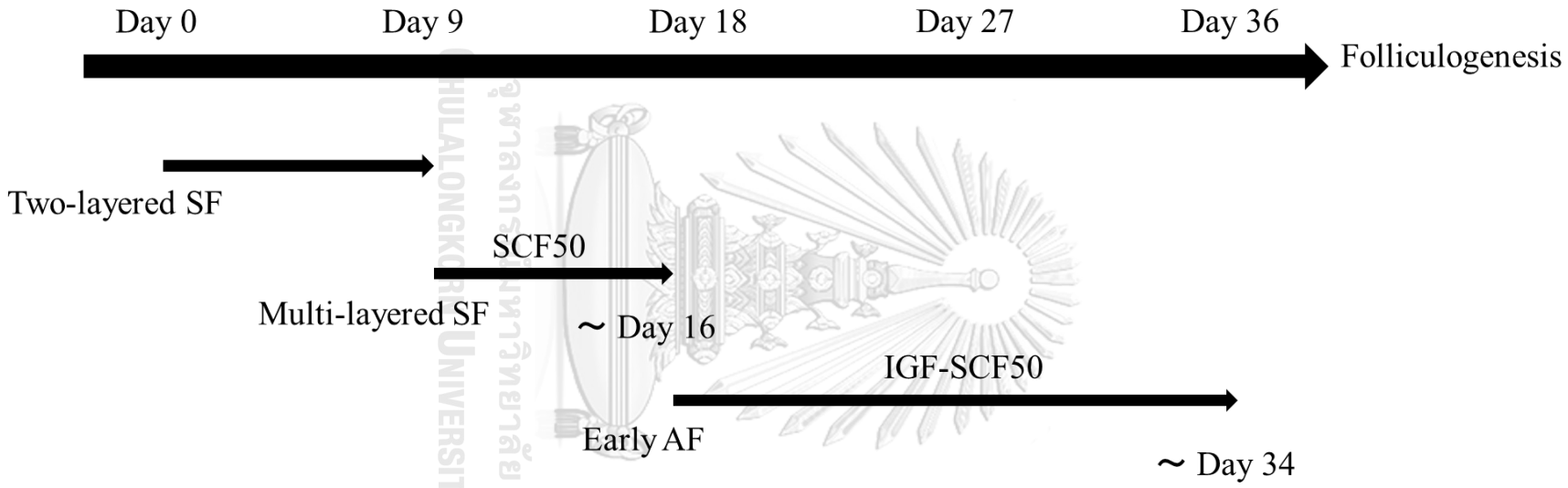


Figure 8. Proposed schematic diagram of IGF-I and SCF supplementation guideline for the multi-step of follicles culture the in cat.

After the follicle developing to the multi-layered SF stage, they need SCF50 for supporting growth and survival. Moreover, the addition of SCF50 promoted the antral cavity formation and upregulated the folliculogenesis related mRNA expressions. The multi-layered SF required 3 to 15 days (Average ~ 7.5 days) for antrum formation. Then, the multi-layered SF developed into the early AF stage. The IGF-SCF50 supplemented was crucial for ongoing growth of early AF and was also important for stimulating follicular function. However, further investigations on *in vitro* follicle culture requirements in order to improve oocyte quality are required.

Chapter V

General discussion and conclusion

In vivo folliculogenesis has been investigated for more than forty years. There have been numerous attempts to induce estrus by mimicking gonadotropin hormone secretion patterns in felids (Swanson et al., 1996; Swanson et al., 1997). Nowadays eCG is commonly used in the domestic cat to treat infertility problems or manage breeding programs (Kutzler, 2007). However, these estrus induction protocols elucidated inconsistent results in wild felids-maintained *ex situ*. The success of hormonal estrus induction depends on timing (Howard et al., 1997) and ovarian-responses (Miller et al., 1990; Howard et al., 1992; Howard et al., 1997). Furthermore, the success rate of AI after estrus induction in felids is low which may be due to the failure of estrus induction associated with dissimilarity in ovarian sensitivity to exogenous gonadotropin treatment among different felid species (Donoghue et al., 1990; Barone et al., 1994; Donoghue et al., 1996). Therefore, the discovery of gonadotropin responsiveness of the domestic cat would provide the basic information for future studies in other endangered felids species.

Testing of the ovarian responsiveness in the wild animals is not possible due to the limited number of the populations. Therefore, the *in vitro* culture served as an alternative tool in this study.

Our findings showed that the responsiveness of domestic cat follicles to eCG varied upon the developmental stages (Chapter II). The domestic cat follicles responded to eCG when they reached the multi-layered SF stage. Moreover, a low dosage of eCG (0.05 IU/mL) promoted the growth of multi-layered SF and sustained oocyte viability possibly through *STAR*.

With the same concentration as treated in Chapter II, tiger antral follicles isolated from the vitrified-warmed ovarian tissue after long-term storage were cultured. The results showed that follicles were increased in size after 3 days of culture.

eCG increased oocyte recovery rate from incubated follicles compared to those cultured without gonadotropin supplementation probably due to eCG's ability to suppress granulosa cell apoptosis and follicular atresia of antral follicle (Li et al., 1998). Therefore, the follicle can maintain bi-directional communication between granulosa cells and oocyte (Russell et al., 2016) resulting in the normal folliculogenesis and oogenesis.

In this study (Chapter III), the oocytes in the antral follicles of the tiger were found larger than the domestic cats (141.8 ± 0.9 µm vs 120 µm) (Izumi et al., 2012). All of the oocytes were remained in the germinal vesicle stage. Therefore, the oocytes from our study may resume meiosis if performed *in vitro* maturation. Since the relation between full size of the oocyte diameter and meiotic competence of the domestic cat has been reported (Otoi et al., 2001).

In chapter IV, it was apparent that there was stage-specific requirement of growth factor supplementation in supporting growth of follicles cultured in medium contained 0.05 IU/mL eCG. Growth factors supplementation did not show satisfactory results in the present study. IGF-I did not exert beneficial effects on follicle and oocyte growth in all developmental stages. In contrast, IGF-I reduced the viability of the enclosed oocytes, especially in the two-layered SF. Although IGF-I has been reported to have positive roles in early follicle development and steroid production (Sharma et al., 2010; Magalhaes-Padilha et al., 2012; Stubbs et al., 2013; Jimenez et al., 2016; Alves et al., 2017), some authors reported no beneficial effects of IGF-I supplement on

follicle development (Brito et al., 2012). In our study, the lower dosage of IGF-I and the longer duration of culture resulted in slightly growth of the follicle and also reducing oocyte diameter especially in two-layered SF may be due to the ideal concentration of IGF-I may be species-specific. The concentration of IGF-I (1 ng/mL) in our study may have been insufficient to ensure the efficient removal of those undesirable factors that would maintain cat two-layered SF viability during *in vitro* culture. In this present study, the multi-layered SF and the enclosed oocyte did not grow when supplement with IGF-I, SCF and their combination compared to previous studies (Lima et al., 2011; Magalhaes-Padilha et al., 2012). Although the follicle survival was enhanced when supplemented IGF-I and SCF (Sharma et al., 2009; Lima et al., 2011), they showed no effect to the survival rate compared to control in this present study.

In the present study, SCF at 50 ng/mL concentration also stimulated the mRNA expression of *FSHR, LHCGR, GDF9, BMP15, CYP17A1, CYP19A1* and *STAR* in the multi-layered SF, implying that folliculogenesis and steroidogenesis have occurred. The role of the SCF on antrum formation was emphasized an earlier observation reported in the mouse which antrum formation was inhibited after received an antibody of SCF (Yoshida et al., 1997).

For the early AF, the combination of 1 ng/mL IGF-I and 50 ng/mL SCF increased the transcription levels of *FSHR, GDF9* and steroidogenic genes (*CYP17A1, CYP19A1* and *STAR*) genes. These mRNA expressions demonstrated that the granulosa cells, theca cells and the oocyte of early AF were proliferated, differentiated and functioned.

In conclusion, eCG responsive stage in the domestic cat was at multi-layered SF onward with the concentration of 0.05 IU/mL being the most efficient. In this present

study, the tiger follicles responded to 0.05 IU/mL eCG. The growth factor supplementations to cat *in vitro* follicle culture illustrated the positive impact to the multi-layered SF and early AF of the domestic cat. The paracrine effect from SCF combined with eCG maintained follicle growth and survival and also promoted antral cavity formation of multi-layered SF. In contrast, the cat early AF required eCG, IGF-I and SCF for stimulate follicle growth and steroid production. However, further investigations on *in vitro* follicle culture requirements in order to improve oocyte quality are required. Finally, this study provides the information of gonadotropin (eCG) and growth factors regulations ovarian follicles in cat species, knowledge that is important to develop strategies to encourage *in vivo* and *in vitro* folliculogenesis.

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