การพัฒนาของตัวอ่อนระยะแรกหลังการฉีดตัวอสุจิเข้าไปในโอโอไซต์กระบือปลัก

นางสาววิบัณฑิตา จันทร์กิติสกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาวิทยาการสืบพันธุ์สัตว์ ภาควิชาสูติศาสตร์ เธนุเวชวิทยาและวิทยาการสืบพันธุ์
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ปีการศึกษา 2554

EARLY EMBRYONIC DEVELOPMENT AFTER INTRACYTOPLASMIC SPERM INJECTION OF SWAMP BUFFALO (*Bubalus bubalis*) OOCYTES

Miss Vibuntita Chankitisakul

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy Program in Theriogenology
Department of Obstetrics, Gynaecology and Reproduction
Faculty of Veterinary Science
Chulalongkorn University
Academic Year 2011

Thesis Title	EARLY EMBRYONIC DEVELOPMENT AFTER INTRACYTOPLASMIC SPERM INJECTION OF SWAMP BUFFALO (*Bubalus bubalis*) OOCYTES
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Field of Study	Theriogenology
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วิบัณฑิตา จันทร์กิติสกุล: การพัฒนาของตัวอ่อนระยะแรกหลังการฉีดตัวอสุจิเข้าไปในโอโอไซต์กระบือปลัก (EARLY EMBRYONIC DEVELOPMENT AFTER INTRACYTOPLASMIC SPERM INJECTION OF SWAMP BUFFALO (*Bubalus bubalis*) OOCYTES)

อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.นสพ.ดร. มงคล เตชะกำพุ, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ.นสพ.ดร. ธีรวัฒน์ ธาราศานิต, 72 หน้า.

สรีรวิทยาของการพัฒนาตัวอ่อนระยะแรกเป็นองค์ความรู้ที่สำคัญต่อการพัฒนาเทคโนโลยีชีวภาพทางระบบสืบพันธุ์ขั้นสูง อย่างไรก็ตามการศึกษาในกระบือปลักในปัจจุบันยังมีจำนวนน้อยและขาดข้อมูลพื้นฐานหลายประการด้วยกัน การศึกษาแบ่งเป็น สามการทดลองดังนี้

การทดลองที่ 1 มีวัตถุประสงค์เพื่อศึกษาการพัฒนาของตัวอ่อนระยะแรกโดยดูผลการเปลี่ยนแปลงของเซลล์โครงร่างซึ่งประกอบด้วยแอกตินไมโครฟิลาเมนต์ ไมโครทิวบูล และโครมาตินระหว่างการแบ่งเซลล์ระยะแรกในตัวอ่อนของกระบือปลัก ทำการเลี้ยงโอโอไซต์กระบือปลักให้พร้อมปฏิสนธิและทำการปฏิสนธิกับอสุจิในหลอดทดลอง จากนั้นนำไซโกตและตัวอ่อนที่ชั่วโมงต่างๆ กัน มาตรวจดูการเปลี่ยนแปลงของโครมาตินและเซลล์โครงร่างภายใต้กล้องจุลทรรศน์หลังการย้อมสีเรืองแสงฟลูออเรสซีนดังต่อไปนี้คือ ย้อมไมโครทิวบูลด้วย monoclonal-α-tubulin-TRIT C แอกตินไมโครฟิลาเมนต์ด้วย Alexa 488 phalloidin และโครมาตินด้วย DAPI ผลการศึกษาพบว่าเส้นใยโปรตีนที่ถูกสร้างขึ้นจากไมโครทิวบูลบริเวณฐานของหัวอสุจิในระหว่างการปฏิสนธิมีบทบาทสำคัญในการช่วยให้เกิดการเคลื่อนที่เข้ามาใกล้กันของโปรนิวเคลียสของเซลล์สืบพันธุ์เพศผู้และเพศเมีย ขณะที่เส้นใยโปรตีนชนิดแอกตินไมโครฟิลาเมนต์มีความเกี่ยวข้องกับการแบ่งเซลล์ของตัวอ่อน ความล้มเหลวของการปฏิสนธิที่เกิดขึ้น น่าจะมีสาเหตุมาจากความสามารถในการเจาะผ่านผนังโอโอไซต์ของตัวอสุจิ นอกจากนี้ การย่อยเปลือกหุ้มโอโอไซต์ให้บางลงไม่ส่งผลต่ออัตราการปฏิสนธิ

การทดลองที่ 2 มีวัตถุประสงค์เพื่อ 1) ศึกษาผลการฉีดตัวอสุจิเข้าไปในโอโอไซต์ระยะพร้อมปฏิสนธิของกระบือปลักร่วมกับการกระตุ้นหรือไม่กระตุ้นโดยสารเคมี โดยดูการเปลี่ยนแปลงโปรนิวเคลียสของเซลล์สืบพันธุ์เพศผู้ 2) เปรียบเทียบการเจริญของตัวอ่อนภายหลังการฉีดตัวอสุจิเข้าไปในโอโอไซต์ร่วมกับจากการกระตุ้นโดยสารเคมี การฉีดเข็มเข้าไปในโอโอไซต์โดยไม่มีตัวอสุจิร่วมกับการกระตุ้นด้วยสารเคมี กับกลุ่มควบคุมได้แก่การปฏิสนธิกับตัวอสุจิในหลอดทดลอง 3) ศึกษาการแสดงออกของยีน *Nnat* ที่ควบคุมโดยเซลล์สืบพันธุ์เพศผู้ เพื่อแยกแยะว่าตัวอ่อนที่เจริญถึงระยะบลาสโตซีสเกิดขึ้นจากการปฏิสนธิจากตัวอสุจิหรือจากการกระตุ้นด้วยสารเคมี ผลการศึกษาพบว่าอัตราการเกิดโปรนิวเคลียสในกลุ่มที่ฉีดตัวอสุจิเข้าไปในโอโอไซต์ร่วมกับจากการกระตุ้นโดยสารเคมี และกลุ่มที่ฉีดเข็มเข้าไปในโอโอไซต์โดยไม่มีตัวอสุจิร่วมกับการกระตุ้นด้วยสารเคมี สูงกว่ากลุ่มที่ฉีดตัวอสุจิเข้าไปในโอโอไซต์เพียงอย่างเดียวอย่างมีนัยสำคัญทางสถิติ อย่างไรก็ตามเนื่องจาก 90.9% ของโปรนิวเคลียสที่เกิดขึ้นในกลุ่มที่ฉีดตัวอสุจิเข้าไปในโอโอไซต์ร่วมกับจากการกระตุ้นโดยสารเคมีนั้นไม่มีการเปลี่ยนแปลงของอสุจิไปเป็นโปรนิวเคลียส จึงอาจเป็นไปได้ว่าการเปลี่ยนแปลงที่เกิดขึ้นเป็นผลมาจากการกระตุ้นของสารเคมี อีกทั้งการพัฒนาของตัวอ่อนไปถึงระยะโมรูล่าและบลาสโตซีส ร่วมกับจำนวนเซลล์ในระยะบลาสโตซีสไม่มีความแตกต่างกันทางสถิติระหว่างกลุ่มที่ฉีดตัวอสุจิเข้าไปในโอโอไซต์ กลุ่มที่ฉีดเข็มเข้าไปในโอโอไซต์โดยไม่มีตัวอสุจิ และกลุ่มที่เกิดจากการปฏิสนธิกับตัวอสุจิในหลอดทดลอง อีกทั้งไม่พบการแสดงออกของยีนในตัวอ่อนที่เกิดจากการฉีดตัวอสุจิเข้าไปในโอโอไซต์บ่งชี้ถึงความล้มเหลวของการฉีดตัวอสุจิเข้าไปในโอโอไซต์ในกระบือปลัก

การทดลองที่ 3 มีวัตถุประสงค์เพื่อเพิ่มการเกิดโปรนิวเคลียสของเซลล์สืบพันธุ์เพศผู้ภายหลังจากเตรียมตัวอสุจิก่อนฉีดเข้าไปในโอโอไซต์ด้วยวิธีการต่างๆกัน คือ (1) 0.1% Triton-X 100,(2) 10 μM calcium ionophore (CaI), (3) แช่แข็งที่อุณหภูมิ -20°C และ (4) กลุ่มควบคุม จากนั้นตัวอสุจิดังกล่าวจะถูกแบ่งออกเป็นสองกลุ่ม โดยกลุ่มหนึ่งจะถูกนำไปเตรียมต่อด้วย 5 M dithiothreitol (DTT) จากนั้นทำการประเมินความสมบูรณ์ของอะโครโซมและดีเอนเอด้วยสีฟลูออเรสซีน (FITC-PNA) และ TUNEL ตามลำดับ นำตัวอสุจิแต่ละกลุ่มมาฉีดเข้าไปในโอโอไซต์ ผลการศึกษาพบว่าตัวอสุจิในกลุ่มที่เตรียมด้วย Triton-X 100 และ CaI ส่งผลให้เกิดการสูญหายไปของอะโครโซมอย่างมีนัยสำคัญกว่ากลุ่มที่แช่แข็งที่อุณหภูมิ -20 °C และกลุ่มควบคุมที่เกิดเพียงอะโครโซมรีแอคชั่น การเตรียมตัวอสุจิด้วยDTT ไม่มีผลต่อการเปลี่ยนแปลงของอะโครโซม การเตรียมตัวอสุจิแต่ละวิธีไม่มีผลกระทบต่อความเสียหายของดีเอนเอ ผลประเมินการเปลี่ยนแปลงของตัวอสุจิที่ชั่วโมงที่ 18 หลังการฉีดพบว่าโปรนิวเคลียสของเซลล์สืบพันธุ์เพศเมียเกิดขึ้นเฉพาะกลุ่มที่ได้รับการกระตุ้นด้วยสารเคมีภายหลังการฉีดตัวอสุจิเข้าไปในโอโอไซต์ อย่างไรก็ตาม ส่วนใหญ่พบว่าตัวอสุจิไม่เกิดการเปลี่ยนแปลงไปเป็นโปรนิวเคลียส การเจริญของตัวอ่อนปกติที่ประกอบด้วยโปรนิวเคลียสของเซลล์สืบพันธุ์เพศผู้และเพศเมีย โดยไม่มีตัวอสุจิอยู่ข้างใน พบเฉพาะกลุ่มที่ตัวอสุจิถูกเตรียมด้วยCaI กลุ่มที่แช่แข็งที่อุณหภูมิ -20 °C และกลุ่มควบคุม ร่วมกับการเตรียมด้วย DTT (8.9, 23.5, และ 31.0% ตามลำดับ) การศึกษานี้สรุปได้ว่าการเตรียมตัวอสุจิให้เกิดอะโครโซมรีแอคชั่นร่วมกับการเตรียมโดยใช้ DTT ก่อนการฉีดตัวอสุจิเข้าไปในโอโอไซต์และกระตุ้นด้วยสารเคมีหลังการฉีด ส่งผลให้เกิดการเปลี่ยนแปลงตัวอสุจิไปเป็นโปรนิวเคลียสของเซลล์สืบพันธุ์เพศผู้ได้

งานวิจัยฉบับนี้ถือเป็นครั้งแรกที่ทำการศึกษากระบวนการพัฒนาตัวอ่อนระยะแรกในกระบือปลัก โดยรายงานการเปลี่ยนแปลงของโครมาติน และเซลล์โครงร่างซึ่งประกอบด้วย แอกตินไมโครฟิลาเมนต์และไมโครทิวบูล และยังได้บ่งชี้ถึงช่วงเวลาการเกิดการเปลี่ยนแปลงต่างๆในกระบวนการปฏิสนธิในระยะแรกของกระบือปลัก ความรู้และเทคนิคที่ใช้สามารถนำไปประยุกต์ใช้เป็นเครื่องมือในการติดตามกระบวนการพัฒนาของตัวอ่อนต่อไป นอกจากนั้นงานวิจัยฉบับนี้ยังเป็นครั้งแรกที่ยืนยันความล้มเหลวของการฉีดตัวอสุจิเข้าไปในโอโอไซต์ในกระบือปลัก การเตรียมอสุจิก่อนการฉีดเข้าไปในโอโอไซต์ถือว่ามีความสำคัญต่อความสำเร็จของการเกิดการปฏิสนธิที่เกิดจากเซลล์สืบพันธุ์เพศผู้และเพศเมีย

ภาควิชา...สูติศาสตร์ เธนุเวชวิทยาและวิทยาการสืบพันธุ์..... ลายมือชื่อนิสิต.....

สาขาวิชา ...วิทยาการสืบพันธุ์สัตว์..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา ..2554..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

5075560931: MAJOR THERIOGENOLOGY
KEYWORDS: INTRACYTOPLASMIC SPERM INJECTION/EARLY
EMRYODEVELOPMENT/PARTHENOGENESIS/ GENE EXPRESSION / SWAMP BUFFALO

VIBUNTITA CHANKITISAKUL: EARLY EMBRYONIC DEVELOPMENT AFTER INTRACYTOPLASMIC SPERM INJECTION OF SWAMP BUFFALO (*Bubalus bubalis*) OOCYTES.ADVISOR: PROF. MONGKOL TECHAKUMPHU, D.V.M., DOCTORAT 3^{e} CYCLE, CO-ADVISOR: ASST. PROF. THEERAWAT THARASANIT, D.V.M., Ph.D.72 pp.

Physiology of early embryonic development is required for a further application of reproductive biotechnology in swamp buffalo. However nowadays a few studies were reported and basic knowledge is limited. This thesis composes of three parts as follows:

EXP.1aimed to study the dynamics of early embryonic development, in terms of redistribution of cytoskeleton (microtubules, actin microfilaments) and chromatin configurations during the first cell cycle in swamp buffalo embryos. Swamp buffalo oocytes were matured and fertilized *in vitro*, presumptive zygotes and embryos were fixed at various time points post-IVF. Microtubules, microfilaments and chromatin were fluorescently labeled using monoclonal-α-tubulin, Phalloidin and DAPI, respectively. The redistribution pattern of cell cytoskeleton and chromosome of the zygotes and embryos was examined under an epifluorescent microscope. The results indicated that a dense network of microtubules or sperm aster in which radiating from the base of the decondensing sperm head plays a crucial role in the fertilization events about migration and apposition of male and female pronuclei in a normal fertilization process whereas microfilaments are considerably required for contractile ring formation during cleavage. Fertilization failure, at least in our current culture system, is predominantly caused by poor sperm penetration. However, partial digestion of ZP did not improve fertilization rate.

EXP. 2 aimed to : 1) examine the efficiency of intracytoplasmic sperm injection (ICSI) technique, with or without chemical activation of *in vitro* matured buffalo oocytes, on sperm head decondensation; 2) compare the subsequent development of embryos following activation of ICSI (ICSI (+) activation group) and sham injection (Sham (+) activation group) oocytes (embryos obtained by *in vitro* fertilization of IVM oocytes served as a control group); and 3)clarify whether blastocysts were derived from syngamy or parthenogenesis, expression of *Nnat*, a paternally-expressed gene in blastocysts derived from IVF, ICSI and oocyte activation without sperm or sham injection was additionally examined using RT-PCR. Pronuclear formation rates in ICSI (+) activation and Sham (+) activation groups were higher than that of ICSI without activation ($P<0.05$). However, since 90.9% of presumptive zygotes in ICSI (+) activation group demonstrated pronuclear formation with an intact sperm head, we inferred that most were parthenotes. Neither developmental competence (morula and blastocyst formation rates) nor mean total cell number of blastocysts was significantly different among ICSI (+) activation, Sham (+) activation and IVF groups. Expression of *Nnat* mRNA was not detected in ICSI (+) activation blastocysts, indicating failure of male genome activation.

EXP. 3 aimed to improve sperm head decondensation by pretreating spermwith various chemicals before ICSI. Sperm were treated with the following protocols; (1) 0.1% Triton-X 100 (TX), (2) 10 μM calcium ionophore (CaI), (3) freezing and thawing (FT) without any cryoprotectant, and (4) untreated control. In each treatmentsperm were then either treated or not with 5 mMdithiothreitol (DTT). Acrosome integrity and DNA fragmentation were evaluated in sperm before ICSI by staining of sperm with fluorescein isothiocyanate–labeled peanut agglutinin and TUNEL, respectively. Then *in vitro* matured oocytes were subjected to ICSI using sperm pretreated as described above. The results revealed significantly increased rates of acrosome-lost sperm cells after TX and CaI treatments, whereas FT treatment and no-treatment (control) significantly increased the proportion of acrosome-reacted sperm. DTT treatment had no significant effect on acrosome configuration of sperm. DNA fragmentation was not significant difference among treatments. At 18 h post-ICSI, female pronucleus (PN) formation was found only in activated oocytes. However, among all the activated ICSI oocytes, the majority of them contained intact sperm heads. Normal fertilization characterized by two PNs without intact sperm head was only observed in CaI and FT treatment and control groups when sperm were treated with DTT before ICSI. In conclusion, these results indicated that DTT treatment of sperm with reacted acrosome before ICSI together with an additional activation of the resultant ICSI oocytes are important for successful sperm head decondensation resulting in male pronuclear formation.

This study is the first report to examine the redistribution of cytoskeleton (microtubules, actin microfilaments) and indicates the dynamic of early embryo development during the first cell cycle in swamp buffalo. The fundamental knowledge and techniques from our study can be used as a tool for further investigating the embryonic development. In addition, this study confirms for the first time about the failure of traditional ICSI technique in swamp buffalo oocytes. Sperm treatment before ICSI is nescessory for successful production of normally fertilized embryos.

Department: Obstetrics, Gynaecology and Reproduction…….. Student's Signature…….....
Field of Study: Therigenology…….. Advisor's Signature……..……
Acedemic Year..2011……..……. Co-advisor's Signature……...……..

ACKNOWLEDGEMENTS

This research was conducted at the Department of Obstetrics, Gynecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. Financial support was received from the Chulalongkorn University (CU) graduate scholarship (commemorating the 72nd anniversary of his majesty King BhumibolAdulyadej), the Royal Golden Jubilee PhD program (Thailand Research Fund), the CU CHE-TRF Senior Research Fund (RTA 5080010), a Graduate school thesis grant, the 90th anniversary of Chulalongkorn university fund (Ratchadaphiseksomphot Endowment Fund), and the Master Research Grants Window II (TRF-WindowII).

I would like to express my sincere gratitude to my advisor, Professor Mongkol Techakumphu, who kindly accepted me to work under his guidance and generously supported me during my course of study. I would also like to thank my co-advisor, Assistant Professor Theerawat Tharasanit, for his moral support and valuable suggestions on my work, as well as another co-advisor, Dr. Takashi Nagai, for the opportunity to learn advanced *in vitro* embryo production technologies and for taking care of me during the time I was in Japan. He posseses an ideal mix of science and humanity, and I do love his way. I'm very grateful to Dr. Nawapen Phutikanit for instruction and assistance in setting up the molecular laboratory, as well as her patience in listening to and helping me addresses the many problems that occurred along the way. I am indebted to Dr. Tamas Somfai for valuable advice on the manuscript and to Dr. Michiko Nakai for instruction on ICSI technique. Also my special thanks to the staff of NILGS who took care of me and showed me the friendship while I was living in Japan. I also would like to thank Associate Professor Daungsmorn Suwattana and Ms. Jutarat Jirasupphachok for allowing me to perform my experiments in their laboratory and for many useful suggestions.

I would like to thank Mr. Kriengsak Tasripoo for his initial guidance in *in vitro* production and ICSI technique, as well as Mr. Somsak Binson and the staffs at the innovation center for veterinary science for their assistance in the collection of ovaries throughout the study and for their hospitality in welcoming me to perform the experiment in their facilities.

Many thanks to Dr. Nutthee Am-in, whom spent many late nights standing beside me always during conducting the experiments and also, helped me to analyze the thesis data. His warm friendship always give me the confidence to overcome every obstacle. Also, I was surrounded by many lovely friends, Dr. Panida Chanapiwat, Dr. Sirirak Buapeung, Dr. Thanida Sanunmuang, and Dr. Paweena Thuwanut, who shared my ups and downs in daily life. I'm really happy to have them as my friends. Special thanks to all who directly or indirectly helped me to achieve my success in the end. Finally, last but most important, my sincere thanks to my family for their support. Without their encourage, today would not have been possible. Please know that I have this success because of you and for you.

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A	adenine
AI	artificial insemination
AII	anaphase II
ARTs	assisted reproductive technologies
B	blastocyst
BCB	brilliant cresyl blue
BES	buffalo estrous serum
bFF	buffalo follicular fluid
BSA	bovine serum albumin
C	cytosine
CaI	calcium ionophore
Ca^{2+}	calcium ions
cDNA	complementary DNA
CHX	cycloheximide
Cl	cleavage
COCs	cumulus oocyte complex
CO_2	carbon dioxide
CSF	cytostatic factor
DAPI	4', 6-diamidino- 2-phenylindole
DMAP	6-dimethylaminopurine
DNA	deoxyribonucleic acid
dNTP	the mixture of four deoxyribonucleotides: dATP (deoxy adenine triphosphate), dCTP (deoxy cytosine triphosphate), dGTP (deoxy guanine triphosphate), dTTP (deoxy thymine triphosphate)
DTT	dithiothreitol
eCG	equine chorionic gonadotropin
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EthD-1	Ethidium homodimer-1
FCS	fetal calf serum
FGF	fibroblast growth factor
FITC-PNA	fluorescein isothiocyanate–labeled peanut (*Arachishypogaea*) agglutinin
FP	forward primer
FSH	follicle stimulating hormone
FT	freezing and thawing
G	guanine
GAPDH	glyceraldehyde 3-phosphate dehydrogenase

GSH	glutathione
h	hour
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICSI	intracytoplasmic sperm injection
IGF	insulin-like growth factor
IGF-IIR	insulin-like growth factor II receptor
IU	international unit
IVEP	*in vitro* embryo production
IVF	*in vitro*fertilization
IVM	*in vitro* maturation
M	morula
MAPK	mitogen activated protein kinases
MEG3	maternal expressed gene3
mg	milligram
MI	metaphase I
min	minute
MII	metaphase II
mL	milliliter
mm	millimeter
mM	millimole
MPN	male pronuclear
MPF	M-phase promoting factor/mitosis promoting factor
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NaH_2PO_4	monosodium phosphate
NaOH	sodium hydroxide
NEAA	nonessential amino acids
ng	nanogram
Nnat	neuronatin
NT	nuclear transfer
N/A	not available information
N_2	nitrogen
OA	oocyte activation
OH^-	hydroxide
OPU	ovum pick up
PA	parthenogenesis

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGF2α	prostaglandin F2α
PHE	penicillamine, hypotaurine and epinephrine
PLCζ	phospholipase lipase C zeta
PMSG	pregnant mare serum gonadotropin
PN	pronuclear formation
PT	perinuclear theca
PVP	poly-vinylpyrrolidone
P1	protamine-1
rhFSH	recombinant human follicle-stimulating hormone
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
RP	reverse primer
RT	reverse transcriptase
s	second
SOAF	sperm-borne oocyte-activating factor
SOF	synthetic oviductal fluid
S.D.	standard deviation
T	thymine
TBE	tris, Borate, EDTA
TCM-199	tissue culture medium 199
TCN	total cell number
TdT	terminal deoxynucleotidyltransferase
TII	telophase II
TRITC	tetramethylrhodamineisothiocyanate
TUNEL	terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick end labeling
TX	triton-X 100
Tyrode	tyrode's balanced salt solution
v/v	volume/volume
WGA	wheat germ agglutinin
w/v	weight/volume
XIST	X-inactive specific transcript
ZP	zona pellucida
µg	microgram
µL	microliter
µM	micromole
C	degree Celsius
β-ME	β-Mercaptoethanol

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Swamp buffalo (*Bubalus bubalis*) is closely related with Thai farmers' lifestyle as complementary with their crop production for several centuries. However, nowadays the numbers of swamp buffalo in Thailand have decreased tremendously due to an invasion of the industry into Thai rural areas. In addition, buffalo is known for poor reproductive due to delayed puberty, silent estrus, and long postpartum period to estrus, poor conception rate and seasonal anestrus. It is well accepted that assisted reproductive technologies (ARTs) are effectively concerns in breeding and disease control. They have been well developed and applied in cattle, sheep and goat. However, in buffalo, the ARTs have some obstracles to implement due to a lack of some knowledge. To increase their numbers and espeacially to improve reproductive efficiency and conserve the greater genetic, the applications of reproductive technologies which are widely used in cattle are considered. The cattle have been served as a model for developing the biotechnology in swamp buffalo as they have the similarity of reproductive physiology species. Recently, reproductive technologies in swamp buffalo have been therefore increasingly studied and used in livestock production in Thailand even the numbers of studies performed on this species are very few when compared with cattle. For instance, artificial insemination (AI), a basic reproductive technique, was studied by Chantaraprateep et al. since 1979. This technique is also more effective if it is used with precise estrus detection. Estrus cycle control by estrus synchronization has been recognized as an important tool for improving reproductive performance in buffaloes. The PGF2α (Chantaraprateep et al., 1981) and intravaginal impregnated with progesterone (PRIDs and CIDRs) as well as ear implants with potent progestagen (Norgestomet) have been successfully used to synchronize estrus cycle in swamp buffaloes (Chantaraprateep et al., 1983). In addition, embryo transfer technique has also been attempted by Lohachit et al., (1987). The first pregnancy after transfer fresh embryos has been reported by Chantaraprateep et al. (1989) and later the successful transfer of frozen-thawed embryos to a recipient was reported by Techakumphu et al. (1998), although the fetus was resorbed at the 3rd month of gestation. However, the overall success rate in terms of calves born is still low for these techniques.

An increased interest in *in vitro* embryo production (IVEP) technologies for achieving rapid genetic improvement was increasing. Madan et al. (1994) firstly reported the success production of buffalo calves derived from *in vitro* maturation and fertilization techniques (IVM-IVF). Thereafter, IVEP techniques have been continuously investigated which aimed to serve as a fundamental research and also to improve breeding strategies as well as to provide routinely applies on the farm. Unfortunately, there are many experiments in which the success rates of IVEP are markedly unsatisfied comparing with cattle, and the majority of these studies used protocols already established for cattle. The problems are usually initiated with the failure of oocytes to develop to cleavage stage following *in vitro* fertilization (9%: Totey et al., 1992; 23.4-36.5%: Nandi et al., 2002; 17.9-31.8%; Songsasen and Apimeteetumrong, 2002). Fundamental study of these aspects is required for understanding more events and characterizing the factors associated with fertilization failure providing a new knowledge of early embryo

development and also facilitating the improvement of *in vitro* embryo production techniques in this species in the future. Therefore, our studies were focused on basic events that associated with gamete interaction as well as dynamics of swamp buffalo early embryo development during fertilization.

In addition to conventional IVF, intracytoplasmic sperm injection (ICSI) is an alternative tool to conventional IVF especially in case of human infertility. More importantly, ICSI can be applied with sex-sorted sperm, transgenic production, and endangered species conservation. Some evidences in bovine species indicated that *in vitro* development into blastocyst embryos obtained using ICSI was either not different or higher than IVF (Li et al., 1999). It was convincingly concluded that intracytoplasmic sperm injection can be successful at least in producing normal embryos of swamp buffalo or providing normal calves. However, nowadays only few studies of ICSI in buffaloes are reported (Liang et al., 2011a; Liang et al., 2011b). In order to develop this technique, more fundamental studies on the process of ICSI, including sperm preparation, sperm injection, oocyte activation and sperm development following ICSI were also interesting to be explored.

In human, ICSI provide a high fertilization rate and high pregnancy outcomes (Palermo et al., 1992) however this technique is far less efficient in livestock species (Yanagimachi, 2005). It appears to be species differences that determine whether an oocyte tolerates sperm injection and whether sperm injection alone can lead to fertilization and activation of an oocyte, resulting in embryonic development (Matsukawa et al., 2007). In rabbits, mice and human, ICSI alone is sufficient to activate oocytes for further development (Keefer, 1989; Tesarik et al., 1994). In contrast, additional activation of oocytes after sperm injection is necessary to obtain cleavage in cattle and pigs (Lee et al., 2002; Liu and Yang, 1999; Rho et al., 1998b). Moreover, there is evident that sperm pretreatment may facilitate the release of sperm-borne oocyte-activating factor (SOAF), resulting in improve the pronuclear formation rate for ICSI in mice, pigs, and cattle (Galli et al., 2003; Lee et al., 2004; Tian et al., 2006). Unfortunately, the technology of ICSI embryo producing is poorly investigated in swamp buffalo. In the present study, it is therefore essential to examine sperm preparation and oocyte activation affecting to ICSI buffalo embryo development.

Although the majority of studies demonstrated that artificial activation of bovine oocytes after conventional ICSI clearly improved fertilization and blastocyst rates (Fujinami et al., 2004; Suttner et al., 2000), the report of birth calves is restricted following transfer embryos to recipient females (Galli et al., 2003; Horiuch et al., 2002; Oikawa et al., 2005). It is hypothesized that blastocyst embryos may be largely derived from parthenogenesis. However, it has to be clarified. The molecular techniques can be used to identify gene expression of *in vitro* culture embryos facilitating the identification of the normality of embryos ICSI produced.

Therefore, the basic information in this species has not well elucidated which is needed to know before applying a further advanced technology in the species. Our overall

objectives aimed to examine and describe the gamete interaction and chronology of early embryonic development, in particular the redistribution of cell cytoskeleton and chromatin configurations after *in vitro* fertilization in swamp buffalo as well as to develop efficiency of ICSI technique which are focused on sperm preparation and oocyte activation affecting to ICSI embryo development. Molecular techniques were also applied to determine gene expression profiles of embryos developed between sexual and asexual reproduction. The knowledge acquired from this study would provide the basic information during fertilization and early embryo development *in vitro*. It would principally facilitate the development of IVEP technique and help to characterize the factors associated with fertilization failure in IVEP production. Besides, ICSI would be at least in producing normal embryos of swamp buffalo or providing high develop into normal offspring after transfer embryos to recipients as well. The research frameworks provide ‚a new window' of basic embryological physiology and also facilitate the improvement of *in vitro* embryo production technique in this species in the future.

1.2 Literature reviews

1.2.1 The first cell cycle of embryo development

In mammal, fertilization is a physiological event by the fusion of capacitated acrosome reacted sperm and matured oocyte, after allowing sperm to pass through the zona pellucida (ZP) into ooplasm (Sutovsky et al., 1997; Yanagimachi, 2005). There are several hypothesis that the penetration of sperm initiates important signaling molecules that trigger the meiosis resumption by increasing in cytosolic free Ca^{2+} within oocyte (Jaffe, 1980; Jones and Whittingham, 1996; Swann et al., 1989), eventually leading to female pronuclear formation. Meanwhile, the disruption of disulfide bonds in the DNA packaging protamines guiding by the oocyte-synthesized tripeptide glutathione (GSH) is a prerequisite for decondensation of the fertilizing sperm nucleus, later become to male pronuclear (Perreault et al., 1984). At the beginning of pronuclear development, microtubules have been demonstrated to play an important role that facilitates the migration, apposition as well as syngamy of male and female pronuclei, resulting in diploid zygote nucleus and subsequently to cleave to 2 cells (Figure 1).

These microtubules are paternally inherited in most mammalian species, including human (Gook et al., 1998; Sathananthan, 1998), sheep (Le Guen and Crozet, 1989), rabbit (Yllera-Fernandez et al., 1992), porcine (Kim et al., 1996), bovine (Alomar et al., 2008; Long et al., 1993; Sathananthan et al., 1999) and rhesus monkey (Hewitson et al., 2000; Prather and Day, 1998). Contrastly, the paternal centrosome in the ooplasm is functionally absent in mice and thus the syngamy of the two pronuclei requires the maternal centrosome (Schatten et al., 1985). In addition to the vital role of microtubules, actin microfilaments, another major component of the cytoskeleton, also play an important part in the syngamy and cell division in embryos during the first cell cycle of

embryo development as reported in Xenopus (Noguchi and Mabuchi, 2001), mouse (Schatten and Schatten, 1986) porcine (Kim et al., 1997) and bovine (Rivera et al., 2004) embryos. However, the events of gamete interaction and early embryo development especially during fertilization have poorly reported in the swamp buffalo. Morphological studies had been performed only *in vivo*, showed the difference of chronology of early embryo development between cattle and swamp buffalo (Chantaraprateep et al., 1989). Understanding the redistribution patterns and role of microtubules and actin microfilaments during fertilization *in vitro* will provide fundamental knowledge of early embryo development in swamp buffalo and may improve *in vitro* embryo production techniques principally by the characterization of factors associated with fertilization failure in this species.

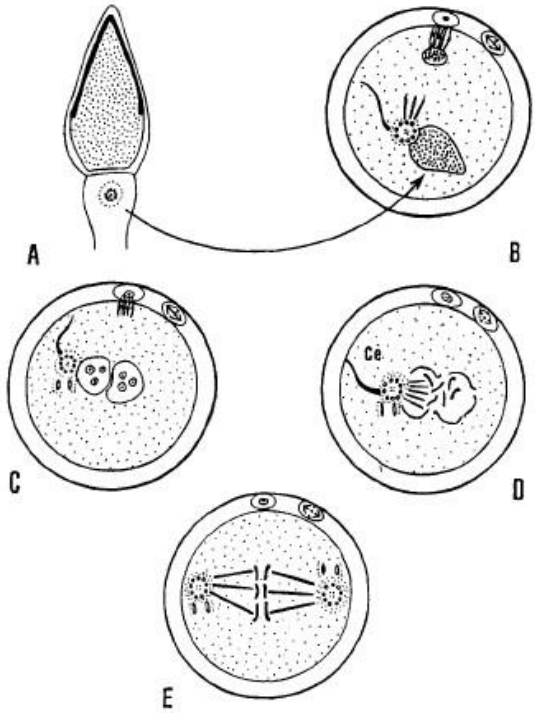

Figure1. Paternal centrosomal dynamics at fertilization. (A) Sperm proximal centriolar inheritance in sperm neck. (B) Incorporated sperm in ooplasm showing developing sperm aster. (C) Duplicating sperm centriole associated with the male pronucleus. Note the close association between male and female pronuclei. (D) Sperm aster (centrosome with duplicated centriole) organizing the first mitotic spindle at prometaphase. Pronuclear envelopes are disorganizing and the chromosomes are condensed. (E) The first bipolar mitotic spindle at syngamy showing duplicated centrioles (diplosomes) at either pole. Note the increase in pericentriolar material around centrioles and chromosomes at the equator (metaphase) (Sathananthan, 1998)

1.2.2 Oocyte recovery

Cattle or buffalo oocytes can be collected either by collection from ovaries of slaughtered animals or by ovum pick up (OPU) from live animals (Boni et al., 1996; Manjunatha et al., 2007; Promdireg et al., 2005). Neglia et al. (2003) reported that the sources of buffalo oocytes from OPU have higher blastocyst yield than slaughtered-ovaries. However, slaughtered-ovaries provide a cheap and abundant source of oocytes (Nandi et al., 2002). Oocyte collection from slaughtered animals was attempted by using techniques of follicle puncture or aspiration or ovarian slicing. Though the oocyte recovery rate was higher using ovarian slicing (Boni et al., 1996), follicle aspiration was the most widely accepted technique due to the easiness and speed of operation (Nandi et al., 2004). However, there no significant differences among three methods as long as only good quality oocytes were selected (Boni et al., 1996).

The OPU technique is a non-invasive and repeatable for recovery a large numbers of meiotically competent oocytes from antral follicles in live animals. Ultrasound-guided transvaginal aspiration usually involves superovulation protocol in young prepubertal calves in which donors were hormonal-stimulated. The OPU would be performed in various statuses of animal, non-lactating, cycling and lactating postpartum buffalo which were possible to harvest the oocytes and matured and fertilized *in vitro* (Promdireg et al., 2005)

It is generally accepted that low oocyte numbers are obtained per ovary in buffalo. According to Madan and et al. (1994), only 0.42 good oocytes per ovary was recovered in buffalo, compared unfavorably with up to average of 10 oocytes per ovary reported among exotic breed of bovines. The low recovery rate resulted from (1) a considerably lower number of primordial follicle reserve; (2) the lower number of antral follicle at all stages of estrous cycle; (3) a high incidence of follicle atresia; and (4) slaughtering of buffaloes at a sub-fertile, unproductive state, with aged and detrimental body condition. This poor oocyte recovery of the buffalo has become the major impediment in multiplication and genetic improvement of this species (Nandi et al., 2004)

1.2.3 Oocyte selection

Normally, the standard of grading oocytes is done by morphological appearance of cumulus cell investments and homogeneity of ooplasm as the following criteria (Chauhan et al., 1997; Ravindranatha et al., 2003):

Grade A: good-oocytes with more than four layers of compact cumulus cells and with granular homogenous oolpasm

Grade B: fair-oocytes with two to three layers of compact cumulus cells and with granular homogenous oolpasm

Grade C: poor-oocytes with one layer of cumulus cells or denuded oocytes or oocytes with scattered cumulus cells and with irregular dark ooplasm

Madan et al. (1994) reported in buffalo the average number of good-quality COCs with more than 3 layers of compact cumulus cells is 0.42 for high-quality COCs per ovary. The oocytes with intact layers of cumulus cells showed better maturation rate or cleavage rate than oocytes without cumulus cells; 64% with ≥ 3 layers of cumulus cells vs. 8.6% with partial or no cumulus cells (Warriach and Chohan, 2004), 64% for grade 1 vs. 46% for grade 4 (Pavasuthipaisit et al., 1992), 70% for good quality vs. 14% for poor quality (Abdoon et al., 2001). However, some oocytes with apparently normal morphology were in the early stage of degeneration. This may reduce the yield of transferable embryos. To establish a non-invasive method for selecting more homogenous and developmentally competent oocytes for IVEP, brilliant cresyl blue (BCB) staining has been used to identify the most competent oocytes (Manjunatha et al., 2007). The BCB is a vital blue dye, which determines the intracellular activity of glucose-6-phosphate dehydrogenase (*G6PDH*), an enzyme synthesized in growing oocytes but with less activity in grown oocytes. The result showed percentage of blastocysts development in BCB+ was higher than BCB-, thus staining of oocytes with BCB before IVM may be useful for oocyte selection in order to increase the *in vitro* embryo production. Moreover, buffalo oocytes displayed a size-dependent ability to undergo meiotic maturation; oocytes from > 4 mm follicles were at the final stage of nuclear maturation and should be considered for *in vitro* production (Yousaf and Chohan, 2003). Therefore the numbers of good quality oocytes depends on the method of oocytes retrieval, reproductive status, stages of estrous cycle, presence of corpus luteum and follicle size.

1.2.4 *In vitro* maturation (IVM)

Normally, the oocytes recovered from the growing follicles are arrested at the immature stage which is not suitable for fertilization. The step of oocyte maturation is prerequisite before fertilization. The maturation medium and the selection of protein supplements and hormones for IVM play an important role in the subsequent fertilization and development of mammalian oocytes during *in vitro* culture (Totey et al., 1993b). The standard media for IVM were tissue culture medium-199 (TCM-199) and Ham's F10 (Totey et al., 1993b). Recent studies demonstrating a higher IVF embryo production efficacy was obtained by using a maturation medium as CR1aa (Abdoon et al., 2001) which showed a significantly higher cleavage rate than TCM-199 (56 vs. 32% respectively). Oocytes were mostly incubated under paraffin oil at 38.5°C in a 5% CO_2 and 90-95% relative humidity during IVM which found to be optimum for *in vitro* embryo production (Ravindranatha et al., 2003). Maturation medium may be supplemented with serum such as fetal calf serum (FCS) and buffalo estrous serum (BES) which were found to be suitable culture system for IVM buffalo oocytes than medium alone (80.0, 76.0 and 34.5%, respectively) (Totey et al., 1993b). Addition of gonadotropins (FSH or eCG) (Abdoon et al., 2001; Totey et al., 1992), PMSG (Gupta et al., 2001) in maturation medium has been demonstrated to improve resumption of

meiosis, produced higher cleavage rate and developmental rate of IVF than control. In addition, positive effect of gonadotropin and serum in the IVM medium could be mimicked by 20% (v/v) buffalo follicular fluid (bFF) (Chauhan et al., 1997) but follicular fluid gave inferior embryo development when compared with serum (Nandi et al., 2004). The supplementation of cysteamine (Gasparrini et al., 2000; Gasparrini et al., 2003) or beta-mercaptoetanol (Songsasen and Apimeteetumrong, 2002) which were thiol compounds in protecting cells from oxidative damages also increased cleavage rate and transferable embryos. Furthermore, growth factors such as insulin-like growth factor I (Pawshe et al., 1998), insulin-like growth factor II (Chauhan et al., 1998), epidermal growth factor (EGF) which also helped improving the maturation, cleavage and blastocyst formation rates. When compared between 20 ng/ml EGF and 20 ng/ml fibroblast growth factor (FGF) supplemented into TCM-199 medium, Nandi et al. (2003) found that EGF was more benefits on buffalo oocyte maturation and embryo cleavage than FGF (83.0 vs. 66.6% and 52.2 vs. 40.2%, respectively). It was inferred that the majority of buffalo oocytes accomplish nuclear maturation (metaphase II) between 21 and 24 hrs of *in vitro* maturation. Increasing duration of IVM (from 18 to 30 hr) linearly decreased both cleavage and blastocyst rates (Gasparrini et al., 2008)

1.2.5 *In vitro* fertilization (IVF) and *in vitro* culture (IVC)

The matured oocytes can be fertilized and matured in *in vitro* in buffalo as demonstrated in other species. However, the quality of oocytes plays an important role of IVF success in cattle. Oocyte surrounded with multilayer cumulus cells so-called "cumulus oocyte complexes (COCs)" is classified as a grade A oocyte. It has been reported a positive effect of cumulus cells on IVF rates in cattle (Chian et al., 1995; Nandi et al., 1998; Younis and Brackett, 1991). For example, the proportion of fertilized oocytes was significantly higher in cumlus-intact (52%) than in cumulus-free group (30%) at 1-2 × 10^6 sperm/ml in buffalo (Nandi et al., 1998). Converstly, Ball et al. (1983) was demonstrated a negative effects of cumulus cells, which were found to interfere with sperm penetration (Chian et al., 1995; Younis and Brackett, 1991). Buffalo oocytes were usually co-incubated with frozen-thawed sperm in Tyrode's modified medium (TALP, Totey et al., 1996) or Brackett and Oliphant (BO, Nandi et al., 1998) medium for fertilization. Sperm capacitation was carried out by incubating motile sperm separately by swip-up (Nandi et al., 1998) or Percoll gradient techniques (Totey et al., 1996) from frozen-thawed semen in medium containing sperm motility enhancer such as heparin and caffeine (Chauhan et al., 1998; Nandi et al., 1998), or a mixure of penicillamine, hypotaurine and epinephrine (PHE; Totey et al. 1996). Even though fresh semen gives better fertilization rates than frozen-thawed semen (Totey et al., 1992), it is not practically as quality of semen is depended on season. A marked difference in sperm concentration and oocyte-sperm co-incubation period during *in vitro* gamete interaction have been reported affected embryo formation; an increase of sperm concentration gave a

high cleavage rate. Totey et al. (1993a) found that a concentration of 2×10^6 sperm/ml yielded the highest fertilization rate in IVF in the buffalo. Contrastly, Nandi et al. (1998) indicated that the morula and blastocyst rate were not different between sperm concentration even though the incidence of penetration without cleavage was lower at 1 - 2×10^6 sperm/ml than other groups. Nevertheless, a higher sperm concentration resulted in a higher oocyte penetration rates and also gave rise to polyspermy (Totey et al., 1993a), similarly to Chain et al. (1995) demonstrated that polyspermy increased when sperm and oocytes were incubated more than 12 h. Moreover, it is known that a short-time exposure of oocytes to sperm improves IVF results, mainly because a long-time exposure of oocytes to a large number of sperm would create suboptimal conditions due to excessive generation of ROS which induce peroxidation of the membrane lipids so reducing membrane fluidity and impairing sperm function.

The most common defined medium for *in vitro* culture is synthetic oviductal fluid (SOF; Tervit et al., 1972) or a modified from of SOF (mSOF; Takahashi and First 1992). The blastocyst formation rate is approximately 15-30% of inseminated oocytes depended on variations of basic techniques. Even though the pattern of embryonic development to the blastocyst stage in buffalo is similar in cattle, buffalo embryos had a faster rate of development to the blastocyst stage than cattle embryos (Jian et al., 1998). The evaluation of the quality of blastocyst can be carried out by morphological assessment such as total cell numbers, ratio between inner cell mass and trophectoderm cells.

1.2.6 Intracytoplasmic sperm injection (ICSI)

Intracytoplasmic sperm injection is a technique being established in human and other animal species to generate live offspring when their sperm lack motility. This technique has also become a technique of choice for oligozoospermic patients and in the case that conventional IVF fails to produce an acceptable rate of embryo development. In human, the ICSI has been successfully used to treat infertility as an alternative to conventional IVF (Yanagimachi, 2005). In animals, the ICSI is not a tool for an infertility treatment as in human, special objectives as sex-sorted sperm injection or sperm-oocyte interaction studies are the key element. The ICSI has been reported in various kinds of animals since 1976. The success was first reported in hamster in terms of embryo development by injecting the nuclei of fresh, frozen-thawed and freeze-dried sperm heads into oocytes (Uehara and Yanagimachi, 1976) even though a number of live hamster offsprings were later successfully produced in a few years ago (Yamauchi et al., 2002). Rodents were used as a model for understanding pronuclear formation after ICSI (Westhusin and Kraemer, 1986). Meanwhile, rhesus monkeys were the excellent animal models for examining whether novel methods of assisted reproduction are safe and effective without confounding variables for human (Hewitson et al., 2000). Rabbit has been also used to produce ICSI offspring but is not routinely because of its low survival rate (Deng and Yang, 2001; Ogonuki et al., 2005). Sperm injection has been also studied

in the domestic cat presumably as a model for captive wild felid species (Bogliolo et al., 2001; Gomez et al., 2000; Pope et al., 1998). Many researchers have done experiments in pigs due primarily to high percentage of polyspermic fertilization during IVF (Coy and Romar, 2002; Kolbe and Holtz, 2000; Nakai et al., 2003; Prather and Day, 1998). In cattle, the first ICSI calves following ICSI were reported by Goto et al. (1990) who used a dead sperm to inject into oocytes. However, it was not for routine offspring production in cattle due to a low success rate (Goto et al., 1990; Keefer et al., 1990). Also, even the ICSI has been successful in producing live offspring in several species (Table 1), this technique is far less efficient in animals than in human. It probably dues to inappropriate sperm capacitation/ decondensation or oocyte activation, resulting in non-pronuclei formation. Different techniques for each species have been investigating, including sperm preparation or oocyte activation.

Table1. Comparison of ICSI efficiency in mammals

Species	% Cl	%M/B	% offspring	Type of sperm	References
Mouse	99	91/68	30	Caudal epididymal	Kimura and Yanagimachi, 1995
Rat	39	N/A	10	Frozen-thawed epididymis	Hirabayash et al., 2002
Hamster	91	49	19	Sperm head	Yamauchi et al., 2002
Rabbit	78	39	N/A	Fresh sperm	Deng and Yang, 2001
Cat	58.1	42.9	2.5	Frozen-thawed	Pope et al., 1998
	41.4	19	N/A	N/A	Gomez et al., 2000
Horse	47	N/A	75*	N/A	Cochran et al., 1998
Pig	26	N/A	0.3	Sex-sorted	Probst and Rath, 2003
	69	31	1.1	Ejaculated	Martin, 2000
Sheep	N/A	N/A	0.39	Sex-sorted	Catt et al., 1996
Cattle	72	20.3	10	Immobilized	Horiuch et al., 2002
Monkey	50	N/A	20	Testiscular	Hewitson et al., 2002

N/A = not available information
%Cl: cleavage; M/B: morula/blastocyst
*from grade 1 embryos

1.2.6.1 Sperm decondensation

For fertilization, sperm firstly undergo a process called capacitation, resulting in sperm becoming competence to undergo the acrosome reaction. During acrsome reaction the membrane surrounding the acrosome fuses with the plasma membrane of sperm, releasing acrosome contents and rendering the sperm to be capable of zona pellucida penetration. The result of acrosome reaction is that the equatorial region of the sperm

membrane is exposed and fuses with the oolemma, facilitating introduction of the sperm nucleus into the oocyte. Once in the ooplasm, the sperm nucleus undergoes several structural changes, known as chromatin remodeling which consists of three phases: decondensation, recondensation, and male pronucleus formation (Adenot et al., 1991). During sperm decondensation, disulfide bonds in protamines reduce due to the activity of glutathione, a reducing factor present in the ooplasm (Perreault et al., 1988; Sutovsky and Schatten, 1997). Reduction of the disulfide bridges enables protamine removal from sperm chromatin (Perreault, 1992). The last stage of sperm chromatin remodeling is the decondensation of tightly recondensed sperm chromatin, leading to the formation of male pronucleus. In IVF, capacitation and acrosome reaction are achieved through maintaining specific culture conditions during gamete co-incubation (Fraser, 1982; Fraser, 1987; Fraser, 1987). The sperm fuses with the oocyte in a similar manner, as it takes place during *in vivo* fertilization. In ICSI, capacitation, acrosome reaction, and membrane fusion are bypassed. Sperm chromatin enters ooplasm together with the perinuclear material, acrosome and cell membrane. Although these components eventually disintegrate inside the oocyte, it was suggested that they might interfere with sperm chromatin remodeling (Hewitson et al., 1996; Sutovsky et al., 1996; Sutovsky et al., 2003; Ramalho-Santos et al., 2000).

Sperm-borne oocyte-activating factor (SOAF) is an important signaling molecule that triggers oocyte activation and further embryo development (Kimura et al., 1998; Parrington et al., 1996; Perry et al., 1999). Alternatively, this sperm component is called sperm-specific phopholipase C zeta (PLCζ) (Cox et al., 2002). The SOAF is localized in the perinuclear theca (PT) membrane especially in the post-acrosomal segment and under the plasma membrane over the equatorial segment of the acrosome. It was hypothesized that the releasing of SOAF initiates important signaling molecules by increasing in cytosolic free Ca^{2+} within oocyte eventually leading to the mechanism of oocyte activation and sperm nuclear decondensation (Sutovsky et al., 2003). In case of ICSI, sperm chromatin enters ooplasm together with the perinuclear material, acrosome and cell membrane (Ajduk et al., 2006). This evidence has been suggested to prevent the male pronucleus formation (Sutovsky et al., 1997). Fortunately, the disintegration of the sperm plasma membrane and removal of acrosome could be performed before sperm injecting by varieties of physical treatments as well as by chemical stimulations (Morozumi et al., 2006). They allow the release of the sperm-borne oocyte-activating factor to activate oocyte in human, rodents and rabbits. Therefore, removal of the plasma membrane has an important role not only in accelerating the onset of oocyte activation but this results in improved embryonic development as well.

Contrast with above mentioned, bovine and porcine sperm nuclear membrane still intact after microinjection in cytoplasm even though the activation of oocyte took place. This is likely due to a higher rigidity of perinuclear theca (Perreault et al., 1988; Sutovsky et al., 1997), together with a large amount of acrosome and acrosomal enzymes entered in

the ooplasm after sperm injection (Morozumi and Yanagimachi, 2005). It is possibly that only physical damage seems to insufficient to damage membrane. Thus, SOAF could not release into the ooplasm, resulting in blockage of oocyte activation and sperm head decondensation. In order to improve pronuclear formation following ICSI of bovine and porcine oocytes, it was suggested that sperm membrane removal prior to injection using calcium ionophore (Galli et al., 2003), Triton-X 100 (Lee and Yang, 2004; Tian et al., 2006) are preferable to treat the sperm in order to remove of sperm plasma membrane. This would allow the release of the SOAF more easily to the cytoplasm of the oocyte.

Additionally, pigs and bull sperm chromatins are tightly packaged and more stable than other species. This is possibly due to a type of protamines (protamine-1, P1) which is highly enriched of cysteine, resulting in the stability of disulfide bonds in bull sperm chromatin (Bedford, 1988). Contrast with pigs and bull, human and mouse sperm nucleus contains both protamine-1 and protamine-2. The protamine-2 is deficient in cysteine residues. Consequently, the number of disulfide bonds is diminished in human sperm in comparison to species expressing P1 alone (Jager, 1990). Dithiothreitol (DTT) is an agent that specifically reduces disulfide bonds in sperm. It could be used for promote the decondensation of nuclear chromatin in mammalian sperm and hence subsequence leads to more male PN formation outcomes in cattle (Rho et al., 1998a; Suttner et al., 2000) and pigs (Tian et al., 2006; Yong et al., 2005).

1.2.6.2 Oocyte activation

In normal fertilization, after sperm/oocytes fusion, oocytes undergo a series of morphological and biochemical alterations in a process known as activation. Oocyte activation is usually categorized as entailing two major events including cortical granule reaction and release from MII arrest and completion of the second meiotic division (Swain and Pool, 2008). The first reponses in a few minutes following sperm/oocytes fusion is a rapid increase in intracellular calcium levels, followed by rapid and repetitive oscillations. Calcium originates from endoplasmic reticulum within oocytes. Evidence in human supports the theory that sperm introduce a cytostolic component directly into oocytes cytoplasm to trigger oscillations (Homa and Awann, 1994). Calcium oscillation appears to be instrumental in destruction of cytostatic factor (CSF), the cytoplasmic component of oocytes responsible for maintaining MII arrest (Masui and Markert, 1971). It is now known that CSF consists of Mos protein, which regulates mitogen-activated protein kinase (MAPK) activity, and stabilizes maturation promotion factor (MPF) (Tunquist and Maller, 2003). Decreased MAPK and MPF activities are required for MII meiotic resumption.

During ICSI procedure in rabbit and human oocytes, puncture of the oolemma or aspiration of cytoplasm with micropipette develops calcium response similar to normal fertilization (Keefer, 1989; Tesarik et al., 1994). In contrast, bovine ICSI is very different from other species, sperm injection is not sufficient to activate the oocytes. In order to

improve oocyte activation, ICSI has been combined with exogenous chemical activations which accelerates an increase of calcium in the ooplasm. Calcium ionophore (CaI) induces a rise of Ca^{2+} concentration in oocytes and with strontium induces calcium-oscillation (Kolbe and Holtz, 2000). 6-dimethylaminopurine (DMAP), a phosphorylation inhibitor inhibits the activities of MPF and CSF, which in turn leads to oocyte activation. It was evidence that DMAP can inhibit the extrusion of the second polar body, due to inhibition of phosphoryration necessary for the spindle apparatus and causes the oocytes to enter directly into interphase with only one diploid pronucleus in pathenogenetic activation (Liu et al., 1998; Machaty and Prather, 1998). Cycloheximide (CHX), a protein synthesis inhibitor, does not block the extrusion of the second polar body and pronuclear formation (Liu and Yang, 1999). The result showed significantly lower rate of haploid parthenogenesis development after activation (4% versus 17% DMAP; Suttner et al., 2000). Aged bovine oocytes could easily be activated by CaI alone (Ware et al., 1989). Unlike the aged bovine oocyte, activation of younger bovine oocytes (e.g. matured *in vitro* for 22-24 h) could be induced by combination of CaI and others (Rho et al., 1998b; Susko-Parrish et al., 1994).

1.2.7 Gene expression during embryo development

Most genes in somatic cells are expressed from both alleles of maternal and paternal chromosomes however some are only expressed from one allele depending on its parental origin and the cell only uses the copy either from the mother or father to make proteins (Khatib et al., 2007; Miozzo and Simoni, 2002). By definition, the allele that is silenced is called “imprinted”. Genomic imprinting is a phenomenon leading to sex-specific monoallelic expression of gene (Surani et al., 1984). Hence, it is important that oocytes need both paternal and maternal chromosomes combining to give rise normal embryos. In case of gynogenetic zygotes, they have twice the normal expression level of maternally derived genes, and lack expression of paternally expressed genes, and vice versa for androgenetic embryos. Thus both gynogenetic and androgenetic zygotes are failed to develop to term and usually die at early postimplantation stages (Surani et al., 1984). This finding provides strong evidence that the imprinted genes are the main stumbling block preventing such asexual reproduction.

It is now known that there are at least 80 imprinted genes in humans and mice, many of which are involved in fetal development, in both growth regulation and brain development. A few imprinted genes were reported in cattle such as *IGFIIR* (Killian et al., 2001), *IGF*, *MEG3*, *XIST* (Dindot et al., 2004) and *Nnat* (Ruddock et al., 2004). *Nnat* or neuronatin is involved in the central nervous system during early hindbrain development (Wijnholds et al., 1995). It is only expressed from the allele inherited from father. Fortunately, *Nnat* is expressed by the blastocyst stage of development only in IVF and not in parthenogenesis (PA) embryos (Ruddock et al., 2004). Therefore, we can use

the imprinted gene expression patterns in order to classify the differences between IVF and PA blastocysts stage in our experiments by using RT-PCR.

1.3 Objectives of the thesis

1. To examine and describe the gamete interaction and chronology of early embryonic development, in particular the redistribution of cell cytoskeleton and chromatin configurations after *in vitro* fertilization in swamp buffalo
2. To examine the efficiency of ICSI technique combined with or without chemical activation of oocytes, in terms of oocyte activation, male pronuclear formation, cleavage, and blastocyst formation rates and total cell number of obtained blastocysts
3. To examine the gene expression of embryo derived from ICSI/oocyte activation to distinguish the absence or presence of male genome activation normally occurring during early embryo development
4. To examine the effect of sperm pretreatment prior to ICSI on sperm decondensation and male pronuclear formation

1.4 Hypothesis

1. Actin microfilaments and microtubules involve the gamete interaction and early embryo development in swamp buffalo
2. Buffalo oocytes can be fertilized and developed by sperm injection
3. Gene expression evaluation can be used to clarify whether blastocystsare derived from normal fertilization between male and female gamete or induced parthenogenesis
4. Membrane-damaged sperm using chemical pretreatments or frozen/thawed without any cryoprotectants and reduction of disulfide bonds in protamines using Dithiothreitol improve decondensation and male pronuclear formation following ICSI

1.5 Keywords: embryonic development, fertilization, intracytoplasmic sperm injection, parthenogenesis, gene expression, decondensation, swamp buffalo

1.6 Research merits:

1. The fundamental knowledge about fertilization and early embryo development is able to apply for improving *in vitro* embryo production in swamp buffalo
2. Buffalo oocytes will be able to apply for ICSI technique and classify between sexual and asexual reproduction by difference of gene expression
3. The knowledge about the alteration of gene expression profile during embryo development of buffalo embryos produced *in vitro*

CHAPTER II

CHRONOLOGICAL REORGANIZATION OF MICROTUBULES, ACTIN MICROFILAMENTS, AND CHROMATIN DURING THE FIRST CELL CYCLE IN SWAMP BUFFALO (BUBALUS BUBALIS) EMBRYOS

2.1 Abstract

This study aimed to study the dynamics of early embryonic development, in terms of redistribution of cytoskeleton (microtubules, actin microfilaments) and chromatin configurations during the first cell cycle in swamp buffalo embryos. Swamp buffalo oocytes were matured and fertilized *in vitro*, presumptive zygotes and embryos were fixed at various time points post-IVF. At 6 h post-IVF, 44.4% matured oocytes were penetrated by sperm. Partial ZP digestion however did not improve fertilization rate compared to control (60.3 vs. 54.8%, respectively; $P>0.05$). At 12 h post-IVF, the fertilized oocytes progressed to the second meiotic division and formed the female pronucleus simultaneously with the paternal chromatin continued to decondense. A sperm aster was observed radiating from the base of the decondensing sperm head. At 18 h post-IVF, most presumptive zygotes had reached the pronuclear stage. The sperm aster was concurrently enlarged to assist the migration and apposition of pronuclei (syngamy). Cell cleavage was facilitated by actin microfilaments and firstly observed by 30 h post-IVF. In conclusion, this study demonstrated that microtubules and actin microfilaments actively involve with the process of fertilization and cleavage in swamp buffalo oocytes. The centrosomal material is paternally inherited. Fertilization failure, at least in our current culture system, is predominantly caused by poor sperm penetration. However, partial digestion of ZP did not improve fertilization rate.

Keywords: microtubules, microfilaments, embryo development, fertilization, swamp buffalo

2.2 Introduction

Fertilization in mammals requires a successful series of events involving a profound remodeling of the nucleus and cytoplasm of both sperm and oocytes. Microtubules and actin microfilaments have been demonstrated to dynamically play an important role during fertilization and cleavage in a number of species. The microtubules actively involve in the process of fertilization by the formation of microtubule networks that facilitate the migration and apposition of male and female pronuclei. These microtubules are paternally inherited in most mammalian species, including human (Gook et al., 1998; Sathananthan, 1998), sheep (Le Guen and Crozet, 1989), rabbit (Yllera-Fernandez et al., 1992), porcine (Kim et al., 1996), bovine (Alomar et al., 2008;

Long et al., 1993; Sathananthan et al., 1999) and rhesus monkey(Hewitson et al., 2000). Contrastly, the paternal centrosome in the ooplasm is functionally absent in mice and thus the syngamy of the two pronuclei requires the maternal centrosome (Maro et al., 1985; Schatten et al., 1985). In addition, the evidence that a reversible microfilament depolymerizer (cytochalasin B) fails to inhibit the movement of male and female pronuclei but it adversely affects the syngamy and cell division (Kim et al., 1997; Rho et al., 1998b), suggests an important role of actin microfilaments during cellular cleavage (Le Guen and Crozet, 1989; Yllera-Fernandez et al., 1992). However, these events on gamete interaction and early embryo development especially during fertilization have poorly reported in the swamp buffalo. It has only been morphologically studied *in vivo* (Chantaraprateep et al., 1989). Understanding the redistribution patterns and role of microtubules and actin microfilaments during fertilization *in vitro* provide fundamental knowledge of early embryo development and may improve *in vitro* embryo production techniques principally by the characterization of factors associated with fertilization failure in this species. The present research was designed to study the dynamics of early embryonic development, in terms of redistribution of cytoskeleton (microtubules, actin microfilaments) and chromatin configurations during the first cell cycle in swamp buffalo embryos.

2.3 Materials and Methods

2.3.1 Chemicals

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

2.3.2 In vitro maturation (IVM)

Swamp buffalo ovaries were obtained from animals of unknown reproductive status at a local slaughterhouse, then they were transported to the laboratory within 4 h in 0.9% (w/v) normal saline supplemented with 100 IU/ml penicillin G and 100 μg/ml streptomycin at 28-35 ^{o}C. The ovaries were washed once in 70% (v/v) alcohol and 0.9% (w/v) normal saline (Das et al., 1996). The oocytes were later aspirated from 2–8 mm antral follicles with an 18-gauge needle attached to a 10 ml syringe. The cumulus oocyte complexes were morphologically selected under a stereomicroscope at 400x magnifications. Cumulus-oocyte complexes (COCs) with homogenous ooplasm and surrounded by compact multiple layers of cumulus cells were submitted to *in vitro* maturation. Groups of 10 COCs were cultured in 50 μL droplets of $NaHCO_3$ buffered tissue culture medium 199 covered with mineral oil supplemented with 10% (v/v) buffalo follicular fluid, 50 IU/mL human chorionic gonadotropin (Intervet, Boxmeer, The Netherlands), 0.02 IU/mL follicle stimulating hormone, 1 μg/mL estradiol-17β, 100 μM cysteamine, 20 ng/mL epidermal growth factor, 100 IU/mL penicillin G and 100 μg/mL

streptomycin. Three pools of follicular fluid were obtained from 2- 8 mm follicles and then sterilized by filtering through the 0.22 μm syringe driven filter and then stored in sterile microcentrifuge tubes at -80 °C. IVM was performed at 38.5 °C for 22 h in a humidified atmosphere of 5% CO_2 in air.

2.3.3 Partial digestion of zona pellucida (ZP)

After *in vitro* maturation, oocytes were denuded and were transferred into 30 μL droplet of an acid Tyrode's solution (pH 3.1) for 45 s at room temperature (28-30 ^oC). They were washed immediately two times with 2 mL of TALP medium. ZP-digested oocytes were submitted to fertilization and culture procedures as mentioned above. Percentage of pronuclear formation was recorded at 18 h post-IVF. Non-Tyrode treated oocytes served as control.

2.3.4 In vitro fertilization and in vitro culture (IVF and IVC)

Frozen semen from a fertile bull was used in this study. The semen was thawed at 37 °C for 30 s and then submitted to swim-up procedure for 45 min in a modified Tyrode's medium (TALP) supplemented with 10 μg/mL heparin as described by Parrish et al. (Parrish et al., 1988). Groups of 10-15 COCs in TALP medium, supplemented with 20 μM penicillamine, 10 μM hypotaurine and 1 μM epinephrine were fertilized with sperm at a final concentration of $2x10^6$ sperm/mL (Totey et al., 1993a). IVF was performed at 38.5 °C in a humidified atmosphere of 5% CO_2, 5% O_2 for 12 h. Excessive cumulus cells and sperm were then removed by repeated pipetting in culture medium containing 1 mg/ml hyaluronidase. Ten to fifteen presumptive zygotes were then cultured in 50 μL droplets of synthetic oviductal fluid containing 1% (v/v) fetal calf serum, 100 IU/ml penicillin G and 100 μg/ml streptomycin at 38.5 ^oC in an atmosphere of 5% CO_2, 5% O_2.

The presumptive zygotes were randomly fixed and examined at 6, 12, 18, 24, and 30 h post-IVF. Prior to fixation, they were incubated for 45 min at 37 ^oC in a glycerol-based microtubule-stabilizing solution that contained 25% (v/v) glycerol, 50 mM $MgCl_2$, 0.1 mM EDTA, 1 mM 2-mercaptoethanol, 50 mM imidazol, 4% Triton-X-100 and 25 μM phenylmethylsulphonyl fluoride at pH 6.7 (Simerly and Schatten, 1993). Subsequently, they were fixed and stored in 4% (w/v) paraformaldehyde in PBS until analysis.

2.3.5 Fluorescent-labeling of oocytes and presumptive zygotes

To label the microtubules, the presumptive zygotes and embryos were first incubated at 25 ^oC for 1 h in a 1:100 solution of monoclonal anti-α-tubulin (clone B1-5-1-2) in 0.1% (v/v) Triton-X-100 in PBS-BSA. They were subsequently washed in PBS-BSA and incubated for 1 h in a 1:100 solution of a goat anti-mouse second antibody

conjugated with tetramethylrhodamineisothiocyanate (TRITC) in PBS-BSA. After washing twice in PBS-BSA, the actin microfilaments were stained by incubation for 30 min in a solution of 0.165 μM Alexa Fluor® 488 phalloidin (Molecular Probes, Invitrogen, OR, USA) in PBS-BSA. In addition, they were subsequently stained for 10 min with Alexa Fluor® 594 wheat germ agglutinin (WGA; Molecular Probes) to locate lectin-riched ZP. Finally, the presumptive zygotes and embryos were incubated for 15 min with 20 μM DAPI to label the chromatin. Labeled samples were mounted on a glass microscope slide in a 2 μL droplet of antifade medium (Vectashield™, Vector Lab, CA, USA) to retard photobleaching.

2.3.6 Confocal laser scanning microscopy

Confocal laser-scanning microscopy (C1, Nikon, Japan) was used to demonstrate the presence or absence of sperm within ooplasm (sperm penetration rate) at 6 h post-IVF. Three laser sources from Diote 408 nm, Argon 488 nm and HeNe 543 nm were used to simultaneously excite the fluorescent signals from DAPI, Alexa Fluor® 488 phalloidin (microfilaments) and Alexa Fluor® 594 (ZP), respectively. The digital-micrographs produced using the sequential scanning mode for the 3 separate colors were merged into single panel using EZ-C1 software (Nikon, Japan). The resulting multi-color micrographs were subsequently examined using Adobe Photoshop CS (Adobe System Inc., Mountain View, CA, USA).

2.3.7 Experimental design

A total of 63 presumptive zygotes were examined at 6 h post-IVF for sperm penetration using confocal laser scanning microscopy. Phalloidin and WGA were used to localize the boundary of the ooplasm and lectin-riched ZP in order to facilitate the visualization of sperm within the ooplasm, and DAPI was used to label the chromatin. The presence of a spermatozoon within ooplasm indicated the sperm penetration, and the oocytes having sperm head (s) bound onto or within the ZP were considered as non-fertilized oocytes.

To test whether partial digestion of ZP prior to IVF would improve the sperm penetration rate, thereby improving IVF efficiency, a total of 64 oocytes were treated with acid Tyrode's solution and then fertilized *in vitro*. Fertilization rate was assessed by percentage of male and female pronuclear formation at 18 h post-IVF.

For the distribution pattern of the cell cytoskeleton and chromatin configurations during fertilization and early embryo development, 378 presumptive zygotes and/or embryos were fixed at 12, 18, 24, and 30 h post-IVF. Following immunolabeling with monoclonal anti-α-tubulin-TRITC, Phalloidin, and DAPI to identify microtubules, microfilaments, and chromatin, respectively, they were examined using immunofluorescent microscopy (BX51, Olympus, Tokyo, Japan). The characteristics of the chromatin, polar body, cytoskeleton, sperm heads and pronuclear formation were

recorded. Oocyte activation was defined by the progression of chromosomal development after IVF from metaphase II (MII) through telophase II and also the formation of female pronucleus. Activated oocytes having decondensing sperm head and/or formation of male pronucleus and cell cleavage were identified as fertilized oocytes/zygotes. Metaphase I (MI), anaphase I or telophase I oocytes were classified as non-matured oocytes. The oocytes that had a dispersed pattern of chromatin and cytoskeleton were classified as degenerate oocytes.

2.3.8 Statistical analysis

Descriptive data was used to describe the chronology of early embryo development in terms of the redistribution of cytoskeleton (microtubules and actin microfilaments) and chromatin configurations. Differences in percentage data of maturation and fertilization among stages were presented as mean and analyzed by Fisher's exact test (SAS 9.1, The SAS Institute Inc., Cary, NC, USA). P-values less than 0.05 were interpreted as significant.

2.4 Results

2.4.1 Sperm penetration

Sperm penetrated into ZP and were found in the cytoplasm of MII oocytes by 6 h post-IVF (28/63, 44.4%, Fig. 2A; Table 2). Although a large proportion of these penetrated oocytes were still arrested at MII stage, 2 of the 28 oocytes (7.1%) had already resumed the second meiosis and were in telophase II. Eighteen MII oocytes (18/63, 28.6%) were classified as non-fertilized oocytes as determined by the absence of a spermatozoon within the ooplasm or sperm were only attached at the ZP of the oocytes. Two of 12 MI oocytes were also penetrated by sperm.

Table2. The sperm penetration of swamp buffalo MII oocytes at 6 h post-IVF analyzed by confocal laser scanning photomicrography

No. of oocytes examined	No. of MII oocytes (%*)		Non MII/Non fertilized (%*)
	Sperm penetrated	Non-sperm penetrated	
63	28 (44.4)	18 (28.6)	17 (27.0)

*Percentages are expressed in relation to the total number of oocytes used for *in vitro* maturation.

2.4.2 In vitro fertilization of partial zona digested oocytes

Tyrode's solution dissolved the ZP of buffalo oocytes. After exposure for 45 s, approximately one-third of ZP was digested. Fertilization rate, in terms of pronuclear formation, of ZP-digested oocytes (60.3%), was not significantly different compared to 54.8% of control (P>0.05, Table 3).

Table3. Percentage of maturation and fertilization of swamp buffalo oocytes treated with Acid Tyrode's solution at 18 h post-IVF

Type of oocytes	No. of oocytes	No of MII oocytes (%)*	Fertilization rate (%)**
Partial zona digested	64	42 (65.6)	60.4[a]
COCs	43	31 (72.1)	54.8[a]

* Percentage is expressed in relation to the total number of oocytes used for *in vitro* maturation.

** Percentage is expressed in relation to the number of MII oocytes.

[a] Within a column, differences were considered to be significant at P<0.05.

2.4.3 Redistribution of cytoskeleton and chromatin configurations

Percentage of maturation and fertilization of swamp buffalo oocytes at 12, 18, 24, and 30 h post-IVF are shown in Table 4, the maturation rate did not differ among groups (P>0.05). However, the rate of fertilization at 12 h post -IVF was lower than the other time-points (P<0.05). The results demonstrated that the presumptive zygotes showed decondensation of both male and female chromatin by 12 h after IVF. A small proportion of MII oocytes (18/63, 28.6%) underwent activation. Of these activated oocytes, 5 and 13 oocytes were in telophase II (Fig. 2B) and pronuclear stage, respectively. During decondensation of sperm chromatin, densely stained microtubules were observed and continually elongated to form the sperm aster that radiated from the nucleation site of the sperm centrosome at the base of thedecondensing sperm head (Fig. 2C). It revealed that the developmental rate of the male and female pronuclei was asynchronous at 12 h post-IVF (a lack of synchrony between the male and female pronuclei). By 18 h post-IVF, 33 of 65 MII oocytes (50.8%) were recorded as being fertilized, 30 of them had already reached the pronuclear stage. At this time, microtubule networks of sperm aster simultaneously increased in size and extended throughout the ooplasm of the fertilized oocytes.

The percentage of fertilized oocytes at 24 h post-IVF was 49.2 % (29/59). Most of fertilized oocytes (25/29) were also in pronuclear stage. Apposition of the pronuclei (syngamy) was observed in 14 of these 25 fertilized oocytes. At this stage, microtubules were intensely stained between the two pronuclei (Fig. 2D). Cleavage was firstly observed by 30 h post-IVF (14 of 27 fertilized oocytes). A dense array of microfilaments formed between two presumptive blastomeres (Fig. 2E) and later formed an intensely labeled layer beneath the plasma membrane of each blastomere after cell cleavage (Fig. 2F). In addition, the percentage of „non-fertilized' MII oocytes was clearly demonstrated in the present study. Most MII oocytes that failed to progress through the second meiotic division had only a number of sperm bound onto or within the zona pellucida, suggesting the importance role of sperm on the failure of fertilization.

Table4. Percentage of maturation and fertilization of swamp buffalo oocytes at 12, 18, 24, and 30 h post-IVF

Time after IVF(h)	No. of oocytes (replicates)	No. of MII oocytes (%)*	No. of fertilization (%)**	Embryo development (% of fertilized oocytes)		
				OA(%)	PF(%)	CC(%)
12	103(9)	63(61.2)	28.6 [a]	5(27.8)	13(72.2)	0(0)
18	97(7)	65(67.0)	50.8 [b]	3(9.1)	30(90.9)	0(0)
24	91(7)	59(64.9)	49.2 [b]	4(13.8)	25(86.2)	0(0)
30	87(8)	56(64.4)	48.2 [b]	4(14.9)	9(33.3)	14(51.6)

[a, b] Within a column, differences were considered to be significant at $P<0.05$. OA= oocyte activation; PF=pronuclear formation; CC= cell cleavage

* Percentage is expressed in relation to the total number of oocytes used for *in vitro* maturation.

** Percentage is expressed in relation to the number of MII oocytes.

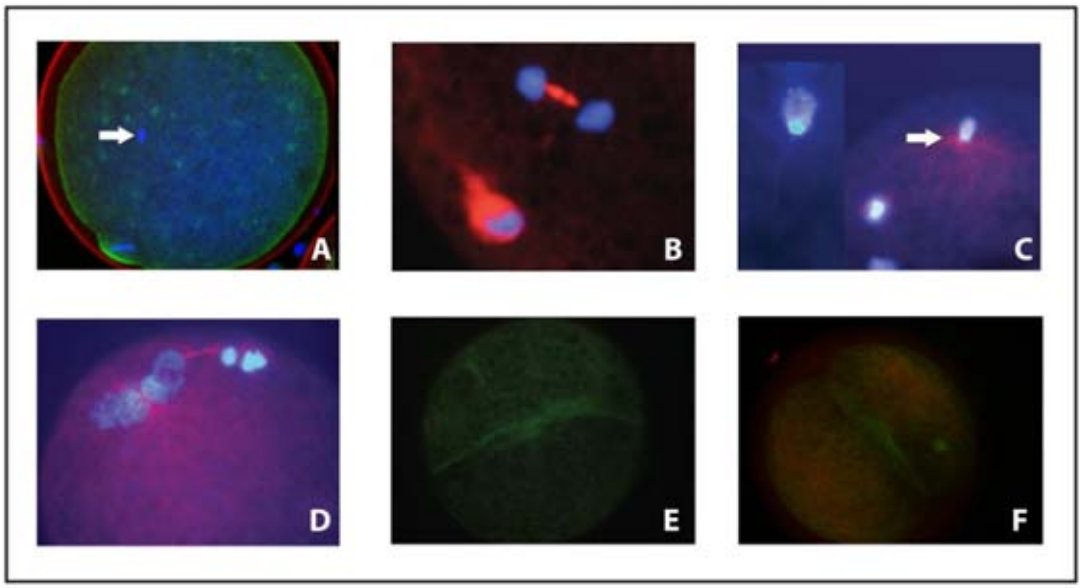

Figure2. Images of swamp buffalo oocytes after staining with wheat-germ agglutinin (red) to localize the outline of the ZP, monoclonal-α-tubulin-TRIT C (red) to demonstrate microtubules, Alexa 488 phalloidin (green) to identify microfilaments and DAPI (blue) to label the chromatin. Fig. A was illustrated using confocal microscopy. Fig. B- F were illustrated by immunofluorescent microscopy. An intact spermatozoon (arrow) was present within the cytoplasm of the MII oocytes at 6 h post IVF (A). Progression of chromosomal development after IVF from MII the oocytes entered telophase II with one polar body, the condensed chromatin were found at the pole of the astral microtubule (B). Sperm aster extended (arrow) from the base of the decondensed sperm head (C). Migration and apposition of male and female pronuclei were assisted by the dense array of microtubules found between pronuclei (D). Microfilaments were condensed in the middle line of the cell furrow during cleavage (E). A two-cell embryo with a dense array of microfilaments formed intensely beneath the plasma membrane of each blastomere (F).

2.5 Discussion

The chronology of early embryonic development in terms of cytoskeleton redistribution and chromatin configurations in swamp buffalo embryos during the first cell cycle was examined and firstly described in this study. Until recently, overall success of *in vitro* embryo production in swamp buffalo has remained relatively poor when compared to the results obtained from riverine buffaloes and bovines, for example due to the limitation of fundamental knowledge associated with gamete interaction at fertilization. Oocyte maturation rates in this study (Table 3) were similar to the range of 47 to 85% in other observations (Nandi et al., 2001) and fertilization rates of these MII oocytes (ranging from 28.6-50.8 %) in terms of oocyte activation and cell cleavage were variable, which is also in accordance with other previous studies (Nandi et al., 1998;

Nandi et al., 2002; Songsasen and Apimeteetumrong, 2002; Totey et al., 1993b; Totey et al., 1992). While a number of factors have been demonstrated to involve in the fertilization rate of swamp buffalo oocytes including sperm quality (Selvaraju et al., 2009), concentration (Totey et al., 1993a)and culture media used (Abdoon et al., 2001; Ravindranatha et al., 2003), intrinsic factors within the cytoplasm of MII oocytes (as usually referred to as cytoplasmic maturation) also play a critical role during the activation of both male and female gametes during fertilization and early embryo development (Krisher, 2004; Laurincik et al., 1994; Leibfried-Rutledge et al., 1987). During IVF, sperm rapidly undergo several modifications including a remodeling of the sperm plasma membrane, and sperm then become hyperactivated (Liu et al., 2007)and capable of binding to mature oocyte via specific sperm-binding ZP3 receptors (Litscher et al., 2009). As a consequence, sperm undergo the acrosome reaction and finally penetrate the ZP and fuse with the oolemma (Yanagimachi, 2005). In this study, sperm penetrated through the ZP of mature oocytes by 6 h post-IVF which was similar to previous reports in bovines (Walters et al., 2006; Xu and Greve, 1988). However, it has been reported that a spermatozoon was already present in the cytoplasm of bovine oocytes as early as 2-3 h post-IVF (Ward et al., 2002). Many factors were likely involved in the difference in speed of sperm penetration among studies such as type of sperm (fresh or frozen), co-incubation time and capability of sperm to respond to the capacitating medium during IVF (Alomar et al., 2008; Ward et al., 2002; Xu and Greve, 1988). In addition, our study demonstrated that sperm penetration in MI swamp buffalo oocytes was also possible which similar to reports in bovine (Abeydeera et al., 1993; Chian et al., 1992) and canine (Yamada et al., 1992). However, it appears that intrinsic factors within the ooplasm of oocyte also play a crucial role in determining the fate of sperm decondensation such as immature oocytes cannot support post-fertilization events of sperm head decondensation (Lacham-Kaplan and Trounson, 1995).

This study revealed that a dense network of a thread-like structure of tubulin (referred to as the sperm aster) was formed at the base of decondensing sperm head during gamete activation. The evidence that elongated radial sperm aster was involved in the movement and apposition of male and female pronuclei of buffalo's zygote, suggested that the centrosomal material is primarily paternally inherited and is similar to previous reports in other mammalian species such as human (Gook et al., 1998; Sathananthan, 1998), sheep (Le Guen and Crozet, 1989), rabbit (Yllera-Fernandez et al., 1992), porcine (Kim et al., 1996), bovine (Alomar et al., 2008; Long et al., 1993; Sathananthan et al., 1999) and rhesus monkey (Hewitson et al., 2000). However, this is different in mouse because cytoplasmic microtubules in the cytoplasm originate from maternal centrosomes and sperm astral microtubules were not detected in the decondensing paternal chromatin (Hewitson et al., 1997; Maro et al., 1985).

We found the significant differences in the percentage of oocytes being fertilized between 12 h and the others ($P<0.05$). The low numbers of fertilized oocytes presented in

Table4 indicate that a large proportion of penetrated sperm underwent pronuclear formation during 12-18 h post-IVF, while formation of female pronucleus in oocytes occurred before the decondensation of sperm head. After decondensation of sperm chromatin, the proportion of pronuclear stage embryos was asynchronously observed by 12-18 h post-IVF. Although not exhaustively examined, this asynchronous development of zygotes has been postulated to be caused by several factors such as delayed decondensation of sperm chromatin (Xu and Greve, 1988), variation of individual animals (Alomar et al., 2008; Ward et al., 2001) and variation of cell cycle transition (M phase to G2 stage) following sperm entry (Capmany et al., 1996; Nagy et al., 1998). In this study, the development of buffalo zygotes was more synchronized around 18 h which was similar to a report in bovine (Alomar et al., 2008).

In buffalo, pronuclei movement was clearly influenced by the sperm aster. We found that the male and female pronuclei were positioned at the center of the oocyte, in which the sperm aster mediated by microtubules was concentrated between the two pronuclei until the zygote entered the first mitotic phase and cleaved to the two-cell stage. This result is in agreement with previous report in sheep (Le Guen and Crozet, 1989), but in contrast to rabbit and mouse. The sperm aster of the latter species was a transitory structure that dispersed rapidly around the male pronucleus (Schatten et al., 1985; Yllera-Fernandez et al., 1992). In addition to the role of microtubules during fertilization and early embryo development, this study also indicated that microfilaments also played an important part in the fertilization and cleavage in swamp buffalo embryos. These filaments were concentrated as a cell furrow predominantly in the middle line of the dividing cell and subsequently located just beneath the plasma membrane as previously reports in Xenopus (Noguchi and Mabuchi, 2001), mouse (Schatten and Schatten, 1986), porcine (Kim et al., 1997), and bovine (Rivera et al., 2004) embryos. Actin microfilaments were regulated by actin-related protein such as profilins (Rawe et al., 2006) and have also been documented to be actively involved in the redistribution of mitochondria (Albertini et al., 1987; Barnett et al., 1996), polarization of embryos and also pronuclear apposition (Maro and Pickering, 1984).

In our study, we firstly found that fertilization failure was associated with the absence of sperm in the MII ooplasm, even though a number of sperm were tightly bound to the zonapellucida. Although partial digestion of ZP using Tyrode's acid did not affect sperm penetration, this approach also failed to improve fertilization rate when compared with non-Tyrode treated control. It is postulated that other factors such as quality of frozen-thawed sperm (Mishra et al., 2008) and culture environment (low versus high oxygen tension) should be taking into an account rather than only assessing the sperm motility prior IVF. Other advance sperm parameters, such as functional membrane integrity, mitochondrial membrane potential, and acrosome integrity may be additionally required (Selvaraju et al., 2009).

In summary, this study is the first report to examine and describe the chronology of swamp buffalo embryo development in terms of redistribution of cytoskeleton and chromatin configurations during the first cell cycle. The study demonstrates that a microtubule organizing center is formed at the area of sperm centrosome and plays an important role in the migration and apposition of pronuclei whereas actin microfilaments actively involves in cellular cleavage. Fertilization failure of buffalo oocytes, at least in our current culture system, is predominantly caused by poor sperm penetration. However, partial digestion of ZP did not improve fertilization rate in this species. Other factors associated with fertilization failure in buffalo' oocytes are needed to be characterized.

CHAPTER III

LACKING EXPRESSION OF PATERNALLY-EXPRESSED GENE CONFIRMS THE FAILURE OF SYNGAMY FOLLOWING INTRACYTOPLASMIC SPERM INJECTION IN SWAMP BUFFALO (BUBALUS BUBALIS)

3.1 Abstract

The objectives were to: 1) examine the efficiency of intracytoplasmic sperm injection (ICSI) technique, with or without chemical activation of *in vitro* matured buffalo oocytes, on sperm head decondensation; and 2) compare the subsequent development of embryos following activation of ICSI (ICSI (+) activation group) and sham injection (Sham (+) activation group) oocytes (embryos obtained by *in vitro* fertilization of IVM oocytes served as a control group). Pronuclear formation rates in ICSI (+) activation and Sham (+) activation groups were higher than that of ICSI without activation ($P<0.05$). However, since 90.9% of presumptive zygotes in ICSI (+) activation group demonstrated pronuclear formation with an intact sperm head, we inferred that most were parthenotes. Neither developmental competence (morula and blastocyst formation rates) nor mean total cell number of blastocysts was significantly different among ICSI (+) activation, Sham (+) activation and IVF groups. To clarify whether blastocysts were derived from syngamy or parthenogenesis, expression of *Nnat*, a paternally-expressed gene in blastocysts derived from IVF, ICSI and oocyte activation without sperm or sham injection was additionally examined using RT-PCR. Expression of *Nnat* mRNA was not detected in ICSI (+) activation blastocysts, indicating failure of male genome activation. Although blastocyst development after ICSI combined with chemical activation was similar to IVF oocytes, these blastocysts were generated by parthenogenesis, due to failure of male pronucleus formation.

Keywords: Intracytoplasmic sperm injection, gene expression, parthenogenesis, swamp buffalo

3.2 Introduction

Intracytoplasmic sperm injection (ICSI) is a micro-fertilization technique that bypasses the interaction between sperm and oocyte at the level of the zonapellucida and oolemma. It has become the technique of choice for treating infertility in human (Palermo et al., 1992), especially when sperm quality and numbers are insufficient for conventional *in vitro* fertilization. This technique also allows the use of immotile or dead sperm following cryopreservation (Goto et al., 1990; Horiuch et al., 2002), freeze-drying (Keskintepe et al., 2002; Kwon et al., 2004), and heat-drying (Lee and Niwa, 2006). In addition, ICSI is a useful method for producing live offspring from sex-sorted sperm (Hamano et al., 1999; Probst and Rath, 2003) or genetically modified animals via sperm

mediated gene transfer (Lee et al., 2009; Perry et al., 1999) and to avoid polyspermy, such as in pigs (Coy and Romar, 2002; Kolbe and Holtz, 2000; Nakai et al., 2003; Prather and Day, 1998). Although live offspring from several species have been born from this technique, the overall success of ICSI remains poor in species such as cattle and pigs in which ICSI *per se* is insufficient to fully activate oocytes for further development (Garcia-Rosello et al., 2009; Nakai et al., 2011; Yanagimachi, 2005). Consequently, injected sperm often fail to undergo sperm head decondensation (Kren et al., 2003; Lee et al., 2003; Wijnholds et al., 1995), and chemical activation of oocytes after sperm injection is required to improve fertilization and blastocyst formation rates (Fujinami et al., 2004; Liu and Yang, 1999; Rho et al., 1998a; Suttner et al., 2000). However, poor *in utero* development of embryos transferred to the recipient females may be due to a high rate of parthenogenetic activation (Galli et al., 2003; Horiuch et al., 2002; Nakai et al., 2003; Oikawa et al., 2005). In buffalo, ICSI has been far less investigated than in cows and pigs; studying the efficiency of ICSI has been restricted to only *in vitro* morphological assessment of blastocyst development (Liang et al., 2011b), and pregnancy after transferring ICSI derived embryos to recipients has apparently not been reported. The aim of this study was to examine the efficiency of ICSI, with or without chemical activation of oocytes, in terms of oocyte activation, male pronuclear formation, cleavage, and blastocyst formation rates and total cell number of obtained blastocysts. Embryos derived from ICSI/oocyte activation were also examined for paternal gene expression to distinguish the presence or absence of male genome activation during early embryo development.

3.3 Materials and methods

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

3.3.1 Oocyte recovery and in vitro maturation

Swamp buffalo ovaries were obtained from animals of unknown reproductive status at a local slaughterhouse and transported to the laboratory within 4 h in 0.9% (w/v) normal saline supplemented with 100 IU/mL penicillin G and 100 μg/mL streptomycin at 28-30 ^{o} C. Then the ovaries were washed once in 70% (v/v) alcohol and 0.9% (w/v) normal saline (Das et al., 1996). The oocytes were subsequently aspirated from 2–8 mm antral follicles with an 18-gauge needle attached to a 10-mL syringe. The oocytes were morphologically selected under a stereomicroscope (Nikon SMZ645, Tokyo, Japan) at a magnification: ×400. Only cumulus-oocyte complexes (COCs) with homogenous ooplasm and surrounded by compact multiple layers of cumulus cells were submitted to IVM. Groups of 10 COCs were cultured in 50 μL droplets of $NaHCO_3$ buffered tissue culture medium 199 (with Earle's salts) covered with mineral oil supplemented with 10% (v/v) buffalo follicular fluid (bFF), 50 IU/mL human chorionic gonadotropin (Intervet-

Schering-Plough, Boxmeer, The Netherlands), 0.02 IU/mL follicle stimulating hormone, 1 μg/mL estradiol-17β, 100 μM cysteamine, 20 ng/mL epidermal growth factor, 100 IU/mL penicillin G and 100 μg/mL streptomycin. The bFF was pooled, sterilized by filtering through the 0.22 μm syringe driven filter and then stored at -80 °C until use. IVM of COCs was performed for 21 h at 38.5 °C in a humidified atmosphere of 5% CO_2 in air. After IVM, COCs selected for ICSI were treated with 1 mg/mL hyaluronidase for 3 min and cumulus cells were thoroughly removed by gentle repeat pipetting. Only oocytes with the visible first polar body were selected and maintained in Hepes-buffered synthetic oviductal fluid (Hepes-SOF) until further treatment.

3.3.2 Sperm preparation

Frozen semen from a swamp buffalo bull routinely used at the National AI center (Ratchaburi, Thailand) was used in this study. The semen was thawed at 37 °C for 30 s and then submitted to swim-up procedure for 45 min in a modified Tyrode's medium (TALP) supplemented with 10 μg/mL heparin. Motile sperm to be used for IVF were collected and a final sperm concentration was adjusted to $2x10^6$ sperm/mL. For ICSI, 5 μL of sperm suspension was mixed with 95 μL Hepes-SOF containing 10% (w/v) poly-vinylpyrrolidone (PVP).

3.3.3 Intracytoplasmic sperm injection

Conventional ICSI was performed using an inverted microscope (IX71; Olympus, Tokyo, Japan) equipped with a micromanipulator (IM-9B; Narishige, Tokyo, Japan). The inner diameter of the injection needle was 9-11 μm (OrigioHumagen Pipets Inc., Charlottesville, VA, USA; REF No. MIC-9-30), and the inner diameter of the holding pipette was 10–20 μm. A motile spermatozoon was immobilized by tail breaking with the tip of the injection needle immediately before injection. The oocyte was oriented with the holding pipette so that the polar body was presented at the 12 or 6 O'clock position. After the injecting needle had been advanced into the oocyte, a small amount of cytoplasm was aspirated to ensure the penetration of the oolemma. Finally, a spermatozoon was directly injected into the ooplasm with minimal volume of PVP. Sham injection of oocytes was conducted using the same procedure as sperm injection, except that no sperm were loaded into the injection pipette.

3.3.4 Oocyte activation (OA)

For OA, the oocytes were exposed to 5 μM calcium ionophore (A23187) in SOF medium supplemented with 1% (v/v) fetal calf serum (FCS) (as referred to SOF1) for 5 min and then incubated for 5 h with 10 μg/mL cycloheximide in SOF1 at 38.5 °C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

3.3.5 In vitro fertilization

After 21 h of IVM, groups of 10 COCs were fertilized with sperm in 50 μL droplet of TALP medium, supplemented with 20 μM penicillamine, 10 μM hypotaurine and 1 μM epinephrine. The IVF was performed for 12 h at 38.5 °C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. Excessive cumulus cells and sperm were then removed by repeat pipetting in culture medium containing 1 mg/mL hyaluronidase. Ten to fifteen presumptive zygotes were then transferred to and cultured in SOF1.

3.3.6 In vitro embryo culture

Presumptive zygotes were cultured in 50 μL droplets of SOF1 covered with mineral oil. The FCS was then increased to 5% and 10% (v/v) on Days 3 and 5, respectively (Day 0 = day of IVF/ICSI). All cultures were performed at 38.5 ^{o}C in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

3.3.7 Fixation and fluorescent-labeling of oocytes/embryos

At 18 h after IVC, oocytes/embryos were fixed and their DNA and microtubules were fluorescently labeled to precisely examine the nuclear status of oocyte and zygote. In brief, they were first incubated for 45 min at 37 ^{o}C in a glycerol-based microtubule-stabilizing solution that contained 25% (v/v) glycerol, 50 mM $MgCl_2$, 0.1 mM EDTA, 1 mM 2-mercaptoethanol, 50 mMimidazol, 4% Triton-X-100 and 25 μM phenylmethylsulphonyl fluoride at pH 6.7 (Simerly and Schatten, 1993). Subsequently, they were fixed and stored in 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS) until analysis.

To label the microtubules, the oocytes were first incubated at 25 ^{o}C for 1 h in a 1:100 solution of monoclonal anti-α-tubulin (clone B1-5-1-2) in 0.1% (v/v) Triton-X-100 in PBS supplemented with 0.1% (w/v) bovine serum albumin (BSA) (PBS-BSA). They were subsequently washed in PBS-BSA and incubated for 1 h in a 1:100 solution of a goat anti-mouse second antibody conjugated with tetramethylrhodamine isothiocyanate (TRITC) in PBS-BSA. The chromatin in oocytes/zygotes was labeled with 0.1 μg/mL 4′6-diamidino-2-phenylindole (DAPI) staining. The fluorescently labeled samples were then mounted on a glass microscope slide in a 2 μL droplet of antifade medium (Vectashield^{TM}, Vector Lab, Burlingame, CA, USA) to retard photobleaching, and then they were visualized with an epifluorescent microscope (BX51, Olympus, Shinjuku, Japan).

The oocytes were classified as metaphase II (MII) when the chromatin was aligned in the center of the barrel-shaped metaphase plate with an extrusion of the first polar body. Anaphase II (AII) and telophase II (TII) were classified when the sister chromatids were segregated and the condensed chromatin was found at the end-pole of the astral microtubule with the first polar body, respectively. Oocytes with one or two pronuclei and polar body (bodies) were considered to be activated. The presence of both

male and female pronuclei in the absence of sperm head were considered to be normally fertilized. The presence of a sperm head within the activated oocyte was considered to have been activated parthenogenetically.

3.3.8 Messenger RNA extraction, reverse transcription polymerase chain reaction

3.3.8.1 Sample preparation and RNA extraction

On Day 7 all the blastocysts obtained from each treatment were stored in 2-5 μL of sterile PBS and kept at -80 °C for examination of paternally expressed *Nnat* gene using RT-PCR technique. A total of 15 blastocysts were used in each experimental group. Total RNA was extracted from a pooled sample of five blastocysts in one replicate. Three replicates were performed in each experiment. Fetal brain tissue was collected from the slaughterhouse and used as Neuronatin (*Nnat*) positive control since *Nnat* is expressed in the central nervous system during early hindbrain development (Wijnholds et al., 1995). Total RNA was extracted from blastocysts and brain tissue using the Absolutely RNA®Nanoprep Kit (Stratagene, Agilent Technologies, San Diego, CA, USA) according to the manufacturer's instructions. Genomic DNA residue was removed during the purification steps by DNase I enzyme provided with the kit. RNA was eluted from the purification column with sterile distilled water.

3.3.8.2 Oligonucleotide primers

The paternally expressed gene (*Nnat*) was selected to represent the paternal genome activity in embryos since it shows monoallelic expression at the blastocyst stage (Ruddock et al., 2004), and a housekeeping gene (glyceraldehyde 3-phosphate dehydrogenase, *GAPDH*) was used as the internal control (Aswal et al., 2008). The primer information is summarized in Table5.

Table5. Forward (F) and reverse (R) primers for RT-PCR used to assess the expression of interested genes.

Gene	Primer sequence (5′→3′)	GeneBank accession no.	Size (bp)	Temp (ºC)	References
Nnat	F:CCTCGGCWGAACTGCTCATC R:GCGKTGCCTRTGCCCAGAT	AY360450	517	56	(Ruddock et al., 2004)
GAPDH	F:CTGACCTGCCGCCTGGAGAAA R:GTAGAAGAGTGAGTGTCGCTGT	U39091	189	55	(Aswal et al., 2008)

3.3.8.3 Reverse transcription polymerase chain reaction (RT-PCR)

Complementary DNA was synthesized using the Superscript III Kit (Invitrogen, Carlsbad, CA, USA) following the product's recommendations. The PCR reaction consisted of 12.5 µL of GoTaq® Green Master Mix (Promega, Madison, WI, USA), 12.5 µM each of forward- and reverse primer, 3 µL of RT reaction, and sterile distilled water. The PCR was carried out using an Amplitronyx™6 thermo cycler machine (NyxTechnik, San Diego, CA, USA). The cycle condition for *Nnat* was 95 °C 1 min, followed by 30 rounds of 95 °C 30 s, 56 °C 30 s, 72 °C 1 min, plus final extension at 72 °C for 1 min. The cycle condition for *GAPDH* was 95 °C 2 min, followed by 30 rounds of 95 °C 30 s, 55 °C 30 s, 72 °C 30 s, plus final extension at 72 °C for 2 min.

The PCR products of the embryo samples, along with positive (fetal brain) and negative (distilled water) controls were analyzed by electrophoresis on 2% (w/v) agarose gel (Bio-Rad, CA, USA) mixed with 0.5 µg/mL of ethidium bromide (Promega). The gel images were taken using the Gene Flash Gel Documentation (Syngene, Cambridge, UK).

3.3.8.4 Sequencing of partial buffalo Nnat

The PCR products acquired from gel electrophoresis were collected for gene sequence and the sequence was confirmed in the GenBank® (National Center for Biotechnology Information, Maryland, USA). The amplicons were purified from agarose gel using NucleoSpin® Extract II kits (Macherey-Nagel, Düren, Germany). The eluted products were confirmed their purity by running in a 1.5% agarose gel. After confirming the eluted products, both strands of each amplicons were sequenced using the same *Nnat* primers. The sequences derived from both strands were assembled using BioEdit Version 7.0.8.0 (T.A. Hall Software, Raleigh, NC, USA). Assembled sequences were then blasted in the GenBank to determine the nucleotide similarity to other species.

3.3.9 Experimental design

3.3.9.1 Experiment 1

This experiment aimed to examine the efficiency of ICSI technique combined with or without chemical activation of oocytes on the resumption of second meiotic division of oocytes and sperm head decondensation leading to male pronucleus formation. A total of 227 matured oocytes with the first polar body were randomly divided into three groups: 1) ICSI without oocyte activation (ICSI (–) activation group; n=74), 2) ICSI plus oocyte activation (ICSI (+) activation group; n=79) and 3) sham injection plus oocyte activation (Sham (+) activation group; n=74). The oocytes were fixed at 18 h of IVC to assess the activation of oocytes, sperm head decondensation and male pronucleus formation.

3.3.9.2 Experiment 2

This experiment was designed to compare the subsequent development ofembryos derived from ICSI (+) activation group (n=148) and Sham (+) activation group (n=60). Embryos obtained by *in vitro* fertilization of IVM oocytes served as a control group (IVF group; n=149). The rates of cleavage and morula and blastocyst formation were assessed on Days 2, 5 and 7 or 8, respectively; blastocysts obtained on Day 7 were separated for fixation immediately and number of blastocysts newly formed on Day 8 was counted. Their development was evaluated by stereomicroscopy at a magnification: ×400. Samples of embryos at the blastocyst stage were fixed for the evaluation of total cell number (TCN). They were stained with DAPI and assessed by the epifluorescent microscope.

3.3.9.3 Experiment 3

This experiment was designed to test the presence of a paternally expressed gene (*Nnat*) in blastocysts derived from IVF, ICSI (+) activation and OA groups to clarify whether the embryos were obtained from syngamy or parthenogenesis. On Day 7 all the blastocysts obtained from each treatment were kept at -80 °C for examination of paternally expressed *Nnat* gene using RT-PCR technique. The gene expression study was performed for three replicates in each experimental group.

3.3.10 Statistical analysis

Data obtained from this study was pooled from at least three independent replicates and expressed as the percentage of oocytes with pronuclear formation, cleaved and subsequently developed to the blastocyst stage. The pronuclear formation, cleavage and blastocyst rates were compared among experimental groups by chi-square and fisher's exact statistical tests using SAS 9.2 (The SAS Institute Inc., Cary, NC, USA). Variations in total cell number of blastocyst were tested for the normal distribution of residuals from the statistical models using the UNIVARIATE procedure option NORMAL. The GLM procedure was subjected for effect from treatments and days. LSMEANS statement was used to compare resultsamong days, treatments and interaction between days and treatments. $P< 0.05$ was defined as the significance level.

3.4 Results

3.4.1 Experiment 1

The nuclear status of oocytes after ICSI/ ICSI plus activation/ sham injection plus activation is shown (Table6). In theICSI (-) activation group, most oocytes remained at MII (56/74; 75.7%), whereas 13.5% (10/74) of them were at AII and TII and 2.7% (2/74) had a female pronucleus; thus 16.2% of them were activated and underwent the 2nd meiotic progression. The pronuclear formation rate, irrespective of the number of

observed pronucleus, was significantly increased when activation was performed following ICSI (55/79; 69.6%) or sham injection (52/74; 70.3%) compared to ICSI (-) activation group. A large proportion of activated oocytes had pronuclear formation after ICSI plus activation with intact sperm head, whereas only five of the zygotes (9.1%) exhibited male pronucleus formation.

Table6. Morphological features of presumptive buffalo zygotes derived from intracytoplasmic sperm injection (ICSI (-) activation group) or ICSI followed by activation (ICSI (+) activation group) or sham injection followed by activation (sham (+) activation.

Treatment	No. of injected oocytes	No. of (%) oocytes not activated (without PN)		No. (%) of oocytes activated (with PN)		
		MII	AII/TII	Total	with intact sperm head**	with male PN (as % successfully activated)**
ICSI (-) activation	74	56(75.7) [a]	10(13.5) [a]	2(2.7) [a]	2 (100)	0
ICSI (+) activation	79	17(21.5) [b]	2(2.5) [b]	55(69.6)[b]	50 (90.9)	5 (9.1)
Sham(+) activation	74	13(17.6) [b]	5(6.6)[a,b]	52(70.3)[b]	-	-

* Zygotes were examined at 18 h of *in vitro* culture.

**Percentages of oocytes with intact sperm head/ with male PN were calculated from a total number of activated oocytes.

[a,b]Within a column, means without a common superscript differed ($P<0.05$)

PN: pronuclear formation; MII: metaphase II; AII: anaphase II; TII: telophase II.

3.4.2 Experiment 2

Following ICSI + activation, Sham + activation, and IVF, oocytes were further cultured *in vitro* and examined for developmental competence (Table7). Cleavage rate did not significantly differ between ICSI (+) activation and Sham (+) activation groups, but they were higher than that of IVF group ($P<0.05$). However, further development in terms of morula and blastocyst formation rates was similar among experimental groups ($P\geq0.05$). Also the mean (± S.D.) TCN of blastocyst embryos was not significantly different among groups. Conversely, IVF embryos were more advanced in development to the blastocyst stage than the others on Day 7, whereas most of ICSI + activation and Sham + activation embryos developed to the blastocyst stage on Day 8 ($P\leq0.05$; Fig.3).

Table7. The developmental competence of buffalo embryos and total cell numbers (TCN) in blastocysts derived from ICSI (+) activation, Sham (+) activation, and IVF groups.

Treatment	No. of oocytes examined	No.(%) of embryos cleaved*	No.(%) of embryos developedto the stage of**		Mean±S.D. of TCN in blastocysts
			Morula	Blastocyst	
ICSI (+) activation	148	118(79.7)[a]	65(55.1)	50(42.4)	87.5±25.8
Sham (+) activation	60	46(76.7)[a]	23(50.0)	18(39.1)	90.8±35.6
IVF	149	62(41.6)[b]	29(46.8)	22(35.5)	109.6±41.3

*Calculated from the number of cleaved embryos per total number of oocytes used.
**Calculated from the number of morula/blastocyst per number of cleaved embryos.
a,b Within column, means without a common superscript differed (P<0.05).

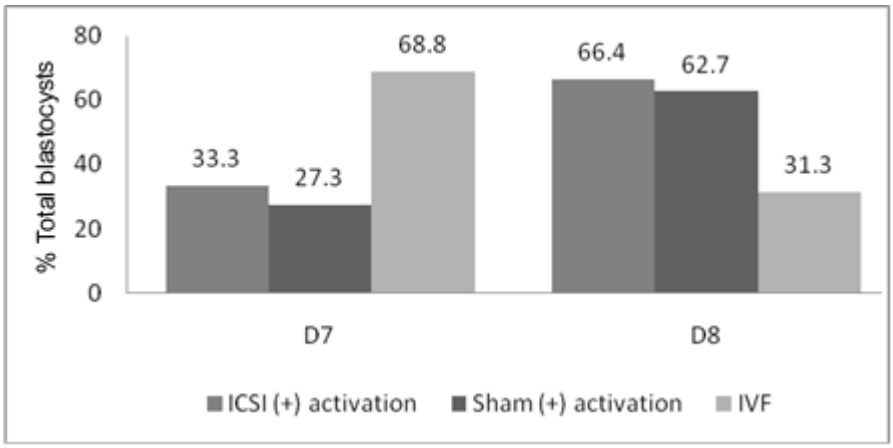

Figure3. Timing of blastocyst development on Day 7 and Day 8 in Experiment 2. [a,b] Values with different superscripts are significantly different (P<0.05). Percentage of blastocysts was expressed in a relation to a total number of blastocyst on Day 7 and Day 8 in each group.

3.4.3 Experiment 3

Electrophoregrams of RT-PCR products are shown (Fig. 4). All samples were positive for *GAPDH*. Neuronatin mRNA was expressed in all samples (3/3) of blastocystsfrom IVF group. Conversely, *Nnat* mRNA was not expressed in any samples (3/3) from ICSI (+) activation group and OA group (3/3).

The PCR product of *Nnat* primers from IVF embryos was sequenced. The sequence had 99% similarity compared to the reported *Nnat* sequence in *Bos taurus* (accession number AY 360450; Table8).

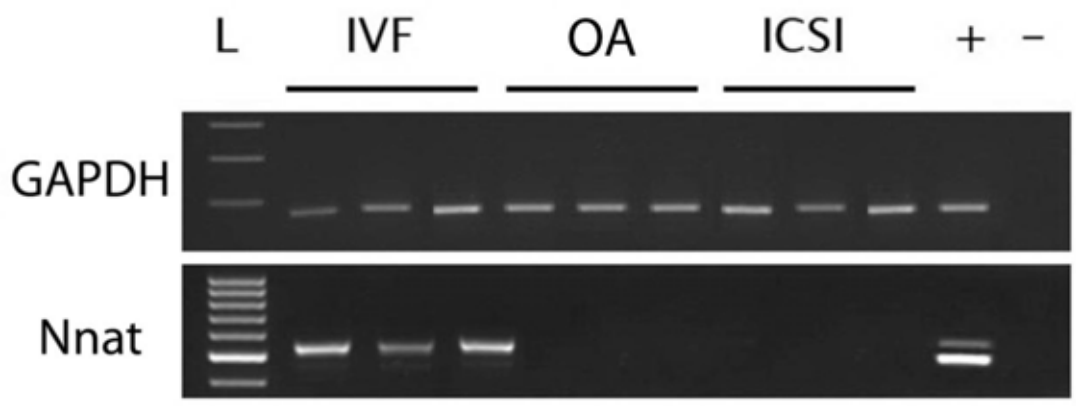

Figure4. Electrophoregram of *GAPDH* and *Nnat* in blastocystsfrom IVF, OA and ICSI (+) activation groups. L: 100 bp DNA marker; IVF: *in vitro* fertilization; OA: oocyte activation; ICSI: intracytoplasmic sperm injection plus activation; +: positive control; -: negative control

Table8. Sequence alignment between *Bos taurus*'s *Nnat* sequence (AY 360450 from base number 217 to 439) and PCR product of *Nnat*primers from buffalo IVF embryos (from base number 207 to 429). Abbreviations and symbols in nucleotide sequence: A=adenine, C=cytosine, G=guanine, T=thymine. Asterisks (*) indicate different nucleotides between the two sequences.

Subject	Base	Nucleotide sequence (5′→3′)	Base
Bostaurus	217	CCCCTCCCCTGGGCGGCCGTGTCACCAGGTGCTC	250
PCR product	207	CCCCTCCCCTGGGCGGCCGTGTCACCAGGTGCTC	240
	251	CTGTGCCATTCCACCAGCATGGGAGCCAGCGCCGC	285
Bostaurus	241	CTGTGCCATTCCACCAGCATGGGAGCCAGCGCCGC	275
PCR product			
	286	GCAGGAATGGGGCGTCCCCTGTGCCCTCTCGCCAG	320
Bostaurus	276	GCAGGAATGGGGCGTCCCCTGTGCCCTCTCGCCAG	310
PCR product			
	321	AGGAGCACTTGCCAAGGTCAGCGAGGGGCTGGTAG	355
	311	AGGAGCACTTGCCAAGGTCAGTGAGGGGCTGGTAG	345
Bostaurus		*	
PCR product	356	GCCCCTGGAGAAGCAGCACCGACAATGACGACAAC	390
	346	GCCCCCGGAGAAGCAGCACCGACAATGACGACAAC	380
		*	
Bostaurus	391	AAGAGATCCCTTCCCCACCCCTTTGCACCCCTCCC	425
PCR product	381	AAGAGATCCCTTCCCCACCCCTTTGCACCCCTCCC	415
	426	ACTGCGGGTGTCCT	439
Bostaurus	416	ACTGCGGGTGGCCT	429
PCR product		*	

3.5 Discussion

In the present study, injection of sperm into cytoplasm of buffalo oocytes alone was not sufficient to activate oocytes and start syngamy. Although some ICSI oocytes were capable of resuming the second meiotic division, embryo development to the blastocyst stage was obtained only when they were additionally activated with calcium ionophore and cycloheximide. This activation protocol is widely used for oocyte activation in cattle (Galli et al., 2003; Rho et al., 2004; Suttner et al., 2000) to elevate cytosolic calcium and also inhibit the activity of mitogen-activated protein kinase (MAPK) and maturation-promoting factor (MPF) which are required during fertilization

and pronuclear formation (Chian and Sirard, 1996; Liu and Yang, 1999). Interestingly, in the present study (Experiment 1) most of activated ICSI oocytes contained two pronuclei together with an intact sperm head inside the ooplasm. The presence of more than one female pronucleus may raise the possibility of the failure of the second polar body extrusion in ICSI oocytes; in case of normal fertilization, this would result in abnormal embryo ploidy. Nevertheless, in the majority (73.8%) of ICSI embryos, extrusion of the second polar bodywas confirmed. The reason for more than one female pronucleus in ICSI oocytes might be attributed to a side effect of the activation procedure. Sham injection followed by oocyte activation also provided a comparable result (Table6). Therefore, we inferred that the chemicals predominantly stimulated female pronuclear formation rather than sperm head decondensation or male pronuclear formation. This circumstance alerted the awareness of parthenogenesis occurring in the ICSI system used in this study.

In Experiment 2, there were two major significant differences among ICSI (+) activation, Sham (+) activation and IVF groups. First, developmental competence at cleavage stage in ICSI (+) activation and Sham (+) activation groups was significantly higher than that of IVF group, although subsequent embryo development of cleaved oocytes had a similar capability. Therefore, we inferred that activated cleaved ICSI embryos had similar competence to develop to the blastocyst stage compared with cleaved IVF embryos in our IVC system. Low cleavage rates after IVF have been associated with several factors, including poor sperm quality (Selvaraju et al., 2009), improper sperm concentration (Totey et al., 1993a), and condition of culture media used for IVF (Abdoon et al., 2001; Ravindranatha et al., 2003). In our study, fertilization failure of buffalo oocytes during IVF was predominantly caused by defective sperm penetration (Chankitisakul et al., 2010). Second, the interval required for blastocyst development of IVF was significantly faster as compared to the ICSI (+) activation and Sham (+) activation groups. It was suggested that parthenogenetic embryos took longer than IVF embryos to reach the blastocyst stage (Narula et al., 1996). In this study, delayed blastocyst formation in the sham injection (+) activation group was in agreement with the report of Narula et al. (1996). However, delayed development in the ICSI (+) activation group was questionable and raisedthe possibility of parthenogenesis due to the activation process.

Total cell number has been used as an indicator for identification of quality and viability of preimplantation embryos (Leoni et al., 2008; Matsuura et al., 2010; Papaioannou and Ebert, 1988). Therefore, TCN of blastocysts produced in ICSI (+) activation, Sham (+) activation and IVF groups was determined. The TCN of blastocysts was not significantly different among groups. Even though only a few embryos produced by ICSI and Sham injection reached the blastocyst stage on Day 7, the embryos had the same quality as those produced by IVF. Besides, the range of the TCN in each group in our study was similar to other reports (Gasparrini et al., 2004; Narula et al., 1996).

Therefore, we concluded that the *in vitro* embryo production protocol used in this study produced and supported development of IVF and parthenogenetic embryos to blastocysts of similar quality. Based on normal blastomere numbers despite a high frequency of male pronucleus formation failure in ICSI embryos, we inferred that TCN was not a reliable indicator of normal fertilization.

It was important to clarify whether the buffalo ICSI embryos in this study were generated from syngamy or parthenogenesis. Therefore, imprinted genes were used to confirm activity ofthe paternal genome. Genomic imprinting is a phenomenon leading to sex-specific monoallelic expression of genes (Goto et al., 1990; Nakai et al., 2011; Surani et al., 1984). A few imprinted genes have been reported in cattle, e.g. *IGFIIR* (Killian et al., 2001), *IGF*, *MEG3*, *XIST* (Dindot et al., 2004) and *Nnat* (Ruddock et al., 2004). In this study *Nnat* was used, since it is a maternally-imprinted-paternally-expressed gene; therefore it is only expressed from the allele inherited from the father. It has been also reported to be actively expressed in the blastocyst stage of IVF bovine embryos, but not in embryos derived by parthenogenesis (Ruddock et al., 2004). This was apparently the first report that the *Nnat* expression in IVF buffalo blastocysts was similar to that found in IVF bovine embryos. However, *Nnat* mRNA was absent from blastocysts in ICSI (+) activation group, similar to blastocysts obtained by OA. The absence of the RT-PCR product in ICSI and OA embryos signified the locus where this gene lies may have not been remodeled and/or made accessible for transcription in ICSI embryos. This was true for the OA embryos for which no paternal genome was included in the generation process, but was unlikely for ICSI. Conversely, the lack of *Nnat* expression confirmed that the specific oocyte activation protocol used resulted in parthenogenetic activation in a high rate of ICSI oocytes. Therefore, we inferred that only morphological evaluation of blastocyst development following ICSI (+) activation was not adequate evidence to conclude that male gamete activation had occurred. Since 9.1% of ICSI buffalo embryos were syngamic, perhaps some of the blastocysts could have expressed the *Nnat* transcript; however it could have remained undetected in the pooled samples using our PCR settings. Nevertheless, it must be noted that only 35% of cleaved IVF embryos formed blastocysts. Therefore, we inferred that no more than 3.5% of blastocyst embryos in ICSI (+) activation group were generated by normal fertilization. Thus even if someICSI embryos were due to syngamy, the vast majority of blastocysts from ICSI group in our experiment must have been parthenotes and therefore the probability of detecting these blastocysts was very low.

Increased cytoplasmic calcium at fertilization is recognized as a primary signal for resumption of the cell cycle, as calcium releasing is related to the transition of MPF activity in the ooplasm, resulting in successful pronuclear formation (Malcuit et al., 2006). However, we proposed that chemical activation following ICSI directly affected female pronuclear formation, but was not associated with male pronuclear formation.The failure of male pronuclear formation after ICSI was also reported in other domestic

animals, e.g. pigs and cattle (Li et al., 1999; Nakai et al., 2011), probably due to a higher rigidity of sperm plasma membrane (Perreault et al., 1988; Sutovsky et al., 1997), together with a large amount of acrosome and acrosomal enzymes present in the ooplasm after sperm injection in these species (Morozumi and Yanagimachi, 2005). Perhaps only physical causes were insufficient to damage the membrane, therefore preventing sperm-borne oocyte-activating factor, located in perinuclear theca membrane, to be released into ooplasm, resulting in a blockage of sperm head decondensation and oocyte activation (Kimura et al., 1998; Parrington et al., 1996; Perry et al., 1999; Sutovsky et al., 2003). It is therefore not surprising that success of full-term pregnancy from transferred ICSI embryos has been remarkably limited in livestock species, probably due to embryos that lack the paternal genome (Surani et al., 1984). It is not possible to conclude that all activation protocols could lead to parthenogenetic activation. However, we inferred that the activation protocol in this study apparently increased the rate of parthenogenetic activation.

To improve syngamy following ICSI, sperm treatments prior to injection are thought to enhance sperm nucleus decondensation. For example, removing the sperm plasma membrane by freezing/thawing without cryoprotectant (Goto et al., 1990), or sperm membrane treatments using Triton-X 100 (Lee et al., 2004; Tian et al., 2006) facilitated the release of oocyte activation factors from sperm into oocyte. Dithiothreitol, a chemical that specifically reduces disulfide bonds of sperm protamines, promoted the decondensation of male nuclear chromatin in cattle (Rho et al., 1998a; Suttner et al., 2000) and improved the normal fertilization rate in pigs (Tian et al., 2006; Yong et al., 2005). In addition, application of piezo pulses for sperm immobilization also induced sperm head decondensation and improved normal fertilization rates without chemical activation in many species (Galli et al., 2003; Katayama et al., 2005; Wang et al., 2003; Wei and Fukui, 2002). In this respect, application of such sperm head pretreatments might improve the efficacy of ICSI technology in buffaloes.

In summary, ICSI alone was insufficient to induce sperm and oocyte activation in buffalo, whereas the oocyte activation protocol used in our study (calcium ionophore and cycloheximide) induced parthenogenetic activation. The chemical activation after ICSI directly affected the female pronuclear formation resulted in embryo development to the blastocyst stage; however, it was not associated withmale pronuclear formation. Our results confirmed that buffalo embryos derived from ICSI combined with oocyte activation were obtained from parthenogenetic activation, since the rates of oocytes with sperm head decondensation and male genome remodeling were so low that the embryos did not express the paternally inherited gene in this study. Further study is required to examine the pattern of calcium oscillation following oocyte activation and also to test whether sperm treatment prior to ICSI can improve male pronuclear formation in buffalo oocytes.

CHAPTER IV

SPERM PRETREATMENT WITH DITHIOTHREITOL INCREASES MALE PRONUCLEUS FORMATION RATES AFTER INTRACYTOPLASMIC SPERM INJECTION (ICSI) IN SWAMP BUFFALO OOCYTES

4.1 Abstract

Failure of sperm head decondensation has been hampered the success of intracytoplasmic sperm injection (ICSI) in swamp buffalo. The aim of the present study was to improve sperm head decondensation by pretreating the sperm with various chemicals before ICSI. In Experiment 1 and 2, the sperm were treated with the following protocols;(1) 0.1% Triton-X 100 (TX) for 1 min,(2) 10 μM calcium ionophore (CaI) for 20 min, (3) freezing and thawing (FT) without any cryoprotectant, and (4) untreated control. These sperm treatments were further combined with or without additional sperm treatment with 5 mM dithiothreitol (DTT) for 20 min. Acrosome integrity and DNA fragmentation were evaluated in the sperm before ICSI. Staining of the spermwith fluorescein isothiocyanate–labeled peanut agglutinin revealed significantly increased rates of acrosome-lost sperm cells after TX and CaI treatments, whereas FT treatment and no-treatment (control) significantly increased the proportion of acrosome-reacted sperm. DTT treatment had no significant effect on the acrosome configuration of sperm. Sperm staining with TUNEL did not indicate significant differences in DNA fragmentation among treatments. In Experiment 3, *in vitro* matured oocytes were subjected to ICSI using pretreated sperm as described above, and then they were stimulated with or without artificial activation. At 18 h post-ICSI, 6-diamidino-2-phenylindole staining revealed female pronucleus formation only in activated oocytes (about 60-80%). However, of all the activated ICSIoocytes, the majority of them contained intact sperm heads. Normal fertilization characterized by the presence of male and female pronuclei without an intact sperm head was only observed in CaI and FT treatment and the control groups when sperm were treated with DTT before ICSI (8.9, 23.5, and 31.0%, respectively). In conclusion, these results indicate that DTT treatment of sperm with reacted acrosome before ICSI together with an additional activation of resultant ICSI oocytes are important for successful sperm head decondensation resulting in male pronuclear formation.

Keywords: Intracytoplasmic sperm injection, dithiothreitol, male pronuclear formation, swamp buffalo

4.2 Introduction

In buffalo, knowledge of intracytoplasmic sperm injection (ICSI) is still limited because of the low number of studies in this field. We have previously reported that an injected sperm itself poorly stimulate the activation process in buffalo oocytes. Althoughan additional chemical activation following ICSI can promote a development of ICSI oocytes to the blastocyst stage, a failure of sperm head decondensation has been mainly observed (Chankitisakul et al., 2012). A possible reason for the failure may be the lack of an important signaling molecule when a sperm head is injected into an oocyte. In case of ICSI, the sperm nucleus is injected into ooplasm together with the perinuclear material, acrosome, and plasma membrane (Ajduk et al., 2006). Theoretically, phospholipase C zeta (PLCζ), a sperm-borne oocyte-activating factor (SOAF) and is localized in the acrosomal and post acrosomal regions of the perinuclear theca (PT) membrane (Dozortsev et al., 1998; Sutovsky et al., 1997), may not be able to penetrate through the sperm plasma membrane resulting in a limited amount of PLCζ available for oocytes activation. This event differs from normal fertilization where the PT is rapidly solubilized following fusion of the sperm plasma membrane with the oolemma, leading to the release of SOAF and other factors in the oocyte cytoplasm (Parrington et al., 1996). To increase the successful rate of ICSI, the disintegration of the sperm plasma membrane and the removal of acrosome have been performed before sperm injection by mechanical stimulus of an injection pipette in humans and some mammalian species such as mice and rabbits (Keefer, 1989; Tesarik et al., 1994). These studies have shown evidence that the removal of the sperm plasma membrane and acrosome has an important role; it does not only accelerate the onset of oocyte activation but also improves embryonic development after ICSI.

Compared to laboratory animals, ICSI is far less efficient in livestock species (Yanagimachi, 2005). Simply physical damage seems not to be sufficient enough to damage the membrane. As a result, SOAF cannot be released into the ooplasm resulting in the blockage of sperm head decondensation and oocyte activation (Kimura et al., 1998; Parrington et al., 1996; Perry et al., 1999; Sutovsky et al., 2003). This is likely due to the higher rigidity of the perinuclear theca (Perreault et al., 1988; Sutovsky et al., 1997), together with the large number of acrosome and acrosomal enzymes which enter the ooplasm after sperm injection (Morozumi and Yanagimachi, 2005). In order to improve normal fertilization following ICSI, sperm pretreatment(s) before injecting have been successfully performed to improve the developmental rates of ICSI oocytes in cattle (Galli et al., 2003; Liu and Yang, 1999; Rho et al., 1998a) and pigs (Lee and Yang, 2004; Lee et al., 2002; Nakai et al., 2003; Tian et al., 2006). Various sperm pretreatment protocols have been used to disrupt the acrosomal and sperm plasma membrane such as using Triton-X 100 (Lee and Yang, 2004; Tian et al., 2006), calcium ionophore(Galli et al., 2003; Lacham-Kaplan and Trounson, 1995; Nakai et al., 2003), and freezing/thawing without cryoprotectant (Goto et al., 1990). Additionally, it has been suggested that bull

and boar sperm chromatin are tightly packaged and more stable than other species (Bedford, 1988; Jager, 1990). Therefore, dithiothreitol (DTT), a chemical that specifically reduces disulfide bonds of sperm protamines, has been demonstrated to promote the decondensation of sperm chromatin in ICSI oocytes in cattle (Rho et al., 1998a; Suttner et al., 2000) and also to improve their developmental capacity in pigs (Tian et al., 2006)

Although this approach has not been investigated in the buffalo, it seems possible that the presence of sperm plasma membrane and acrosome and the tightly packaged status of sperm chromatin could contribute to the failure of sperm head decondensation and oocyte activation in swamp buffalo after ICSI. Therefore, the present study was designed to investigate the efficacy of various chemical and physical treatments of sperm prior to ICSI in order to improve male pronuclear formation for the first time in buffalo. The quality of sperm after sperm treatments was examined in terms of acrosome and DNA integrity of buffalo sperm after treatment with Triton-X 100, calcium ionophore, freezing and thawing and DTT. Also we examined the effects of sperm pretreatments on sperm head decondensation and male pronuclear formation in ICSI oocytes.

4.3 Materials and Methods

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

4.3.1 Oocyte recovery and maturation

Swamp buffalo ovaries were obtained from animals of unknown reproductive status at a local slaughterhouse and were transported to the laboratory within 4 h in 0.9% (w/v) of normal saline supplemented with 100 IU/mL penicillin G and 100 μg/mL streptomycin at 28-30 ^{o}C. The ovaries were later washed twice in phosphate buffered saline supplemented with 100 IU/mL penicillin G and 100 μg/mL streptomycin. The oocytes were subsequently aspirated from 2 to 8 mm antral follicles with an 18-gauge needle attached to a 10-mL syringe. The oocytes weremorphologically selected under a stereomicroscope (Nikon SMZ645, Tokyo, Japan) at a magnification: ×400. Only cumulus-oocyte complexes (COCs) with homogenous ooplasm and surrounded by multiple compact layers of cumulus cells were submitted for *in vitro* maturation. Groups of 25-30 COCs were placed in 4-well plastic dishes (Nunc, Roskilde, Denmark), with each well containing 500 μL of $NaHCO_3$ buffered tissue culture medium 199 (with Earle's salts) supplemented with 10% (v/v) fetal calf serum (FCS), 50 IU/mL human chorionic gonadotropin (Intervet-Schering-Plough, Boxmeer, The Netherlands), 1μl/mL insulin–transferrin–selenium, 0.05 IU/mL recombinant human follicle-stimulating hormone (Organon, Bangkok, Thailand), 100 μM cysteamine, 20 ng/mL epidermal growth factor, 100 IU/mL penicillin G and 100 μg/mL streptomycin. *In vitro* maturation was performed for 21 h at 38.5 °C in a humidified atmosphere of 5% CO_2 in air. Then,

the cumulus cells were completely removed by gentle repeated pipetting. Only oocytes with a visible first polar body were selected and they were maintained in Hepes-buffered synthetic oviductal fluid (Hepes-SOF) until further treatment.

4.3.2 Sperm preparation and treatment

Frozen semen from the single batch of a buffalo bull provided by the Thai Swamp Buffalo Conservation and Development Center (TSBCDC; Charoen Pokphand, Thailand) was used in this study. The semen was thawed at 37 °C in a water bath for 30 s. The thawed semen was layered on top of apercoll gradient density consisting of 1 mL each of 45% and 90% Percoll in a 15-mL plastic conical centrifugetube. The tube was then centrifuged at 800 × g for 15 min. The top layers were then removed immediately after centrifugation and only the sperm pellet was subsequently used. Sperm were washed with 1 mL modified Tyrode's medium (TALP) at 600 × g for 5 min. The supernatant was then removed until 100 μL containing the sperm suspension was left in the tube for further treatment.

The sperm suspension was subjected to the following treatments: 1) TX treatment; 50 μL of sperm suspension was diluted in 50 μL of 0.2% (v/v) TX and incubated for 1 min according to Lee and Yang (2004), 2) CaI treatment; 50 μL of sperm suspension was exposed to 10 μM CaI for 20 min at 37 °C according to Nakai et al. (2003), 3) FT treatment; sperm were frozen without any cryoprotectant using -20 °C refrigerators and then thawed in 37 °C water according to Goto et al. (1990), and 4) control; sperm without any treatments. In each treatment sperm were then either treated or not treated with 5 mM dithiothreitol (DTT) for 20 min at room temperature according to Rho et al. (1998a). Following each sperm treatment, the treated sperm were washed once with 5 mL of TALP by centrifugation at 800 × g for 5 min. The sperm pellets were then resuspended in Hepes-SOF and used either for further examination of acrosome and DNA integrity or ICSI, according to the experimental design.

4.3.3 Sperm assessment

4.3.3.1 Acrosome integrity

The integrity of sperm acrosome was evaluated with fluorescein isothiocyanate–labeled peanut (*Arachishypogaea*) agglutinin (FITC-PNA) staining. In brief, 10 μL sperm suspension was mixed with 10 μL of Ethidiumhomodimer-1 (EthD-1) and incubated at 37 ºC for 15 min. Then, 5 μL mixtures was smeared on a glass slide and air-dried. Then the samples were fixed with 95 % ethanol for 30 s and air-dried again. In the next step 50 μL FITC-PNA (dilute FITC-PNA: PBS = 1:10 (v/v)) was spread over the slide and the slides were incubated in a dark moist chamber at 4 °C for 30 min. After that, the slides were rinsed with cold PBS and air-dried. Sperm were assessed under a fluorescent microscope at a magnification: ×1,000 and categorized as three groups; A) acrosome-

intact sperm having bright fluorescence over the acrosomal cap indicating the presence of the outer acrosomal membrane, B) acrosome-reacted sperm having a patchy disrupted fluorescence over the acrosome or demonstrating fluorescence limited only to some segments of the acrosomal cap, and C) acrosome-loss sperm displaying no FITC-PNA staining and showing red fluorescence due to counterstaining with EthD-1 (Cheng et al., 1996).

4.3.3.2 DNA fragmentation

The DNA fragmentation was assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. In brief, the sperm suspension was first smeared on to a glass microscopic slide coated with aminopropyltriethoxysilane and allowed to air dried at room temperature. Each slide containing sperm was fixed with 4% paraformaldehyde for 30 min.After a brief washing with PBS, the sperm was permeabilized on ice with 0.1% (v/v) Triton X-100 in PBS for 5 min. Detection of DNA fragmentation was performed using an *in situ* cell death detection kit (Roche, Mannheim, Germany) following the manufacturer's instructions. Briefly, the slides containing sperm were washed in PBS and incubated with TUNEL reaction mixture (TdT enzyme: label solution = 1 : 10 (v/v)) for 1 h at 37°C in a humidified chamber. Then the chromosome was labeled with 5 pg/mL of 4',6-diamidino-2-phenylindole (DAPI). The antifade mounting medium (vectashield ™, Vector labs, California, USA) was used to retard photo bleaching that might occur during sperm examination by a fluorescent microscope. The TUNEL-negative sperm fluorescently were stained with red color and TUNEL-positive sperm fluorescently were stained brightly in green color. Sperm positive to TUNEL was classified as apoptotic or DNA fragmented sperm.

4.3.4 Intracytoplasmic sperm Injection (ICSI)

Conventional ICSI was performed using an inverted microscope (IX71; Olympus, Tokyo, Japan) equipped with a micromanipulator (IM-9B; Narishige, Tokyo, Japan). The inner diameter of the injection needle was 9-11 μm (OrigioHumagen Pipets Inc., Charlottesville, VA, USA; REF No. MIC-9-30), and the inner diameter of the holding pipette was 10–20 μm. The treated sperm were diluted with 10% polyvinylpyrrolidone in Hepes-SOF. Sperm were injected as described previously (Chankitisakul et al., 2012). Injection was conducted within one hour of each sperm treatment. Sham injection of oocytes was conducted using the same procedure as sperm injection, except that no sperm was loaded into the injection pipette.

4.3.5 Oocyte activation

Injected oocytes were exposed to 5 μM calcium ionophore (A23187) in SOF medium supplemented with 3 mg/mL bovine albumin serum (SOF-BSA) for 5 min and then incubated for 5 h with 10 μg/mL cycloheximide in SOF-BSA at 38.5 °C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

4.3.6 In vitro embryo culture (IVC)

Presumptive zygotes were cultured in SOF supplemented with 3 mg/mL BSA for 18 h post ICSI. *In vitro* culture was performed at 38.5 ^{o}C in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

4.3.7 Assessment of oocytes

At 18 h after IVC, the oocytes were fixedand stored in 4% (w/v) paraformaldehyde in PBS until analysis. The chromosomes in the oocytes and the state of sperm heads were labeled by DAPI staining in order to precisely examine the nuclear status. The fluorescently labeled samples were then mounted on a glass microscope slide in a 2 μL droplet of antifade medium to retard photobleaching and visualized with an epifluorescent microscope (BX51, Olympus, Tokyo, Japan). Oocytes with one or two pronuclei and polar body (bodies) were considered activated, whereas those with both male and female pronuclei in the absence of sperm head were considered normally fertilized. An activated oocyte containing a sperm head was considered to have been activated parthenogenetically but not fertilized.

4.3.8 Experimental design

4.3.8.1 Experiment 1: Effect of sperm treatments on acrosome integrity

To assess acrosome integrity after treatment with following protocols: (1) 0.1% TX; (2) 10 μM CaI; (3) FT; (4) untreated control. Sperm in each group were either treated or not with 5 mM DTT and were fixed and evaluated for acrosome integrity by FITC-PNA. The experiment was replicated at least three times.

4.3.8.2 Experiment 2: Effect of sperm treatments on DNA fragmentation

To examine the effect of sperm treatment on DNA fragmentation, DNA fragmentation of sperm treated in each group (according to Experiment 1) was evaluated by TUNEL assay. The experiment was replicated at least three times.

4.3.8.3 Experiment 3: Effect of sperm treatments on pronuclear formation

The objective of this experiment was to examine sperm head decondensation and male pronuclear formation (MPN) in presumptive zygotes obtained by ICSI using pretreated sperm. Matured oocytes were subjected to ICSI using sperm pretreatments

according to Experiment 1 followed by oocyte activation or nothing. Nuclear changes of the presumptive zygotes were morphologically evaluated for pronuclear formation using DAPI and epifluorescent microscopy at 18 h post-ICSI. The experiment was replicated three times.

4.3.9 *Statistical analysis*

In Experiment 1 and 2, all the data on acrosome and the DNA integrity of sperm from each treatment before subjection to ICSI was tested for the normal distribution of residuals from the statistical models using the UNIVARIATE procedure option NORMAL. The GLM procedure was subjected for effect from treatments. LSMEANS statement was used to compare the results among treatment groups. In Experiment 3, the percentage of MPN defined as the successful fertilization was compared among treatment groups by chi-square and fisher's exact statistical tests using SAS 9.2 (The SAS Institute Inc., Cary, NC, USA). $P< 0.05$ was defined as the significant level.

4.4 Results

4.4.1 Experiment 1: Effect of sperm treatments on acrosome integrity

The distributions of sperm with different acrosome status in various pretreatments are shown in Figure 5. The TX and CaI treated sperm caused an increase in the number of sperm without acrosome (acrosome-loss sperm) when compared with sperm treated by FT or control ($P<0.05$). Contrastly, the proportion of acrosome-reacted sperm was higher in FT treated sperm and the control groups than that of other groups ($P<0.05$). The DTT had no significant effect on the acrosome status of buffalo sperm. The morphology of sperm acrosome is shown in Figure 6(a).

4.4.2 Experiment 2: Effect of sperm treatments on DNA fragmentation

The DNA fragmentation following treatments of sperm in each group was less than 3% and it did not differ ($P>0.01$) among treatments. The morphology of sperm DNA fragmentation is shown in Figure 6(b).

4.4.3 Experiment 3: Effect of sperm treatments on pronuclear formation

As shown in Table 9, oocyte activation occurred only in ICSI (+) activation groups at similar rates (about 60-80%). None of ICSI (-) activation oocytes showed meiotic resumption (Data not shown). Irrespective sperm pretreatment, all of ICSI activated oocytes contained intact sperm heads when DTT was not applied. Normally fertilized oocytes with 2 PN (one MPN) without intact sperm head was observedin CaI (+) DTT, FT (+) DTT and DTT treatment groups (8.9, 23.5, and 31.0%, respectively).

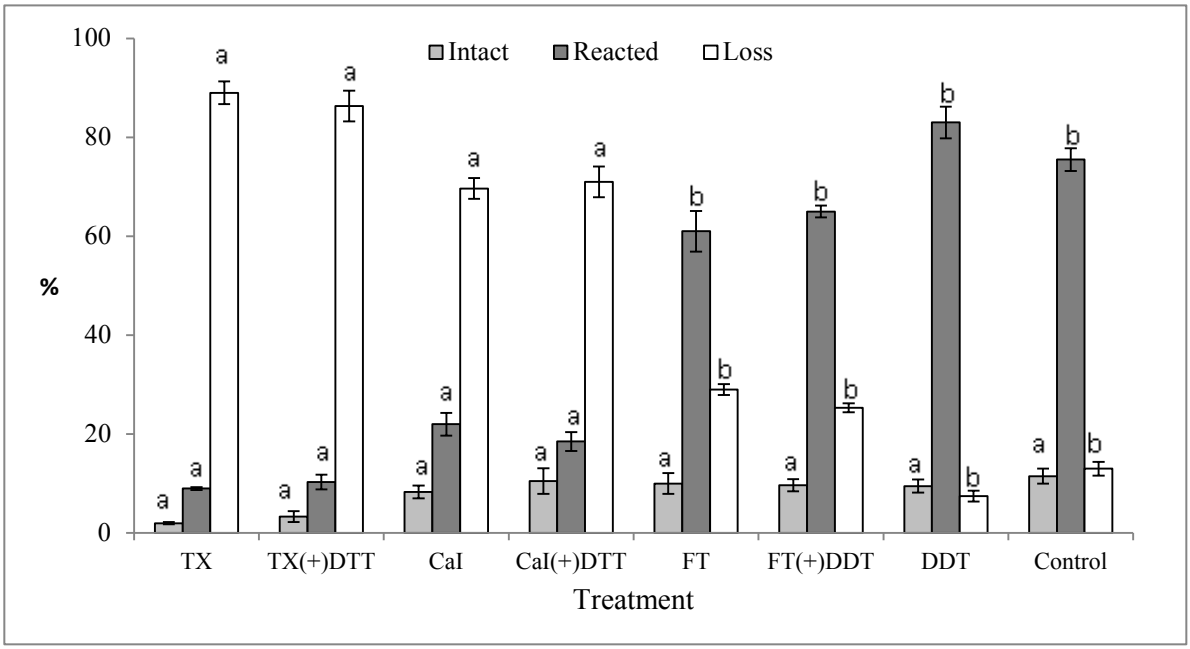

Figure5. Percentages of bu ffalo sperm with various acrosome configurations in different pretreatments combined with DTT treatment ([a,b] within the same color bar differ P<0.05). (TX: Triton-X 100; TX (+) DTT: Triton-X 100 + DTT; FT: freezing and thawing without any cryoprotectant; FT (+) DTT: freezing and thawing + DTT; DTT: dithiothreitol; control: untreated sperm)

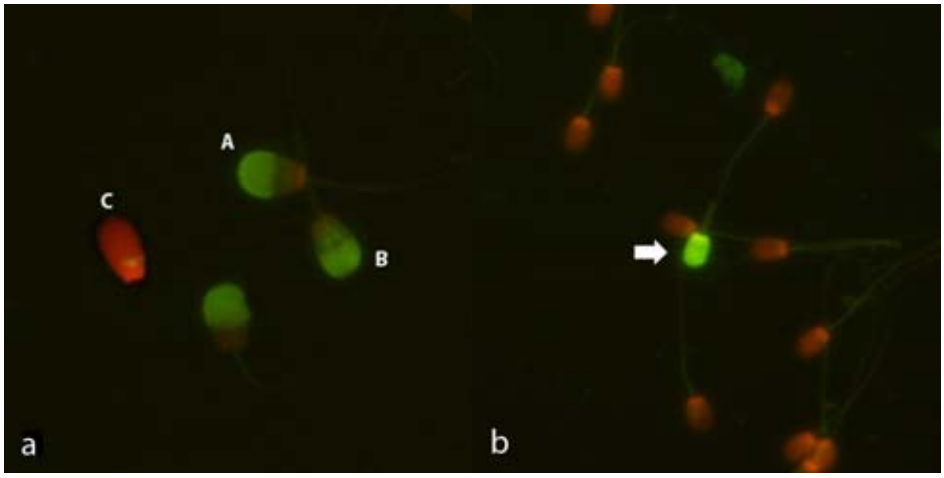

Figure6. (a) Acrosome (green: FITC-PNA) and DNA (Orange: EthD-1) of the buffalo sperm. (A) acrosome-intact sperm: having a bright fluorescence over the acrosomal cap; (B) acrosome-reacted sperm: having a patchy disrupted fluorescence over the acrosome; (C) acrosome loss sperm: no FITC-PNA staining. (b) Buffalo sperm stained with TUNEL: the DNA fragmentation was identified bya bright green fluorescence overthe sperm head (an arrow).

Table9. Activation and male pronuclear formation of buffalo oocytes injected with sperm after various treatments followedby oocyte activation

Sperm pretreatment	DTT treatment	No. of oocytes injected	No. of oocytes activated[a]	
			Total (%)[b]	With MPN (%)[c]
Tx-100	+	57	42(73.4)	0[g]
	–	33	28(84.8)	0
CaI	+	63	45(66.2)	4(8.9)[f,g]
	–	45	39(86.7)	0
FT	+	46	34(74.0)	8(23.5)[e,f]
	–	64	54(84.4)	0
Untreated	+	46	29(63.0)	9(31.0)[e]
	–	40	32(80.0)	0
Sham	–	51	41(80.4)	N/A

[a] oocytes were examined 18 h post-ICSI

[b] Percentage of oocytes injected

[c] Percentage of total oocytes activated

[e,g,f] Different superscript letters within column are significantly different ($P \leq 0.05$)

N/A no sperm was injected

4.5. Discussion

Our previous study revealed the failure of sperm head decondensation after ICSI in swamp buffalo oocytes (Chankitisakul et al., 2012). In the present study, we investigated the effects of sperm pretreatments on sperm head decondensation and pronuclear formation following ICSI. Our results demonstrate that the existence of reacted acrosome together with the reduction of disulfide bonds in sperm nucleus by DTT treatment can promote sperm head decondensation whereas the loss of acrosome induced by TX or CaI cannot induce sperm head decondensation even after DTT treatment.

It is well-known that the injection of an intact sperm allows decondensation of sperm chromatin in humans and some mammalian species. In contrast, the decondensation of sperm heads has been reported to be very low after injection of the untreated sperm in cattle and pigs; this difference in sperm decondensation in these species was suggested to be due to the higher rigidity of the sperm plasma membrane entered in the ooplasm after sperm injection in cattle and pigs (Perreault et al., 1988; Sutovsky et al., 1997). Most of the studies found an improvement of sperm head decondensation when sperm were freed from the plasma membrane before ICSI because the damage to the sperm plasma membrane might have contributed to the release of factors involved with sperm head decondensation and oocyte activation (Galli et al., 2003; Kolbe and Holtz, 2000; Probst and Rath, 2003). In addition, a large amount of acrosomal contents and enzymes have appeared to be harmful to oocytes in mice,

therefore prior to ICSI removal of acrosome from the sperm is essential for successful ICSI (Lacham-Kaplan and Trounson, 1995; Morozumi and Yanagimachi, 2005). The disintegration of the sperm plasma membrane and the removal of acrosome simultaneously have been studied using various treatments (Galli et al., 2003; Morozumi and Yanagimachi, 2005; Suttner et al., 2000). In our study, the sperm plasma membrane and acrosome damage were effectively induced by TX, CaI and FT treatments. Evaluation of sperm acrosome integrity indicated that sperm treatment with TX and CaI apparently causes a significant increase in the number of acrosome-loss sperm. However, in this study the removal of the sperm plasma membrane and acrosome did not promote sperm head decondensation and oocyte activation after ICSI in swamp buffalo.

Contrastly, the decondensation of sperm chromatin was enhanced when sperm were treated with DTT before ICSI in CaI (+) DTT, FT (+) DTT, and DTT treatment groups. The DTT is an agent that specifically reduces disulfide bonds in sperm chromatin. It is evident in bull and boar sperm that the disulfide bonds of protamines in sperm chromatin are tightly packaged and more stable than other species (Bedford, 1988). This chemical reagent, therefore, was suggested to be able to access the sperm nucleus via perinuclear theca to reduce disulfide bonds of protamine resulting in the decondensation of the chromosomes (Rho et al., 1998a; Sutovsky et al., 1997). Interestingly, sperm treatment with DTT following Triton X (TX (+) DTT) pretreatment could not induce sperm head decondensation in our study. This outcome is not in accordance with previous reports in cattle and pigs in which TX (+) DTT sperm treatment accelerated sperm head decondensation after injection (Chung et al., 2000; Nakai et al., 2006). Also, in our study we noticed that even though CaI (+) DTT sperm treatment could promote sperm head decondensation at a low rate (8.9%), the majority of activated oocytes contained intact sperm heads. This rate also did not differ significantly from that of TX (+) DTT. Contrastly, when treatments resulting in high rates of sperm with reacted acrosome (ie. control and FT groups) were combined with DTT treatment, the highest male pronuclear formation rates were obtained after ICSI. The high rate of sperm with reacted acrosome in the control group in this study can be explained by the damage to the sperm plasma membrane during freezing and thawing in diluents containing cryoprotectant under standard procedures for frozen semen at TSBCDC. Therefore, we speculated that the status of acrosome integrity had an effect on sperm head decondensation after ICSI. Our results have demonstrated that the proportion of sperm without acrosome was significantly increased in TX (+) DTT and CaI (+) DTT treatment groups, and differed significantly from those of FT (+) DTT and DTT treatment groups. One might think that suppressed MPN formation by TX treatment might be caused by a side effect on the nuclear structure. However, TUNEL staining has demonstrated no difference on DNA integrity among the treatment groups. It can therefore be speculated that the damage methods for buffalo sperm plasma membrane and acrosome using TX and CaI, at least in our study, might have either caused a premature release of acrosomal

matrix containing specific substances or perhaps removal of some extra-nuclear components that probably participate in the activation process. Such side effects may lead to the failure of sperm chromatin decondensation (Gutierrez-Perez et al., 2011; Yan et al., 2008). In accordance with our results Nakai et al (2003) has reported that acrosome loss of porcine sperm induced by CaI treatment does not affect embryo development. They found that the rate of acrosome loss in CaI treated sperm was significantly higher than that of untreated sperm. Even though the embryo could develop to the blastocyst stage following oocyte activation, the recipients that received blastocysts derived from embryos obtained by ICSI using CaI treated sperm were not pregnant, whereas the transfer of control blastocysts into recipients resulted in pregnancy.

In this study, we also demonstrated that a significantly higher rate of oocytes was activated in the activation group compared with the non-activated group (Data not shown). It has been reported that sperm introduced by ICSI in oocytes generates insufficient calcium oscillation to activate the oocytes (Malcuit et al., 2006). We therefore suggested that additional activation is required to compensate for insufficient stimulation for the buffalo oocytesto be activated and from pronucleus, in accordance with the effect of additional activation following ICSI on oocytes activation in cattle (Chung et al., 2000) and pigs (Lee et al., 2003; Nagashima et al., 2003); however, the proportion of normal fertilization (2PN, without sperm intact) still remained low. Additionally, it was noticed that the incident of activation at 18 h post ICSI in the experimental and control groups was similar to that following a sham injection. So, it should be aware of parthenogenesis.

In conclusion, we improved the efficiency of ICSI in buffalo using a combination of sperm treatments and oocyte activation. These results show that the disintegration of sperm plasma membrane and removal of acrosome in buffalo sperm can be performed by various methods which do not significantly affect the DNA integrity. We report for the first time that buffalo sperm pretreatments with DTT following additional activation promoted sperm head decondensation. However, the overall efficiency of ICSI in buffalo oocytes in terms of male PN formation is still poor. Further research is needed to study methods of increasing normal fertilization after ICSI in buffaloes.

CHAPTER V

GENERAL DISCUSSION AND CONCLUSIONS

It is well known that buffalo are generally regarded as having low reproductive efficiency. This is largely due to a number of inherent problems. Also, the application of reproductive biotechnologies has not been developed as much as in cattle. *In vitro* embryo production remains in the experimental stages due to poor yield of embryos. Several problems need to be resolved before *in vitro* embryo production technology can be used commercially for producing buffalo breeding stock. The problems are usually initiated with the failure of oocytes to develop to cleavage stage following *in vitro* fertilization (IVF). However, only a few reports have studied the early embryo development after fertilization in buffalo, especially in swamp buffalo. A considering to fundamental knowledge associated with gamete interaction at fertilization is therefore attempted to investigate in the present study. We focused on basic events that associated with gamete interaction in particular the redistribution of cell cytoskeleton and chromatin configurations after IVF in swamp buffalo. Besides IVF, intracytoplasmic sperm injection (ICSI) is also another tool for studying the fundamental aspects of gamete interaction during fertilization. Unfortunately, a few similar works have been performed in swamp buffalo (Liang et al., 2011a; Liang et al., 2011b). Also, the main problem of ICSI as reported in other livestocks species was a very low success rate of live offspring. However, exact reason for this remains entirely clear. Thus our study aimed to efficiently develop ICSI technique which was focused on sperm preparation and oocyte activation. Molecular techniques were also applied to determine gene expression profiles of embryos developed between sexual and asexual reproduction. The knowledge acquired from this study would provide the basic information during fertilization and early embryo development *in vitro*.

Chronological reorganization of microtubules, actin microfilaments, and chromatin during the first cell cycle

Fertilization in mammals requires a successful series of events involving a profound remodeling of the nucleus and cytoplasm of both sperm and oocytes. Microtubules and actin microfilaments have been demonstrated to dynamically play an important role during fertilization and cleavage in many species. We firstly examined the chronology of early embryonic development in terms of cytoskeleton redistribution and chromatin configurations in swamp buffalo embryos during the first cell cycle (at 6, 12, 18, 24, and 30 h post-IVF). The study exhibited that sperm penetrated through the ZP of mature oocytes by 6 h post-IVF. Also sperm penetration in MI swamp buffalo oocytes was possible which was similar to reports in bovine (Abeydeera et al., 1993; Chian et al.,

1992) and canine (Yamada et al., 1992). Partial ZP digestion however did not improve fertilization rate. A microtubule organizing center is formed at the area of sperm centrosome and plays role in the migration and the apposition of pronuclei. This evidence suggested that the centrosomal material is primarily paternally inherited and is similar to previous reports in other mammalian species such as human (Gook et al., 1998; Sathananthan, 1998), sheep (Le Guen and Crozet, 1989), rabbit (Yllera-Fernandez et al., 1992), porcine (Kim et al., 1996), bovine (Alomar et al., 2008; Long et al., 1993; Sathananthan et al., 1999) and rhesus monkey (Hewitson et al., 2000). However, this is different from mouse because cytoplasmic microtubules in the cytoplasm originated from maternal centrosomes and sperm astral microtubules were not detected in the decondensing paternal chromatin (Hewitson et al., 1997; Maro et al., 1985). This study also indicated that microfilaments played an important part in the fertilization and cleavage in swamp buffalo embryos. These filaments were concentrated as a cell furrow predominantly in the middle line of the dividing cell and subsequently located just beneath the plasma membrane as previously reports in Xenopus (Noguchi and Mabuchi, 2001), mouse (Schatten and Schatten, 1986), porcine (Kim et al., 1997), and bovine (Rivera et al., 2004) embryos. Fertilization failure was associated with the absence of sperm in the MII ooplasm, even though a number of sperm were tightly bound to the zonapellucida. It is postulated that other factors such as quality of frozen-thawed sperm (Mishra et al., 2008) and culture environment (low versus high oxygen tension) should be taking into an account rather than only assessing the sperm motility prior IVF. Other advance sperm parameters, such as functional membrane integrity, mitochondrial membrane potential, and acrosome integrity may be additionally required (Selvaraju et al., 2009).

Intracytoplasmic sperm injection in swamp buffalo

The knowledge of ICSI is still limited because the restricted numbers of studies have been reported (Liang et al., 2011a; Liang et al., 2011b). The success of ICSI depends on the protocol used for each species. In sheep, rabbit, goat and horse, conventional ICSI seems sufficient to activate the oocytes for further embryo development. In cattle and pig, the majority of studies demonstrate that artificial activation of oocytes after conventional ICSI improved the fertilization rates. In buffalo, success rate of pronuclear formation and further development to blastocysts seemed to be occurred when oocytes were activated following ICSI with an additional chemical activation (Liang et al., 2011b). No buffalo calve have been born by ICSI. Our results also exhibited that injection of sperm into cytoplasm of buffalo oocytes alone was not sufficient to activate oocyte and start syngamy. The second meiotic resumption and pronuclear formation were mostly occurred when the oocytes were additionally activated with chemicals after ICSI. In cattle and pig, the majority of studies demonstrated that the additional activation of bovine oocytes after ICSI improved their development (Horiuch

et al., 2002; Kolbe and Holtz, 2000; Lee et al., 2003). In contrast, in hamsters (Uehara and Yanagimachi, 1976), rabbits (Keefer, 1989) and humans (Tesarik et al., 1994), the mechanical processes during ICSI, penetration of an ICSI needle through the zona pellucida and cytoplasmic suction, were reported to be enough to activate oocytes.

Interestingly, however we inferred that most of activated ICSI oocytes contained two pronuclei together with an intact sperm head inside the ooplasm. The chemicals predominantly stimulated female pronuclear formation rather than sperm head decondensation or male pronuclear formation. Also, neither embryo development to the blastocyst stage nor mean total cell number of blastocysts in ICSI (+) activation group was significantly different among ICSI (+) activation and Sham (+) activation. This circumstance alerted the awareness of parthenogenesis occurring in the ICSI system used in this study.

It was important to clarify whether the buffalo ICSI embryos in this study were generated from syngamy or parthenogenesis. Therefore, imprinted genes were used in our study to confirm activity of the paternal genome. Neurotonin (*Nnat*), a maternally-imprinted-paternally-expressed gene, was selected in our experiment as *Nnat* is only expressed from the allele inherited from the father, also it has been reported to be actively expressed in the blastocyst stage of IVF bovine embryos, but not in embryos derived by parthenogenesis (Ruddock et al., 2004). This was apparently reported that *Nnat* expression in IVF buffalo blastocysts was similar to that found in IVF bovine embryos. However, *Nnat* mRNA was absent from blastocysts in ICSI (+) activation group, similar to blastocysts obtained by oocytes activation. The absence of the RT-PCR product in ICSI and OA embryos signified the locus where this gene lies may have not been remodeled and/or made accessible for transcription in ICSI embryos. This was true for the OA embryos for which no paternal genome was included in the generation process, but was unlikely for ICSI. Conversely, the lack of *Nnat* expression confirmed that the specific oocyte activation protocol used resulted in parthenogenetic activation in a high rate of ICSI oocytes. Due to failure of sperm head decondensation and also lack of the paternal gene expression therefore, we inferred that only morphological evaluation of blastocyst development following ICSI (+) activation was not adequate evidence to conclude that male gamete activation had occurred.

Sperm head decondensation

The reason why only mechanical activation and the existence of spermatozoon in the cytoplasm could not induce extrusion of the second polar body of oocytes to complete the meiosis processes in cattle, pig and swamp buffalo is not clear. A possible reason for the failure may be the lack of an important signaling molecule when a sperm head is injected into an oocyte. In natural fertilization, after the penetration of zona pellucida, sperm without outer-acrosomal membrane and acrozomal enzymes reach to the ooplasm. However, by ICSI, sperm do not lose those constructions and those with their complete

form are injected into the ooplasm. Therefore, it is important to discuss the effects of acrosome, acrosomal enzymes and sperm membranes on fertilization by ICSI procedure. It has been reported that a large amount of acrosomal contents and enzymes have appeared to be harmful to oocytes in mice (Lacham-Kaplan and Trounson, 1995; Morozumi and Yanagimachi, 2005). The disintegration of the sperm plasma membrane and the removal of acrosome performed before sperm injection by mechanical stimulus of an injection pipette increase the successful developmental rates in humans and some mammalian species such as mice and rabbits (Keefer, 1989; Tesarik et al., 1994). However, applying physical damage seems not to be sufficient enough to damage the membrane in cattle (Galli et al., 2003; Liu and Yang, 1999; Rho et al., 1998a) and pigs (Lee and Yang, 2004; Lee et al., 2002; Nakai et al., 2003; Tian et al., 2006). This is likely due to the higher rigidity of the perinuclear theca (Perreault et al., 1988; Sutovsky et al., 1997), together with the large number of acrosome and acrosomal enzymes which enter the ooplasm after sperm injection in these species (Morozumi and Yanagimachi, 2005). Thus phospholipase C zeta, a sperm-borne oocyte-activating factor and is localized in the acrosomal and post acrosomal regions of the perinuclear theca membrane (Dozortsev et al., 1998; Sutovsky et al., 1997), may not be able to penetrate through the sperm plasma membrane resulting in a limited amount of C zeta available for oocytes activation. In order to damage the sperm plasma membrane and acrosome as well as to improve the developmental rates of ICSI oocytes, sperm pretreatment(s) before injecting have been successfully performed in cattle (Galli et al., 2003; Liu and Yang, 1999; Rho et al., 1998a) and pigs (Lee and Yang, 2004; Lee et al., 2002; Nakai et al., 2003; Tian et al., 2006). Various sperm pretreatment protocols have been used to disrupt the acrosomal and sperm plasma membrane such as using Triton-X 100 (TX)(Lee and Yang, 2004; Tian et al., 2006), calcium ionophore (CaI) (Galli et al., 2003; Lacham-Kaplan and Trounson, 1995; Nakai et al., 2003), and freezing/thawing without cryoprotectant (FT) (Goto et al., 1990). We inferred that the sperm plasma membrane and acrosome damage were effectively induced by TX, CaI and FT treatments. Sperm treatment with TX and CaI apparently causes a significant increase in the number of acrosome-loss sperm while FT treated sperm and untreated sperm apparently causes a significant increase in the number of acrosome-reacted sperm. However, in this study the removal of the sperm plasma membrane and acrosome did not promote sperm head decondensation and oocyte activation after ICSI in swamp buffalo.

Besides above mentioned factors which involve during fertilization in under normal conditions, the decondensation of sperm nucleus that entered cytoplasm of an oocyte is considered to be a critical importance. Sperm nucleus is condensed highly and stabilized by formation of disulfide bonds between protamines. For formation of pronucleus, sperm nucleus has to decondense with reduction of those disulfide bonds by ooplasmic glutathione and replacement of the protamine (Yanagimachi, 2005). A failure of decondensation in sperm nucleus was observed in livestock species (Catt et al., 1996;

Kren et al., 2003). It was inferred that those sperm chromatins are tightly packaged and more stable than other species (Bedford, 1988). Therefore, the injection of sperm after treatment with disulfide bond reducing agents, dithiothreitol (DTT), has been considered to promote the decondensation of sperm nucleus in cattle (Rho et al., 1998b; Suttner et al., 2000). However in pigs, the effects of DTT treatment seem to be markly different among each paper. It was reported that DTT treatment increased the rate of normal fertilization and blastocyst formation (Tian et al., 2006) on the one hand, and have no significantly effect on pronuclear formation, fertilization and embryonic development on the other (Nakai et al., 2006; Yong et al., 2005). In this study, we have first investigated the effects of DTT on sperm head decondensation or male pronuclear formation after ICSI in swamp buffalo. We inferred that DTT treatment together with the existence of reacted acrosome is important to promote sperm head decondensation whereas the loss of acrosome cannot induce sperm head decondensation even after DTT treatment. Alternatively, the status of acrosome integrity is important for successful sperm head decondensation resulting in male pronuclear formation. Our interpretation was therefore the damage methods for buffalo sperm plasma membrane and acrosome using TX and CaI, resulting in loss of acrosome, might have either caused a premature release of acrosomal matrix containing specific substances or perhaps removal of some extra-nuclear components that probably participate in the activation process.Similarly, it was reported in pig that treatment of sperm before ICSI caused loss of PLCζ, localized in the post-acrosomal and tail regions of pig sperm, resulting in a weakened sperm activation signal (Nakai et al., 2011). Such side effects may lead to the failure of sperm chromatin decondensation (Gutierrez-Perez et al., 2011; Yan et al., 2008).

Conclusion and further studies

While *in vitro* embryo production technologies can be achieved in human and various animal species including cattle whose reproductive physiology is comparable to buffalo, the success rate of *in vitro* embryo production is markedly unsatisfied and also have not developed as far as in cattle. In order to improve *in vitro* embryo production, more fundamental studies during fertilization in buffalo are necessary. This study is the first report to examine and describe the chronology of swamp buffalo embryo development in terms of redistribution of cytoskeleton and chromatin configurations during the first cell cycle. The study demonstrates that a microtubule organizing center is formed at the area of sperm centrosome and plays an important role in the migration and apposition of pronuclei whereas actin microfilaments actively involves in cellular cleavage. The fundamental knowledge and techniques from our study can be used as a tool for further investigating the embryonic development. Moreover, the results from gamete interaction demonstrated failure of fertilization is caused by poor sperm penetration, therefore, in order to improve the fertilization and developmental rate, sperm quality besides motility before fertilization should be examined, also the culture condition

of *in vitro* system should be further investigated. With regard to intracytoplasmic sperm injection, we inferred that injection sperm into cytoplasm of buffalo oocytes alone was not sufficient to activate oocytes and start syngamy. Further study is required to examine the pattern of calcium oscillation following oocyte activation as increasing in calcium ion within oocyte leading to the mechanism of oocyte activation. In addition, ICSI with piezo-driven needle system was reported to be an alternative option to chemical activation because good developmental rates were obtained in some mammalian species such as cows, pig and goats when the mechanical pulses were used to induce activation of oocytes without chemical activations. However with oocyte activation following ICSI with untreated-sperm, the chemical at least were used in our study predominantly stimulated only female pronucleus rather than male pronucleus. This study confirms for the first time about the failure of traditional ICSI technique in swamp buffalo oocytes by utilizing the special trait of imprinted genes to distinguish the absence or presence of male genome activation normally occurring during early embryo development. Only morphological evaluation of blastocyst development was not adequate evidence to conclude that male gamete activation had occurred. The efficiency of intracytoplasmic sperm injection in terms of sperm head decondensation in buffalo was improved whether using a combination of sperm chemical treatments and oocyte activation. Anyway it should be noted that the status of acrosome is important for suscessful sperm head deconndensation as the loss of acrosome was negative effectively whereas the existence of reacted acrosome together with the reduction of disulfide bond can induce sperm head decondensation. From out results, it was suspected that acrosomal contents may contain some specific substances which are thought to be the oocyte-activating factor in mammalian sperm such as PLCζ, that probably participate in the activation process hence injection of acrosomal contents only into oocytes is interesting to test the hypothesis.

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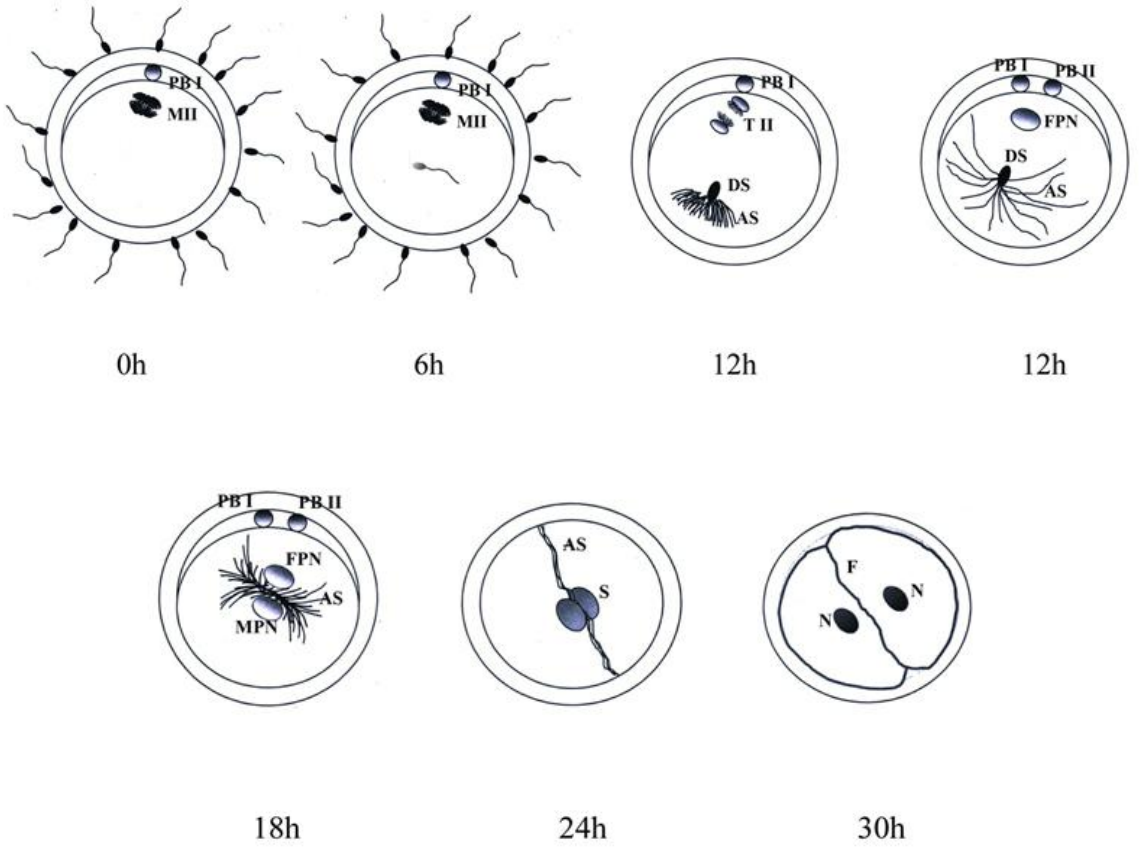
APPENDIX A



Supplementary figure. Chronological of embryo development during the first cell cycle in swamp buffalo. At 0 h: oocyte was inseminated with sperm at 0 h. At 6 h post insemination: sperm had penetrated into oocyte. At 12 h post insemination, MII was resumed and transformed into FPN, together with sperm chromatin decondensed and formed sperm aster. At 18 h after IVF, MPN and FPN were synchronously, and sperm aster increased in size and extended through cytoplasm to assist the migration and apposition of pronucei. The apposition and syngamy occurred at 24 h post insemination. Cell cleavage was facilitated by microfilament and firstly observed by 30 h after IVF. AS: sperm aster; DS: sperm head decondensation; F: actin microfilaments; FPN: female pronucleus; MPN: male pronucleus; M II: metaphase II; N: nucleus; PB I: first polar body; PB II: second polar body; T II: telophase II; S: syngamy. (Chankitisakul et al., 2010).

APPENDIX B

List of publications and conferences

1. Chankitisakul V, Tharasanit T, Tasripoo K, Techakumphu M. 2010. Chronological reorganization of microtubules, actin Microfilaments, and chromatin during the first cell cycle in swamp buffalo (*Bubalus bubalis*) Embryos. **Vet Med Int.** 2010: 382989.
2. Chankitisakul V, Tharasanit T, Phutikanit N, Tasripoo K, Nagai T, Techakumphu M. 2012. Lacking expression of paternally-expressed gene confirms the failure of syngamy following intracytoplasmic sperm injection in swamp buffalo (*Bubalus bubalis*). **Theriogenology.** 77(7): 1415-1424.
3. Chankitisakul V, Somfai T, Inaba Y, Techakumphu M, Nagai T. 2011. ATP content in bovine oocytes may not determine the timing of first cleavage and further developmental competence after IVF. **J Reprod Dev.** 57: 80.
4. Chankitisakul, V.,Tharasanit, T., Tasripoo, K. and Techakumphu, M. **2009.** Redistribution of cytoskeleton and chromatin configulations during early embryo development in swamp buffalo (*Bubalus bubalis*). **The 8th Proc. Ann. Conv. Vet. Sci. Chula. Meeting**/ 3 April 2009.
5. Chankitisakul, V.,Tharasanit, T., Tasripoo, K. and Techakumphu, M. **2010.** Failure of sperm decondensation after intracytoplasmic sperm injection in swamp buffalo (*Bubalus bubalis*). **The 36th Annual Conference of the IETS/ 23rd Annual Meeting SBTE**/ 9-13 January 2010.
6. Techakumphu, M., Chankitisakul, V.,Tasripoo, K. and Tharasanit. **2010.** Redistribution of cytoskeleton and chromatin configulations during early embryo development in swamp buffalo (*Bubalus bubalis*). **The 36th Annual Conference of the IETS/ 23rd Annual Meeting SBTE**/ 9-13 January 2010.
7. Chankitisakul, V., Tharasanit, T., Tasripoo, K. and Techakumphu, M. **2010.** Acrosome integrity and DNA integrity of swamp buffalo sperm treated by triton-X 100, freezing and thawing and dithiothreitol. **The 13th AITVM 2010 International Conference**/ 23-26 August 2010.
8. Chankitisakul, V., Tharasanit, T., Phutikanit, N., Tasripoo, K. and Techakumphu, M. 2010. Lacking expression of paternally-expressed gene confirms the failure of normal fertilization following intracytoplasmic sperm injection in swamp buffalo (*Bubalus bubalis*). **The 36th ICVS 2010 International Conference on Veterinary Science**/ 2-5 November 2010.
9. Chankitisakul, V., Tharasanit, T., Tasripoo, K. and Techakumphu, M.The dynamics of early embryo development during *in vitro* fertilization in swamp buffalo (*Bubalus bubalis*). **The 6th KU-KPS Conference, 8-9 December 2009.**

10. กระบวนการพัฒนาของตัวอ่อนระยะแรกระหว่างการปฏิสนธิภายนอกร่างกายในกระบือปลัก.การประชุมวิชาการโครงการทุนวิจัยมหาบัณฑิตสกว. สาขาวิทยาศาสตร์และเทคโนโลยีครั้งที่ 4 ณ โรงแรมจอมเทียนปาล์มบีชรีสอร์ท เมืองพัทยา จ.ชลบุรี ระหว่างวันที่ 28-30 มี.ค. 2553

CURRICULUM VITAE

Vibuntita Chankitisakul was born on October 25th 1981 in Chiang Mai province, Thailand. She graduated with Degree of Doctor of Veterinary Medicine (D.V.M.) with the 2nd honour from Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai, Thailand in 2006. In 2007, she received a scholarship from Commemorate the 72nd Anniversary of His Majesty King BhumibolAdulyadej to perform a Master programme at the Department of Obstetrics Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. In 2009, she was continuing on for a Ph.D. after changed from a Master degree program to a Ph.D. program as she got a scholoarship from the Royal Golden Jubilee PhD program of Thailand Research Fund. During her study, she has focused on *in vitro* embryo production in cattle, and especially on aspects of ontracytoplasmic sperm injection in swamp buffalo.