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FOLLICULAR DYNAMICS, OOCYTE PICK UP – *IN VITRO FERTILIZATION* IN SWAMP BUFFALOES (*Bubalus bubalis*)

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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การศึกษานี้มีวัตถุประสงค์เพื่อ ศึกษาการพัฒนาของฟอลลิเคิลบนรังไข่กระบือปลักภายหลังการเหนี่ยวนำการเป็นสัด ประเมินประสิทธิภาพในการนำเทคนิคการเก็บโอโอไซต์ด้วยวิธีโอพียูมาใช้ในแม่กระบือปลักที่มีวงจรการเป็นสัดปกติ และแม่กระบือในช่วงให้นมหลังคลอด ทั้งที่กระตุ้น และไม่ได้กระตุ้นด้วยฮอร์โมนโกนาโดโทรปิน และศึกษาการพัฒนาของตัวอ่อนจากโอโอไซต์ที่เก็บได้ด้วยวิธีโอพียู โดยแบ่งเป็น 3 การทดลอง
การทดลองที่ 1 ใช้แม่กระบือปลักจำนวน 9 ตัว ฝังฮอร์โมนโปรเจสเตอโรนชนิดฝังใบหูเป็นเวลา 10 วัน ฉีดฮอร์โมนโพรสตาแกรนดิน เอฟทูอัลฟา ครั้งเดียว ในวันที่ถอดฮอร์โมนที่ฝังหูออก ทำการตรวจด้วยเครื่องอัลตร้าซาวด์ และเก็บเลือดเพื่อตรวจวัดระดับฮอร์โมนโปรเจสเตอโรน หนึ่งวันหลังถอดฮอร์โมน โดยทำการศึกษาเป็น 2 ช่วงในหน้าร้อน (มีนาคม ถึง มิถุนายน 2547) และในหน้าหนาว (พฤศจิกายน 2547 ถึงกุมภาพันธ์ 2548) ผลการศึกษาแสดงให้เห็นว่าวงจรการเป็นสัดในกระบือมีการเจริญของฟอลลิเคิลที่มีลักษณะเป็นคลื่น จาก 22 วงจรการเป็นสัด พบว่า 5(22.7%) ของวงจรการเป็นสัดมีหนึ่งคลื่น และ17(77.3%) มีสองคลื่นต่อวงจรการเป็นสัด โดยลักษณะการเจริญของฟอลลิเคิลในรอบวงจรการเป็นสัดไม่มีความแตกต่างกันระหว่างหน้าร้อนและหน้าหนาว
การทดลองที่ 2 แม่กระบือที่มีวงจรการเป็นสัดปกติ 5 ตัว และแม่กระบือให้นมหลังคลอด 6 ตัว ได้รับการกระตุ้นด้วยฟอลลิเคิล สติมูเลติง ฮอร์โมน ขนาด 400 มก. ร่วมด้วย 100 ไมโครกรัมของ ฮอร์โมน จีเอ็นอาร์เอช 24 ชั่วโมงภายหลังฉีดฮอร์โมน เอฟเอสเอชเข็มสุดท้าย ภายหลังจากสิ้นสุดการทำโอพียูในแม่กระบือที่กระตุ้นฮอร์โมน จะพักกระบือเป็นเวลา 1 เดือน หลังจากนั้นแม่กระบือทั้ง 2 กลุ่มจะทำการเก็บโอโอไซต์ด้วยวิธีโอพียู โดยไม่มีการกระตุ้นฮอร์โมนเพิ่มอีก 6 ครั้ง ในกลุ่มแม่กระบือที่กระตุ้นด้วยฮอร์โมน จำนวนฟอลลิเคิลที่สามารถทำการเจาะไม่แตกต่างกันระหว่างแม่กระบือที่มีวงจรการเป็นสัดปกติ และแม่กระบือให้นม (7.2 ± 3.7 และ 9.0 ± 3.2 ฟอลลิเคิลต่อครั้ง ตามลำดับ)($P > 0.05$) นอกจากนี้ยังไม่มีความแตกต่างกันของจำนวนโอโอไซต์ที่เก็บได้ระหว่าง 2 กลุ่ม (3.7 ± 2.7 และ 5.9 ± 3.5 โอโอไซต์ต่อครั้ง ตามลำดับ) ($P > 0.05$) ในแม่กระบือทั้งสองกลุ่มที่ไม่ได้รับการกระตุ้นด้วยฮอร์โมน จำนวนฟอลลิเคิลที่สามารถทำการเจาะไม่แตกต่างกันระหว่างแม่กระบือที่มีวงจรการเป็นสัดปกติ และแม่กระบือให้นม (2.1 ± 1.4 และ 1.4 ± 0.7ฟอลลิเคิลต่อครั้ง ตามลำดับ)($P > 0.05$) และไม่มีความแตกต่างกันของจำนวนโอโอไซต์ที่เก็บได้ระหว่าง 2 กลุ่ม (1.4 ± 1.3 และ 0.7 ± 0.8 โอโอไซต์ต่อครั้ง ตามลำดับ) ($P > 0.05$) โอพียูสามารถที่จะทำได้สำเร็จในแม่กระบือที่มีสถานภาพทางระบบสืบพันธุ์ที่แตกต่างกัน และการกระตุ้นด้วยฮอร์โมน เอฟเอสเอช สามารถเพิ่มจำนวนของฟอลลิเคลที่สามารถเจาะได้ ทั้งในแม่กระบือที่มีวงจรการเป็นสัดปกติ และแม่กระบือให้นมหลังคลอด
การทดลองที่ 3 ใช้แม่กระบือ 5 ตัว ที่ได้รับการกระตุ้นด้วยฮอร์โมน เอฟเอสเอช ขนาด 400 มก. ทำการเก็บโอโอไซต์ด้วยวิธีโอพียู จำนวนรวม 60 ครั้ง ได้โอโอไซต์ทั้งหมด 265 โอโอไซต์ เฉลี่ย 4.4 ± 3.4 โอโอไซต์ต่อตัว จาก 475 ฟอลลิเคิล (7.9 ± 3.5 ฟอลลิเคิลต่อตัว) ล้างโอโอไซต์ด้วยน้ำยา TCM 199 2.5 Hepes 2 ครั้ง หลังจากนั้นนำโอโอไซต์ที่มีเซลล์คิวมูลัสหุ้มรอบไปเลี้ยงต่อในน้ำยา ทีซีเอ็ม 199 โซเดียมไบคาร์บอเนต + 10% ฟิตัล คาร์ฟ ซีรั่ม เป็นเวลา 24 ชั่วโมง ที่อุณหภูมิ 38.5 °C ภายใต้สภาวะ 5% CO_2 หลังจากนั้นทำการปฏิสนธิ และ เลี้ยงต่อในน้ำยา B2 + 2.5% ฟิตัล คาร์ฟ ซีรั่ม ร่วมกับเซลล์เวโร เป็นระยะเวลา 7 วัน และสังเกตการพัฒนาของตัวอ่อน ผลการศึกษาพบว่าอัตราความสมบูรณ์พร้อมในการปฏิสนธิ เท่ากับ 62.8%(49/78) อัตราการแบ่งตัวของตัวอ่อนเป็นระยะ 2-4 เซลล์ ตัวอ่อนระยะมอรูล่า และบลาสโตซีส เท่ากับ 40.5% (34/84), 15.5% (13/84) และ 1.2%(1/84) ตามลำดับ จากการศึกษาสรุปได้ว่ามีความเป็นไปได้ในการผลิตตัวอ่อนจากโอโอไซต์กระบือปลักที่เก็บด้วยวุธีโอพียู แต่อัตราการพัฒนาของตัวอ่อนถึงระยะมอรูล่า และบลาสโตซีสยังต่ำอยู่ ทั้งนี้อาจเนื่องจากโอโอไซต์ที่มีคุณภาพดีมีเปอร์เซ็นต์ที่ต่ำ

ภาควิชาสูติศาสตร์ เธนุเวชวิทยาและวิทยาการสืบพันธุ์
สาขาวิชา วิทยาการสืบพันธุ์สัตว์
ปีการศึกษา 2548

ลายมือชื่อนิสิต...เอกชาติ พรหมดิเรก
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KEY WORD: FOLLICULAR DYNAMICS/ OPU/ IVF/ SWAMP BUFFALO

AKACHART PROMDIREG :(FOLLICULAR DYNAMICS, OOCYTE PICK UP – *IN VITRO FERTILIZATION* IN SWAMP BUFFALOES (*Bubalus bubalis*). THESIS ADVISOR : PROF. MONGKOL TECHAKUMPHU, Ph.D., THESIS ADVISOR : ASSOC. PROF. SUDSON SIRIVAIDYAPONG, Ph.D. 72 pp.

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The objectives of the studies were to elucidate ovarian follicular dynamics in swamp buffalo cows (*Bubalus bubalis*) following an estrous synchronization protocol (EXP 1), to evaluate the efficiency of Ovum Pick Up (OPU) in cycling and lactating postpartum swamp buffaloes with and without gonadotropin stimulation (EXP 2) and to develop the embryo production from oocytes retrieved by ovum pick up (EXP 3).

Exp. 1. A total of 9 cyclic buffalo cows, received a progesterone ear implant for 10 days; and a single PGF2α at the day of ear implant removal. Daily ultrasound monitoring and blood collection were performed to determine serum concentration progesterone starting one day after implant removal. Data analysis was carried out for the first 5 days since the ear implant removal and at least two consecutive cycles in each buffalo with estrous sign. This study was performed during hot season (March to June 2004) and cool season (November 2004 to February 2005). The results showed that the estrous cycles of swamp buffalo presented the characteristic pattern of follicular growth waves. From 22 estrous cycles, 5(22.7%) presented one follicular wave; 17(77.3%) presented two follicular waves. The characteristics of the follicular dynamics in the swamp buffaloes; one follicular wave, the wave emerged on day 2.3 ± 0.5 and 1.8 ± 0.4 of the cycle in hot and cool respectively. The dominant follicle reached its maximum diameter on day 13.5 ± 1.2 versus 12.6 ± 1.5 with the maximum diameter was 14.5 ± 2.1 versus 16.4 ± 2.7 mm. in hot and cool seasons, respectively. No difference of follicular development between hot and cool season.

EXP. 2. Cycling (n=5) and lactating postpartum (n=6) cows received hormonal stimulation were given a total of 400 mg. follicle stimulating hormone (FSH), together with 100 μg of GnRH, 24 h after the last FSH injection. Following a resting period of 1 month, the two groups of buffaloes, were subjected to the same OPU regimen, but without any hormonal treatment for an additional six OPU sessions. The number of aspirated follicles recorded from the hormonal stimulated, cycling animal and lactating, postpartum buffaloes was not significantly different, 7.2 ± 3.7 and 9.0 ± 3.2, respectively ($P > 0.05$). Recovered oocytes collected from the two groups of hormonally stimulated animals were also not statistically different: 3.7 ± 2.7 in the cycling and 5.9 ± 3.5 in the lactating postpartum group ($P > 0.05$). In the two groups of buffaloes not receiving hormonal stimulation, the number of aspirated follicles was not significantly different: 2.1 ± 1.4 and 1.4 ± 0.7 in cycling and lactating postpartum buffaloes respectively ($p > 0.05$). Recovered oocytes in the non-treated groups were also similar: 1.4 ± 1.3 vs 0.7 ± 0.8 in cycling and lactating buffaloes ($p > 0.05$). OPU can be performed successfully in swamp buffalo in different reproductive status and FSH administration was shown to increase the number of aspirated oocytes in both cycling and lactating, postpartum buffaloes.

EXP 3. The OPU was performed in 5 buffalo cows, administered with 400 mg (NIH unit) of follicle stimulating hormone. The recovered oocytes were washed in TCM 199 2.5 Hepes 2 times and then incubated in TCM 199 $NaHCO_3$+10% fetal calf serum for 24 h at 38.5°C, in 5% CO_2 in air. fertilization and culture with B2 (Menezo) +2.5% fetal calf serum on vero cells for 7 days and observation the developmental of embryo. The COC and single layer cumulus oocytes were submmited for IVF. The results showed that the maturation rate were 62.8% and the cleavage rate of 40.5% (34/84), but only 1 oocyte(1.2%) was developed until blastocyst. In conclusion, it is possible to produce embryos from OPU in swamp buffalo, however the morula and blastocyst rates were poor due to the low percentage of good quality oocytes.

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Academic year: 2005

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LIST OF ABBREVIATION

bST	bovine somatotropin
CL	corpus luteum, corpora lutea
COC	cumulus-oocyte complexes
d	day
D	denuded oocyte
Deg	degenerated oocyte
Exp	expanded cumulus oocyte
FCS	fetal calf serum
FSH	follicle stimulating hormone
FZ	free zona without cytoplasm
Hepes	N-(2-hydroxyethyl)-1-piperazine-N'-(2-ethanesulfonic acid)
g	gram
GnRH	Gonadotropin releasing hormone
hrs	hours
IGF-I	insulin like growth- I
i.m.	intramuscular
IVEP	*in vitro* embryo production
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
LH	luteinizing hormone
M199	Medium 199
MII	metaphase of meiosis II
ml	milliliter
ML	multi-layer oocyte
mm	millimeter
OPU	ovum pick up
P4	progesterone
PBS	phosphate buffer saline
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$

SEM	standard error of the mean
SL	single layer oocyte
yrs	year

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

The swamp buffalo has traditionally been used for draft purposes in Asian agriculture and forestry since time immemorial. It has survived to date because it adapted so well to its humid tropical environment. In earlier times, buffaloes multiplied to abundance, naturally and without assistance. The farmer could choose freely from amongst them and, in those days, there was no special need for their reproductive improvement. However, after the Second World War, the socio-economic situation changed rapidly due to the human population increase and to the food and energy crisis. The buffalo, principally used as a beast of burden, has become more and more important as a food animal. The natural reproductive rate of the buffalo has become progressively less competitive with the increasing need. In countries of south-east Asia, it is clearly evident that the swamp buffalo has declined alarmingly both in size and number during the last few decades due to illegal as well as legal slaughtering, exporting, replacement by mechanization and by lack of planning for reproductive improvement (Bodhipaksha, 1976)

It is only during the last few decades that scientists have been studying reproductive physiology and pathology in the swamp buffalo. The interest in swamp buffaloes has been growing worldwide including Europe and USA. The progressive advancement in scientific research in swamp buffalo reproduction seems promising in relation to quick improvement of the breed. The ultimate success in reproductive improvement of the animal depends very much on regional and international cooperation and coordination of all concerned, especially by those working in the field. Exchange of knowledge among scientists is essential to success.

In buffalo, it has been accepted that the low reproductive efficiency of swamp buffalo is generally considered as a major problem. The low fertility rate about 35-40% and late maturity in male and female (Na-Chiengmai, 2002). The general data of buffalo raised in seven buffalo research stations in Thailand, reviewed the average age of first

calving was 4.19 yrs varying from 3.58 to 4.91 yrs and the average calving interval of 573.3 days, varying from 489.7 to 700 days (Na-Chiengmai, 2002). It means that the long days open is one of the factors involving the low reproductive efficiency in this species.

Since the discovery of ultrasound technology, it was applied into animal reproduction. An amount of informations has been collected especially on ovarian follicular development observation in domestic animals. The pattern of follicle changes during estrus cycle called “follicular wave” representing by the recruitment of small follicles, the selection of the dominant follicles and the regression of other follicles in each cycle was observed. A silent heat with a high variation of estrous period was found in the species. Danell (1987) and Singh *et al*,. (2000) indicated that the number of primordial follicle pool in ovaries are ten folds less than in those of cattle. The study of follicular dynamics in swamp buffalo is not yet explored, this will help to have a more understanding of estrus cycle, to improve the estrus induction and superovulation in the species.

The OPU-IVF technique is a tool to produce embryos for transferring into recipients, which can be accelerate the genetic drift and increase the reproductive efficiency of females. This technique was first reported in human for IVF-ET program and later in animals. In cattle OPU-IVF-ET is widely used to produce calves in known genetical donor. For buffalo basing on the previous experience, it was shown that OPU has been applied also repeatedly in prepubertal buffalo calves and this has significant implications for reducing generation intervals and enhancing rates of genetic gain. Moreover, if OPU was applied in buffalo during non productive period such as during non-pregnancy and postpartum period, it will be maximally utilize the female during these two periods.

Repeated use of the ovum pick up technique yields a large quantity of meiotically competent oocytes from individual bovine donors (Pieterse *et al.,*1988; Bungartz *et al.,* 1995). These oocytes are suitable for *in vitro* embryo production programs that supply transferable embryos and live calves (Kruip *et al*., 1991; Kruip *et al.,*1994; Looney *et al*.,1994). The advent of ovum pick up has boosted *in vitro* embryo production programs. In fact, early *in vitro* embryo production technologies using slaughterhouse ovaries as oocytes donors have had very little impact on genetic improvement programs

because the ovaries of slaughtered animals supply very few oocytes and, hence, the number of transferable embryos yielded is low. Repeated ovum pick up represents an opportunity to apply *in vitro* embryo production technologies to animal breeding. The combination of ovum pick up and *in vitro* embryo production has a higher potential than superovulation for embryo production in cattle (Kruip *et al*.,1994). Ovum pick up and *in vitro* embryo production supplies more transferable embryos than superovulation. Given the rapidly increasing efficiency of *in vitro* embryo production and the lack of improvement in the superovulation technique, the ovum pick up and *in vitro* embryo production procedure may become the technique of choice for embryo production in cattle.

In the buffalo species, embryo production with *in vivo* and *in vitro* techniques is not very efficient. There is a low response to superovulation, and the number of collected embryos per donor is low. The response to superovulation is affected by parameter such as season, number of days open, as well as dosage and dose schedule of FSH administration. The low number of embryos collected is related to the low number of follicles and to method of follicle recruitment. Reproductive seasonality results in an acyclic condition which rarely responds for hormone treatments. Because ovum pick up can be utilized regardless of the estrous cycle, embryos can be produced during any period of the year. In swamp buffalo, the reproductive technology has not been studied as extensively as those of cattle and river buffalo. The limiting factors are poor *in vivo* embryo production and a very low recovery rate of immature oocytes from slaughterhouse ovaries which make it difficult to apply the technology in this species. Ovum pick up has been used with success on swamp buffalo of different ages and physiological conditions (Pavasuthipaisit *et al*., 1995)

1.2 Literature review

1.2.1 Follicular dynamics during estrous cycle in swamp buffalo

Since the advent of ultrasound technology and applied to animal reproduction, a large amount of information has been collected about ovarian follicular dynamics and related hormonal profiles in domestic animals as well as other species. One of the basic

information from study called “follicular wave”, it meaned that each estrous cycle possessed the recruitment of small follicles and selecting the dominant follicles and later regress of other follicles. Many studies have been performed in prepuberal and peripuberal cattle. River buffalo both of buffalo calves and cows (Sirois and Fortune, 1988; Knopf *et al.,* 1989; Ginther *et al*. 1989; Lean *et al.,* 1992; Sunderland *et al.,* 1994) whereas a little studies in the buffalo specie have been made. Presicce *et al.,*(2003) studied in Mediterranean buffalo calves aged between 5-9 months, he found that buffalo calves had the follicular developmental pattern similar to buffalo and cattle cows. The estrous cycle composed of the recruitment, dominance and regression phases in every 9.9 $\pm$ 2.8 days. The estrous cycle of buffalo is 20-22 days with a duration of estrus period 41.62 hrs and the time of ovulation was 6-21 hrs after estrus with a silent estrus and a high individual variation of estrous cycle (Bodhipaksha, 1987). The number of primodial follicles was significant lower than those of cattle (Danell, 1987; Singh *et al*,. 2000). Baruselli *et al*. (1997) performed the studies of folllicular wave in 30 Murrah buffalo, it was found that Murrah buffalo had 1-3 wave per estrous cycle, predominantly of 2 waves(63.3%), 3 waves(33.3%) and 1 wave(3.3%). The number of wave has correlated to the luteal phase and the length of estrous cycle, however no report of follicular wave in swamp buffalo is noted. The studies of follicular wave will be an interesting topic and a basic knowledge for a development of reproductive technology.

1.2.2 Ovarian anatomy

In buffaloes, the ovaries are oval and smaller than in cattle, however, there is considerable changes in ovarian weight and dimensions during the different phases of estrus cycles. Maximum size and weight were observed when a fully developed corpus luteum presented on the ovary. The minimum and maximum average weights being 2.9 g and 6.1 g in buffalo and 3.9 g and 9.9 g in cattle (Tian and Zhao, 2000). Danell (1987) reported that the average weight of the left and right ovaries in 30 normally cycling buffaloes were 3.4 ± 1.3 g and 3.6 ± 1.5 g, whereas in non-cycling animals, the average weights were 2.5 ± 1.2 g and 2.5 ± 0.9 g, respectively. In Thailand, Lohachit *et al.* (1981) reported that the left and right ovaries were similar in size. The average length, width, thickness and weight were 2.12 cm, 1.38 cm, 0.97 cm and 2.24 g respectively for

the left ovary, and 2.19 cm, 1.43 cm, 1.1 cm and 2.28 g for the right ovary. The size and weight of corpus luteum were also similar for both the left and right ovaries.

1.2.3 Estrous behavior and pattern

The duration of estrous symptoms ranges from 11.9 to 28.5 hrs in buffaloes compared with 12.5 to 27.8 hrs in cows but the intensity of signs were lower in buffaloes (Kanai and Shizumi, 1983). Singh *et al*. (1984) suggested that vulva discharge of clear mucus, especially in the recumbent animal, was the most reliable single sign of estrus available to the buffalo stockman.

Estrus commences toward late evening with a peak sexual activity between 6.00 P.M. and 6.00 A.M. (Jainudeen and Hafez, 1992). Diurnal estrous behavior is common in buffalo and the majority breeds in the cooler hours of morning and evening, and sometimes during night (Pathak, 1992). On the other hand, more animals have been reported to be in heat at daylight than at night. However, this is still controversial and need more definite proof (Fischer and Bodhipaksha, 1992). Mating continues until late morning in the river buffaloes but usually ceases during daylight hours in the swamp buffaloes (Tailor *et al*., 1990). Barkawi *et al.* (1993) proved that copulation may took place at any time throughout the day. The main problem that found in the swamp buffaloes was silent heat and difficult to detect only by visual observation. According to the report of Kanai and Shizumi (1983) that the estrous characteristics can be affected by various intrinsic and extrinsic factors such as breeds, climatic conditions, level of feeding and other management practices. Furthermore, the accuracy of estrous detection is a major problem since the signs of estrous are not easily observable in buffaloes.

1.2.4 Estrous cycle

The studies of ovarian changes during estrous cycle in swamp buffalo was conducted for the first time in Thailand in 1974 by Bodhipaksha *et al.* (1978). Ten healthy, non-pregnant postpuberty females were selected at random from 700 animals. The examination per rectum was performed daily for 200 consecutive days for determining of ovarian changes. A blood sample was also collected everyday from the

jugular vein of each animal for radioimmunoassay of serum progesterone, 17-hydroxy progesterone and 17-beta estradiol. The ovarian changes were determined by rectal palpation showed an individual variation with irregular patterns, which corresponded to the findings by the corresponding hormonal radioimmunoassay. The estrous cycle was found to be 22.1 d while it was reported to be 21.3 d by Toelihere (1977), 20 days by Wetchrutpimon and Mongkonpunya (1978), 21.3 d by Jellinek and Avenell (1980) and 25 days by Kanai and Shimizu (1983).

The estrous period was stated to last 41.6 hrs by Toelihere (1977), 32 hrs by Wetchrutpimon and Mongkonpunya (1978) and 19 hrs by Kanai and Shimizu (1983). Ovulation occurred at 6-21 hrs (averagely 13.3 hrs) after the end of estrus (Kanai and Shimizu, 1983). Hafez (1992) concluded that, the duration of estrus and ovulation time are influenced by species, breed, climate, and management.

Buffalo is characterized by a marked seasonal pattern of breeding. The female is sexually active from March to June. However the duration of estrus in buffaloes during early summer is shorter and anoestrus can be found. Buffalo females come into estrus and conceive (calving percentage as high as 80%) under rigorous summer conditions, provided that the animals are well sluiced, allowed to wallow and provided with shade. Swamp buffaloes of Malaysia have estrous periods all year but more regularly in hot weather, April to July (Dobson and Kamonpatana, 1986).

1.2.5 Ovarian observation by ultrasonography

Ginther (1986) stated that “gray-scale diagnostic ultrasonography is the most profound technological advance in the field of large animal research and clinical reproduction since the introduction of transrectal palpation and radioimmunoassay of circulating hormones.” It is hard to imagine that many discoveries and procedures related to ovarian, uterine and fetal functions that we use today would have been considered without the development of real-time ultrasound. The research and commercial applications of ultrasound developed for reproduction over the last 15 years would support the statement by Ginther (1986).

Ovarian stroma, ovarian vessels, follicles, cysts, corpolar haemorrhagica (CH), and corpora lutea (CL) are all structures that can be identified by real-time

ultrasonography. The most distinguishable ovarian structures are antral follicles because the follicles are fluid-filled structures, they absorb ultrasound waves and are displayed as black on the screen (i.e., anechoic or non-echoic). In contrast, the ovarian stroma, CH, and CL all contain varying degrees of dense cells, which reflected the ultrasound waves and resulted in a gray image on the screen.

Routine reproductive examinations should include visualization of the major structures on both ovaries. Although rectal palpation can be an accurate method for diagnosing pregnancy, it can not be used to count small growing ovarian follicles (3-8 mm in diameter). By contrast, ultrasonic imaging is a highly accurate and rapid method for assessing all ovarian structures. The ovaries contain a wealth of information that can be used to help in diagnosing the reproductive status of the cow, and for selecting appropriate therapies or reproductive interventions.

Manual palpation or ultrasonographic examination of the cow's genital tract are currently used by veterinarians involved in reproductive management. Ultrasound is effective at identifying small follicles (< 10 mm in diameter) than rectal palpation technique. Follicles with 10-15 mm in diameter can be detected in 90% of cases using ultrasonography versus 62% by using rectal palpation. Nevertheless, both ultrasonography and rectal palpation can be used to detect follicles with $\geq$ 15 mm in diameter.

The corpus luteum (CL) is a transient endocrine gland that forms after ovulation. Thus, CL can be regarded as a terminal stage of follicular development. CL appears as distinctly echogenic areas within the ovarian stroma. Corpora lutea appear as a solid tissue masses but may also contain fluid-filled cavities. Basing on ultrasonographic examinations in dairy heifers, 79% of otherwise normal CL contains cavities ranging from less than 2 to greater than 10 mm in diameter at some time during the estrous cycle and early pregnancy (Kastelic *et al.,* 1990). Spell *et al.* (2001) determined that luteal diameter was not associated with concentrations of progesterone on d 7 of estrous cycle, but area and volume were correlated to concentrations of progesterone.

1.2.6 Follicular growth

In a number of species, follicle growth is characterized by a follicle wave pattern; with two or three waves occurring during the normal course of estrous cycles in cattle (Evans, 2003) and the average length of the 2- waves estrous cycle was significantly shorter than that of the 3-waves estrous cycle. A follicle wave is characterized by a synchronous emergence of a group or cohort of follicles; as the wave progresses one of the members of the cohort is selected to become the dominant follicle whilst the remainders of the group undergo atresia (subordinate follicles). The day of follicle emergence is considered the first day of the follicular wave when a growing cohort of follicles can be firstly detected using ultrasonography at about 5 mm in diameter. This is also often described as follicle recruitment. Selection is the process that results in the decrease in the number of growing follicles to the species specific. The number of follicles that ovulate, usually one in cattle, and ends when the dominant follicle has been selected from the subordinate follicles as seen by a difference in follicle size (Evans, 2003). This divergence in growth rate is referred to as deviation (Ginther *et al.,* 2003) and is coincident with decreasing plasma FSH concentrations and the acquisition of luteinising hormone(LH) responsiveness by the dominant follicle (Mihm and Bleach, 2003). Whilst all dominant follicles are capable of ovulating, their ability to ovulate is dictated by hormonal environment. Progesterone prevents the ovulation of dominant follicles that matured during the luteal phase through its negative feedback on the regulation of LH. Dominant follicle that develops under this condition regresses like subordinate follicles. Once progesterone concentration decreases, the follicular phase ensures and LH pulsatility increases and under the influence of oestradiol, a gonadotrophin surge leads to the ovulation of the dominant follicle.

In cattle, both 2- and 3- wave estrous cycles, emergence of the first follicular wave occurs on the day of ovulation (d0), while the second wave emerges on Day 9 or 10 in 2- wave cycles, and on d 8 or 9 in 3- wave cycles, with a third wave emerging on d 15 or 16. Duration of the estrous cycle is approximately 20 days in 2- wave cycles and 23 days in 3- wave cycles. The dominant follicle present at the time of luteolysis becomes the ovulatory follicle and emergence of the next wave is delayed until the ensuing ovulation. The proportion of animals with 2- versus 3- wave cycles is probably more or

less equally distributed; fertility is not affected by the number of follicular waves per cycle (Martinez *et al.,*2001). It has been shown that cattle fed a low energy ration had a greater proportion of 3- wave cycles than those fed higher energy ration (Murphy *et al.,* 1991). Follicular waves have also been reported in heifers before puberty (Evans *et al.,* 1994) and in postpartum cow before the first ovulation (Savio *et al.,* 1990).

In riverine buffalo, like in cattle, during each follicular wave, one follicle is selected, becomes larger and dominates the other follicles. After the dominance phase, this follicle either ovulates or become atretic, depending on whether the phase of dominance is associated with luteolysis or not (Taneja *et al.,* 1996). Litteratures on physiological aspects of the follicular growth and regression in buffaloes remain sparse (Dobson and Kamonpatana, 1986; Baruselli *et al*., 1997 Singh *et al.,* 2000; Manik *et al.,* 2002) and no report in swamp buffalo.

1.2.7 Follicular population in buffaloes

In water buffaloes, as with other livestock species, the ovarian follicular population has been a subject of interest for some years. This is because the ovarian follicular pool determines how many follicles are available as potential sources of oocytes using in assisted reproductive biotechnologies as ovum pick up and *in vitro* fertilization. In swamp buffaloes, Smith (1990) reported the number of ovarian follicles of various sizes in animals with different ages. Ovaries obtained from 2-year old swamp buffaloes indicated a relatively high rate of transformation from the primordial follicles to growing follicles and up to tertiary follicles. In 7-8 year-old buffaloes, this massive transformation was not noticeable, and an average primordial follicular population was high as 5,997. Among riverine buffalo heifers, the number of primordial follicles per ovary ranges from 6,000 (Danell, 1987) to 19,000 (Samad and Nasseri, 1979).

The number of secondary follicles in the pubertal buffaloes indicates that transition of the growing follicles to secondary follicular stage was at a slower rate. The number of secondary follicles was only 7.56% of the growing follicles. The average numbers of secondary follicles in the adult and old animals were 14 and 8, respectively, and were 77.7% and 47.0%, respectively, of the number of growing follicles counted.

A decline in the number of tertiary follicles was decreased with age. The number of the tertiary follicles present in pubertal buffaloes was 62.7. This was significantly greater than those in adult and old buffaloes, at 9.0 and 6.7, respectively.

The transformation of primordial follicles to the growing stage and finally to the tertiary stage appears to be very inefficient. This inefficiency can also be seen in the relatively high level of atretic follicles measured as a percentage of the total follicles $\geq$ 1.0 mm diameter. This pattern was also observed among riverine buffaloes (Danell, 1987), and is higher than the levels of atretic follicles reported in cattle (Settergren, 1994)

1.2.8 Hormonal levels during estrous cycle

In cycling Murrah buffaloes, plasma progesterone concentration reaches its peak at d13 to d15 of the cycle (Bachlus *et al.,* 1979; Arora and Pandey, 1982; Thankkar *et al*., 1983; Palta *et al.,* 1996). However, Kumud (1999) showed that plasma progesterone concentration culminated at d 9 of the estrous cycle in Murrah buffaloes (Kumud, 1999). The concentration in the peripheral blood progesterone of Swamp and Murrah buffaloes is low at estrus (d 0: 0.13-0.27 ng/ml) and does not rise until after d5. The values increase until d14-16 with a lower mean value of 1.51-2.6 ng/ml in swamp buffalo compared to value of 4.0-4.26 ng/ml in Murrah buffaloes.

The concentration of progesterone in milk was related to that in plasma. In Murrah buffaloes, whole milk P4 values at estrus ranges from 0.5 to 4.17 ng/ml and luteal phase P4 values range from 18.0 to 24.75 ng/ml, where in estrous cows whole milk P4 values range from 0.1 to 4 ng/ml, with luteal phaseP4 values of 10-60 ng/ml (Dobson and Kamonpatana, 1986).

Kanai and Shimizu (1983) found the secretory patterns of luteinizing hormone (LH), progesterone and estradiol during the estrous cycle in the swamp buffalo basically similar to those in cattle except that the actual concentration of progesterone during the luteal phase was low in the buffalo. Plasma progesterone concentration decreased rapidly during 4 days prior to estrus, followed by a sustained increase in estradiol and LH concentrations. Preovulatory surge of the LH was initiated in association with the onset of the behavioral estrus and lasted for about 12 hrs. Ovulation took place approximately 30 hrs after the peak. Alejardrino *et al.* (1981) found that the serum

progesterone values in ten swamp buffalo heifers, also measured by radioimmunoassay, to be below 0.5 ng/ml during estrus and 4 ng/ml as the highest in the cycle. Chua *et al.* (1983) studied in normally cycling and anestrous animals, found that the plasma progesterone level of 0.24 ng/ml which gradually increased and reached the peak of 1.76 ng/ml on d12-14 in the luteal phase of the cycle and declined to the lowest level again at the next estrus. Two of the anestrous buffaloes were found to have low progesterone level of 0.16 ng/ml throughout the period. Jainudeen *et al.* (1981), in their study of plasma profiles of progesterone in relation to postpartum ovarian activities in the swamp buffalo, found that during estrus (d0), plasma P4 levels was low (0.24 ng/ml). It rose to the level of 0.42 ng/ml by d6 and reached the peak of 1.51 ng/ml on d15. Then it declined to the basal level of 0.20 ng/ml on d22. Jellinek and Avenell (1980) stated that the progesterone profiles in the natural estrous cycle of the swamp buffalo in Indonesia rose to detectable levels on d4 and reached the peak of 2.80 ng/ml by d12-14. After d19 of the cycle, the progesterone values declined to less than 0.10 ng/ml.

In buffalo, there are lower plasma progesterone values and a lower LH response to gonadotrophin-releasing hormone (GnRH) injections in the hotter months (Dobson and Kamonpatana, 1986). A heat stress influence on peripheral plasma progesterone may be mediated by the level of nutrition. Kaur and Arora (1984) found lower progesterone values and an onset of ovarian inactivity during hotter months in buffalo fed sub-optimally, compared with similar animals receiving adequate nutrients. Further efforts appear necessary to understand the mechanisms by which heat stress reduces reproductive efficiency in buffaloes.

1.2.9 Factors affecting ovarian function

The number of ovarian follicles were affected by the reproductive status, stage of estrous cycle, and the presence of a CL (Ahmed and Omaima, 2001). Montgomery *et al.* (1985) found that season of calving influenced a resumption of ovarian cycles even at a constant high plane of nutrition and that season of calving interacts with nutrition such that effects of season are more likely to be expressed under conditions of low nutrition. From this study according to the report of Villa-Godoy *et al*. (1990) and Lucy *et al.* (1992) suggested that fat body condition, coincident with negative energy balance, may reduce

an accuracy of artificial insemination timing relative to ovulation and may consequently reduce fertility in cattle.

Pancarci (1999) monitored and compared follicular and luteal function between genetically high- and low producing dairy cattle cows by ultrasonography. From this study found that there was a positive correlation between products of lengths and widths, diameters and averages of lengths and widths of CL, and mean plasma P4 concentrations. Higher-producing dairy cows have lower reproductive efficiency than lower-producing dairy cows (McClary, 1991). This suboptimal reproductive performance is thought to be caused by a negative energy balance due to superior genetics or bST treatment. In this regard, a slight retardation of cyclic ovarian activity and decreased first service conception rates contribute to increased days open in the higher-producing dairy cows.

Wettemann and Bossis (1999) reported that nutrient intake and body energy reserves are major regulators of reproductive performance of beef cows. Reduced body weight causes cessation of estrous cycles, and inadequate body energy stores at parturition prolong the postpartum anestrous interval. Nutritionally induced reduction in follicular growth is a result of decreased secretion of GnRH and LH. During anestrous, ovarian follicular waves are recurrent, but inadequate estradiol is secreted by the dominant follicle to cause estrous and ovulation. Realimentation of nutritionally induced anovulatory cows results in larger dominant follicles and ovulation were occurs when body energy stores are adequate. Increased follicular growth rate is associated with increased concentration of LH, astradiol, and IGF-I in plasma. When nutritional induced anovulatory cows are realiment, ovulation and a corpus luteum with shorter than normal function occurs, usually without estrous, before the first normal cycle. In addition, exogenous GnRH infusion induces ovulation in nutritionally induced anestrous cows. From this studied conclude that prolonged restriction of nutrient intake reduces secretion of LH and IGF-I, less estradiol is produced by the dominant follicle, and ovulation ceases. In another study (Dominguez, 1995), showed that small and large follicles were affected by body condition score, and cows with poor body condition showed fewer follicles. And he found also that reproductive status did not affect the number of small follicles, but it did show a significant effect on the number of medium and large follicles.

Cows in the last trimester of gestation exhibited fewer medium follicles than non-pregnant cows or cows in the second trimester. The population of large follicles showed a clearer effect of pregnancy, and cows in the second and third trimesters of gestation exhibited fewer large follicles than cyclic or early pregnant cows.

Heat stress alters the follicular development pattern in cattle. Exposure of cows to heat stress led to a reduction in the size of the dominant follicles of the first and second follicular wave of the estrous cycle. Depression of follicular dominance by heat stress was indicated by the absence of a decrease in medium sized follicles during the first follicular wave or during the follicular phase of the estrous cycle, a large size and a slow decrease in the size of the second largest follicle; an in crease in the number of large follicles during the first follicular wave; and an early emergence of the preovulatory follicle (Wilson *et al*., 1998). According to report of Roth *et al*. (2000) found that during the follicular phase (d17-20 of the treated cycle), heat stress cows showed an increase in the number of large follicles ($\geq$ 10 mm), and the preovulatory plasma FSH surge was significantly higher in the heat stress cows than in cooled cows. The effect of heat stress was also observed during the first follicular wave of the subsequent cycle: the postovulatory plasma FSH concentration was higher, but fewer medium follicles developed, and first follicular wave decreased at a slower rate in previously heat stress cows than in cooled cows. This study showed both of immediate and delayed effects of heat stress on follicular dynamics, which were associated with high FSH and low inhibin concentrations in plasma. These alterations may have physiological significance that could be associated with low fertility of cattle during the summer and autumn. The mechanism by which heat stress reduces the intensity of estrous signs may be hormonal change such as the decrease in circulating estradiol concentration. Some recent studies indicated that plasma estradiol concentration was reduced during summer by heat stress. Wilson *et al*. (1998) suggested that heat stress inhibited follicular growth during the preovulatory period, and abnormal ovarian function in heat stressed cows was manifested as a decrease in the proestrous period of estradiol. The decrease in estradiol secretion from the dominant follicle may cause poor expression of estrus (Wolfenson *et al*., 1997). Whereas the reported of Walker *et al*. (1996) reported that season did not alter estrous behavior, and the increase in temperature did not influence the duration and

intensity of estrous in dairy cows. This was an evidence supported by White *et al.* (2002) found that size of the ovulatory follicle was not influenced by season and season did not influence time of ovulation, somehow beef cows were mounted more frequently during estrus in winter compared to in summer. Seasonal effects on estrous behavior may differ among studies due to breed of cows, climatologically variations, or managed mental factors such as frequency of milking, feeding, movement of cows, etc.

In buffaloes, other factors are responsible for the resumption of cyclicity, like the light-darkness shift over the months together with cold or hot climatic conditions (Zicarelli, 1997). Reproductive efficiency and postpartum resumption of ovarian activity and estrous cycle in buffaloes are conditioned by season and reproductive maturity of the animals (Esposito *et al*., 1992 ; Campo *et al*., 2002) .

1.2.10 Ovum Pick Up and *in vitro f*ertilization

In the early 1990s, the introduction of ovum pick up followed by *in vitro* embryo production (OPU-IVP) opened up even greater possibilities. Using these technologies, we can challenge biological mechanisms in reproduction. Where normally only one oocyte per estrous cycle will develop to ovulation, now numerous oocytes that otherwise would have degenerated are expected to develop into an embryo. Completion of oocyte growth and re-maturation *in vivo* before a final maturation both appear to be essential phases in order to obtain competence to develop into an embryo and finally a healthy offspring (Merton *et al*., 2003).

In vitro fertilization (IVF) is the technique that keeps the excess oocytes for embryo production. The oocyte can be recovered from ovary of slaughterhouse or the new technique by aspirated pass the vaginal wall called " ovum pick up" (Kriup *et al*,. 1994). *In vitro* fertilization is the tool for produced embryo for research or embryo transfer for produced calves. The processes of IVF are an oocyte collection from ovary and brings them it to *in vitro* maturation (IVM) then fertilized with capacitated sperm in the tube. Embryos from IVF will be cultured in a suitable condition in petridish for development to morula or blastocyst and later transferred into recipient. The IVF technique was world wide used in cattle.

OPU technique was previously in cattle by Peterse *et al.* (1988) and then applied to many species including riverine buffalo (Boni, 1994; Boni *et al*,. 1996; Neglia *et al*,. 2003). In swamp buffalo, there were a few reports (Kitiyanant *et al*.,1995 ; Pavasuthipaisit *et al*,. 1995; Promdireg *et al.,* 2000; Promdireg *et al.,* 2004; Promdireg *et al.,* 2005). In swamp buffalo, it was found that oocytes can be collected from prepubertal buffalo calves, buffalo heifers and cows. From these studies 5-6 oocytes can collected per animal with 40% of good quality after stimulated with 280-400mg of Follicle Stimulating Hormone(FSH) (Promdireg *et al*., 2000). The results were similar to that of cattle *et*(Looney *et al*,. 1994; Bungart *et al*,. 1995) and river buffalo (Boni *et al*,. 1996). The study to ability of *in vitro* fertilization is the one way for produce embryo and the next step of ovum pick up research.

1.2.11 Factors affecting oocyte quality and quantity

In the case of OPU-IVEP, the frequency of OPU clearly affects quantity and quality of the collected oocytes and FSH stimulation prior to OPU every 2 weeks resulted in 3.3 embryos per session. Analysis of 7,800 OPU sessions demonstrated that the oocyte yield is dependent on the operators, in particular, the technicians who manipulate the ovaries. It can be concluded that an increased understanding of the process of oocyte growth, pre- and final maturation will help to improve the efficiency of embryo technologies (Merton *et al*, 2003).

There are many factors affects the quantity and quality of oocytes collected by OPU such as twisting and type of aspiration needle, frequency of OPU, operators etc. Sasamoto *et al* (2003) studied about effects of twisting and type (single- and double-lumen) of aspiration needle on the efficiency of transvaginal ultrasound-guided ovum pick up. The first study using slaughterhouse ovaries revealed that twisting of the needle during follicle aspiration improved the oocyte recovery rate without deleterious effects on the attachment of cumulus layers. Vacuum pressure affected the oocyte recovery and cumulus attachment, regardless of the needle type. The needle type did not affect the oocyte recovery or cumulus attachment with an optimized vacuum pressure. In the second study, OPU was performed in live cows using two types of needles with a vacuum pressure of 75 mmHg. The needle type did not affect the ovary or cumulus

attachment of the recovered oocytes. The results revealed that twisting of the needle is effective in follicle aspiration, and suggested that a single-lumen needle is as useful as a double-lumen needle for OPU in cattle. The frequency of oocyte collection is another factor concern OPU. Petyim *et al*. (2003) compared two different schemes of twice-weekly ovum pick up, continuous and discontinuous, with special emphasis on differences in oocyte yield and quality, estrous cyclicity, ovarian dynamics, and progesterone production. The mean number of punctured follicles and recovered oocytes per session was slightly higher using the discontinuous scheme, but in total, similar number of oocytes were obtained. The quality of the oocytes as well as cleavage rate after *in vitro* fertilization of the oocytes did not differ between the two OPU schemes. There was no influence of a corpus luteum presented on the ovary at the time of puncture on the oocyte yield and quality, whereas the presence of dominant follicles appeared to decrease the number of recovered oocytes. In the discontinuous scheme, the heifers showed regular and normal cyclic activity throughout the puncture period, with one to two complete follicular waves during the interval from the last OPU to the next estrus. In the continuous scheme, the heifers occasionally revealed cyclicities with irregular interestrous intervals and weaker signs of estrous. No complete follicular waves were seen during the OPU period in this scheme. The CL developed from the ovulation of the preovulatory follicles in the discontinuous scheme showed similar characteristics to the CLs of the pre-OPU period; however, the CL-like structures from the puncture of follicles, in both the discontinuous and the continuous schemes, revealed a shorter life span and inferior competence in producing progesterone. The studied indicated that the discontinuous OPU sheme, which allows animals to go into natural ovulation prior to the first OPU, does not affect their ovulation function, whereas the continuous OPU scheme does.

1.3 Rationale and objectives

1.3.1 Objectives

1. To study the follicular development and hormonal profile during estrous cycle on swamp buffalo cows.
2. To evaluate the yield and quality of oocytes recovered from buffaloes in different reproductive status, cycling and lactating, postpartum after hormonal stimulation and without hormonal stimulation
3. To study the *in vitro* embryo production from oocyte, retrieved by ovum pick up

1.3.2 Hypothesis

1. The follicle development in swamp buffalo presents in wave pattern
2. The buffalo cows that treated with FSH have the ovarian responses and recovered oocytes more than non-treated buffalo cows
3. No difference of the yield and quality of oocytes that recovered from different status, postpartum and non-pregnant
4. The embryos can be produced by in vitro fertilization of oocytes recovered by ovum pick up technique

1.3.3 Research merit

1. The basic knowledge on follicular dynamics will be useful to control estrous cycle in swamp buffalo.
2. The oocyte collection in postpartum and/or non-pregnant swamp buffalo animal is to maximize the reproductive potential of buffalo cows when combined with *in vitro* fertilization.
3. The recovered oocytes by OPU technique in buffalo cows and conjunction with IVF technique can produce embryos and reduce the problem for long calving interval.
4. The results from this experiment help to provide a suitable ovarian superstimulation program in the buffalo and to improve ovarian response and provide large number of good quality oocytes for *in vitro* embryo production.

CHAPTER II

FOLLICULAR DYNAMICS DURING ESTRUS CYCLE IN SWAMP BUFFALO

2.1 Abstract

The objective was to elucidate ovarian follicular dynamics in swamp buffalo cows (*Bubalus bulalis*) following an estrous synchronization protocol. Nine buffalo cows, received a progesterone ear implant for 10 days; and a single PGF2α at the day of ear implant removal. Daily ultrasound monitoring and blood collection were performed to determine serum concentration progesterone starting one day after implant removal. Data analysis was carried out for the first 5 days since the ear implant removal and at least two consecutive cycles in each buffalo with estrous sign. This study was performed during hot season (March to June 2004) and cool season (November 2004 to February 2005), The results showed that the estrous cycles of swamp buffalo presented the characteristic pattern of follicular growth waves. From 22 estrous cycles, 5(22.7%) presented one follicular wave; 17(77.3%) presented two follicular waves. The characteristics of the follicular dynamics in the swamp buffaloes; one follicular wave, the wave emerged on day 2.3 ± 0.5 and 1.8 ± 0.4 of the cycle in hot and cool respectively. The dominant follicle reached its maximum diameter on day 13.5 ± 1.2 versus 12.6 ± 1.5 with the maximum diameter was 14.5 ± 2.1 versus 16.4 ± 2.7 mm. in hot and cool season, respectively. Two follicular waves during the estrous cycle. In hot season, first wave emerged on day 1.2 ± 0.3 of the cycle. The second wave emerged on day 11.4 ± 0.8 and reaching its maximum diameter on day 19.7 ± 1.1 with the maximum diameter was 10.3 ±1.2 mm. In cool season, first wave emerged on day 0.9 ± 0.4 of the cycle and onset of atresia on day 12.4 ± 1.6. The second wave emerged on day 10.7 ± 0.9 and reaching its maximum diameter on day 18.9 ± 1.7 with the maximum diameter was 12.8 ± 1.2 mm. The levels of progesterone level observed on the day of estrous was 0.07 ± 0.03 ng/ml, which reaching a peak value of 1.7 ± 0.8 ng/ml on Day 15 of the estrous cycle.

2.2 Introduction

Since the discovery of ultrasound technology, it was applied into animal reproduction. An amount of informations has been collected especially on ovarian follicular development observation in domestic animals. The pattern of follicle changes during estrus cycle called " follicular wave" representing by the recruitment of small follicles, the selection of the dominant follicles and the regression of other follicles in each cycle was observed. These have been done in prepubertal heifers and pubertal cows (Sirois and Fortune, 1988; Knopf *et al,* 1989; Ginther *et al*,1989 ; Lean *et al,*1992 ; Sunderland *et al,* 1993) whereas there are only a few studies in buffalo. Most studies were performed in Riverine buffalo, both in calves and cows. Presicce *et al* (2003) studied in Mediterranean buffalo calves aged 5-9 months, they found that the buffalo calves possessed the follicular development in the same pattern as found in buffalo and cattle cows. Baruselli *et al* (1997) studied of folllicular wave in 30 Murrah buffalo from the report, it was found that Murrah buffalo possessed 1-3 waves per estrous cycle, composing 2 waves for 63.3%, 3 waves for 33.3% and 1 wave only 3.3%. The number of wave correlated to the luteal phase and the length of estrous cycle in buffalo. The recruitment of follicles occurred every 9.9 $\pm$ 2.8 days. In swamp buffalo, some information such as the estrous cycle was 20-22 days with estrous duration of 41.62 hours and the time of ovulation was 6-21 hrs after the end of estrus (Bodhipaksha, 1987). A silent heat with a high variation of estrous period was found in the species. Danell (1987) and Singh *et al*,. (2000) indicated that the number of primordial follicle pool in the ovaries are ten fold less than in those of cattle. In Thailand, the reproductive performance of swamp buffalo was different between summer and winter. The average of temperature in summer. The study of follicular dynamics in swamp buffalo is not yet explored, this will help to improve the estrus induction and superovulation in the species. The objective of the study was to observe the development of follicles in ovary and hormonal dynamics in estrous cycle on swamp buffalo cows.

2.3 Materials and Methods

2.3.1 Animals

Nine buffalo cows, aged between 3-10 yrs, weighing around 400-600 kg, were selected from the National Breeding Center, Department of Livestock Development and from the herd of Department of Obstetrics Gyneacology and Reproduction, Faculty of Veterinary science, Chulalongkorn university. They were housed at the veterinary student training center at Nakorn Pathom province and fed with 4 kg of concentrate, 14% of protein daily and roughage and water in ad libitum. This study was conducted during March to June 2004 (hot season with average outdoor temperature of 31.7 ^{o}C (range 26.0-35.1), THI = 84) and November 2004 to January 2005 (cool season with average outdoor temperature of 31.3 ^{o}C (range 22.7-33.9), THI= 81). In order to synchronize the estrus, each buffalo received an progesterone ear implant (Crestar®, Intervet, The Netherlands) for 10 days and one injection of 25 mg PGF2α injected at the day of implant removal. Ovulation was monitored within 5 days and identified when the dominant follicle disappeared. In case that no ovulation in 5 days after implant removal, a single injection of 2,000 iu hCG will be given.

2.3.2 Follicular development observation

The follicle development was daily observed since 24 hr after PGF2α injection by real time B-mode ultrasound equipped with 5 MHz transvaginal probe (Aloka, SSD-210 Tokyo, Japan). The ovarian observation was performed by the same person. Ovarian follicles with a diameter of $\geq$ 2 mm were measured by using the built-in electronic calipers after freezing the image on the screen. These follicles were classified into three classes, Class I (2-5 mm), Class II (6-9 mm) and Class III ($\geq$ 10 mm). They were then recorded on the follicular map. Non-spherical follicles were measured by averaging the largest and the widest diameters. Moreover, the size (length and width) and the number of corpora lutea were recorded. Heat detection performed at least twice daily (6.00, 18.00, 24.00) by epididydectomized bull. Follicular wave will be observed until at least 2 consecutive estrous cycles.

2.3.3 Blood collection for hormonal profile

Ten millimeters of blood were collected from a jugular vein of each buffalo once a day. They were stored into sterile plastic tubes without anticoagulant and later, centrifuged at 1500g for 10 min, then serum were kept in ependorf tube, stored at -20^{0} C until progesterone analysis.

2.3.4 Hormonal assay

Analysis level of hormone progesterone(P4) used RIA technique by progesterone test kit (Hegstag, 1992)

2.3.5 Statistical analysis

In this study, the day of maximum diameter for a follicle was defined as the day at which the maximum diameter was observed. When a follicle had the same diameter for more than 1 day the first day was taken as the day of maximum diameter. The maximum size was the largest diameter attained by a follicle. The persistence of a dominant follicle within the ovary was defined as the interval of time (days) elapsed between its appearance and disappearance as a follicle $\geq$4 mm. The growth rate (mm/d) of each dominant follicle was calculated by the maximum size divided by the number of days between its appearance as a follicle $\geq$ 4 mm and its maximum size. The following transformation of the data were made to normalize error distribution: 1) log for maximum size and persistence of follicle, and 2) square root for growth rate. Differences between duration of estrous cycle, maximum size of dominant follicles, persistence of follicle, growth rate of dominant follicles and mean number of follicles were tested using one-way analysis of variance. The level of hormone calculated are mean $\pm$ SD.

2.4 Results

Nine estrous cycles from 4 cows were observed during hot season while 13 cycles in cool season from 5 cows. The estrous cycle during both season presented in follicular growth waves pattern, with the initial development of a group of 3 to 5 mm

follicles, followed by the selection, development and atresia of a dominant follicle. It was found that during hot season, 77.8%(7/9) of estrous cycle were two-wave cycle and 22.2%(2/9) were one-wave cycle. The length of estrous cycle was 22.6 ±1.9 days in two-wave cycle and 25.5±3.5 days in one-wave cycle. In cool season, 76.9%(10/13) of estrous cycle were two-wave and 23.1%(3/13) were one- wave. The length of estrous cycle was 22.5 ±1.9 days in two-wave cycle and 20.7 ± 2.8 in one- wave cycle (Table 1 and 2) From these estrous cycles (n=22), five (22.7%) presented one follicular wave (Fig. 1); seventeen (77.3%) presented two follicular waves). Then, a higher prevalence of cycles were two follicular waves.

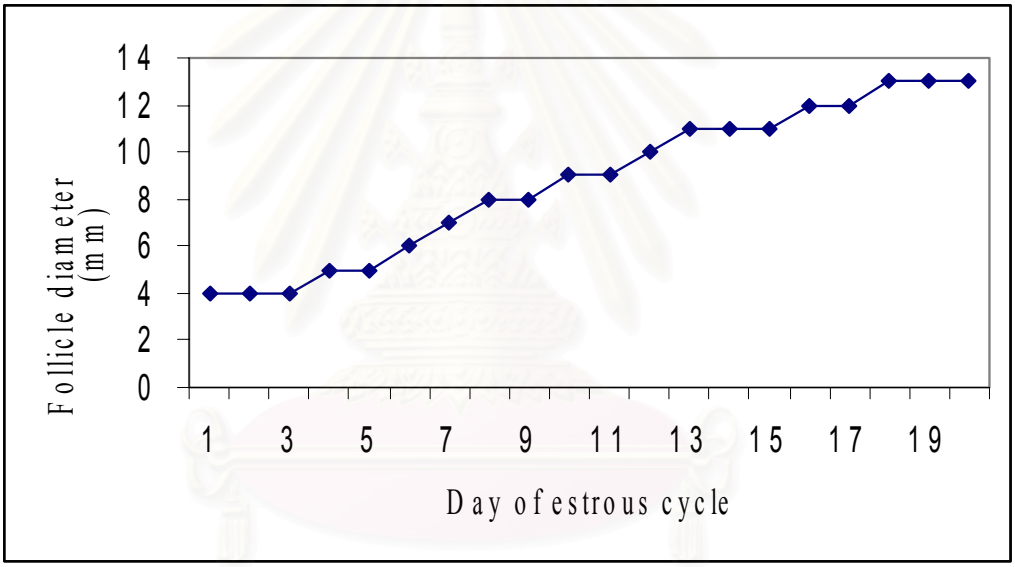

Fig. 1. Pattern of growth of dominant follicle from swamp buffalo cow with one follicular wave during estrous cycle (mean ± SD, N=5).

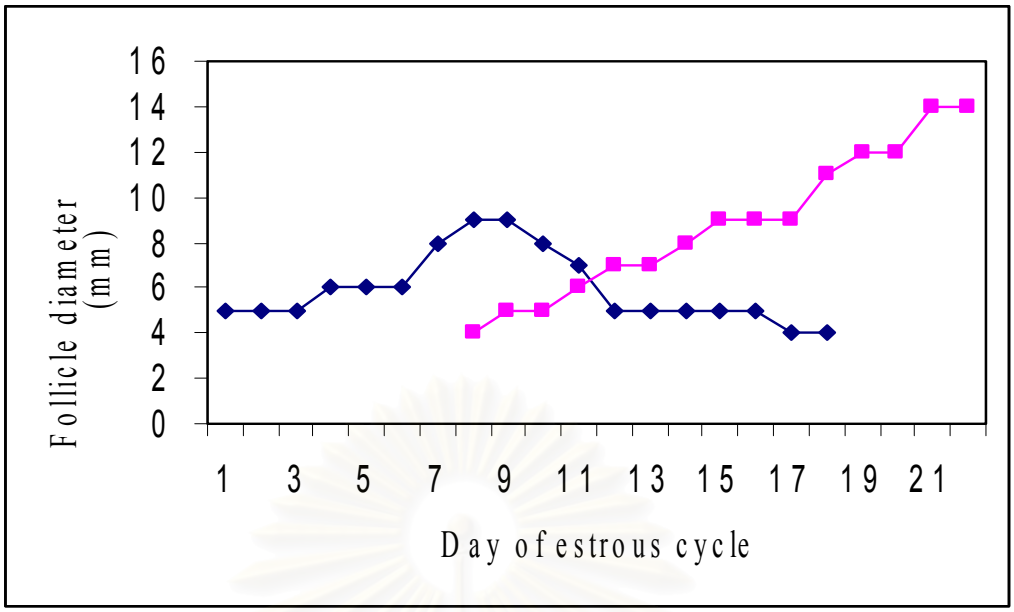

Fig. 2. Pattern of growth and atresia of dominant follicles from swamp buffalo cow with two follicular waves during estrus cycle (mean ± SD, N=17).

The characteristics of the follicular dynamics in the buffaloes with one follicular wave in hot and cool season are shown on Table 1 and 2, respectively. The wave emerged on day 2.3±0.5 and day 1.8 ± 0.4 of the cycle in hot and cool season, respectively. The ovulation time is assigned as day 0. The selection of the dominant follicle, characterized the divergence among the dominant and subordinate follicles growth rate, occurred on day 7.6±1.8 and day 8.2±1.7 in hot and cool season, respectively. The maximum diameter of the subordinated follicles were 8.7±1.7 vs 9.3±2.4 mm in hot and cool season, respectively and most of them become atresia. Only one dominant follicle reached its maximum diameter on day 13.5±1.2 versus day 12.6±1.5 in hot and cool season, respectively with the maximum diameter was 14.5±2.1versus 16.4±2.7 mm. in hot and cool season, respectively.

Table 3 and 4 shows the characteristics of follicular dynamics in buffaloes with two follicular waves (Fig.2) during the estrous cycle in hot and cool season, respectively. In hot season, the first wave emerged on day 1.2±0.3 of the cycle and was followed by selection of the dominant follicle on day 7.4±1.3, growed until day 9.8±0.9 and become atresia on day 11.3±0.8. The second wave emerged on day 11.4± 0.8, with the ovulatory

follicle being selected on day 15.8±2.4, and reaching its maximum diameter on day 19.7±1.1 with the maximum diameter of 10.3 ±1.2 mm. In cool season, the first wave emerged on day 0.9±0.4 of the cycle and was followed by selection of the dominant follicle on day 7.8±0.8, developed until day 10.6±1.3 and become atresia on day 12.4±1.6. The second wave emerged on day 10.7±0.9, with the ovulatory follicle on day 15.4±1.6, and reaching its maximum diameter on day 18.9±1.7 with the maximum diameter of 12.8 ±1.2 mm.

The dominant follicle found in the right ovary than the left ovary 68.7% vs 31.3% during the anovulatory cycle and 62.3% vs 37.7% ovulatory cycle, respectively. A higher incidence of ovulated dominant follicles in the right ovary of all the follicular waves was coherent with the high frequency of corpora lutea presented of 64.5%.

Table 1. The characteristics of follicular waves, growth and atresia of dominant follicles and of the largest subordinate follicles in buffalo cows with one follicular wave during estrous cycle in hot season (mean ± standard deviation)

Characteristics	Follicular wave
	One wave
No. of estrous cycle (%)	2/9(22.2%)
Wave onset (day)	2.3 ± 0.5
Wave length (days)	25.5 ± 3.5
Dominant follicle	
Maximum diameter (mm)	14.5 ± 2.1
Day of maximum diameter	13.5 ± 1.2
Growth rate (mm/day)	0.6 ± 0.3
Day of divergence	7.6 ± 1.8
Length of growth phase (days)	11.2 ± 0.8
Largest subordinate follicle	
Maximum diameter (mm)	8.7 ± 1.7
Growth rate (mm/day)	0.5 ± 0.1
Atresia rate (mm/day)	0.7 ± 0.2
Persistence (days)	5.2 ±1.9

Table 2. Characteristics of follicular waves, growth and atresia of dominant follicles and of the largest subordinate follicle in cows with one follicular wave during estrous cycle in cool season (mean ± standard deviation)

Characteristic	Follicular wave
	One wave
No. of estrous cycle (%)	3/13(23.1)
Wave onset (day)	1.8 ± 0.4
Wave length (days)	20.7 ± 2.8
Dominant follicle	
Maximum diameter (mm)	16.4 ± 2.7
Day of maximum diameter	12.6 ± 1.5
Growth rate (mm/day)	0.7 ± 0.2
Day of divergence	8.2 ± 1.7
Length of growth phase (days)	10.8 ± 0.9
Largest subordinate follicle	
Maximum diameter (mm)	9.3 ± 2.4
Growth rate (mm/day)	0.6 ± 0.1
Atresia rate (mm/day)	0.9 ± 0.3
Persistence (days)	4.8 ± 1.4

Table 3. Characteristics of follicular waves, growth and atresia of dominant follicles and of the largest subordinate follicle in cows with two follicular waves during estrous cycle in hot season (mean ± standard deviation)

Characteristics	Follicular wave	
	First wave	second wave
No. of estrous cycle (%)	-	7/9(78.8%)
Wave onset (day)	1.2 ± 0.3	11.4 ± 0.8
Wave length (days)	11.8 ± 2.3	10.6 ± 2.6
Dominant follicle		
Maximum diameter (mm)	10.9 ± 2.1	10.3 ±1.2
Day of maximum diameter	9.8 ± 0.9	19.7 ± 1.1
Growth rate (mm/day)	0.7 ± 0.2	0.7 ± 0.1
Day of divergence	7.4 ± 1.3	15.8 ± 2.4
Length of growth phase (days)	8.4 ± 0.7	8.2 ± 1.3
Onset of atresia (day)	11.3 ± 0.8	-
Atresia rate (mm/day)	0.9 ± 0.2	-
Length of atresia (days)	9.9 ± 1.4	-
Largest subordinate follicle		
Maximum diameter (mm)	7.7 ± 1.6	7.9 ± 1.3
Growth rate (mm/day)	0.6 ± 0.3	0.6 ± 0.2
Atresia rate (mm/day)	1.0 ± 0.5	1.0 ± 0.4
Persistence (days)	6.3 ± 1.2	5.9 ± 1.8

Table 4. Characteristics of follicular waves, growth and atresia of dominant follicles and of the largest subordinate follicle in cows with two follicular waves during estrous cycle in cool season (mean ± standard deviation)

Characteristic	Follicular wave	
	First wave	Second wave
No. of estrous cycle (%)	-	10/13(76.9)
Wave onset (day)	0.9 ± 0.4	10.7 ± 0.9
Wave length (days)	12.2 ± 2.1	10.4 ± 1.8
Dominant follicle		
Maximum diameter (mm)	8.7 ± 1.8	12.8 ± 1.2
Day of maximum diameter	10.6 ± 1.3	18.9 ± 1.7
Growth rate (mm/day)	0.7 ± 0.2	0.8 ± 0.1
Day of divergence	7.8 ± 0.8	15.4 ± 1.6
Length of growth phase (days)	9.5 ± 0.9	8.1 ± 1.3
Onset of atresia (day)	12.4 ± 1.6	-
Atresia rate (mm/day)	1.0 ± 0.4	-
Length of atresia (days)	11.2 ± 1.9	-
Largest subordinate follicle		
Maximum diameter (mm)	8.1 ± 1.5	8.6 ± 1.4
Growth rate (mm/day)	0.6 ± 0.4	0.6 ± 0.3
Atresia rate (mm/day)	1.1 ± 0.5	1.2 ± 0.3
Persistence (days)	7.3 ± 1.7	7.1 ± 1.6

Progesterone levels during estrous cycle

The progesterone level on the day of estrus was 0.07 ± 0.03 ng/ml and increase between Day 4 and 7 of estrous cycle, reaching a peak value of 1.7 ± 0.8 on Day 15 of the estrous cycle. Thereafter, the level began to decrease, and dropped to 0.08 ± 0.04 ng/ml on the day of estrus. The cyclic pattern of progesterone concentration observed in this study was in line with the changes found in corpus luteum functioning during the estrous cycle.

Increasing progesterone levels observed in present study appeared to be the result of luteinization after ovulation and subsequent secretion from the functional corpus luteum. Between Day 16 and 21 of estrous cycle, the expected process of luteolysis resulted in a precipitous fall in progesterone to a concentration ranging between 0.04 and 2.94 ng/ml in this study. Progesterone levels during estrous cycle in swamp buffalo in hot and cool season show in Table 5 and 6.

Progesterone profiles clearly indicated that swamp buffalo showed ovarian cyclicity without apparent signs of estrous. These cases may have been either silent or weak estrous. The detection of silent estrous cycles at an early stage of estrous period through progesterone assay would be substantially reduce the long inter-calving interval. In present study, two of the anestrous buffaloes (Fig. 3) were found to have low progesterone level of 0.04-0.18 ng/ml throughout the period.

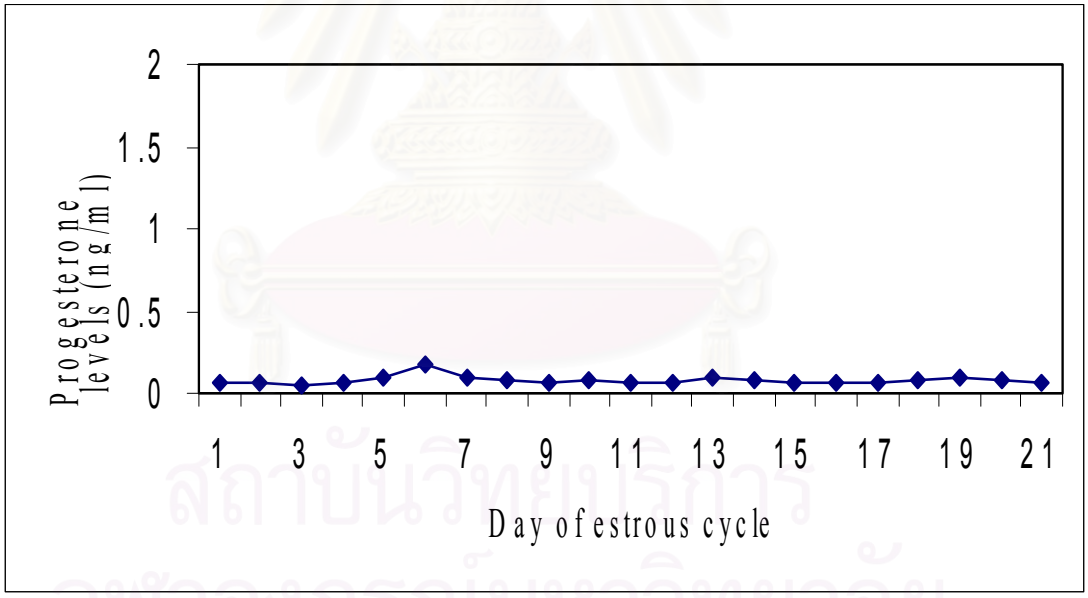

Fig. 3. Progesterone levels of swamp buffalo cow that show anestrous cycle (number 116)

Table 5. Progesterone levels during estrous cycle in swamp buffaloes in hot season

	1-wave	2-wave	
		1st wave	2nd wave
Length of days	25.5 ± 3.5	11.8 ± 2.3	10.6 ± 2.6
Maximum follicular diameter (mm)	14.5 ± 2.1	10.9 ± 2.1	10.3 ± 1.2
Average progesterone concentration (ng/ml)	0.9 ± 0.09	1.1 ± 0..05	1.3 ± 0.21

Table 6. Progesterone levels during estrous cycle in swamp buffaloes in cool season

	1-wave	2-wave	
		1st wave	2nd wave
Length of days	20.7 ± 2.8	12.2 ± 2.1	10.4 ± 1.8
Maximum follicular diameter (mm)	16.4 ± 2.7	8.7 ± 1.8	12.8 ± 1.2
Average progesterone concentration (ng/ml)	1.2 ± 0.19	0.89 ± 0.11	1.17 ± 0.14

2.5 Discussion

In this study, estrous behavior started toward late evening until early morning with peak sexual activity between 6.00 PM and 6.00 AM. Diurnal estrous behavior is common in buffalo and the majority breeds in the cooler hours of morning and evening, and sometimes during the night (Pathak, 1992; Jainudeen and Hafez, 1992). Tailor *et al* (1990) suggested that in swamp buffalo, mating behavior usually ceases during daylight hours. The intensity of estrous behavior has been found to be much less than cattle. The observed of estrous signs from this study found that homosexual activity in buffaloes is not as pronounced as in cattle (Kanai and Shimizu, 1983), but vulva discharge of clear mucus, especially in the recumbent animal, is the most reliable single sign of estrous in buffalo similar to reported of Singh *et al*. (1984). Using a epididymectomized bull for heat

detection is an appropriate technique in this study. In many areas, vasectomized bulls are not available and an intact animal that has been suitably trained and accustomed to control can be used. From the present study, estrous behavior was little affected in the hot summer season, especially during daylight. It is remarked that the swamp buffalo responds poorly to estrus synchronization, only one from nine buffaloes either during hot or cool season ovulated within 5 days after progesterone implant removal.

In order to observe the follicle development, a linear-array transducers of 5.0 or 7.5 MHz frequency ranges are most commonly used in cattle to perform reproductive ultrasound examinations, and most veterinary ultrasound scanners are compatible with probes of different frequencies. An ultrasound scanner equipped with a 5.0 MHz transducer is most useful for bovine practitioners conducting routine reproductive examinations, however, small ovarian structures such as developing follicles are best imaged with a 7.5 MHz transducer.

Pieterse et al. (1990) detected the corpora lutea and follicles in cows by using transavaginal ultrasonography and rectal palpation. From these studies, they found that the detection of a mid-cycle corpus luteum, the sensitivity and predictive value of rectal palpation were 83.3% and 73.2% and for ultrasonography the sensitivity and predictive value were 80.6% and 85.3%, respectively. However, both techniques were in accurate for the detection of young and old corpora lutea. For detecting follicles ultrasonography was a significantly better method than a rectal palpation. Ultrasonography can be used to detect about 95% of follicles with a diameter > 10 mm whereas rectal palpation detected only 71% of these follicles. Both techniques failed to detect for follicles of 5 to 10 mm in diameter; only 21.5% were detected by rectal palpation and 34.3% by ultrasonography. In this study, a human transvaginal probe can be used to observe the follicles development in swamp buffalo and it is appropriate to the genital tract of swamp buffalo which is smaller and shorter than that in the cattle (Lohachit, 1981). When operator used transvaginal probe to observe ovary the buffaloes less excited and pain and easy for practitioner to control ovary and probe from contraction of intestine, but this probe have disadvantage in clear detected of small follicular size.

In present study, only one from nine buffalo cow ovulated (11.1%) within 5 days after estrus synchronization both in summer and winter season. Presicce et al. (2004)

synchronized Mediterranean Italy buffaloes with a progesterone releasing intravaginal device(PRID) inserted for 10 days; a luteolytic dose of synthetic prostaglandin was given 7 days after PRID insertion. Only one from 11 buffalo heifers ovulated within 5 days after PRID removal and seven from 10 in buffalo cows. Supplement 2,000 iu of hCG, single injection in protocol of synchronization 48 hours after PGF2α injected. Can improve the percentage of number of ovulated buffalo cows within 5 days to five of nine cows (55.6%). From this results, the possibility of the reason that increase rate of ovulation because of increase level of LH and frequency of LH pulse to stimulated ovulation after injected hCG.

The length of the estrous cycle in buffaloes are generally more variable than those observed in cattle (Bhattacharya et al., 1974). The cycles of 18-26 days were considered as normal cycles in buffaloes and 15-29 days reported in riverine buffalo (Kanai and Shimizu, 1983) with the average 21.6 ± 0.23 days (Asdell, 1964). In the present study, the buffaloes have the length of estrous cycle within the normal range. The average length of estrous cycle was 25.5 ± 3.5 in 1- wave cycle and 22.6 ±1.9 days in 2- wave cycle in hot season and was 20.7 ± 2.8 in 1- wave cycle and 22.5 ±1.9 days in 2- wave cycle in cool season. The average length of estrous cycle similar to literature (Toelihere, 1977; Bodhipaksha et al., 1978; Wetchrutpimon and Mongkonpunya,1978; Jellinek and Avenel, 1980; Kanai and Shimizu,1983). In this study, the length of estrous cycle not different between in summer and winter.

The number of follicular wave per cycle 77.27% (17/22) was two-waves and 22.73(5/22) was one-wave according to the reported of Presicce et al. (2004) in Mediterranean Italy buffaloes found that after induced ovulation, buffalo heifers exhibited a one- wave (5 from 10 buffaloes; length of cycle 8-12 days), two-wave (4 from 10 buffaloes; 20-26 days) and three –wave cycle (1 from 10 buffaloes; 25 days). In buffalo cows, all of non-pregnant(n=8) had a two-wave cycle (range; 19-25 days). And also presicce et al. (2003) studied follicular turnover in prepubertal Mediterranean Italian buffaloes found that in buffalo prepubertal calves had a range of two-six regular follicular waves was reported among calves with an average of 4 ± 1.1 waves.

The development pattern of dominant and subordinate during estrous cycle was not different between hot and cool season. The wave length of first wave longer than

second in both season. There was not significant different between characteristic of follicular dynamics in summer and winter season. The possibility reason to explain this result may be the climate in this year not different between two seasons and in hot the buffalo received nutritional in energy balance level. But from observation of estrous signs in present study showed that expression of estrous signs in cool season more than hot season, especially on night time. Seasonal effects on estrous behavior may differ among studies due to breed of cows, climatologically variations, or manage mental factors such as frequency of milking, feeding, movement of cows.

From the result of progesterone level in this study, according to the previous report of Dobson and Kamonpatana (1986) that the concentration in the peripheral blood of Swamp and Murrah buffaloes is low at estrous and does not rise until after Day 5. Values increase until Day 14-16 with a lower mean value of 1.51-2.6 ng/ml in swamp buffalo. This findind accordance to other report of Jellinek and Avenell (1980), Alejardrino et al. (1981), Jainudeen et al. (1981) and Kanai and Shimizu (1983).

In this study, it was found the differences in the plasma progesterone levels among the normally cycling and anestrous animals similar to Chua et al. (1983) found that the anestrous buffaloes were found to have low progesterone level of 0.16 ng/ml throughout the period, according to this study that found anestrous buffaloes have progesterone level less than 0.18 ng/ml throughout the period.

In conclusion, the estrous cycles in swamp buffalo presented the characteristic pattern of follicular growth waves, with the initial development of small follicles, followed by selection, development and atresia of a dominant follicle similar in cattle. Follicular dynamics is characterized by one and two of follicular waves during the estrous cycle. The cyclic pattern of progesterone concentration observed was in line with the changes found in corpus luteum functioning during the estrous cycle. The detection of silent estrous cycles at an early stage of estrous period through progesterone assay would be reduce the long inter-calving interval.

CHAPTER III

OVUM PICK UP IN CYCLING AND LACTATING POSTPARTUM SWAMP BUFFALOES (*Bubalis bubalis*)

3.1 Abstract

The objective of this study was to evaluate the efficiency of ovum pick up (OPU) in cycling (n=5) and lactating postpartum swamp buffaloes (n=6) with and without gonadotropin stimulation. The OPU was performed every two weeks in all groups of animals, for a total of six sessions. Thirty collections were performed in five cycling and 36 collections in six lactating postpartum buffaloes. Buffaloes that received hormonal stimulation were given a total of 400 mg, follicle stimulating hormone (FSH), administered twice daily over 3 days in decreasing doses, together with 100 μg of GnRH, 24 hrs after the last FSH injection. Following a resting period of 1 month, the two groups of buffaloes, were subjected to the same OPU regimen, but without any hormonal treatment for an additional six OPU sessions. The number of aspirated follicles recorded from the hormonal stimulated, cycling animal and lactating, postpartum buffaloes was not significantly different, 7.2 ± 3.7 and 9.0 ± 3.2, respectively ($P > 0.05$). Recovered oocytes collected from the two groups of hormonally stimulated animals were also not statistically different: 3.7 ± 2.7 in the cycling and 5.9 ± 3.5 in the lactating postpartum group ($P > 0.05$). In the two groups of buffaloes not receiving hormonal stimulation, the number of aspirated follicles was not significantly different: 2.1 ± 1.4 and 1.4 ± 0.7 in cycling and lactating postpartum buffaloes respectively ($p > 0.05$). Recovered oocytes in the non-treated groups were also similar: 1.4 ± 1.3 vs 0.7 ± 0.8 in cycling and lactating buffaloes ($p > 0.05$). Among stimulated buffaloes, most aspirated follicles were small in size (≤ 5 mm), whereas they were mostly medium and large sizes in the non-treated buffaloes. The oocyte recovery rate in both the groups, cycling and lactating postpartum, were 51.6% and 69.5% in stimulated groups and 55.0% and 53.1% in non-stimulated groups ($p> 0.05$). The majority of recovered oocytes were single- and multi-layered, and the number was greater in the cycling than in the lactating, postpartum buffaloes. The number and quality of recovered oocytes was similar in all groups of buffaloes whether

they were received or did not receive hormonal stimulation. Moreover no difference was found in multi- and single-layered oocytes between cycling and lactating, postpartum buffaloes. In conclusion, OPU can be performed successfully in swamp buffalo in different reproductive status and FSH administration was shown to increase the number of aspirated oocytes in both cycling and lactating, postpartum buffaloes.

3.2 Introduction

Reproductive efficiency in swamp buffaloes is affected by a number of variables among which are late maturity in both genders and reduced fertility after natural mating (Bodhipaksha et al., 1978; Chantarakhana et al., 1981; Fischer and Bodhipaksha, 1992; Na-Chiengmai, 2002). A review of the management data among buffalo research stations in Thailand reports: (i) an average age at first calving of 4.19 $\pm$ 0.82 yrs, ranging from 3.58 to 4.91 yrs, and (ii) an average calving interval of 573.3 $\pm$ 117.13 days, ranging from 489.7 to 700 days (Na-Chiengmai, 2002). The weaning age of swamp buffalo in the National Breeding Station in Thailand is around 8 months after calving. From this data it follows that a long period of days open is an important factor affecting the reproductive efficiency in this species. The combination of recently developed reproductive technologies such as OPU and – *In vitro* Embryo Production (IVEP), enables the acceleration of the genetic improvement for production traits and the reproductive efficiency of females. The feasibility of OPU has already been reported in prepuberal buffaloes, heifers and cows (Boni, 1994; Kitiyanant *et al.,*1995; Pavasuthipaisit *et* al.,1995; Boni, 1997; Promdireg *et al*., 2000; Techakumphu et al. 2000a,b; Presicce *et al.* 2002; Techakumphu *et al.,* 2004a,b). The reproductive potential of females can be maximized, if OPU and IVEP are applied in the course of non-productive periods such as anestrus and postpartum. Therefore the aim of this study was to evaluate the yield and quality of oocytes recovered from buffaloes in different reproductive status, cycling and lactating, postpartum after hormonal stimulation and without hormonal stimulation.

3.3 Materials and Methods

3.3.1 Animals

This study was carried out from May to November 2003. The swamp buffalo cows were selected from a breeding herd at the National Buffalo Breeding Center, Buriram province and the research was performed at the university research unit at Chulalongkorn University, Nakorn Pathom. They were given roughage and water ad libitum together with 2 kg of concentrate daily. Buffaloes were divided into two groups according to their reproductive status: (i) five cyclic buffalo cows, with an average age of 6.2 years and 436 $\pm$ 26.4 kg average weight. One month before the experiment started, signs of their estrus cycle were observed daily (a.m./p.m.), using an epididydectomized bull and (ii) six lactating postpartum buffaloes, 3 months after calving at the beginning of the study, with an average age of 5.6 years and 419$\pm$20.8 kg average weight. Lactating females were 6 months postpartum at the end of the hormonal stimulated trial and 9.5 months at the end of the non-hormonal trial. Calves were left with their respective mothers during the period of study.

3.3.2 Hormonal stimulation

Both cycling and lactating, postpartum buffaloes were hormonally treated and oocytes were collected by OPU every 2 weeks for six sessions. Following a rest of 1 month, the same two groups of buffaloes were used as non-treated groups for the same number of sessions at the same scheduled interval. For hormonal stimulation, a modified FSH (Folltropin®-V, Vetrepharm Canada Inc., London, Ontario, Canada) protocol was used (Techakumphu et al. 2004a). To synchronize the cycle, at the beginning of experiment, each buffalo received a 3 mg of norgestomet ear implant together with 5 mg of estradiol valerate im (Crestar®, Intervet, The Netherlands) 12 days before FSH administration (day-10). Synthetic prostaglandin (Lutalyse®, Upjohn, USA) was administered im (25 mg) at the time of implant removal (day 0). A total of 400 mg of FSH was then administered in decreasing doses 80/80 mg, 70/70 mg and 50/50 mg twice a day (8 a.m./6 p.m.), over a period of 3 days starting from day 4 to day 6.

Twenty-four hours after the last FSH administration, 100 μg of Gonadotropin Releasing Hormone (GnRH, Fertagyl®, Intervet, and The Netherlands) was given on day 7 and OPU was performed on day 8 (Fig. 4).

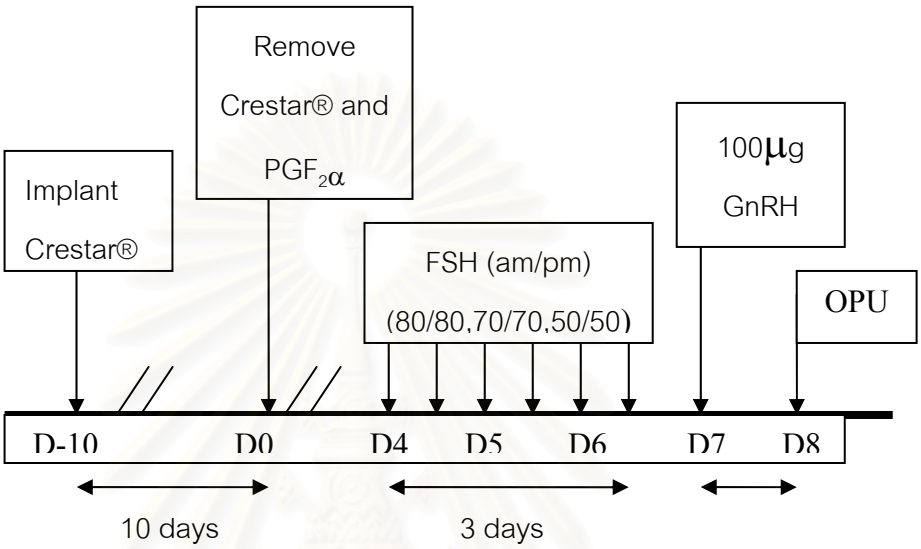

Fig. 4. Ovarian stimulation program in cycling and lactating postpartum buffaloes (D = day).

3.3.3 OPU

Before OPU, the buffaloes were restrained in a squeeze chute and tranquilized with 1 ml of Xylazine HCL (Rompun®, Bayer Korea Ltd. Seoul, Korea), followed 10 min later, by 2 ml 2% lidocaine hydrochloride, given epidurally (OLIC, Sweden). OPU was performed as described in our previous study (Techakumphu et al. 2004a,b). The ultrasound unit was equipped with a 5 MHz sector scanner probe (Aloka Co. Ltd., Tokyo, Japan). Follicles were divided into three categories according to their size as small (2-5 mm), medium (6-9 mm) and large (≥ 9 mm). Only follicles ≥ 3 mm were aspirated by using a vaginal probe fitted with a 34.5 cm 17G single lumen needle. The negative pressure of the vacuum pump (Cook, Australia) was set between 60 and 80 mmHg. As a prophylactic measure and following OPU, buffaloes were injected im with 100,000 IU penicillin-streptomycin.

3.3.4 Oocyte processing

Recovered oocytes were searched immediately under a stereomicroscope after filtering the aspirated follicular fluid. Oocytes were washed twice in TCM 199 2.5 mMHepes (Gibco, USA) and classified, according to the degree of cumulus cell investment and the quality of the cytoplasm (Loos et al.,1989 ; Techakumphu et al. 2004a), into four groups: (i) multilayered oocyte (ML, >4 layers of cumulus cells and homogeneous ooplasm); (ii) single layer oocyte (SL, 1-4 layers and homogeneous ooplasm but with a coarse appearance and dark zone at the periphery of the oocyte) ; (iii) expanded cumulus oocyte (EXP) + denuded oocyte (D); (iv) degenerated oocyte (Deg) + free zona pellucida without cytoplasm (FZ).

3.3.5 Statistical Analysis

The number and size of the follicles as well as the number of recovered oocytes are expressed as mean $\pm$ S.E.M. and differences between mean of groups have been compared by analysis of variance (ANOVA). Differences were considered significant at $P < 0.05$.

3.4 Results

In FSH-treated animals, a similar number of aspirated follicles were recorded in both between cycling and lactating, postpartum buffaloes: 7.2 $\pm$ 3.7 and 9.0 $\pm$ 3.2, respectively ($P > 0.05$). The number of aspirated follicles in the non-treated animals was also similar, 2.1 $\pm$ 1.4 and 1.4 $\pm$ 0.7 in cycling and lactating, postpartum buffaloes, respectively ($P > 0.05$). Most oocytes, from all buffaloes were single-layered (70%). Multi-layered oocytes were also found in all animals. The rate of recovered oocytes was similar in both cycling and lactating, postpartum buffaloes (Table 7). In treated cycling and lactating, postpartum buffaloes, most of aspirated follicles were small, 77.8 and 65.6% respectively, while in the non-treated cycling and lactating, postpartum buffaloes, they were mostly in the range of medium to small, 57.1 and 28.6% in cycling and medium to large, 42.9 and 42.8% in lactating, postpartum buffaloes (Table 8). The oocyte recovery

rates in both the groups, were 51.6 and 69.5% with an average of 61.8% in treated groups and 55.0 and 53.1% in the non-treated groups, which averaged 54%.

Table 7. Mean (±SEM) and total number (n) of aspirated follicles, recovery and quality of oocytes between cyclic and lactating, postpartum buffaloes.

Buffaloes	FSH	Follicles animal/session (number)	Oocyte recovery Animal/session	Classification of oocytes			
				ML(%)	SL (%)	EXP & D(%)	Deg + FZ(%)
Cycling	+	7.2±3.7 (217)	3.7 ± 2.7(112) [51.6]	22 (19.6)	75 (67.0)	13 (11.6)	2+55 (1.8)
Lactating	+	9.0±3.2 (285)	5.9 ± 3.5(198) [69.5]	19 (9.6)	143 (72.2)	36 (18.2)	0+20
Cycling	-	2.1±1.4 (64)	1.4 ± 1.3(35) [55.0]	5 (14.3)	24 (68.6)	2 (5.7)	4 (11.4)
Lactating	-	1.4±0.7 (49)	0.7 ± 0.8(26) [53.1]	-	18 (69.2)	8 (30.8)	-

[ ] aspiration rate

ML, multi-layered oocyte; SL, single layer oocyte; EXP, expanded cumulus oocyte; D, denuded oocyte; Deg, degenerated oocyte; FZ, free zona with out cytoplasm.

Note: no significant differences were found between the means.

Table 8. Size of punctured follicles from cyclic and lactating,postpartum buffaloes.

Buffaloes	FSH	Session	Aspirated follicles (follicle/ session)	Size of follicle (follicle/animal/session)		
				Small (3-5mm)	Medium (6-9mm)	Large (>9mm)
Cycling	+	30	217	5.6 ± 3.0 (77.8%)[a]	1.5 ± 1.5 (20.8%)[a]	0.1± 0.3 (1.4%)[a]
Lactating	+	36	285	5.9 ± 1.9 (65.6%)[b]	3.0 ± 2.6 (33.3%)[b]	0.1± 0.3 (1.1%)[b]
Cycling	-	30	64	0.6 ± 1.5 (28.6%)[a]	1.2 ± 1.3 (57.1%)[a]	0.1± 0.4 (14.3%)[a]
Lactating	-	36	49	0.2± 0.9 (14.3%)[b]	0.6 ± 0.9 (42.9%)[a]	0.6 ± 0.5 (42.8%)[b]

[a,b] Different superscripts indicate significant differences within columns at $p < 0.05$.

3.5 Discussion

In this study it has been shown that OPU can be successfully performed in cycling and lactating, postpartum swamp buffaloes. Results in terms of recovered oocytes and oocyte quality are not significantly different. In agreement with previous studies in heifers and prepubertal buffalo calves (Promdireg *et al.,* 2000; Techakumphu *et al*. 2000a,b; 2004a, b), the administration of FSH before OPU can improve the yield of oocytes. In swamp buffaloes a prolonged postpartum period is considered a major obstacle to the improvement of reproductive efficiency. The combined application of OPU and IVEP could be used to make a more productive and shorter time interval.

Previous studies in riverine (Taneja *et al.,* 1996; Baruselli *et al.,* 1997) and swamp buffalo (Bodhipaksha *et al.,* 1978; Danell, 1987) have reported an ovarian follicle wave turnover similar to cattle. Moreover, in both cattle and buffaloes follicle wave development resumes immediately after calving (Murphy *et al*., 1990; Henao *et al.,* 2000; Presicce *et al.,* 2004), and this is in agreement with our recent studies (Promdireg *et al.,* 2004). Collectively these informations leads to the possibility to performing OPU in cyclic,

non-pregnant as well as lactating, postpartum buffaloes and to use exogenous gonadotropins to increase the development of follicles. This study shows that oocytes can be successfully collected by OPU in both physiological status with similar results in terms of number of follicles and oocytes, as well as oocyte quality, confirming the report of Bungart *et al*. (1995); Kitiyanant *et al.* (1995) and Pavasuthipaisit *et al.* (1995).

FSH treatment increased the number of aspirated follicles and oocytes in both reproductive status, confirming most previous studies in buffaloes (Boni, 1994; Boni *et al.,* 1997; Techakumphu *et al*., 2000a, b; 2004a, b) and cattle (reviewed by Faber *et al.,* 2003). According to these studies, FSH administration increases the availability of antral follicles, collected oocytes, oocyte quality and transferable embryos per OPU session. This is confirmed in the current study, where both antral follicles and recovered oocytes were higher in treated buffaloes compared to non-treated animals. The exogenous FSH helps to rescue small healthy and early atretic follicles from atresia by providing the appropriate environment to develop (Taneja *et al*., 1995; Reis *et al*., 2002). In this study, the majority of follicles in treated animals were small which is different from the report of Presicce *et al.* (2002), who found that the majority of follicles in treated animals were of medium to large in size. The use of GnRH 24h before OPU may be cause of the difference. According to our previous studies, GnRH administration following hormonal stimulation resulted in a higher number of small follicles compared to hormonal treatment without GnRH (Techakumphu *et al.,* 2000b). The frequency of OPU sessions may also an additional reason for the different findings. Weekly or twice weekly intervals were proposed for OPU in Mediterranean Italian buffalo (Gasparrini, 2002), swamp buffalo (Pavasuthipaisit *et al.,* 1995) and cattle (Hanenberg and Van Wagtendonk-de leeuw, 1997; Garcia *et al*., 1998). It was reported that the mean number of available follicles did not differ when collected at 2, 3 and 4 day intervals. However, a decrease in the number of small follicles and an increase in large follicles were found when the interval was more prolonged and this is in agreement with our findings in cyclic non-pregnant and postpartum buffaloes. In swamp buffaloes, Pavasuthipaisit et al. (1995) reported a high number of aspirated follicles and recovered oocytes obtained from weekly OPU in non-treated cycling and non-cycling, pre-pubertal and pregnant animals, which was not seen in this study. In this study, OPU was performed at two-

weekly intervals, which corresponds to the time of new follicle recruitment (Baruselli *et al.,* 1997; Promdireg *et al*., 2004). The basic information on follicular recruitment during the estrus cycle and the frequency of OPU will be an interesting subject for further study, before applying the OPU technique on the buffalo production.

Lactating, postpartum buffaloes 3 months after calving responded to FSH treatment in a similar manner as cycling buffaloes. However, the number of aspirated follicles and recovered oocytes per animal, non-treated, postpartum buffaloes, appeared to be lower than in the cycling ones. In postpartum cattle, it has been shown that the resumption of follicular growth and ovulation mechanisms are not well established from some time, due to the irregularity of endocrine function (Murphy *et al.,* 1990; Peters and Lamming, 1990). This may be related to the environmental factors such as suckling, milk yield, energy imbalance and season (Peters, 1984; Murphy *et al.,* 1990). In a previous pilot study involving four postpartum swamp buffaloes, monitored daily by ultrasound, it was found that for 4 months after calving, follicle development is characterized by an short cycle and the first ovulation without estrus (unpublished data), which is similar to that of cattle (reviewed by Yavas and Walton, 2000). In swamp buffaloes, Kamonpatana *et al.* (1980) revealed that the level of luteinizing hormone during the postpartum period was low, caused by the suppressive effect of prolactin, actively secreted in response to suckling stimulus, which is known to affect ovarian function (Jainudeen *et al.,* 1984). The study carried out in Italian buffaloes by Boni *et al.* (1997) revealed that the increase of days open had a negative influence on follicular population possibly related to a negative energy balance (Rensis and Scaramuzzi, 2003). In our study, OPU in non-treated animals was performed during 7-9 months after calving which is the age of pre-weaning in swamp buffalo in Thailand.

The quality of oocytes in treated and non-treated, cycling or lactating, post-partum, buffalo cows was similar. Collectively the majority of recovered oocytes were single- and multilayered and at a higher rate in cycling than in lactating, postpartum buffaloes. This contradicts our finding in previous studies in prepuberal buffaloes treated with FSH and heifers treated with PMSG, where only a low numbers of good quality oocytes was recovered (Promdireg *et al.,* 2000; Techakumphu *et al.,* 2004a, b).

In conclusion, oocyte retrieval by OPU can be continously performed on a once every 2 weeks collection schedule for at least 6 months in both cycling and lactating, postpartum buffaloes. The administration of FSH can increase the number of antral follicles as well as recovered oocytes.

CHAPTER IV

IN VITRO FERTILIZATION OF OOCYTE RECOVERED BY TRANSVAGINAL ULTRASOUND-GUIDED FOLLICLE ASPIRATION TECHNIQUE IN SWAMP BUFFALO

4.1 Abstract

The objective of the study was to develop embryo production from oocytes retrieved by ovum pick up. Oocyte quality and embryo development rates were recorded. OPU was performed in 5 buffalo cows, aged 2-5 yrs, after the administration of 400 mg (NIH unit) of follicle stimulating hormone in order to induce multiple follicle development. The recovered oocytes were washed in TCM 199 2.5 Hepes, twice, and then incubated in TCM 199 $NaHCO_3$+10% fetal calf serum for 24 hrs, at 38.5 ^{o}C in an atmosphere containing 5% CO_2 . There were later fertilized and cultured with B2 (Menezo) +2.5% fetal calf serum on vero cells for 7 days. The average number of follicles per cow were calculated using sixty sessions of OPU. Two hundred and sixty five oocytes (4.4$\pm$3.4 per animal) were collected from 45 follicles (7.9±3.5 per animal) which represented a 55.8% of recovery rate. Most of recovered oocytes, 67.9% (180/265) were single layered cumulus oocytes 15.5% (41/265) were EXP+D, 14.7%(39/265) were COC and 1.9%(5/265) were degenerate. COC and single layer cumulus oocytes were submmited for IVF. The results showed that the maturation rate was 62.8%(49/78) and the embryos could develop to the 2-4 cell stage 40.5% (34/84), to morula (>16 cell), 15.5%(13/84) and 1.2 (1/84) to blastocyst. In conclusion, it is possible to produce embryos from oocytes collected by OPU in swamp buffalo, however the rate of morula and blastocyst development was poor.

4.2 Introduction

The primodial follicle in the ovary of cattle contains more than 130,000 follicles, but in buffalo, the number is less than in cattle and is approximately, 20,000 follicles (Singh *et al*,. 2000). Many calves can be produced by the IVF-ET technique. The oocytes

can be recovered from ovaries in a slaughterhouse or by a new technique called "ovum pick up" (OPU) (Kriup *et al*,. 1994). *In vitro* fertilization is the tool for producing embryos for research or embryo production for embryo transfer. The processes of IVF are oocyte collection, *in vitro* maturation (IVM) of oocytes, followed by fertilization with capacitated sperm. Embryos at the morula or blastocyst stages can be tranfered to appropriate recipients to produce calves. The OPU technique was developed previously in cattle by Peterse *et al*. (1988) and then applied to many other species including riverine buffalo (Boni,1994; Boni *et al*,. 1996; Neglia *et al*,. 2003). In swamp buffalo, there have been a few reports on OPU in different reproductive states (Kitiyanant *et al*,. 1995; Pavasuthipaisit *et al*,. 1995; Promdireg *et al.,* 2000; Promdireg *et al.,* 2004; Promdireg *et al.,* 2005). The studies in swamp buffalo showed it possibly to collect oocytes from prepubertal buffalo calves, buffalo heifers and cows. As seen in chapter III, oocytes can be collected successfully from postpartum and cyclic buffalo cows. An average of 5-6 oocytes per animal were collected, with 40% of good quality (Promdireg *et al* ,. 2000), which was similar to that of cattle (Looney *et al*,. 1994; Bungart *et al*,. 1995) and river buffalo (Boni *et al*,. 1996). The objective of the study was to study *in vitro* embryo production from oocytes collected by ovum pick up.

4.3 Materials and Methods

4.3.1 Animals

Five swamp buffalo heifers (2-5 yrs old) were selected from the National Breeding Center, Burirum, fed 2 kg of concentrates daily with ad libitum roughage and water. The buffalo received a progesterone ear implant 11 days. Before ovarian stimulation was done, using a total of 280 mg (NIH unit) Follicle Stimulating Hormone in decreasing doses over three days (60x60,50x50,30x30) (a.m/p.m). One hundred micrograms of Gonadotropin Releasing Hormone (GnRH) were given at 24 hrs after the last FSH injection, and then 24 hrs later ovum pick up performed. Ovum pick up was performed every 2 wks during a 6 months period, as described by Promdireg *et al* .(2000).

4.3.2 Oocyte collection by OPU

The ovaries were examined using an Aloka SSD-550V, ultrasound unit, equipped with a 5 MHz sector scanner, human, vaginal probe (Aloka Co. Ltd., Tokyo, Japan). Before OPU the follicles were scanned to investigate the number and size of the follicles in each ovary. Follicle that had a diametter >2 mm were punctured. The technique of OPU was described in chapter III.

4.3.3 Oocyte Classification

Immediatedly following aspiration, the follicular fluid collected, during OPU, was washed with phosphate buffer saline(PBS) and filtered, using an Emcon embryo filter (45 μm). A small amount of fluid was left to be searched for oocytes, under a stereomicroscope. Oocytes were placed in TCM 199 2.5 mMHepes(Gibco, USA) and classified, according to the degree of cumulus cell investment and the quality of the cytoplasm, into 3 grades, a modification of Loos *et al.(*1989)

Grade I : Compact multilayered cumulus investment (COC, >4 layers of cumulus cells) with homogenous cytoplasm.

Grade 2 : Single layered cumulus oocyte (S, 1-4 layers), with homogenous Cytoplasm.

Grade 3 : Oocytes with incomplete cumulus mass, with dark clusters, or an irregular color of the cytoplasm. The oocytes in this grade comprised of expanded cumulus oocytes, or denuded cumulus oocytes without cumulus mass, or degenerated oocytes (Deg) as well as a free zona pellucida, without cytoplasm.

4.3.4 *In vitro* oocyte maturation

Only good quality COC and S oocytes were submitted for *in vitro* maturation. The maturation medium was TCM199, supplemented with 25 mM Sodium bicarbonate, 10 μg/ml FSH/LH, 1 μG/ml Estradiol-17β and 10% fetal calf serum. The oocytes were cultured for 24 hrs at 38.5 ^{o}C, in a humidified atmosphere containing 5% CO_2. The maturation stage was observed by rapid staining (Apimeteethumrong *et al.,* 1999).

4.3.5 *In vitro* fertilization

The media and the procedures of *in vitro* fertilization were a modification of that of Techakumphu *et al.* (1993). One lot of frozen, buffalo bull semen, provided by The Artificial Insemination Research Center, Rachaburi Province, was used for *in vitro* fertilization. Sperm capacitation was achieved by the swim up method using TALP medium supplemented with bovine serum albumin fraction V at 38.5 ^{o}C in 5% CO_2 and air for 1 hr. The sperm concentration was calculated and adjusted to $1x10^6$ /ml for insemination (Gordon, 1994).

The fertilization medium was TALP medium supplemented with bovine serum albumin fraction V, without fatty acid, PHE (Penicillamine, Hypotaurine, Epinephrine) and heparin The oocytes recovered from the *in vitro* maturation process were washed and put into 500 μl of the sperm solution (Gordon, 1994) and the co-culture wias carried out at 38.5 ^{o}C, in a humidified atmosphere, with 5% CO_2, for 18 hrs.

4.3.6 *In vitro* embryo culture

The cumulus cells of the presumptive one cell zygote were removed and the zygotes put into B2 medium (Menezo, France), supplemented with 10% fetal calf serum and co-culture with vero cells, 18 hrs after fertilization. The culture was carried out at 38.5 ^{o}C, in a humidified atmosphere with 5% CO_2, and the evaluation of the embryo development was performed as followed :

1. 48 h after culture, the cleavage of the embryos was evaluated. Then the fertilization rate and the cleavage rates were calculated.
2. Six days after culture, the morula formation rate was calculated
3. 7-10 days after culture, the blastocyst formation rate was evaluated

4.3.7 Statistical Analysis

The number and the size of the follicles and the number and the classification of the oocytes were expressed as means (± SD). The oocyte recovery rates were calculated as the ratio between the recovered oocytes and the number of aspirated follicles.The difference in the quality of the recovered oocyte was evaluated by Chi-

square testing. The fertilization rate, cleavage rates and the morula/blastocyst rate, were calculated.

4.4 Results

Table 9 showed the results of OPU in 60 sessions with 5 buffalo cows. The treatment session did not influence the average number of follicles and recovered oocytes per cow. The mean number of follicle per animal was 7.9±3.5 (n=475) per session and the average diameter was 5.0±1.4 mm.

Table 9. The results of oocyte recovery by OPU in five swamp buffalo cows

Sequence of OPUs	Aspirated follicles* (follicle/animal/session)	Diameter of follicles (mm.)**
1	51	4.9±1.2
2	48	4.8±1.4
3	39	5.1±1.6
4	40	4.9±1.3
5	38	5.3±1.5
6	42	5.3±1.4
7	35	5.1±1.4
8	33	5.2±1.2
9	39	4.9±1.5
10	41	4.7±1.6
11	42	4.6±1.3
12	27	5.3±1.2
Total	**475 (7.9±3.5)**	**5.0±1.4**

* number of follicle size >3 mm.

** (mean± SD)

Most of the follicles were small, 69.7% (331/475) with an average of 5.5±2.5 follicles per animal while the medium size ones totaled 29.1%(138/475, 2.3±2.1 follicle per animal) and the largest totaled 1.2%(6/475, 0.1±0.3 folicle per animal) (Table 10).

Table 10. Classification of follicle size

Number of OPU	aspirated follicles per animal	Size of follicle (follicle/animal/session)		
		small (3-5 mm.)*	medium (6-9 mm.)*	large (>9 mm.)*
60	475	331[a](69.7%)	138[b](29.1%)	6[c](1.2%)
	7.9±3.5	5.5±2.5	2.3±2.1	0.1±0.3

* (Mean± SD)

[a,b,c] P< 0.05

The average number of recovered oocytes was 4.4±3.4 per animal. A total of 265 useable oocytes were collected from 60 sessions which represented a 55.8% recovery rate (Table 11).

Table 11. The mean number of follicles and oocytes recovered from OPU in swamp buffalo cows

Sequence of OPUs	Follicles per animal per session	Oocytes per animal per session	Recovery rate (%)
1	51[a]	39[c]	39/51
	8.5±2.7	6.5±2.7	(76.5)
2	48[a]	29[d]	29/48
	8.0±3.2	4.8±3.1	(60.4)
3	39[a]	22[d]	22/39
	6.5±1.4	3.7±1.3	(56.4)
4	40[a]	20[d]	20/40
	6.7±1.4	3.3±2.1	(50.0)
5	38[a]	18[d]	18/38
	6.3±2.2	3.0±1.5	(47.3)
6	42[a]	25[d]	25/42
	7.0±2.8	4.2±3.1	(59.5)
7	35[a]	22[d]	22/35
	5.8±2.3	3.7±2.2	(62.9)
8	33[a]	16[d]	16/33
	5.5±2.3	2.7±2.0	(48.5)
9	39[a]	16[d]	16/39
	6.5±2.7	2.7±2.7	(41.0)
10	41[a]	26[d]	26/41
	6.8±2.4	4.3±3.1	(63.4)
11	42[a]	19[d]	19/42
	7.0±2.9	3.2±2.7	(45.2)
12	27[b]	13[d]	13/27
	4.5±4.0	2.2±2.6	(48.1)
Total	475	265	265/475
	7.9±3.5	4.4±3.4	(55.8)

[a,b,c,d] $P < 0.05$

Most quality oocytes were single (S) 180 oocyte (67.9%) and (EXP+D) 41 oocyte(15.5%), with COC 39 oocytes(14.7%) and Deg 5 oocytes (1.9%). (Table 12).

Table 12. The quality of oocytes recovered by OPU.

No of OPU sessions(n=5)	60
Recovered oocytes per animal	265(4.4±3.4)
Oocyte quality	
- grade I	99(37.4%)
- grade II	120(45.3%)
- grade III	46(17.3%)

The *in vitro* maturation rate of buffalo oocytes recovered by OPU was 62.8% (48/79). The cleavage rates at day 2, 2-4 cell, 8-cell, morula and blastocyst are presented in Table 13 and Fig. 5-7.

Table 13. *In vitro* development of embryos fertilized in vitro in ration to the number of oocytes recovered by OPU

Number of IVF	84
Cleavage rate(%)	34(40.5)
2-4 cell(%)	10(11.9)
8-cell(%)	10(11.9)
morula(%)	13(15.5)
blastocyst(%)	1(1.2)

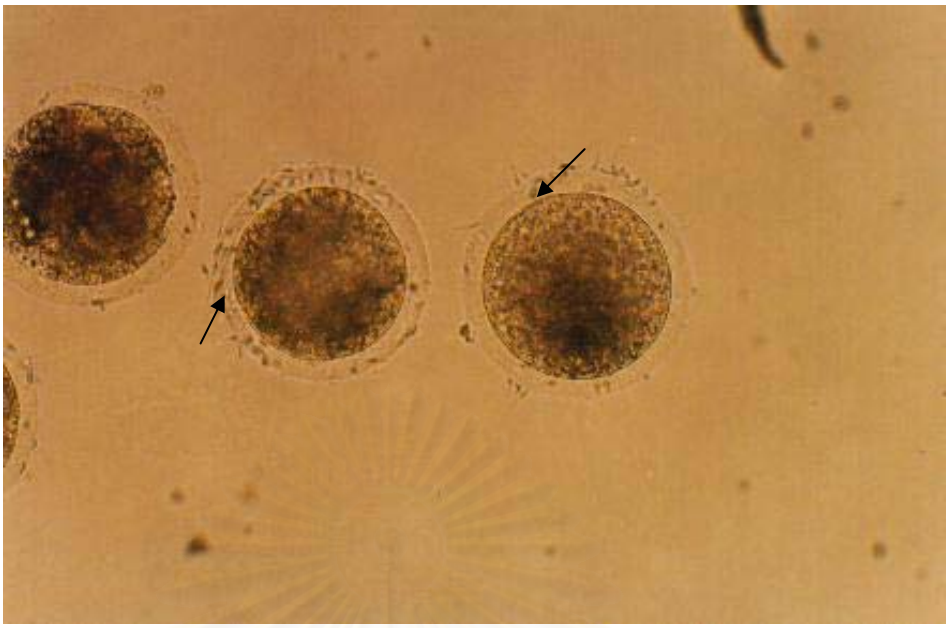

Fig. 5 The attachment of a spermatozoa on the zona pellucida of an oocyte, arrow indicated sperm attachment (x200)

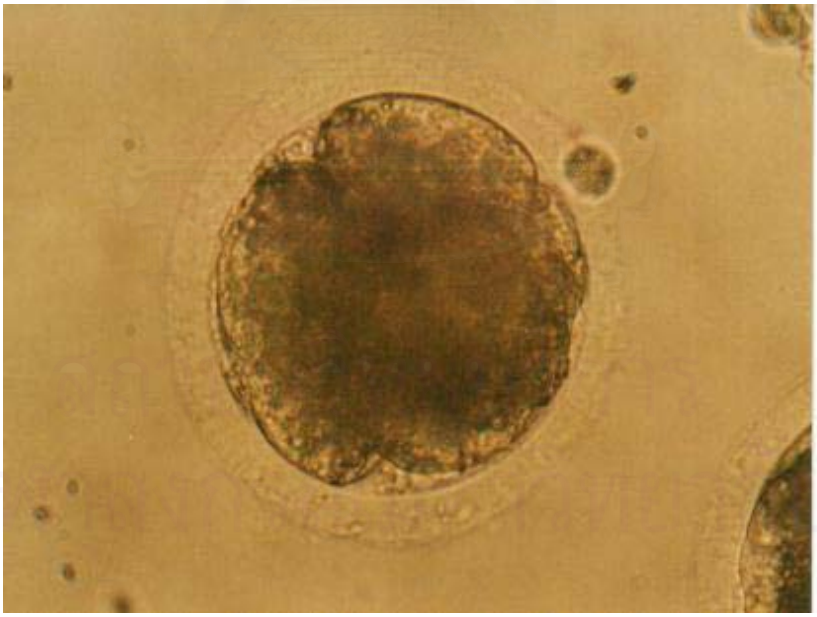

Fig. 6 A four-cell stage embryo after *in vitro* fertilization on D2 (x400)

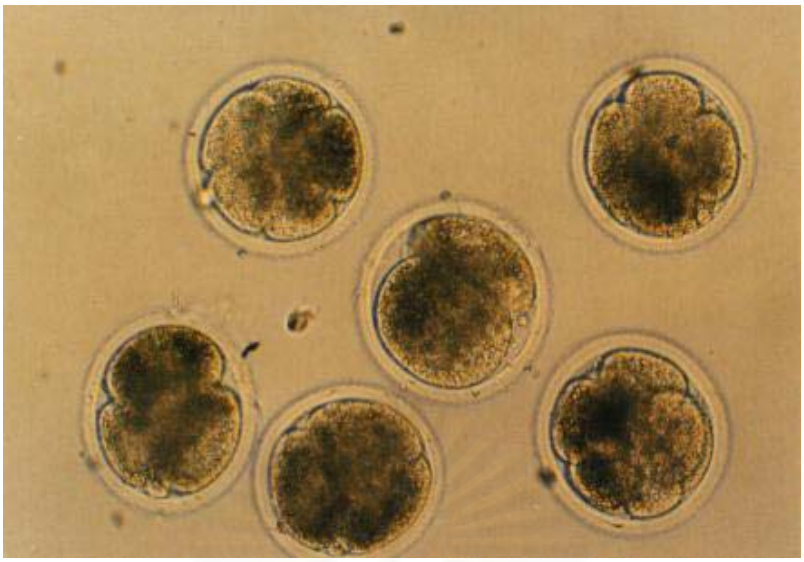

Fig. 7 Embryos at different stages, after fertilization of oocytes recovered from OPU, in swamp buffalo cows (x200)

4.5 Discussion

The number of OPU oocytes in the study that were appropriate for *in vitro* maturation and fertilization was only 37%. The quality of the oocytes was lower than that described by Pavasuthipaisit et al.(1995), Kitiyanant *et al.(*1995) and Gupta *et al.*(2005), who found that 60-70% of recovered oocytes, from OPU, were appropriate for *in vitro* embryo production. This may be a major cause of the low morula and blastocyst formation seen in this study. The maturation rate of recovered oocytes from slaughterhouse ovaries from dairy cattle showed that 70-90% achieve maturation and 40-50% cleave, after *in vitro* maturation and fertilization (Barile *et al.,* 1990; Chohan and Hunter, 2004). In swamp buffalo, Techakumphu *et al.(*2000) found that oocytes recovered from laparotomy in hormone treated buffalo, after *in vitro* maturation in buffalo cows had a greater maturation rate than those from buffalo calves (49/78; 62.8%) vs 20/38; 52.6% , respectively). Nandi *et al*. (2004) showed that the effect of follicular fluid on different diameter follicles, in maturation media, produced varying embryo development. The follicular fluid effect divided was into 3 groups, depend on the size of the follicle, small (>3 mm), medium (3-8 mm) and large (>8 mm). The study found that the development of oocytes to embryos, after in vitro fertilization show that oocyte maturation using follicular fluid was lower than that using serum. Follicular fluid from

small follicles showed more oocyte development than when using follicular fluid from medium and large follicles. In this study, it was found that the development of embryos to the blastocyst stage was low. Correction of this lower rate of development would allow for *in vitro* fertilization to produce embryos for embryo transfer and embryo freezing. Pavasuthipaisit *et al. (*1992) studied the *in vitro* maturation and fertilization of recovered oocytes from slaughterhouse ovaries in swamp buffalo, without co-culture media They found that more than 60% of COC and EXP, developed to 2 cells (64% and 68%, respectively). Partial and denuded oocytes only developed to 2-cell only 53% and 46%, respectively. The development of embryos 6-8 cells stage were 32%, 24%,10% and 6% in EXP, COC, partial, and denuded oocytes, respectively. They concluded that buffalo oocytes could develop to 8 cells after fertilization and culture in media without using co-culture. Acorrding to the results in cattle there is a block at the 8 cell stage as the embryos require maternal substances for the next step of development, such as from the oviductal cells or other sources for support. Abdoon *et al*.(2001) studied the development of embryos after *in vitro* fertilization of oocytes from slaughterhouse ovaries. It was found that supplementation with FSH or eCG in the maturation media can increase the cleavage rate and the development rate of embryos. They also found that good and fair quality buffalo oocytes produced a higher cleavage rate than poor quality ones. The morula production rate was also higher for good ones as compared with fair quality oocytes and embryo development with poor quality oocytes became arrested at the two to sixteen cell stage. Gasparrini *et al*. (2000) found that adding a thiol compound (such as cysteamine) to the IVM medium improved buffalo in vitro embryo production efficiency.

Neglia *et al.* (2003) studied the effect of oocyt source on subsequent embryo development in buffalo. The percentage developing to blastocyst were 29.7% and 19.9% in oocytes recovered by the OPU technique and the abattoir ovaries, respectively. They concluded that in buffalo, the source of oocytes significantly affected post-fertilization embryo development.

Galli et al.(2004) reported that many factors influence in vitro embryo production, but the most important factor is the physiology of the donor and the culture system. According to basic factors such as age, body condition, farm management, the special

factors are efficiency of the reproductive system and ovarian activity. The culture system and the composition of medium, can also affect embryo quality. In fact, various studies have been shown that while the innate qualiy of the oocyte is the major factor that determines the blastocyst yield, the in vitro culture environment, which the embryos are exposed to after fertilization, is the key determinant of blastocyst quality(Pereira *et al*., 2005).

In conclusion, it is possible to produce embryo by *in vitro* fertilization with oocytes recovered by the OPU technique, but the developmental rate after fertilization was low, due to principally to the quality of OPU oocytes and the *in vitro* culture process.

CHAPTER V

GENERAL DISCUSSION AND CONCLUSIONS

Follicular dynamics

The estrous cycle in swamp buffalo presents the characteristic pattern of follicular growth waves similar in cattle. Follicular dynamics is characterized by either one or two follicular waves during the estrous cycle.

The pattern of ovarian follicle recruitment and selection in buffaloes could provide additional insights, and lead to a better refinement of protocols for follicle synchronization and induction of ovulation for successive AI. A complete lack of information in swamp buffalo regarding reproductive biotechnology, such as the ovarian stimulation protocol and oocyte retrieval by the Ovum Pick Up(OPU) technique.

The basic information on follicular recruitment during the estrus cycle and the frequency of OPU will be an interesting subject for further study, before it can be applying for buffalo production.

Ovum pick up

In this study it has been shown that OPU can be successfully carried out in cycling and lactating, postpartum, swamp buffaloes. The results in terms of recovered oocytes and oocyte quality are not significantly different. In agreement with previous studies in heifers and prepubertal buffalo calves (Promdireg *et al.*, 2000; Techakumphu *et al*. 2000a,b; 2004a, b), the administration of FSH before OPU can improve the yield of oocytes. In swamp buffaloes a prolonged postpartum period is considered a major obstacle to the improvement of reproductive efficiency. The combined application of OPU and IVEP could be used to make a would be more productive and shorter pospartum time interval.

FSH treatment increased the number of aspirated follicles and oocytes in which, confirming most previous studies in buffaloes (Boni, 1994; Boni *et al.*, 1997; Techakumphu *et al*., 2000a, b; 2004a, b) and cattle (reviewed by Faber *et al*., 2003).

In conclusion, oocyte retrieval by OPU can be performed on a continuous basis every 2 weeks for at least 6 months, in both cycling and lactating, postpartum buffaloes. The administration of FSH can increase the number of antral follicles as well as recovered oocytes.

In vitro fertilization

In this study, the success rate for the IVF technique in term of swamp buffalo oocytes recovered using OPU was low. One of the influencing factors was the low quality of oocytes. An interesting subject for further study would be to improve the quality of oocyte for in vitro fertilization, using the protocols such as ovarian stimulation and puncture dominant follicles before hormonal stimulation.

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APPENDICES

APPENDIX A

List of publications and conferences

Local :

1. Promdireg, A., Na-Chiangmai and A., Techakumphu, M., 2004. Follicular dynamics in swamp buffalo cows (*Bubalus bubalis*). The 30th Veterinary Medicine and Livestock Development Annual Conference, The Thai Veterinary Medical Association Under the Royal Patronage, 10-12 November, 2004, Bangkok, p. 74.

2. Promdireg, A. and Techakumphu, M., 2004. Follicular dynamics in swamp buffalo cows (*Bubalus bubalis*). The symposium on biotechnology in breeding and nutrition of cattle and swamp buffalo, 23-24 August 2004. Faculty of Veterinary Science, Chulalongkorn University, Thailand. p. 125-129.

3. Promdireg, A., Adulyanubap, W. and Techakumphu, M. 2005. Usins early pregnant swamp buffalo cows for oocyte collection by OPU. The 4th Chulalongkorn University Annual Conference, 60th Vet Anniversary Building, Chulalongkorn University, 15 Febuary 2005.

4. Punquejana, W., Wachum, W., Chalermwanij, I., Promdireg, A. and Techakumphu, M. 2005. The detection of ovulation time in swamp buffaloes using real time B-mode ultrasound. The 4th Chulalongkorn University Annual Conference, 60th Vet Anniversary Building, Chulalongkorn University, 15 Febuary 2005.

International :

1. Promdireg, A., Techakumphu, M., Adulyanubap, W. and Na-Chiangmai, A. and 2003. *In vitro* fertilization of buffalo oocytes obtained by ovum pick up. Asia link symposium on animal reproduction in South-East Asia, 23-24 June 2003, Faculty of Veterinary Science, Chulalongkorn University, Thailand. p. 27.

2. Promdireg, A., Adulyanubap, W., Singlor, J., Na-Chiangmai, A. and Techakumphu, M. 2005. Ovum pick–up in cycling and lactating postpartum swamp buffaloes(*Bubalus bubalis*). Reprod. Dom. Anim. 40: 145-149.

3. Promdireg, A. 2006. Ovum pick up and *in vitro* fertilization in a Montbeliard cow. The 2nd symposium on animal reproduction: Trend of biotechnology for animal reproduction:

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4. Techakumphu, M., Promdireg, A. and Yindee, M. 2006. Ovarian activity during estrus cycle and early postpartum. International course of buffalo reproduction and reproductive biotechnology. Faculty of Veterinary Science, Chulalongkorn university, Thailand. p. 39-46.

5. Techakumphu, M. and Promdireg, A. 2006. Ovum pick up in Thai swamp buffaloes(*Bubalus bubalis*). International course of buffalo reproduction and reproductive biotechnology. Faculty of Veterinary Science, Chulalongkorn university, Thailand. p. 100-108.

6. Promdireg, A., Techakumphu, M., Singlor, J. and Presicce, G. 2006. Follicular dynamics during estrous cycle in swamp buffalo (in preparation)

BIOGRAPHY

Mr Akachart Promdireg was born in Bangkok province, Thailand, on May 23rd , 1975. He graduated with DVM from Khon Kaen University, Faculty of Veterinary Medicine, in 1999 and M.Sc. (Veterinary Science) from Chulalongkorn University in 2001. In 2002, he attended his Ph.D. study under the Royal Golden Jubilee Program, Thailand Research Fund, at the Department of Obstetrics Gynaecology and Reproduction, Faculty of veterinary Science, Chulalongkorn University.