การเจริญภายนอกร่างกายของพรีแอนทรัลฟอลลิเคิลในสัตว์ตระกูลแมว

นายกฤษณรงค์ วงศ์บ้านดู่

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาวิทยาการสืบพันธุ์สัตว์ ภาควิชาสูติศาสตร์ เธนุเวชวิทยาและวิทยาการสืบพันธุ์
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IN VITRO GROWTH OF PREANTRAL FOLLICLES IN CATS

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A Dissertation Submitted in Partial Fulfillment of the Requirements

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Thesis Title	*IN VITRO* GROWTH OF PREANTRAL FOLLICLES IN CATS
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กฤษณรงค์ วงศ์บ้านดู่: การเจริญภายนอกร่างกายของพรีแอนทรัลฟอลลิเคิลในสัตว์ตระกูลแมว. (*IN VITRO* GROWTH OF PREANTRAL FOLLICLES IN CATS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. สพ.ญ. ดร. เกวลี ฉัตรดรงค์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. แคทารินา เยฟเกนอฟ, 70 หน้า.

การทดลองที่ 1 วัตถุประสงค์เพื่อศึกษาอิทธิพลของไทร็อกซิน และแอคติวิน เอต่อการเจริญและลักษณะโครงสร้างของพรีแอนทรัลฟอลลิเคิลในแมวบ้าน ทำการแยกฟอลลิเคิลจากเนื้อเยื่อรังไข่ของแมวบ้านและเลือกฟอลลิเคิลที่มีลักษณะสมบูรณ์ไปแยกเลี้ยงในหยดน้ำยาเลี้ยงปริมาตร 20 ไมโครลิตร ซึ่งมีส่วนประกอบของไทร็อกซินเข้มข้น 0.5 1.0 หรือ 2.0 ไมโครกรัม/มิลลิลิตร หรือ แอคติวิน เอเข้มข้น 10 100 หรือ 200 นาโนกรัม/มิลลิลิตร เป็นเวลา 14 วัน วัดขนาดเส้นผ่านศูนย์กลางของฟอลลิเคิลในวันที่ 0 3 7 และ 14 ประเมินลักษณะโครงสร้างจากความสมบูรณ์ของเยื่อหุ้มฟอลลิเคิล การงอกขยายของแกรนูโลซ่าเซลล์ และการแยกตัวของโอโอไซต์ออกจากฟอลลิเคิล ตรวจการมีชีวิตของฟอลลิเคิลในวันที่ 14 ผลการศึกษาพบว่าฟอลลิเคิลทุกกลุ่มมีขนาดใหญ่ขึ้นในวันที่ 3 และมีเส้นผ่านศูนย์กลางใหญ่ที่สุดในวันที่ 7 น้ำยาเลี้ยงที่มีส่วนประกอบของแอคติวิน เอเข้มข้น 10 และ 200 นาโนกรัม/มิลลิลิตร กระตุ้นให้ฟอลลิเคิลมีขนาดใหญ่ขึ้นและใหญ่กว่ากลุ่มควบคุมในวันที่ 7 ของการเลี้ยง ($P < 0.05$) นอกจากนี้แอคติวิน เอความเข้มข้น 10 นาโนกรัม/มิลลิลิตร เพิ่มอัตราการรอดชีวิตของฟอลลิเคิลหลังเลี้ยงสูงขึ้น (46.9%, $P < 0.05$) พบการงอกขยายของแกรนูโลซ่าเซลล์ในฟอลลิเคิลซึ่งเลี้ยงในน้ำยาที่มี แอคติวิน เอสูงกว่ากลุ่มอื่นในช่วงวันที่ 7 ขณะที่การเติมไทร็อกซินในน้ำยาเลี้ยงไม่ส่งผลกระตุ้นการเจริญของพรีแอนทรัลฟอลลิเคิลในแมวบ้าน

การทดลองที่ 2 วัตถุประสงค์เพื่อศึกษาหาความเข้มข้นที่เหมาะสมของอัลจิเนตเจลสำหรับหุ้มพรีแอนทรัลฟอลลิเคิลของแมวบ้านในระบบการเลี้ยงแบบสามมิติ คัดเลือกพรีแอนทรัลฟอลลิเคิลที่มีลักษณะสมบูรณ์ซึ่งแยกได้จากรังไข่แมวบ้าน นำฟอลลิเคิลที่เลือกเข้าสู่กระบวนการเอนแคปซูเลชั่นเพื่อหุ้มด้วยอัลจิเนตเจลความเข้มข้น 0.25% 1.0% หรือ 2.0% ก่อนนำไปเลี้ยงนาน 14 วัน ประเมินการเจริญจากขนาดของเส้นผ่านศูนย์กลางในวันที่ 0 3 7 และ 14 นอกจากนี้ทำการสังเกตลักษณะโครงสร้างของฟอลลิเคิลตลอดระยะเวลาของการเลี้ยง ผลการศึกษาพบว่าฟอลลิเคิลในกลุ่มที่หุ้มด้วยอัลจิเนตเจลความเข้มข้น 0.25% และ 2.0% มีขนาดใหญ่ขึ้นภายหลังเริ่มต้นเลี้ยงและมีขนาดใหญ่ที่สุดในวันที่ 7 ขณะที่กลุ่มที่หุ้มด้วยอัลจิเนตเจลความเข้มข้น 1.0% มีการเจริญอย่างต่อเนื่องโดยมีขนาดเส้นผ่านศูนย์กลางใหญ่ที่สุดในวันที่ 14 ซึ่งมากกว่ากลุ่มที่ใช้อัลจิเนตเจลความเข้มข้น 0.25% และ 2.0% ($P < 0.05$) ไม่พบความเสียหายของเยื่อหุ้มฟอลลิเคิลขณะที่มีการงอกขยายของแกรนูโลซ่าเซลล์ในทุกกลุ่มทดลอง ผลการศึกษาสรุปได้ว่าการใช้อัลจิเนตเจลความเข้มข้น 1.0% สนับสนุนการเจริญและมีความเหมาะสมใการนำมาใช้หุ้มฟอลลิเคิลของแมวบ้านเพื่อเลี้ยงในระบบสามมิติ

การทดลองที่ 3 วัตถุประสงค์เพื่อศึกษาผลการแช่แข็งเนื้อเยื่อรังไข่ต่อการรอดชีวิตของพรีแอนทรัลฟอลลิเคิล และศึกษาการเจริญภายนอกร่างกายของฟอลลิเคิลที่แยกได้จากเนื้อเยื่อรังไข่ส่วนคอร์เท็กซ์แช่แข็งของเสือชีตาร์หลังตาย รังไข่ของเสือชีตาร์ถูกเก็บแช่เย็นและ ขนส่งไปยังห้องปฏิบัติการภายในเวลา 29 ชั่วโมง ตัดเนื้อเยื่อรังไข่ส่วนคอร์เท็กซ์เป็นชิ้น ขนาด 2.0 x 2.0 x 1.0 มิลลิเมตร นำชิ้นเนื้อผ่านกระบวนการแช่แข็งด้วยวิธีลดอุณหภูมิแบบช้าในภาชนะสำหรับแช่แข็ง และแช่แข็งด้วยวิธีวิทริฟิเคชั่น หลังการเก็บชิ้นเนื้อไว้ 1 ปี นำชิ้นเนื้อที่ผ่านกระบวนการแช่แข็งทั้งสองวิธี ๆ ละ 3 ชิ้นเนื้อ มาละลายและแยกพรีแอนทรัลฟอลลิเคิลออกจากชิ้นเนื้อ พบว่าแยกฟอลลิเคิลได้จากเนื้อเยื่อรังไข่ที่ผ่านการแช่แข็งด้วยวิธีลดอุณหภูมิแบบช้าจำนวน 23 ฟอลลิเคิล และจากเนื้อเยื่อรังไข่ที่ผ่านการแช่แข็งด้วยวิธีวิทริฟิเคชั่นจำนวน 58 ฟอลลิเคิล ฟอลลิเคิลที่รอดชีวิตและนำไปเลี้ยงในน้ำยามีจำนวน 10 และ 12 ฟอลลิเคิลจากเนื้อเยื่อที่ผ่านการแช่แข็งสองวิธีดังกล่าวตามลำดับ ขนาดเส้นผ่านศูนย์กลางของฟอลลิเคิลที่ได้จากเนื้อเยื่อที่แช่แข็งด้วยวิธีลดอุณหภูมิแบบช้าค่อย ๆ ลดลงจาก 53.5 ± 14.2 ไมโครเมตรในวันที่ 0 เป็น 50.9 ± 17.1 ไมโครเมตรในวันที่ 7 และมีฟอลลิเคิลที่มีชีวิต 2 จาก 10 ฟอลลิเคิล ขนาดเส้นผ่านศูนย์กลางของฟอลลิเคิลที่ได้จากเนื้อเยื่อที่แช่แข็งด้วยวิธีวิทริฟิเคชั่น รักษาขนาดเส้นผ่านศูนย์กลางไว้ได้คงเดิมคือ 50.7 ± 15.6 และ 50.5 ± 17.9 ไมโครเมตรในวันที่ 0 และ 7 ตามลำดับ โดยมีฟอลลิเคิลมีชีวิต 2 จาก 12 ฟอลลิเคิล โดยสรุปพรีแอนทรัลฟอลลิเคิลที่เก็บได้จากเนื้อเยื่อรังไข่แช่แข็งหลังเสือชีตาร์ตายสามารถเจริญภายนอกร่างกายได้นาน 7 วัน อย่างไรก็ตามควรมีการศึกษาหากระบวนการแช่แข็งเนื้อเยื่อรังไข่และส่วนประกอบของน้ำยาที่ใช้เลี้ยงพรีแอนทรัลฟอลลิเคิลที่เหมาะสม เพื่อเพิ่มอัตราการรอดชีวิตและอัตราการเจริญเติบโต

ภาควิชา สูติศาสตร์เธนุเวชวิทยาและวิทยาการสืบพันธุ์ ลายมือชื่อนิสิต.....

สาขาวิชา วิทยาการสืบพันธุ์สัตว์ ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา 2555 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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KEYWORDS: FELIDS / FEMALE GAMETE / GROWTH FACTORS / BIOENGINEERING / *IN VITRO* CULTURE / OVARIAN FOLLICLE

GRISNARONG WONGBANDUE: *IN VITRO* GROWTH OF PREANTRAL FOLLICLES IN CATS. ADVISOR: ASSOC. PROF. KAYWALEE CHATDARONG, Ph.D. CO-ADVISOR: PROF. KATARINA JEWGENOW, Ph.D., 70 pp.

EXP. 1 The objective was to determine effects of thyroxin (T_4) and activin A on *in vitro* growth and morphology of preantral feline ovarian follicles. Preantral follicles (86.3± 18.7 µm) were isolated from fresh ovaries of domestic cats. Healthy follicles were cultured individually for 14 d in 20-µL microdrops of basic culture medium supplemented with various concentrations of T_4 (0.5, 1.0 or 2.0 µg/mL) or activin A (10, 100 or 200 ng/mL). Follicle diameter was measured on Days 0, 3, 7, and 14 of culture. Follicle morphology was characterized based on granulosa cell proliferation, dissociation of somatic cells, and detachment of oocytes from follicles. On Day 14, follicles were assessed for viability using ethidium homodimer-1 staining. In controls, diameters of follicles increased from initial sizes on Day 3, and peaked on Day 7. This pattern was also observed in both T_4-and activin A-treated follicles. On Day 7, diameters and diameter gains of follicles treated with 10 and 200 ng/mL activin A were larger than those of the controls ($P < 0.05$). Furthermore, 10 ng/mL activin A increased percentage of viable follicles on Day 14 (46.9 % viable; $P < 0.05$). Follicles treated with activin A had rapid granulosa cell proliferation until Day 7. In conclusion, activin A promoted growth of preantral feline follicles and supported follicle viability during a14-d culture, whereas T_4 supplementation had no beneficial effects.

EXP. 2 The objective was to optimize alginate gel concentrations for feline preantral follicle culture. Preantral follicles with round or oval shape were mechanically isolated from ovaries of domestic cats, and individually encapsulated with 0.25% (n = 15), 1.0% (n = 31) or 2.0% alginate gel (n = 22), respectively. Each encapsulated follicle was cultured in a 96-well plate containing 100 µL medium comprised of M199 supplemented with 0.23 mM sodium pyruvate, 2 mM L-glutamine, 12.5 mM Hepes, 0.3% BSA, 1% ITS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1.0 mIU/ml growth hormone, 2.3 µg/ml FSH, 10 ng/ml IGF-I and 10 ng/ml activin A. Follicle morphology and diameter were determined on Days 0, 3, 7 and 14. The follicles encapsulated in 0.25% and 2.0% alginate gel reached their maximal diameters on Day 7 and maintained their sizes till Day 14. In contrast, diameters of the follicles encapsulated in 1.0% alginate increased continuously from Day 0 to 14 and finally reached greater size than follicles in the other alginate concentrations ($P<0.05$). Most cultured follicles exhibited intact basement membrane throughout culture. Our findings suggested that 1% alginate gel is optimal supporting cat preantral follicle growth in the 3-D culture system.

EXP. 3 This study aimed to investigate freezing effects of ovarian tissues on survival of preantral follicles and observe *in vitro* growing of preantral follicles retrieved from cryopreserved ovarian cortical tissues of a cheetah post-mortem. After 29 hours cold storage, ovarian cortices were cut into small pieces (2.0 x 2.0 x 1.0 mm^3) and allocated to be frozen using a passive cooling container (n=3 pieces) or vitrification (n=3 pieces). After one year of storage, 23 and 58 preantral follicles were mechanically isolated from ovarian tissues cryopreserved using a passive cooling container and vitrification, respectively. Of 23 and 58 isolated follicles, 10 and 12 morphologically intact and viable were selected to be *in vitro* grown in a culture medium for 7 days. Diameters and diameter gains were examined on Days 0, 3 and 7. Follicle viability was assessed on Day 7. Diameters of follicles retrieved from the slow freezing ovarian tissues decreased gradually from 53.5 ± 14.2 µm on Day 0 to 50.9 ± 17.1 µm with 2 out of 10 viable on Day 7 whereas those frozen using vitrification maintained their diameters between 50.7 ± 15.6 µm and 50.5 ± 17.9 µm on Days 0 and 7, respectively, with 2 of 12 viable. In conclusion, preantral follicles obtained from cryopreserved cheetah ovarian tissues can be grown *in vitro* for 7 days. However, optimization of freezing protocol and culture medium are required to improve the viability and growing rate.

Department: Obstetrics Gynaecology and Reproduction Student's Signature .....

Field of Study: Theriogenology Advisor's Signature .....

Academic Year: 2012 Co-advisor's Signature .....

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CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

The mammalian ovary contains abundant of preantral follicles which are the major source of oocytes (Jewgenow and Stolte, 1996; Lucci et al., 1999). Of those germ cells, a few numbers leave dormant stage and grown to ovulatory stage during lifespan, while the other encounters with degeneration and become atresia (Oktem and Urman, 2010). Consequently, the attempts to utilize these ovarian follicles to improve reproductive capacity and fertility in human and animals attract studies in the fields of reproductive biotechnology and assisted reproductive technology (ART) for decades.

In human, acute ovarian failure and ovarian insufficiency is induced unintentionally by gonadotoxic treatment from chemotherapy or radiation, which destroys immature follicle pool of female cancer patients (Xu et al., 2011). Although a well-established protocol of ovarian stimulation, *in vitro* fertilization (IVF) and embryo cryopreservation prior cancer treatment provides some child–bearing potential, but it requires a period of time and available of sperm donor (Wang et al., 2011). Therefore, the technique is not suitable for all patients, especially in the pre-pubertal girls and patients who required immediate treatment without delayed. An alternative approach is the ovarian tissue cryopreservation followed by ovarian tissue retransplantation or *in vitro* follicle culture to restore fertility at a suitable time for the women after recovering from cancer. To date, this combined technique has been proved to be effective with successes of 18 births human babies (Andersen et al., 2012; Wiedemann et al., 2012)

In animals, the death of an individual or an ovariectomy for treatment of reproductive diseases is accompanied with the loss of the female gametes (Johnston et al., 1991). Although protocols applying IVF and embryo cryopreservation are available for many animals, preservation of female germ cells, in particular of a genetically valuable animal, requires spermatozoa of the same species at the time when female

pass away or is spayed. Therefore, rescue female gametes in combination with growth to maturity at the proper time provide the last opportunity for preservation of fertility in animals, particularly in those of valuable and endangered species.

The Felidae family consists of 41 members that widely divergent in phenotype and geographic range (Howard and Wildt, 2009). The tropical forest of Thailand is a natural habitat for 9 felids, comprising tiger (*Panthera tigris*), leopard (*Panthera pardus*), clouded leopard (*Neofelis nebulosa*), asian golden cat (*Felis temmincki*), fishing cat (*Felis viverrina*), leopard cat (*Felis bengalensis*), jungle cat (*Felis chaus*), flat-headed cat (*Felis planiceps*), and marbled cat (*Felis marmorata*) (Srisamoot et al., 2007). According to the accelerated rate of habitat destruction and poaching, populations of these felids are declining in its natural range (Bristol-Gould and Woodruff, 2006). Hence, almost felids, except domestic cats, are listed as endangered, vulnerable, or near threatened with extinction (International Union for Conservation of Nature; IUCN Red Lists of Threatened Species, 2012). Establishment of reservoir population in captivity with effective breeding management program in conjugated with assisted reproductive technology (ART) is considered enhancing existence of these valuable species, as well as offering research opportunity that is not be available in nature (Pelican et al., 2006). To improve the knowledge of non-domestic felids, domestic cat is considered a suitable model for reproductive biological studies in the family, contributing to the understanding of felid reproductive physiology despite considerable species differences (Swanson, 2003).

Interestingly, around 38,000 preantral follicles with oocytes are found in cat ovaries (Carrijo et al., 2010), but only 2% of them leave their dormant stage and start growing (Jewgenow and Paris, 2006). Although additional numbers of ovulatory oocytes can be theoretically induced by superovulation using exogenous hormones, factors of species-specific and dose-dependent responses to hormones promoting follicular development and ovulation in felids are unpredictable (Pelican et al., 2006). Therefore, beside mature and immature oocyte retrieval, oocytes enclosed in follicles are worth preserved for further restoration of fertility in a recent study. Becuase feline mature

oocytes exhibit a high sensitivity to cryoprotectant and chilling process (Luvoni, 2006), and small preantral follicles are better survive cryopreservation, the great potential of preserving huge number of oocytes within preantral follicles was emphasized (Jewgenow and Paris, 2006).

In vitro follicle culture as a part of ART was first demonstrated in mouse by Eppig (1977). This technique potentially supports growth and development of preantral follicles derived from fresh or cryopreserved ovarian tissues in humans and domestic animals. It not only aims on the production of fully grown oocytes for fertility restoration, but also facilitates comprehensive research on the complex regulatory process of follicle development (West et al., 2007a). Currently, achievements of *in vitro* follicle culture to produce fertilized oocytes from preantral follicles have been demonstrated in laboratory animals and domestic species including mice (Eppig and Schroeder, 1989), pigs (Wu et al., 2001), sheep (Arunakumari et al., 2010) and goats (Magalhaes et al., 2011a). The full developmental course starting with *in vitro* growth of oocytes from preantral follicles by culture to maturity, followed by *in vitro* fertilization and embryo transfer till life birth of viable offspring has been reported only in the mice (Eppig and Schroeder, 1989; Wang et al., 2011). It is important to mention, that in mice the growth period in vivo lasts only 3 weeks, whereas in non-rodent species, including human and felids it is suggested to be about 3 months.

To date, few extrinsic and intrinsic factors are known, which promote cat preantral follicle development *in vitro*. Among others, the insulin-like growth factor I (IGF-I) and basic fibroblast growth factor (bFGF) enhance the oocyte metabolism *in vitro* and promote proliferation of somatic cells in small follicles, respectively (Jewgenow, 1996). In addition, Jewgenow and Pitra (1993) demonstrated the role of follicle stimulating hormone (FSH) that stimulated growth rate of secondary follicles and finally provided competent oocytes. Many other signals, which can influence follicular survival, growth, and development are reported in human and other domestic species, i.e. activins, anti-Mullerian hormone (AMH), growth differentiation factor 9 (GDF-9), growth hormone (GH), and thyroxin (T_4). None of those factors have been investigated in

felids. It is noteworthy that recent studies of *in vitro* follicle culture revealed the potential role of activin A which is the most abundant activin isoform, and thyroxin hormone which regulates follicle development in human and domestic species. While activin A promoted granulosa cell differentiation in rat (Findlay, 1993), and encouraged sheep oocyte and preantral follicle growth and development (Thomas et al., 2003), activin A inhibited recruitment of human primordial follicle (Ding et al., 2010).

The positive effects of T_4 are found to promote follicle development and antral formation in a dose-dependent manner in sheep (Arunakumari et al., 2007). In addition, the combination of T_4 with FSH, GH, and IGF-I supplemented to culture medium resulted in the best development of ovine follicular growth and oocyte maturation rate (Arunakumari et al., 2010). There is no report on the roles of activin A and T_4 during follicle growth in the cats. Besides, while the three-dimensional follicle culture system has been currently applied and displayed advantages for follicular development in mouse (Xu et al., 2006) and human (Oktem et al., 2011), studies in felids are scarce. Hence, additional study of the influence of extrinsic and intrinsic factors promoting *in vitro* growth and development of preantral follicle in felids, as well as of optimal culture system are essential keys to promote cat preantral follicle growth and development.

1.2 Literature Review

1.2.1 Classification of follicle stages

Initiation of ovarian follicle commences with primary oocytes enveloped by a single layer of flattened granulosa cells. In rodents, follicle formation occurs synchronously during the first few days after birth. By contrast, follicles are formed during fetal life with less synchronous manner in primates and other domestic animals (Fortune, 2003). Therefore, the fetal ovaries of animals contain both growing preantral and antral follicles. Preantral follicle stages have been classified by several means that vary among and within species. The perspicuous classification of follicle stages is presented by Fortune (2003) (Table 1). In details, primordial follicles are formed with a single layer of flattened pregranulosa cells that differentiate to cuboidal when follicles

leave the resting pool. Transition of primordial follicle to primary stage is prolonged *in vivo*. In the late stage of primary follicle, the number of granulosa cells reaches 60 with the first presence of theca interna recruited from ovarian stromal cells. The theca layers of ruminant and primate follicles can be observed at late preantral stage. The secondary follicle commences with a formation of second layer of granulosa cells, and progresses through the addition of up to 6-7 layers. Termination of the secondary follicle occurs when entering antral stage characterized by gradual development of an antral cavity to follicle diameter of about 250 μm (Fortune, 2003).

In domestic cats, primordial follicles are the smallest follicles which are covered by 1-8 flattened squamous granulosa cells (Bristol-Gould and Woodruff, 2006), ranging in the size from 40 to 50 μm (Jewgenow and Göritz, 1995). Primary follicles are of the same size but surrounded by cuboidal granulosa cells. Secondary follicles greatly vary in size due to multiple layers of granulosa cells, ranging from 100 to 400 μm. In addition, the secondary follicle is the first stage that zona pellucida is observed through histological examination (Reynaud et al., 2009). Early antral follicles are characterized by the presence of antral cavity containing follicular fluid with the size of 120 to 200 μm. The antral follicles increase in size up to 3000 μm with accumulation of follicular fluid (Bristol-Gould and Woodruff, 2006).

Table 1. Classification of stages of preantral follicles in mice (Fortune, 2003)

Follicle type	Layer of granulosa cells	Number of granulosa cells (range)	Clearly defined theca interna
Type 1	0	0	-
Type 2 (primordial)	1	<4	-
Type 3a (early primary)	1	≤20	-
Type 3b (late primary)	1	21-60	+
Type 4 (early secondary)	2	61-100	+
Type 5a (mid secondary)	3	101-200	+
Type 5b (late secondary)	4-6	201-400	+

1.2.2 Regulation of preantral follicle growth and development

In all mammals, follicle and oocyte development starts with the establishment of ovary shortly after conception and terminates with the ovulation of fertilizable oocytes (Picton et al., 2008). This unique process, in the sequence of events, requires complex regulatory mechanisms involving both extrinsic (endocrine) and intrinsic signaling pathways in the follicle stage-related manner as illustrated in Figure 1. In the facet of intrinsic signaling pathways, numerous peptides locally produced from oocyte, granulosa cell, theca cell, and ovarian stromal cell appear to activate or suppress follicle growth through the autocrine and paracrine activity (Kidder and Mhawi, 2002). In addition, it is proposed that an important pathway for intercellular communication between granulosa cells and the oocyte is mediated through gap junction (Diaz et al., 2007). Beside the influences of those signals, supplementation of follicle promoting substance, for example insulin-transferrin-selinium (ITS) also expressed positive impacts on preantral follicle viability and growth *in vitro* (Wright et al., 1999; Demeestere et al., 2005).

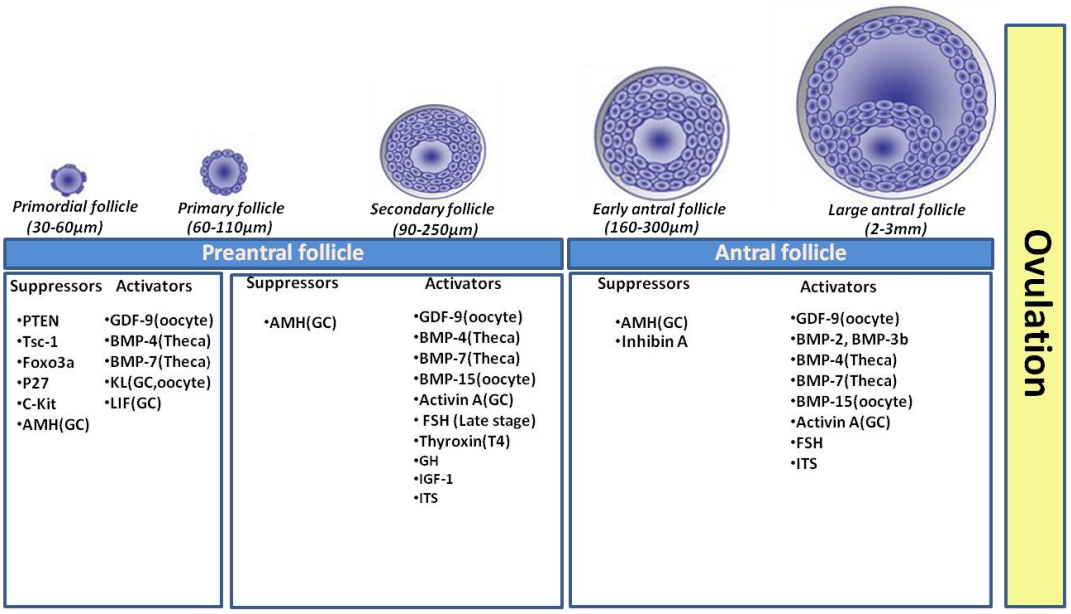

Figure 1. Schematic representation of intraovarian factors, endocrines, and follicular promoting substances, that suppress or activate follicle growth and development at different stages (Jewganow, 1998; Webb et al., 2004; Demeestere et al., 2005; Arunakumari et al., 2007; Fortune, 2003; Reynaud et al., 2009; Oktem and Urman, 2010).

Transition of primordial follicle to primary follicle

The earliest follicle growth stage, the transition of primordial to primary follicle, is gonadotropin-independent and mainly regulated by intraovarian factors. Although triggering of this growth stage has not been clarified, it has been proposed that it initiates within the oocyte or in the surrounding cells (Smitz and Cortvrindt, 2002). This might be a reason that explains why isolated primordial follicles do not survive in culture, but grow *in situ* in ovarian tissue culture (O'Brien et al., 2003). According to studies of animal models, several members of the transforming growth factor-beta (TGF-β) superfamily, such as BMP-4 and BMP-7 (expressed on ovarian stromal cells and/or theca cells) and GDF-9 (expressed on oocytes) play a critical role on the transition of primordial follicles (Oktem and Urman, 2010). In addition, kit-ligand (KL) and the leukemia inhibitory factor (LIF) are other factors which recognized acting at paracrine level on the promotion of primary follicles formation are (Nilsson and Skinner, 2002).

Studies on genetic modified mice indicated that some inhibitory signals are able to maintain primodial follicle in the dormant stage. These include, for instance, tumor suppressor tuberous sclerosis complex I (Tsc-I), phosphatase and tensin homolog deleted on chromosome 10 (PTEN), Foxo3a, p27, and FoxI2. Knock-out of these inhibitory molecules leads to premature activation of primordial follicle pool (Adikari et al., 2010). Beside those inhibitory signals, an increased recruitment of primordial follicle into growing pool was observed in anti-Mullerian hormone (AMH) null mice suggesting a negative effect of AMH on primordial to primary follicle transition (Durlinger et al., 2002). AMH also attenuates preantral follicle response to FSH in advance stages of follicle development (Knight and Glister, 2006).

Development of primary follicle to early antral stage

The further follicle development is characterized by the proliferations of granulosa cells, thus transforming single-layered primary follicles into multi-layered \secondary follicles. In addition to the granulosa cell proliferation, an permanent increase in oocyte diameter, formation of basal lamina, zona pellucida, and theca cell

layer are observed during this stage of development (Knight and Glister, 2006). This process lasts months in human (Oktem and Urman, 2010). The role of follicle stimulating hormone (FSH) is still unclear, although the receptor of FSH first expressed in the secondary follicles. Thus, supplementation of FSH alone increases survival, proliferation of granulosa cells, and antrum formation in isolated mice follicles ranging from 95 to142 µm in diameter (Cortvrindt et al., 1997). FSH has also been shown to enhance the survival of ovarian graft transplanted into immune-deficient mice (Oktay et al., 1998). In contrast, McGee et al. (1997) found out that FSH is not expression a positive effect on growth and survival *in vitro* when isolated preantral follicles are cultured in the absence of serum. Their findings suggest that FSH may have a permissive role, or synergized positive effect with other intra-ovarian regulators, rather than being essential for preantral follicle growth.

Beside the influence of gonadotropins, altering level of 3,3',5'-tetra-iodothyronine (T_4) and 3,3',5-triiodothyronine (T_3) can influence mammalian fertility (Burrow, 1993). Although T_3 negatively affected *in vitro* antral formation in mouse preantral follicle via a mechanism involving granulosa cell ability response to FSH (Cecconi et al., 2004), T_4 supplementation to culture medium of sheep preantral follicle had positive effects on *in vitro* growth in both small and large preantral follicles, and antrum development together with subsequent maturation of oocyte to the MII stage was shown (Arunakumari et al., 2007). In addition, the mixture of T_4, FSH, growth hormone (GH) and IGF-1 supplementation provide the best development of *in vitro* sheep preantral follicle and oocyte maturation (Arunakumari et al., 2010). Nevertheless, the effects of T_4 supplemented to culture medium on follicle growth and development in other domestic species, including cats, are scarcely recognized.

As for the intraovarian factors, the member of TGF-β superfamily which are locally produced from granulosa cells (activins), theca cells (BMP-4 and BMP-7), and oocytes (GDF-9), play a crucial role in the growth of primary follicle transformation to antral stage (Oktem and Urman, 2010). GDF-9 has been shown to initiate progression of *in vitro* follicle growth in human and rodents (Wang and Roy, 2004). Arrest of follicle

growth at the primary stage in GDF-9 gene null mice and sheep inactivating mutation GDF-9 confirmed the role of GDF-9 in promoting follicle growth beyond the primary stage (Hanrahan et al., 2004). Similarly to the GDF-9, the BMP-4 and BMP-7 of thecal origin are able to promote follicle growth in rodents beyond primary stage in both *in vivo* and *in vitro* (Lee et al., 2004).

Among the intraovarian regulators, activins are thought to play an autocrine/paracrine role in controlling early follicular development together with promoting follicular growth and differentiation (Findlay, 1993). Activins are homodimers composed of two inhibin β subunits (βA βA as activin A, βB βB as activin B, and βA βB as activin AB). The most bioactive and functioning form is activin A which regulates the target cell function through membrane-bound heteromeric complexes of serine-threonine kinase receptors and intracellular Smad protein (Figure 2) (Horbelt et al., 2003; Pangas et al., 2003). Zhao et al. (2001) demonstrated the activin A mRNA expression and immunoractivity in rat preantral follicle assuring that activin A is biosynthesized by oocytes or somatic cells of such follicles. In addition, the same authors reported that activin A not only did stimulate proliferation of follicle cells but also induced antral formation in a dose-dependent manner (Telfer et al., 2008). Influences of activin A stimulating *in vitro* preantral follicle growth and development has been further reported in medium-sized ovine preantral follicles (Thomas et al., 2003), caprine follicles (Silva et al., 2006), rat follicles (McGee et al., 2001), and human follicles (Telfer et al., 2008). However, it has been discovered that activin A inhibited primordial follicle development in human (Ding et al., 2010). Moreover, activin derived from secondary mouse follicles suppresses the *in vitro* growth of primary follicle (Thomas et al., 2003). Thus, the precise role of activin is still unclear. Interestingly, in felids, the role of activin A in preantral follicles development *in vitro* has not been investigated.

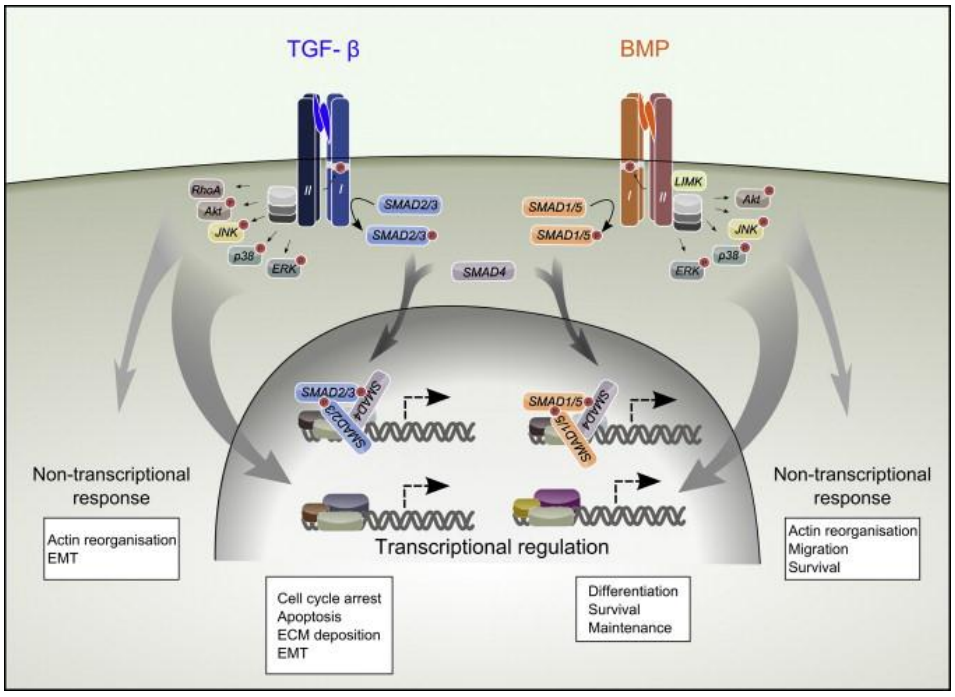

Figure 2. Pathway of activin A regulates cell function through transmembrane receptors (activin receptor type I and II) and cytoplasmic Smad proteins. As a member of TGF-β, activin A-receptor complex phosphorylates Smad2/3 protein which accumulates in the nucleus and forms post-receptor transduction (Horbelt et al., 2003).

1.2.3 Assessment of *in vitro* follicle growth and development

Assessment of *in vitro* growth and development of preantral follicle can be operated in various approaches. Measurement of follicle diameter is a basic technique used to investigate follicle and oocyte growth, by comparing the follicle diameter at the first day of cultivation to its size at the terminal date. Increasing of follicle diameter, as a quantitative data, appeared to relate to the number of granulosa cells and enlargement of oocyte (McGee et al., 2001). This technique is extensively applied in human (Telfer et al., 2008), mice (Xu et al., 2006; Oktem et al, 2011), and sheep (Thomas et al., 2003). An advantage of follicle diameter assessment is that data are chronologically retrieved along the culture period. Other assessments indicating follicle growth and development are expression of mRNA encoding for AMH and Oct-4. AMH, a glycoprotein hormone, is a member of TGF-β superfamily which is produced from granulosa cells of growing preantal and early antral follicle (Fortune, 2003). Locally produced AMH can be applied

as valuable criteria for evaluation of human ovarian reserve (Oktem and Urman, 2010). In addition, expression of AMH mRNA is utilized as a marker for follicle growth because it is detected only in proliferating granulosa cells (Muruvi et al., 2005). Oct-4 gene is a nuclear transcriptional factor belonging to the POU family in which its acronym has been named from the first four members; Pit-1, Oct-1. Oct-2, and Unc-86 (Phillips and Luisi, 2000). The Oct-4 regulates the expression of developmental genes and is required for maintenance of cell pluripotency (Monti et al., 2006). The expression of Oct-4 was first observed in the inner cell mass of mouse embryo during early pre-implantation stage and ceased when oocytes enter meotic division. Interestingly, reappearing of Oct-4 begins soon before initiation of oocyte growth and persists through the folliculogenesis (Monti et al., 2006). Therefore, using of RT-PCR detecting the expression of Oct-4 could be a potential tool to investigate the oocyte viability and development *in vitro*.

1.2.4 *In vitro* follicle culture system

In vitro follicle culture systems can be categorized into 2 approaches (West et al., 2007a). The first is the 2-dimensional follicle culture system in which isolated follicles are placed and allowed to attach on the surface of culture dishes. In growing follicles, proliferating granulosa cells break through the basement membrane and migrated onto the culture surface, leading to diffuse morphology of follicles. Although this system has been proven successful in producing oocytes and subsequently giving live offspring in mice (Eppig and Schroeder, 1989; Wang et al., 2011), there is no report of supporting normal follicle development in human (Abir et al., 2001) and other domestic animals (West et al., 2007a). Maintaining of association between granulosa cells and oocytes is difficult in large animal species due to their large size of the follicles and the required long-term period of culture (Ksiazkiewicz, 2006). The second approach is the 3-dimension follicle culture system which maintains follicle architecture by preventing follicle from adhesion by encapsulating preantral follicle in a three-dimensional matrix. There are many potential materials available that meet a majority of these requirements, including polyethylene glycol (PEG), collagen, matrigel, and alginate. Among them, alginate is a promising material to be applied for preantral follicle culture because of its

gentle gelation and straightforward dissolution (West et al., 2007b). Successful 3-dimantional follicle culture system with alginate hydrogel has been reported in mice (Xu et al., 2006), pigs (Wu et al., 2001), and human (Telfer et al., 2008). However, these systems have not been used for culture of cat preantral follicles.

1.3 Objectives of the thesis

1. To investigate growth and development of cat preantral follicles after culture in medium in the presence or absence of thyroxin (T_4) or activin A
2. To assess the optimize concentration of alginate gel on viability and growth of preantral follicles cultured in three-dimension system
3. To determine viability and growth of wild felid preantral follicles derived from cryopreserved ovarian tissues after *in vitro* culture using the system translated from domestic cat model

1.4 Hypothesis

1. Supplementation of T_4 or activin A to culture medium potentially promote cat preantral follicle growth and development *in vitro*
2. The optimal concentration of alginate gel applied for three-dimensional culture system could improve *in vitro* growth and development of cat preantral follicles
3. Preantral follicles recovered from cryopreserved ovarian tissues of wild felids can be grown and survived *in vitro* under the culture system developed in domestic cat

1.5 Keywords: biomaterial matrix, feline species, female gamete, growth factors, hormones, *in vitro* culture, preantral follicles, ovarian tissues

1.6 Research merits

1. The knowledge in the effects of thyroxin (T_4) and activin A provides additional information on the roles of hormones and growth factors during folliculogenesis in felids
2. The knowledge in optimal alginate concentration could be an essential part for improvement of culture condition in cats, which finally may succeed in the production of fully grown and fertilizable oocyte
3. Culture of cryopreserved ovarian follicles recovered from animal post-mortem conduct to the possibility of translation of gamete rescue and culture technique from the animal model to endangered wildlife species

CHAPTER II

EFFECTS OF THYROXIN (T4) AND ACTIVIN A ON IN VITRO GROWTH OF PREANTRAL FOLLICLES IN DOMESTIC CATS

2.1 Abstract

Preantral follicle culture is a promising technique for rescuing gametes from endangered animals that die abruptly. The objective was to determine effects of thyroxin (T_4) and activin A on *in vitro* growth and morphology of preantral feline ovarian follicles. Preantral follicles (86.3± 18.7 µm) were isolated from fresh ovaries of domestic cats. Healthy follicles were cultured individually for 14 d in 20-µL microdrops of M199 supplemented with 0.23 mM sodium pyruvate, 2 mM L-glutamine, 12.5 mM Hepes, 0.3% (wt/vol) BSA, 1% (vol/vol) ITS, 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 1.0 mIU/mL growth hormone, 2.3 µg/mL FSH, and 10 ng/mL IGF-I. The effect of various concentrations of T_4 (0.5, 1.0 or 2.0 µg/mL) or activin A (10, 100 or 200 ng/mL) on follicle growth and follicular integrity were assessed. Follicle diameter was measured on Days 0, 3, 7, and 14 of culture. Follicle morphology was characterized based on granulosa cell proliferation, dissociation of somatic cells, and detachment of oocytes from follicles. On Day 14, follicles were assessed for viability using ethidium homodimer-1 staining. In the controls, diameters of follicles increased from initial sizes on Day 3, and peaked on Day 7. This pattern was also observed in both T_4-and activin A-treated follicles. On Day 7, diameters and diameter gains of follicles treated with 10 ng/mL (mean±SEM; 170.8 ± 7.6 and 35.9 ± 5.1µm, respectively) and 200 ng/mL activin A (165.2± 10.4 and 32.8 ± 5.5 µm, respectively) were larger than those of the controls ($P < 0.05$). Furthermore, 10 ng/mL activin A increased percentage of viable follicles on Day 14 (46.9 % viable; $P < 0.05$). Follicles treated with activin A had rapid granulosa cell proliferation until Day 7. In conclusion, activin A promoted growth of preantral feline follicles and supported follicle viability during a14-d culture, whereas T_4 supplementation had no beneficial effects.

2.2 Introduction

Combining ovarian tissue cryopreservation and *in vitro* culture of ovarian follicles is a promising approach for fertility restoration in both humans and endangered wildlife species. In women with cancer, cryopreservation of ovarian tissue before radiation or chemotherapy has been used to preserve fertility (Xu et al., 2009c). To date, at least 13 children have been born after transplantation of cryopreserved ovarian strips (Donnez et al., 2011). Despite this success, apprehension concerning possible re-introduction of cancer cells remains (Xu et al., 2009c). Therefore, the technique of *in vitro* follicle culture after cryopreservation was proposed, in order to obtain fully grown oocytes for IVF and thus preclude any risk of cancer cell transfer.

Almost all felid species are listed as endangered, vulnerable, or nearly threatened with extinction on the International Union for Conservation of Nature (IUCN) Red List for endangered species (IUCN, 2012). Assisted reproductive techniques are considered an integral part of conservation efforts within conservation breeding programs of endangered felids (Swanson, 2006). Amongst others, cryopreservation of ovarian cortex followed by *in vitro* growth of preantral follicles to maturity has potential to preserve fertility of cats who die abruptly or undergo an ovariohysterectomy for medical reasons (Jewgenow and Paris, 2006; Jewgenow et al., 2011). To achieve this goal, culture conditions must be developed for each particular species. The domestic cat is considered a suitable model for reproductive biological studies of non-domestic felids, contributing to the understanding of felid reproductive physiology despite considerable species differences (Swanson, 2003). Ovaries from domestic cats, which are routinely available after ovariohysterectomy, are used to develop basic *in vitro* techniques, e.g.*in vitro* maturation (IVM), *in vitro* fertilization (IVF), and embryo culture (Goodrowe et al., 1988; Pope, 2004; Ringleb et al., 2011). However, *in vitro* growth of isolated follicles from domestic cat, has apparently not been reported since earlier work of Jewgenow and Pitra (1993), although substantial progress has been made in other species, including humans (Telfer et al., 2008; Xu et al., 2009b), non-human primates (Xu et al., 2009c;

Hornick et al., 2012), pigs (Wu et al., 2007), sheep (Arunakumari et al., 2010; Magalhaes et al., 2011b), goats (Huanmin and Yong, 2000), dogs (Serafim et al., 2010) and mice (Wang et al., 2011; Desai et al., 2012)

Successful *in vitro* culture of isolated follicles requires determination of essential factors which promote early follicular development. Recent studies revealed the potential role of activin A for ovarian follicular growth in humans (Telfer et al., 2008), mice (Cossigny et al., 2012) and sheep (Thomas et al., 2003; Choi et al., 2008). Activin A, the most abundant activin isoform (McLaughin et al., 2010), promotes granulosa cell differentiation in mice (Findlay, 1993) and growth of oocytes and preantral follicles in sheep (Thomas et al., 2003), but it inhibits recruitment of human primordial follicles (Ding et al., 2010). Furthermore, thyroxin (T_4) also promotes follicle development and antrum formation in a dose-dependent manner in sheep (Arunakumari et al., 2007). In that regard, adding T_4 to culture medium supplemented with FSH, growth hormone (GH) and insulin-like growth factor I (IGF-I) resulted in the highest rates of follicle development and oocyte maturation in sheep (Arunakumari et al., 2010).

The present study aimed to develop a culture system for isolated preantral follicles of domestic cats. Follicle growth, as well as oocyte/ follicle viability, were monitored during 14 d of culture. In addition, the effects of activin A and T_4 supplementation on culture success were also determined.

2.3 Materials and methods

All chemicals and reagents used in this present study were purchased from Sigma-Aldrich Company (St. Louis, MO, USA), unless otherwise stated.

2.3.1 Collection of ovaries

Fresh ovaries were collected from domestic cats that underwent routine ovariohysterectomy at private veterinary clinics or public animal shelters. Tissues were placed in 50 mL Greiner tubes containing a transport medium of Minimum Essential

Medium Eagle Hepes Modification supplemented with 0.3% (wt/vol) BSA and 1x Antibiotic Antimycotic Solution and shipped at 4 °C in a styrofoam box to the laboratory within 2 to 4 h after removal. Upon arrival, connective tissues and vessels were removed from the ovaries, and the latter were washed and stored in transport medium overnight at 4 °C before isolation of preantral follicles.

2.3.2 Isolation of preantral follicle

Preantral follicles were mechanically isolated from ovarian tissues in M199 supplemented with 25 mM HEPES, 0.23 mM sodium pyruvate, 2 mM L-glutamine, 0.3% (wt/vol) bovine serum albumin (BSA), 100 IU/mL penicillin, and 0.1 mg/mL streptomycin (holding medium). Each ovary was dissected in half and laid with its medulla surface down on a 60-mesh cell dissociation sieve (Sigma-Aldrich) which was placed in a petri dish with holding medium. The ovarian tissue was sliced with surgical blades and subsequently pressed through the cell dissection sieve. The resulting ovarian cell suspension was immediately filtered through a 100-µm Falcon cell strainer (BD Bioscience Discovery Labware, Durham, NC, USA). Thereafter, the filtered suspension containing preantral follicles (diameter < 100 µm) was transferred through a 40 µm strainer. The fragments remaining on the 40 µm nylon sieve were flushed with 10 mL fresh holding medium and transferred to a siliconized glass tube for centrifugation at 80 x g for 3 min at room temperature. After removing the medium, the pellet was re-suspended in 4 mL of fresh holding medium. The suspension was transferred to a culture dish pending collection of preantral follicles.

2.3.3 Selection of preantral follicles

Isolated preantral follicles with normal morphology, characterized by an intact basement membrane, round or oval in shape, and absence of pigmented granulosa cells were selected under a stereomicroscope (Stemi 200-C, Zeiss, Germany) at X 50 magnification. In addition to morphological assessment, viability was determined by subsequent staining with 50 µg/mL neutral red (38 °C, 20 min). This staining technique was developed in our laboratory, based on a protocol for ovarian tissue staining

(Chambers et al., 2010) with shorter incubation. Only morphologically normal and viable follicles stained red were used for our experiments.

2.3.4 Culture of preantral follicles

Selected preantral follicles were washed three times in a control culture medium (CCM) composed of M199 supplemented with 12.5 mM HEPES, 0.23 mM sodium pyruvate, 2 mM L-glutamine, 0.3% (wt/vol) bovine serum albumin (BSA), 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 1% (vol/vol) ITS, 2.13 μg/mL FSH, 10 ng/mL IGF-I, and 1.0 mIU/mL growth hormone, and subsequently allocated to Experiments 1 or 2. In Experiment 1, follicles were cultured in CCM (control) or CCM containing 0.5, 1.0, or 2.0 μg/mL T_4 (T0397). In Experiment II, follicles were cultured in CCM (control) or CCM containing 10, 100, or 200 ng/mL activin A (rhAct A; R & D Systems, Abingdon, UK). In each group, follicles were individually placed into 20-μL droplet of medium, which was prepared in tissue culture dishes and pre-equilibrated for 2 h at 38.5 °C, 5% CO_2 in air. Thereafter, droplets containing follicles were overlaid with mineral oil and incubated at 38.5 °C, 5% CO_2 in air for 14 d. Every second day, half of the medium was replaced with freshly prepared medium.

2.3.5 Assessment of preantral follicle growth

Follicle diameter

Follicle diameters were assessed with ProgRes Capture Pro 2.0 (Jenoptik, GmbH, Jena, Germany) by measuring both the maximum and perpendicular to the maximum diameter through the center of each follicle. The mean diameters of each follicle on Days 0, 3, 7, and 14 were recorded. In addition, diameter gain was determined by consecutive subtractions of the follicle diameters between Days 0 and 3, Days 3 and 7, and Days 7 and 14.

Follicle morphology

Preantral follicles were observed for morphological changes under an inverted microscope (Axiovert 100, Carl Zeiss AG, Germany). To document development and facilitate further assessment, photographs were taken on Days 0, 3, 7, and 14 with a digital microscopic camera (ProgRes10, Jenoptik). Preantral follicles that decreased in size and had degenerative signs (shrunken shape and darkened granulosa cells) on Day 3 were defined as atretic follicles and excluded from further incubation. The remaining follicles were designated as healthy and were cultured further.

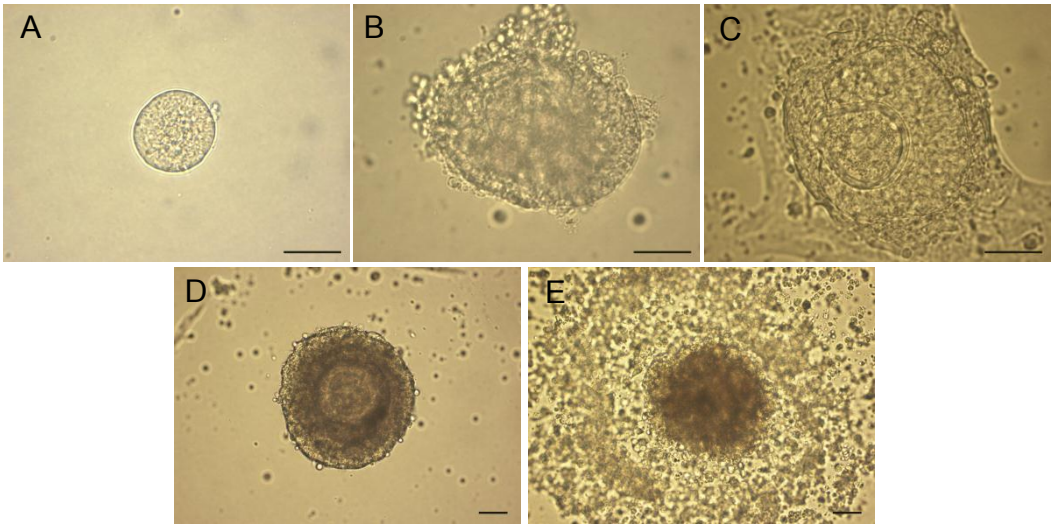

Figure 3. Morphology of preantral follicles from domestic cats during culture were categorized into five types; Type I: follicles with intact basement membrane containing granulosa cells and oocyte (A); Type II: follicles with initiation of granulosa cells proliferating through the basement membrane (B); Type III: follicles with granulosa cells proliferating through the basement membrane together with adhering and expanding on culture dish surface (C); Type IV: follicles with granulosa cells proliferating through basement membrane and creating dome-like structure surrounding an oocyte (D); and Type V: preantral follicles with dispersing of granulosa cells (E). Bar = 50 µm.

According to the phenotypic classification of *in vitro* growing preantral follicles described in the previous study (Martinez et al., 2004), morphological characteristics of preantral follicle on Days 3, 7, and 14 were adaptively categorized into five types (Figure 3), namely preantral follicles with: I) intact basement membrane (A); II) some granulosa cells proliferating outward through basement membrane (B); III) broken basement membrane and proliferating granulosa cells adhered and expanded on culture surface (C); IV) broken basement membrane and proliferating granulosa cells developing dome-like shape structure covering oocyte (D); and V) broken basement membrane and granulosa cells detached from oocyte-granulosa cell complex (E), indicating atresia characteristic of cultured follicles. The five characteristics were reported as percentages of follicles observed on Days 3, 7, and 14.

2.3.6 Viability assessment

On Day 14, the cultured follicles were examined for viability by staining with 4 μM ethidium homodimer-1 (EthD-1) in PBS for 10 min and immediately assessed under an inverted fluorescence microscope (Aviovert 200M, Carl Zeiss AG). The red fluorescence signal of EthD-1 emitted from dead cells was 617 nm. Follicle viability was defined according to percentages of damaged granulosa cells in viable and dead follicles. Viable follicles were categorized as: 1) non-damaged follicle (0% dead granulosa cells); 2) minimally damaged follicles (<10% dead granulosa cells); and 3) moderately damaged follicles (10-50% dead granulosa cells). Follicles with >50% dead granulosa cells were defined as dead follicles (Amorim et al., 2009; Hartshorne, 1997).

2.3.7 Experimental design

The effect of follicle size on potential growth was evaluated by dividing the control follicles cultured in CCM into five groups based on initial diameter at Day 0; 50 - 70 μm (n = 12), 71 - 85 μm (n = 14), 86 - 100 μm (n = 11), 101 - 115 μm (n = 12) and 116 -130 μm (n = 4). Diameter gains of all size groups were determined between Days 0 and 3, 3 and 7, and 7 and 14.

Experiment 1: Effects of T_4 on growth of preantral follicles were tested by culturing follicles in CCM supplemented with T_4 at 0.5 (n = 26), 1.0 (n = 26), or 2.0 µg/mL (n = 22). Follicles cultured in CCM served as controls (n = 26). Follicle diameters and morphological characteristics were determined on Days 0, 3, 7, and 14. On Day 14, all preantral follicles were stained for viability assessment. Three replicates were performed.

Experiment 2: To test the effects of activin A, preantral follicles were cultured in CCM supplemented with 0 (n = 32), 10 (n = 32), 100 (n = 32), or 200 ng/mL activin A (n = 32). Follicle diameters and morphological characteristics were observed on Days 0, 3, 7 and 14. Evaluation of follicle viability was performed on Day 14. Three replicates were performed.

2.3.8 Statistical analyses

Data were analyzed using SPSS Statistics version 20.0 (IBM SPSS, New York, NY, USA). Follicle diameters and diameter gains (growth) were presented as mean ± SEM (calculated from three repeated experiments) and compared by ANOVA and Turkey-Kramer test. Follicle morphology was presented as percentages of Types I, II, III, IV, and V. Percentages of follicle viability (viable and dead follicles) on Day 14 were compared using Chi-square. Differences were considered significant when $P < 0.05$.

2.4 Results

A total of 231 preantral follicles, ranging from 50.9 to 136.1 µm, were included in this study. There were no apparent effects of initial follicle size on potential growth (Figure 4). Between Days 0 and 3, diameter gains of all follicle sizes were highest ($P < 0.05$). Diameter gains decreased slowly until Day 7 ($P < 0.05$). Thereafter, follicle diameter diminished until Day 14 ($P < 0.05$). There was a difference of diameter gains between the group of 50-70 µm follicles and the 116-130 µm follicles from Days 3 to 7 ($P < 0.05$). However, the largest follicles were not different between Days 3 to 7 and 7 to 14 ($P > 0.05$).

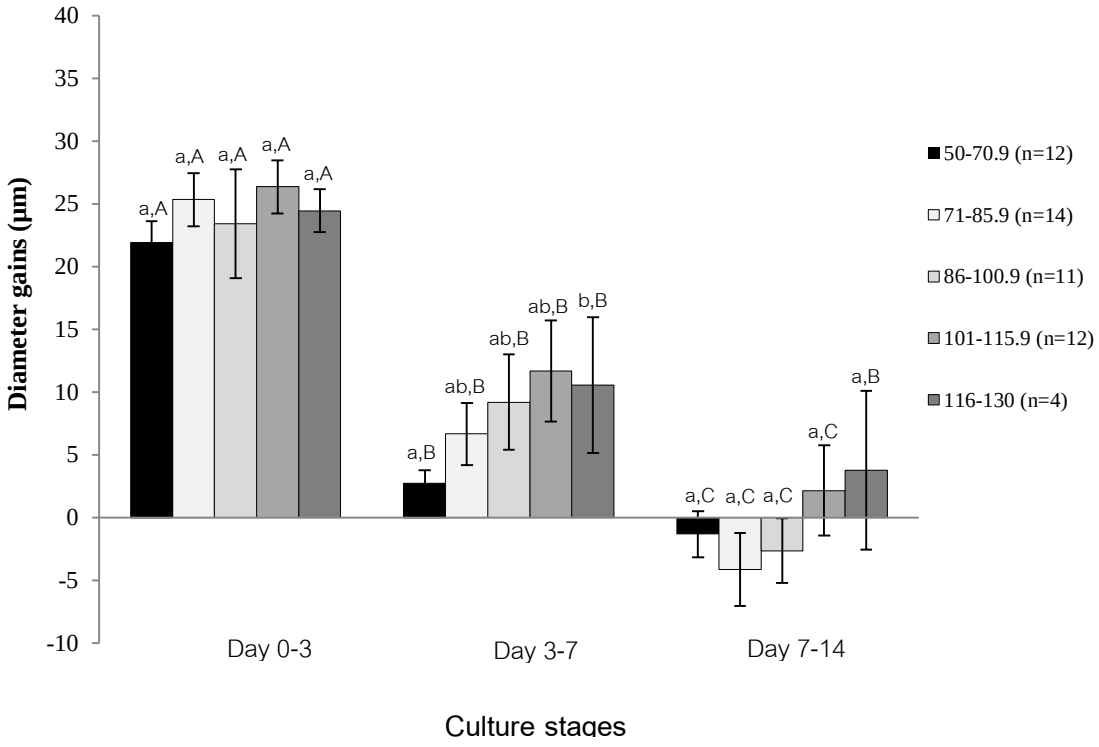

Figure 4. Diameter gain of preantral follicles from domestic cats cultured between Days 0 and 3, Days 3 and 7, and Days 7 and 14. Follicles (50 – 136 µm) were grouped into five size classes. [a-c]Within a culture stage, means without a common letter (a-c) differed ($P < 0.05$). [A-C]Within a treatment, means without a common letter differed ($P < 0.05$).

2.4.1 Growth and survival rates of preantral follicles in the presence of T_4

Diameters and diameter gains did not differ between control and treatment groups ($P > 0.05$; Figure 5). Diameter of preantral follicles cultured in T_4 supplemented medium increased from 90.0 ± 19.2 to 113 ± 22.4 µm during the first 3 d, independent of T_4 concentrations (Figure 5A). Thereafter, follicle growth weakened and diameter gains decreased towards Day 14 ($P < 0.05$). Diameter gains reflected dynamics of follicular growth, with an increase during the first 3 day, followed by a slight growth until Day 7, and regression after Day 7 of culture (Figure 5B).

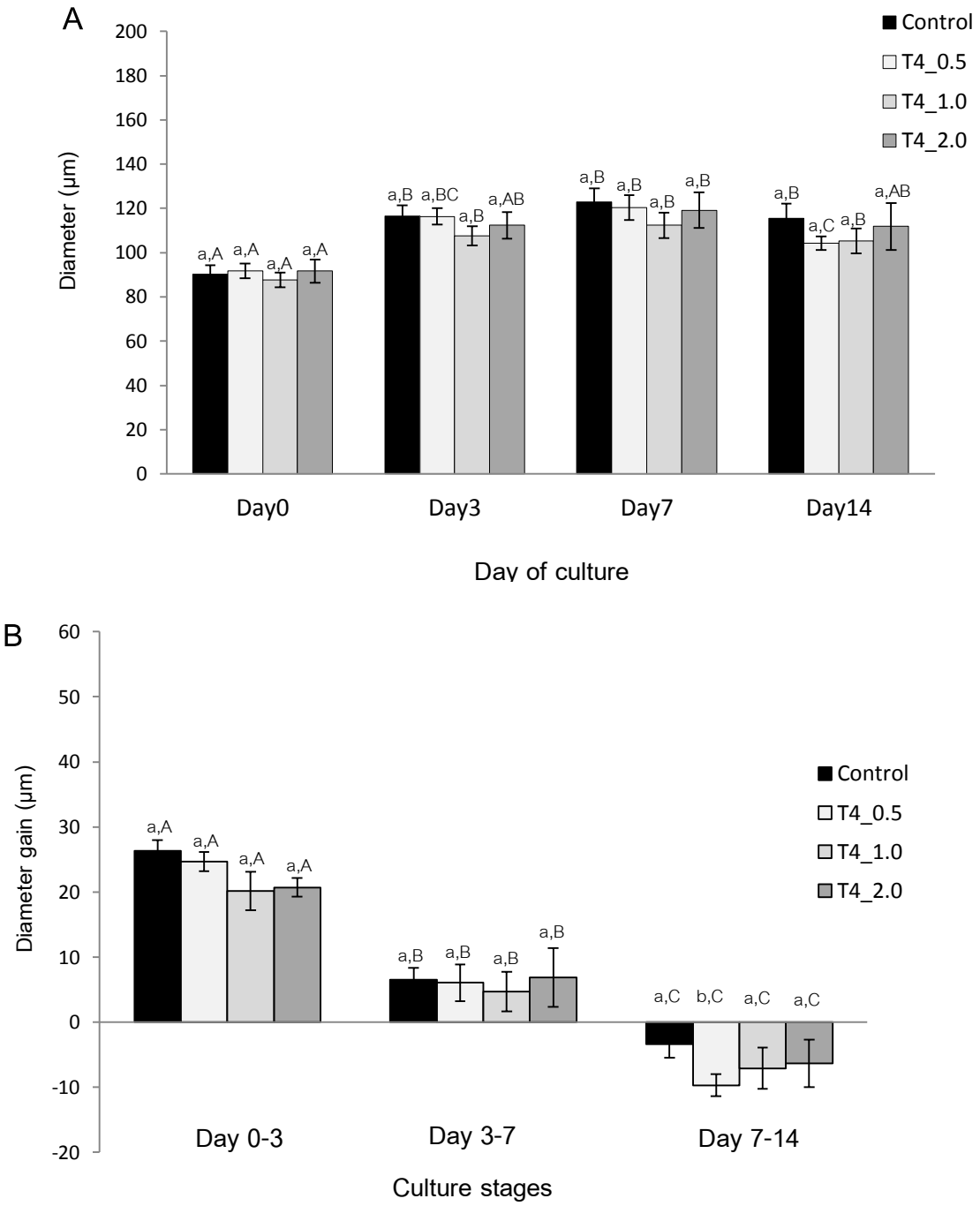

Figure 5. Follicle diameter (A) and diameter gains (B) of preantral follicles from domestic cats cultured in the absence (control) and presence of different concentrations of T_4 (µg/mL). [a-c]Within a culture stage, means without a common letter (a-c) differed ($P < 0.05$). [A-C]Within a treatment, means without a common letter differed ($P < 0.05$).

Morphological development of preantral follicles in the T_4 supplemented medium was similar to the control (Table 2). On Day 3, most follicles (70-95%) grew with intact basement membranes (Type I), whereas some (17-29%) had broken basement membranes with granulosa cell proliferation (Type II). Types III and IV follicles containing highly proliferated granulosa cells were initially seen on Day 7. On Day 14, a few T_4 supplemented follicles (4-5%) underwent dissociation of granulosa cells and oocytes (Type V).

During the first 3 day of culture, 7.7% and 15.5 ± 3.8% of controls and T_4-treated follicles, respectively, had degenerative signs and were excluded from further culture (Table 3). On Day 14, none of the follicles contained 100% live granulosa cells (non-damaged follicle). The percentages of viable follicles in T4-supplemented medium were not different from the controls ($P > 0.05$) and similar among groups ($P > 0.05$).

2.4.2 Growth and survival rates of preantral follilcles in the presence of activin A

Diameters of follicles cultured in the presence of 10 and 200 ng/mL activin A were higher than the controls on Days 3, 7, and 14 ($P < 0.05$) (Figure 6). During the first 3 d, the diameter of activin A-treated follicles increased from 86.2 ± 1.8 μm to 126.8 ± 3.2 μm with a diameter gain of 40.7 ± 2.0 μm. The largest follicles were observed on Day 7 in culture medium supplemented with 10 or 200 ng/mL activin A (Figure 6A). Diameter gains of follicles cultured in the presence of activin A at all concentrations were higher than the controls during 7 d of culture ($P < 0.05$) (Figure 6B). Interestingly on Day 14, the diameter gains of follicles cultured in 100 or 200 ng/mL activin A decreased at a higher rate than the controls ($P < 0.05$).

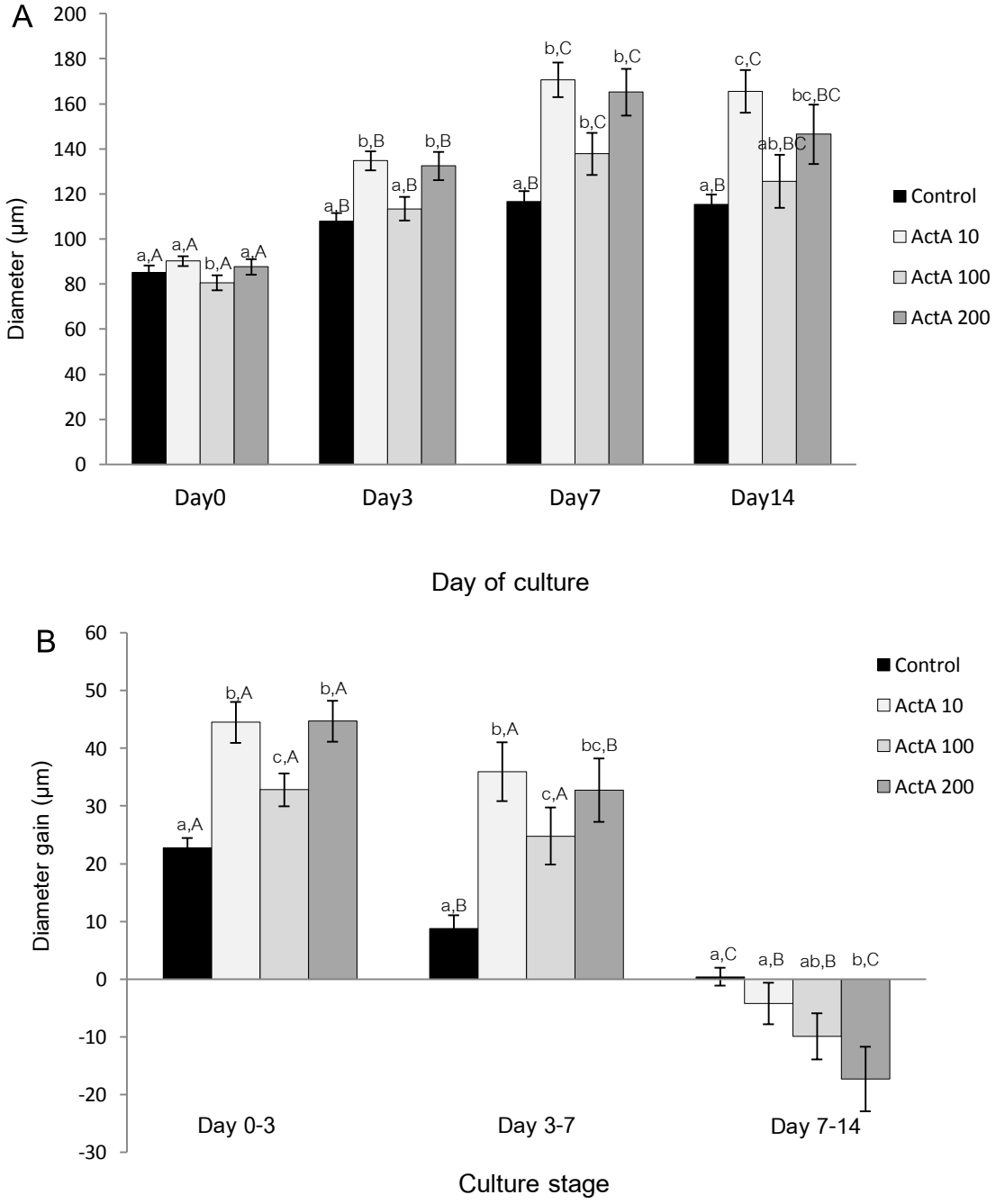

Figure 6. Follicle diameter (A) and diameter gains (B) of preantral follicles from domestic cats cultured in the absence (control) and presence of various concentrations of activin A (ng/mL). [a-c]Within a culture stage, means without a common letter differed ($P < 0.05$). [A-C]Within a treatment, means without a common letter differed ($P < 0.05$).

At Day 3, the majority of follicles (75-81%) cultured in the medium with activin A supplementation exhibited morphology Type II (Table 2). Follicles of morphology Types IV and V were first observed on Day 7. In medium supplemented with 10 ng/mL activin A, half of the follicles developed their morphology to Type IV, whereas without activin A (control) most follicles remained Types I or II (>90%) until Day 7. On Day 14, the majority of the follicles cultured in 100 or 200 ng/mL activin A had morphology Type V, whereas follicles cultured in 10 ng/mL activin A conserved the adherent granulosa cell (Type IV), as present on Day 7.

None of the follicles cultured in activin A-supplemented medium had degenerative signs on Day 3 (Table 3). There was a higher percentage of viable follicles in the control and 10 ng/mL activin A-treated medium than 100 or 200 ng/mL activin A-supplemented medium ($P < 0.05$).

Table 2. Classification of morphology of domestic cat preantral follicle during 14-day culture. Presented percentages of follicles with different morphology types cultured in T_4 or activin A supplemented medium (Type I: intact basement membrane; Type II: granulosa cells proliferating through basement membrane; Type III: broken basement membrane and proliferating granulosa cells expanded on culture surface; Type IV: broken basement membrane and granulosa cells developing dome-like shape and Type V: broken basement membrane and granulosa cells detached from oocyte).

Group	N	Classification of follicle morphology (%)														
		Day3					Day7					Day14				
		I	II	III	IV	V	I	II	III	IV	V	I	II	III	IV	V
Control T_4	24	70.8	29.2	0.0	0.0	0.0	62.5	16.7	12.5	8.3	0.0	50.0	25.0	20.8	4.2	0.0
T_4 0.5	23	82.6	17.4	0.0	0.0	0.0	56.5	26.1	17.4	0.0	0.0	47.8	17.4	30.4	0.0	4.4
T_4 1.0	20	95.0	5.0	0.0	0.0	0.0	70.0	25.0	5.0	0.0	0.0	65.0	20.0	5.0	5.0	5.0
T_4 2.0	22	77.3	22.7	0.0	0.0	0.0	63.6	22.7	0.0	13.7	0.0	54.5	18.2	9.1	18.2	0.0
Control ActA	29	82.8	17.2	0.0	0.0	0.0	65.5	31.0	0.0	3.5	0.0	62.1	24.1	6.9	3.4	3.4
ActA 10	32	18.8	81.3	0.0	0.0	0.0	3.1	31.3	0.0	46.9	18.8	0.0	6.3	6.3	56.3	31.3
ActA 100	32	25.0	75.0	0.0	0.0	0.0	6.3	28.1	0.0	34.4	31.3	6.3	9.4	6.3	21.9	56.3
ActA 200	32	25.0	75.0	0.0	0.0	0.0	0.0	53.1	0.0	34.4	12.5	0.0	3.1	3.1	25.0	68.8

Table 3. Viability of domestic cat preantral follicles cultured in the presence or absence of $T_4$and activin A at different concentrations determined at Day 14.

Groups	N	Healthy follicle at Day 3 (%) (n)	Viable follicle at Day 14 (%) (n)			Dead (%) (n)
			Viable			
			Minimally damaged	Moderate damaged	Total	
Control T_4	26	92.3 (24)	20.8 (5)	12.5 (3)	33.3 (8)	66.7 (16)
T_4 0.5	26	88.5 (23)	8.7 (2)	34.8 (8)	43.5 (10)	56.5 (13)
T_4 1.0	26	76.9 (20)	10.0 (2)	25.0 (5)	35.0 (7)	65.0 (13)
T_4 2.0	25	88.0 (22)	13.6 (3)	18.2 (4)	31.8 (7)	68.2 (15)
Control actA	32	90.6 (29)	10.3 (3)	31.0 (9)	41.4 (12)^{a,b}	58.6 (17)
actA 10	32	100.0 (32)	18.8 (6)	28.1 (9)	46.9 (15)^{a}	53.1 (17)
actA 100	32	100.0 (32)	12.5 (4)	9.4 (3)	21.9 (7)^{b,c}	78.1 (25)
actA 200	32	100.0 (32)	6.3 (2)	12.5 (4)	18.8 (6)^{c}	81.2 (26)

Within a column of each experiment, means without a common superscript differed ($P < 0.05$).

2.5 Discussion

The primary objective of this study was to investigate effects of activin A and T_4 supplementation on growth and viability of cat preantral follicles. This was apparently the first report of this kind in felids. There was a beneficial influence of low activin A concentrations on preantral follicle growth *in vitro*, whereas T_4 treatment had no apparent benefits. Furthermore, the diameter gain of cultured small cat preantral follicles was independent of their original sizes (50 – 130 μm, 86.3± 18.7 μm). In addition, greatest increase in follicle diameter occurred during Days 0 to 3 of culture; thereafter, follicle growth diminished or follicles underwent regression, with a broken basement membrane during prolonged culture (until Day 14).

By grouping follicles into five size groups, the control follicles of all size classes potentially grew equivalently in our control medium to Day 14. Similarly, in a previous study, secondary follicles (120-300 μm) underwent equivalent growth (enlargement of

diameter) after being cultured in M 199 medium supplemented with FSH, hydrocortisone and insulin-transferrin-selenite solution (ITS) (Jewgenow and Pitra, 1993).

This study demonstrated changes in the morphology of feline follicles, indicating the dynamics of growth in a two dimensional (2-D) culture system. At an early stage of culture, follicles grew in diameter and sustained their intact architecture (Type I). Only the follicles treated with activin A showed an alteration in morphology indicated by proliferating granulosa cells outwards and broken membranes (Type II). During later stages, the growing follicles classified as Type II potentially developed into Type III through irregular expansion of proliferating granulosa cells around the oocytes. This irregular proliferation of granulosa cells altered the spherical shape and obscured follicles on borders, contributing to inaccurate diameter measurement. Similar findings were previously observed in mice (Mitchell et al., 2002; Hirao et al., 2004) and cattle (Akers and Denbow, 2008). In contrast, almost all Type II follicles treated with activin A continuously grew with a morphological transformation to Type IV, characterized by marked proliferating granulosa cells which formed dome-like cover over oocytes (Martinez et al., 2004). However, some were unable to conserve their structure, manifested by detached and dissociated granulosa cells (Type V) by Day 14. The presence of Type V follicles in activin A treated medium was consistent with poor viability on Day 14, suggesting a negative consequence of activin A supplementation in the present culture system.

As a member of TGF-β superfamily, activin A acts via a sequence of binding to the activin I and II receptor and intracellular signaling by Smad protein transducers (Harrison et al., 2004). In several species, activin A promotes preantral follicle and oocyte growth, antrum formation and supports follicle health in rats (Jiang et al., 2000), sheep (Thomas et al., 2003) and humans (Telfer et al., 2008). Accordingly, in this study, the addition of activin A to the culture medium markedly increased the diameter of preantral feline follicles throughout the 14-day culture period. However, whether the growing oocyte or proliferating granulosa cells contributed to the increase in follicle diameter was unclear, due to the limitations of the assessment used in this study.

Immunohistological labeling of the proliferating granulosa cells and growing oocytes with proliferating cell nuclear antigen (PCNA) and OCT-4 gene, respectively, are suggested.

Furthermore, activin A supported granulosa cell proliferation in the present study, which resulted in a broken basement membrane and an outspread of cells on the culture dish. In contrast to the controls and T_4-treated follicles, some activin A-supplemented follicles with a loss of intact architecture were still accompanied by adherent granulosa cells and live oocytes (Fig. 1C). However, if the dispersal of granulosa cells and oocyte dislocation from the surrounding follicle were too severe, follicle atresia was initiated, and the oocyte degenerated (Type V). The latter findings accounted for the decrease of mean diameter at the end of culture in activin A-supplemented groups. Similar evidence was reported for human follicles (Telfer et al., 2008). The best strategy for prolonged culture of preantral follicles is prevention of granulosa cell outspreading on culture dishes by a three-dimensional (3-D) culture system; this system was capable of maintaining intact follicle morphology and supporting oocyte-granulosa cell interaction during prolonged culture of follicles from mice (Kreeger et al., 2006; West et al., 2007b; Xu et al., 2009a), rhesus monkeys (Xu et al., 2009c), baboons (Xu et al., 2011), and humans (Xu et al., 2009b). In future studies, this system could be used for culture of preantral feline follicles.

Thyroid hormones are major regulators of cellular functions; they increase oxygen consumption, enhance glycogen storage, and promote glucose uptake (Akers and Denbow, 2008). In the reproductive system, thyroid hormones play a role in oocyte maturation, estradiol secretion and differentiation of granulosa cells (Cecconi et al., 1999; Jiang et al., 2000). Furthermore, in hypothyroid mice, exogenous thyroxin improved folliculogenesis and estradiol secretion (Jiang et al., 2000). The benefits of T_4 supplementation for preantral follicles were also demonstrated in humans (Wakim et al., 1995) and sheep (Arunakumari et al., 2007). In contrast to previous results from other species, in the present study, T_4 had no apparent benefits for induction and maintenance of growth of preantral feline follicles. Arunakumari et al. (2007) demonstrated that larger ovine preantral follicles (250-400 μm) responded more

markedly to T_4 with not only diameter increase, but also antrum formation, when compared to the smaller diameter group (150-200 μm). The initial size of selected follicles in our study was only 56-130 μm; perhaps expression of thyroid receptors in granulosa cell develops at late preantral or early antral stages. In addition, T_4-treated follicles had a higher proportion of Type III morphology than the controls on Days 7 and 14; these irregular shaped follicles may also have affected mean diameters and diameter gains, which were reflected in follicle growth.

In conclusion, growth of preantral ovarian follicles during *in vitro* culture was stimulated by activin A. In contrast, there were no apparent beneficial effects of T_4. Whereas follicles had considerable diameter enlargement during the first 3 d of culture (independent of their original size) when treated with activin A, prolonged culture (14 day) was accompanied by increased follicle regression due to a failure in the maintenance of intact follicle architecture. Perhaps this would be overcome with a 3-D culture system. Although further investigations into follicle and oocyte health, maturation competence and communication between cellular compartments within follicles are needed, our study provided valuable information, to expand and improve the efficacy of preantral culture of feline ovarian follicles.

CHAPTER III

OPTIMIZATION OF ALIGINATE GEL CONCENTRATION FOR *IN VITRO* GROWING OF CAT PREANTRAL FOLLICLE IN THREE-DIMENSIONAL CULTURE SYSTEM

3.1 Abstract

Ovarian cortex contains a huge number of female germ cells which can be used to restore fertility after cryopreservation if *in vitro* growth of preantral follicles will be achieved. Recently, a three-dimensional culture system (3-D) has been introduced with promising results. Our aim was to optimize alginate gel concentrations for feline preantral follicle culture. Preantral follicles with round or oval shape (diameter 98.4 ± 2.7 µm) were mechanically isolated from ovaries of domestic cats. They were identified as viable by neutral red and individually encapsulated with 0.25% (n = 15), 1.0% (n = 31) or 2.0% alginate gel (n = 22), respectively. Each encapsulated follicle was cultured in a 96-well plate containing 100 µL medium M199 (0.23 mM sodium pyruvate, 2 mM L-glutamine, 12.5 mM Hepes, 0.3% BSA, 1% ITS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1.0 mIU/ml growth hormone, 2.3 µg/ml FSH, 10 ng/ml IGF-I and 10 ng/ml activin A). Follicle morphology and diameter were determined on Days 0, 3, 7 and 14. The follicles encapsulated in 0.25% and 2.0% alginate gel reached their maximal diameters on Day 7 and maintained their sizes till Day 14. In contrast, diameters of the follicles encapsulated in 1.0% alginate increased continuously from Day 0 to 14 and finally reached greater size than follicles in the other alginate concentrations ($P<0.05$). Most cultured follicles exhibited intact basement membrane throughout culture. Our findings suggested that 1% alginate gel is optimal supporting cat preantral follicle growth in the 3-D culture system.

3.2 Introduction

The mammalian ovary contains abundant of primordial and preantral follicles that potentially grow and ultimately produce numbers of fertilizable oocytes when develop to maturation stage (Jewgenow and Paris, 2006). In cancer patients, follicle and oocytes constituted in the ovaries would be destroyed by radiation or chemotherapy during cancer treatment leading to reproductive failure and infertility in women (Meirow and Nugent, 2001). Loss of female gamete also appears in endangered animals that die abruptly or underwent with ovariectomy for life saving from reproductive tract infections (Santos et al., 2010). Therefore, freezing of ovarian tissue in subsequent with *in vitro* culture of preantral *in vitro* provides a great opportunity to fertility restoration in both human and other valuable endangered.

Three-dimensional follicle culture system (3-D) is a novel approach for growing ovarian follicles *in vitro* (Nayudu et al., 2001). To maintain the intact architecture and cell-cell communication, follicles cultured in 3-D are encapsulated in a biomaterial matrix preventing attachment of follicle basement membrane to culture surface, which resulted in broken basement membrane and spread of granulosa cells of growing follicles (West et al., 2007a). The concentration of alginate utilized for encapsulation indicates gel rigidity which is an important factor affecting growth and survival rate of preantral follicles reported in mice (West et al., 2007b) and monkeys (Xu et al., 2009c). Although, domestic cat preantral follicles grew and survived after *in vitro* culture in conventional two-dimensional culture system (2-D), they encountered broken basement membrane leading to degeneration after 14-day culture (Wongbandue et al., 2013). Therefore, 3-D may potentially promotes growth of preantral follicle and improves morphology of growing follicles in domestic cats.

The present study aimed to determine optimal concentration of alginate gel to promote growth of preantral follicles in cats. In addition, morphological changes of preantral follicle were characterized.

3.3 Materials and methods

3.3.1 Collection of ovaries

Ovaries were collected from domestic cats that underwent routine ovariohysterectomy at private veterinary clinics or public animal shelters. The samples were placed in 50 mL centrifuge tubes containing a transport medium 0.9% sodium chloride supplemented with 100 IU/mL penicillin and 0.1 mg/mL streptomycin, and then shipped to laboratory after removal. Upon arrival, ovaries were freed of connective tissues and vessels. Thereafter, they were washed and preceded for follicle isolation.

3.3.2 Alginate gel preparation

Preparation of alginate gel for encapsulation was modified according to Xu et al. (2009a). In brief, sodium alginate (FMC BioPolymers, Philadelphia, USA) was dissolved in deionized water to a concentration of 1% (wt/vol) and treated with activated charcoal (0.5g charcoal/ 1g alginate) to remove organic impurities. Thereafter, alginate solution was sterilized by filtering through 0.22 μm membrane filters and lyophilized within a wide neck filter bottle (127405, Christ, Germany). The freeze-dried alginate was reconstructed with sterile 1 x PBS to concentrations of 0.25%, 1.0%, or 2.0% (wt/vol), respectively.

3.3.3 Isolation of preantral follicle

Preantral follicles were mechanically isolated from ovarian tissues in a holding medium comprised of M199 supplemented with 25 mM HEPES, 0.23 mM sodium pyruvate, 2 mM L-glutamine, 0.3% (wt/vol) bovine serum albumin (BSA), 100 IU/mL penicillin, and 0.1 mg/mL streptomycin. In brief, an ovary was dissected in half and laid with its medulla surface down on a 60-mesh cell dissociation sieve (Sigma-Aldrich) which was placed in a petri dish with holding medium. The ovarian tissue was sliced with surgical blades and subsequently pressed through the cell dissection sieve. The ovarian cell suspension was filtered through a 100-μm Falcon cell strainer (BD Bioscience Discovery Labware, Durham, NC, USA). Thereafter, the filtered suspension containing preantral follicles (diameter < 100 μm) was transferred through a 40 μm strainer. The

fragments remaining on the 40 µm nylon sieve were flushed with 10 mL fresh holding medium and transferred to a siliconized glass tube for centrifugation at 80 x g for 3 min at room temperature. After removing of the medium, the pellet was re-suspended in 4 mL of fresh holding medium. The suspension was transferred to a culture dish waiting for preantral follicles collection.

3.3.4 Selection of preantral follicles

Isolated preantral follicles with normal morphology, characterized by intact basement membrane, round or oval in shape, and absence of pigmented granulosa cells were selected under a stereomicroscope (SMZ645, Nikon, Japan) at X 50 magnification. In addition to morphological assessment, viability was determined by subsequently staining with 50 µg/mL neutral red at 38 °C for 20 min (Wongbandue et al., 2013). Only morphologically normal and viable follicles stained red were collected and washed in the holding medium. These selected follicles were transferred to 50-µl drops of culture medium (10 follicles/ drop) and incubated at 38 °C, 5% CO_2 for 30 min before encapsulation.

3.3.5 Follicle encapsulation and culture

The selected follicles were transferred into a 50 µl drop of alginate gel. Thereafter, each follicle was pipetted with 5µl of alginate gel and subsequently dropped into encapsulating solution (140 mmol/L Nacl, 50 mmol/L $CaCl_2$ and 5% (vol/vol) fetal bovine serum). Gels containing follicles were then left in the solution for 2-3 min to cross-linkage and transformed to alginate beads. Afterward, they were rinsed with holding medium and placed individually in a well of 96-well plates containing 100 µl culture medium (M199 supplemented with 12.5 mM HEPES, 0.23 mM sodium pyruvate, 2 mM L-glutamine, 0.3% (wt/vol) bovine serum albumin (BSA), 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 1% (vol/vol) ITS, 2.13 µg/mL FSH, 10 ng/mL IGF-I, 1.0 mIU/mL growth hormone and 10 ng/ml activin A). Encapsulated follicles were cultured in the medium at 38.5 °C, 5% CO_2 in air for 14 days. Every second day, half of the medium was replaced with freshly prepared medium.

3.3.6 Assessment of preantral follicle growth and morphology

To determine follicle growth and morphology, photographs of each follicle were taken on Days 0, 3, 7 and 14 using an inverted microscope (CKX41, Olympus, Japan) installed with a digital microscopic camera (DP20, Olympus, Japan). The mean diameters of follicles were assessed with assistance of the program DP2-BSW (Olympus, Japan) by measuring both the maximum and perpendicular to the maximum diameter through the center of each follicle. In addition to diameters, morphology of each encapsulated follicles were observed.

3.3.7 Experimental design

To determine the optimal concentration of alginate on preantral follicle growth, follicles of domestic cats were encapsulated in 0.25% (n = 15), 1.0% (n =31), or 2.0% (wt/vol) (n = 22) of sodium alginate before culture for 14 days. Follicle diameters were assessed on Days 0, 3, 7, and 14. Four replicates were performed.

3.3.8 Statistical analysis

Data were analyzed using SPSS Statistics version 20.0 (IBM SPSS, New York, NY, USA). Follicle diameters were presented as mean ± SEM (calculated from three repeated experiments) and compared by ANOVA and Turkey-Kramer test. Differences were considered significant when $P < 0.05$.

3.4 Results

Sizes of follicles encapsulated in 0.25% and 1.0% were increased on Day 3 and reached their maximum diameters on Day 7 ($P < 0.05$) (Figure 7). Thereafter, the follicle diameter was slightly decreased to day 14 ($P > 0.05$). In group of 1% alginate, follicle diameter enlarged from 103.18 ± 3.8 μm at Day 0 to 152.73 ± 5.9 μm at Day 7 ($P < 0.05$). At Day 14, follicles continuously grew to the largest diameter of 159.01 ± 7.0 μm ($P > 0.05$). In addition, morphological assessment revealed all encapsulated follicles

maintained their intact morphology with non-broken basement membrane during the 14-day culture (Figure 8).

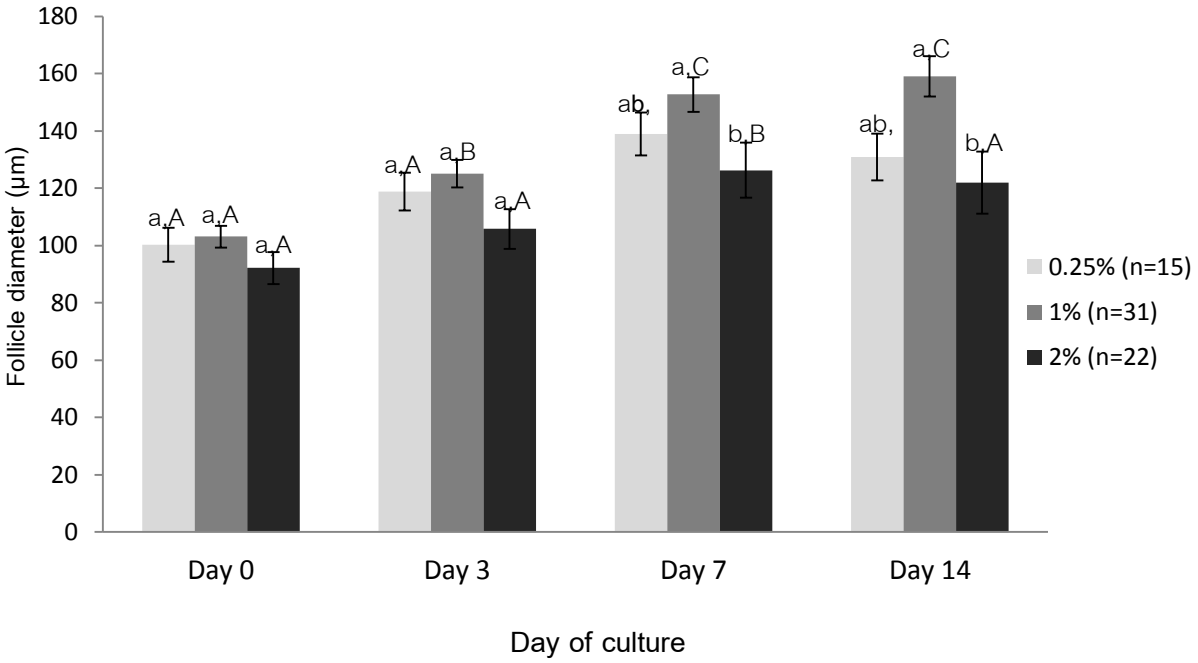

Figure 7. Diameter of cat preantral follicles encapsulated in different concentrations of alginate gels. Within a culture stage, means without common lowercase letter (a and b) differed ($P < 0.05$). Within a treatment, means without capital latters (A-C) differed ($P < 0.05$). $^{a\text{-}c}$Within a culture stage, means without a common letter (a-c) differed ($P < 0.05$). $^{A\text{-}C}$Within a treatment, means without a common letter differed ($P < 0.05$).

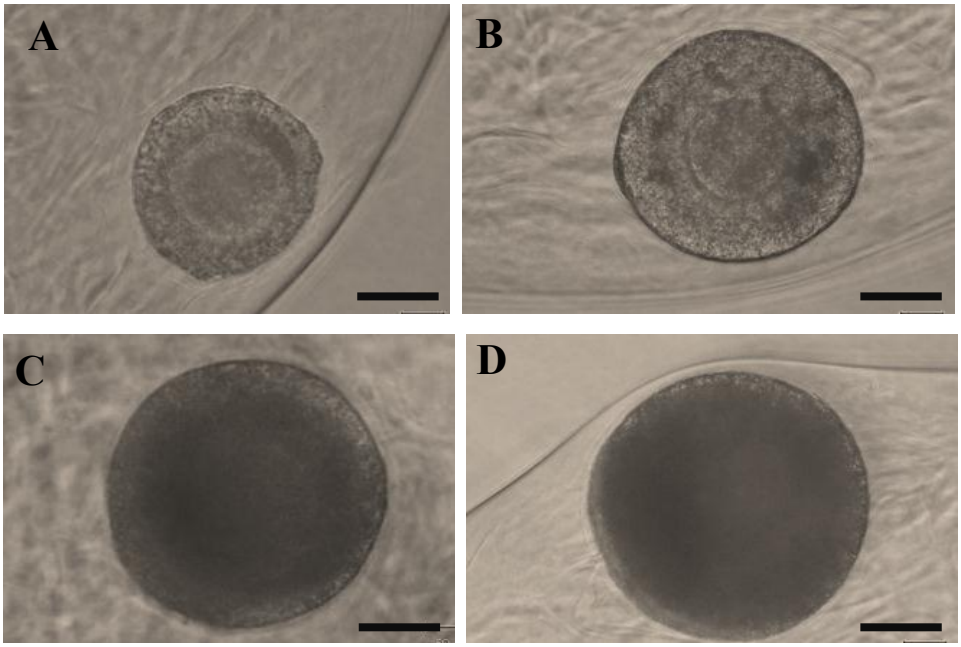

Figure 8. A preantral follicle encapsulated in 1% alginate gel. The follicle grew from their original size at Day 0 (A) through Day 3 (B), Day 7 (C) and Day 14 (D) with preserved intact morphology. Bar = 50 μm.

3.5 Discussion

This study presented the first attempt to optimize *in vitro* 3-D culture in felids. While preantral follicles of domestic cats encapsulated within alginate gel were able to grow in 3-D, the concentration of 1.0% alginate gel maintained follicle growth through 14-day culture period. In addition, encapsulation of alginate gel performed the benefit on preserving intact morphology of follicles in 3-D culture.

The advantages of three-dimensional follicle to maintain the intact morphology of growing follicle have been demonstrated in mice (Xu et al., 2006; West et al., 2007b), rhesus monkeys (Xu et al., 2009c) and baboon (Xu et al., 2011). Although alginate encapsulation promotes follicles growth *in vitro*, rigidity of gel reconstructed from different concentration affected to follicle growth and survival rate (West et al., 2007b).

In our study, 1% alginate gel performed the best support domestic cat preantral follicle growth for 14 day culture. This optimal concentration is higher than those of mice, indicating that 1% concentration of alginate may mimic to physical environment of cat ovarian stroma which is presumably more rigid than stromal tissue from mice. In addition, 3-D was capable to maintain intact morphology of cat growing follicles which substantial for oocyte-granulosa cell communication. Although this study revealed the positive effect of 3-D with alginate gel encapsulation in domestic cats, health and survival of the oocytes still need to be further investigated.

CHAPTER IV

VIABILITY AND GROWTH OF PREANTRAL FOLLICLES DERIVED FROM CRYOPRESERVED OVARIAN TISSUE OF A CHEETAH (*Acinonyx jubatus*) POST-MORTEM

4.1 Abstract

This study aimed to investigate freezing effects of ovarian tissues on survival of preantral follicles and observe *in vitro* growth of preantral follicles retrieved from cryopreserved ovarian cortical tissues of a cheetah post-mortem. After 29 hours cold storage, ovarian cortices were cut into small pieces (2.0 x 2.0 x 1.0 mm^3) and allocated to be frozen using a passive cooling container (n=3 pieces) or vitrification (n=3 pieces). After one year of storage, 23 and 58 preantral follicles were mechanically isolated from ovarian tissues cryopreserved using a passive cooling container and vitrification, respectively. Of 23 and 58 isolated follicles, 10 and 12 morphologically intact and viable (positively stained with neutral red) were selected for *in vitro* growth in a culture medium containing M199 supplemented with 0.23 mM sodium pyruvate, 2 mM L-glutamine, 12.5 mM HEPES, 0.3% (w/v) bovine serum albumin, 1% (v/v) insulin-transferrin-selenite solution, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1.0 mIU/ml growth hormone, 2.3 µg/ml follicular-stimulating hormone, 10 ng/ml insulin-like growth factor I and 10 ng/ml activin A for 7 days. Diameters and diameter gains were examined on Days 0, 3 and 7. Follicle viability was assessed on Day 7. Diameters of follicles retrieved from the slow freezing ovarian tissues decreased gradually from 53.5 ± 14.2 µm on Day 0 to 50.9 ± 17.1 µm with 2 out of 10 viable on Day 7 whereas those frozen by vitrification maintained their diameters between 50.7 ± 15.6 µm and 50.5 ± 17.9 µm on Days 0 and 7, respectively, with 2 of 12 viable. In conclusion, preantral follicles obtained from cryopreserved cheetah ovarian tissues can be grown *in vitro* for 7 days. However,

optimization of freezing protocol and culture medium are required to improve the viability and growing rate.

4.2 Introduction

Preantral follicle culture is a promising approach for fertility restoration in human and endangered animals (Smitz et al., 2010). This tool provides great opportunity to preserve child-bearing potential in cancer patients, particularly pre-pubertal women prior to receive a radiation or chemotherapy for treatment of cancers (Rodriguez-Wallberg and Oktay, 2012). Restoration of fertilizable follicles from cryopreserved ovarian tissues is feasible through a tissue re-implantation or follicle *in vitro* culture. Although ovarian implantation has exhibited achievements with birth of 18 human babies (Andersen et al., 2012; Wiedemann et al., 2012), risk of reintroduction of malignant cells back to recipients has been concerned (Shaw et al., 1996). In rare animal species, female gametes may be recovered when animals die accidentally or underwent spaying for medical reasons (Jewgenow and Paris, 2006). Recue of gamete and ovarian cryopreservation followed by *in vitro* follicle culture offers promising approach for fertility preservation in these valuable animals. Live birth productions have been demonstrated from *in vitro* culture of cryopreserved ovarian follicles in mice (Wang et al., 2011).

The cheetah (*Acinonyx jubatus*) is classified as vulnerable by International Union for Conservation of Nature (IUCN, 2012) because of decreasing of populations caused by habitat destruction and poaching. Although practical efforts with assisted reproductive techniques (ART) including induction of ovulation using hormones, *in vitro* oocyte maturation (IVM), *in vitro* fertilization (IVF), artificial insemination (AI) and embryo transfer (ET) have been utilized to improve reproductive capacity in captive population, successes of giving live offspring of this vulnerable species are low (Pelican et al., 2006). Therefore, additional approaches like conserving immature follicles in ovarian tissues to enhance genetic diversity in future breeding attempts are helpful to ensure survival of the species. Current protocols for ovarian tissue cryopreservation have been categorized into 2 methods; the conventional slow freezing and rapid freezing or

vitrification (Isachenko et al., 2007). While slow freezing has been employed as a standard protocol for preserving oocytes and various somatic tissues, vitrification is an alternative technique requiring lesser steps of freezing and freezing equipment, consequently simplifying cryopreservation process. Comparative outcomes of slow freezing and vitrification have been shown in monkeys (Yeoman et al., 2005) and mice (Kim et al., 2011) by ovarian graft survival after transplantation. In felids, successful ovarian cryopreservation has been demonstrated in domestic cats (Lima et al., 2006) and several felid species (Wiedemann et al., 2012; Wiedemann et al., 2013) using programable slow freezing machine. Recently, a passive freezing container has replaced a programmable freezer to convey the slow freezing rate. However, application of the device has not been investigated in cats.

In vitro follicle culture in felids has been reported in the domestic cats indicating beneficial effects of culture medium, proteins, gonadotropins and ovarian growth factors on promoting preantral follicle growth and viability (Jewgenow and Pitra, 1993; Jewgenow, 1996; Wongbandue et al., 2013). In addition, secondary follicles were shown to be capable to develop to antral stage *in vitro* (Jewgenow and Pitra, 1993). However, these investigations are limited on preantral follicles collected from fresh ovarian tissues of the domestic cats, and have not been studied in other wild species. This study aimed to: 1) compare two freezing methods for ovarian tissues retrieved from a cheetah post-mortem, and 2) determine *in vitro* growing of preantral follicle extracted from cryopreserved ovarian tissues.

4.3 Materials and methods

All chemicals and reagents used in this study were purchased from Sigma-Aldrich Company (St. Louis, MO, USA), unless otherwise stated.

4.3.1 Animal and collection of ovaries

A pair of ovary was obtained from a cheetah died in captivity at Khaokeow Open Zoo (Chonburi, Thailand). Ovaries were removed after 3 hours of necropsy, stored in

0.9% (v/w) normal saline solution (NSS) at 4°C, and shipped to the laboratory in 29 hours. Upon arrival, connective tissues and blood vessels were trimmed off and ovaries were washed 3 times in NSS supplemented with 100 IU/ml penicillin and 0.1 mg/ml streptomycin. From each ovary, ovarian cortex was dissected and cut into small pieces of 2.0 x 2.0 x 1.0 mm^3. Pieces of cortical tissues were then allocated to be frozen using a passive cooling container (n=3) or vitrification (n=3).

4.3.2 Cryopreservation

4.3.2.1 Slow freezing and thawing

Ovarian cortices were incubated in cryopreservation medium containing 1.5 M dimethyl sulphoxide (DMSO) and 0.1 M sucrose in phosphate buffered solution (PBS) at 4°C for 15 min. Thereafter, the tissues were placed into cryovials and incubated for 15 min at 4°C. Next, vials containing ovarian cortices were transferred into pre-cooled passive cooling device (Coolcell, Biocision, Lakspur, CA, USA) and placed in -80°C freezer for 24 hours to achieve cooling rate of -1°C/min. Afterward, frozen vials were stored in liquid nitrogen (-196 °C) until analyzed. Thawing of cryopreserved tissues was performed according to Cleary et al. (2001). In brief, cryovials were removed from liquid nitrogen and placed in a water bath at 37°C for 3 min. The tissues were then incubated in a thawing medium (PBS added with 0.75 mol/l DMSO and 0.2 mol/l sucrose) for 10 min at room temperature before transferred to a dissection medium (M199 supplemented with 25 mM HEPES, 0.23 mM sodium pyruvate, 2 mM l-glutamine, 0.3% (wt/vol) bovine serum albumin (BSA), 100 IU/ml penicillin, and 0.1 mg/mL streptomycin).

4.3.2.2 Vitrification and thawing

Vitrification of ovarian tissues was modified from the previous report used for freezing of testicular tissue (two-step freezing technique) (Thuwanut and Chatdarong, 2012). The tissues were incubated in an equilibration medium containing HEPES M199 supplemented with 7.5% (w/v) DMSO, 7.5% (w/v) ethylene glycol (EG) and 20% (v/v) fetal calf serum (FCS) at room temperature for 15 min, followed by transferring into a vitrification medium (HEPES M199 supplemented with 15% (w/v) DMSO, 15% (w/v) EG

and 0.5 M sucrose) at 4°C for 15 min. Subsequently, the ovarian tissues were immersed into liquid nitrogen. For thawing, cryovials were immersed in a water bath at 37°C for 3 min. Thawed tissues were then placed into a warming medium containing 1 M sucrose and 20% FCS in HEPES M199 at 37°C for 10 min.

4.3.3 Preantral follicle isolation and selection

Preantral follicles were isolated from ovarian tissues by mechanical technique. In brief, frozen-thawed ovarian tissues were placed on a petri-dish containing the dissection medium. Thereafter, they were hold with surgical forceps and finely sliced with surgical blades and needles. Isolated preantral follicles of normal morphology, characterized by an intact basement membrane, round or oval in shape, and absence of pigmented granulosa cells were selected under a stereomicroscope (SMZ645, Nikon, Japan) at X 50 magnification. Follicle viability was determined by staining with 50 µg/ml neutral red (38°C, 20 min) (Wongbandue et al., 2013). Only morphologically normal and viable follicles stained red were chosen for *in vitro* culture.

4.3.4 Culture of preantral follicles

Selected preantral follicles were washed in the dissection medium before transferring to a culture medium that composed of M199 supplemented with 12.5 mM HEPES, 0.23 mM sodium pyruvate, 2 mM L-glutamine, 0.3% (wt/vol) BSA, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 1% (vol/vol) insulin-transferrin-selenite solution (ITS), 2.13 µg/ml follicle stimulating hormone (FSH), 10 ng/ml insulin-like growth factor I (IGF-I), and 1.0 mIU/ml growth hormone (GH) and 10 ng/ml activin A (rhAct A; R&D Systems, Abingdon, UK) (Wongbandue et al., 2013). The follicles were individually placed into 20-µL drops of medium, which were prepared in tissue culture dishes and pre-equilibrated for 2 hours at 38.5°C, 5% CO_2 in air. Thereafter, the droplets containing follicles were overlaid with mineral oil and incubated at 38.5°C, 5% CO_2 in air for 7 days. Every second day, half of the medium was replaced with freshly prepared medium.

4.3.5 Assessment of preantral follicle growth

To allow assessment of follicle growth, photographs of each follicle were taken on Days 0, 3 and 7 using an inverted microscope (CKX41, Olympus, Japan) installed with a digital microscopic camera (DP20, Olympus, Japan). Mean diameters of follicles were determined with assistance of the program DP2-BSW (Olympus, Japan) by measuring maximum and perpendicular to the maximum diameters through the center of each follicle. In addition, diameter gains were calculated by subtractions of the follicle diameters between Days 0 and 3, and Days 3 and 7.

4.3.6 Viability assessment

Day 7, follicles were examined for viability by staining with 50 µg/ml neutral red for 20 min and immediately assessed under an inverted microscope (CKX41, Olympus, Japan) at X 400 magnification. Follicles with all granulosa cells stained red in cytoplasmic lysosome were classified as viable. Follicles with >50% unstained granulosa cells were defined as dead.

4.3.7 Statistical analyses

Data were analyzed using SPSS statistics version 20.0 (IBM SPSS, New York, NY, USA). Follicle diameters and diameter gains were presented as mean ± SEM (calculated from three repeated experiments). Comparisons of diameters among culture periods (Day 0, Day 3 and Day 7) within the same freezing method were performed by ANOVA and Turkey-Kramer test. Diameters of follicles extracted from tissues frozen using slow freezing and vitrification were compared by t-test. Percentages of viable follicles on Day 7 were compared between freezing methods using Chi-square. Differences were considered significant when $P<0.05$.

4.4 Results

A total of 23 and 58 preantral follicles were collected from cryopreserved ovarian tissues underwent slow freezing and vitrification, respectively. The pattern of

preantral follicle growing was represented in Figure 9. The preantral follicles retrieved from slow freezing exhibited viability of 43% (10 of 23) compared to 21% (12 of 58) of that vitrified (Table 4).

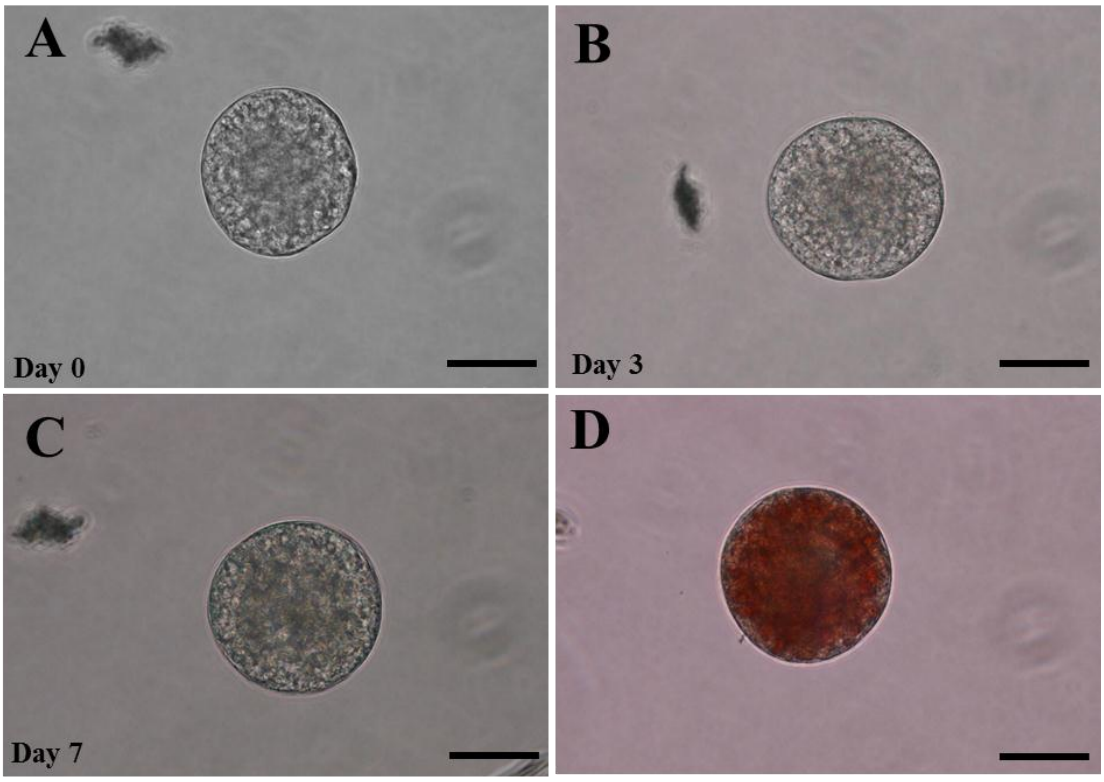

Figure 9. Vitrified cheetah preantral follicle after frozen-thawed (A), after 3 days (B) and 7 days of *in vitro* (C) and stained red with neutral red on Day 7 of culture (D).

On Day 0, the initial diameters of the selected follicles were 53.4 ± 4.5 μm and 50.7 ± 4.5 in slow freezing (n = 10) and vitrification group (n = 12), respectively (Figure 10A). The diameters were not different between the slow freezing and vitrification groups throughout the culture period ($P > 0.05$). The follicles in the slow freezing group showed diameter decrease during Day 3 to 7 compared to the vitrification group ($P < 0.05$) (Figure 10B). After thawing, follicles recovered from ovarian tissues cryopreserved using the slow freezing method presented higher percentages of viability than the vitrification

group ($P < 0.05$) (Table 4). However, after 7 days of culture, the percentages of viability were similar between the two groups ($P > 0.05$).

Table 4. Percentages of viable preantral follicles extracted from ovarian tissues cryopreserved using slow freezing and vitrification before and after cultured for 7 days.

Freezing techniques	Isolated follicles (N)	% Viable follicles (N)	
		Day 0	Day 7
Slow freezing	23	43 (10) [a]	20 (2) [a]
Vitrification	58	21 (12) [b]	17 (2) [a]

[a,b] values with different superscripts within column differ significantly ($P < 0.05$)

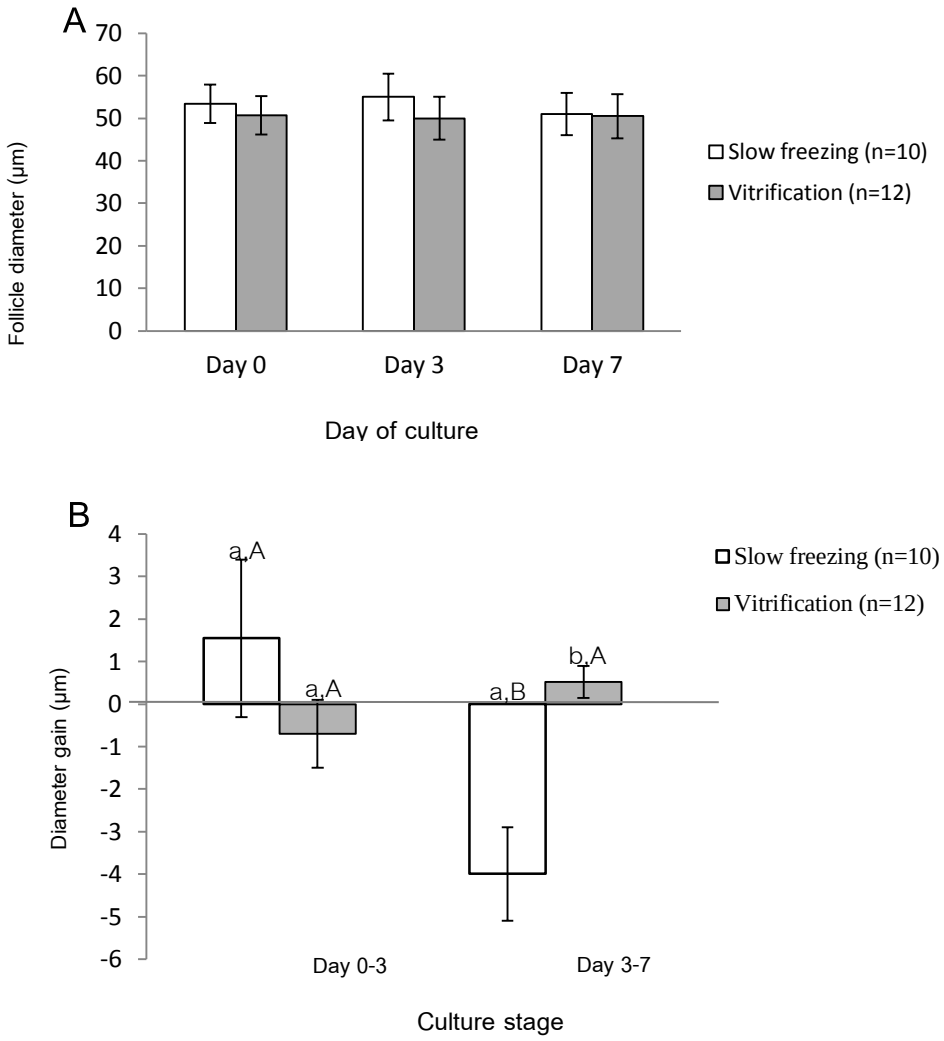

Figure 10. Diameter (A) and diameter gain (B) of cryopreserved cheetah preantral follicles during 7 days of *in vitro* culture. [a,b]Within a culture stage, means without a common letter differed (P< 0.05). [A,B]Within a treatment, means without a common letter differed (P < 0.05).

4.5 Discussion

The present study was the first to demonstrate survival of the cheetah preantral follicles after ovarian tissue cryopreserved and cultured for 7 days.

Ovarian tissue cryopreservation has been performed in wildlife post-mortem, including elephant (Gunasena et al., 1998), wombat (Cleary et al., 2004) and lion

(Wiedemann et al., 2012). The constraints of this technique in wild animals usually involve health of the animals prior to ovaries recovery (Johnston et al., 1991), duration of organ transportation and storage temperature. It has been suggested that time delay between animals die and ovarian collection resulted in dramatically decrease of survived follicles (Cleary et al., 2001). Miao et al. (2007) revealed an increase of granulosa cell apoptosis leading to follicle and oocyte degeneration when excision of ovaries delayed up to 30 minutes in mice carcasses. Moreover, duration and temperature during transportation affected viability and morphology of preantral follicles was demonstrated in dogs (Lopes et al., 2009). Interestingly, follicle development can be suppressed by reduction of food consumption through the mechanism of metabolic hormone leptin inhibition (Sirotkin, 2010). This condition would appear when animals were stressed or sick. In case of the cheetah, all together, health status, time between death of the animals and ovaries recovery and transportation duration of cheetah ovaries to the laboratory were responsible for the low viability of preantral follicle already before cryopreservation, and may account for small numbers of follicles recovered from ovarian tissues after thawing.

In the present study, the proportion of viable cheetah follicles in the slow freezing group (43%) was comparable to that in the lions (37-59%) (Wiedemann et al., 2012) and domestic cats (39%) (Lima et al., 2006). In addition, the survival rates of preantral follicles isolated from vitrified-warmed ovarian tissues were slightly higher than the previous study in the cats (21% vs 18%, respectively) (Galiguis et al., 2012). However, assessment of follicle viability in those studies was based on histological morphology and sizes of preantral follicles within ovarian tissues, whereas the follicle viability in our study indicated physiological function by cell uptake of non-toxic dye neutral red.

The advances of *in vitro* preantral follicle culture have been reported in various species including human (Telfer et al., 2008), primate (Xu et al., 2009c), ovine (Arunakumari et al., 2010) and murine (Wang et al., 2011) whereas developments in felids are limited. In felid species, achievements of *in vitro* follicle culture were demonstrated only in the domestic cats (Jewgenow and Pitra, 1993; Wongbandue et al.,

2013). According to a previous study (Wongbandue et al. 2013), the use of FSH and growth factors (IGF-I and activin A) in the culture medium was essential for the domestic cats. The beneficial effects of these supplements could not be proven for cheetah follicles during 7-day culture period, because of the impaired viability before cryopreservation. The survival rate of thawed cryopreserved ovarian follicles (17% and 20%) were lower than in domestic cats for fresh follicles (46.9%) after 7 days of culture (Wongbandue et al. 2013). The results were likely contributed to the damage caused by cryopreservation process. The communication between oocytes and granulosa cells via gap junctions has been recognized as an important factor in development of growing follicles (Navarro-Costa et al., 2005). While proliferation and differentiation of granulosa cells controlled by the oocytes, development and meiotic transcription of oocytes are regulated by the granulosa cells (Matzuk et al., 2002). The recent study exhibited decrease expression levels of gap junction protein genes; connexin 37 (Gja4) and connexin 43 (Gja1) which facilitated interaction of granulosa-granulosa cells and oocyte-granulosa cells, respectively (Xu et al., 2009a). In addition, the freezing impaired cellular organelles and caused failure of meiotic spindle stabilization in the oocytes (Camboni et al., 2008).

In conclusions, preantral follicles retrieved from ovarian tissues of the cheetah 29 hours post-mortem survives cryopreservation using the slow freezing and vitrification. Moreover, the study presents the ability of cheetah preantral follicles to grow *in vitro* up to 7 days. The report represents possibility of female gamete rescue in the felid species post-mortem.

CHAPTER V

GENERAL DISCUSSION AND CONCLUSION

Growth and development of ovarian preantral follicle to fully-grown stage has been recognized that regulated by extrinsic and intrinsic ovarian factors including gene expression, endocrine hormones and growth factors, in stage-specific manner (Fortune, 2003). In addition, the communication between granulosa cell and granulosa cell-oocyte via gap junction plays a critical role on growth and survival (Xu et al., 2009a). The present study revealed beneficial effects of growth factor activin A supplemented at low concentration in culture medium enhancing growth and survival rate of domestic cat preantral follicles *in vitro*. Nonetheless, the conventional two-dimension culture system could not support intact morphology of growing follicles during 14 day culture. To solve this problem, a novel technique of three-dimension culture system (3-D) was firstly applied in felids. As in other species, 3-D preserves intact architecture of growing follicle through 14-day culture period. In addition, the rigidity of 1% alginate gel encapsulation provides the best growth and survival of cat follicles. Moreover, the successful model of *in vitro* follicle culture in domestic cat has been translated to a wild felid, the cheetah. Viability of cryopreserved preantral follicles up to 7-day culture indicating on the possibility of female gamete rescue and growth *in vitro* after death of the animal.

In domestic cats, the beneficial effects of FSH, growth factor IGF-I and protein supplementation on preantral follicle development have been previously reported (Jewgenow and Pitra, 1993; Jewgenow, 1998). The positive influences of activin A promoting growth of preantral follicles presented in this present study, therefore, improves our fundamental knowledge of folliculogenesis in cats. For the further details, the average original size of follicles in the first experiment was around 86.3 μm. Thus, the follicles were classed as secondary follicles (Reynaud et al., 2009). The cat secondary follicles were well-responding to low-dose activin A supplementation. During prolonged culture, however, these growing follicles underwent basement membrane destruction

and the proliferating granulosa cells markedly affect the intact follicle architecture and cell communication. These findings may account for damages of oocytes and granulosa cells leading to low survival rate of follicles during the second week of culture. Furthermore, decreasing of follicle growth rate from Day 7 to Day 14 suggests that modification of culture system are required, which might include (i) supplementation of other growth promoting factors or hormones and/or (ii) preserving follicle morphology by three-dimension (3-D) matrices.

The 3-D culture system was shown to be successful in supporting growth and physiological function of growing follicles in mice (Xu et al., 2006), pig (Wu et al., 2001) and human (Xu et al., 2009b). According to biomaterial gel encapsulation, enclosed preantral follicles are prevented to attach culture surface and grow within a mimic environment of ovarian tissue. The alginate gel concentration influences to follicle growth and morphological integrity in stage-specific manner. This was demonstrated in non-human primate (Hornick et al., 2012). In the present study, the concentration of 1% alginate gel supports growth and viability of secondary cat preantral follicles beyond 0.25% and 2%, respectively. This optimal concentration of alginate is higher than former reported for mice (0.7%) (West et al., 2007b) but lower than those for primates (2.0%) (Hornick et al., 2012), indicating different physiological rigidity of cat ovaries compared to the both other species. Although the follicle morphology of cats is well-preserved in 3-D, growth of preantral follicle is retarded after the first week of culture, thus indicating for missing growth supporting factors in the *in-vitro* system. Together with the investigation in 2-D, the pattern of cat follicle growth during 14-day culture emphasized that *in vitro* development of cat follicle from the secondary to preovulatory stage requires other critical factors which need to be elucidated in future experiments.

According to considerable outcome of preantral follicles culture in domestic cats, the final part of the thesis attempt to apply the basic culture system to a wildlife feline species. It was shown that viable preantral follicles could be recovered from cryopreserved ovarian tissues of cheetah, and that some of the follicles survived for 7-day in culture. However, growth and survival rate was considered lower than

determined for fresh ovarian follicles of domestic cats. This can be explained by the effects of cryopreservation damages of intracellular structure and intercellular communication between oocytes and granulosa cells (Xu et al., 2009a). In addition, the species-specific factor for growth induction and hormones response must be considered.

Further prospective

Retrieval of healthy fertilizable oocytes from *in vitro* follicle culture is a promising process that contributes to reproductive efficacy and improving assisted reproductive technology (ART) in endangered species. Although the technique of *in vitro* follicle culture has been suggested for felids several time ago (Jewgenow, 1993), no progress in producing mature oocytes has been achieved yet. Beside investigations on other growth promoting factors and on physical integrity, physiological functions and health status of cultured follicles future study will profoundly profit from gene expression approaches. In this respect, the expression of mRNA encoding anti-Mullerian hormone (AMH) and Oct-4 would be suitable representatives. AMH is produced from proliferating granulosa cells of growing preantral follicles (Fortune, 2003). It is used to evaluate the ovarian reserve in human (Oktem and Urman, 2010), but AMH mRNA expression can also utilize as the marker for health evaluation of somatic cells within growing follicles. Oct-4, a nuclear transcriptional factor, appears during oocyte growth in folliculogenesis (Monti et al., 2006). Therefore, detection of Oct-4 mRNA expression will be valuable criteria indicating oocyte health and viability.

At present, successful follicle culture to term (fully grown and fertilizable oocytes) can only achieved by starting the culture with late secondary preantral follicles. Nevertheless, feline ovary contains around 99% of primordial follicles, whereas only 1% leaves the dormant stage and develops to antral follicles (Jewgenow and Paris, 2006). Therefore, the further research on follicle culture in cat should focus on the major follicle population. To date, activation of primordial follicle was shown to be regulated by

specific genes, including *Pten* and *Foxo3*, in PI3K signaling pathway (Reddy et al., 2008). In addition, recent study revealed successful activation of primordial follicles using *Pten* inhibitor in mouse (Li et al., 2010). However, the roles of *Pten* and *Pten* inhibitor to primordial follicle in felids have not been investigated.

Conclusion

In vitro follicle culture is a promising technique for producing fertilizable oocytes from preantral follicles. Growth and survival of cultured follicles *in vitro* requires sufficient growth factor and hormone supplementations in dose- and stage specific manner. In addition, the optimal culture system is important for preserving intact morphology which plays a role for the necessary communication between oocyte and granulosa cells. The present study indicates the beneficial effects of activin A, but not thyroxin. If supplemented to culture medium, activin A promotes growth and survival of domestic cat preantral follicles. In addition, the benefit of novel 3-D culture system which supports cat follicle integrity over at least two weeks was exhibited. Futhermore, the effective approach for culture domestic cat follicles was translated to cheetah. Although growth of cryopreserved cheetah follicles was not comparative to fresh follicle of domestic cats, survival of those cultured follicles suggest the possibility to rescue female gamete and produce fetilizable oocyte in feline species *in vitro*.

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APPENDIX

APPENDIX

List of publication and conferences

1. Wongbandue, G., Jewgenow, K. and Chatdarong, K. 2013. Effects of thyroxin (T4) and activin A on in vitro growth of preantral follicles in domestic cats. Theriogenology 79: 824-832.
2. Wongbandue, G., Tanpradit, N., Thongthainun, D., Thuwanut, P. and Chatdarong, K. 2013. Viability and growth of preantral follicles derived from cryopreserved ovarian tissues of a cheetah (*Acinonyx jubatus*) post-mortem. Thai J. Vet. Med. 43(3): (In press)
3. Wongbandue, G., Thuwanut, P., Tanpradit, and Chatdarong, K. 2011.*In vitro* growth of preantral follicles derived from fresh and vitrified Burmese eld's deer ovarian tissue. The 2nd PhD Student Symposium: Biostatistical analysis-the magic tool in wildlife biology and conservation medicine?. Berlin, Germany, 12-13 September 2011, p53. (Poster presentation)
4. Wongbandue, G. and Chatdarong, K. 2011. Thyroxin (T4) supplementation impairs *in vitro* growth of cat preantral follicles. 8th International Conference on Behavior, Physiology and Genetics of Wildlife. Berlin, Germany,14-17 September 2011. p201. (Poster presentation)
5. Wongbandue. G. and Chatdarong, K. 2012. Effects of activin A on in vitro growth of cat preantral follicles. Royal Golden Jubilee (RGJ) Seminar Siries LXXXVII, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. 17 May 2012. (Oral presentation)
6. Wongbandue. G., Jewgenow, K. and Chatdarong, K. 2013. Optimization of alginate gel concentration for *in vitro* growing of cat preantral follicle in three-

dimensional culture system. The 3th International Congress on Controversies in Cryopreservation of Stem Cells, Reproductive Cells, Tissues and Organs. Berlin, Germany, 21-21 March 2013. p A-17 . (Poster presentation)

7. กฤษณรงค์ วงศ์บ้านดู่ และ เกวลี ฉัตรดรงค์. 2555. การเจริญภายนอกร่างกายของไพรมารี่และเซเคิลดารี่ฟอลลิเคิลในสัตว์ตระกูลแมว. สัมนาทางวิชาการสัตว์ป่าสวนสัตว์ครั้งที่ 6, โรงแรมอิมพีเรียลแม่ปิง จังหวัดเชียงใหม่, 18-19 กรกฎาคม พ.ศ. 2555: 83-86. (Poster presentation)

VITAE

Grisnarong Wongbandue was born on November 16th 1979 in Mahasarakam, Thailand. He graduated with Degree of Doctor of Veterinary Medicine (DVM) from Chulalongkorn University, in 2004. In the same year 2004, he continue studied in Master degree program of Theriogenolology at Department of obstetrics Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. In 2006-2009 he had earned research experience in faculty of Agricultural at Gifu University in Japan in the field of reproductive physiology. Unfortunately, he discontinued the program due to private reason. In 2010, he continue his research passion by applied for PhD program of Theriogenolology at Department of obstetrics Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University. His researches have been focused on Reproductive biotechnology and Assisted reproductive techniques (ART). In addition, he also has special attention on reproductive and fertility clinic in small animal practices.