EFFECT OF VARIOUS GROWTH FACTORS ON THE *IN VITRO* DEVELOPMENT OF SWAMP BUFFALO EARLY ANTRAL FOLLICLE

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ผลของโกรทแฟ็กเตอร์ต่อการเจริญของฟอลลิเคิลขนาดเล็กในกระบือปลัก

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EFFECT OF VARIOUS GROWTH FACTORS ON THE *IN VITRO* DEVELOPMENT OF SWAMP BUFFALO EARLY ANTROL FOLLICLE

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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ขวัญฤดี แก้วมุงคุณ : ผลของโกรทแฟ็กเตอร์ต่อการเจริญของฟอลลิเคิลขนาดเล็กในกระบือปลัก (EFFECT OF VARIOUS GROWTH FACTORS ON THE *IN VITRO* DEVELOPMENT OF SWAMP BUFFALO EARLY ANTRAL FOLLICLE)
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การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาผลของโกรทแฟ็กเตอร์ต่อการเจริญของฟอลลิเคิลขนาดเล็กของกระบือปลักและศึกษาการเจริญของไข่ที่ได้จากการเลี้ยงฟอลลิเคิลหลังจากเลี้ยงไข่ในน้ำยา IVM โดยชนิดของโกรทแฟ็กเตอร์ที่ทำการทดลองคือ basic fibroblast growth factor (bFGF) insulin-like growth factor-I (IGF-I) และ epidermal growth factor (EGF) โดยแบ่งฟอลลิเคิลออกเป็น 3 กลุ่ม ตามขนาดเส้นผ่านศูนย์กลาง: กลุ่มที่ 1 ขนาด 200-399 μm, กลุ่มที่ 2 ขนาด 400-599 μm และ กลุ่มที่ 3 ขนาด 600-799 μm ฟอลลิเคิลเหล่านี้ถูกเลี้ยงในคอลลาเจนเจลนาน 14 วัน โดยวัดอัตราการเจริญในวันที่ 7 และ 14 จากการทดลองพบว่าฟอลลิเคิลในกลุ่มที่ 1 สามารถเจริญได้ เมื่อเลี้ยงในน้ำยาที่เติมโกรทแฟ็กเตอร์ bFGF, bFGF+IGF-I และฟอลเคิลในทรีทเม้นท์ควบคุมซึ่งไม่มีการเติมโกรทแฟ็กเตอร์ โดยมีการเจริญเติบโตเท่ากับ 8.7, 7.5, และ 6.8 % ตามลำดับ ส่วนที่เติม IGF-I มีการเจริญได้เพียง 1.7% และฟอลลิเคิลซึ่งเลี้ยงในน้ำยาที่มี EGF, bFGF+EGF, IGF-I+EGF และ bFGF+IGF-I+EGF ไม่สามารถเจริญได้ ส่วนการเลี้ยงฟอลลิเคิลในกลุ่มที่ 2 พบว่าเมื่อเลี้ยงในน้ำยาที่เติม bFGF (44.8%) และ bFGF+IGF-I (37.3%) ไม่มีความแตกต่างกันทางสถิติ ฟอลลิเคิลซึ่งเลี้ยงในน้ำยาที่เติม bFGF สามารถเจริญได้ดีกว่าฟอลลิเคิลซึ่งเลี้ยงในน้ำยาที่เติม IGF-I (21.6%) และ ทรีทเม้นท์ควบคุม (26.1%) อย่างมีนัยสำคัญทางสถิติ และ EGF, bFGF+EGF, IGF-I+EGF และ bFGF+IGF-I+EGF ไม่สามารถทำให้ฟอลลิเคิลเจริญได้ การเลี้ยงฟอลลิเคิลในกลุ่มที่ 3 พบว่าการเจริญของฟอลลิเคิลซึ่งเลี้ยงในน้ำยาที่เติม bFGF (32.7%) สูงกว่ากลุ่มที่เติม IGF-I (19.0%), bFGF+IGF-I (11.8%) และ ทรีทเม้นท์ควบคุม (12.8%) อย่างมีนัยสำคัญทางสถิติ ส่วนฟอลลิเคิลซึ่งเลี้ยงในน้ำยาที่มี EGF, bFGF+EGF, IGF-I+EGF และ bFGF+IGF-I+EGF ไม่สามารถเจริญได้ ส่วนผลของการอยู่รอดของไข่และการศึกษาระยะการเจริญของไข่หลังจากเลี้ยงในน้ำยา IVM พบว่าไข่ที่ได้จากการเลี้ยงฟอลลิเคิลจากการทดลองนี้ไม่สามารถบอกได้ถึงผลที่ชัดเจนของผลของโกรทแฟ็กเตอร์ที่มีต่อการอยู่รอดและระยะการเจริญของไข่ การทดลองนี้สามารถสรุปได้ว่า bFGF เป็น โกรทแฟ็กเตอร์หลักที่ช่วยทำให้มีการเจริญของฟอลลิเคิลขนาดเล็ก ส่วน EGF และโกรทแฟ็กเตอร์อื่นๆ ที่เติมร่วมกันมีผลยับยั้งการเจริญของฟอลลิเคิล

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____
ปีการศึกษา 2553 ลายมือชื่ออาจารย์ที่ปรึกษา_____
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KWANRUDEE KAEWMUNGKUN : EFFECT OF VARIOUS GROWTH FACTORS ON THE *IN VITRO* DEVELOPMENT OF SWAMP BUFFALO EARLY ANTRAL FOLLICLE. THESIS ADVISOR : ASST. PROF. RANGSUN PARNPAI, Ph.D., 54 PP.

GROWTH FACTOR/SWAWP BUFFALO/FOLLICLE CULTURE/ *IN VITRO* DEVELOPMENT

This study was carried out to examine the effect of growth factors on swamp buffalo's early antral follicle growth, and to study oocytes development retrieved from *in vitro* grown early antral follicles after being cultured in IVM medium. The follicles were divided into 3 groups, depending on their diameters, group I: 200-399 µm, group II: 400-599 µm, and group III: 600-799 µm. The follicles had been cultured in collagen gel dish for 14 days. The diameters of follicles were measured at days 7 and 14. The results indicated that follicles cultured in group I were able to grow after being cultured in medium supplemented with bFGF, bFGF+IGF-I and in control treatment (no supplements). The percents of follicle development were at 8.7, 7.5, and 6.8 respectively. The growth rate of follicles in medium supplemented with IGF-I was only 1.7%, whereas follicles cultured in medium supplemented with EGF, bFGF+EGF, IGF-I+EGF, and bFGF+IGF-I+EGF were unable to grow. The developmental rate of follicles in group II cultured in medium supplemented with bFGF (44.8%) and bFGF+IGF-I (37.3%) were not significantly different, but the development rate of follicles in medium supplemented with bFGF was significantly higher than that of IGF-I (21.6%) and control treatment (26.1%). Follicles cultured in

medium supplemented with EGF, bFGF+EGF, IGF-I+EGF and bFGF+IGF-I+EGF were unable to grow. Follicles in group III which were cultured in medium supplemented with bFGF (32.7%) showed a significantly higher growth rate when compared with IGF-I (19.0%), bFGF+IGF-I (11.8%), and control treatment (12.8%). Lastly, follicles cultured in medium supplemented with EGF, bFGF+EGF, IGF-I+EGF and bFGF+IGF-I+EGF were unable to grow. However, the effect of growth factors on oocytes viability and meiotic stage could not be described. In conclusion, bFGF might be a key growth factor that promotes buffalo's early antral follicle growth; EGF supplementation and different combination of growth factors could suppress the development of the follicles.

School of Biotechnology

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Kwanrudee Kaewmungkun

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LIST OF ABBREVIATIONS

bFGF	=	basic fibroblast growth factor
°C	=	degree celsius
EGF	=	epidermal growth factor
FDA	=	fluorescein diacetate
FGF	=	fibroblast growth factor
FSH	=	follicle stimulating hormone
Gn-RH	=	gonadotropin releasing hormone
GV	=	germinal vesicle
GVBD	=	germinal vesicle break down
hCG	=	human chlorionic gonadotropin
HEPES	=	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
ICSI	=	intracytoplasmic sperm injection
IGF-I	=	insulin-like growth factor-I
IGF-II	=	insulin-like growth factor-II
ITS	=	insulin-transferin-selenium
IVM	=	*in vitro* maturation
IVG	=	*in vitro* growth
IVF	=	*in vitro* fertilization
LH	=	Luteinizing hormone
mDPBS	=	Dulbecco's phosphate buffer saline
MI	=	metaphase I

LIST OF ABBREVIATIONS (Continued)

MII	=	metaphase II
ml	=	milliliter
OGC	=	oocyte granulosa cell complex
OCCG	=	oocyte-cumulus complexes with pieces of parietal granulosa
PVP	=	polyvinylpyrrolidone
TGF-α	=	transforming growth factor-α
µm	=	micromater
VIP	=	vasoactive intestinal polypeptide

CHAPTER I

INTRODUCTION

1.1 Introduction

Before birth mammalian ovaries consist of large numbers of oocytes. However, after birth the number of oocytes loss during meiotic division has been observed (ven den Hurk and Zhou, 2005). The process involved the degenerate of follicles is apoptosis which occurs in oocytes during fetal development and present in granulosa cells among adult life (Hussein, 2005). Mature oocytes are able to ovulate less than 1 percent in many mammalian species (Clark *et al*, 2004). Follicle atresia that occur during estrous cycle may be because of FSH decrease or/and the larger follicle directly inhibit development of smaller follicle. (Driancourt, 2000; Evans, 2003). The follicle atresia is important problems which result in limited number of mature oocytes to be useful in reproduction (Clark *et al*, 2004). The alternative way to preserve population of aviable follicle to produce mature oocytes is follicle culture *in vitro*. The first follicle culture *in vitro* was successful in mouse by Eppig and O'brien in 1996. They produce a live pup born from mouse primordial follicle culture. Moreover, Yamamoto and co-worker in 1999 able to produce a live calf born from culture of small oocytes that were isolated from early antral follicles. After the first farm animals were born, many researchers try to improve small oocytes and small follicle culture techniques in many species. However, the knowledge of follicle development is still unclear. Additional growth factor and nutrient mey be

requied during the development of the follicle (Webb *et al*, 2004). Growth factor is important to stimulate follicle growth, such as IGF-I that is able to improve the differentiation of granulosa cells and stimulate DNA synthesis of porcine follicle (Zhou and Zhang, 2005). ITS stimulates granulosa cells growth and selenium acts as acceptor of free radicals during cell metabolism. EGF supports preantral follicle growth by increasing proliferation of granulosa cells and changes granulosa cells morphology (Saha *et al*, 2000). FGF is able to stimulate buffalo follicle growth (Gupta *et al*, 2002) and bFGF presented in granulosa cell of preantral follicle (Smitz and Cortvrindt, 2002) involved granulosa cells function and stimulate DNA synthesis of preantral follicle (Zhou and Zhang, 2005).

Previous reports tried to improve the development of small oocytes and follicle culture by supplemented with growth factor in many farm animals such as ovine (Tamilmani *et al*, 2005), caprine (Zhou and Zhang, 2005) and bovine (Alm *et al*, 2006). Buffalo is the one of farm animal whose mature oocyte is limited and low quantity. Buffalo ovaries contain about 12,000-19,000 of primordial follicle (Nandi *et al*, 2009). In Thai swamp buffalo, estrous cycle presents one or two waves during follicular development (Yindee *et al*, 2010). Estrous cycle of buffalo starts round the day of ovulation which follicle smaller than 5 mm initial grown and increase large number of follicle. However, the number of follicle decreased because it underwent atresia at the beginning of the second follicular wave. Then at the end of estrous cycle, number of follicle decreased again until the dominant follicle ovulated (Ali *et al*, 2003). Buffalo follicle undergo atresia during the estrous cycle about 92% - 95% (Feranil *et al*, 2005). The alternative way to produce more mature oocytes for reproductive system is follicle culture *in vitro*.

1.2 Research objective

1.2.1 To study the effects of growth factors (IGF-I, EGF and bFGF) on *in vitro* growth of swamp buffalo early antral follicles.

1.2.2 To study meiotic division of oocytes development retrieved from *in vitro* grown early antral follicles after cultured in IVM medium.

1.3 Research hypothesis

1.3.1 Growth factors could support the development of early antral follicles.

1.3.2 Oocytes retrieved from *in vitro* grown early antral follicles will be develope to MII stage after cultured in IVM medium.

1.4 Scope of the study

1.4.1 The effects of growth factors on the growth of buffalo early antral follicles buffalo were examined by isolation early antral follicles from buffalo ovaries by enzymatical and mechanical techniques. Follicles were divided into 3 groups depending on their diameters (group I: 200-399 µm, group II: 400-599 µm and group III: 600-799 µm). Follicles were embedded in collagen gel and treated with *in vitro* growth medium containing different types of growth factors (IGF-I, EGF and bFGF) for 14 days. The diameter of follicles was measured every 7 days using a video micrometer (Olympus) on a screen connected to a CCD camera on an inverted microscope.

1.4.2 The meiotic division of oocytes development retrieved from *in vitro* grown early antral follicles after cultured in IVM medium were examined by picking

out oocytes from the follicles after 14 days of culture and sequentially cultured in IVM medium for another one day. The oocytes were stained with fluorescein diacetate and observed under the ultraviolet light to examine viability. The oocytes nucleus were stained with Hoechst 33342 and observed under the ultraviolet light. The meiotic stages of oocytes were checked by staining with aceto-orcine dye.

CHAPTER II

LITERATURE REVIEW

2.1 Follicles and oocytes development

Mammalian ovaries divide into 2 parts including medulla and cortex. Medulla contains many kind of connective tissues and blood vessel. On the other hand, cortex composes of many stages of follicle pool including primordial follicles, primary follicles, secondary follicles and graafian follicles. Each ovarian follicle contains one oocyte and follicular cells, such as granulosa cells, theca cells and cumulus cells. During folliculogenesis, oocytes increase in size and resume to either meiotic division or degenerate as show in figure 2.1. The patterns of folliculogenesis in mammalian species are similarly morphology. However, times cales of follicle development are different depending on species. The time span of primordial follicle form in sheep fetal is around day 75 (McNatty *et al*, 2007); 154 days in human fetal (Picton, 2000). Follicles and oocytes initialy developed at 3 weeks after fertilization (Smitz and Cortvrindt, 2002). Primordial germ cells are rapidly proliferating by mitotic divisions and increase in number. During the development, primordial germ cells differentiate to oogonia (Adams *et al*, 2008). After that, oogonia enter meiosis division and transits to form primary oocytes. The first meiotic division starts at leptotene, zygotene, and arrests at diplotene stage of meiotic prophase I. In addition, the sizes of primary oocytes increased during diplotene stage arrest and developed to primordial follicle by surrounded with a single layer of flattened pre-granulosa cells (Picton, H.M., 2000).

Primordial follicle initially develops to primary follicle, determined by the shape of pre-granulosa cells from flattened to cuboidal and growing oocytes diameters. In contrast, follicle at this phase is without theca cell layer and oocytes without zona pellucida. When granulusa cells rapidly proliferate, follicle will be surrounded by layers that contain at least two layers of granulose cells, zona pellucida, and a theca layer developed from interstitial stroma cells called secondary follicle. The next step for follicle development is antral follicle characterized by follicle contain with many layers of granulosa cells and present antral cavity which full-fill with follicular fluid (Cortvindt and Smitz, 2001). The antrum capacity space increased is caused by follicle growth to graafian follicle. At this stage, the arrested oocytes at diplotene stage start meiotic division until oocytes develop to metaphase II (M II). Mature oocytes arrest at M II until ovulation. In case of oocytes activated by sperms, oocytes will be continued with a meiotic division to produce ovum for fertilization (ven den Hurk and Zhou, 2005).

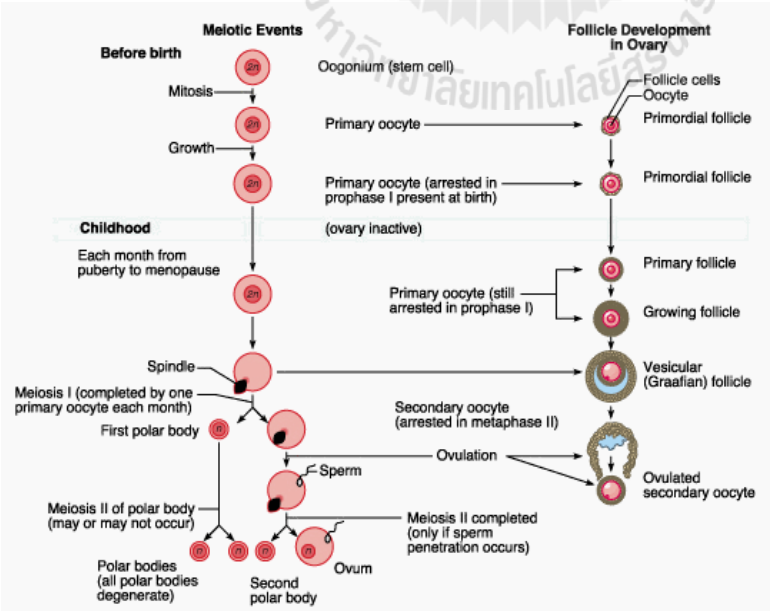

Figure 2.1 Follicles and oocytes development (Cumming, 2001).

Follicle development in some species depends on hormonal wave. This pattern is called follicular wave (Baruselli *et al*, 1996; Evans, 2003). Follicular wave causes by the released of gonadotropin releasing hormone (Gn-RH) from hypothalamus to stimulated the anterior pituitary gland to secreted FSH (Barnett *et al*, 2006). After that, the follicle is stimulated by FSH to initially grow and form antrum cavity. Moreover, estrogen and activin which produced by granulosa cells will stimulate their rapid proliferation. On the other hand, activin will restrain small follicle growth. Only dominant follicle is able to continue growing (Mizunuma et al, 1999; Webb et al, 2004). Dominant follicle produces estrogen and activin, and then negative feedback is sent to suppress FSH.

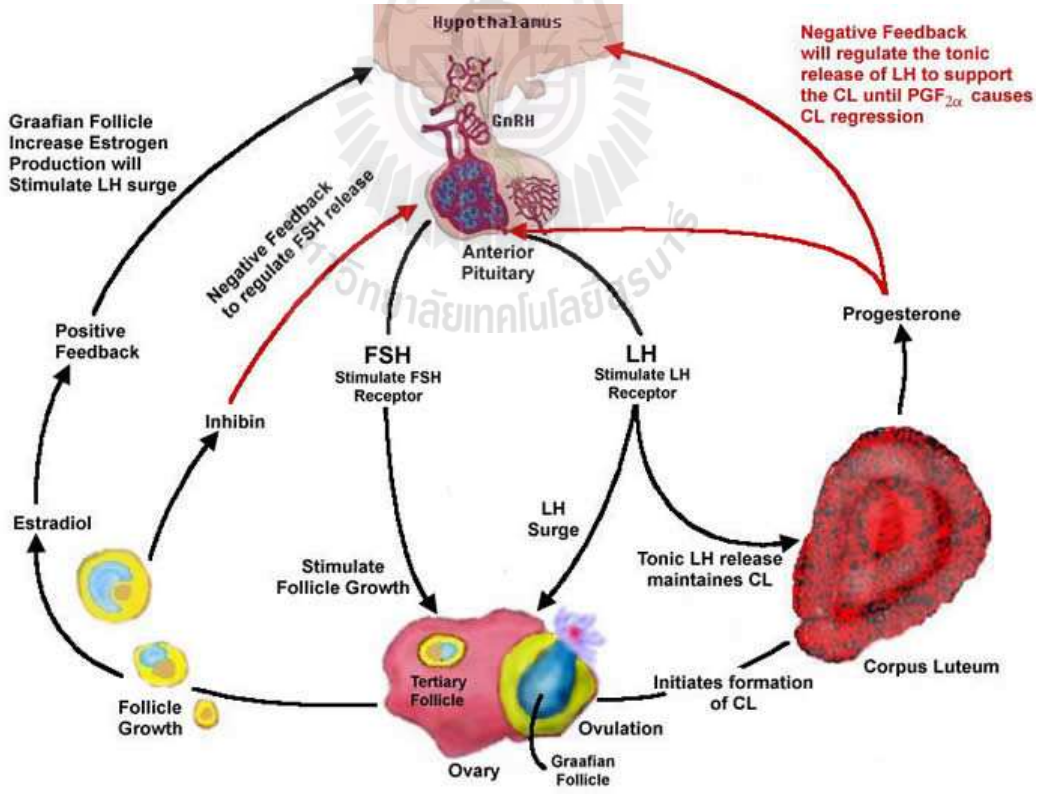

Figure 2.2 Hormonal regulation of reproduction (www.partnersinreproduction.com).

FSH decrease is causes smaller follicle that still require FSH become atresia (Webb *et al*, 2004). During dominant follicle growth, anterior pituitary gland releases LH to stimulate ovulation. In fact, dominant follicle from first follicular wave is unable to be ovulated because the ovary still contains corpus luteum (CL) that produced progesterone to inhibit LH pulse. After dominant follicle in the first wave die, estrogen that produced from granulose cells will drop. Then, the initial released FSH stimulates small follicle growth and continues to the next follicular wave. Dominant follicle from a new wave will be able to ovulate if CL disintegrate as show in figure 2.2 (Azawi *et al*, 2009). Normally, number of follicular wave was different depending on species, such as 2-3 waves in bovine, 3-4 waves in goat, and 1-2 waves in horse (Evans, 2003).

During follicle develop in follicular wave; there are three important events (recruitment, selection, dominant) for the selection of growing follicle as show in figure 2.3. The first event is recruitment which occurs when FSH increases and stimulates primordial follicle growth. However, the oocytes are still arrested at prophase of meiosis. When oocytes grow until zona pellucida exist. There are initial resume to meiosis (McGee and Hsueh, 2000). However, at selection process, most of follicles undergo atresia. This may because of the decrease of FSH and/or the larger follicle directly inhibit development of smaller follicle. The last event of follicle selection is dominant, at this event, dominant follicle is fully grown and contain with a mature oocyte and all of smaller follicles are completely atresia (Driancourt, 2000).

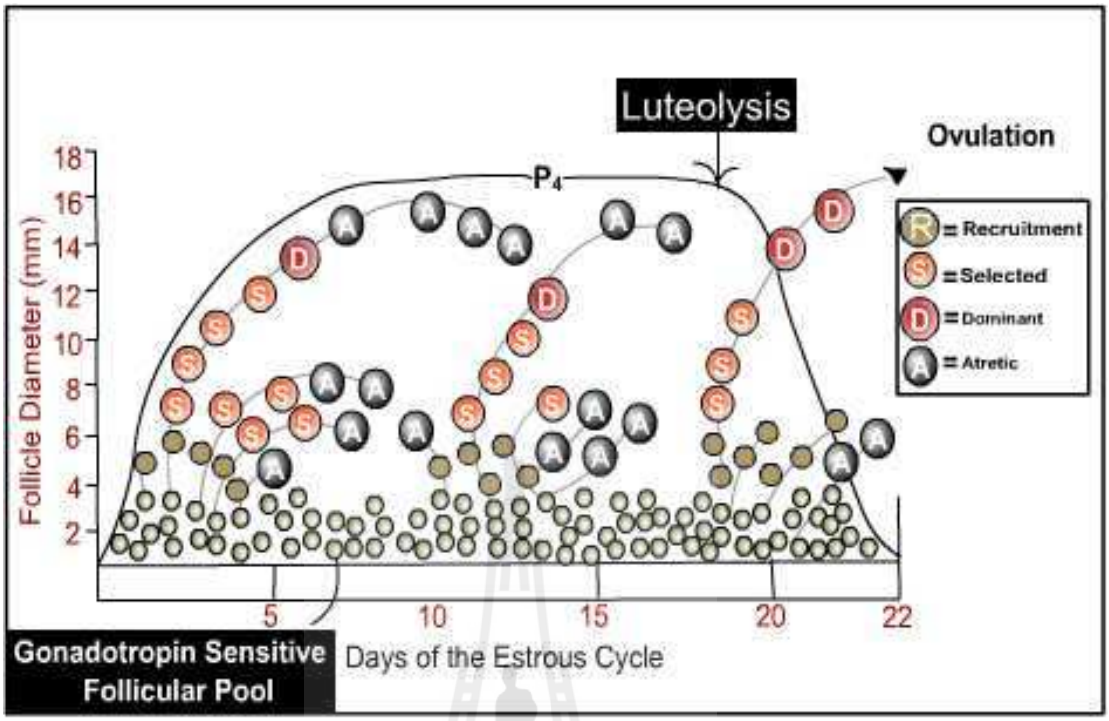

Figure 2.3 Follicle developments during estrous cycle (http://beef.unl.edu).

2.2 Follicles culture *invitro*

The mammalian ovaries consist of many stages of follicle pool. However, most of the mammalian follicles can not get ovulated because of undergo atresia, that cause limited number of mature oocytes ovulated. In addition, follicle that is able to ovulate is less than 1 percent in many species (Clark *et al*, 2004). Before birth, mammalian ovaries consist of a large number of oocytes, such as in rodents with 50,000-75,000 oocytes; in human with 7,000,000 oocytes and 2,700,000 oocytes in cows. On the other hand, after birth the number of oocytes loss during meiotic division, such as 10,000-15,000 remaining oocytes in rodents, 700,000 remaining oocytes in human and 135,000 remaining oocytes in cows (ven den Hurk and Zhou,2005). Moreover, during folliculogenesis, most of follicles become atresia because larger follicle inhibits smaller follicle growth. In other word, it is the negative

feedback that decreases FSH level during follicle development (Driancourt, 2000) and mature oocytes could not get ovulated because progesterone form CL inhibits LH release (Azawi *et al*, 2009). The process involves degeneration of follicle is apoptosis which occur in oocytes during fetal live and presented in granulosa cells in adult live (Hussein, 2005). Ovarian follicles could be classified into 4 groups following the follicular histology: 1) Healthy follicles consist of intact granulosa layer complexs which are well organized, 2) Early atretic follicles which have less number of degenerated cells of granulosa layer, 3) Advanced atretic follicles have a lot of degenerated cells of granulosa layer and 4) late atretic follicles which do not have any granulosa layer (Nakao *et al*, 2005). Apoptosis occurred in oocytes and follicular cells resulted in the limited number of mature oocytes that are useful sources for reproduction. Therefore, techniques for small follicle culture before becoming atresia have been used as an alternative way to produce new source of oocytes for livestock production such as *in vitro* fertilization (IVF), cloning and intracytoplasmic sperm injection (ICSI). Additionally, another advantage of small follicle culture is to understand the mechanism of follicle and oocyte growth.

Culture systems of small oocytes or small follicles could be divided into 4 methods; 1) organ culture (Eppig and Brien, 1996), 2) oocyte granulosa cell complex (OGC) culture (Yousaf and Chohan, 2003; Santos *et al*, 2006), 3) oocyte-cumulus complexes with pieces of parietal granulosa (OCCG) 4) and follicle culture (Yamamoto *et al*, 1999; Miyano, 2005; Kreeger *et al*, 2006). Techniques that are wildly used to isolate small follicle can be divided to two techniques. First, a mechanical technique using fine forceps (Yamamoto *et al*, 1999), shape needle (Gutierrez *et al*, 2000; Alm et al, 2006) and tissue chopper (Saha *et al*, 2000; Santos et

al, 2006) to insolate follicle. Second, an enzymatic technique which can be done by used enzymes to digest ovarian tissue from follicle. In additional, the important aspect for use enzyme is a suitable time for digestion which is varied by types of enzyme and species. collagenase type I and IV (Eppig and O'Brien, 1996; Zhou and Zhang, 2005).

The first follicle culture *in vitro* was successful in mouse by Eppig and O'brien in 1996. They used organ culture system and OGC culture system to culture mouse primordial follicles. After *in vitro* culture, oocytes develop to germinal vesicle break down (GVBD) 32% and metaphase II (MII) 22% and cleavage to 2-cell stage 21%. In turn, one living pup delivered from embryo transfer and the other died soon after delivery. The successful primordial follicles culture in mouse enlightens the idea of small oocyte cultures in many mammals. In 1999, the first farm animal born from OCCG culture by Yamamoto and co-workers. They cultured bovine early antral follicles with the diameter of 0.5-0.7 mm by embedding the follicle in collagen gel. The result of this research was that 27% of oocytes developed to MII stage. After IVF, 3.7% of the embryos developed to blastocyst stage and after transferred embryos to recipients a live calf was born. After the first farm animal born, many researchers have been trying to improve small oocytes and small follicles culture technique in many species including bitch (Bolamba *et al*, 2002), bovine (Tamilmani, *et al*, 2005), goat (Zhou and Zhang, 2005), and buffalo (Gupta *et al*, 2002). It has been several years that researchers are trying to understand the important factors that are involved with oocytes and follicles growth. However, these factors that effect oocytes and follicles development remain unclear. Growth factor is one factor which is important for follicle development. Preantral follicle and early antral follicle presented insulin-like growth factor-I (IGF-I) during development (McGee and Hsueh, 2000; Picton,

2000) which involved with follicle sensitive to FSH caused by IGF-I stimulate FSH receptors on granulosa cells and enhance activin A and theca cells for initialed antrum formation (van den Hurk and Zhao, 2005). In the recruiting event, the increase of IGF-I concentration has an effect on an increasing number of follicle. IGF-I and IGF-II are able to make follicle sensitive to LH (Driancourt, 2000). Nevertheless, higher concentration of IGF-I may decrease the follicle growth (Webb *et al*, 2003). Additionally, basic fibroblast growth factor (bFGF) presented in granulosa cells of preantral follicle (Smitz and Cortvrindt, 2002) which involved granulosa cells function and stimulates DNA synthesis of preantral follicle (Zhou and Zhang, 2005). Epidermal growth factor (EGF) is found in primary follicle, which have function on granulosa cells proliferation (Picton, 2000). EGF stimulates granulosa cell proliferation in bovine and porcine and involve primary follicle development to secondary follicle in porcine; although it is unable to stimulate granulosa cell proliferation in rat and cat (Zhou and Zhang, 2005). Additionally, EGF could be involved in DNA synthesis, increased follicle diameter and able to induce follicular cells proliferation in goat and sheep (Rajarajan *et al*, 2006). insulin-transferin-selenium (ITS) is able to preserve follicle culture. Selenium functions like an accepter of free radicals generated by cell metabolism (Saha *et al*, 2000). Insulin is able to increase production of estradiol from granulosa cells in small antra follicle (Webb *et al*, 2003) which increases absorption of amino acids and glucose for metabolism (Demeestere *et al*, 2005) and fibroblast growth factor (FGF) is able to stimulate buffalo follicle growth (Gupta *et al*, 2002). The function of many growth factors is important to preserve viable follicle during culture. However, the optimal concentration of growth factor for follicle culture in different mammalian species is

still unclear and requires a continuous research to investigate a suitable concentration of growth factor for follicle culture. In 2005, Zhang and Zhou studied the effect of EGF and IGF-I on caprine preantral follicle. The results showed that EGF (50 mg/l) increased the survival rate of oocytes, but the growth rate of oocytes decreased. In addition, IGF-I (100 mg/l) preserved the survival rate of oocytes and increased the growth rate of oocytes. The result showed that oocytes cultured in EGF and IGF-I together had higher survival and growth rates than either EGF or IGF-I treatment alone. Bolamba and co-workers (2006) reported that EGF had no effect on both of granulosa cells expansion and nuclear maturation. On the other hand, medium containing EGF (50 ng/ml), follicle-stimulating hormone (FSH; 0.5 µg/ml) and luteinizing hormone (LH; 5 µg/ml) significantly improved granulosa cells expansion in dog oocytes. Additionally, the study of effect of growth factors on goat preantral follicles indicated that EGF (50 ng/ml) and FSH (1 µg/ml) were able to increase follicular diameter by inducing new DNA synthesis in the follicular cells and increased follicular cell numbers. In contrast, transforming growth factor-α (TGF-α; 10 ng/ml) and insulin-like growth factor-II (IGF-II; 20 ng/ml) were unable to increase follicle diameter and follicular cell numbers of goat preantral follicle (Rajarajan *et al*, 2006). Moreover, the results of bovine preantral follicles cultured in medium supplemented with FSH (100 ng/ml) alone, FSH plus EGF (100 ng/ml), FSH plus ITS (1%; 6.25 µg/ml insulin, 6.25 µg/ml transferring, 6.25 ng/ml selenium) or FSH plus hypoxanthine (4 mM), showed that follicles cultured in medium supplemented with FSH alone or FSH plus EGF was significantly larger than follicles cultured in other supplements (Saha *et al*, 2000). In contrast, the results of culture preantral follicle in sheep showed that TGF-α (2.5 ng/ml) and FSH (1 and 2 µg/ml) were suitable for

sheep preantral follicles culture and oocytes could develop to MII stage. Oocytes cultured in medium containing EGF (50 ng/ml) did not mature to MII stage (Tamilmani *et al*, 2005). However, the present studies of small follicle culture are still limited and are unable to produce good quantity of mature oocytes. Additional research is needed in small follicle to understand factors that are suitable for culture condition.

2.3 Buffalo

Buffalo (*Bubalus bubalis*) could be classified into two sub-species depending on number of diploid chromosome (2n); the riverine buffalo contains 50 couple of diploid chromosome and the swamp buffalo consist of 48 couple of diploid chromosome. The buffalo that are found in Southeast Asia are swamp buffalo, which remain less number when compared with riverine buffalo. Buffalo ovarians are smaller than bovine (Presicce, 2007) and contain 12,000-19,000 primordial follicle (Nandi *et al*, 2009). Thai swamp buffalo estrous cycle contains one or two follicular wave during the follicle development (Yindee *et al*, 2010). Estrous cycle of buffalo starts around the day of ovulation which the first follicular wave occurs during day 1-4 by follicle that smaller than 5 mm initial grown. At this wave buffalo ovarian contain with large number of follicle and dominant follicle present at day 4 or 5 of estrous cycle. However, the number of follicle decrease during day 5-10 and then increase again during day 11-17 that mean dominant follicle at first wave undergo atresia and the second follicular wave start. In turn, during end of estrous cycle at day 18-21 the number of follicle decrease again until dominant follicle become ovulation (Ali *et al*, 2003). Moreover, during estrous cycle, buffalo follicle becomes to undergo

atresia 92% – 95% (Feranil *et al*, 2005). In Thai swamp buffalo, the follicle develops in estrous cycle and they form one or two follicular waves (Yindee *et al*, 2010). However, the mature oocytes that are able to get ovulated and used for reproductive system are still low because of atresia. The alternative way to produce more mature oocytes for reproductive system is follicle culture *in vitro*. Gupta and coworkers (2002) studied buffalo preantral follicle culture. They compared individual culture of buffalo preantral follicle with group culture (2-4 preantral follicles in a group) and examined the effects of FSH (0.05 IU/ml), ITS (1%), EGF (50 ng/ml), FGF (50 ng/ml) and vasoactive intestinal polypeptide (VIP; 50 ng/lm) on buffalo preantral follicle culture. They found that preantral follicles cultured in groups had higher growth rate than individual culture and all of the supplementation were able to increase follicle size. Additionally, ITS was able to increase buffalo follicle growth. FSH and FGF alone are involved with follicle growth. EGF and VIP require FSH for stimulate follicle grow. Furthermore, the studied of the culture of preantral follicle from buffalo fetuses showed that FSH (0.5 mg/ml) plus EGF (100 ng/ml) and ITS (1%) improved the survival rate and the growth rate of buffalo fetal preantral follicles, whereas follicles that cultured in medium supplemented with EGF plus ITS were unable to increase in size and became degenerated (Santos *et al*, 2006). Moreover, Gupta and coworkers (2008) found that cumulus cells and ovarian mesenchymal cells are able to increase preantral follicle diameter when co-cultured with buffalo follicle. However, the previous reports of buffalo small follicle culture were unsuccessful. The percent of small oocytes developed to MII was still low and unable to produce calf born from small oocytes or small follicles culture. Moreover, previous reports showed that several growth factors could support small follicles growth in culture. However,

the growth factors used depended on species. The method for cultured buffalo small follicles is needed to be improving. The suitable growth factors are needed to be investigated for culture buffalo small follicle.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials and methods

3.1.1 Isolation of early antral follicles

Buffalo ovaries were obtained from a slaughterhouse and placed in isotonic sodium chloride solution (0.9% NaCl) at room temperature to transport to the laboratory. The ovaries were washed in 70% ethanol for 1 min and then washed once in 0.9% NaCl for 1 min. The washed ovaries were excised into small pieces (1-2 mm) by a surgical blade. The minced tissue were washed in 0.9% NaCl 3 times and placed in HEPES (H-4034, Sigma) -buffered TCM 199 (M-5017, Sigma) containing 0.1% collagenase type IV (17104-019, Gibco) and 40 units/ml DNase (10 104 159 001, Roche), then incubated at 37ºC for 1 hour. After the digestion, the healthy follicles (Figure 3.2) were isolated by using fine forceps in HEPES-buffered TCM 199 supplemented with 300 IU/ml penicillin G (P-3032, Sigma) and 0.3 mg/ml streptomycin (S-9137, Sigma) under a stereomicroscope (Figure 3.1). The diameter of follicles were measured with a video micrometer (Olympus) on a screen connected to a CCD camera on an inverted microscope to categorize into 3 groups depending on their diameters (group I: 200-399 µm, group II: 400-599 µm, group III: 600-799 µm) as showed in figure 3.3.

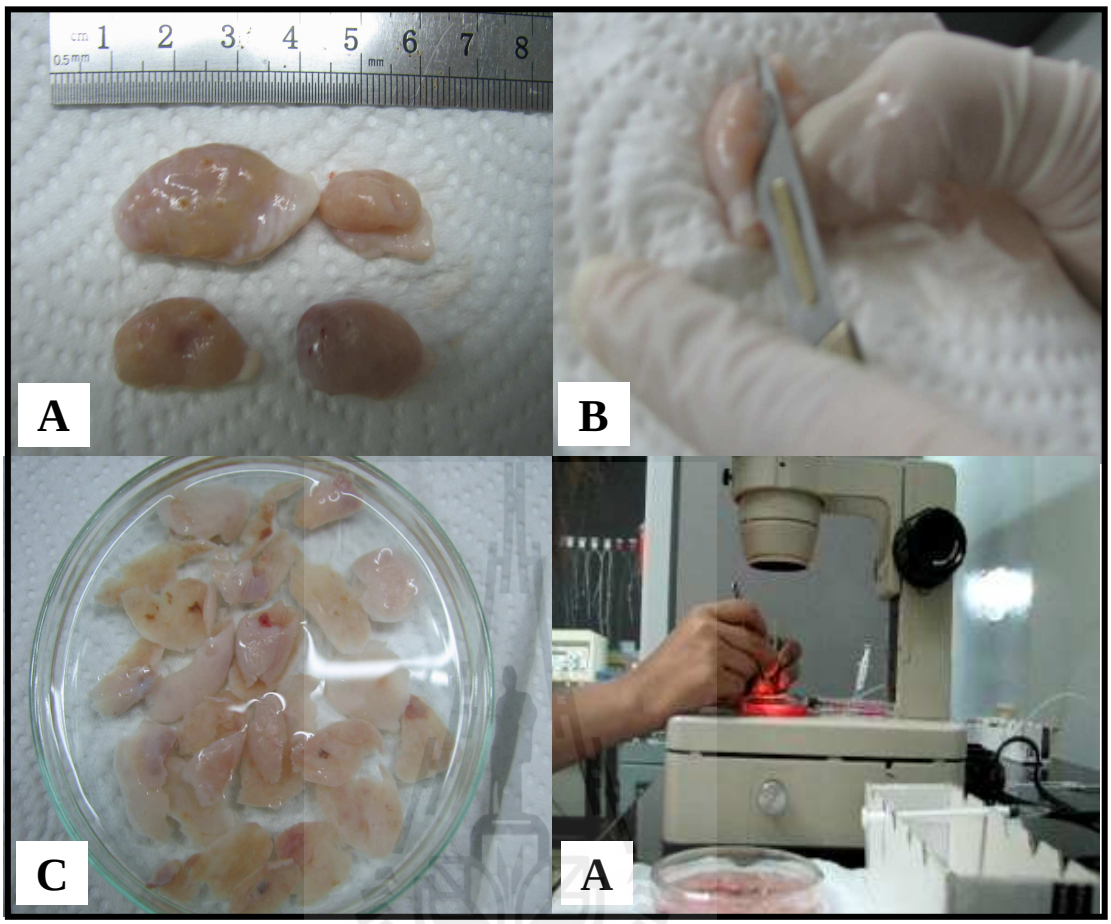

Figure 3.1 Early antral follicles isolation of buffalo ovaries (A). Ovaries were excised by a surgical blade (B). Small pieces of ovarian tissues after excision (C). Follicles were isolated by using fine forceps (D).

3.1.2 *In vitro* growth on early antral follicles

Early antral follicles were embedded in collagen gel. A collagen mixture was made by mixing a 0.3% acid collagen solution (Cellmatrix Type I-A, Nitta Gelatine, Japan), 10 time-concentrated TCM 199, and 0.05 N sodium hydroxide solution (Carlo Erba) containing 22 mg/ml $NaHCO_3$ (S-5761, Sigma) and 47.7 mg/ml HEPES, at a ratio of 8:1:1 (v:v:v) on ice. Base layer was made by using 0.4 ml collagen mixture aliquoted into 4–well dish (Nunc) and placed on a warm plate

at 35ºC for 5 minutes. Four early antral follicles were placed onto the base layer. The remained collagen mixture was placed into the water at 25ºC for 5 minutes and then 0.4 ml of the mixture was poured on the base layer contained four early antral follicles to make the top layer and the dishes were incubated at 37ºC for 10 minutes. After the gelatinization, 5 ml of the *in vitro* growth medium (IVG); TCM 199 supplemented with 2 mM L-glutamine (G -5763, Sigma), 0.23 mM sodium pyruvate (P-5280, Sigma), 2 mM Hypoxanthine (P-5280, Sigma) 1% ITS (I-1884, Sigma: Insulin 6.25 µg/ml, Transferrin 6.25 µg/ml, Selenium 6.25 ng/ml), 100 IU/ml penicillin G, 0.1 mg/ml streptomycin, 1 µg/ml FSH (Folltropin-v@ Bellevi, Ontario, Canada) was poured onto the gel containing the follicles, and then cultured at 38.5ºC under a humidified atmosphere of 5% CO_2 in air for 14 days. Half volume of the IVG medium was replaced every 2 days. The diameters of the follicles were measured at day 7 and 14 with a video micrometer on a screen connected to a CCD camera on an inverted microscope.

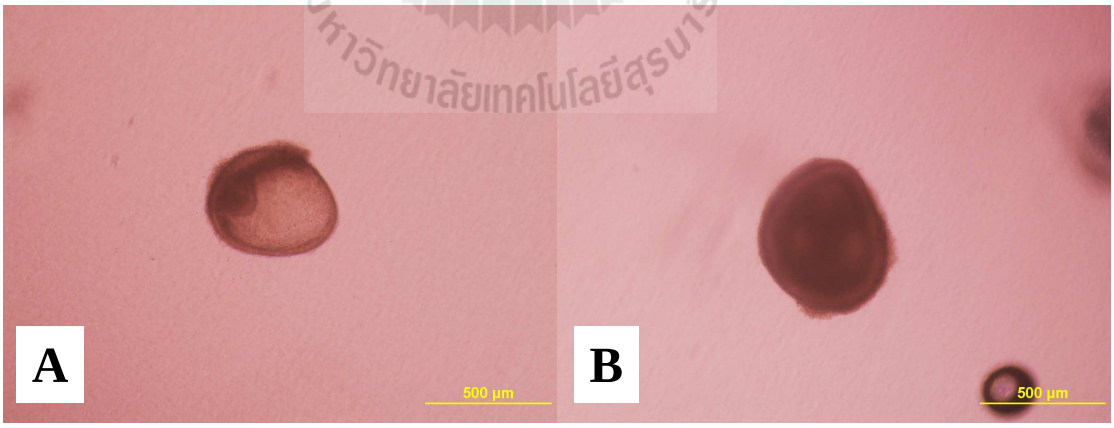

Figure 3.2 Healty follicle consisted of intact granulosa layer complex and well organized arrangement (A) and atretic follicle had plentiful degenerate cells of granulosa layer (B, magnification: 40x).

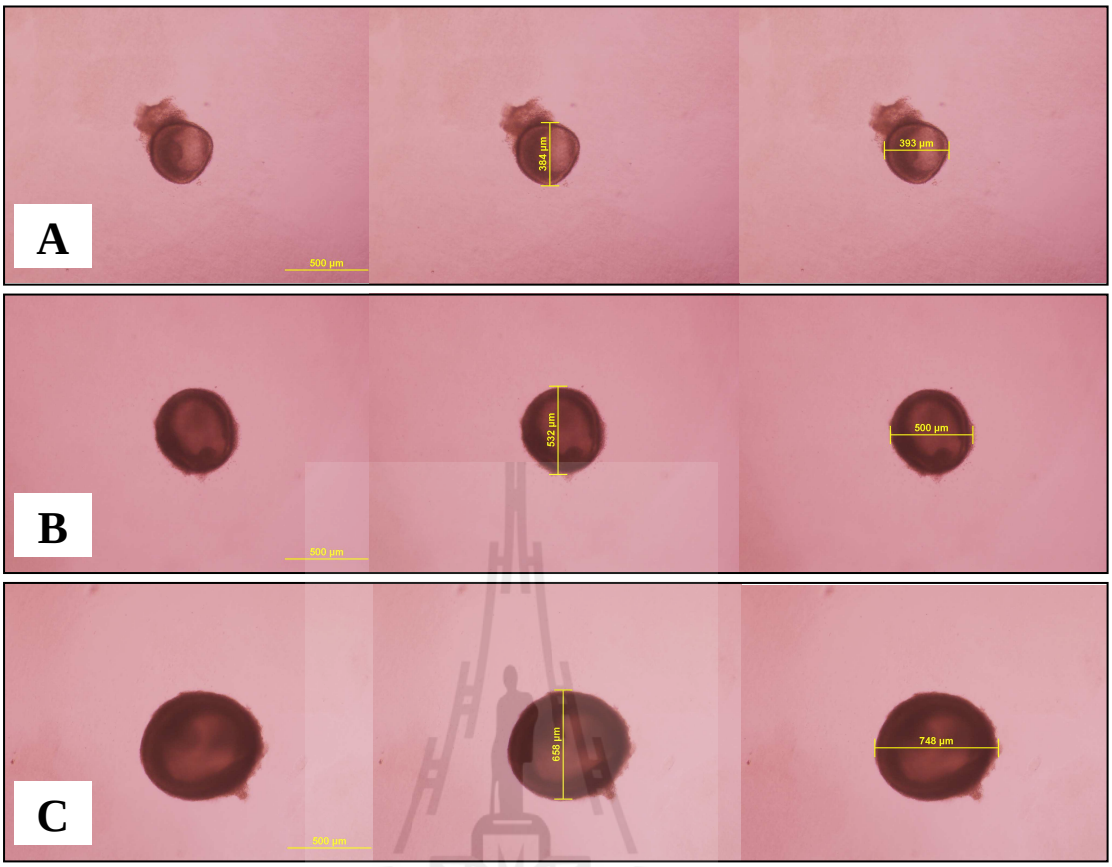

Figure 3.3 The diameters of the follicles were measured with a video micrometer on a screen connected to a CCD camera on an inverted microscope. The categorization of follicles into 3 groups at day 0 ; group I diameters 200-399 µm (A), group II 400-599 µm (B) and group III 600-799 µm (C, magnification: 40x)

3.1.3 Experimental design

The experiment was designed to evaluate the effect of growth factors, IGF-I (I-3769, Sigma), EGF (E-9644, Sigma) and bFGF (13256-029, Invitrogen) on the *in vitro* growth and the viability of the 3 groups of buffalo follicles. The selected early antral follicles were cultured in 8 different treatments as described in Table 3.1.

Table 3.1 Buffalo early antral follicles cultured in to 8 treatments.

Treatments	bFGF (50 ng/ml)	IGF-I (100 ng/ml)	EGF (50 ng/ml)
1	-	-	-
2	+	-	-
3	-	+	-
4	-	-	+
5	+	+	-
6	+	-	+
7	-	+	+
8	+	+	+

3.1.4 Maturation of *in vitro* growth on buffalo oocytes

The cultured early antral follicles were recovered from the collagen gels with fine forceps. The follicles were opened to collect the oocytes and were placed in Dulbecco's phosphate buffer saline (mDPBS) supplemented with 0.1% polyvinylpyrrolidone (PVP) (P 0950, Sigma) under a stereomicroscope (Figure 3.3). The oocytes were washed 3 times in *in vitro* maturation medium (IVM) consisting of TCM 199 medium supplemented with 10% fetal bovine serum (FBS, 10270-098, Gibco), 50 IU/ml hCG (CDN781851, Intervet), 0.02 AU/ml FSH (Antrin®, Denka Pharmaceutical, Japan) and 1 μg/ml 17β-estradiol (E-8875, Sigma). Twelve oocytes were transferred to 50 μl drops of IVM medium under mineral oil (M-8410, Sigma) and were cultured at 38.5ºC under humidified atmosphere of 5% CO_2 in air for 24 hours.

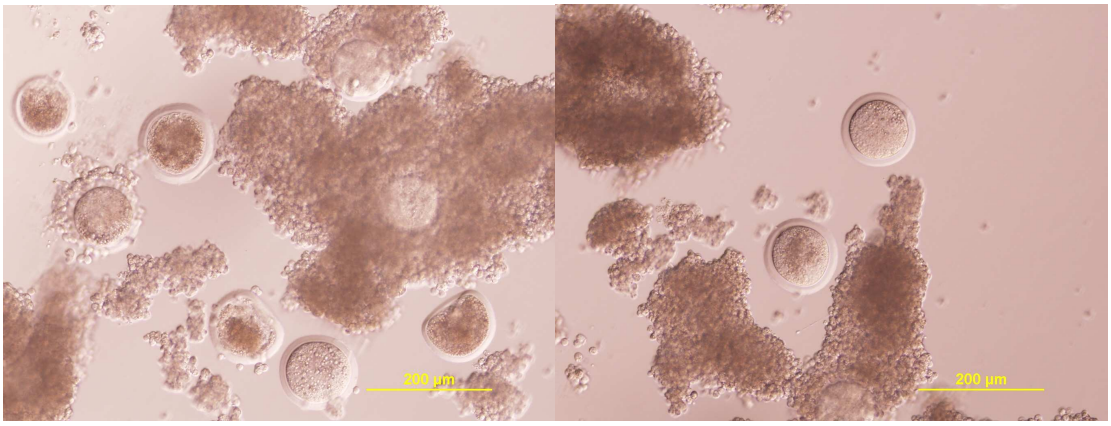

Figure 3.4 Oocytes retrieved from *in vitro* growth on early antral follicles before being cultured in IVM medium (magnification: 100x).

3.1.5 Oocytes viability staining

The cumulus cells of cultured oocytes were removed by repeated pipetting in 0.2% hyaluronidase (H-3506, Sigma) and then washed 3 times in mDPBS+PVP. The denuded oocytes were stained with 2.5 ug/ml fluorescein diacetate (FDA, F7378, Sigma) for 2 minutes and washed 3 times in mDPBS+PVP. Later on, the oocytes viability were examined under ultraviolet light with the video micrometer on a screen connected to a CCD camera on an inverted microscope.

3.1.6 Oocytes nucleus staining

After viability staining, the oocytes were washed 3 times in mDPBS+PVP and then stained with 5 ug/ml Hoechst 33342 (C-2261, Sigma) for 15 minutes. After stained, oocytes were washed 3 times in mDPBS+PVP. The oocyte nucleus was checked under the ultraviolet light with the video micrometer on a screen connected to a CCD camera on an inverted microscope.

3.1.7 Fixing and staining oocytes

The oocytes were mounted on slides and then fixed by incubation into acetic alcohol solution containing gracial acetic acid and 95% ethanol alcohol (1:3, v:v) for 1 day. After that, oocytes were stained with aceto-orcein dye for 10 minutes and washed in washing solution containing glycerol, 95% ethanol and water (1:1:1, v:v:v). The stages of oocyte were determined with a video micrometer on a screen connected to a CCD camera on an inverted microscope (Figure 3.4).

3.1.8 Statistical analysis

Effects of growth factors (IGF-I, EGF and bFGF) on *in vitro* growth of swamp buffalo's early antral follicles were determined. The experiments were performed for at least 10 replications. The experiments were designed in Completely Randomized Design (CRD). Analyses of variance were analyzed and comparisons of means were done by Statistical Analysis System (SAS Institute, Inc., 1993). Oocytes viability (FDA) and oocytes stages results were evaluated by Chi-square.

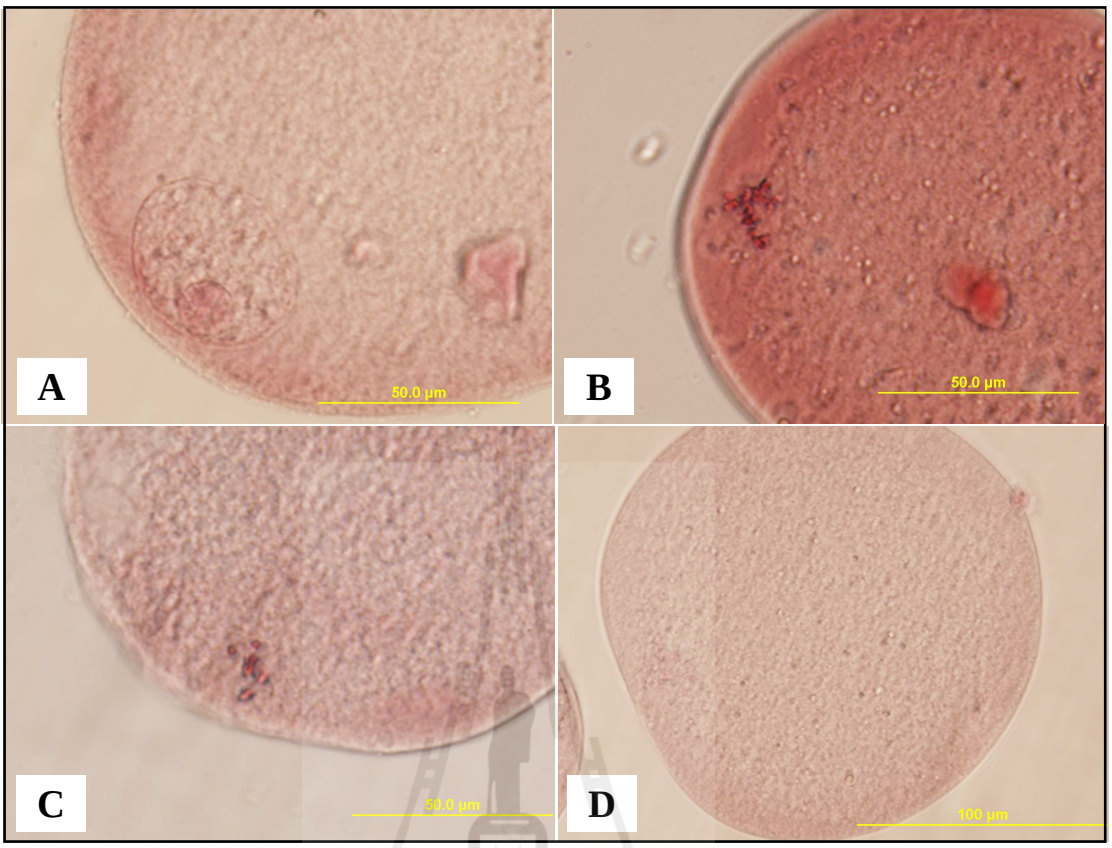

Figure 3.5 Meiotic stage of oocytes after stained with aceto-orcein dye. GV stage (A), MI (B), degenerate chomosome (C, magnification 400x) and absence of chromosome (D, magnification 200x)

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Results

4.1.1 Effects of growth factors on the growth of swamp buffalo's early antral follicles *in vitro*.

The results showed that buffalo follicle in group I (Ø 200-399 µm) were able to grow when cultured in medium supplemented with bFGF, bFGF+IGF-I, and either were the follicles in a controlled treatment (no supplementation). The percent of follicular development are 8.7, 7.5 and 6.8 respectively. The statistical analysis showed that the percent development of follicle in 3 treatments had no significant difference, whereas the follicular development rate of bFGF, bFGF+IGF-I and control treatment was significantly difference when compared with follicles cultured in medium supplemented with IGF-I, EGF, bFGF+EGF, IGF-I+EGF and bFGF+IGF-I+EGF. Follicle cultured in medium supplemented with IGF-I had only 1.7% of follicular development and follicle cultured in medium supplemented with EGF, bFGF+EGF, IGF-I+EGF and bFGF+IGF-I+EGF were unable to grow after the treatment. The buffalo follicle in group II (Ø 400-599 µm) which were cultured in medium supplemented with bFGF, bFGF+IGF-I, control treatment and IGF-I were able to grow after culture. The statistical analysis revealed that the percent development were not significantly difference but the percent development of

follicle in medium supplemented with bFGF was significantly higher than IGF-I (21.6%) and controlled treatment (26.1%). None of any follicle cultured in medium supplemented with EGF, bFGF+EGF, IGF-I+EGF and bFGF+IGF-I+EGF was able to grow . Lastly, the buffalo follicles in group III (Ø 600-799 µm) that were cultured in medium supplemented bFGF (32.7%) had a significantly higher growth rate than IGF-I (19.0%), bFGF+IGF-I (11.8%) and controlled treatment (12.8%). However, follicles cultured in medium supplemented with EGF, bFGF+EGF, IGF-I+EGF and bFGF+IGF-I+EGF were unable to grow (Table 4.1).

4.1.2 Effects of growth factors on the morphology of buffalo ‘s early antral follicles after *in vitro* culture

Every group of follicles cultured in medium supplemented with bFGF, bFGF+IGF-I and control treatment were able to grow after being cultured at day 7 and 14. The follicles increased in diameter and able to form an antrum-like structure. The developmental follicles consisted of antrum space was filled with follicular fluid inside. Moreover, the follicular cells inside and outside of the follicles were able to divide which resulted to the increase of the number of cells. The follicles in group I, group II and group III cultured in medium supplemented with EGF, bFGF+EGF, IGF-I+EGF and bFGF+IGF-I+EGF were unable to grow after being cultured at day 7 and 14. Those follicles were flat in shape and attached to the collagen gel. The follicles were unable to produce follicular fluid while follicular cells were able to multiply themselves (Figure 4.1, 4.2, and 4.3).

Table 4.1 Effects of growth factors on buffalo's early antral follicles development *in vitro*.

Growth factors	Follicle development from day 0 – day 14 (%)		
	Group I (ø 200-399 µm)	**Group II (ø 400-599 µm)**	**Group III (ø 400-599 µm)**
Control	8/117[a] (6.8)	30/115[b] (26.1)	14/109[b] (12.8)
bFGF	9/103[a] (8.7)	52/116[a] (44.8)	35/107[a] (32.7)
IGF-I	2/115[b] (1.7)	25/116[b] (21.6)	20/105[b] (19.0)
EGF	0/107[b] (0.0)	0/119[c] (0.0)	0/96[c] (0.0)
bFGF+IGF-I	9/120[a] (7.5)	44/118[ab] (37.3)	13/110[b] (11.8)
bFGF+EGF	0/115[b] (0.0)	0/116[c] (0.0)	0/108[c] (0.0)
IGF-I+EGF	0/110[b] (0.0)	0/110[c] (0.0)	0/116[c] (0.0)
bFGF+IGF-I+EGF	0/108[b] (0.0)	0/119[c] (0.0)	0/93[c] (0.0)

[a,b,c] Mean within columns with different superscripts differ ($P<0.05$, Chi-square).

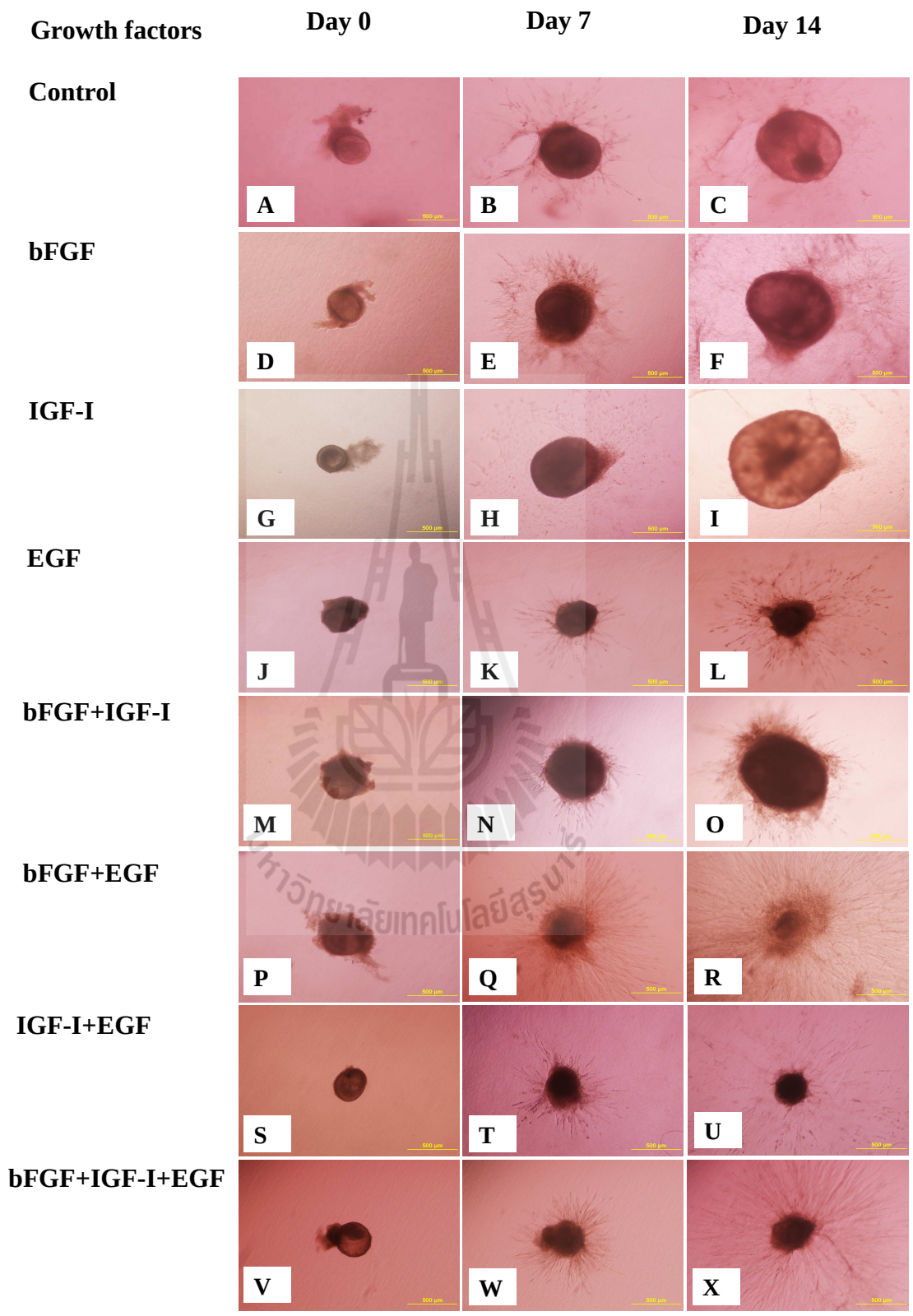

Figure 4.1 Morphology of early antral follicles from buffalo in group I (diameters 200-399 µm) after *in vitro* culture (magnification: 40x).

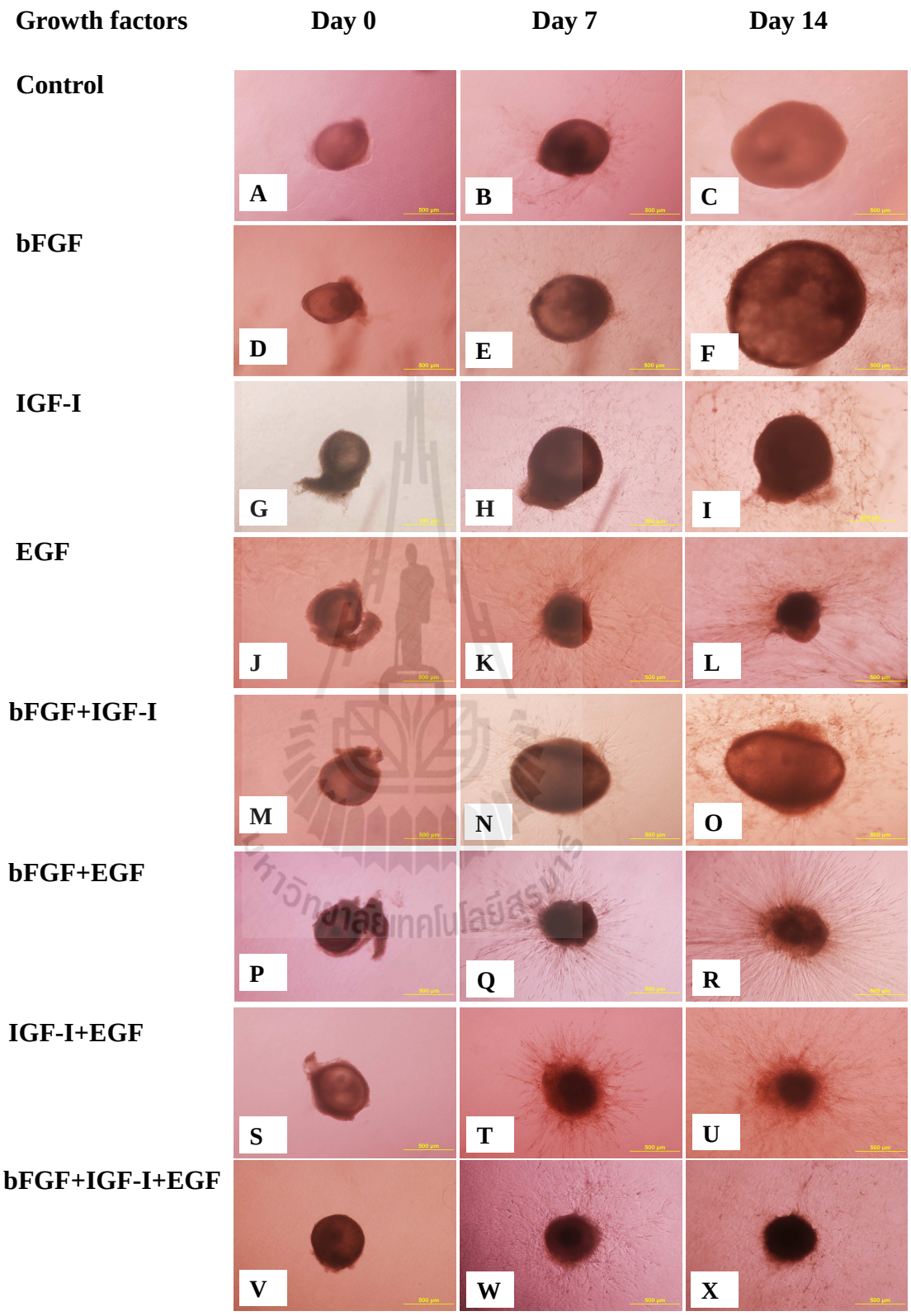

Figure 4.2 Morphology of early antral follicles from buffalo in group II (diameters 400-599 µm) after *in vitro* culture (magnification: 40x).

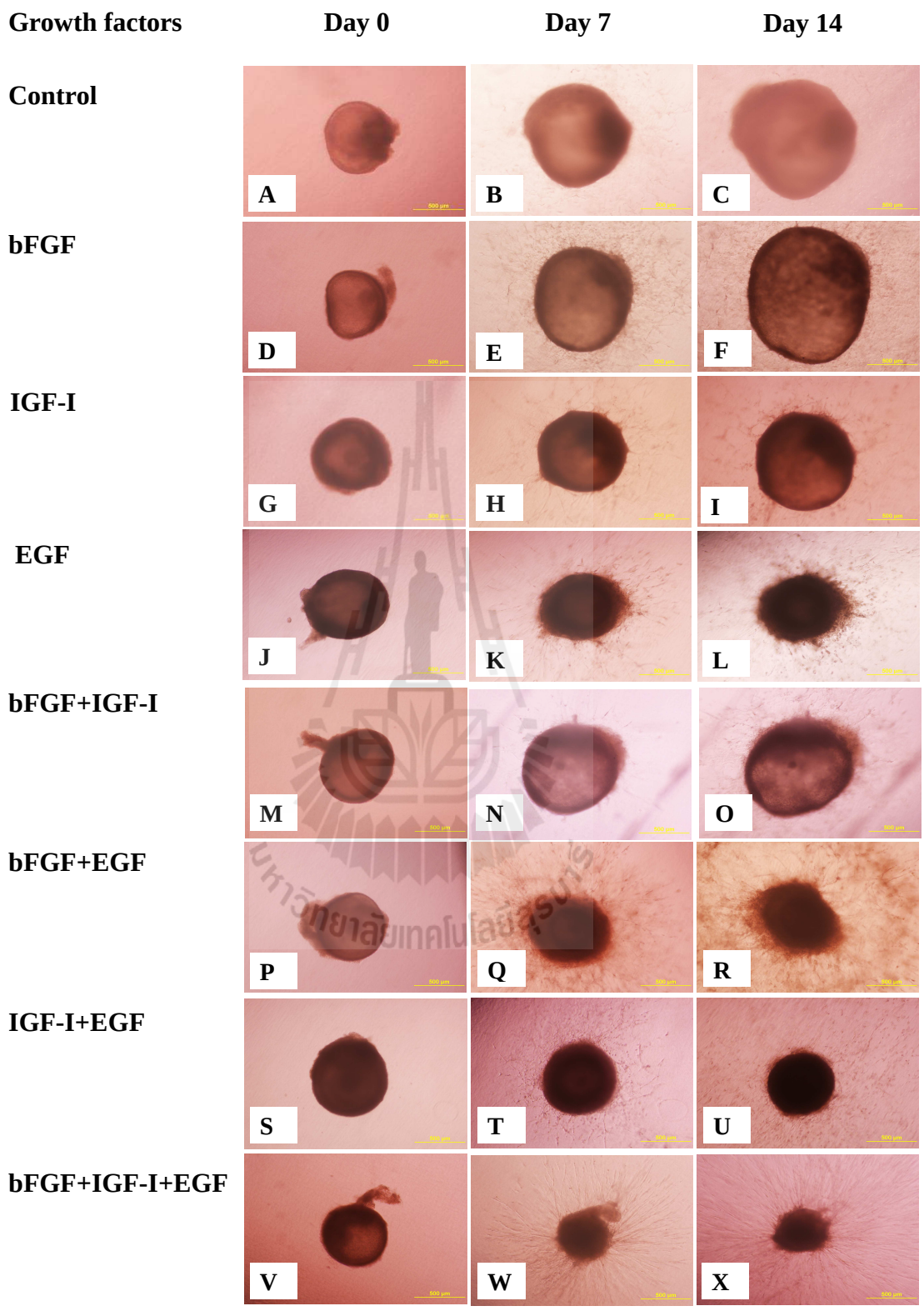

Figure 4.3 Morphology of early antral follicles from buffalo in group III (diameters 600-799 µm) after *in vitro* cultured (magnification: 40x).

4.1.3 Effects of growth factors on follicular diameter after *in vitro* culture.

The diameters of follicles that were treated with bFGF+IGF-I in group I measured at day 7 significantly increased against the follicles cultured in medium supplemented with bFGF and controlled treatment. The average diameter of follicles from day 0 - day 7 were at 212.2 µm, 110.1 µm and 66.4 µm, respectively. However, the follicular diameters of bFGF treatment in group I measured at day 14 significantly increased in diameter when compared with follicles in controlled treatments and follicles cultured in medium supplemented with bFGF+ IGF-I. The average follicular diameters from day 7- day 14 were at 304.3 µm, 157.5 µm and 100.6, µm respectively (Figure 4.4 and table 4.2).

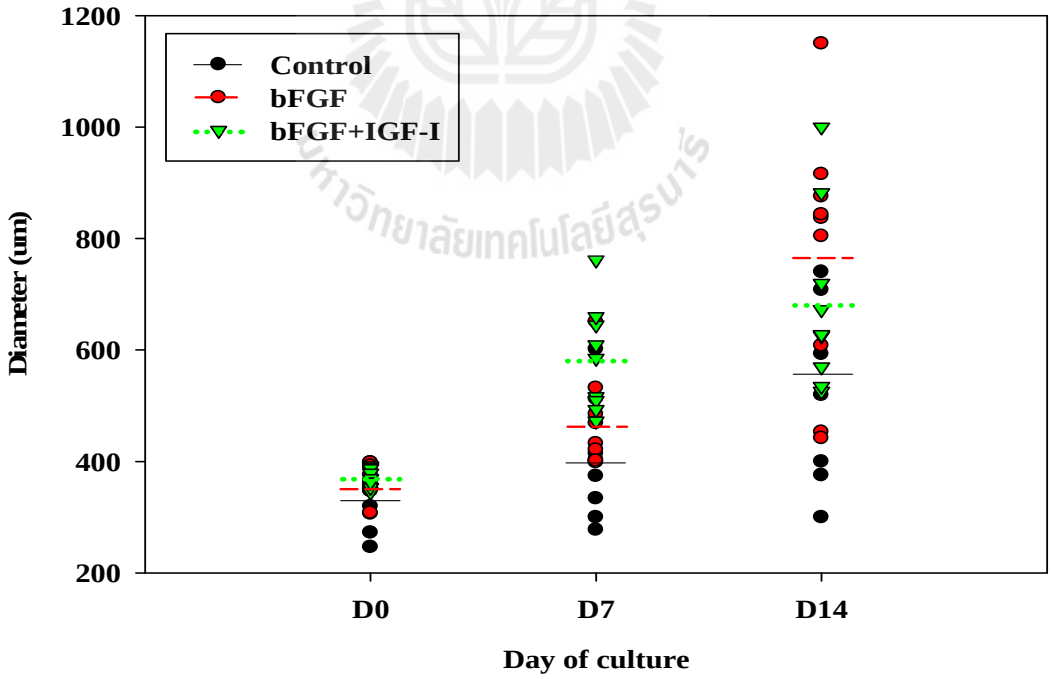

Figure 4.4 The growth of early antral follicles from buffalo in group I (diameters 200-399 µm) after *in vitro* culture.

Table 4.2 Effects of growth factors on the growth of early antral follicles from buffalo in group I (diameters 200-399 µm).

Growth factors	Follicle diameter (µm) (mean ± S.M.E.)			Increasing follicle diameter (µm) (mean ± S.M.E.)	
	Day 0	Day 7	Day 14	Day 0-Day 7	Day 7-Day 14
Control	335.8±4.9[a]	402.2±10.1[b]	559.7±17.9[a]	66.4±7.6[b]	157.5±9.3[a]
bFGF	355.6±3.2[a]	465.6±8.18[b]	769.9±22.6[a]	110.1±7.1[b]	304.3±17.5[b]
bFGF+IGF-I	371.3±1.5[a]	583.6±8.7[a]	684.2±14.7[a]	212.2±8.3[a]	100.6±7.2[a]

[a,b] Mean within columns with different superscripts differ (P<0.05, CDR).

The increasing average follicular diameters in group II measured at day 7 bFGF+IGF-I, IGF-I, bFGF and controlled treatment were not significantly different. The averages of follicular diameters from day 0 - day 7 were at 220.3 µm, 210.3 µm, 196.5 µm and 165.8 µm respectively. However, the diameters of follicles treated with bFGF in group II measured at day 14 were significantly higher than that of follicles cultured in medium supplemented with IGF-I, bFGF+ IGF-I and controlled treatment. The averages of follicular diameters from day 7- day 14 were at 387.6 µm, 260.1 µm, 192.1 µm and 228.0 µm, respectively (Figure 4.5 and table 4.3).

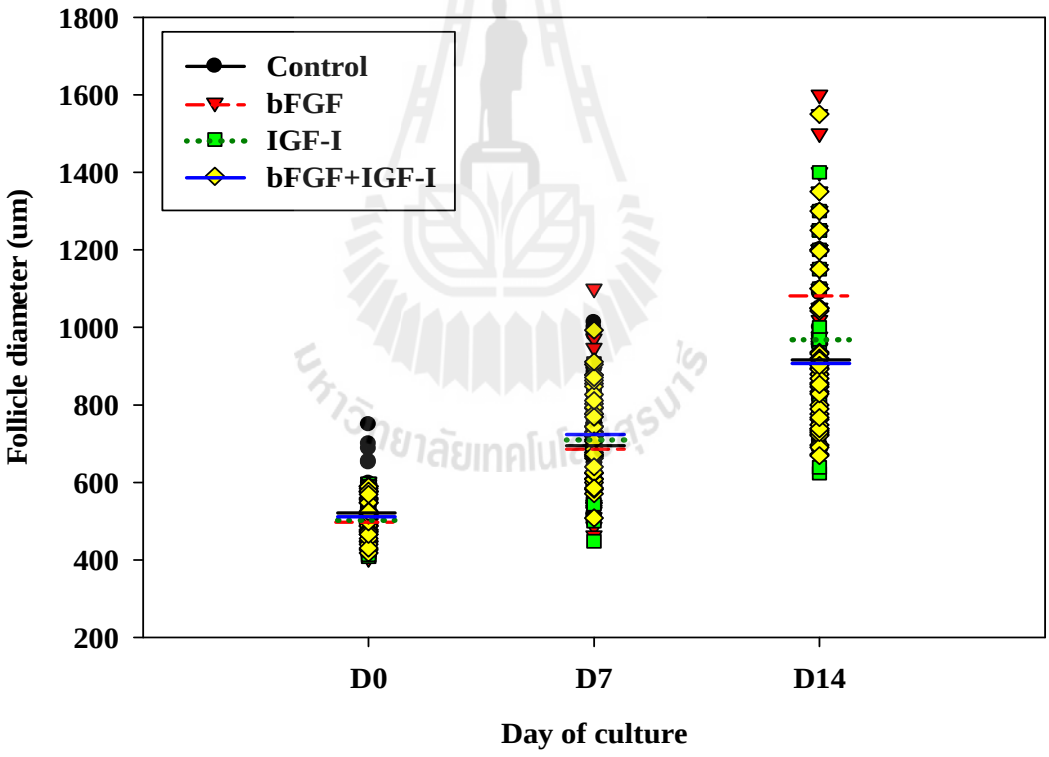

Figure 4.5 The growth of early antral follicles from buffalo in group II (diameters 400-599 µm) after *in vitro* culture.

Table 4.3 Effects of growth factors on the growth of early antral follicles from buffalo in group II (diameters 400-599 µm).

Growth factors	Follicle diameter (µm) (mean ± S.M.E.)			Increasing follicle diameter (µm) (mean ± S.M.E.)	
	Day 0	Day 7	Day 14	Day 0-Day 7	Day 7-Day 14
Control	532.3±8.1[a]	698.1±13.9[a]	926.0±16.3[b]	165.8±9.5[a]	228.0±10.6[b]
bFGF	500.5±5.9[a]	697.0±12.7[a]	1084.6±19.4[a]	196.5±9.9[a]	387.6±15.9[a]
IGF-I	502.6±6.4[a]	712.8±9.9[a]	973.0±21.0[ab]	210.3±9.0[a]	260.1±15.0[ab]
bFGF+IGF-I	512.6±4.6[a]	732.9±9.9[a]	925.0±19.1[ab]	220.3±8.0[a]	192.1±14.3[b]

[a,b] Mean within columns with different superscripts differ (P<0.05, CDR).

The follicular diameters in group III supplemented with bFGF measured at day 7 were found to be largely increased in diameters than that of follicles in controlled treatment and cultured follicles in medium supplemented with IGF-I and bFGF+IGF-I. The averages of follicle diameters from day 0 - day 7 were 259.1 µm, 211.7 µm, 184.8 µm and 107.5 µm, respectively. Moreover, follicular diameters in group III measured at day 14 were similar to day 7. The follicles cultured in medium supplemented with bFGF were able to increase in follicular diameters, larger than the follicles in controlled treatment and the follicles cultured in medium supplemented with IGF-I and bFGF+IGF-I. The averages of follicle diameters from day 7 - day 14 were 230.4 µm, 152.5 µm, 101.3 µm and 60.0 µm, respectively (Figure 4.6 and table 4.4).

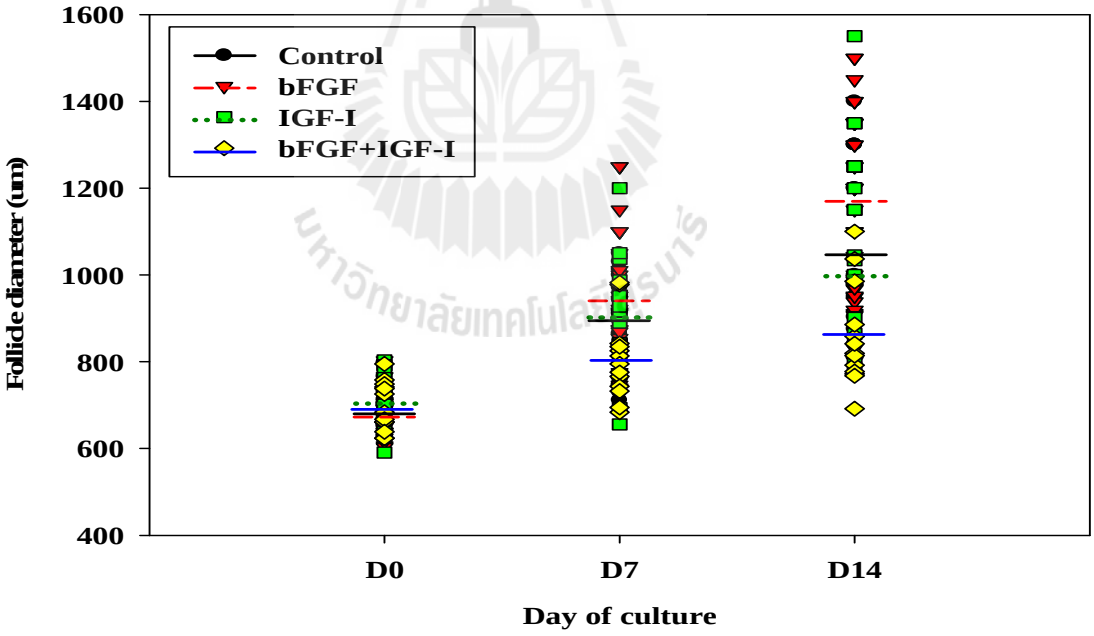

Figure 4.6 The growth of early antral follicles from buffalo in group III (diameters 600-799 µm) after *in vitro* culture.

Table 4.4 Effects of growth factors on the growth of early antral follicles from buffalo in group III (diameters 600-799 µm).

Growth factors	Follicle diameter (µm) (mean ± S.M.E.)			Increase in follicle diameter (µm) (mean ± S.M.E.)	
	Day 0	Day 7	Day 14	Day 0-Day 7	Day 7-Day 14
Control	686.1±4.8[a]	897.8±10.7[a]	1050.3±19.0[a]	211.7±9.6[ab]	152.5±12.9[ab]
bFGF	684.7±4.7[a]	943.9±14.2[a]	1174.3±19.8[a]	259.1±14.1[a]	230.4±13.8[a]
IGF-I	714.3±5.8[a]	899.0±12.0[a]	1000.3±19.6[ab]	184.8±9.7[ab]	101.3±14.7[ab]
bFGF+IGF-I	697.5±5.4[a]	805.0±8.8[a]	865.0±11.2[b]	107.5±5.6[b]	60.0±4.2[b]

[a,b] Mean within columns with different superscripts differ (P<0.05, CDR).

4.1.4 Effects of growth factors on oocytes viability and meiotic division

The oocytes viability rate in all the treatments in group I that were able to grow and increase in diameters after being cultured for 14 days was not significantly difference except with the follicles cultured in medium supplemented with bFGF+IGF-I whose oocytes viability rate was significantly lower than bFGF treatment and controlled (Table 4.5 and figure 4.7). However, checking the meiotic stage by staining the viable oocytes from follicle in control treatment, bFGF and bFGF+IGF-I were found to be arrested at germinal vesicle stage (GV).

In group II, the viability rate of oocytes cultured in medium supplemented with bFGF was significantly higher than follicle cultured in medium supplemented with bFGF+IGF-I. However, the viability rate of oocytes from follicles in controlled treatment, IGF-I and bFGF+IGF-I were not significantly difference (Table 4.6 and figure 4.8). The results of meiotic stage found that only oocytes from follicle cultured in medium supplemented with bFGF were able to development to metaphase I (MI). Oocytes from other treatment were arrested at GV stage.

The viability rate of oocytes from follicle cultured in controlled and IGF-I treatment in group III had a significantly higher viability rate than the follicles cultured in medium supplemented with bFGF and bFGF+IGF-I (Table 4.7 and figure 4.9). Additionally, the oocytes from follicles in controlled treatment and in medium supplemented with IGF-I and bFGF were able to develop to MI stage oocytes. However, oocytes from 4 treatments were arrested at GV stage.

Table 4.5 Effects of growth factors on oocytes viability and meiotic stages after IVM of buffalo's early antral follicles in group I (diameter 200-399 µm)

Growth factors	Oocytes viability (%)	meiotic stages			
		GV (%)	MI (%)	Degenerate (%)	Chromosome not found (%)
Control	9/9[a] (100)	7/9 (78)	0	0	2/9 (22)
bFGF	8/9[a] (89)	6 (67)	0	0	2/8 (25)
bFGF+IGF-I	5/9[b] (56)	4 (80)	0	0	1/5 (20)

[a,b] Mean within columns with different superscripts differ ($P<0.05$, Chi-square).

Table 4.6 Effects of growth factors on oocytes viability and meiotic stages after IVM of buffalo's early antral follicles in group II (diameter 400-599 µm)

Growth factors	Oocytes viability (%)	meiotic stages			
		GV (%)	MI (%)	Degenerate (%)	Chromosome not found (%)
Control	16/30 [ab] (53.3)	15/16 (93.8)	0	0	1/16 (6.3)
bFGF	32/52 [a] (61.5)	28/32 (87.5)	2/32 (6.3)	1/32 (3.1)	1/32 (3.1)
IGF-I	13/25 [ab] (52.0)	13/13 (100)	0	0	0
bFGF+IGF-I	14/44 [b] (31.8)	14/14 (100)	0	0	0

[a,b] Mean within columns with different superscripts differ (P<0.05, Chi-square).

Table 4.7 Effects of growth factors on oocytes viability and meiotic stages after IVM of buffalo's early antral follicles in group III (diameter 600-799 µm)

Growth factors	Oocytes viability (%)	meiotic stages			
		GV (%)	MI (%)	Degenerate (%)	Chromosome not found (%)
Control	7/14[a] (50)	2/7 (28.6)	2/7 (28.6)	2/7 (28.6)	1/7 (14.3)
bFGF	5/35[b] (14.3)	3/5 (60)	1/5 (20.0)	1/5 (20.0)	0
IGF-I	8/21[a] (38.1)	6/8 (75)	2/8 (25.0)	0	0
bFGF+IGF-I	1/13[bc] (7.7)	1/1 (100)	0	0	0

[a,b,c] Mean within columns with different superscripts differ (P<0.05, Chi-square).

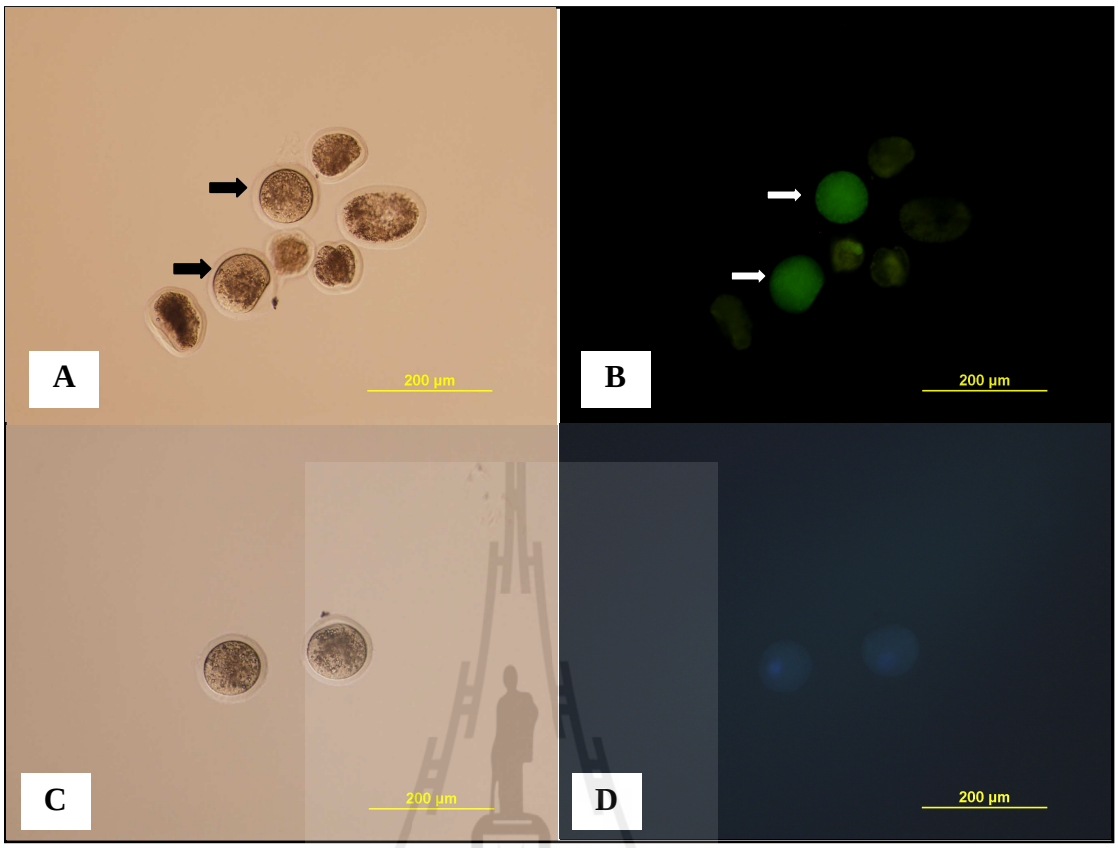

Figure 4.7 Oocytes retrieved from follicles in group I (diameters 200-399 µm). Cumulus cells were removed after being cultured for 24 hours (A), oocytes viability after stained with FDA (B), oocytes before stained with Hoechst 33342 (C) and oocytes nucleus after stained with Hoechst 33342 (D). The arrows indicated the viable oocytes (magnification: 100x).

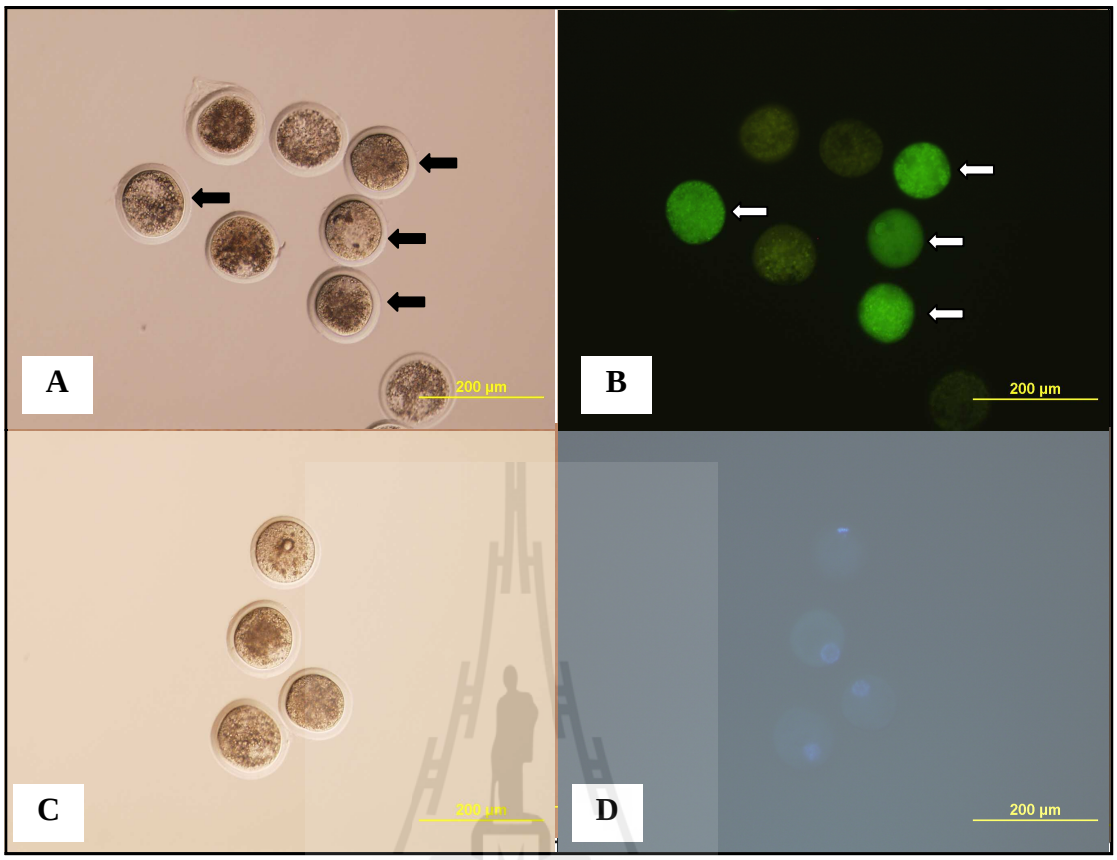

Figure 4.8 Oocytes retrieved from follicles in group II (diameters 400-599 µm). Cumulus cells were removed after being cultured for 24 hours (A), oocytes viability after stained with FDA (B), oocytes before stained with Hoechst 33342 (C) and oocytes nucleus after stained with Hoechst 33342 (D). The arrows indicated the viable oocytes (magnification 100x).

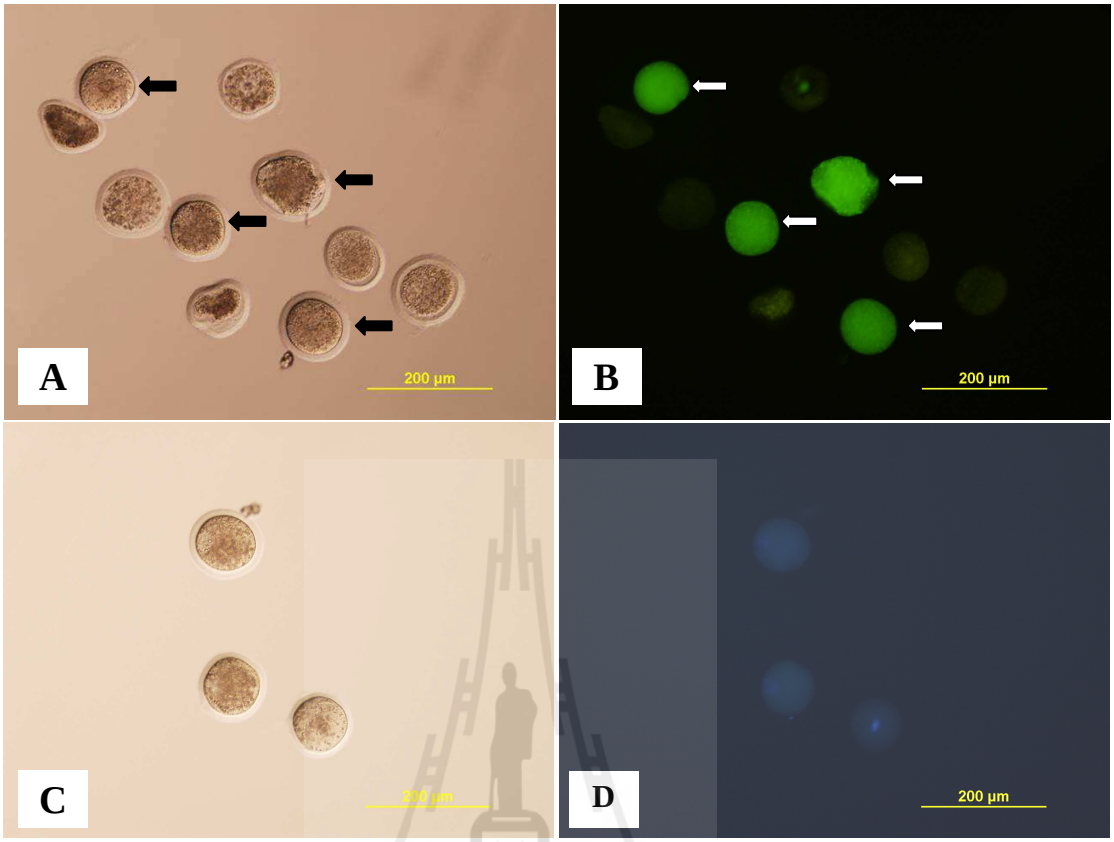

Figure 4.9 Oocytes retrieved from follicles in group III (diameters 600-799 µm). Cumulus cells were removed after being cultured for 24 hours (A), oocytes viability after stained with FDA (B), oocytes before stained with Hoechst 33342 (C) and oocytes nucleus after stained with Hoechst 33342 (D). The arrow indicated the viable oocytes (magnification 100x).

4.2 Discussions

Early antral follicles from buffalo cultured in medium without any supplementation (controlled treatment) in all 3 groups were able to grow after being cultured for 14 days as indicated by the increased follicular diameter, produced follicular fluid, and increased a number of follicular cells. However, a few percents of follicles developed in group I was lower than group II and group III respectively.

This incidence might be because the follicles in group I have a smaller size, which resulted in an early formation of antrum space that contains a fewer of follicular fluid and follicular cells. Therefore, almost all follicles in group I were unable to develop, increased antrum space, follicular cells and produced follicular fluid after *in vitro* culture. Additional growth factors, hormones and nutrients may be required to complete the processes mentioned earlier. The follicles in group II were able to develop better than that in group I because the size of initial follicle was bigger than that of group I. Moreover, follicles contain a larger antrum space, more follicular cells and more follicular fluid. Base on these facts, the follicles in group II were able to increase antrum space, follicular cells and follicular fluid better than those of group I. The follicles in group III had the largest size among other groups, which made the follicles in this group contain a larger antrum space and produce more follicular cells and follicular fluid than group I and group II. However, a few percent of follicular development in this group was lower than group II which may be as a result of the absence of some essential growth factors, hormones and nutrient. In addition, the cocktail used in this experiment might not be suitable for development of follicles in group III. Therefore, a few percent of follicular development in group III was lower than that of group II.

The early antral follicles from buffalo cultured in medium supplemented with bFGF in all groups of follicles were able to grow after being cultured for 14 days. The follicles in group II and group III had a significant higher percent of follicular development than other treatments, but a percent of follicular development in group I was not significantly difference as compared with the controlled treatment. The result of this study is inconsistent with previous reports by Zhou and Zhang in 2005. They

cultured small follicles from goat and found that bFGF were able to increase survival rate of follicles but did not have any effects on the follicular development. However, the results of this study showed that bFGF is suitable for follicles cultured in group II and group III. The follicles in group I may require additional growth factors, hormones and nutrients to support the follicular development.

The early antral follicles from buffalo that were treated with IGF-I in group II and group III were able to develop after cultured but a percent of follicular development was not significantly difference from controlled treatment. In contrast, a few percent of follicular development in group I were significantly lower than follicles in controlled treatment. The results of this experiment indicated that IGF-I was not suitable to culture follicles in group I and IGF-I had no effect on follicular development in group II and group III. The results of this experiment is also different from Zhou and Zhang's reported in 2005. They found that IGF-I was able to increase developmental and survival rate of follicles in goat.

Since, there are not any previous reports of follicles culture in bFGF+IGF-I, so this is the first report about small follicles of buffalo cultured in bFGF+IGF-I. The follicular development at all groups of follicles in this study increased but not significantly difference from controlled treatment. The results of this study indicated that bFGF+IGF-I had no effect on follicle development in all groups.

The early antral follicles from buffalo cultured in medium supplemented with EGF in all 3 groups were unable to grow. The morphology of follicles in this treatment was flat and attached to collagen gel. Follicles were unable to produce follicular fluid but follicular cells were able to divide themselves and increased its cells number. Moreover, the buffalo follicles cultured in medium supplemented with

EGF combined with other growth factors (bFGF+EGF, IGF-I+EGF, and bFGF+IGF-I+EGF) were unable to grow after being cultured. The results of this study indicated that EGF could suppress follicular development. The result of this study is consistent with the results of previous reported by Shen and co-work in 1998. They found that EGF caused granulosa cells division and spread out from oocyte. Therefore, follicles were unable to maintain their round shapes and inhibit antrum formation.

In group I, the oocytes viability from follicles in controlled treatment was not significantly different from oocytes from follicle cultured in medium supplemented with bFGF, whereas the follicles cultured in medium supplemented with bFGF+IGF-I had a significantly lower oocytes viability than bFGF treatment and control. The result of meiotic stage found that oocytes from 3 treatments arrested at GV stage. The oocytes viability from follicle in group II was not significantly different in controlled treatment and follicles cultured in medium supplemented with bFGF, IGF-I. The follicles cultured in medium supplemented with bFGF+IGF-I had a significant lower oocytes viability rate than bFGF. The result of meiotic stage found that the oocytes from 4 treatments were arrested at GV stage. Only oocytes from follicles cultured in medium supplement with bFGF were able to develop to MI. The oocytes viability rate of follicles in group III were significantly lower in bFGF and bFGF+IGF-I treatment when compared with IGF-I and controlled. The result of meiotic stage found that oocytes from 4 treatments were arrested at GV stage and oocytes from follicle cultured in medium supplemented with bFGF and controlled treatment were able to develop to MI stage oocytes. The result of in this study was unable to indicate the effects of growth factor on oocytes viability and meiotic stage because of limited number of oocytes from follicle. The number of the oocytes should be more than this

experiment (around 100 oocytes) to indicate the effect of growth factor on oocytes viability and meiotic stage. However, this study indicated that bFGF was able to support the buffalo's early antral follicle culture in group II and group III. This basic knowledge may be useful to develop efficient method for follicle culture in the future.

CHAPTER IV

CONCLUSION

The effect of growth factors on buffalo early antral follicle culture in vitro was examined in this study. bFGF was found to be most suitable for follicle culture referred from the growth rate of follicles in group II and group III. Follicles in group I may require additional growth factors or nutrient to induce the follicle growth in this group. IGF-I could suppress follicle development in group I but IGF-I have no effected on follicles development in group II and group III. Additionally, bFGF+IGF-I have no effect on follicles development in all groups as well. The results indicated that EGF could suppress follicle development, even when combined with other growth factor (bFGF+EGF, IGF-I+EGF, and bFGF+IGF-I+EGF). EGF causes granulosa cells division and spreading out of the oocyte. Therefore, follicles were unable to maintain a round shape and disrupt antrum formation. The effect of growth factor on oocytes viability and meiotic division could not be measured in this study because of limited number of oocytes. However, this study indicated that bFGF was able to support buffalo early antral follicle growth cultured in group II and group III. EGF supplementation and different combination of growth factors could suppress the development of the follicle. This basic knowledge will be useful to develop the most efficient method for follicle culture in the future.

REFERENCES

Adams, G.P., Jaiswal, R., Singh, J. and Malhi, P. 2008. Progress in understanding ovarian follicular dynamic in cattle. **Theriogenology** 69: 72-80.

Ali, A., Abdel-Razek, A.K., Abdel-Ghaffar, S. and Glatzel, P.S. 2003. Ovarian follicular dynamics in buffalo cows (Bubalus bubalis). **Reprod. Dom. Anim.** 38: 214-218.

Alm, H., Katska-Książkiewicz, L., Ryńska, B. and Tuchscherer. 2006. Survival and meiotic competence of bovine oocytes orinating from early antral ovarian follicle. **Theriogenology** 65: 1422-1434.

Barnett, K.R., Schilling, C., Greenfeld, C.R., Tomic, D. and Flaws, J.A. 2006. Ovarian follicle development and transgenic mouse models. **Hum. Reprod Update** 12: 537-555.

Clark, L.J., Irving-Rodgers, H.F., Dharmarajan, A.M. and Rodgers, R.J. 2004. Theca Interna: The other side of bovine follicular atresia. **Biol. Reprod.** 71: 1071–1078.

Cortvrindt, R. and Smitz, J. 2001. *In vitro* follicle growth: achievement in mammalian species. **Reprod. Dom. Anim.** 36: 3-9.

Cumming, B. 2001. Events of oogenesis. An imprint of Addison Wesley Longman. **www.tarleton.edu/~anatomy/oogenesis.html**.

Demeestere, I., Delbaere, A., Gervy, C., Van den Bergh, M., Devreker, F. and Englert, Y. 2002. Effect of preantral follicle isolation technique on in-vitro follicular growth, oocyte maturation and embryo development in mice. **Hum. Reprod.** 17: 2152-2159.

Driancourt, M.A. 2000. Regulation of ovarian follicular dynamics in farm animals implications for manipulation of reproduction. **Theriogenology** 55: 1211-1239.

Eppig, J.J. and Brien, M.J. 1996. Development in vitro of mouse oocytes from primordial follicles. **Biol. Reprod.** 54: 197-207.

Evans, A.C.O. 2003. Characteristics of ovarian follicle development in domestic animals. **Reprod. Dom. Anim.** 38: 240-246.

Feranil, J.B., Isobe, N. and Nakao, T. 2005. Apoptosis in the antral follicles of swamp buffalo and cattle ovary: TUNEL and caspase-3 histochemistry. **Reprod. Dom. Anim.** 40: 111-116.

Gupta, P.S.P., Nandi, S., Ravindranatha, B.M. and Sarma, P.V. 2002. *In vitro* culture of buffalo (*Bubalus bubalis*) preantral follicles. **Theriogenology** 57: 1839-1854.

Gupta, P.S.P., Ramesh, H.S., Manjunatha, B.M., Nandi, S. and Ravindra, J.P. 2008. Production of buffalo embryos using oocytes from in vitro grown preantral follicles. **Zygote** 16: 57-63.

Hussein, H.R. 2005. Apoptosis in the ovary: molecular mechnisms. **Hum. Reprod. Update**. 11: 162-178.

McGee, E.A. and Hsueh, A.J.W. 2000. Initial and cyclic recruitment of ovarian follicles. **Endocrine review** 21: 200-214

McNatty, K.P., Reader, K., Smith, P., Heath, D.A. and Juengel, J.L. 2007. Control of ovarian follicular development to the gonadotrophin-dependent phase: a 2006 perspective. **Soc. Reprod. Fertil. Suppl.** 64: 55-68.

Mizunuma, H., Lui, X., Andoh, K., Abe, Y., Kobayashi, J., Yamada, K., Yokota, H., Ibuki, Y. And Hasegawa, Y. 1999. Activin from secondary follicles cause small preantral follicles to remain dormant at the resting stage. **Endocrinol.** 140: 37-42.

Nandi, S., Kumar, V.G., Ramesh, H.S., Manjunatha, B.M. and Gupta, P.S.P. 2009. Isolation and culture of ovine and bubaline small and large pre-antral follicle: effect of cyclicity and presence of a dominant follicle. **Reprod. Dom. Anim.** 44: 74-79.

Pang, X., Yang, M., Wang, L., Tong, C. and Guo, Z. 2010. *In vitro* culture of sheep lamb ovarian cortical tissue in a sequential culture medium. **J. Assist. Reprod. Genet** 27: 247-257.

Picton, H.M. 2001. Activation of follicle development: the primordial follicle. **Theriogenology** 55: 1193-1210

Presicce, G.A. 2007. Reproduction in the water buffalo. **Reprod. Dom. Anim.** 42: 24-32.

Rajarajan, K., Roa, B.S., Vagdevi, R., Tamilmani, G., Arunakumari, G., Sreenu, M., Amarnath, D., Naik, B.R. and Rao, V.H. 2006. Effect of various growth factors on the *in vitro* development of goat preantral follicles. **Small. Rumin. Res.** 63: 204-212.

Santos, S., Biondi, F., Cordero, M., Mirando, M., Dantas, J., Figueiredo, J. and Ohashi, O. 2006. Isolation, follicular density, and culture of preantral follicles of buffalo fetuses of different ages. **Anim. Reprod. Sci.** 95: 1-15.

Saha, S., Shimizu, M., Geshi, M. and Izaike, Y. 2000. *In vitro* culture of bovine preantral follicles. **Anim. Reprod. Sci.** 63: 27-39.

Schering-plough animal health. 2010. Hormonal regulation of reproduction in cattle. **www.partners-in-reproduction.com**.

Shen, X., Miyano, T. and Kato, S. 1998. *In vivo* and *in vitro* antrum formation of pig ovarian follicles. **Reprod. Biol. Update** : 73-79.

Smitz, J.E.J. and Cortvrindt, R.G. 2002. The earliest stages of folliculogenesis *in vitro*. **Reproduction** 123: 185-202.

Tamilmani, G., Rao, B.S., Vagdevi, R., Amarnath, D., Naik, B.R., Mutharao, M. and Rao, V.H. 2005. Nuclear maturation of ovine oocytes in cultured preantral follicles. **Small. Rumin. Res.** 60: 295-305.

Talfer, E.E., Binnie, J.P., McCaffery, F.H., and Campbell, B.K. 2000. In vitro development of oocytes from porcine and bovine primary follicle. **Mol. Cell. Endocrinol.** 163: 117-123.

Van den Hurk, R. and Zhao, J. 2005. Formation of mammalian oocytes and their growth, differentiation and maturation within ovarian follicles. **Theriogenology** 63: 1717-1751.

Webb, R., Garnsworthy, P.C., Gong, J.G. and Armstrong, D.G. 2004. Control of follicular growth: local interaction and nutritional influences. **J. Anim. Sci.** 82: 63-74

Yamamoto, K., Otoi, T., Koyama, N. Horikita, N., Tachikawa, S. and Miyano, T. 1999. Development to live young from bovine small oocytes after growth, maturation and fertilization *in vitro*. **Theriogenology** 52: 81-89.

Yindee, M., Techakumphu, M., Lohachit, C., Sirivaidyapong, S., Na-Chiangmai, A., Rodriguez-Martinez, H., ven der Weyden, G.C. and Colenbrander, B. 2010. Follicular dynamics and oestrous detection in Thai postpartum swamp buffaloes (Bubalus bubalis). **Reprod. Dom. Anim.** Doi:10.1111.

Zhou, H. and Zhang, Y. 2005. Effect of growth factor on *in vitro* development of caprine preantral follicle oocytes. **Anim. Reprod. Sci.** 90: 265-272.

Zhou, H. and Zhang, Y. 2005. Regrulation of *in vitro* growth of preantral follicles by growth factors in goats. **Domest. Anim. Endocrinol.** 28: 235-242.

University of Nebraska-Lincoln, Institute of agiculture and natural resources. 2010. Physiology of estrous cycle. **http://beef.unl.edu/learning/estrous.shtml**.

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