การเก็บรักษาตัวอสุจิจากเนื้อเยื่ออัณฑะและการพัฒนาของตัวอ่อน
ภายหลังการฉีดตัวอสุจิเข้าสู่ไซโตพลาสมของโอโอไซต์แมวบ้าน

นางสาวศิริรักษ์ บัวพึ่ง

TESTICULAR SPERM PRESERVATION AND EMBRYO DEVELOPMENT FOLLOWING

INTRACYTOPLASMIC SPERM INJECTION INTO OOPLASM OF DOMESTIC CATS

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ศิริรักษ์ บัวพึ่ง: การเก็บรักษาตัวอสุจิจากเนื้อเยื่ออัณฑะและการพัฒนาของตัวอ่อนภายหลังการฉีดตัวอสุจิเข้าสู่ไซโตพลาสมของโอโอไซต์แมวบ้าน (TESTICULAR SPERM PRESERVATION AND EMBRYO DEVELOPMENT FOLLOWING INTRACYTOPLASMIC SPERM INJECTION INTO OOPLASM OF DOMESTIC CATS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.นสพ.ดร.มงคล เตชะกำพุ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ.นสพ.ดร.ธีรวัฒน์ ธาราศานิต, ดร.ปิแอร์ โคมิซโซลิ, 151 หน้า.

เนื้อเยื่ออัณฑะเป็นแหล่งอสุจิที่สำคัญของสัตว์ที่ตายหรือถูกทำหมัน การเก็บรักษาเนื้อเยื่ออัณฑะอย่างเหมาะสมด้วยการแช่เย็นและแช่แข็งจะเป็นประโยชน์ต่อการนำไปใช้ผลิตตัวอ่อนในอนาคต การศึกษาแบ่งเป็น 4 การทดลองดังนี้

การทดลองที่ 1 มีวัตถุประสงค์เพื่อศึกษาผลกระทบของการแช่เย็นที่มีต่อเยื่อหุ้มเซลล์ ดีเอนเอ และความสามารถในการปฏิสนธิของอสุจิจากลูกอัณฑะ โดยทำการแช่เย็นลูกอัณฑะในสารละลาย Dulbecco's Phosphate Buffered Saline (DPBS) นาน 1-7 วัน และทำการตรวจคุณภาพของเยื่อหุ้มเซลล์และดีเอนเอของอสุจิด้วยการย้อมสี Ethidium homodimer-1 และ TUNEL ตามลำดับ อสุจิที่ได้จากลูกอัณฑะแช่เย็นถูกฉีดเข้าสู่เซลล์ไข่ของแมวบ้านเพื่อดูความสามารถในการปฏิสนธิของอสุจิ โดยประเมินจากลักษณะของโครมาตินของโอโอไซต์ที่ถูกย้อมด้วย DAPI ภายหลังการฉีดอสุจิ 18 ชม. พบว่าในช่วง 4 วันแรกของการแช่เย็น อสุจิที่เยื่อหุ้มเซลล์ยังสมบูรณ์(อสุจิที่ยังมีชีวิต)มีจำนวนไม่ต่างจากอสุจิก่อนเก็บรักษา (P > 0.05) หลังจากแช่เย็น 7 วัน อสุจิมีชีวิตรอดประมาณ 50% และมีการเสียหายของดีเอนเอเพียง 1 % อย่างไรก็ตามระยะเวลาในการแช่เย็นไม่มีผลต่อดีเอนเอและความสามารถในการปฏิสนธิของอสุจิอย่างมีนัยสำคัญ (P > 0.05)

การทดลองที่ 2 มีวัตถุประสงค์เพื่อ (1) เปรียบเทียบความสามารถของสารละลาย DPBS และ HEPES-contained medium (HM) ในการรักษาสภาพของเยื่อหุ้มเซลล์ของอสุจิระหว่างการแช่เย็น (2) ศึกษาผลของความเข้มข้นของ bovine serum albumin (BSA) ที่มีต่อเยื่อหุ้มเซลล์อสุจิ (3) ประเมินคุณภาพดีเอนเอของอสุจิ และความสามารถในการเจริญภายในห้องปฏิบัติการของตัวอ่อนที่ได้จากการฉีดอสุจิที่แช่เย็นในสารละลาย HM ที่มีส่วนผสมของ BSA 1.6 % นาน 7 วัน โดยทำการแยกอสุจิออกมาจากเนื้อเยื่ออัณฑะก่อนแช่เย็นใน DPBS และHM นาน 7 วันแล้วทำการตรวจคุณภาพของเยื่อหุ้มเซลล์ทุกวันด้วยการย้อมสี Ethidium homodimer-1 พบว่า DPBS และ HM มีความสามารถในการรักษาสภาพของเยื่อหุ้มเซลล์อสุจิไม่แตกต่างกันอย่างมีนัยสำคัญ อย่างไรก็ตาม HM ให้ผลที่ดีกว่า DPBS เมื่อนำอสุจิไปแช่เย็นใน HM ที่มี BSA เข้มข้น 0.4, 0.8 และ 1.6 % นาน 1 สัปดาห์ พบว่า BSA ที่ความเข้มข้น 0.4 % มีจำนวนอสุจิมีชีวิตน้อยกว่าที่ความเข้มข้น 0.8 และ 1.6 % เนื่องจาก HM ที่มี BSA 1.6 % ให้ผลดีที่สุดจึงถูกนำมาใช้แช่เย็นอสุจิเพื่อดูความเสียหายของดีเอนเอด้วยการย้อม TUNEL และประเมินความสามารถในการพัฒนาของตัวอ่อนในห้องปฏิบัติการภายหลังฉีดอสุจิเข้าสู่เซลล์ไข่ ถึงแม้ว่าคุณภาพดีเอนเอของอสุจิที่แช่เย็นใน HM ที่มี BSA 1.6 % นาน 1 สัปดาห์จะต่ำกว่าอสุจิที่ไม่ผ่านการแช่เย็นอย่างมีนัยสำคัญ (P < 0.05) แต่ตัวอ่อนที่ได้จากการฉีดอสุจิแช่เย็นสามารถเจริญในห้องปฏิบัติการไปสู่ระยะคลีเวจ (44.7 %) มอรูลา (13.6 %) และบลาสโตซีสต์ (7.3 %) เทียบเท่ากับการฉีดอสุจิที่ไม่ได้แช่เย็น (P > 0.05)

การทดลองที่ 3 มีวัตถุประสงค์เพื่อ (1) เปรียบเทียบประสิทธิภาพของสารป้องกันความเสียหายจากการแช่แข็ง 4 ชนิด ได้แก่ glycerol, ethylene glycol, 1, 2-propanediol และ dimethyl sulphoxide (2) เปรียบเทียบคุณภาพอสุจิจากการแช่แข็ง 2 วิธี (3) ศึกษาการเปลี่ยนแปลงของเซลล์สืบพันธุ์เพศผู้และเพศเมียภายใน 18 ชม.หลังการฉีดอสุจิจากเนื้อเยื่ออัณฑะแช่แข็งเข้าสู่เซลล์ไข่ (4) ศึกษาการพัฒนาในห้องปฏิบัติการของตัวอ่อนที่ได้จากการฉีดอสุจิจากเนื้อเยื่ออัณฑะแช่แข็งเข้าสู่เซลล์ไข่ พบว่าที่ความเข้มข้น 5 % (v/v) เท่ากันการแช่แข็งเนื้อเยื่ออัณฑะโดยการลดอุณหภูมิ 2 ขั้นตอนด้วย glycerol ช่วยรักษาสภาพของเยื่อหุ้มอสุจิได้ดีที่สุด (เทียบเท่ากับอสุจิก่อนทำการแช่แข็ง) ชนิดของสารป้องกันความเสียหายจากการแช่แข็งและเทคนิคในการแช่แข็งไม่ได้มีผลต่อความเสียหายของดีเอนเอ อสุจิจากเนื้อเยื่ออัณฑะแช่แข็งที่ถูกฉีดเข้าสู่เซลล์ไข่สามารถปฏิสนธิเซลล์ไข่ได้โดยไม่ต้องใช้การกระตุ้นจากภายนอก ทั้งนี้เซลล์สืบพันธุ์เพศผู้และเพศเมียมีการเปลี่ยนแปลงอย่างเป็นอิสระต่อกัน ตัวอ่อนที่ได้สามารถพัฒนาในห้องปฏิบัติการได้ถึงระยะบลาสโตซีสต์ โดยมีอัตราการพัฒนาเป็นระยะคลีเวจ (32.7 %) มอรูลา (6.5 %) บลาสโตซีสต์ (4.4 %) และจำนวนเซลล์ของตัวอ่อนระยะบลาสโตซีสต์ เทียบเท่ากับอสุจิจากเนื้อเยื่ออัณฑะที่ไม่ได้แช่แข็ง (P > 0.05)

การทดลองที่ 4 มีวัตถุประสงค์เพื่อศึกษาการพัฒนาภายในห้องปฏิบัติการและภายในตัวสัตว์ ของตัวอ่อน (ระยะ 2-8 เซลล์) แช่แข็งที่ได้จากการฉีดอสุจิจากเนื้อเยื่ออัณฑะแช่แข็ง พบว่าการฉีดอสุจิจากเนื้อเยื่ออัณฑะแช่แข็งเข้าสู่เซลล์ไข่ได้ตัวอ่อนในระยะคลีเวจน้อยกว่าการผลิตตัวอ่อนด้วยวิธีไอวีเอฟ ตัวอ่อนที่ได้จากการฉีดอสุจิแช่แข็งภายหลังการแช่แข็งและทำละลายสามารถพัฒนาไปสู่ระยะมอรูลา (22.6 %) และบลาสโตซีสต์ (21.3 %) น้อยกว่าตัวอ่อนที่ไม่ได้แช่แข็งอย่างมีนัยสำคัญ (45.2 และ 38.7 % ตามลำดับ) แต่จำนวนเซลล์ของตัวอ่อนระยะบลาสโตซิสต์ไม่แตกต่างกันระหว่างตัวอ่อนแช่แข็งและไม่แช่แข็งได้จากการฉีดอสุจิจากเนื้อเยื่ออัณฑะแช่แข็งเมื่อย้ายฝากตัวอ่อนแช่แข็งภายหลังการทำละลายเข้าสู่ท่อนำไข่ของแมวตัวรับซึ่งถูกกระตุ้นด้วยฮอร์โมน eCG และ hCG พบว่าแมวตัวรับ 3 ตัวจาก 7 ตัวตั้งท้อง อย่างไรก็ตามมีแมวเพียงตัวเดียวคลอดลูกเพศผู้ 2 ตัวออกมาในวันที่ 64 หลังการย้ายฝาก

งานวิจัยฉบับนี้ชี้ให้เห็นว่าการแช่เย็นและแช่แข็งสามารถช่วยยืดระยะเวลาการเก็บรักษาอสุจิภายในเนื้อเยื่ออัณฑะเพื่อนำมาใช้ในการผลิตตัวอ่อนภายในห้องปฏิบัติการได้ ตัวอ่อนที่ได้จากการฉีดอสุจิจากเนื้อเยื่ออัณฑะแช่แข็งสามารถพัฒนาต่อได้ทั้งภายในห้องปฏิบัติการและภายในแมวตัวรับ โดยลูกแมวที่ถือกำเนิดจากการศึกษาครั้งนี้ถือเป็นลูกแมวคู่แรกที่ได้จากการย้ายฝากตัวอ่อนแช่แข็งซึ่งผลิตโดยการฉีดอสุจิจากเนื้อเยื่ออัณฑะแช่แข็ง ความรู้ที่ได้จากงานวิจัยครั้งนี้จะเป็นพื้นฐานที่นำไปสู่การพัฒนาเทคนิคการเก็บรักษาเนื้อเยื่อสืบพันธุ์และการผลิตตัวอ่อนในสัตว์ที่ใกล้สูญพันธุ์ต่อไป

ภาควิชา	สูติศาสตร์ เธนุเวชวิทยาและวิทยาการสืบพันธุ์	ลายมือชื่อนิสิต ....
สาขาวิชา	วิทยาการสืบพันธุ์สัตว์	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.................................
ปีการศึกษา	2555	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.................................
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      COLD STORAGE / CRYOPRESERVATION
      SIRIRAK BUARPUNG: TESTICULAR SPERM PRESERVATION AND EMBRYO DEVELOPMENT FOLLOWING
INTRACYTOPLASMIC SPERM INJECTION INTO OOPLASM OF DOMESTIC CATS. ADVISOR: PROF. MONGKOL
TECHAKUMPHU, D.V.M., DOCTORAT 3e CYCLE, CO-ADVISOR: ASST.PROF. THEERAWAT THARASANIT, D.V.M., Ph.D.,
PIERRE COMIZZOLI, D.V.M., Ph.D., 151 pp.

Testicular tissue is an important sperm source of dead or castrated animals. Optimal preservation of testicular tissue by cold storage and cryopreservation would be beneficial for future embryo production. This thesis composes of 4 parts as follows:

EXP.1 aimed to examine the effect of cold storage on plasma membrane integrity, DNA integrity and fertilizing ability of testicular spermatozoa. Intact testes were cold stored in Dulbecco's Buffered Saline (DPBS) at 4ºC for 1 - 7 days. Sperm membrane and DNA integrities were examined by Ethidium homodimer-1 and TUNEL staining, respectively. Spermatozoa from cold stored testes were injected into cat oocytes to evaluate their fertilizing ability, indicated by chromatin configuration of injected oocytes which were stained by DAPI at 18 h after injection. The data showed that, in the first 4 days, the number of spermatozoa with intact plasma membrane (alive spermatozoa) was comparable to non-preserved control (Day 0) (P > 0.05). After cold storage for 7 days, viability of preserved testicular spermatozoa remained up to approximately 50%, while the incidence of DNA fragmented spermatozoa was approimately1 %. However, duration of cold storage did not significantly impact on sperm DNA integrity and fertilizing ability (P > 0.05).

EXP.2 aim to (1) compare the efficiency of DPBS and HEPES-containing medium (HM) to protect sperm membrane integrities during cold storage (2) examine the effects of bovine serum albumin (BSA) concentration on sperm membrane integrity during cold storage (3) examine the DNA integrity and fertilizing ability of spermatozoa cold stored within HM supplemented with 1.6 % BSA for 1 week. Spermatozoa were extracted before cold storage in DPBS and HEPES-contained medium (HM) for 7 days. Sperm plasma membrane was daily examined by Ethidium homodimer-1. The protective efficiencies of DPBS and HM on sperm membrane integrity were not significant different (P > 0.05), though HM provided the better result than DPBS. Testicular spermatozoa were then cold stored within HM supplemented with BSA at different concentrations (0.4, 0.8 and 1.6 %) for 1 week. The number of alive testicular spermatozoa from HM supplemented with 0.4 % BSA was lower than that of 0.8 % and 1.6 % through 1 week of storage. Due to HM with 1.6 % BSA gave the best result it was therefore selected to be a preserving medium for assessment of DNA integrity (by TUNEL assays) and in vitro embryo development after intracytoplasmic sperm injection (ICSI). Although DNA integrity of testicular spermatozoa stored in HM supplemented with 16% BSA significantly declined after cold stored for 7 days, the embryos produced by ICSI with chilled spermatozoa could develop in vitro to cleavage (44.7 %) morula (13.6 %) and blastocyst (7.3 %) stages at the similar rates with those of fresh spermatozoa (P > 0.05).

EXP.3 aimed to (1) compare the protective efficiency on sperm membrane and DNA integrities of 4 different cryoprotectants including glycerol, ethylene glycol, 1, 2-propanediol and dimethyl sulphoxide (2) compare the effects of 2 different freezing techniques on sperm membrane and DNA integrities (3) determine gamete activation up to 18 h after ICSI with spermatozoa recovered from cryopreserved testicular tissues (4) evaluate in vitro development of embryos produced by ICSI with spermatozoa recovered from cryopreserved testicular tissues. The result revealed that at the same concentration of cryoprotectants, 5 % (v/v), testicular tissue cryopreservation by 2-step freezing with glycerol maintained the best sperm membrane integrity. This parameter was comparable to that of spermatozoa from non frozen tissues. Cryopreservation did not impact on sperm DNA integrity (irrespective of cryoprotectants and freezing techniques). Spermatozoa from frozen testicular tissues can fertilize cat oocytes without external oocyte activation. However, male and female activation occurred in an asynchronous and independent manner. The percentages of cleavage (32.7 %), morula (6.5 %) and blastocyst (4.4 %), and also the blastocyst cell number of embryos produced by ICSI with cryopreserved testicular spermatozoa were comparable to non-crypreserved control (P > 0.05).

EXP.4 aimed to study in vitro and in vivo development of frozen cleaved embryos (2- to 8 cell stage) derived by ICSI with spermatozoa from frozen-thawed testicular tissues. The result revealed that ICSI with frozen-thawed testicular spermatozoa yielded the lower cleavage rate than conventional IVF. Frozen-thawed ICSI embryos developed to morula (22.6 %) and blastocyst (21.3 %) stage at the lower percentages than that of non-frozen ICSI embryos (45.2 and 38.7 %, respectively, P < 0.05). However, the blastocyst cell numbers of frozen and non-frozen ICSI embryos were not different. After transfer of frozen-thawed embryos produced by ICSI with cryopreserved testicular tissues into the oviducts of eCG/hCG treated recipient cats, 3 out of 7 queens were pregnant. Only one of these cats delivered 2 male kittens on day 64 after transfer.

This study showed that cold storage and cryopreservation of testicular tissue could prolong storage time of spermatozoa for subsequent embryo production. Frozen embryos produced by ICSI with spermatozoa from cryopreserved testicular tissues can develop in vitro and in vivo. In addition, this is the first report of the births of kittens after transfer of frozen-thawed embryos produced by ICSI with sperm recovered from cryopreserved testicular tissues. This study will be the fundamental knowledge for development of gamete preservation and embryo production for endangered wild felid conservation.

Department: _____Obstetrics, Gynaecology and Reproduction_____ Student's Signature……………………..……..………………….……

Field of Study: ___Theriogenology_____ Advisor's Signature……………………….....………………….…....

Acedemic Year ___2012_____ Co-advisor's Signature……………..……………………………….….

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AI	artificial insemination
ANOVA	Analysis of Variance
ARTs	assisted reproductive technologies
AV	artificial vagina
B	blastocyst
BSA	bovine serum albumin
Ca2+	calcium ions
°C	degree celcius
CL	corpus luteum
COCs	cumulus oocyte complexes
CO2	carbon dioxide
CPA	cryoprotectant
D	day
DAPI	4', 6-diamidino- 2-phenylindole
DMAP	dimethylaminopurine
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
ds	decondensing sperm head
DSH	decondensing sperm head
DTT	Dithiothreitol
dUTP	Deoxyuridine Triphosphate
eCG	equine chorionic gonadotropin
EDTA	ethylenediaminetetraacetic acid
EE	electroejaculation
EG	ethylene glycol
EGF	epidermal growth factor
EGTA	Ethylene glycol tetraacetic acid

ET	Embryo transfer
EthD-1	Ethidium homodimer-1
EX	extender
f	female pronucleus
FCS	fetal calf serum
FIT-PNA	fluorescein isothiocyanate–labeled peanut (*Arachishypogaea*) agglutinin
FM	Freezing medium
FSH	follicle stimulating hormone
FT	frozen-thawed
g	gram
GnRH	Gonadotropin-Releasing Hormone
GPX	glutathione peroxidase
GSR	glutathione reductase
GST	glutathione S-transferase
h	hour
hCG	human chorionic gonadotropin
H blast	hatching blastocyst
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HM	holding medium
Hoechst-33342	2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloride trihydrate
hpi	hour post injection
HTS	HypoThermosol solution-FRS
ICSI	intracytoplasmic sperm injection
ISH	intact sperm head
IU	international unit

IVC	*in vitro* culture
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
KCl	potassium chloride
kg	kilogram
KSOMaa	potassium simplex optimization medium with amino acids
L	liter
L15	Leibovitz L15
LH	luteinizing hormone
M	molar
M199	medium 199
MI	metaphase I
MII	metaphase II
MAPK	Mitogen activated protein kinase
MEM	minimum essential medium
mg	milligram
MgCl$_2$	Magnesium chloride
min	minute
ml	milliliter
mm	millimeter
mM	milimolar
MP	metaphase plate
MPF	maturation promoting factor
MPN	male pronuclear
MTOC	microtubule-organizing center
n	number
N	number
NaCl	sodium chloride

NaHCO$_3$	sodium bicarbonate
NaH2PO$_4$	monosodium phosphate
NaOH	sodium hydroxide
NEAA	nonessential amino acids
ng	nanogram
NIM	nuclear isolation medium
NSS	normal saline solution
OVH	ovariohysterectomy
PB	polar body
PBS	phosphate buffered saline
PCC	premature chromosome condensation
PF	paraformaldehyde
PI	propidium iodide
PLCζ	Phospholipase C-zeta
PMSG	pregnant mare serum gonadotropin
PN	pronucleus / pronuclei
PrOH	1, 2-propanediol
PT	perinuclear theca
PVP	poly-vinylpyrrolidone
P1	protamine-1
rhFSH	recombinant human follicle-stimulating hormone
ROS	reactive oxygen species
RT	room temperature
SAS	Statistical Analysis System
SOAF	sperm-borne oocyte-activating factor
SOD	superoxide dismutase
SOF	synthetic oviductal fluid
sec	second

SEM	standard error of the mean
SMI	sperm membrane integrity
TCM-199	tissue culture medium 199
TCN	total cell number
TdT	Terminal Deoxynucleotidyl Transferase
TRITC	tetramethylrhodamine isothiocyanate
TUNEL	terminal deoxynucleotidyltransferase (TdT)-mediated dUTP nick end labeling
v/v	volume/volume
w/v	weight/volume
wt	weight
yr	year
ZP	zona pellucida
µg	microgram
µl	microliter
µm	micrometer
µM	micromole

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

During the past few decades, many wild animals have gradually decreased in numbers, and many of which are being classified as threatened, vulnerable, endangered or even extinction due to extensive deforestation, habitat loss and illegal wild animal trading (Nowell and Jackson, 1996; Holt and Pickard, 1999). Of 36 wild felids worldwide, nine species have been found in Thailand; *Panthera tigris* (tiger), *Panthera pardus* (leopard), *Neofelis nebulosa* (clouded leopard), *Felis bengalensis* (leopard cat), *Felis viverrina* (fishing cat), *Felis temmincki* (Asian golden cat), *Felis chaus* (jungle cat), *Prionailurus planiceps* (flat-headed cat), and *Pardofelis marmorata* (marbled cat). According to the International Union for Conservation of Nature (IUCN, 2008), these 9 wild cats are being concerned with high risk of losing their genetic diversity.

Assisted reproductive technologies (ARTs) such as artificial insemination (AI), *in vitro* fertilization (IVF), embryo transfer (ET) and gamete cryopreservation have been used to support perpetuation of genetically valuable endangered felids. Due to the limitation of specimens from these animals, the domestic cat (*Felis catus)* is logically used as a model for development of these techniques. Semen of desired cats is usually collected by electrical stimulations using an electroejaculator (EE). The electroejaculation technique is however no longer be used when animals would die unexpectedly or testicles/reproductive tracts are obstructed and/or injured. Alternatively, spermatozoa can be harvested from existed testicles of unexpectedly dead animals via either epididymis or testicular parenchyma. Testicular parenchyma is a valuable source of male germ cells because it contains abundant numbers of germ cells at various stages of development. Immature and mature forms of spermatozoa within the testis have been used to produce embryos and offspring principally via intracytoplasmic sperm injection (ICSI). More interestingly, the spermatogonial stem cells resided in the

seminiferous tubule have also been isolated and cultured *in vitro*. These pleuripotent cells have indeed held a great promise of sperm production *in vitro* (Kubota and Brinster, 2006).

In many circumstances animals would have died quite far from fully equipped laboratory, the *in vitro* embryo production using testicular spermatozoa is therefore not possible. The optimal handling and storage technique of male gametes by means of cold storage and cryopreservation of testicular tissue is becoming an important issue for rescue the genetic potentials of male animals. In domestic cat, the studies on embryo production by testicular spermatozoa as well as the effects of cold storage and freezing on testicular sperm quality (Comizzoli et al., 2006a,b; Thuwanut and Chatdarong, 2012) are still limited. Moreover, the information on fertilizing ability of preserved testicular spermatozoa and embryo development after ICSI has not been reported. In fact, testicular spermatozoa obtained via fine needle biopsy and testicular sperm extraction have been routinely used to produce embryo in men suffering from azoospermia to achieve pregnancies and normal life birth (Devroey et al., 1994; Silber et al., 1995; Windt et al., 2002; Schwarzer et al., 2003; Meseguer et al., 2003). The overall aim of this study is therefore to develop techniques for testicular sperm extraction, preservation and embryo production by ICSI using domestic cat as a model. The techniques obtained from this study would principally apply to short- and long-term preservation of testicular spermatozoa and tissue for male germ line preservation and subsequent use in genome resource banking program of genetically valuable wild animals.

1.2 Literature reviews

1.2.1 The reproductive organs of male cats

The reproductive organs of male cats include testes, epididymides, ductus deferens, prostate glands, bulbourethral glands, penis and prepuce (**Figure 1**). The spermatogenic function of male cats becomes mature at 8 to 10 months of age and average testicular weight of adult male cat is around 1.5 grams (Tsutsui et al., 2004).

The mean daily sperm production per gram of testis (efficiency of spermatogenesis) is approximately 16 x 10^6 spermatozoa (França and Godinho, 2003).
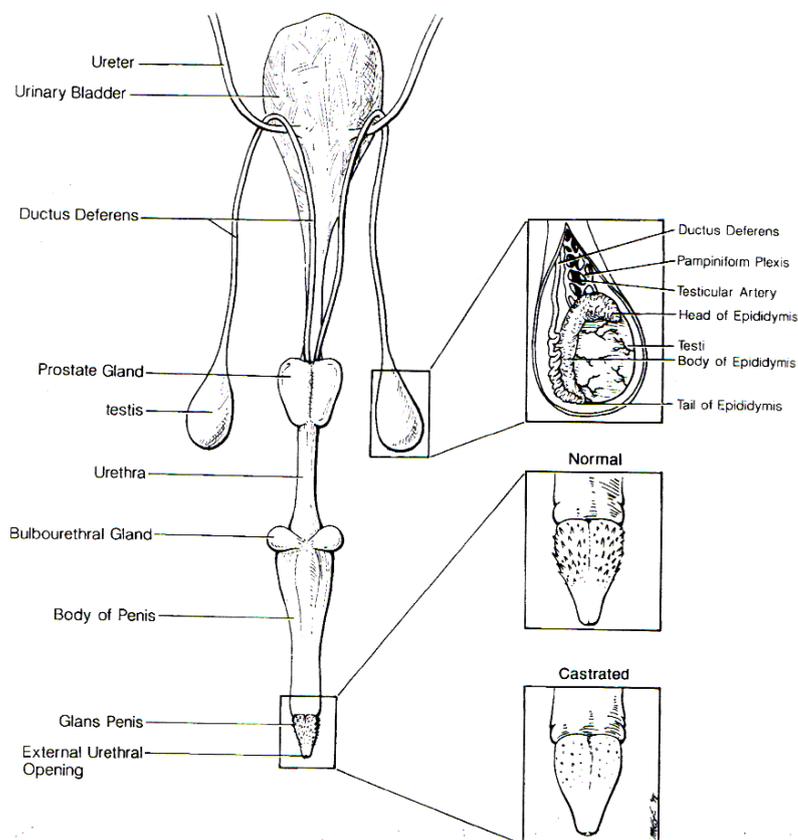
Figure 1 Gross anatomy of the male reproductive organs of the cat. (Johnston et al., 2001)

1.2.2 Spermatogenesis and sperm maturation

Spermatogenesis (consisting of spermatocytogenesis and spermiogenesis) takes place in seminiferous tubules within testicular parenchyma. This process is controlled by testosterone secreted by interstitial Leydig cells. During spermatocytogenesis, primitive cells called spermatogonia (2N) undergo mitotic divisions and yield primary spermatocytes. The diploid number of primary spermatocytes is then halved during meiosis. A primary spermatocyte is transformed into two secondary spermatocytes during meiosis I. These cells in turn are converted into (1N) spermatids during meiosis II

(**Figure 2A**). Spermatids undergo dramatic changes in form during spermiogenesis into the streamline spermatozoa adapted for fertilization (**Figure 2B**). Spermiogenesis involves nuclear condensation, formation of the acrosomal cap, and development of a tail. In cat, the duration of each cycle of seminiferous epithelium is 10.4 days and the total duration of spermatogenesis based on 4.5 cycles is 46.8 days (França and Godinho, 2003). The sperm production occurs throughout the year (Blottner and Jewgenow, 2007).
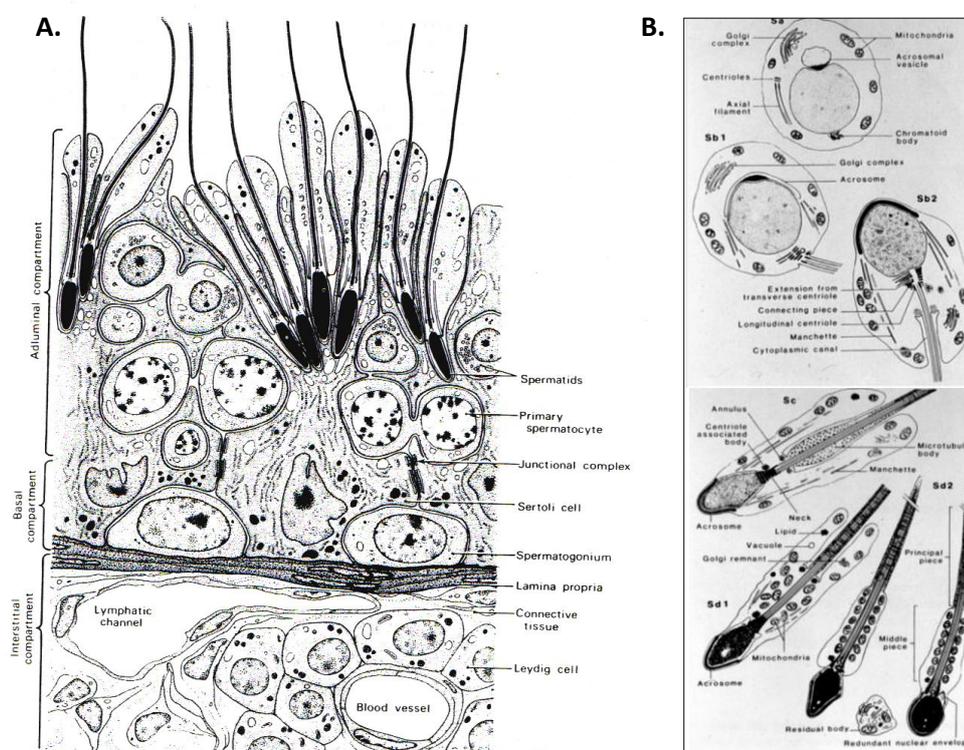
Figure 2 (A) Structure of the seminiferous tubule representing the spermatogenesis (Amann, 1986). (B) Diagrammatic presentation showing various stages of spermatids during spermiogenesis; Sa and Sb1 = round spermatids, Sb2 = elongating spermatids, Sc and Sd1 = elongated spermatids and mature spermatids (immature spermatozoa) = Sd2 (De Kretser and Kerr, 1994).

Following the spermatogenesis, spermatozoa are released from the seminiferous tubules to rete testis and efferent ducts before being transported to epididymis (caput, corpus and cauda regions, respectively) for maturation. Most of fluid secreted from the

testis as spermatozoa is reabsorbed in the efferent ducts. The remaining testicular fluid is absorbed again in caput epididymis resulting in an increase of concentrations of specific organic compounds and spermatozoa (Clulow et al. 1994; Axnér et al., 1999). During epididymal transit, spermatozoa acquire the capability for motility and fertilization such as migration of the cytoplasmic droplet, morphological changes of acrosome, stabilization of sperm nucleus by disulfide bonds formation in the nuclear protamines, changes in lipid contents and glycoproteins of sperm plasma membrane, increasing of cAMP contents of spermatozoa and phosphorylation of sperm proteins followed by increasing of motility (Crabo et al, 1975; Amann et al., 1982; Rodriguez-Martinez et al., 1990; Amann et al., 1993; Jones R, 1998; Lewis and Aitken, 2001; Axnér, 2006). After maturation, spermatozoa are stored in cauda epididymis (and proximal ductus deferens) until ejaculation. In mammals, the estimate transit time of spermatozoa through the epididymis is 10-12 days (Courot, 1981).

1.2.3 Structure of feline spermatozoa

The main structure of spermatozoa is separated into 4 parts including head, neck, middle piece and a tail (**Figure 3A**). Cat spermatozoa have elongated, oval shaped heads with two planes of symmetry (longitudinal and lateral) (**Figures 3B, C**) (Root et al., 1996). The neck tapers inwardly between the head and midpiece (**Figure 3C**) (Swalec and Smeak, 1989; Root et al., 1996). The two major components of the head are the nucleus and the acrosome containing with hydrolytic enzymes which is important for sperm-oocyte interaction at fertilization. The mid-piece contains large helical mitochondria that generate the energy for sperm movement. The tail contains microtubules. Sperm plasma membrane composes of lipids and proteins arranged as a bilayer with the hydrophilic ends of the lipids externally and the hydrophobic fatty chains internally.
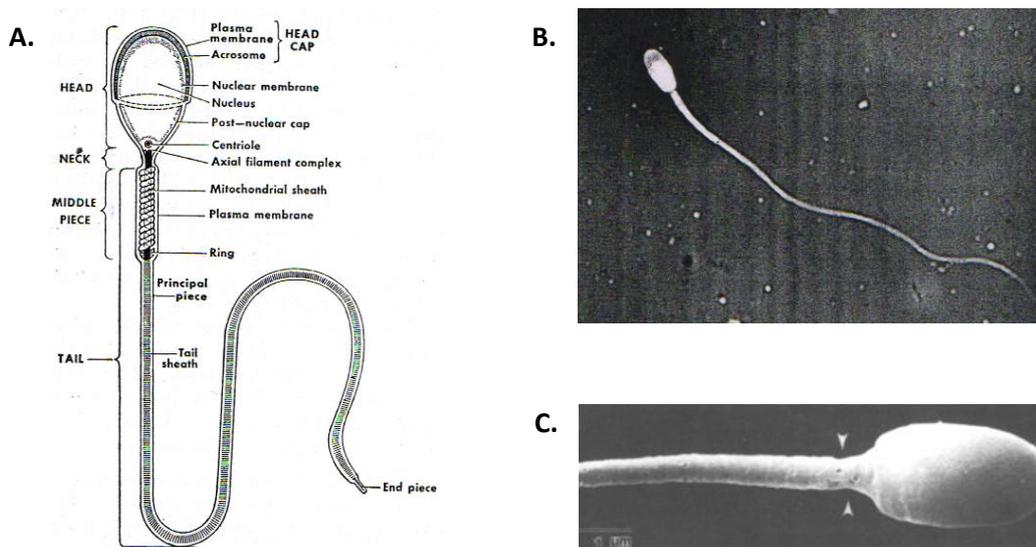
Figure 3 (A) Diagrammatic representation of a mammalian spermatozoon (Hamner, 1970). (B) Normal feline spermatozoon examined by light microscopy using eosin-negrosin stain (1000x). (C). A scanning electron micrograph of a spermatozoon of domestic cat demonstrating 'the midpiece tapers' inward (arrow heads) in the neck area (Schmehl and Graham, 1989).

1.2.4 Sperm transport and natural fertilization

The stored spermatozoa (from epididymis) as well as surrounding fluid are mixed with the alkaline secretions from the accessory sex glands, to protect spermatozoa from acidic environment of vagina, and deposited in the cranial vagina of the queens during natural coitus (Tsutsui, 2006; Axnér, 2008). Spermatozoa are rapidly passed via the cervical canal and transported through the uterus by uterine contraction (Chatdarong et al., 2002). Spermatozoa are stored in the uterotubal junction and uterine crypts which are the initial sperm reservoirs before redistributed to the isthmus at 48 h after mating (Swanson et al. 1994; Chatdarong et al., 2004; Axnér, 2008). During transportation in the female reproductive tract, spermatozoa undergo a further period of maturation which has been defined as "capacitation". This process involves changes in sperm plasma membrane (including hyperpolarization, phospholipid redistribution, cholesterol outflow),

adenylate cyclase-cAMP system, intracellular ions, metabolism and acrosome (Suzuki and Yanagimachi, 1989; Suarez et al., 1993; Yanagimachi 1994; Patrat et al., 2000; Naz and Rajesh, 2004). The change of calcium signal during capacitation contributes to sperm hyperactivation, the state that the ejaculated spermatozoa swim vigorously with high-amplitude and asymmetrical flagellar beating (Yanagimachi, 1970; Chang and Suarez, 2011), in the oviduct. This movement of mammalian spermatozoa enhances the sperm capacity to detach from sperm reservoirs (Ho and Suarez, 2001; Suarez and Ho, 2003), to penetrate viscous oviductal fluid and a viscoelastic cumulus matrix surrounding the oocyte (Suarez et al., 1991) and, eventually, to approach the zona pellucida of the oocytes (Stauss et al., 1995). The biding of mammalian spermatozoa to zona pellucida glycoproteins, especially ZP3 (Florman et al., 2008), triggers "acrosome reaction" (usually occurs in the ampulla of the oviduct) in which sperm plasma membrane and the outer membrane of acrosome are fuse with oolemma. Spermatozoon then release acrosomal enzymes, including hyaluronidase and acrosin, to break egg plasma membrane and allow sperm head content penetrate through the egg for fertilization. The cortical reaction occurs after sperm penetration. This process leads to a modification of the zona pellucida that blocks polyspermy.

1.2.5 Sperm collection

Electroejaculation (EE) and the use of artificial vagina (AV) are the most common methods for sperm collection in felid species (Zambelli and Cunto, 2006). The quality of cat semen collected by EE is comparable to semen collected by AV, although EE semen yields a higher pH and lower sperm concentration than another. EE is more practical for wild species than semen collection by AV due to it can be accomplished in any male that can be safely anesthetized and does not require a trained male and teaser queen (female in heat or spayed queen treated with estrogen) which are essential for semen collection using an AV (Sojka, 1986). Spermatozoa can also be collected by vaginal lavage from post-coital queens (Sojka, 1986) or by urethral catheterization in male cats (Filliers et al., 2010) and from epididymis and testicle after routine castration or postmortem (Axnér, 2004; Comizzoli et al., 2006a,b; Buarpung et al., 2012). In human,

testicular spermatozoa can be achieved by fine needle aspiration (Hewitson et al., 2002) and tissue biopsy combined with testicular sperm extraction (Friedler et al., 1997). Although the spermatozoa within the body of animals (testis, epididymis and vas deference) degenerate quickly after death as demonstrated in mouse (Kishikawa et al., 1999), the reports in deer indicated that recovering functional spermatozoa from testis of death animal is also possible (Soler et al., 2003; Martínez et al., 2008).

1.2.6 Intracytoplasmic sperm injection (ICSI)

Since, spermatozoa obtained from testicular tissue are not fully maturated and most of them are incapable of effective forward motility and usually show only a slowly twitching motility or completely immotile (Jow et al., 1993; Schiewe et al., 1997), testicular spermatozoa are therefore not to be used for conventional *in vitro* fertilization (IVF) program. Fortunately, fertilization and pregnancy could be achieved from testicular spermatozoa via intracytoplasmic sperm injection (ICSI) combined with embryo transfer such as in human (Devroey et al., 1994, 1995; Silber et al., 1995; Konc et al., 2008), rhesus monkey (Hewitson et al., 2002), mouse (Ogonuki et al., 2006) and pig (Nakai et al., 2010). ICSI is an *in vitro* fertilization procedure in which a single spermatozoon is directly injected into cytoplasm of a matured oocyte (**Figure 4**). Except the cortical reaction which was observed after ICSI similar to that after IVF in human and pig (Bourgain et al., 1998; Katayama et al., 2002), ICSI bypass capacitation, acrosome reaction and fusion which depend on the sperm integrity. ICSI is therefore an available technique for *in vitro* production using poor quality spermatozoa (Pope et al., 2006). Although most of testicular spermatozoa are morphologically abnormal mainly because they have not yet completed epididymal maturation (Cosentino and Cockett, 1986; Yeung et al., 1997), spermatozoa morphology appeared not to involve poor fertilizability of testicular spermatozoa (Human: Yavetz et al., 2001) after ICSI.
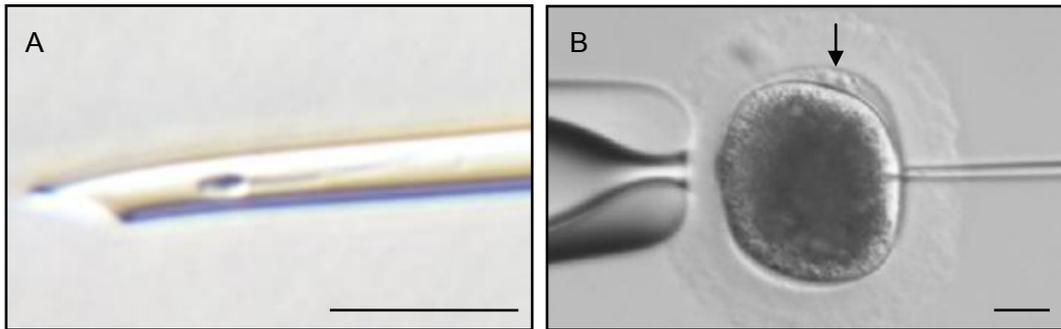
Figure 4 ICSI procedure A) Loading of a spermatozoon in the ICSI micropipette, B) A spermatozoon is injected into an oocyte with a visible first polar body (arrow). Bar = 20 µm.

The success of ICSI is influenced by many factors such as source of spermatozoa, sperm quality, injection technique and oocyte activation protocol (Pope et al., 1998; Nasr-Esfahani et al., 2005; Ito et al., 2009), but not the quality of oocytes (sheep: Catalá et al., 2012). In human, testicular spermatozoa yields similar fertilization, cleavage and pregnancy rates to epididymal spermatozoa (Silber et al., 1995) but is lower than ejaculated spermatozoa (Nagy et al., 1995; Qian, 2005). This poor *in vitro* development of ICSI derived embryos was possibly due to centrosomal immaturity of testicular spermatozoa, as demonstrated in domestic cat (Comizzoli et al., 2006b), rather than the differences in the nuclear status (Dadoune, 1995). It is in accordance with the report in human that the timing of the fertilization process, indicated by pronuclear formation, was not significantly affected by the sperm origin (ejaculated or testicular) (Nagy et al., 1998). Several previous reports suggested that the selection of motile spermatozoa is a key to successful ICSI because movement of spermatozoa allows easier identification of vital spermatozoa (Yanagimachi, 2005; Kovacic et al., 2006). It was comfirmed by the study in human indicated that fertilization rate of immotile testicular spermatozoa was significantly lower than that of motile spermatozoa (Stalf et al., 2005). In human, sperm motility can be increased by preincubation of spermatozoa in culture medium before ICSI (Windt et al., 2002; Wu et al., 2005) or by adding pentoxifylline (PF) or the other chemicals such as 2-deoxyadenosine to the testicular spermatozoa (Angelopoulos et al., 1999; Terriou et al., 2000; Griveau et al., 2006).

Nevertheless, PF and 2-deoxyadenosine appeared to be toxic to the oocytes or resulting in poor embryo development (Lacham-Kaplan and Trounson, 1993). In deed, motility of spermatozoa is not essential for fertilization by ICSI. Immotile human spermatozoa, whatever their origin, have the capacity to fertilize an oocyte after ICSI (Nijs et al., 1996). It was supported by the observations that comparable fertilisation and pregnancy rates can be achieved from ICSI with motile and immotile testicular spermatozoa (Moghadam et al., 2005), and mouse offsprings can be produced from the injection of nonviable spermatozoa (Wakayama and Yanagimachi, 1998; Kishikawa et al., 1999). Intracytoplasmic morphologically selected sperm injection (IMSI), the technique which human spermatozoa were selected at a high magnification under an inverted microscope before injection, has been proposed to increase the results of ICSI (Garolla et al., 2008). Although the recent study demonstrated that IMSI did not enhance embryo development and pregnancy rate in human (De Vos et al., 2013), it may be used to improved pregnancy and delivery rates following the failure of ICSI (Klement et al., 2013). However, this technique is time-consuming and also expensive (Ubaldi and Rienzi, 2008).

In the past decade, gamete micromanipulation for embryo production using ICSI has been gradually developed in canine and feline species. This technique allows matured oocytes to be fertilized with immotile spermatozoa and to develop to blastocysts (Farstad, 2000; Comizzoli et al., 2006a). Cat embryos could be produced by ICSI with ejaculated (Pope et al., 1998; Gómez et al., 2000), epididymal (Bogliolo et al., 2001) and testicular spermatozoa (Comizzoli et al., 2006a). Live kitten were deliverred after transfer of ICSI embryos (Pope et al., 1998; Gómez et al., 2000), though the studies about ICSI using testicular spermatozoa in cat still limited only *in vitro* development to blastocyst stage (Comizzoli et al., 2006a). Beside testicular spermatozoa, the other testicular cells such as spermatid also have fertilizing ability for embryo production as reports in monkey (Ogonuki et al., 2003), hamster (Haigo et al., 2004) and mouse (Miki et al., 2004; Jiang et al., 2005). In mouse, the live-birth outcomes were not significantly different to those obtained by mature spermatozoa (Loren and Lacham-Kaplan, 2006).

There are many reports showing that embryo formation and normal births can be produced via ICSI using elongated spermatids and testicular spermatozoa of mouse (Yanagimachi, 1998), rat (Said et al., 2003) and rhesus monkey (Hewitson et al., 2002). Therefore, ICSI cooperated with testular tissue or cell preservation is a useful technique for embryo production for valuable animal.

1.2.7 Gamete activation after *in vitro* fertilization (IVF) and ICSI

Gamete activation in female gamete involves with the structural and biochemical changes in the oocytes. Mature mammalian oocytes arrested at metaphase of the second meiotic division (MII). They contain a second meiotic spindle and a first polar body (**Figure 5A**). After a single spermatozoon is injected into an oocyte, the arrested oocyte reactivates and progresses through anaphase and telophase to complete the second meiotic division. One set of sister chromatids is extruded and becomes the second polar body (**Figure 5B**). The remaining haploid set of chromosomes transforms to female pronucleus, while a haploid sperm nucleus decondenses to form male pronucleus (**Figure 5C**). A microtubule aster forms at the base of the decondensing sperm head and expands toward the female pronucleus for the migration and apposition of two pronuclei (**Figures 5C, D**). During syngamy, male and female nuclei fuse together and then migling their chromosomes results in a diploid zygote. The parental chromosomes are then aligned in the middle of the zygote while the centrosome (containing a pair of centriole) is separated to form the two poles of the mitotic spindle and distributes chromosomes to daughter cells during the first mitotic division (**Figures 5E, F**).
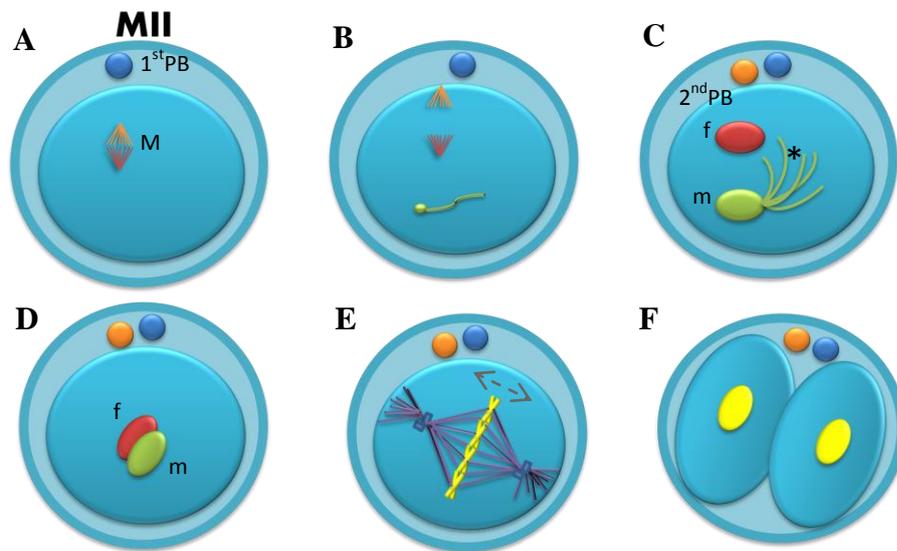
Figure 5 The sequence of early events in mammalian fertilization. Matured oocytes arrested at the metaphase II (MII) stage, indicated by the present of metaphase plate and a first polar body (A). After sperm penetration, one set of sister chromatids is extruded (B) and becomes the second polar body, while the remaining chromosomes transform to female pronucleus (C). The spermatozoon forms the aster adjacent to the base of sperm head while the sperm head decondenses to form male pronucleus (C). The sperm aster brings the two pronuclei together for syngamy (D). The parental chromosomes line up along the middle of the zygote, move toward the poles of the mitotic spindle (E) and then distribute to daughter cells (F). M = metaphase plate; PB = polar body; female pronucleus = f; male pronucleus = m; asterisk = sperm aster.

These processes are similar either after IVF or ICSI. In human, the timing of second polar body extrusion after ICSI with testicular spermatozoa was similar to that after IVF or ICSI with ejaculated spermatozoa (Nagy et al., 1998). After ICSI, sperm decondensation will not happen until acrosomal residuals were absent from sperm head, this event and subsequence male-pronucleus formation were therefore delayed when compared with conventional IVF (Ludwig et al., 2001). Decondensation of sperm head and oocyte activation can occur independently after ICSI (Human: Flaherty et al., 1995b) results in asynchronous formations of male and female pronuclei as reports in human and sheep (Nagy et al., 1998; Gómez et al., 1998b). These events

could also be obtained after human IVF (Nagy et al., 1998), whereas simultaneous pronuclear formation was achieved after sheep IVF (Gómez et al., 1998b).

The key of biochemical change is a calcium signal within injected oocyte (Grabiec et al., 2007). Penetration of a spermatozoon into the oocyte initiates a rise of intracellular oocyte calcium (Ca2+) concentration, as called calcium oscillations, by releasing of Phospholipase C-zeta (PLCζ), a considerable candidate of sperm-borne oocyte activating factor (SOAF), from sperm perinuclear theca into ooplasm. Maturation promoting factor (MPF) and Mitogen activated protein kinase (MAPK) which maintain matured oocytes arrest at MII stage (Kubiak et al., 1993; Fan and Sun, 2004) are declined when Ca2+rise leads to the completion of meiosis II and second polar body extrusion (Fan and Sun, 2004; Jones, 2005). The release of MII arrest induces the inactivation of MAPK which is correlated to pronuclear formation (Liu et al., 1998). Although ICSI procedure can provoke Ca2+rise, it is not responsible for oocyte activation (Tesarik et al., 1994). In human (Palermo et al., 1993; Teserik and Mendoza, 2003), mouse (Yanagimachi, 1998) and rabbit (Keefer, 1989), the injected spermatozoa is able to restart the cell cycle of the oocyte without any additional oocyte activation treatment, but this is not the case in several other species that require artilicial activation of the oocyte such as bovine (Chung et al., 2000) and sheep (Gómez et al., 1998a; Shirazi et al., 2009). In cat, oocyte activation by electric current or chemical agents (ethanol, calcium ionophore, cycloheximide) has been reported (Skrzyszowska et al., 2002; Taeyoung et al., 2002; Gómez et al., 2003; Kitiyanant et al., 2003; Comizzoli et al., 2006a). However, Pope et al. (1998) suggested that artificial stimuli used for activation of rabbit (Keefer, 1989) and bovine oocytes (Goto et al., 1990; Keefer et al., 1990) were not necessary to achieve consistent fertilization/cleavage of cat oocytes.

1.2.8 Cold storage of spermatozoa

Cold storage is used to reduce metabolism and to maintain sperm viability over an extended period of time (Bovine: Karunakaran et al., 2007). Refrigeration (5 °C) of epididymal spermatozoa can minimize post-mortem change, prolong sperm viability and yield higher fertilizing capacity when compared to samples stored at room temperature

as reports in red deer (Soler et al., 2003) and ram (Kaabi et al., 2003), although the study in dog revealed that sperm quality was reduced with storage time (England and Ponzio, 1996). Several studies in human (Zavos et al., 1980; Dondero et al., 2006), dog (England and Ponzio, 1996) and cat (Siemieniuch and Dubiel, 2007) suggested that chilling is more suitable for temporary storage than freezing because the quality of chilled spermatozoa was superior to that of the frozen-thawed spermatozoa. However, prolonged cold storage damages the plasma membrane (an important indicator of sperm viability), acrosome and DNA chromatin (Dog: Shahiduzzaman and Linde-Forsberg, 2007). Although the testis contains several proteins actively involved with antioxidative mechanisms such as superoxide dismutase (SOD), glutathione reductase (GSR), glutathione peroxidase (GPX), glutathione S-transferase (GST) (Bauché et al., 1994; Gu and Hecht, 1996) which may be sufficient to protect testicular cells against oxidative stress during short duration of cold storage, spermatozoa should be maintained in suitable extender that can provide sufficient energy, maintain pH and osmolarity, and also has a property to protect spermatozoa from cold shock (Shahiduzzaman and Linde-Forsberg, 2007). Freezing extender, especially egg yolk-based extender, has been applied for cold storage to protect cat spermatozoa from cold shock injury (Hermansson and Axnér, 2007; Siemieniuch and Dubiel, 2007; Gañán et al., 2009), though egg yolk did not show the advantage for maintenance of plasma membrane integrity of refrigerated cat spermatozoa (Hermansson and Axnér E, 2007). Several components such as bovine serum albumin (Matsuoka et al., 2006), skim milk (Abe et al., 2008) and soy bean lecithin (Aires et al., 2003; Vick et al., 2012), have been used for substitution of egg yolk in freezing extender in order to decrease the variation of extender quality, which is depended on individual egg yolk quality, and to resolve the transportation problem during outbreak of avian flu. The advantage of bovine serum albumin (BSA) in protecting membrane integrity and developmental potential of spermatozoa has been reported in dog (Risopatron et al., 2002), mouse (Van Thuan et al., 2005; Li et al., 2011), ram (Matsuoka et al., 2006) and bull (Nang et al., 2012), though high concentration of BSA could negatively affected on structure of bovine

spermatozoa by osmotic stress (Nang et al., 2012; Osman et al., 2012). However, the optimal concentration of BSA for sperm protection may vary among species. Beside freezing extender, basic medium such as potassium simplex optimization medium with amino acids (KSOMaa), HEPES-CZB, K(+)-rich nuclear isolation medium (NIM) and Phosphate buffered saline (PBS) have been used for mouse sperm cold storage (Van Thuan et al., 2005; Li et al., 2011). Full term development of mouse embryos could be generated by ICSI with epididymal spermatozoa cold stored in KSOMaa, NIM and PBS which were supplemented by BSA (Van Thuan et al., 2005; Li et al., 2011). From early studies, testicular cell of primate (Jahnukainen et al., 2007) and pig (Zeng et al., 2009; Yang and Honaramooz, 2010) have been well preserved in Dulbecco's phosphate buffered saline (DPBS, basal salt solution) and complex culture media such as medium 199 (M199), Dulbecco's modified Eagle's medium (DMEM), Leibovitz L15 (L15) and HypoThermosol solution-FRS (HTS) with varying degrees of success. However, the effects of these media in preserving testicular spermatozoa have not been studied yet.

1.2.9 Cryopreservation

Cryopreservation is an application of very low temperature to infinitely store the cells or tissues. Cryopreservation of genetic materials (spermatozoa, oocytes and embryos) from animal has been developed for animal conservation over the past 20 years (Leibo and Songsasen, 2002). However, the cells can be damaged by several events occur during cryopreservation consist of extracellular ice formation, intracellular ice formation, dehydration and solution effects, which related to the migration of water and aqueous solutions. Formation of extracellular ice increases the osmolarity of extracellular fluid result in the efflux of intracellular water to equilibrate the concentrations of intra- and extra- cellular fluid /cryoprotectant, and the cells dehydration. If the temperature is decreased too slowly, water will overflow out of the cells which cause cellular dehydration and the extracellular ice is extensively formed. These result in cell membrane damage by crushing. Furthermore, the high concentration of intracellular salt and cryoprotectant are able to harm the cells by osmotic shock or by chemical toxicity. Conversely, too rapid cooling rate could lead to

intracellular ice formation which also harms the cells by internally mechanical damage. These effects can be reduced by adding cryoprotectants and using optimal cooling rate. Water or aqueous solutions usually cools below their melting point (0 °C) without the initiation of ice nucleation which is often referred to as "supercooling". Lethal intracellular ice crystals will form if cells are supercooled without extracellular formation of an ice nucleus. To minimize the detrimental effects of superculling, "seeding" (usually manipulates by clamping the straw or vial to be frozen with pre-cooled forceps) is performed to initiate ice outgrowth artificially.

1.2.10 Cryopreservation of testicular tissues

Testicular tissue cryopreservation followed by sperm extraction for subsequent uses in assisted reproduction such as *in vitro* embryo production via intracytoplasmic sperm injection (ICSI) has been applied and yielded pregnancy in human since 1996 (Hovatta et al., 1996). However, application and success of this technique for domestic cat and endangered felids have yet to be examined. The success of sperm cryopreservation is affected by several factors including freezing and thawing techniques, cryoprotectants and *in vitro* culture of testicular tissues (England and Ponzio, 1996; Wu et al., 2005; Fernandez-Santos et al., 2006). Cryopreservation induces cold shock, a direct result of sudden cooling that is a cause of disrupted and/or increased permeability of plasma membrane, such as in boar (Buhr et al., 1994), human (Nogueira et al., 1999) and red deer (Fernandez-Santos et al., 2006) spermatozoa. This problem can be reduced by testing the optimal cooling rate before freezing and/or incorporating egg yolk into the extender (White, 1993). The study in mouse indicated that plasma membrane integrity of spermatozoa is not essential for fertilization and full-term development if sperm nuclei are injected directly into oocytes by ICSI procedures (Wakayama and Yanagimachi, 1998), although intact sperm membrane would protect sperm DNA from prolong direct exposure with extracellular environment which can cause DNA deterioration (Tateno et al., 2000), and it has frequently used as viable marker for sperm quality. Testicular spermatozoa of boar appeared to have less cold

sensitivity and also lower level of DNA damage when compared with cauda epididymal and ejaculated spermatozoa (Johnson et al., 1972; Steele et al., 1999, 2000). The pregnancy outcomes in woman receiving embryos derived from ICSI were not significantly different when compared with fresh and frozen-thawed testicular spermatozoa. However the effect of cryopreservation was more pronounced at implantation (Human: Wu et al., 2005). This result suggested that cryopreservation technique is needed to be optimized and embryo transfer of embryo following ICSI of frozen testicular spermatozoa is required.

Human testicular spermatozoa can be frozen as cell suspension, or within a piece of testicular tissue (Allan and Cotman, 1997; Crabbé et al., 1999; Hovatta, 2000) using different freezing regimes. Several cryoprotectants (CPAs) have been used for testicular sperm freezing. These include both non-penetrating and membrane permeable CPAs such as sucrose, trehalose, glyclerol, ethylene glycol (EG), propanediol (PROH) and dimethyl sulphoxide (DMSO). Unni et al. (2012) observed in human that immature testicular tissue was more susceptible to toxic assault by CPAs than adult tissue and DMSO is an effective CPA for immature whereas ethylene glycol for adult testicular tissue. They also demonstrated the cell-specific sensitivity to CPAs that the populations of spermatid, spermatocyte and spermatogonia were well preserved with glycerol, EG and DMSO respectively. Beside spermatogonia, DMSO has also been used to maintain sertoli cells, and stromal compartment during freezing and thawing of human (Keros et al., 2007) and primate (Jahnukainen et al., 2007) testicular tissue. In human, glycerol could maintain the motility of frozen-thawed isolated spermatozoa better than cryopreserved pieces of tissue (Salzbrunn et al., 1996; Crabbé et al., 1999), while DMSO was indicated as the most suitable cryoprotectant for testicular tissue cryopreservation because it has low molecular weight and high tissue penetration efficiency (Keros et al., 2005). However, the study in cattle demonstrated that DMSO could be toxic at high concentration (Chen et al., 2007). The alternative cryoprotectants for cryopreserving testicular spermatozoa and also testicular tissue are needed to be

elucidated especially for domestic cats where as the effect of cryopreservation on testicular spermatozoa had not yet been examined.

1.2.11 Cryopreservation of cat embryos

Embryo cryopreservation has been used for embryo storage, when *in vitro* production has resulted in more embryos than is currently needed or the recipients are not available, and transportation. The duration of storage did not show any significant effect on postthaw survival of human (Riggs et al., 2010) and sheep (Yao et al., 2012) embryos and pregnancy outcome. Pregnancies and deliveries have been reported after transfer of frozen-thawed embryos in several mammalian species (Saragusty and Arav, 2011). Since the first report of kittens birth following transfer of cryopreserved feline embryos (Dresser et al., 1988), the protocols for freezing of domestic and non-domestic felid embryos have been developed continuously (Saragusty and Arav, 2011). Cat embryos at several stages of development could be frozen, e.g., 2- to 4-cell stage (Pope et al., 1994), morula (Day 4 to 5 of *in vitro* culture; IVC) (Gómez et al., 2003) and blastocyst (Day 5 to 6 of IVC) (Pope et al., 2000; Karja et al., 2006), and transfer resulted in pregnancies and deliveries. The reports in sheep (Cocero et al., 1996) and bovine (Leibo et al., 1996) indicated that embryo developmental stages affect the embryo viability after freezing and thawing. The early cleavage stage embryos of several species, such as human (Freitas et al., 1994), mouse (Uechi et al., 1997) and sheep (Garcia-Garcia et al., 2006), are sensitive to cryopreservation. Furthermore, stage of development affect on permeability of mouse embryo to cryoprotective agents (Pedro et al., 2005). However, the previous study in cat demonstrated that the stage of embryos at cryopreservation did not affect on *in vitro* development to blastocyst stage (Gómez et al., 2003).

In human, fertilization methods including IVF and ICSI did not affect on survival rates of thawed embryos, implantation, pregnancy and delivery rates after transfer (Steirteghem et al., 1994; Kowalik et al., 1998; Salumets et al., 2006). These reports were supported by a study in mouse of Lee et al. (1997) that a small penetration generated by ICSI needle did not compromise developmental ability of frozen embryos. Cat embryos

has been used for cryopreservation were mainly produced by IVF. By contrast, cryopreservation and transfer of cat embryo derived from ICSI, especially with spermatozoa from cryopreserved testicular tissue, has not yet been established.

1.2.12 Estrus induction for ARTs in felid species

Follicular development is readily induced by administration of follicle-stimulating hormone (FSH) or equine chorionic gonadotropin (eCG / PMSG; pregnant mare serum gonadotropin) to queens (Kutzler, 2007), while ovulation is induced by luteinizing hormone (LH) or by human chorionic gonadotropin (hCG). The disadvantage of any FSH product is the need of daily or twice daily injections of the hormone for 2-6 consecutive days, requiring frequent animal restraint or darting, thereby increasing stress. Moreover, high dose of FSH (6 IU/d) has also been demonstrated to associate with increased *in vitro* degeneration of cat embryos (Verstegen et al., 1993). Because eCG and hCG have longer half-life than FSH preparation, most protocol in wild felids is therefore considered them to be more desirable than short half-life gonadotropin.

1.2.13 Embryo transfer

Embryo transfer (ET) has been used to produce live offspring from high merit of genetic background or from animals that are valuable or being endangered. This technique has also been used to determine *in vivo* development of *in vitro* produced embryos since *in vitro* culture system has marked influence on embryo quality in terms of morphology, gene expression and also developmental potentials. ICSI-derived domestic cat embryos retained *in vivo* development potential as demonstrated by the birth of live kittens following transfer (Pope et al., 1998). The births after embryo transfer of ICSI derived embryos have demonstrated convincingly that ICSI-generated embryos are viable and not a result of parthenogenesis (Gómez et al., 2000). Several factors have been classified as potential caused that limit the success of ET. These include the techniques for synchronization and ovulation control of recipient, the stage of embryo development at ET and also transfer technique (intraoviduct vs. intrauterine ET) (Gómez et al., 2004; Pelican et al., 2006; Fayrer-Hosken, 2007; Kutzler, 2007; Pope et al., 2009).

In vitro-derived embryo can be transferred either at the morula and blastocyst stages to uterine horn (Pope et al., 1993, 1997 and 1998; Gómez et al., 2003) or at early cleavage stage into oviducts of gonadotropin treated recipients (Goodrowe et al., 1988; Pope et al., 2009). Gómez et al. (2004) reported that African wild cat cloned embryos was higher after laparoscopic oviductal transfer on Day-1 than after uterine transfer on Days 5-7.

1.3 Objectives of the thesis

1. To examine the effect of cold storage on plasma membrane integrity, DNA integrity and fertilizing ability of testicular spermatozoa

2. To examine the effects of preserving medium and BSA concentration on sperm membrane integrity, DNA integrity, and also to assess the developmental competence of embryo derived from ICSI using 7 days cold stored testicular spermatozoa

3. To compare the effects of cryoprotectants and freezing techniques on integrity of sperm membrane and DNA

4. To determine gamete activation up to 18 h after injection of spermatozoa recovered from cryopreserved testicular tissue

5. To evaluate *in vitro* development of embryos produced by ICSI with spermatozoa recovered from cryopreserved testicular tissue

6. To study *in vitro* and *in vivo* development of frozen cleaved embryos (2- to 8 cell stage) derived by ICSI with spermatozoa from frozen-thawed testicular tissue

1.4 Hypothesis

1. Intact testes could be cold stored in basic medium for maintaining testicular sperm quality and viability

2. Different media and concentrations of bovine serum albumin affect on testicular sperm quality during cold storage

3. Cryoprotectants and freezing techniques affect on testicular sperm quality after freezing and thawing

4. Embryos produced by ICSI with cryopreserved testicular spermatozoa can survive after freezing and thawing as well as *in vivo* developmental competence following embryo transfer

1.5 **Keywords**: domestic cat, testicular spermatozoa, intracytoplasmic sperm injection, embryo, cold storage, cryopreservation

1.6 **Research merits:**

1. The development of testicular spermatozoa and tissue preservation for subsequent embryo production will play an important role in genetic management of wild felid.

2. The data acquired from this study would verify optimal technique to improve the applicability of ART for wild felid conservation.

3. National and international publications

1.7   Thesis framework

The thesis was divided into 4 parts as follows
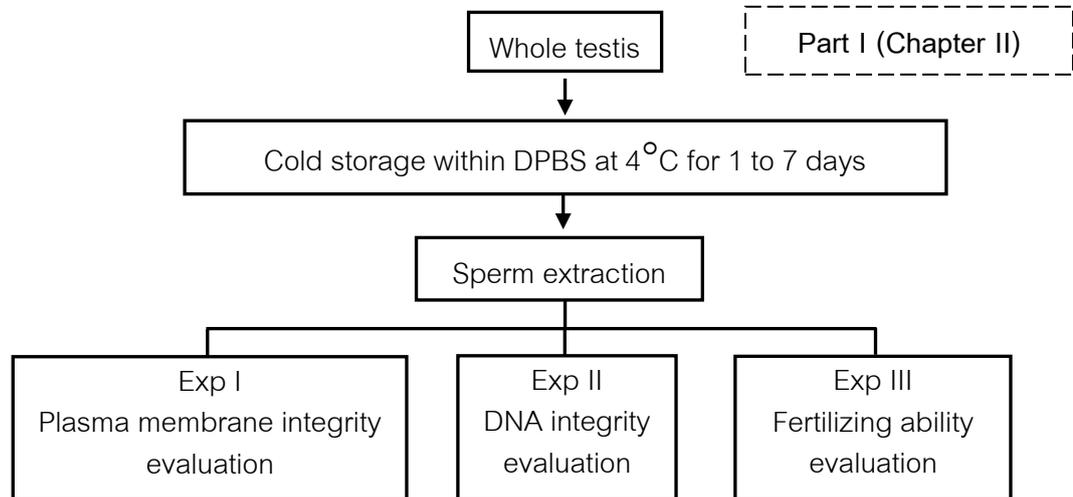
Figure 6 Part I: Effects of cold storage on plasma membrane, DNA integrities and fertilizing ability of feline testicular spermatozoa
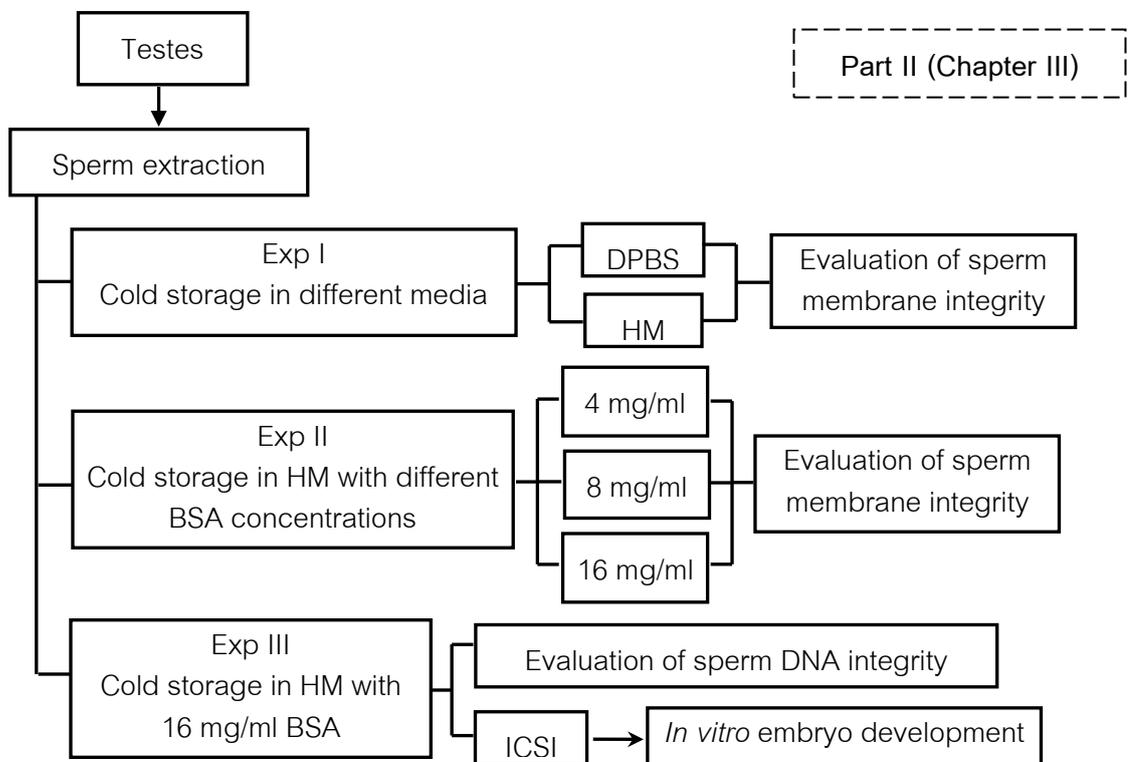
Figure 7 Part II: Spermatozoa isolated from cat testes retain their structural integrity as well as a full developmental potential after refrigeration for up to 7 days

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┌─────────────────────────┐                    ┌─ ─ ─ ─ ─ ─ ─ ─ ─ ─ ┐
│ Testicular tissue pieces│                    │  Part III (Chapter IV)│
└─────────────────────────┘                    └─ ─ ─ ─ ─ ─ ─ ─ ─ ─ ┘
            │
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┌─────────────────────────┐
│   Cryopreservation      │
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Figure flowchart:

- Testicular tissue pieces → Cryopreservation

  - Exp I Different CPAs → Glycerol / EG / PrOH / DMSO → Sperm membrane and DNA integrities evaluation
  - Exp II Different Freezing protocols → Two-step freezing / Controlled slow freezing → Sperm membrane and DNA integrities evaluation
  - Exp III Assessment of gamete activation after ICSI
  - Exp IV Assessment of *in vitro* embryo development after ICSI
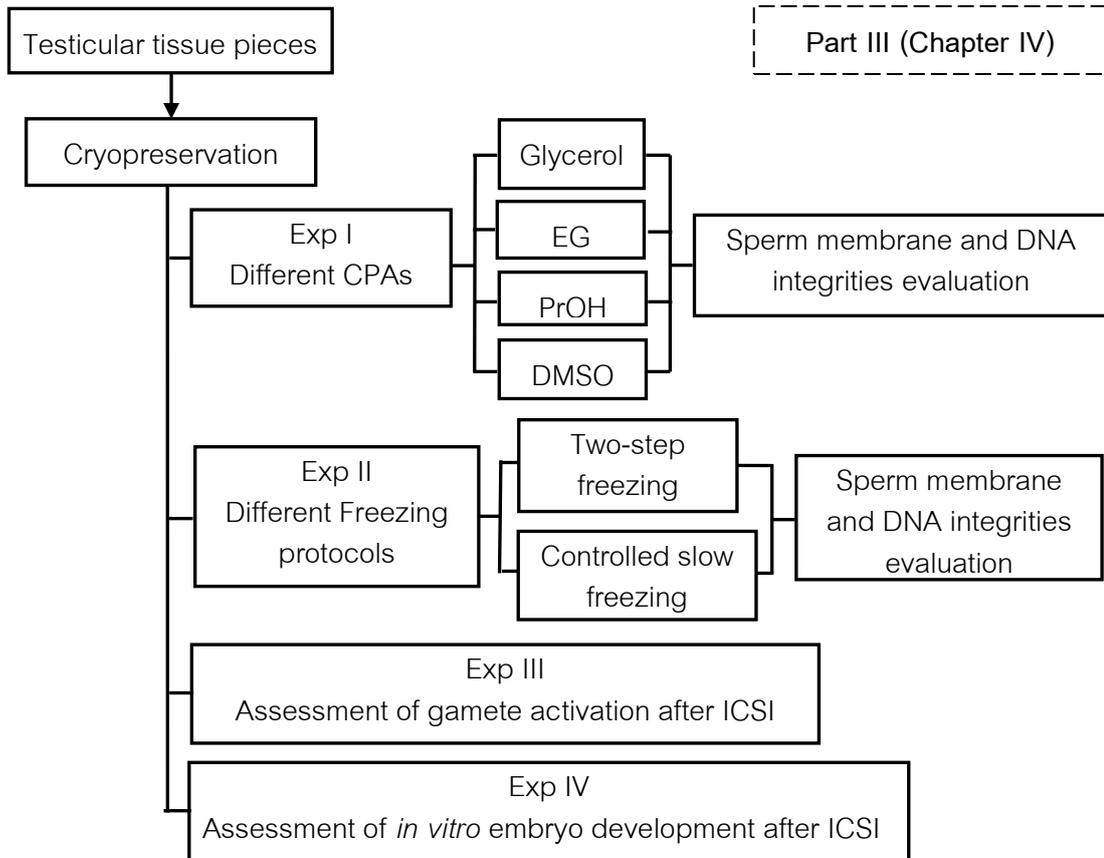
Figure 8 Part III: Feline spermatozoa from fresh and cryopreserved testicular tissues have comparable ability to fertilize matured oocytes and sustain the embryo development after intracytoplasmic sperm injection

Figure flowchart:

- Testicular tissue cryopreservation → ICSI → Embryo cryopreservation
  - Assessment of *in vitro* embryo development
  - Assessment of *in vivo* embryo development
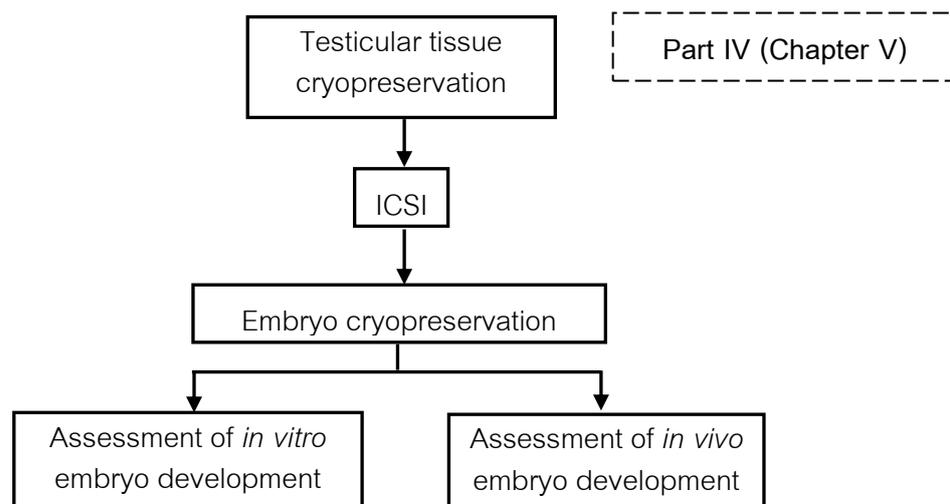
Part IV (Chapter V)

Figure 9 Part IV: *In vitro* and *in vivo* development of frozen embryos produced by ICSI with cryopreserved testicular spermatozoa

CHAPTER II

EFFECTS OF COLD STORAGE ON PLASMA MEMBRANE, DNA INTEGRITY AND FERTILIZING ABILITY OF FELINE TESTICULAR SPERMATOZOA

2.1 Abstract

This study examined the effects of cold storage on plasma membrane, DNA integrity, and fertilizing ability of domestic cat spermatozoa. Intact cat testes were stored at 4 °C in Dulbecco's phosphate buffered saline (DPBS) for 7 days. Membrane integrity (experiment I) and DNA integrity (experiment II) of extracted spermatozoa were assessed over time during storage. Testicular spermatozoa were also tested for their fertilizing ability via intracytoplasmic sperm injection (ICSI) in term of gamete activation and early embryonic development at 18 h (experiment III). The membrane integrity of testicular spermatozoa was well preserved in DPBS for 4 days compared to non-preserved control (Day 0) (P < 0.05). The incidence of testicular sperm DNA fragmentation was < 1 % after 7 days of cold storage and was not significantly affected by the duration of cold storage (P > 0.05). Finally, testicular spermatozoa could form pronuclei and sustain embryo development following ICSI regardless of the storage time (P > 0.05). In conclusion, cat testicular spermatozoa can be preserved at 4 °C for up to 7 days without severely compromising of plasma membrane and DNA integrity while retaining a normal fertilizing ability.

2.2 Introduction

Assisted reproductive technologies (ARTs) such as artificial insemination (AI), and *in vitro* fertilization (IVF) combined with gamete preservation can be used to support the management of wild felid populations (Pukazhenthi et al., 2006a) that rapidly decrease because of many threatening factors. These include illegal hunting, loss of habitats and inbreeding-related problems, such as immunodepletion-related illness (Peña et al., 2006) and infertility due to teratospermia (Pukazhenthi et al., 2006b). Spermatozoa used

for these ARTs in domestic and wild cats are normally collected by electroejaculation (Zambelli and Cunto, 2006). Intracytoplasmic sperm injection (ICSI) with testicular spermatozoa offers many advantages. This technique allows the study and use of immotile intragonadal gametes from genetically valuable: 1) wild animals that die unexpectedly or are castrated for medical reasons, 2) adult males with obstructive and nonobstructive azoospermia (Levine et al., 2003) and 3) prepubertal males after xenografting of testis tissue in immunodeficient mice (Nakai et al., 2010). In humans, testicular spermatozoa yield similar percentages of fertilization, cleavage and pregnancy compared to epididymal spermatozoa (Silber et al., 1995) but these were reported to be slightly lower than ejaculated spermatozoa (Nagy et al., 1995; Qian et al., 2005). Until recently, only a few studies have used testicular spermatozoa for producing feline embryos (Comizzoli et al., 2006a, b).

Cold storage technique is a useful tool for 'short-term' preservation of sperm viability, particularly when transportation was delayed or in conditions that immediate use of testicular spermatozoa (e.g. embryo production and cryopreservation) cannot be performed. Besides, cold storage is more suitable for temporary storage than cryopreservation because it provided less effect on sperm qualities than cryopreservation (Zavos et al., 1980; England and Ponzio, 1996; Dondero et al., 2006; Siemieniuch and Dubiel, 2007). However, prolonged cold storage and suboptimal cold storage conditions also induce sperm damage of critical sperm components, such as plasma membrane, acrosome and chromatin (Shahiduzzaman and Linde-Forsberg, 2007). Although techniques for cold storage of testicular tissue has been developed, the success has been variable between techniques and across species employed (Jahnukainen et al., 2007; Zeng et al., 2009; Yang and Honaramooz, 2010). In domestic cats, testicular spermatozoa recovered from chilled testes (4 ºC) for approximately 6 h have been successfully used to fertilize matured oocytes via intracytoplasmic sperm injection (ICSI) (Comizzoli et al., 2006a,b). However, the effects of prolonged cold storage on quality of feline testicular spermatozoa have not been examined. The objectives of this study were to examine the effects of cold storage on membrane and

DNA integrity of domestic cat testicular spermatozoa, and also to assess the fertilizing ability of preserved testicular spermatozoa.

2.3 Materials and Methods

2.3.1 Chemicals

All chemicals used in the experiments were purchased from Sigma Aldrich (St Louis, MO, USA) unless otherwise specified.

2.3.2 Collection and cold storage of testes

Testes were obtained from adult domestic cats (approximately 1-5 yrs) sterilized at the Fertility and Neutering Clinic, Small Animal Teaching Hospital, the Faculty of Veterinary Science, Chulalongkorn University and The Veterinary Public Health Division of The Bangkok Metropolitan Administration, Bangkok, Thailand. They were maintained in 0.9 % (w/v) saline (NaCl) solution and transported to the laboratory within 3 h at ambient temperature. Upon arrival, external tissues were removed (**Figure 10A**) and only the testes (weighing between 1 - 1.8 g) encapsulated within the tunica albuginea were used in this study. The testes were washed and maintained at 4 °C in Dulbecco's phosphate buffered saline (DPBS) in the 15-ml conical tubes (BD Falcon, Bedford, MA, USA) (**Figure 10B**).
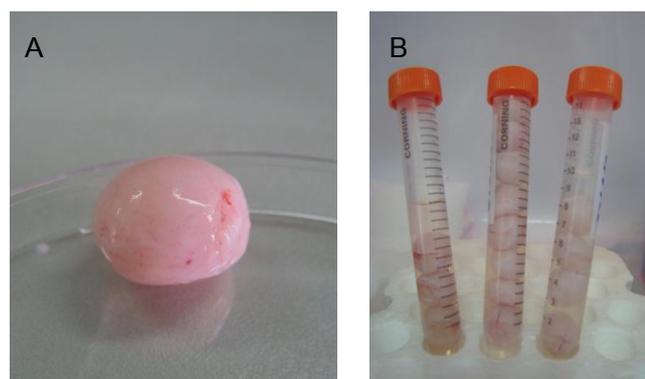
Figure 10 The photographs represent cold storage of intact testes. (A) A feline testis was removed from external tissues and epididymis. (B) The testes were cold stored within the 15-ml conical tubes.

2.3.3 Testicular sperm extraction

To extract the spermatozoa, testes were first decapsulated and then mechanically minced with sharp-ended scissors in DPBS at room temperature (25-27 °C). Spermatozoa were further extracted from minced tissue by gentle pipetting. The sperm suspension was finally filtered through 100 and 40 μm cell strainer (BD Falcon, Bedford, MA, USA), respectively. A total of 100 - 200 spermatozoa per sample were examined for each sperm quality test.

2.3.4 Assessment of sperm plasma membrane integrity

Extracted sperm samples were centrifuged and then stained with 2 μM Ethidium homodimer-1, a membrane impermeable DNA stain (EthD-1: Invitrogen, Oregon, USA). Hoechst-33342 (3.5 μg/ml) was used as a counterstaining. The fluorescently labeled testicular spermatozoa were smeared onto a glass slide and examined with an epifluorescent microscope (BX51; Olympus, Shinjuku, Japan) at 1000x magnification. Bright red sperm head (EthD-1 positive spermatozoa) indicated the loss of plasma membrane integrity (dead spermatozoa, **Figure 11E**), while spermatozoa negative to the EthD-1 were classified as viable spermatozoa (**Figure 11F**).

2.3.5 Assessment of sperm DNA integrity

Detection of DNA fragmentation was performed by terminal deoxynucleotidyl transferase (TdT)- mediated dUTP nick end labeling (TUNEL) assay (In Situ Cell Death Detection Kit; Roche, Mannheim, Germany) following the manufacturer's instructions. Because the late apoptosis (DNA fragmentation) occurs in a specific stage prior to necrosis (cell death), the EthD-1 staining was used to discriminate the dead sperm cells from apoptotic spermatozoa. After staining the extracted spermatozoa with 2 μM EthD-1 for 15 min, the EthD-1 was neutralized by adding excessive amount of salmon sperm DNA. The testicular spermatozoa were subsequently smeared onto a glass microscopic slide coated with aminopropyltriethoxysilane and air dried. The slides containing testicular spermatozoa were fixed with 4 % (w/v) paraformaldehyde for 30 min. After a brief wash with phosphate buffered saline (PBS) supplemented with 0.1 % (w/v) BSA (PBS-BSA), the sperm plasma membrane was permeabilized on ice with 0.1 % (v/v)

Triton X-100 in PBS for 5 min. To perform TUNEL assay, the slides were washed in PBS and incubated with a mixture of TUNEL reaction mix (TdT enzyme and nucleotide) for 1 h at 37 °C in a humidified chamber. Slides were counter stained with 4'6' Diamidino-2-Phenylindole Dihydrochloride (DAPI; 50 ng/ml). The antifade mounting medium (Vectashield™, Vector labs, Burlingame, USA) was used to mitigate the photobleaching. Spermatozoa positive to TUNEL displayed a bright green head under an excitation and emission wavelength of 460–490 nm and 520 nm, respectively (**Figure 11G**). Spermatozoa positive to EthD-1 (dead spermatozoa) exhibited bright red sperm head, while live spermatozoa were negative to EthD-1 (**Figure 11F**). The percentage of DNA fragmented spermatozoa was defined as the number of TUNEL positive spermatozoa in relation to the total number of live spermatozoa (i.e. EthD-1 negative spermatozoa).

2.3.6 Oocyte collection and *in vitro* maturation

Cumulus oocyte complexes (COCs) were recovered after mincing cat ovaries in holding medium (HM) consisted of HEPES-buffered M199, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin and 100 µg/ml streptomycin and 0.1 % (w/v) bovine serum albumin (BSA, embryo tested grade). Only oocytes completely surrounded with more than five layers of compacted cumulus cells and containing homogeneous-dark ooplasm were used. *In vitro* maturation was performed as previously described by Sananmuang et al. (2010). In brief, 30-40 COCs were matured *in vitro* in 500 µl of maturation medium containing $NaHCO_3$ buffered M199 supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 100 IU/ml penicillin, 4 mg/ml BSA, 0.05 IU/ml recombinant human follicle stimulating hormone (rhFSH, Organon, The Netherlands) at 38.5 °C in a humidified condition of 5 % CO_2 in air for 18-24 h. After *in vitro* maturation, cumulus cells were removed by gentle pipetting. The denuded oocytes exhibiting the 1st polar body were submitted to ICSI.
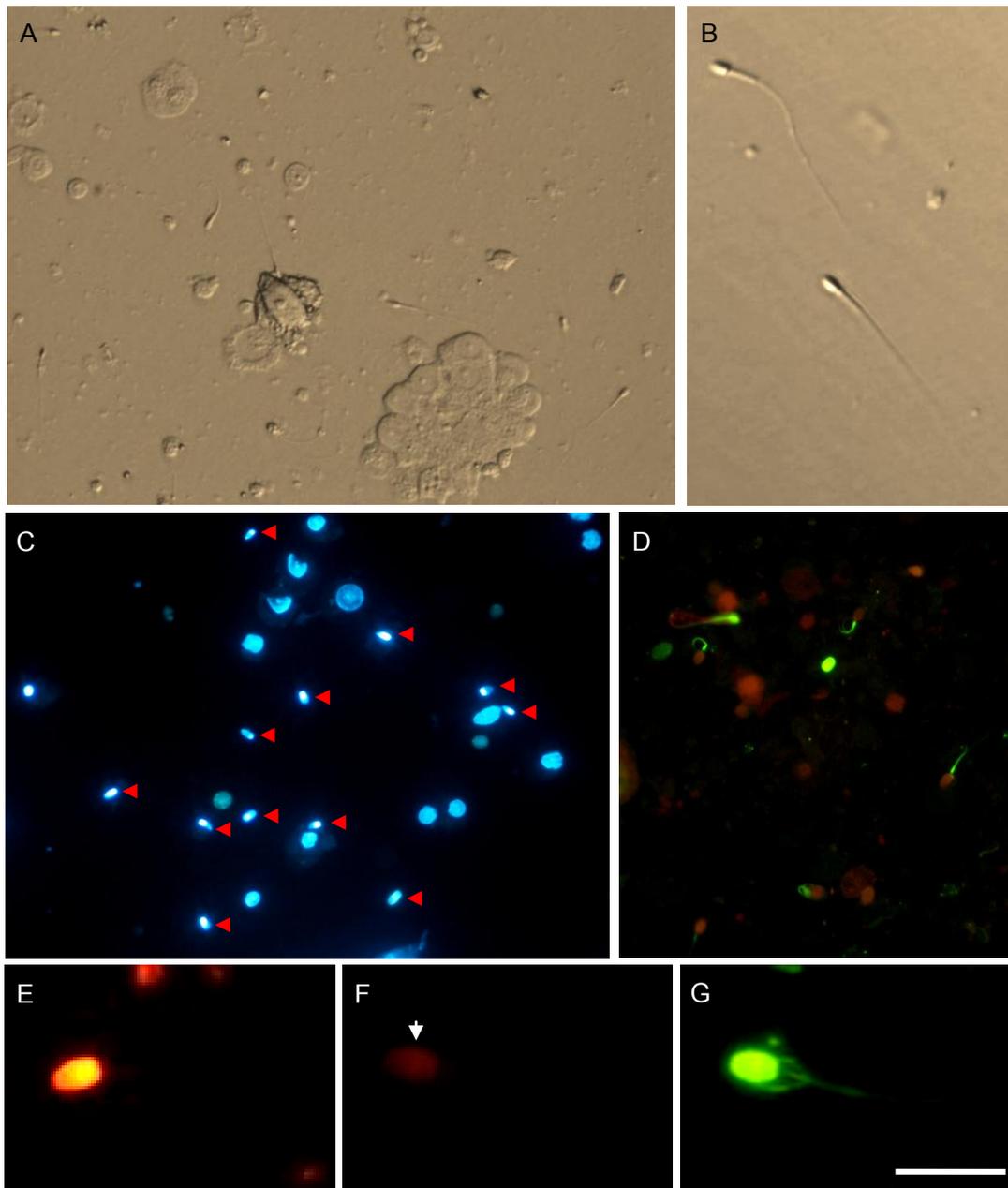
Figure 11 Photomicrographs of sperm suspension (after filtration by cell-strainer) contained testicular cells and spermatid in various stages of development (A). Only mature spermatids (B) were included for evaluation. DNA- specific Hoechst and DAPI stains were used for locating of both live and dead spermatozoa, and presented fluorescent blue (sperm head = red arrow head; C). A population of spermatozoa after staining with TUNEL and EthD-1 (D), spermatozoon positive to EthD-1 (dead spermatozoon) exhibited a bright red head (E), while EthD-1 weakly labeled (negative) sperm head indicated the intactness of sperm membrane integrity (arrow head; F). Spermatozoon with fragmented DNA exhibited a bright green head (G). Bar represents 10 μm.

2.3.7 Sperm preparation and intracytoplasmic sperm injection (ICSI)

Spermatozoa used for ICSI were extracted from the testis after cold storage and maintained in HEPES buffered synthetic oviductal fluid (HEPES-SOF). The sperm suspension was then mixed with 10 % (w/v) polyvinylpyrrolidone (PVP; MediCult a/s, Jyllinge, Denmark). Microinjection was performed using an inverted microscope (IX 70, Olympus, Tokyo, Japan) equipped with a Narishige micromanipulator (Model No. MMO-202D; Narishige Instrument Co., Tokyo, Japan) and a warm stage at 37 °C. Injecting pipettes were 5-6 and 10 μm for inner and outer diameters respectively with 30° beveled end (Humagen Fertility Diagnostics, Inc., Charlottesville, VA, USA). Holding pipettes were 15-20 and 120-150 μm for inner and outer diameter with a fire-polished tip. A morphologically normal immotile spermatozoon (**Figure 12A**) was selected for ICSI. The oocytes were held by negative pressure with the polar body oriented at the six or twelve o'clock position (**Figure 12B**). The injection was performed by advancement of ICSI needle through the zona pellucida at three o'clock position. After a minimal amount of ooplasm had been aspirated into the injection pipette, a spermatozoon was then released into the ooplasm. After ICSI, the oocytes were activated by incubation with 7 % (v/v) ethanol at 38.5 °C for 5 min. The presumptive zygotes were subsequently cultured at 38.5 °C in 50 μl droplets of synthetic oviductal fluid (SOF) medium containing with 4 mg/ml BSA in a humidified condition of 5 % CO_2 in air.
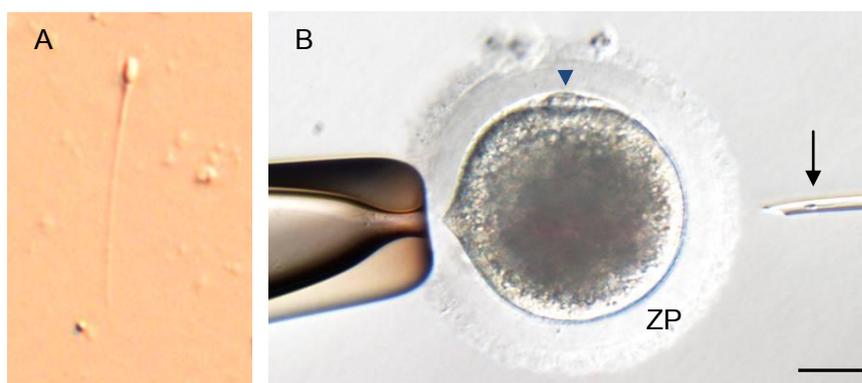
Figure 12 A testicular spermatozoon (A) with morphologically normal head, midpiece and tail was selected for ICSI. The polar body (arrow head) of matured oocyte was oriented at twelve O' clock position (B). An arrow = spermatozoon; ZP = zona pellucida. Bar = 20 μm.

2.3.8 Assessment of testicular sperm fertilizing ability

At 16-18 h after ICSI, presumptive zygotes were incubated for 45 min at 37 °C in a pre-fixation solution containing 25 % (v/v) glycerol, 50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM 2-mercaptoethanol, 50 mM imidazol and 4 % (v/v) Triton-X-100 prior to fixation in 4 % (w/v) paraformaldehyde overnight. The presumptive zygotes were washed twice in PBS-BSA and then stained with 0.1 μg/ml DAPI. The fluorescently labeled zygotes were mounted on a glass microscope slide in a 2 μl droplet of antifade medium and examined with an epifluorescent microscope. To determine the fertilizing ability of testicular spermatozoa, the ICSI oocytes were assigned to 1 of 4 categories including normal fertilized, unfertilized, parthenogenetic and degenerate oocytes. Normal fertilization was identified by the presence of one (female) pronucleus with decondensing sperm head or two pronuclei or cleaved embryo with an extrusion of the second polar body, while unfertilized oocytes were typified by the MII oocytes (MII arrest) that contained with an intact sperm head within the ooplasm. Oocyte activation following sham ICSI and also dividing cells containing an intact sperm head after ICSI indicated parthenogenetic activation. Degenerate oocytes were oocytes with dispersed chromatin within the ooplasm. Percentages were specified as the numbers of oocyte in each type relative to the total number of injected oocytes.

2.3.9 Experimental design

Experiment I: Effect of cold storage on membrane integrity of testicular spermatozoa

In this experiment, a total of 70 testes (encapsulated within tunica albuginea) were used to examine the effect of cold storage on membrane integrity of testicular spermatozoa. They were maintained at 4 °C in DPBS and the testes were daily examined for 7 days (n = 10/day). Testicular spermatozoa extracted within 6 h after castration (n=10) served as a control group.

Experiment II: Effect of cold storage on testicular sperm DNA integrity

This experiment aimed specifically to detect the damage of the DNA integrity after prolonged cold storage (7 days). After cold storage, testicular sperm samples obtained

by sperm extraction were examined for the incidence of DNA fragmentation by TUNEL assay. A total of 50 intact testes were used for this study. DNA integrity was only examined in viable/intact sperm plasma membrane on day 0, 1, 3, 5 and 7 of cold storage (n=10 per storage time).

Experiment III: Effect of cold storage on fertilizing ability of testicular spermatozoa

To test the fertilizing ability of testicular spermatozoa recovered from long-term chilled testes, the testes (n = 20) were first maintained at 4 °C in DPBS and then extracted on 1, 3, 5 and 7 days of cold storage (each group was replicated 5 times). A morphologically normal immotile spermatozoon recovered from chilled testis was directly injected into the ooplasm of metaphase II oocytes (ICSI) to examine the fertilizability of the testicular spermatozoa. After 16-18 h post-ICSI, the presumptive zygotes were fixed, and the gamete activation was determined by means of sperm head decondensation, resumption of the 2nd meiosis, the formation of male and female pronucleus, and also cleavage rates following ICSI. Microinjection without spermatozoon (sham ICSI) and ICSI with fresh testicular spermatozoon (5 replicates) served as controls.

2.3.10 Statistical analysis

The data were analyzed using the Statistical Analysis Systems software package (Version 9.0, SAS Institute Inc., 1996, N.C., USA). Normality of the data was evaluated using UNIVARIATE procedure option NORMAL PLOT. Sperm membrane integrity and DNA integrity were analyzed using the General Linear Mixed Model (MIXED) procedure of the SAS and expressed as mean ± standard error of the mean (SEM). Differences of sperm quality during preservation (Day 0-7) were examined by one-way ANOVA statistical test. The means were separated using a Duncan's multiple range test. The Chi-square test was used to compare the number of inactivated, 1PN, 2PN oocytes and cleaved embryos among ICSI groups (fresh testes, D1-, D3-, D5-, D7- chilled testes and sham ICSI). Differences were considered statistically significant when $P < 0.05$.

2.4 Results

2.4.1 Experiment I: Effect of cold storage on membrane integrity of testicular spermatozoa

The plasma membrane integrity of testicular spermatozoa slowly decreased during cold storage. Cold storage of intact testes preserved the plasma membrane integrity of testicular spermatozoa ($P > 0.05$) similar to control (non-preserved testes), especially during the first 4 days of cold storage (% intact membrane spermatozoa: 68.4 ± 1.8 vs. 67.6 - 57.7 for fresh and chilled testes for 1-4 days, respectively, **Figure 13**). After cold storage for 7 days, viability of preserved testicular spermatozoa remained up to approximately 50 % which was significantly lower than the control ($P < 0.05$).
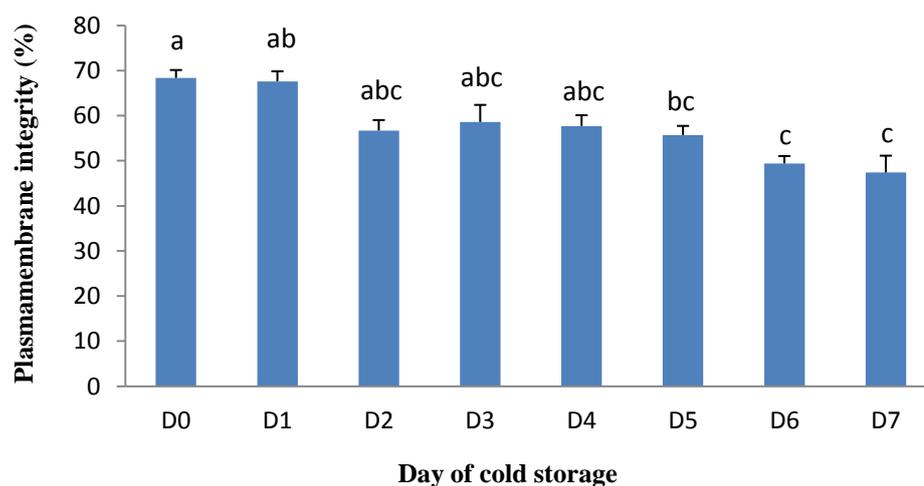
Figure 13 Percentage of spermatozoa with intact plasma membrane (viable) during cold storage for 1 week. Values are presented as Mean ± SEM (n=10 per group). Different superscripts denote values that differ significantly ($P < 0.05$).

2.4.2 Experiment II: Effect of cold storage on testicular sperm DNA integrity

We further analyzed the DNA integrity of viable testicular spermatozoa during cold storage for 7 days. In fresh and preserved samples, the incidence of membrane intact testicular spermatozoa with fragmented DNA (**Figures 11F, G**) during 7 days of cold storage was low (less than 1 %, **Figure 14**), and the percentages were not statistically different compared with the non-preserved (fresh) control and also among the days of

sperm examination (P > 0.05). However, the proportion of DNA fragmentation in testicular spermatozoa tended to increase over 7 days of cold storage (**Figure 14**).
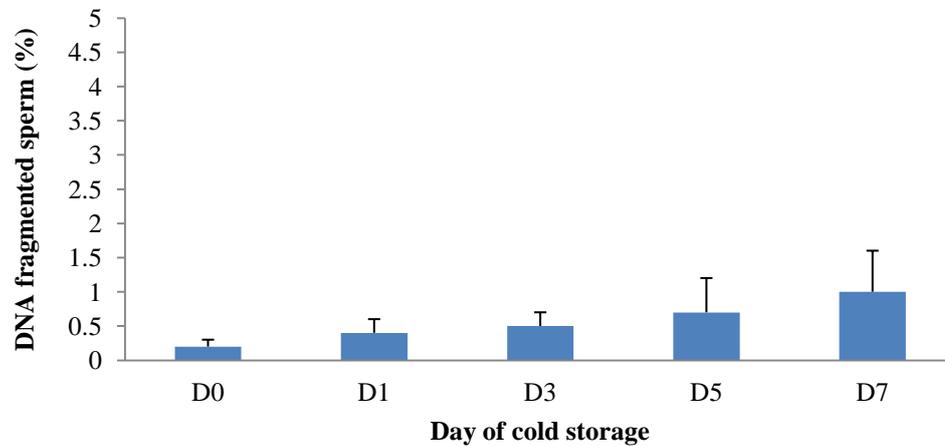
Figure 14 The proportion of viable spermatozoa with fragmented DNA after cold storage for 1, 3, 5 and 7 days versus control (D0). Values are presented as Mean ± SEM

2.4.3 Experiment III: Effect of cold storage on fertilizing ability of testicular spermatozoa

16-18 h post-ICSI, injected oocytes were classified into 4 groups as previously described in materials and methods. MII arrested oocytes could be observed after ICSI with either intact (**Figure 15A**: arrowhead) or decondensing sperm head (**Figure 15B**: asterisk), suggesting that male and female gametes were independently activated. The activated oocytes extruded the second polar body (**Figure 15C-H**) and formed a female pronucleus (**Figure 15C, D**). The intact spermatozoa could also be found within oocytes having one or two pronuclei (**Figure 15F**) and cleaved embryos (**Figure 15H**). Preserved spermatozoa retained their fertilizing ability after cold storage for 7 days similar to fresh testicular spermatozoa (P > 0.05, **Table 1**). The duration of cold storage did not significantly affect the numbers of fertilized oocytes with two pronuclei and cleaved embryos following ICSI, in terms of the percentage of normal fertilization and parthenogenesis (P > 0.05). Percentages of parthenogenesis were the least for ICSI with spermatozoa preserved for 5 and 7 days. In sham ICSI, high proportions of injected oocytes were arrested at MII stage 57.7 % (41/71). However, some of the sham ICSI oocytes (35.3 %) also underwent parthenogenetic activation.

Table 1

In vitro development of cat oocytes at 16-18 h after ICSI using fresh and cold stored testicular spermatozoa (5 replicates/ group)

ICSI Group (days)	Injected oocytes	Non-activated oocytes (MII)			Activated oocytes (with 2PB)								Degenerated
		Without Sperm	+ ISH	+ DSH	Normal fertilization				Parthenogenesis				
					1PN*	2PN	Cleaved	Total	1PN**	2PN	cleaved	Total	
Fresh	83	0	29 (34.9)	2 (2.4)	2 (2.4)	11 (13.3)	6 (7.2)	19 (22.9)	8 (9.6)	5 (6)	12 (14.5)	25 (30.1)[a, c]	8 (9.6)
D1	74	0	26 (35.1)	1 (1.4)	1 (1.4)	16 (21.6)	7 (9.5)	24 (32.5)	3 (4.1)	8 (10.8)	9 (12.2)	20 (27.1)[a, b, c]	3 (4.0)
D3	60	0	14 (23.3)	2 (3.3)	0	10 (16.7)	12 (20)	22 (36.7)	2 (3.3)	5 (8.3)	7 (11.7)	14 (23.3)[a, b, c]	8 (13.3)
D5	65	0	30 (46.2)	1 (1.5)	0	13 (20)	9 (13.8)	22 (33.8)	1 (1.5)	1 (1.5)	8 (12.3)	10 (15.3)[b]	2 (3.1)
D7	76	0	25 (32.9)	2 (2.6)	0	11 (14.5)	17 (22.3)	28 (36.8)	3 (3.9)	4 (5.3)	7 (9.2)	14 (18.4)[a, b]	7 (9.2)
Sham	71	41 (57.7)	0	0	0	0	0	0	1 (1.4)	6 (8.5)	18 (25.4)	25 (35.3)[c]	5 (7.0)

Within a column, different letters (a, b and c) denote values that differ significantly ($P < 0.05$).

ISH= intact sperm head, DSH=decondensing sperm head

*= female pronucleus with a decondensing sperm head

**= female pronucleus after sham ICSI or a female pronucleus presented with an intact sperm head after sperm injection
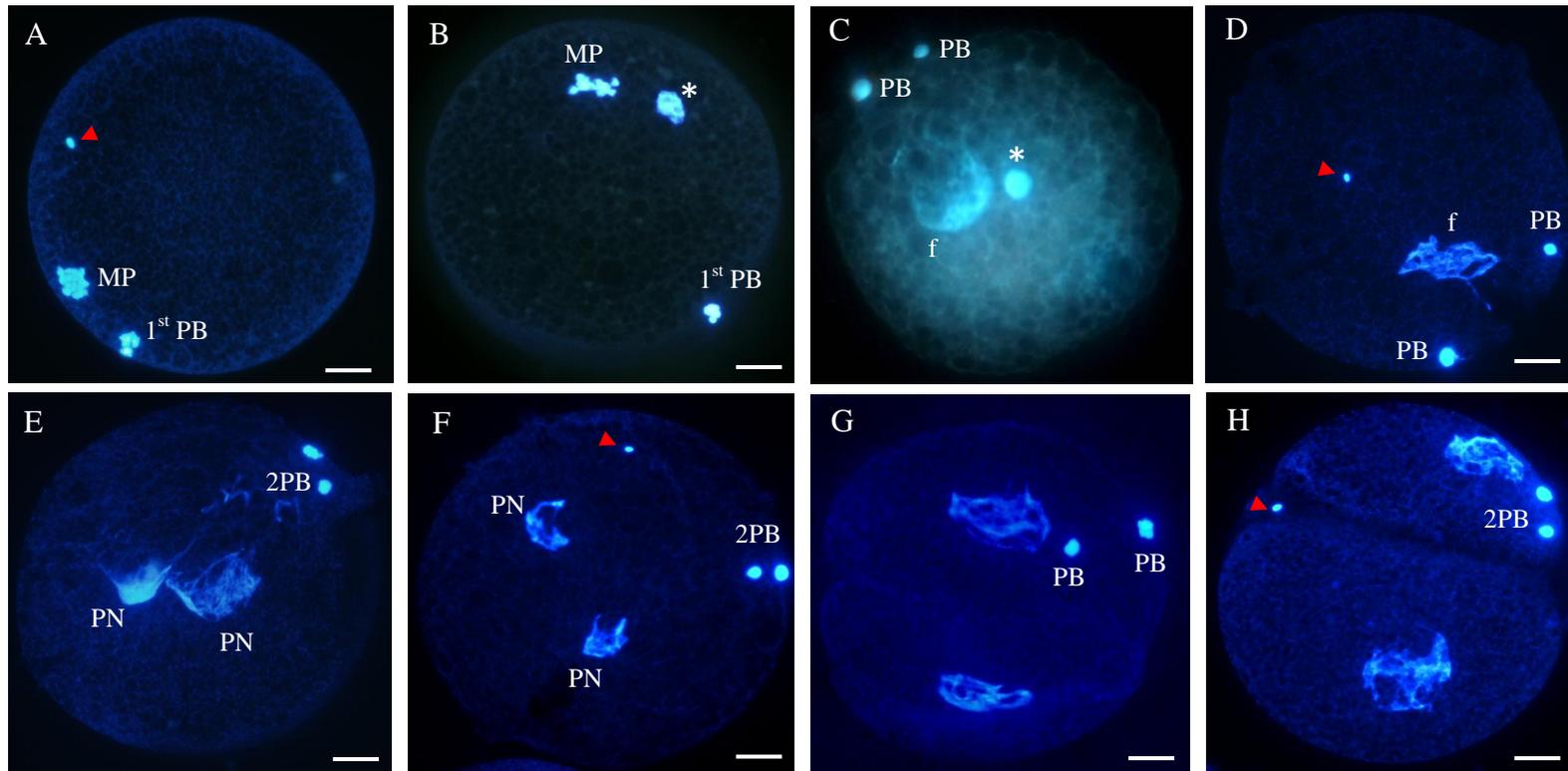
Figure 15 Fluorescent micrographs of chromatin configuration (stained with DAPI) of cat oocytes at 16-18 h post ICSI. MII arrest was identified by the presence of metaphase plate with the first polar body (1st PB; A-B). After sperm injection, non-fertilized oocytes arrested in MII stage could be presented with an intact (A) or with decondensing (B) sperm head. The activated oocytes (C-H) extruded the second polar body (2nd PB), then formed a female pronucleus that could be observed with either decondensing (C) or intact (D) sperm head. Oocytes with two pronuclei could be presented with (F) or without (E) an intact sperm head. Two-cell embryos obtained from normal fertilization (G) or parthenogenesis (H). MP = metaphase plate, PB = polar body, PN = pronucleus/pronuclei, f = female pronucleus, asterisk = decondensing sperm head, red arrowed head = intact sperm head. (Bar = 20 µm; Original magnification 20x-40x)

2.5 Discussion

In the present study, cold storage maintained testicular sperm membrane and DNA integrity for at least 7 days, although differences were found among the days of sperm examination (**Figure 13** and **Figure 14**). Since the data on the effect of prolonged preservation of feline testis on sperm quality has not yet been reported, we therefore examined the plasma membrane (Exp. 1) and DNA integrity (Exp. 2) in order to investigate the possibility to preserve the testes at 4 °C without significantly affecting sperm quality. Our study demonstrated that cooling of testicular spermatozoa within the testicular parenchyma as intact testis in basic medium (DPBS) yielded high sperm membrane integrity similar to non-preserved testes. The proportion of viable spermatozoa with fragmented DNA tended to increase over the time of cold storage but did not different from control. These preserved testicular spermatozoa could fertilize the *in vitro* matured oocyte as well as fresh testicular spermatozoa.

DPBS was preferably used as a preservation medium because it has been reported to maintain the membrane integrity of testicular cells for at least 6 days of cold storage (Yang and Honaramooz, 2010). This basic salt solution can be easily prepared and stored compared to other complex media, although long term preservation of testicular tissue in the DPBS seemed to adversely affect the fate of other testicular cells i.e. porcine testicular germ cells (Zeng et al., 2009).

Although the specific pathways for supporting plasma membrane and DNA integrity of testicular spermatozoa during cold storage of feline testes is not clear, we hypothesized that testicular parenchyma may protect testicular spermatozoa from direct exposure to rapid cooling, there preserving the feline sperm membrane integrity (Pukazhenthi et al., 1999). In addition, the testis contains several proteins actively involved with antioxidative mechanisms such as superoxide dismutase (SOD), glutathione reductase (GSR), glutathione peroxidase (GPX), glutathione S-transferase (GST) (Bauché et al., 1994; Gu and Hecht, 1996). These antioxidative mechanisms may be sufficient to protect testicular cells against oxidative stress during short duration of cold storage. Moreover, supporting effect of testicular tissue on membrane integrity was

confirmed by the survival of spermatozoa as we found a small proportion of twitching testicular spermatozoa (approximately 3.4 ± 1.4 % and 0.5 ± 0.3 % for fresh and 7-day chilled testes, respectively, unpublished data). The high membrane integrity (above 50 %) of testicular spermatozoa following cold storage for 7 days suggests the role of testicular compartments in supporting sperm membrane integrity. Although we did not exam testicular sperm quality if the testes were kept for longer than 7 days, this 'short-term' cold storage would be sufficient for transport the testicular tissues to nearby laboratory.

The DNA integrity of spermatozoa is also one of the most important parameters for ICSI. DNA damaged spermatozoa markedly contribute to the low fertilization rate and abnormal embryo development (Benchaib et al., 2003; Tesarik et al., 2004). In this study, we examined the proportion of DNA fragmentation only in viable spermatozoa because DNA fragmentation occurs by both preprogrammed apoptotic (late stage) and necrotic (dead) spermatozoa. Proapoptotic signals specifically cleave the DNA into internucleosomal fragments, while DNA fragmentation occurs in non-specific manner after cell death. We found that the incidence of DNA fragmentation in feline testicular spermatozoa was low (1.0 ± 0.6 %) after cold storage for 7 days (**Figure 14**). This percentage was also low when intactness of sperm plasma membrane was taken into account (approximately 2.3 ± 1.1 %, unpublished data). These results were in agreement with a report indicating that DNA integrity diminishes slower than plasma membrane integrity of spermatozoa (López-Fernández et al., 2008). DNA of mammalian spermatozoa is different from somatic cells in that the histone protein is replaced by protamine, with disulfide cross linkages between the protamines. Feline spermatozoa contains 2 protamins coupled with a highly condensed chromatin may play a role in protecting against DNA damage during cold storage (Corzett et al., 2002; Aoki et al., 2005). Membranes on the other hand are dynamic structures as opposed to the stable DNA structure. Until recently, the mechanism that induces testicular sperm DNA damage is unclear. Unfavorable environments, for example heat stress, have been demonstrated to activate capase-3 dependent cascades and to induce unrepaired double-strand DNA breaks of the germ cells (Paul et al., 2008, 2009). This effect may be less detrimental to

spermatozoa compared to other types of germ cell due theoretically to the highly condensed sperm chromatin (Ward and Coffey, 1991).

This study revealed that spermatozoa recovered from chilled (up to 7 days) and fresh testes had the same ability to fertilize *in vitro* matured oocytes, although the fertilization rates of testicular spermatozoa recovered from chilled testes tended to be higher than that of non-preserved testicular tissue (**Table 1**). Furthermore, parthenogenetic activation, as indicated by oocyte activation with intact sperm head, was also observed at 18 h post ICSI. This incidence in ICSI-oocytes with D5 and D7 cold stored spermatozoa tended to be lower than other storage times. Although the exact reason of this is still unclear, it is possible that spermatozoa losing membrane integrity (with intact DNA) after prolonged cold storage (i.e. 5-7 days) may facilitate the direct contact between the sperm factors and the ooplasm. This is in an agreement with the finding that demembranization of sperm plasma membrane before ICSI improved the fertilization success compared to intact-membrane spermatozoa (Yanagimachi, 2005). Moreover, parthenogenetic activation seems likely to depend upon the oocyte activation protocol used rather than the effect of cold storage. Our studies indicated that the incidence of parthenogenetic embryos was remarkably low when ICSI was performed without any oocyte activation (unpublished data). In the current study, the percentages of normal fertilization by fresh testicular spermatozoa were lower than a previous report (22.9 vs. 65 %, Comizzoli et al., 2006a), although the oocyte activation protocol was identical. The inter-laboratory variations may be caused by several factors such as temperature at transportation (25-26 °C vs. 4 °C), culture medium and duration of *in vitro* maturation (18-24 h and 30 h). For instance, the ability of oocytes to achieve activation has been demonstrated to correlate with the duration of MII arrest (Kubiak, 1989). In addition, specific requirements for activation protocol may differ between the sources of spermatozoa. In cat, the artificial stimuli for oocyte activation after ICSI were not necessary for consistent achievement of fertilizing ability of ejaculated cat spermatozoa (Pope et al., 1998). Conversely, others studies with testicular and epididymal spermatozoa reported that the mechanical stimulus during ICSI was insufficient to activate MII oocytes (Comizzoli et al., 2006a) and chemical activation of oocytes after

ICSI is necessary (Bogliolo et al., 2001). Similar to many previous studies, our result found that most of non-fertilized oocytes after ICSI were arrested at MII stage with an intact sperm head in the ooplasm (Pope et al., 1998; Bogliolo et al., 2001; Comizzoli et al., 2006a), suggesting that the mechanism of testicular sperm activation following ICSI is compromised.

2.6 Conclusions

Testicular spermatozoa can be preserved at 4 °C but the membrane integrity gradually decreased over time. The DNA integrity of viable testicular spermatozoa is not remarkably affected by cold storage but it tended to increase during the 7-day storage times. These preserved testicular spermatozoa retain their capability of fertilization after ICSI similar to fresh testicular spermatozoa. Further development of these ICSI-derived embryos and optimization of oocyte activation protocol for ICSI oocytes remain to be elucidated.

CHAPTER III

SPERMATOZOA ISOLATED FROM CAT TESTES RETAIN THEIR STRUCTURAL INTEGRITY AS WELL AS A FULL DEVELOPMENTAL POTENTIAL AFTER REFRIGERATION FOR UP TO 7 DAYS

(The work was submitted in Animal Reproduction Science, 2013)

3.1 Abstract

Cat spermatozoa can be refrigerated within intact testes for up to 1 week without remarkable damage to sperm membrane and DNA integrity as well as the potential to fertilize mature oocytes. The objective of this study was to compare the efficiency of preservation medium for isolated feline testicular spermatozoa as well as the concentrations of bovine serum albumin (BSA) on (1) the membrane (SMI) and DNA integrity of spermatozoa and (2) the developmental potential of spermatozoa after intracytoplasmic sperm injection (ICSI). Isolated cat spermatozoa were stored in HEPES-M199 (HM) or Dulbecco's phosphate buffered saline (DPBS) at 4 °C for up to 7 days. Results indicated that HM maintained a better SMI than DPBS throughout storage period (P > 0.05). When spermatozoa were stored in HM supplemented with bovine serum albumin (BSA) at different concentrations (4, 8 and 16 mg/ml), SMI obtained from HM containing 8 and 16 mg/ml BSA was higher than with 4 mg/ml BSA (P < 0.05). DNA integrity of spermatozoa stored in HM with 16 mg/ml BSA for 7 days was poorer than fresh control, but the subsequent percentages of cleavage, morula, blastocyst produced by ICSI as well as their average blastomere numbers of blastocysts were similar (P > 0.05). In summary, cat spermatozoa immediately isolated from testicular tissue could be stored as a suspension in basic buffered medium at 4 °C for up to 7 days. BSA supplementation into this medium improves membrane integrity of the spermatozoa during cold storage. Testicular spermatozoa stored in HM containing 16 mg/ml BSA retain a full *in vitro* developmental potential after ICSI similar to fresh controls even though DNA integrity had slightly declined.

3.2 Introduction

During the past few decades, the number of wild felids has decreased rapidly because of human activities, such as habitat destruction and poaching. In addition to protecting the species in their natural habitats, the captive breeding of animals is critical to maintain a good genetic diversity that will ensure the sustainability of the populations. In this context, the studies of gamete preservation combined with the development of assisted reproductive techniques (ARTs) are powerful conservation tools (Wildt et al., 2010). Because of a similar reproductive physiology and easily obtainable gametes for research, the domestic cat is an ideal model to develop preservation techniques before application to non-domestic species. Although ejaculated spermatozoa from live animal are normally used for ARTs in felid species (Zambelli et al., 2006), preservation and use of testicular tissue are sometimes the only option in case of unexpected death or castration for medical reasons. In human (Zavos et al., 1980; Dondero et al., 2006), dog (England and Ponzio, 1996) and cat (Siemieniuch and Dubiel, 2007), short-term sperm preservation by cold storage (~4 °C) is preferred to cryopreservation because it could maintain a better sperm quality including DNA integrity (Riel et al., 2011). This DNA integrity is essential for the success of intracytoplasmic sperm injection (ICSI) and the embryo quality (Jiang et al., 2011).

Cat spermatozoa can be refrigerated within intact testes for up to 1 week without significant effects on DNA integrity while plasma membrane integrity decreased overtime (Buarpung et al., 2012). Although storage of intact testes is more convenient, this approach may be harmful for germ cells probably because of excessive accumulation of metabolic substances within the cold-stored organs (kidney: Cotterill et al., 1989 and lung: Pickford et al., 1990). In addition, the testicular spermatozoa are protected from preservation medium by dense connective tissue, so-called tunica albuginea. Therefore, preservation of isolated spermatozoa would avoid any detrimental effects and allow the solution to directly preserve testicular cells. This hypothesis is supported by the finding in pig that viability of isolated testicular cells could be maintained for up to 80 % after cold storage for 6 days (Yang and Honaramooz, 2010). Furthermore, cold storage of isolated testicular spermatozoa would be beneficial in case

of sperm extraction for ICSI program (Levran et al., 2001). Testicular spermatozoa should be preserved within medium that has protective effects on sperm structure and functions including sperm plasma membrane and DNA integrity. Although membrane integrity is not an essential sperm parameter for ICSI, intact sperm membrane would protect the spermatozoa against DNA deterioration from prolonged exposure with extracellular environment (Tateno et al., 2000).

Egg yolk-based extender, widely used for semen freezing, has been applied for cold storage of cat ejaculated and epididymal spermatozoa, to protect spermatozoa from cold shock injury (Hermansson and Axnér, 2007; Siemieniuch and Dubiel, 2007; Gañán et al., 2009). However, several components such as bovine serum albumin (Matsuoka et al., 2006), skim milk (Abe et al., 2008) and soy bean lecithin (Aires et al., 2003; Vick et al., 2012), have been used for substitution of egg yolk to decrease the variation of egg yolk quality and also minimize the transportation problems during the outbreak of avian influenza.

In addition to freezing extender, basic medium, such as potassium simplex optimization medium with amino acids (KSOMaa), HEPES-CZB, K(+)-rich nuclear isolation medium (NIM) and phosphate buffered saline (PBS) supplemented with bovine serum albumin (BSA) have been successfully used for cold storage of mouse spermatozoa, in terms of sperm viability and fertilizing ability (Van Thuan et al., 2005; Li et al., 2011). From previous studies, testicular cells have been well preserved in Dulbecco's phosphate buffered saline (DPBS, basal salt solution) and complex culture media such as medium 199 (M199), Dulbecco's modified Eagle's medium (DMEM), Leibovitz L15 (L15), Ham's F12 and HypoThermosol solution-FRS (HTS) with varying degrees of success (Jahnukainen et al., 2007; Zeng et al., 2009; Yang et al., 2010; Yang and Honaramooz, 2010). However, the effects of these media in preserving testicular spermatozoa have not been studied yet. The advantage of bovine serum albumin (BSA) in protecting sperm membrane integrity has been reported in dog (Risopatron et al., 2002), mouse (Van Thuan et al., 2005; Li et al., 2011), bull (Nang et al., 2012) and ram (Matsuoka et al., 2006). A study also found that development of ICSI embryos could

improve when mouse spermatozoa was preserved in medium supplemented with BSA (Li et al., 2011).

The objective of this study was therefore to better understand the influence of the preservation medium as well as the BSA concentrations during 7 days of cold storage on sperm membrane integrity, DNA integrity, and developmental potential after ICSI.

3.3 Materials and Methods

3.3.1 Chemicals

All chemicals used in the experiments were purchased from Sigma Aldrich, St Louis, MO, USA, unless otherwise specified.

3.3.2 Collection of testes

Testes were obtained from adult domestic cats castrated at the Veterinary Public Health Division of The Bangkok Metropolitan Administration, Bangkok, Thailand. They were maintained in 0.9 % (w/v) saline (NaCl) solution supplemented with antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin) and transported to the laboratory within 3 h at ambient temperature. Upon arrival, external tissues were removed and only the testes weighing between 1 and 1.8 g were used in this study. The testes then were washed in saline solution and dried off with clean gauze.

3.3.3 Testicular sperm extraction and cold storage

Testes were decapsulated and mechanically minced with sharp-ended scissors either in Dulbecco's phosphate buffered saline without Ca^{2+} and Mg^{2+} (DPBS; Gibco®) or in holding medium (HM) consisted of HEPES-buffered M199 (M199), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin and 100 µg/ml streptomycin. Spermatozoa were further extracted from minced tissue by gentle pipetting. The sperm suspension was finally filtered through 40 µm cell strainer (BD Falcon, Bedford, MA, USA). One-milliliter aliquots of sperm suspension were stored in 1.5-ml tubes at 4 °C.

3.3.4 Assessment of sperm plasma membrane integrity

Sperm suspension was stained with Ethidium homodimer-1, a membrane impermeable DNA stain (EthD-1: Invitrogen, Oregon, USA) and Hoechst-33342 was

used as a counterstaining. The fluorescently labeled testicular spermatozoa were smeared onto a glass slide and examined under an epifluorescent microscope (BX51; Olympus, Shinjuku, Japan) as described in Chapter I. Spermatozoa with disrupted plasma membrane exhibited bright red at sperm head and were categorized as dead spermatozoa, while spermatozoa negatively labeled with the EthD-1 were classified as viable spermatozoa.

3.3.5 Assessment of sperm DNA integrity

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling assay (In Situ Cell Death Detection Kit; Roche, Mannheim, Germany) was used for DNA integrity evaluation as described in Chapter I, except that the percentage of DNA fragmented spermatozoa was calculated in relation to the total number of spermatozoa.

3.3.6 Oocyte collection and *in vitro* maturation

Cumulus oocyte complexes (COCs) were recovered after mincing of adult cat ovaries, obtained from routine ovariohysterectomy at the Veterinary Public Health Division of The Bangkok Metropolitan Administration, Bangkok, Thailand, in HM supplemented with 0.1 % (w/v) bovine serum albumin (BSA, embryo tested grade). Only oocytes completely surrounded with more than five layers of compacted cumulus cells and containing homogeneous-dark ooplasm were used. *In vitro* maturation was performed as previously described by Sananmuang et al. (2010).

3.3.7 Sperm preparation and intracytoplasmic sperm injection (ICSI)

To perform ICSI, the testicular spermatozoa were first extracted and then preserved in HM containing 16 mg/ml BSA. The preserving medium was then removed by centrifugation at 400 g for 5 min. Sperm samples were washed twice and placed in HEPES buffered synthetic oviductal fluid (HEPES-SOF). The sperm suspension was then mixed with 10 % (w/v) polyvinylpyrrolidone (PVP; MediCult a/s, Jyllinge, Denmark). Microinjection was performed using an inverted microscope (IX 70, Olympus, Tokyo, Japan) equipped with a Narishige micromanipulator (Model No. MMO-202D; Narishige Instrument Co., Tokyo, Japan) as described by Buarpung et al. (2013).

3.3.8 Embryo culture

Two days after ICSI, non-cleaved oocytes were fixed in 4 % (w/v) paraformaldehyde (PF) and then stained with DAPI. The nuclear status was identified using an epifluorescent microscope. Only cleaved embryos were selected for further culture in SOF containing 10 % (v/v) fetal bovine serum (J R Scientific, Woodland, USA). The medium was changed every 2 days. Cleavage, morula and blastocyst percentages (related to the total number of injected oocytes) were determined on Day 2, 5 and 7 after ICSI, respectively. All Day-7 embryos were fixed in 4 % (w/v) PF and stained with DAPI to determine the cell numbers in blastocysts.

3.3.9 Experimental design

Experiment I: Effect of preservation medium (DPBS and HM) on membrane integrity of extracted testicular spermatozoa after cold storage

A total of 10 testes were used in this study (10 replicates). Each testis was cut into 2 equal pieces and the spermatozoa were then extracted in either DPBS or HM. The sperm suspensions were stored at 4 °C for 1 week. Sperm membrane integrity was daily examined. Testicular spermatozoa extracted on the day of castration served as a control group.

Experiment II: Effect of BSA concentration on membrane integrity of extracted testicular spermatozoa after cold storage

As experiment I indicated that HM provided a better result than DPBS, HM was therefore used as a preserving medium for this experiment. To examine whether addition of BSA would improve sperm quality after cold storage, this experiment was designated to determine the protective effects of BSA on sperm membrane integrity. Individual testis (n=10) was divided into 3 equal parts. Testicular spermatozoa were extracted from each fragment and then maintained at 4 °C in HM supplemented with different concentrations of BSA (4, 8 and 16 mg/ml). Sperm membrane integrity was examined daily for 7 days.

Experiment III: Assessment of sperm DNA integrity after 7-day cold storage and embryo development after ICSI

HM supplemented with 16 mg/ml BSA was used as a preserving medium in this experiment because the membrane integrity of testicular spermatozoa was best preserved compared to other BSA concentrations. A total of 8 testes were used in this study. Testicular spermatozoa were first extracted from each testis and then cold stored at 4 °C for 7 days. The DNA fragmentation was assessed by TUNEL assay on Day 0, 1, 3, 5 and 7 of cold storage (8 replicates). To further examination on fertilizing ability of cold-stored spermatozoa, spermatozoa were separately extracted from other 6 testes and cold stored for 7 days. ICSI was used to determine the fertilizing ability of the refrigerated spermatozoa. A total of 206 MII oocytes were injected (6 replicates) with testicular spermatozoa, and the developmental competence of ICSI oocytes was evaluated. The cleavage, morula and blastocyst formation rates were assessed on Day 2, 5 and 7 of *in vitro* embryo culture (Day 0 = ICSI), respectively. ICSI with fresh testicular spermatozoa (n=186) and sham ICSI (injection without spermatozoa, n=82) served as controls. The embryos with more than 30 cells were classified as morulae, and the embryos containing blastocoelic cavity with 50 cells or more were categorized as blastocysts.

3.3.10 Statistical analysis

The statistical analysis was performed using the Statistical Analysis Systems software package (Version 9.0, SAS Institute Inc., 1996, N.C., USA). The statistical differences of sperm qualities among the experimental groups and the days of storage were evaluated by one-way ANOVA and DUNCAN analysis. The Chi-square test was used to compare the embryo development among experimental groups. The total cell numbers of blastocyst were analyzed by one-way ANOVA. In all cases, differences were considered significant when $P < 0.05$.

3.4 Results

3.4.1 Experiment I: Effect of preservation medium (DPBS and HM) on membrane integrity of extracted testicular spermatozoa after cold storage

In general, sperm membrane integrity gradually decreased during 7 days of cold storage (**Figure 16**). HM maintained sperm membrane integrity at higher percentages than DPBS throughout the storage periods, although the results obtained did not significantly differ between the two media ($P > 0.05$) in all time points. Spermatozoa stored within HM preserved the membrane integrity in comparable with fresh spermatozoa up to Day 2 of storage and decreased gradually during 5 days of storage. In contrast, this membrane integrity reduced significantly ($P < 0.05$) after 1 day storage in DPBS. After 1-week cold storage, the number of viable spermatozoa remained up to approximately 26 % and 33 % for DPBS and HM, respectively.
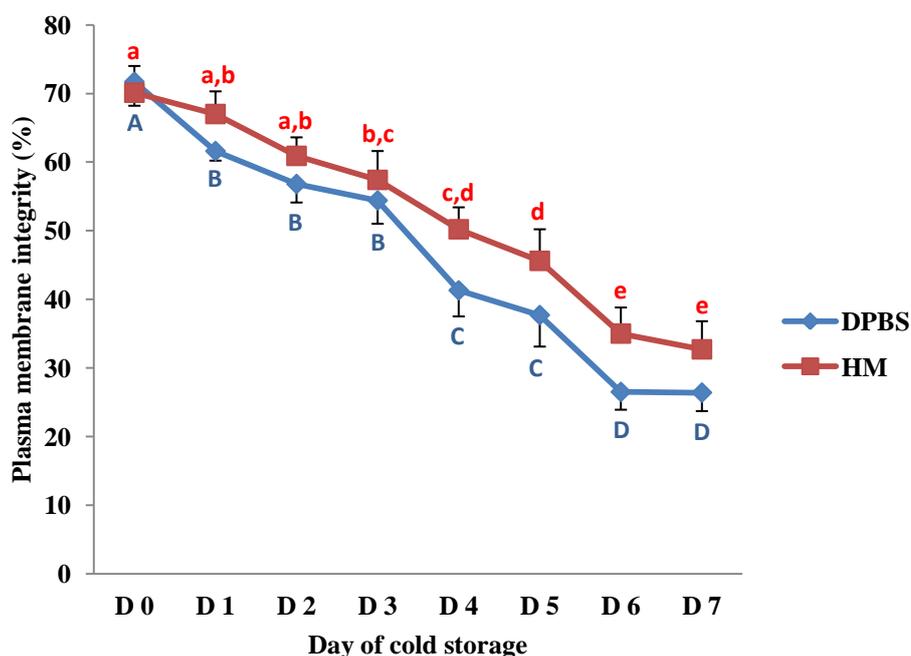
Figure 16 Percentages of testicular spermatozoa with intact plasma membrane during cold storage in DPBS or HM. Values are presented as Mean ± SEM (10 replicates). Different lowercase and uppercase letters denote values that differ significantly ($P < 0.05$) between days of preservation in DPBS and HM, respectively.

3.4.2 Experiment II: Effect of BSA concentration on membrane integrity of extracted testicular spermatozoa after cold storage

The percentages of testicular spermatozoa with intact plasma membrane decreased with storage time, irrespective of BSA concentrations (**Table 2**), though this proportion in HM with 4 mg/ml BSA decreased faster than those of 8 and 16 mg/ml BSA. Overall, membrane integrity of cold stored spermatozoa in HM containing 4 mg/ml BSA was less than other two groups, but it did not show significant difference among groups during the first 3 days ($P > 0.05$). After 1 week, medium containing 16 mg/ml BSA maintained the highest proportion of membrane integrity (47.8 %). However, sperm membrane integrity was not different between 8 and 16 mg/ml BSA groups.

Table 2

The percentage of testicular spermatozoa with intact plasma membrane (Mean ± SEM) during cold storage in HM supplemented with different concentrations of BSA.

Day of cold storage	Testicular spermatozoa with intact plasma membrane (%) in HM with		
	4 mg/ml BSA	8 mg/ml BSA	16 mg/ml BSA
D 0	67.2 ± 2.0^{a}	67.4 ± 2.9^{a}	69.9 ± 2.4^{a}
D 1	$62.2 \pm 1.9^{a,b}$	$63.9 \pm 2.6^{a,b}$	$64.3 \pm 2.5^{a,b}$
D 2	$57.4 \pm 3.1^{b,c}$	$61.9 \pm 2.3^{a,b}$	$60.5 \pm 2.9^{b,c}$
D 3	$51.9 \pm 2.2^{c,d}$	$59.8 \pm 2.1^{a,b,c}$	$59.2 \pm 2.0^{b,c}$
D 4	$46.9 \pm 3.5^{d,A}$	$56.8 \pm 3.2^{b,c,d,B}$	$58.6 \pm 2.1^{b,c,B}$
D 5	$38.1 \pm 2.8^{e,A}$	$53.2 \pm 3.5^{c,d,e,B}$	$58.0 \pm 2.7^{b,c,B}$
D 6	$35.1 \pm 3.7^{e,A}$	$48.2 \pm 3.8^{d,e,B}$	$54.1 \pm 1.9^{c,d,B}$
D 7	$32.8 \pm 4.7^{e,A*}$	$45.8 \pm 3.7^{e,A*,B}$	$47.8 \pm 4.5^{d,B}$

Within a column, different lower case letters denote significantly difference between the duration of cold storage.

Within a row, different upper case letters denote significantly difference between BSA concentrations ($P < 0.05$).

*= the values tended to be difference ($P = 0.0643$).

3.4.3 Experiment III: Assessment of sperm DNA integrity after 7-day cold storage and embryo development after ICSI

The percentage of fresh spermatozoa with DNA fragmentation was approximately 0.8 ± 0.2 %. This proportion increased after cold storage but did not significantly differ ($P > 0.05$) during 5-day of storage, although it significantly increased to 6.21 ± 1.6 after 7 day cold storage ($P < 0.05$; **Figure 17**). ICSI with 7 days cold stored testicular spermatozoa yielded cleavage, morula and blastocyst percentages similar to fresh control ($P > 0.05$, **Table 3**). An average blastomere number of blastocysts were comparable ($P > 0.05$) between the two groups. A small number of sham injections (4 of 82: 4.9 %) were cleaved, and 25 % of the cleaved embryos reached to morula stage. However, they did not develop further to blastocyst.
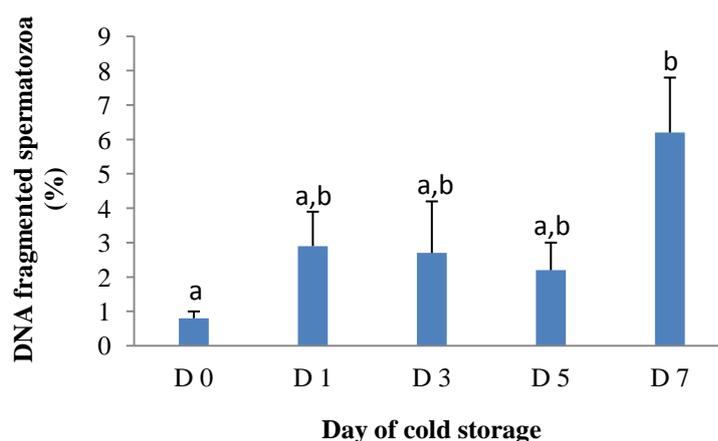
Figure 17 The percentages of spermatozoa with DNA fragmentation after cold storage in HM containing 16 mg/ml BSA for 0, 1, 3, 5 and 7 days. Values are presented as Mean $\pm$ SEM. During the storage time, different superscripts denote values that differ significantly ($P < 0.05$).

Table 3

Developmental competence of feline embryos produced by ICSI with testicular
spermatozoa cold-stored in HM containing 16 mg/ml BSA for 7 days

| Group | No.* | Rep | stage of development | | | Blast. cell no. |
			Cleavage n (%)	Morula n (%)	Blastocyst n (%)	(Mean ± SEM)
Fresh control	155	6	60 (38.7)[a]	26 (16.8)[a]	14 (9.0)	128.2 ± 23.5
7-day storage	206	6	92 (44.7)[a]	28 (13.6)[a]	15 (7.3)	116.1 ± 16.8
Sham	82	3	4 (4.9)[b]	1 (1.2)[b]	0	0

[a, b] Within a column, different superscripts denote values that differ significantly ($P < 0.05$).

No.*= total number of injected oocytes

The large proportions of non-cleaved oocytes/embryos (Day 2 of culture) were arrested at metaphase II (MII), though the ICSI with refrigerated spermatozoa (83.3 %) had significantly higher rate of intact sperm head than that of fresh control (62.1 %, $P < 0.05$). In addition, the number of MII oocytes with decondensing sperm head in cold stored group was lower than fresh control group ($P < 0.05$). However, degenerate oocytes or MII oocytes with degenerate spermatozoa, two or three pronuclei and premature chromosome condensation (PCC) could also be observed (**Table 4**).

Table 4

The nuclear status of noncleaved oocytes (2-day culture) after ICSI

Group	No	MII with ISH, n (%)	MII with DSH, n (%)	Degenerated oocytes/spermatozoa, n (%)	PCC, n (%)	Two PN, n (%)	Three PN, n (%)
Fresh control	95	59 (62.1)[a]	24 (25.3)[a]	7 (7.4)	1 (1.0)	3 (3.2)	1 (1.0)
7-day storage	114	95 (83.3)[b]	6 (5.3)[b]	6 (5.3)	3 (2.6)	4 (3.5)	0
Sham	78	78 (100)[c]	0	0	0	0	0

Within a column, different superscript letters denote values that differ significantly (P < 0.05).

Abbreviations: DSH = decondensing sperm head, FT = frozen-thawed, ICSI = intracytoplasmic sperm injection, ISH = intact sperm head,

MII = metaphase II, PCC = premature chromosome condensation, PN = pronucleus/pronuclei.

3.5 Discussion

Our study demonstrated that isolated testicular spermatozoa could be stored up to 7 days without freezing. Membrane integrity was improved when the spermatozoa were maintained in more complex culture medium supplemented with bovine serum albumin (BSA). Furthermore, the testicular spermatozoa also retained their fertilizing ability after cold storage for 7 days similar to that of fresh testicular spermatozoa.

In this study, we examined sperm plasma membrane integrity during cold storage because impaired plasma membrane may prolong direct contact between sperm chromatin and surrounding medium, resulted in DNA damage (Tateno et al., 2000). This DNA damage could negatively affect to developmental competence of ICSI derived embryos and also ICSI outcomes as previous reports in humans (Nasr-Esfahani et al., 2005; Avendano et al., 2010; Jiang et al., 2011). Comparing to our previous data (Buarpung et al., 2012), plasma membrane integrity of testicular spermatozoa seemed to be better preserved within intact testes than suspension observed in this study. It is possible that the testicular parenchyma could protect the spermatozoa from rapid cooling rate, and testicular cells may retain their functions to support the viability of testicular spermatozoa during preservation. This study also found that types of medium also influenced on the sperm quality during cold storage. HM provided a higher protective effect on membrane integrity of testicular spermatozoa throughout the preservation period than that of DPBS. We presumed that more complex medium could heighten viability of spermatozoa stored without surrounding testicular tissues. Although the study in mouse revealed that HEPES did not improve sperm survival (Sato and Ishikawa, 2004), spermatozoa stored in HM might acquire energy supply from pyruvate which is a substrate for adenosine triphosphate (ATP) production within the spermatozoa (Hereng et al., 2011). In addition, antioxidant properties of pyruvate might protect plasma membrane from oxidative stress injury during cold storage.

Bovine serum albumin (BSA), a multifunctional protein, is routinely used as an additive in culture medium to support oocyte maturation (Wood et al., 1995), fertilization (Eckert and Niemann, 1995) and embryo development (Russler-Long et al., 1991; Varga

et al., 2011). Our study demonstrated that increase of BSA concentration from 4 to 16 mg/ml significantly improved membrane integrity of testicular spermatozoa. The protective properties of BSA on sperm plasma membrane were also reported in canine (Risopatron et al., 2002), mouse (Van Thuan et al., 2005) and bull (Nang et al., 2012). It is possible that BSA may absorb into the sperm plasma membrane thereby increasing the stability of membrane structure (Blank et al., 1976). BSA also acts as an antioxidant to protect spermatozoa from destruction by free radicals produced by oxidative stress during preservation (Uysal and Bucak, 2007). By contrast, beneficial effect of BSA in protecting spermatozoa against cold shock and sperm membrane integrity was not observed in ram (Colás et al., 2009) and extremely high BSA concentrations could negatively affect to bovine spermatozoa from osmotic stress (Nang et al., 2012; Osman et al., 2012). It therefore seems likely that the different results may reflect that cat spermatozoa may be less susceptible to osmotic shock (Pukazhenthi et al., 2000) and the specific requirements of proteins in maintaining sperm membrane integrity and function among species studied and also types of spermatozoa (*i.e* testicular vs. epididymal vs. ejaculated spermatozoa).

In addition to the membrane integrity, our study also found that the percentage of DNA fragmented spermatozoa significantly increased after 7-day cold storage. However, the 7-day cold storage did not impair fertilizing ability of spermatozoa after ICSI similar to that of fresh testicular spermatozoa, though further investigation of these ICSI embryos on in *in vivo* development remains to be studied. Most of non-fertilized oocytes after ICSI were arrested at MII stage with an intact sperm head in the ooplasm similar to previous studies (Pope et al., 1998; Bogliolo et al., 2001; Comizzoli et al., 2006a; Buarpung et al., 2012). Cold storage of testicular spermatozoa had a negative effect on sperm head decondensation (**Table 4**) following ICSI. It is possible that cold storage of spermatozoa may adversely affect to the pathways associated with the mechanisms of sperm head decondensation.

3.6 Conclusions

Spermatozoa could be isolated from testicular tissues before cold storage in simple medium up to 7 days. Plasma membrane integrity of cold stored spermatozoa could be improved by BSA supplementation. Although DNA integrity of testicular spermatozoa stored in HM supplemented with 16 mg/ml BSA significantly declined after cold stored for 7 days, these preserved spermatozoa could fertilize cat oocytes by ICSI resulted in comparable *in vitro* fertilization to fresh testicular spermatozoa. This study provided the basic knowledge which can be applied for rare species conservation. However, the embryo transfer for *in vivo* development is required to determine the efficiency of this technique and the specific development of this technique was necessary for different species.

CHAPTER IV

FELINE SPERMATOZOA FROM FRESH AND CRYOPRESERVED TESTICULAR TISSUES HAVE COMPARABLE ABILITY TO FERTILIZE MATURED OOCYTES AND SUSTAIN THE EMBRYO DEVELOPMENT AFTER INTRACYTOPLASMIC SPERM INJECTION

4.1 Abstract

Cryopreservation of testicular tissue associated with intracytoplasmic sperm injection (ICSI) is a critical tool that still needs to be explored for preserving the fertility of endangered species. Using the domestic cat as a model for wild felids, the study aimed at determining the effect of different cryoprotectants and freezing techniques (two-step freezing vs. controlled slow freezing) on the sperm quality (membrane and DNA integrity). Then, spermatozoa were extracted from frozen-thawed testicular tissues and used for ICSI to assess early gamete activation or developmental competence in vitro. The percentage of spermatozoa with intact plasma membrane was not different (P > 0.05) among nonfrozen control, glycerol-, and ethylene glycol-frozen tissues (63.2 ± 2 %, 58.2 ± 2.6 %, 53.3 ± 2.3 %, respectively). However, these percentages were significantly lower (P < 0.05) in groups of dimethyl sulfoxide (46.3 ± 3.3 %) and 1,2 propanediol (44.3 ± 2.9 %) when compared with control. Two-step freezing combined with 5 % (v/v) glycerol best preserved sperm membrane integrity (55.0 ± 2.7 %) when compared with other freezing techniques. The incidence of DNA fragmentation was found to be low (0.2 %-1.1 %) in all freezing protocols. After ICSI with frozen testicular spermatozoa, male and female gametes were asynchronously activated and the percentages of normal fertilization at 6, 12, and 18 h were 11.2 %, 20.6 %, and 22.1 %, respectively. Metaphase II-arrested oocytes containing or not a decondensed sperm head were predominantly found after ICSI with frozen testicular spermatozoa. Although two-pronucleus formation could be observed as soon as 6 h post ICSI (10 %), the rate increased dramatically after 12 and 18 h post ICSI (17.2 % and 19.5 %, respectively).

ICSI using frozen-thawed testicular spermatozoa yielded cleavage (32.7 %), morula (6.5 %), and blastocyst (4.4 %) percentages similar to nonfrozen control (P > 0.05). It is concluded that two-step freezing technique with glycerol as a principle cryoprotectant is simplified and applicable for cat testicular tissue cryopreservation.We also demonstrate for the first time that feline spermatozoa derived from frozen-thawed testicular tissues retain their fertilizing ability and can be used to produce ICSI-derived embryos.

4.2 Introduction

Most wild felid species are currently classified as threatened or endangered species, because their population rapidly declines primarily because of many threatening factors including illegal hunting, loss of habitats, and inbreeding-related problems, such as immunodepletion-related illness and teratospermic-induced infertility (Wildt et al., 2010). To propagate and preserve the genetic potential of these wild felids, gamete preservation techniques associated with assisted reproductive technologies such as artificial insemination, in vitro fertilization, and intracytoplasmic sperm injection (ICSI) have been developed in domestic and in wild cats (Wildt et al., 2010). Although ejaculated spermatozoa collected by electrostimulation are normally used for assisted reproductive technologies in domestic and wild cats (Zambelli and Cunto, 2006), the use of these spermatozoa is not applicable for valuable animals that would unexpectedly die. Therefore, only spermatozoa from epididymis and testicular tissue recovered from sudden death animals are meaningfully used for embryo production via *in vitro* fertilization technique (Bogliolo et al., 2001; Comizzoli et al., 2006a,b; Cocchia et al., 2010). In addition, these sperm sources also increase the number of spermatozoa that can be collected from a given individual.

In vitro embryo production using cryopreserved-thawed testicular spermatozoa has been successfully performed via ICSI in human (Gil-Salom et al., 1996; Hovatta et al., 1996; Wu et al., 2005) and mouse (Ohta et al., 2008). Though successful application of these techniques, in terms of embryo production and live offspring has not been established in domestic and endangered wild felids, we hypothesized that frozen feline testicular spermatozoa were probable to maintain ability to activate oocytes similar to

other species. However, the ICSI outcome was affected by sperm qualities. Because spermatozoa obtained from testicular tissue are not fully matured and most of them usually show only a slowly twitching movement or are completely immotile, sperm motility is therefore not to be used to assess the postthaw quality of testicular spermatozoa. Although plasma membrane integrity of spermatozoa used for ICSI is not essential for fertilization and embryo development (Wakayama et al 1998), prolonged exposure of plasma membrane-disrupted spermatozoa to unfavorable environment appeared to increase the incidence of sperm DNA damage Tateno et al., 2000). The damaged DNA impairs ICSI outcomes in terms of fertilization and embryo developmental rate (Yildiz et al., 2007). Suboptimal freezing and thawing potentially damage plasma membrane and DNA integrity of spermatozoa (Byer et al 1989; Hammadeh et al., 1999, 2001; Jezek et al., 2001; Axnér et al., 2004; Yildiz et al., 2007; Cocchia et al., 2010; Kim et al., 2010). To improve sperm quality after freezing and thawing, several techniques have been applied. Testicular spermatozoa can be frozen as cell suspension, or as a piece of testicular tissue (Allan and Cotman, 1997; Khalifeh et al., 1997; Crabbé et al., 1999; Hovatta, 2000) using different freezing regimens. Several cryoprotectants (CPAs) have been used to protect testicular tissue against cryoinjury including penetrating and nonpenetrating CPAs such as sucrose, trehalose, glycerol, ethylene glycol (EG), 1, 2-propanediol (PrOH), and dimethyl sulphoxide (DMSO) (Gil-solom et al., 1996; Jezek etal 2001; Keros et al 2005). Nevertheless, the efficacy of these CPAs depends on several factors such as CPA concentration, cooling rate, and also species studied. For example, though DMSO has been demonstrated to be a promising CPA for preserving human testicular tissue (Keros et al., 2007; Wyns et al., 2007, 2008), this CPA was inferior to protect porcine testicular cells when compared with glycerol and EG (Abrishami et al., 2010). Until recently, data on types of CPA and also freezing techniques that affect postthaw testicular sperm quality including the fertilizing ability in terms of successful embryo production via ICSI in domestic cats has been limited. Moreover, the efficient and simplified freezing technique for cat testicular tissue cryopreservation is still required.

The objective of this study was to determine (1) the effects of CPAs and freezing protocols on testicular sperm plasma membrane and DNA integrity, and (2) the fertilizing ability (first cell cycle and early embryo development) after ICSI.

4.3 Materials and Methods

4.3.1 Chemicals

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

4.3.2 Collection of testes

Testes were obtained from adult cats submitted for castration at the Fertility and Neutering Clinic, Small Animal Teaching Hospital, the Faculty of Veterinary Science, Chulalongkorn University, and The Veterinary Public Health Division of The Bangkok Metropolitan Administration, Bangkok, Thailand. After castration, testes were maintained in 0.9 % (w/v) saline (NaCl) solution and transported to the laboratory within 3 h at ambient temperature. Testicular tissues were then prepared at room temperature (approximately 25 °C) before freezing procedures. The extraneous tissues were removed from these testes, later they were washed once in normal saline solution supplemented with antibiotics (100 IU/ml penicillin and 100 mg/ml streptomycin) and dried with clean gauze. Only the testes that weighed between 1 and 2 g were used in this study.

4.3.3 Cryopreservation

The freezing extender including extender1 (EXI) and extender2 (EXII) were prepared according to Axnér et al. (2004). The EXI and EXII contained 3 % (v/v) and 7 % (v/v) CPA (glycerol, EG, PrOH, or DMSO), respectively.

Testicular tissue from individual testis was cut into 20 equal portions (approximately 2 x 3 x 5 mm in size; **Figures 18A, B**). They were equilibrated in EXI at 5 °C for 1 h before adding an equal volume of EXII. The equilibrated testicular tissues were subsequently loaded into the 0.5 ml polyvinyl straws (5 pieces per straw; **Figure 18C**). For two-step freezing, the straws containing testicular tissues were horizontally

placed on a rack, 4 cm above liquid nitrogen surface, for 10 min before plunging into liquid nitrogen for long-term storage. Controlled-rate slow freezing was performed using a programmable CL863 freezer (Cryologic PL, Victoria, Australia). The freezing rate used in this study was performed as previously described by Keros et al. (2007) with minor modifications. The initial temperature was set at 4 °C. After holding the straw at this temperature for 5 min, the temperature was then slowly reduced at a rate of 0.5 °C/min to -8 °C. The straws were held at this temperature for 5 min before seeding by touching the wall of the freezing straw with forceps precooled in liquid nitrogen. The temperature was subsequently reduced from -8 °C to -40 °C at a rate of 0.5 °C/min and then to -70 °C at a rate of 6 °C/min. The straws were finally plunged into liquid nitrogen and stored in liquid nitrogen until analysis
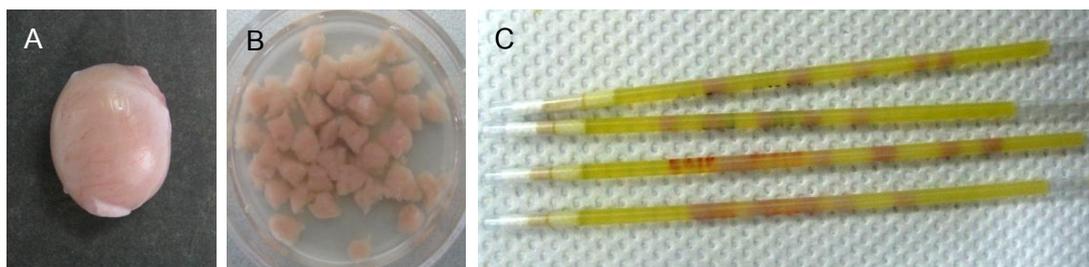
Figure 18 The photographs represent the preparation of testicular tissues for cryopreservation. Each individual testis (A) was cut into equal small pieces (B). After equilibration, testicular tissues were loaded into the straws (C) and then cryopreserved.

4.3.4 Thawing of cryopreserved testicular tissues

The straws containing testicular tissue were thawed in air for 10 seconds, followed by immersion in warm water (37 °C) for 30 seconds. The testicular tissues was then released into a thawing medium (Axnér et al., 2004) and incubated at 37 °C for 5 min before sperm extraction.

4.3.5 Testicular sperm extraction

Testicular tissues were washed and mechanically minced with sharp-ended scissors in a holding medium consisting of HEPES-buffered M199 (M199), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin and 100 µg/ml streptomycin and 0.4 % (w/v) bovine serum albumin (BSA, embryo tested grade). Spermatozoa were

further extracted from minced tissue by repeat pipetting. The sperm suspension was finally filtered through a 40 µm cell strainer (BD-Falcon, Franklin Lakes, NJ, USA).

4.3.6 Assessment of sperm plasma membrane integrity

The Live-Dead sperm Viability Kit (Molecular Probes, Eugene, OR, USA) was used for assessment of sperm plasma membrane integrity. A working solution of SYBR-14 and propidium iodide (PI) was prepared as previously described (Garner and Johnson 1995). In brief, the sperm suspension was incubated with 400 nM SYBR-14 and 20 µM PI at 37 °C for 20 min. An aliquot of 2 µL sperm sample was placed on a prewarmed glass slide covered with cover slip and then examined under a fluorescent microscope (BX51; Olympus, Shinjuku, Japan) at magnification x 1000. The nuclei of live spermatozoa were stained by SYBR-14 and exhibited green fluorescence and PI positive spermatozoa (red fluorescence) indicated the spermatozoa with disrupted plasma membrane.

4.3.7 Assessment of sperm DNA integrity

Detection of DNA fragmentation was performed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling assay (In Situ Cell Death Detection Kit; Roche, Mannheim, Germany) as described in Chapter I.

4.3.8 Oocyte collection and *in vitro* maturation

Cumulus oocyte complexes were recovered after mincing cat ovaries in holding medium. Only oocytes completely surrounded with more than five layers of compacted cumulus cells and containing homogeneous dark ooplasm were used. *In vitro* maturation was performed as previously described by Sanuanmuang et al. (2010).

4.3.9 Sperm preparation and intracytoplasmic sperm injection (ICSI)

Testicular tissues were washed twice and then extracted as previously described in HEPES buffered synthetic oviductal fluid (HEPES-SOF) as previously described in Chapter I. The extracted spermatozoa were then mixed with 10 % (w/v) polyvinylpyrrolidone (MediCult a/s, Jyllinge, Denmark) to aid fluid flow and also to prevent attachment of the spermatozoa to the injecting pipette. Intracytoplasmic sperm

injection was performed using an inverted microscope (IX 70, Olympus, Tokyo, Japan) equipped with a Narishige micromanipulator (Model No. MMO-202D; Narishige Instrument Co., Tokyo, Japan) and a heat stage at 37 °C. Injecting pipettes (30° beveled angle) were 5 to 6 μm and 10 μm for inner and outer diameters, respectively (Humagen Fertility Diagnostics, Inc., Charlottesville, VA, USA). Holding pipettes were 15 to 20 μm and 120 to 150 μm for inner and outer diameters with a fire-polished tip. A morphologically normal immotile spermatozoon was blind-selected for ICSI because the viability (plasma membrane integrity) could not be determined before ICSI. The oocytes were held by negative pressure with the polar body oriented at the six or twelve o'clock position. The injection was performed by advancement the ICSI needle through the zona pellucida at the three o'clock position. After a minimal amount of ooplasm had been aspirated into the injecting pipette, a spermatozoon was then gently released into the ooplasm. All ICSI oocytes were washed and cultured at 38.5 °C under mineral oil in 50 μl droplets of synthetic oviductal fluid (SOF) containing with 4 mg/ml BSA and 100 IU/ml penicillin (IVC-1 medium) in a humidified condition of 5 % CO_2 in air.

4.3.10 Assessment of gamete activation after ICSI

Gamete activation was examined at 6, 12 and 18 h after ICSI. All presumptive zygotes were treated for 45 min at 37 °C with a glycerol-based microtubule stabilizing solution containing 25 % (v/v) glycerol, 50 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM ethylene glycol tetraacetic acid, 1 mM 2-mercaptoethanol, 50 mM imidazol, and 4 % Triton-X-100 (Simerly and Schatten, 1993) before fixation in 4 % (w/v) paraformaldehyde. To label microtubule, the presumptive zygotes were first incubated with a 1:100 solution of a monoclonal anti-α-tubulin antibody (clone B1-5-1-2) and then with a 1:100 solution of a goat anti-mouse secondary antibody conjugated to tetramethylrhodamine isothiocyanate (TRITC). The presumptive zygotes were subsequently labeled with 4',6-diamidino-2-phenylindole (DAPI; 0.1μg/ml) for chromatin configurations. The examination of gamete activation was performed using an epifluorescent microscope (BX51 Olympus, Shinjuku, Japan). Two fluorescent wave lengths obtained from 408 nm and 543 nm filters were used to simultaneously excite the fluorescent signals for DAPI

(DNA) and TRITC (microtubules), respectively. The resulting double-color micrographs were subsequently examined using Adobe Photoshop CS (Adobe System Inc., Mountain View, CA, USA).

4.3.11 Embryo culture

After ICSI, only cleaved embryos on day 2 were washed and cultured in IVC-2 medium (synthetic oviductal fluid containing 10 % v/v fetal bovine serum; JR Scientific). The medium was changed every 2 days. The percentages of cleavage, morula and blastocyst (related to the number of injected oocytes) were observed on day 2, 5 and 7 after ICSI, respectively. All embryos fixed in 4 % paraformaldehyde were stained with DAPI and examined under an epifluorescent microscope to determine the cell numbers of blastocyst.

4.3.12 Experimental design

Experiment I: Effect of CPAs on sperm viability and DNA integrity after freezing and thawing

A total of 10 testes (10 replicates) were used in this study. Testicular tissues from each testis were cut into 20 equal small pieces and frozen by a two-step freezing with a total concentration of 5 % (v/v) CPAs (five tissue pieces per treatment). Four CPAs including glycerol, DMSO, PrOH, and EG were tested in this study. After thawing, spermatozoa were extracted and evaluated for plasma membrane and DNA integrity. Noncryopreserved testicular tissue from each testis (N = 10) served as a control group.

Experiment II: Effect of freezing techniques on sperm plasma membrane and DNA integrity

A total of 10 testes (10 replicates) were used in this study. Each testis was cut into 10 equal portions and cryopreserved by either 2-step freezing or controlled-rate slow freezing methods. The CPA to be used in this study was the best CPA according to the result of experiment 1. Sperm quality was assessed as previously described in experiment 1. Noncryopreserved testicular spermatozoa were used as a control.

Experiment III: Gamete activation after ICSI with spermatozoa recovered from frozen-thawed testicular tissues

After sperm extraction from frozen-thawed testicular tissue, the spermatozoa were injected into *in vitro* matured oocytes. A total of 244 ICSI-oocytes were cultured for 6, 12 and 18 h to examine gamete activation after ICSI. The oocyte activation was not performed in this study to avoid the probable parthenogenetic activation of the ICSI-oocytes. Spermatozoa used in this study were harvested from testicular tissues cryopreserved by the best CPA and freezing technique, according to the results of experiment 1 and 2. Male gamete activation was indicated by decondensation of sperm head with or without sperm aster formation and male pronuclear formation. Female gamete activation typified by oocytes demonstrating the female pronucleus with an extrusion of the second polar body.

Experiment IV: *In vitro* embryo development after ICSI with spermatozoa recovered from frozen-thawed testicular tissues

A total of 294 metaphase II (MII) oocytes were used for ICSI with spermatozoa from testicular tissues, cryopreserved by a technique as described in the previous experiment. The number of cleaved embryos was determined on Day 2 after ICSI. The developmental competence of ICSI oocytes was assessed, in terms of cleavage, morula and blastocyst formation percentages at Days 2, 5 and 7 of *in vitro* embryo culture (Day 0 = ICSI), respectively. Intracytoplasmic sperm injection using nonfrozen testicular spermatozoa (N= 363) and sham ICSI (injection without spermatozoon, N = 157) served as controls. The embryos with 30 to less than 50 cells were categorized as morulae, and the embryos demonstrating blastocoelic cavity with 50 cells or more were classified as blastocysts.

4.3.13 Statistical analysis

The statistical analysis was performed using the Statistical Analysis Systems software package (Version 9.0, SAS Institute Inc., Carey, NC, USA). The statistical differences of sperm plasma membrane and DNA integrity among the experimental groups were evaluated by one-way ANOVA statistical test and DUNCAN analysis. The

Chi-square test was used to compare the percentages of gamete activation and embryo development. The total cell numbers of blastocyst were analyzed by one-way ANOVA. In all cases, differences were considered significant when P < 0.05.

4.4 Results

4.4.1 Experiment I: Effect of CPAs on sperm viability and DNA integrity after freezing and thawing

Cryoprotectants used for freezing feline testicular tissue differentially influenced on the sperm plasma membrane integrity. Glycerol and ethylene glycol preserved the plasma membrane integrity of testicular spermatozoa similar to nonfrozen control (P > 0.05; **Table 5**). In this study, the properties of DMSO and PrOH to preserve the sperm plasma membrane integrity during cryopreservation was significantly poorer than glycerol (P < 0.05, **Table 5**). In contrast to the effect of CPA on sperm plasma membrane integrity, cryopreservation did not have an impact (P > 0.05) on DNA integrity of testicular spermatozoa (**Table 5**). The percentages of testicular spermatozoa with fragmented DNA in frozen groups and nonfrozen control were similar (P > 0.05, **Table 5**), irrespective the types of CPA used.

Table 5 Percentages (Mean ± SEM) of spermatozoa with intact plasma membrane and fragmented DNA after testicular tissue cryopreservation with different cryoprotectants

Type of cryoprotectant	Intact sperm plasma membrane (%)	Fragmented DNA (%)
Nonfrozen control	63.2 ± 2.0[a]	0.9 ± 0.5
Glycerol	58.2 ± 2.6[a]	0.7 ± 0.3
EG	53.3 ± 2.3[a,b]	0.5 ± 0.4
DMSO	46.3 ± 3.3[b]	1.1 ± 0.4
PrOH	44.3 ± 2.9[b]	0.3 ± 0.3

Within a column, different superscripts letters denote values that differ significantly (P < 0.05)

Abbreviations: DMSO = dimethyl sulphoxide, EG = ethylene glycol,

PrOH = 1, 2-propanediol.

4.4.2 Experiment II: Effect of freezing techniques on sperm plasma membrane and DNA integrity

Because of experiment 1 indicated that glycerol was the best CPA for testicular tissue cryopreservation, this study therefore examined whether controlled-rate cryopreservation would improve postthaw sperm quality over the two-step cryopreservation. Testicular tissue cryopreserved by controlled-rate slow freezing technique had significantly lower (P < 0.05) percentage of intact sperm plasma membrane than the control, and tended to be less than that of two-step freezing technique (P = 0.07, **Table 6**). However, the proportion of testicular spermatozoa with fragmented DNA was not different (P > 0.05) among the two freezing techniques and nonfrozen testicular tissue.

Table 6 Percentages (Mean ± SEM) of spermatozoa with intact plasma membrane and fragmented DNA after testicular tissue cryopreservation with glycerol by different freezing techniques

Freezing technique	Intact sperm plasma membrane (%)	Fragmented DNA (%)
Nonfrozen control	60.3 ± 0.9[a]	0.3 ± 0.1
Two-step freezing	55.0 ± 2.7[a, b]	0.6 ± 0.4
Controlled-rate freezing	45.9 ± 3.7[b]	0.2 ± 0.2

Within a column, different superscripts letters denote values that differ significantly (P < 0.05).

4.4.3 Experiment III: Gamete activation after ICSI with spermatozoa recovered from frozen-thawed testicular tissues

According to the results obtained from experiment 1 and 2, two-step freezing with glycerol was therefore used in this study. Overall, normal fertilized zygotes (with male and female gamete activation) were successfully produced by ICSI with frozen-thawed testicular spermatozoa. Male and female activation occurred in an asynchronous and independent manner. Though only few activated oocytes contained with intact sperm head (three of 87 at 12 h post injection [hpi]; **Table 7**, **Figure 19A**), a large number of

ICSI-oocytes arrested at MII stage irrespective the stages of male gamete activation (intact; **Figure 19B**, or decondensed sperm head; **Figure 19C**). The numbers of MII arrest with decondensed sperm head decreased significantly (P = 0.01) at 18 hpi when compared with 6 hpi. In addition, the total number of oocytes which were activated (data included normal fertilization and oocyte activation with intact sperm head; 11.2, 24 % and 22.1 % at 6, 12, 18 hpi, respectively; **Table 7**) was obviously lower than the number of sperm activation (data included normal fertilization and MII arrest with decondensed sperm head [or male pronucleus]; 50 %, 55.1 %, 44.2 % at 6, 12 and 18 hpi, respectively; **Table 7**) suggesting an insufficient sperm factor for stimulation of the second meiosis resumption. This might be in agreement with the finding that short sperm asters (Fig. 1D) were frequently found adjacent to a decondensed sperm head. Nevertheless, decondensed sperm head with absent sperm aster (**Figures 19C, E**) and asterification with incomplete sperm head decondensation (**Figure 19D**) could also be found. Two pronuclei formation (**Figure 19F**) could be observed as soon as 6 h post injection (10 %) and increased noticeably after 12 and 18 hpi (17.2 % and 19.5 %, respectively, P > 0.05), cleaved embryos were first appeared at 18 hpi. The incidence of abnormal fertilization such as ICSI-oocytes with multiple (more than two) pronuclei was found to be low (1 % - 2 %).
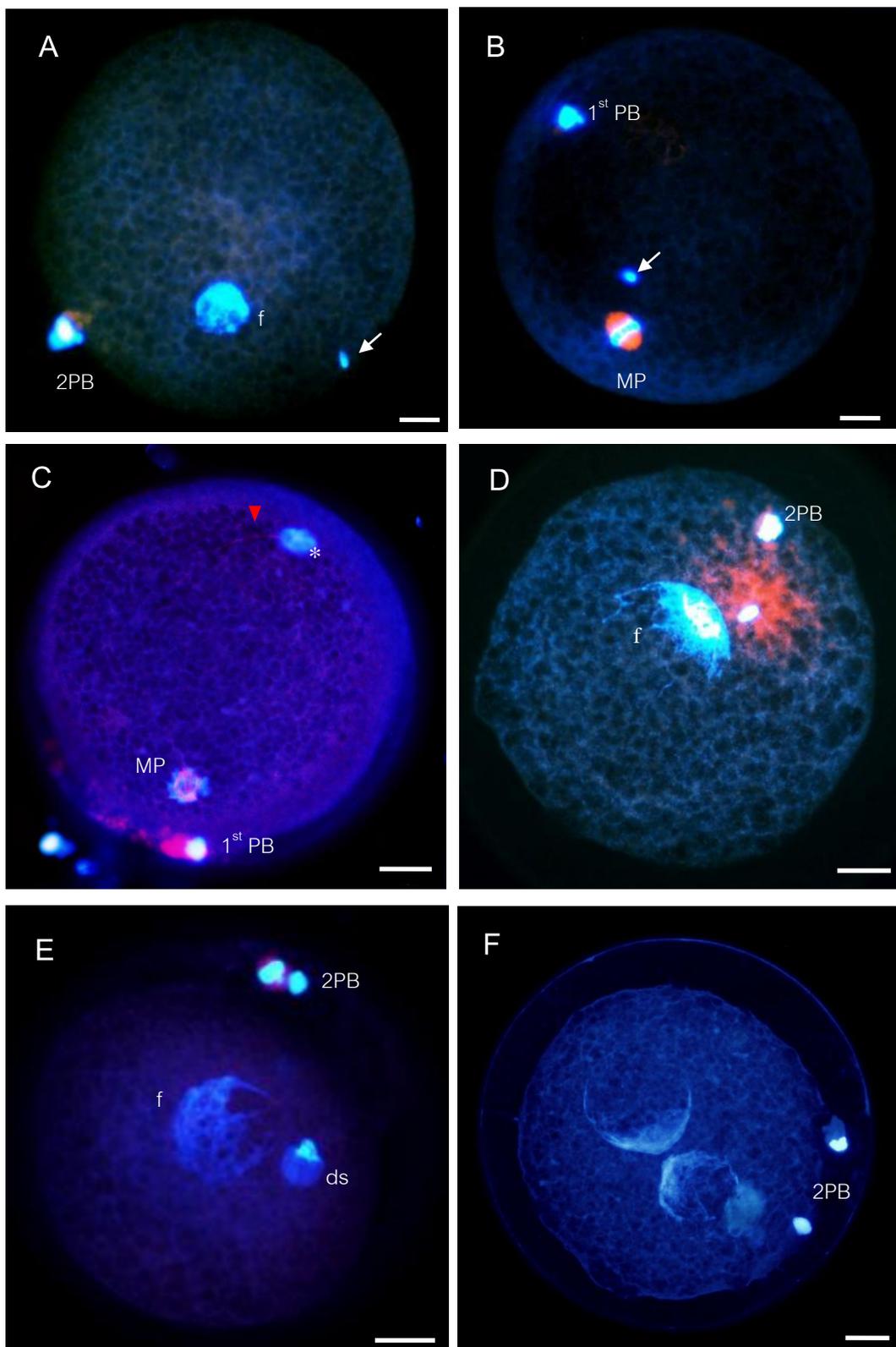
Figure 19 Assessment of gamete activation by chromatin and microtubule configurations at 6, 12 and 18 h after intracytoplasmic sperm injection. (A) Activated female gamete (indicated by 2PB and female pronucleus) with intact sperm head; (B)

nonactivated both male and female gametes indicated by the presence of metaphase plate and the first polar body (1stPB) of metaphase II arrested oocyte with an intact sperm head; (C) metaphase II arrested oocyte contained activated male gamete (indicated by decondensing sperm head) without sperm aster; (D-F) activated gametes; (D) female pronucleus with short asterification before complete decondensation of sperm head; (E) female pronucleus with decondensing sperm head (ds); (F) an oocyte with two pronuclei. Asterisk = decondensing sperm head, white arrow = intact sperm head; red arrowhead = a tail remnant, f = female pronucleus, MP = metaphase plate, PB = polar body, PN = pronucleus/pronuclei. (Bar = 20μm; Original magnification 20x-40x)

Table 7 Gamete activation profiles of oocytes/zygotes at 6, 12 and 18 h after ICSI with spermatozoa recovered from frozen-thawed testicular tissues.

Gamete status	Number (%)		
	6 hpi	12 hpi	18 hpi
Number of ICSI- oocytes	80	87	77
Normal fertilization (both gamete activation)			
Oocyte activation with decondensed sperm head	1 (1.2)	3 (3.4)	0 (0)
Male and female pronuclei formation	8 (10)	15 (17.2)	15 (19.5)
Cleavage	0 (0)	0 (0)	2 (2.6)
Oocyte activation with intact sperm head	0 (0)	3 (3.4)	0 (0)
MII arrest with intact sperm head	36 (45)	30 (34.5)	35 (45.4)
MII arrest with decondensed sperm head	31 (38.8)[a]	30 (34.5)[a,b]	17 (22.1)[b]
Unidentified and degenerated oocytes	2 (2.5)	5 (5.8)	6 (7.8)
Abnormal fertilization[c]	2 (2.5)	1 (1.2)	2 (2.6)

Within a row, different superscript letters denote values that differ significantly (P < 0.05)

Abbreviations: hpi = hour post injection, ICSI = intracytoplasmic sperm injection,

MII = metaphase II.

[c] Abnormal fertilization includes: one pronucleus with three polar bodies, three pronuclei with intact sperm head, or three spindles and two metaphase plates with one polar body.

4.4.4 Experiment IV: *In vitro* embryo development after ICSI with spermatozoa recovered from frozen-thawed testicular tissues

As shown in **Table 8**, the proportions of cleavage, morula and blastocyst in ICSI with fresh and frozen-thawed testicular spermatozoa were similar ($P > 0.05$). Only 30 % to 40 % of ICSI-oocytes were cleaved (**Figure 20A**), but only 14 % of these cleaved embryos developed to blastocysts (**Figure 20B**), irrespective the type of testicular spermatozoa. Additionally, blastocyst quality by means of average cell number did not significantly differ ($P > 0.05$) between blastocyst derived from frozen-thawed and nonfrozen testicular spermatozoa (202.8 ± 36.6 vs. 140.2 ± 14.2, respectively; **Figures 21A, B**). Some sham-injected oocytes (5.1 %) also underwent cleavage but they did not develop to morula and blastocyst stage.

Most of noncleaved oocytes were MII oocytes contained with an intact sperm head within ooplasm (**Table 9**). This evidence in frozen group was significantly higher than that of fresh control. However, the presence of MII oocytes with a decondensing sperm head, in noncleaved oocytes, was not different ($P > 0.05$) between frozen and fresh groups. The numbers of degenerated oocytes with or without fragmented spermatozoon in the frozen group were less than in the control group. The other nuclear statuses of noncleaved oocytes were oocytes that contained one pronucleus and two or three pronuclei; the incidence of one pronucleus was higher ($P < 0.05$) in the frozen group (7.6 %) compared with fresh control (1.4 %).
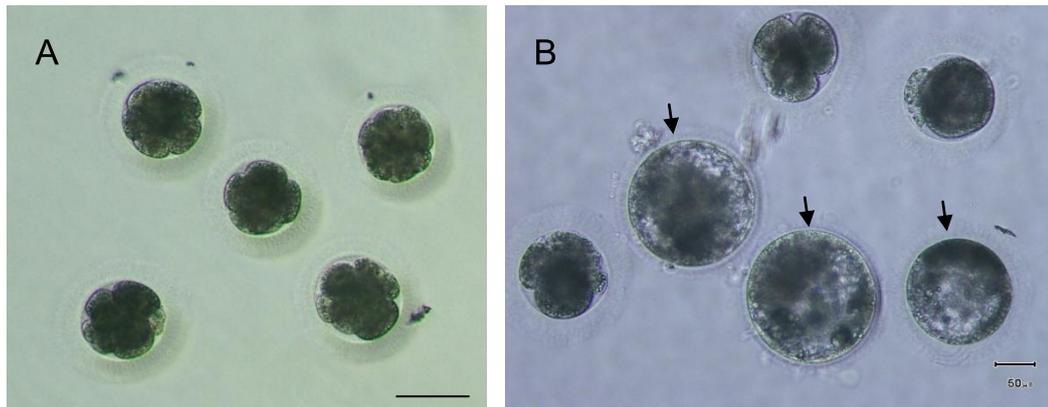
Figure 20 The light microscopic appearance of cat embryos produced by intracytoplasmic sperm injection (ICSI) with spermatozoa recovered from frozen-thawed testicular tissues: (A) cleaved embryos on Day 2 after ICSI. (B) blastocysts on Day 7 after ICSI (arrows). Scale bars, (A) 100 µm, and (B) 50 µm.
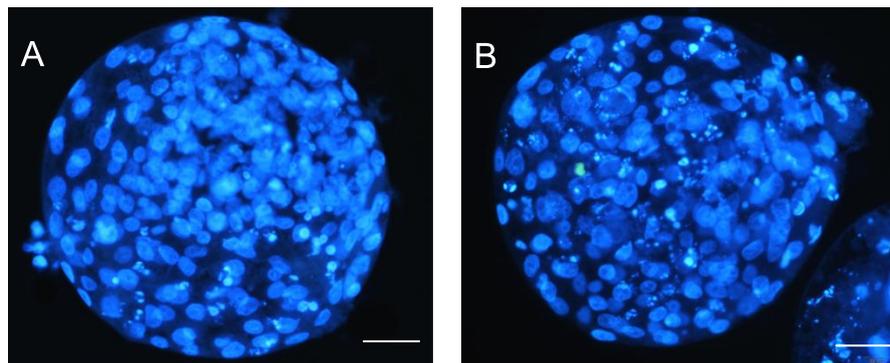
Figure 21 Cat blastocysts stained with 4', 6-diamidino-2-phenylindole (DAPI) were visualized under an epifluorescent microscope (magnification x 400). (A) Blastocyst derived from intracytoplasmic sperm injection with spermatozoa recovered from frozen-thawed testicular tissues. (B) Blastocyst derived from intracytoplasmic sperm injection using spermatozoa recovered from nonfrozen testicular tissues (control group). Scale bars represent 40 µm.

Table 8 Developmental competence of feline embryos after ICSI using spermatozoa recovered from cryopreserved testicular tissue.

Treatment	Number of ICSI-oocyte (replicate)	Stage of development				Blast cell, N (Mean ± SEM)
		Cleavage, N (%)	Morula, N (%)	Blastocyst, N (%)	Blastocyst/cleavage, N (%)	
Nonfrozen	363 (10)	144 (39.7)[a]	34 (9.4)	20 (5.5)	20 (13.9)	140.2 ± 14.2
Frozen-thawed	294 (8)	96 (32.7)[a]	19 (6.5)	13 (4.4)	13 (13.5)	202.8 ± 36.6
Sham	157 (4)	8 (5.1)[b]	0	0	0	0

Within a column, different superscript letters denote values that differ significantly (P < 0.05).

Abbreviation: ICSI = intracytoplasmic sperm injection.

Table 9 The nuclear status of noncleaved oocytes after ICSI and 2-day culture

Group	No.	MII+ISH, N (%)	MII+DSH, N (%)	Degenerated oocytes, N (%)	One PN, N (%)	Two PN, N (%)	Three PN, N (%)
FT	197	132 (67.2)[a]	32 (16.2)	11 (5.6)[a]	15 (7.6)[a]	3 (1.5)	2 (1.0)
Nonfrozen	211	120 (56.9)[b]	46 (21.8)	27 (12.8)[b]	3 (1.4)[b]	6 (2.8)	5 (2.4)

Within a column, different superscript letters denote values that differ significantly (P < 0.05).

Abbreviations: DSH = decondensing sperm head, FT = Frozen-thawed, ICSI = intracytoplasmic sperm injection, ISH = intact sperm head,

MII = metaphase II, PN = pronucleus/pronuclei.

4.5 Discussion

This study demonstrated that types of CPAs and freezing techniques for feline testicular tissues influenced the quality of testicular spermatozoa, in terms of sperm plasma membrane integrity. However, freezing and thawing of testicular spermatozoa, regardless of the techniques used, did not affect the DNA integrity. In addition, spermatozoa recovered from cryopreserved testicular tissue maintained their fertilizing ability similar to noncryopreserved spermatozoa.

The quality of testicular cells and spermatozoa can be affected by several factors during the freezing process, e.g., types of CPA (Keros et al., 2005; Jezek et al., 2001), freezing technique (Keros et al., 2007), and tissue size (Crabbé et al., 1999). We first determined the ability of different CPAs to protect the testicular spermatozoa against cryoinjury that occurs during testicular tissue cryopreservation. From our results, glycerol was found to be the best CPA for plasma membrane integrity of frozen-thawed testicular spermatozoa compared with other CPAs (including EG, PrOH and DMSO). It is therefore postulated that 1 h of equilibration of testicular tissue with glycerol as a sole membrane-permeable CPA coupled with their small size (2 x 3 x 5 mm) used in the current study was sufficient for protecting sperm plasma membrane. In contrast, other lower molecular weight CPAs appeared to permeate at a faster rate than glycerol, and prolonging exposure of particular CPAs may become toxic. However, the toxicity of particular CPAs can be substantially reduced by lowering the CPA concentration and equilibration time (Karlsson and Toner 1996).

This present study is the first to examine the difference between two-step and programmable freezing for testicular tissue cryopreservation. We observed that two-step freezing with glycerol (the method developed for semen freezing) was superior to the controlled slow freezing, although the sperm quality was not significantly different between the two freezing techniques. It is therefore suggesting that further study on the relationship among different CPAs, tissue size, equilibration time, cooling rate and freezing techniques, is required to optimize the viability and fertilizing ability of testicular spermatozoa during cryopreservation.

In the current study, the effects of different freezing regimes on cryoinjury were markedly found only at the level of sperm plasma membrane rather than sperm DNA (**Table 5 and 6**). However, though it is rather difficult to correlate the sperm plasma membrane integrity with the ICSI outcome, disruption of sperm plasma membrane appears to increase the risk of sperm DNA damage (Tateno et al., 2000) that, in turn, affects the embryo development (Seli et al., 2004, Yildiz et al., 2007) and pregnancy rate (Benchaib et al., 2003). In fact, though only less than 1 % of frozen-thawed testicular sperm demonstrated the DNA fragmentation as similar to other reports (Steele et al., 1999, 2000; Kadirvel et al., 2012), this freezing and thawing potentially increase the susceptibility of DNA to damage during sperm preparation for ICSI (Meseguer et al., 2011).

We demonstrated for the first time that testicular spermatozoa recovered from frozen-thawed testicular tissue retained their fertilizing ability after ICSI in terms of gamete activation and also pronuclear formation. We found that spermatozoa underwent gamete activation before oocytes because the proportion of activated spermatozoa was higher than the numbers of activated oocytes when examined at 6 h post ICSI. Our observation supported the earlier study stating that beginning of oocyte activation correlated with decondensation of sperm chromatin (Bourgain et al., 1998). These results confirmed the fact that the sperm-born oocyte activating factor, phospholipase C-zeta (a calcium oscillation stimulator), released from the entered spermatozoa is necessary for oocyte activation (Tesarik et al., 1994; Malcuit et al., 2006; Jones, 2005). Furthermore, our data agree with previous reports that male and female pronucleus formation occurred in an asynchronous manner (Flaherty et al 1995b; Gómez et al., 1998b; Bourgain et al., 1998). The finding that male and female pronuclear formation occurred by 6 h after ICSI using cryopreserved testicular spermatozoa was similar to the results obtained from ICSI with cat ejaculated spermatozoa (Jin et al., 2012). Interestingly, the number of sperm activation as indicated by sperm decondensation and pronuclear formation was high at 6, 12 and 18 h after ICSI; this might be responsible for the finding that the strength of sperm's disulphide bonds was diminished by osmotic stress during freezing and thawing (Flores et al., 2011). By contrast, the

transition between the first observed pronuclear and cleavage stages appeared to be delayed since cleavage was observed only at 18 h post ICSI. Although the reason of this remains unclear, the immaturity of testicular sperm centrosome, expressed by the distinctly short microtubular aster formation, can delay the cleavage and also slow the developmental rate (Comizzoli et al., 2006b). Furthermore, ICSI failure in this study was mostly found to associate with MII arrest (**Tables 7, 9**) as similar to other reports (Sousa and Tesarik, 1994; Flaherty et al 1995a). However, many of these MII arrest oocytes contained decondensed sperm head. Although it is difficult to underpin the exact cause of this MII arrest, diminished sperm factor (e.g. PLCζ concentrations) caused by freezing and thawing (Kashir et al., 2011) and the immaturity of centrosome of intragonadal spermatozoa (Comizzoli et al., 2006b) rather than DNA fragmentation could be the causes. In addition, the high proportion of MII arrest with sperm activation suggests that artificial stimulation of oocytes after ICSI is essential to increase the ICSI outcome. Several chemical agents such as ethanol (Comizzoli et al., 2006a, b) and calcium ionophore (Ahmady et al., 2007) have been used for oocyte activation after testicular sperm injection. However, it is also worth nothing that the artificial oocyte activation used to date potentially induced parthenogenetic activation rather than increase the number of normal fertilization (Buarpung et al., 2012; Chankitisakul et al., 2012).

In the current study, the developmental competence, in terms of cleavage, morula and blastocyst percentages, of ICSI oocytes using testicular spermatozoa in this study were significantly poorer (i.e., cleavage rate) than the results obtained from *in vitro* fertilization using ejaculated spermatozoa in our previous studies (Sanunmuang et al., 2010, Tharasanit et al., 2011). We also did not observe the difference of developmental capability of ICSI derived from either nonfrozen or frozen-thawed testicular spermatozoa as similar to other reports (Gil-Salom 1996; Romero et al 1996; Ben-Yosef et al., 1999). It is therefore suggested that this approach (cryopreservation of testicular spermatozoa plus ICSI) after the death of valuable animal can potentially be used. Though the ability of these ICSI-derived embryos to develop until term remains to be elucidated, the embryo development obtained by ICSI in this study was a result of normal fertilization

since we did not apply any oocyte activation and some sham injected cleaved oocytes (5.1 %) did not develop to morula and blastocyst stage (**Table 8**) as previously reported (Pope et al., 1998; Comizzoli et al., 2006a).

4.6 Conclusions

Cat testicular tissue can be cryopreserved successfully. Types of CPAs and freezing techniques used play a central role in determining the postthaw quality of feline testicular spermatozoa. We first describe that frozen-thawed testicular spermatozoa retain the fertilizing ability, although the development capability of embryos derived from ICSI with frozen-thawed testicular spermatozoa is poor. Development of ICSI-derived embryos to term remains to be studied.

CHAPTER V

IN VITRO AND IN VIVO DEVELOPMENT OF FROZEN EMBRYOS PRODUCED BY ICSI WITH CRYOPRESERVED TESTICULAR SPERMATOZOA

5.1 Abstract

Testicular tissue cryopreservation associated with intracytoplasmic sperm injection (ICSI) and subsequence embryo freezing is a potential strategy for conservation of endangered animal. This study aimed to examine the *in vitro* and *in vivo* development of frozen 2- to 8- cell cat embryos retrieved from ICSI with cryopreserved testicular spermatozoa. Testicular tissues were frozen by 2-step freezing with 5 % (v/v) glycerol before sperm extraction and injection. After ICSI for 2 days, cleaved embryos were cryopreserved in 10 % (v/v) ethylene glycol-based medium by a controlled-rate slow freezing. Frozen cleaved embryos were thawed and cultured *in vitro* until Day 8. Fresh ICSI, Fresh *in vitro* fertilization (IVF) and frozen-thawed IVF embryos were cultured in parallel. The proportions of cleavage, zona pellucida damage after thawing, morula and blastocyst formation, average cell number of blastocyst were compared (Experiment I). An additional group of frozen-thawed embryos (n = 209) were transferred into the oviducts of 7 cat recipients to assess the *in vivo* embryo development (Experminent II). The percentage of cleaved embryo retrieved from ICSI with frozen testicular spermatozoa was less than that of conventional IVF (P < 0.05). ICSI and IVF embryos yielded the similar percentage of damaged zona pellucida after thawing (8 % vs. 5.3 %, respectively). However, frozen ICSI embryos reached to morula (22.6 %) and blastocyst (20.6 %) stage at the lowest proportions when compared to fresh ICSI, fresh IVF and frozen IVF embryos. An average cell number of blastocyst did not difference (P > 0.05) among frozen ICSI, fresh ICSI and fresh IVF embryos (~242 cells, ~320 cells and ~302 cells , respectively), all of them were less (P < 0.05) than those of frozen IVF

embryos (~434 cells). Three out of the seven recipients were pregnant after transfer, but only one queen delivered 2 healthy kittens. In conclusion, domestic cat embryos produced by ICSI using spermatozoa from frozen testicular tissues can be cryopreserved, thawed and cultured resulted in *in vitro* embryo development to blastocyst stage, pregnancy and delivery of healthy kittens.

5.2 Introduction

Testicular tissue cryopreservation combined with intracytoplasmic sperm injection (ICSI) is a technique has been used for embryo production in azoospermia patients (Gil-salom et al., 2000). This procedure would be highly beneficial for genetic conservation of endangered species, including wild felids which are currently classified as threatened or endangered species. Domestic cat (*Felis Catus*) is usually used as a model for development of assisted reproductive technologies (ARTs) before the application to endangered wild felids.

Although ejaculated spermatozoa, collected by electroejaculation from living animal, is generally used for *in vitro* embryo production in felid species, testicular tissues would increase the sperm source for valuable animal that die unexpectedly or are castrated for medical reason. In domestic cat, embryo formation can be achieved after ICSI with fresh (Comizzoli et al., 2006a), cold stored (Buarpung et al., 2012) and frozen testicular spermatozoa (Buarpung et al., 2013). The latter study demonstrated that *in vitro* developmental competence of embryos was comparable between embryos produced by frozen and fresh testicular spermatozoa.

In many conditions, embryos would be transported or long-term storage for subsequent transfer, therefore, embryo freezing has become a beneficial tool for infinite preservation. Since the first report of kittens birth following transfer of cryopreserved feline embryos (Dresser et al., 1988), the protocols for freezing of domestic and non-domestic felid embryos have been developed continuously (Saragusty and Arav, 2011). Cat embryos at several stages of development could be frozen, e.g., 2- to 4-cell stage (Pope et al., 1994, 2012), morula (Day 4 to 5 of IVC) (Gómez et al., 2003) and blastocyst (Day 5 to 6 of IVC) (Pope et al., 2000), and transfer resulted in pregnancies and

deliveries. However, these embryos mainly produced by IVF. By contrast, cryopreservation and transfer of embryo derived from ICSI, especially with spermatozoa from cryopreserved testicular tissue, has not yet been established. The object of this study was therefore to examine *in vitro* and *in vivo* development of frozen cleaved embryo retrieved from ICSI using spermatozoa from cryopreserved testicular tissue.

5.3 Materials and Methods

5.3.1 Chemicals

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

5.3.2 Collection of testes and ovaries

Testes and ovaries of domestic cats, submitted for routine castration and ovariohysterectomy (OVH) at the Veterinary Public Health Division of The Bangkok Metropolitan Administration, Bangkok, Thailand, were maintained in 0.9 % (w/v) saline (NaCl) solution supplemented with antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin) and transported at room temperature (approximately 26-30 $^\circ$C) to the laboratory within 6 h.

5.3.3 Testicular tissue cryopreservation

The freezing extender including extender1 (EXI) and extender2 (EXII) were prepared according to Axnér et al. (2004). The EXI and EXII contained with 3 % (v/v) and 7 % (v/v) glycerol, respectively. At arrival, the extraneous tissues were removed from testes, later they were once washed in normal saline solution and dried off with clean gauze. Testicular tissue from individual testis was cut into 20 equal portions (approximately 2 x 3 x 5 mm in size). They were equilibrated in EXI at 5 $^\circ$C for 1 h before adding an equal volume of EXII. The equilibrated testicular tissues were subsequently loaded into a 0.5 ml polyvinyl straw (5 pieces per straw). Straws containing testicular tissues were horizontally placed on a rack, 4 cm above liquid nitrogen surface, for 10 min before plunging into liquid nitrogen for storage.

5.3.4 Oocyte collection and *in vitro* maturation

Cumulus oocyte complexes (COCs) were recovered after mincing cat ovaries in holding medium (HM) consisted of HEPES-buffered M199, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin and 100 µg/ml streptomycin and 0.1 % (w/v) bovine serum albumin (BSA, embryo tested grade). Only oocytes completely surrounded with more than five layers of compacted cumulus cells and containing homogeneous-dark ooplasm were used. *In vitro* maturation was performed as previously described by Sananmuang et al. (2010).

5.3.5 Thawing of cryopreserved testicular tissues and sperm extraction

The straws containing testicular tissue were thawed in air for 10 seconds, followed by immersion in warm water (37 ºC) for 30 seconds. The testicular tissues was then released into a thawing medium (Axnér et al., 2004) and incubated at 37 °C for 5 min. Testicular tissues were twice washed and mechanically minced with sharp-ended scissors in HEPES buffered synthetic oviductal fluid (HEPES-SOF). Spermatozoa were further extracted from minced tissue by repeat pipetting. The sperm suspension was finally filtered through a 40 µm cell strainer (BD-Falcon[TM], Franklin Lake, NJ, USA).

5.3.6 Intracytoplasmic sperm injection (ICSI)

After 18-24 h of *in vitro* maturation, cumulus cells were removed from oocytes by gentle pipetting. Only denuded oocytes exhibiting the 1st polar body were submitted to ICSI as previously described by Buarpung et al. (2013). Injected oocytes were cultured at 38.5 °C in 50 µL droplets of synthetic oviductal fluid medium containing with 4 mg/ml fatty acid-free BSA and 100 IU/ml penicillin (SOF-BSA) in a humidified condition of 5 % CO_2 in air.

5.3.7 *In vitro* fertilization (IVF)

In vitro fertilization (IVF) was performed 38.5 °C in a humidified atmosphere with 5 % CO_2 as previously described by Sanunmuang et al. (2010).

5.3.8 Embryo freezing

After 24 h of culture, presumptive zygotes from ICSI and IVF were then washed and cultured in SOF-BSA supplemented with 10 μM forskolin (Sigma Aldrich, St. Louis, MO, USA) for 24 h On Day 2, the number of cleaved embryos was observed and 2- to 8-cell stage embryos were selected for freezing with 10 % (v/v) ethylene glycol (EG) -based medium by a controlled-rate slow freezing. SOF-HEPES containing 20 % (v/v) fetal calf serum (FCS; Gibco®, Invitrogen, CA, USA) and 0.125 M Trehalose was used as a freezing medium (FM). Embryos were equilibrated 4 steps including FM without EG followed by FM supplemented with 2.5 %, 5 % and 10 % EG (5 min each), respectively, before loading to the 250 μL mini-straws. Controlled-slow freezing was performed using a programmable CL863 freezer (Cryologic PL, Australia). The initial temperature was set at 24 °C. The straws were slowly cooled at a rate of 2 °C/min to -6 °C. The straws were held at this temperature for 5 min before seeding, induction extracellular ice formation by touching the wall of freezing straws with forceps precooled in liquid nitrogen. After holding at -6 °C for 5 min, the temperature was reduced to -33 ° C at a rate of 0.3 °C/min and held at this temperature for 10 min. The straws were finally plunged into liquid nitrogen and stored in liquid nitrogen until analysis.

5.3.9 Thawing and culture of frozen-thawed embryo

The straws were held in air for 10 seconds and immersed in a water bath at 37 °C for 20 seconds. The embryos were released into a warm thawing solution (37 °C) that was composed of SOF-HEPES, 20 % (v/v) FCS and 0.25 M Trehalose, and then incubated in this solution for 3 min. Cryoprotectant was removed stepwise at 37 °C by transferring embryos into the same medium with 0.125 M, 0.062 M and 0.031 M trehalose (3 min each). Frozen-thawed embryos were then twice washed and cultured in SOF containing 10 % (v/v) FCS (SOF-FCS) until Day 8. This medium was changed every 2 days of culture. The number of embryos with damaged zona pellucida was recorded immediately after thawing. Percentages of morula and blastocyst formation (related to the number of cleaved embryos) were determined on Day 5 and 8 of *in vitro* culture (IVC), respectively. On Day 8 of IVC, all embryos were fixed and stained with

fluorescently DNA labeling (4'6' Diamidino-2-Phenylindole Dihydrochloride; DAPI), by incubation at concentration 1 µg/ml for 10 min, in order to examine the blastocysts (> 50 cells with blastocoel formation; Bogliolo et al., 2001) and embryonic-stage classification (according to the cell number/embryo), as described by Pope et al., 1998 (with minor modification).

5.3.10 Embryo transfer

Estrus induction was performed by intramuscular injection of 150 IU equine chorionic gonadotropin (eCG, Folligon, Intervet-Schering Plough, Boxmeer, The Netherlands). After 96 h of eCG injection, they were checked for estrous signs, and ovulation was induced by intramuscular injection of 100 IU human chorionic gonadotropin (hCG). Embryo transfer was performed on Day 3 after hCG injection (**Figure 22**). The recipients were anesthetized with a combination of 0.04 mg/kg atropine sulphate (A.N.B. Laboratories, Bangkok, Thailand), 3 mg/kg xylazine (Laboratorios calier, Barcelona, Spain) and 10 mg/kg ketamine hydrochloride (Gedeon Richter, Budapest, Hungary). Frozen embryos were thawed and cultured in SOF-FCS for 2 h before transfer. Only morphologically normal embryos with intact zona pellucida were selected and transferred into oviducts of recipients using a tom cat catheter (Buster, Langeskov, Denmark) (**Figure 23**). Gestation period and birth rate were recorded.
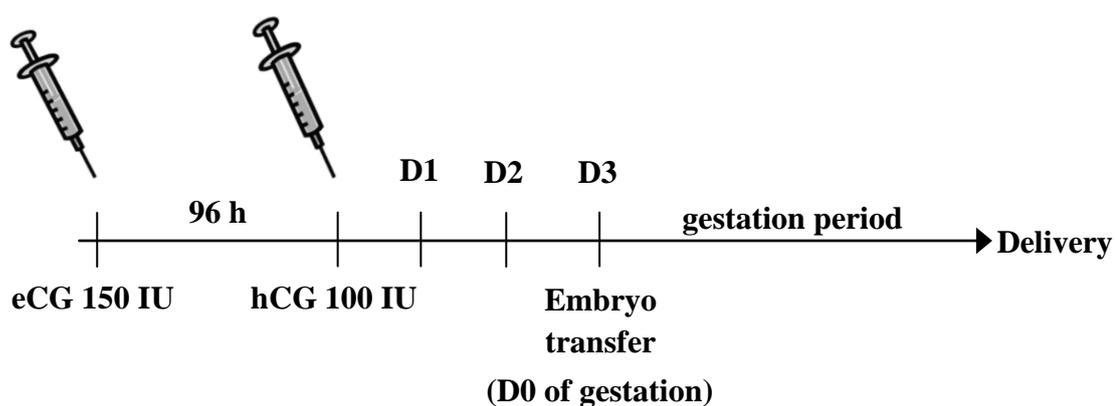
Figure 22 The timeline indicates the schedule for hormonal injections and embryo transfer. The recipient cats were injected with 150 IU eCG to induced estrus cycle. After 96 h of eCG shot, they were injected with 100 IU hCG for ovulation induction. The embryos were transferred into the queens on Day 3 after hCG injection.
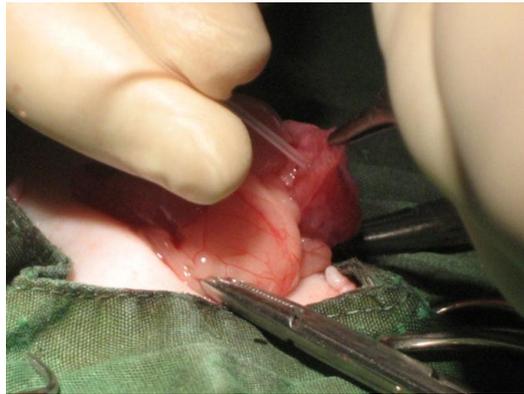
Figure 23 A tom cat catheter was passed into the fimbria, then frozen-thawed embryos were flushed into the oviduct of recipient cats.

5.3.11 Experimental design

Experiment I: *In vitro* development of frozen-thawed embryos derived from ICSI using sperm from cryopreserved testicular tissues

In vitro mature oocytes were injected with spermatozoa from cryopreserved testicular tissue (n = 820; 15 replications), while the other 874 MII oocytes were used for IVF (8 replications). The number of cleaved embryos from ICSI and IVF were recorded at Day 2 of *in vitro* culture (IVC). Only early cleaved (2 to 8-cell stage) embryos were selected for freezing (ICSI = 163; IVF = 287). Parts of frozen ICSI (n = 155) and IVF (n = 151) embryos were thawed, and the number of embryos with damaged zona pellucida was recorded. All frozen-thawed embryos were cultured until Day 8 (the day of thawing = Day 2). Fresh ICSI (n = 93) and Fresh IVF (n = 308) embryos were cultured in parallel and served as controls. The developmental competence was assessed in terms of morula and blastocyst formation on Day 5 and 8 of IVC, respectively. On Day 8 of IVC, all embryos were fixed and stained with DAPI to examine the number of blastomere and embryonic stage.

Experiment II: *In vivo* development of frozen-thawed embryos derived from ICSI using spermatozoa from cryopreserved testicular tissues

MII oocytes were injected with spermatozoa from cryopreserved testicular tissues (n = 706; 10 replications). Only 2 to 8-cell stage embryos (n = 230) were selected for freezing. A total of 209 frozen-thawed embryos were transferred into the oviducts of 7

eCG/hCG treated cat recipients in order to examine their *in vivo* development (approximately 14-25 embryos per oviduct). Pregnancy was detected by radiography on day 49 after transfer. Embryonic vital sign (by mean of kitten heart beat) was observed by ultrasonography on day 62 of gestation. Percentages of pregnancy and birth were particularized as the number of pregnant and parturient queens, respectively, relative to the total number of recipients.

5.3.12 Statistical analysis

The data was statistically analyzed using the Statistical Analysis Systems software package (Version 9.0, SAS Institute Inc., 1996, N.C., USA). Chi-square statistical test was used to compare the percentages of cleavage and post-thawed embryonic development, including zona pellucida-damaged embryos, morula and blastocyst formation, and *in vitro* development to different embryonic stages on day 8 of IVC. Difference between the total cell numbers of blastocyst was analyzed by one-way ANOVA. P-values< 0.05 were considered as statistically significant.

5.4 Results

5.4.1 Experiment I: *In vitro* development of frozen-thawed embryos derived from ICSI using sperm from cryopreserved testicular tissues

ICSI with spermatozoa from cryopreserved testicular tissues yielded a lower percentage of cleavage (34.0 %) than that of conventional IVF with frozen-thawed ejaculated spermatozoa (68.8 %) (P < 0.05; **Table 10**). The number of embryos with damaged zona pellucida (**Figure 24**) after thawing was not different between ICSI and IVF groups (8 % and 5.3 %, respectively, p > 0.05). The percentages of morula and blastocyst (**Figures 25C, D**) derived from IVF (either frozen-thawed or nonfrozen) embryos were higher than ICSI embryos (**Table 11**). The number of frozen ICSI embryos developed to morula and blastocyst (**Figures 25A, B**) were less (P < 0.05) than fresh ICSI embryos, though these proportions did not show significant difference between fresh and frozen IVF embryos. Average cell numbers of blastocyst developed from fresh IVF and ICSI embryos were not different (P > 0.05). The lowest blastocyst cell number was achieved from frozen-thawed ICSI embryos when compared to other groups (P <

0.05) (**Figures 26A-D**). The numbers of degenerated embryos, obtained on D8 of IVC, in frozen groups were higher than fresh controls irrespective of fertilization methods (IVF and ICSI) (**Table 12**). Nearly half of frozen-thawed ICSI embryos (47.1%) degenerated. Most of nondegenerated embryos, which did not reach to blastocyst, arrested at premorula stage. The numbers of fresh ICSI embryos which arrested at premorula stage were higher ($P < 0.05$) than fresh IVF embryos, though this proportion was not significant differ between frozen and fresh embryos (either IVF or ICSI).

Table 10 Percentages of cleaved embryos after IVF with frozen-thawed ejaculated spermatozoa and ICSI with frozen-thawed testicular spermatozoa. Cleaved embryos were categorized for freezing and culture as controls.

Group	N	Rep	Cleavage (%)	Frozen embryos (n)	Cultured embryos (n)
IVF	874	8	601 (68.8)[a]	287	308
ICSI	820	15	279 (34.0)[b]	163	93
Sham ICSI	150	5	8 (5.3)[c]	0	0

Abbreviations: N = number of inseminated/ injected oocytes, Rep = Replications,
IVF = *in vitro* fertilization, ICSI = intracytoplasmic sperm injection
Values with different superscripts in the same column, are statistically different (P < 0.05).

Table 11 Developmental competence *in vitro* of frozen-thawed ICSI and IVF embryos

Group	N	Morula (%)	Blastocyst (%)	Cell no.of blastocyst (MEAN ± SEM)
FT IVF	151*	90 (59.6)[a]	85 (56.3)[a]	439.0 ± 38.7[a]
FT ICSI	155*	35 (22.6)[b]	32 (20.6)[b]	242.5 ± 43.1[b]
Fresh IVF	308	182 (59.1)[a]	160 (51.9)[a]	302.2 ± 15.4[c]
Fresh ICSI	93	42 (45.2)[c]	35 (37.6)[c]	327.9 ± 27.8[a,c]

Abbreviations: N = number of thawed/cultured embryos, FT = frozen-thawed,
IVF = *in vitro* fertilization, ICSI = intracytoplasmic sperm injection
Values with different superscripts in the same column, are statistically different (P < 0.05).
*some embryos were lost during freezing and thawing processes.

Table 12 Developmental stage according to the cell number of nonblastocyst-embryos on Day 8 of *in vitro* culture

Group	N	Premorula (%)	Early morula (%)	Late morula (%)	Degenerated (%)
FT IVF	151	25 (16.6)[a]	13 (8.6)[a,b]	3 (1.99)[a,b]	24 (15.9)[a]
FT ICSI	155	39 (25.2)[a,b]	9 (5.8)[a]	1 (0.6)[a]	73 (47.1)[b]
Fresh IVF	308	61 (19.8)[a]	44 (14.3)[b]	18 (5.8)[b]	25 (8.1)[c]
Fresh ICSI	93	28 (30.1)[b]	6 (6.4)[a]	5 (5.4)[b]	19 (20.4)[a]

Abbreviations: N = number of cultured embryos, FT = frozen-thawed
Premorula = 2- to 15-cell embryos; Early morula = 16- to 32-cell embryos;
Late morula = 33- to 49-cell embryos
Values with different superscripts in the same column, are statistically different (P < 0.05).
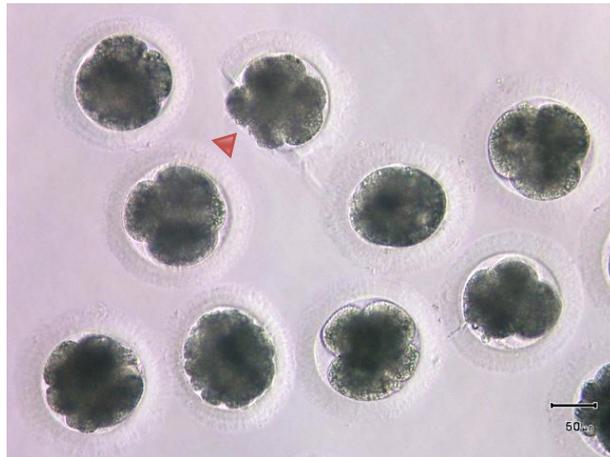
Figure 24 Cleavage-stage embryos of domestic cat after thawing. A damaged-zona pellucida was indicated by a red arrow head. Bars = 50 μm.
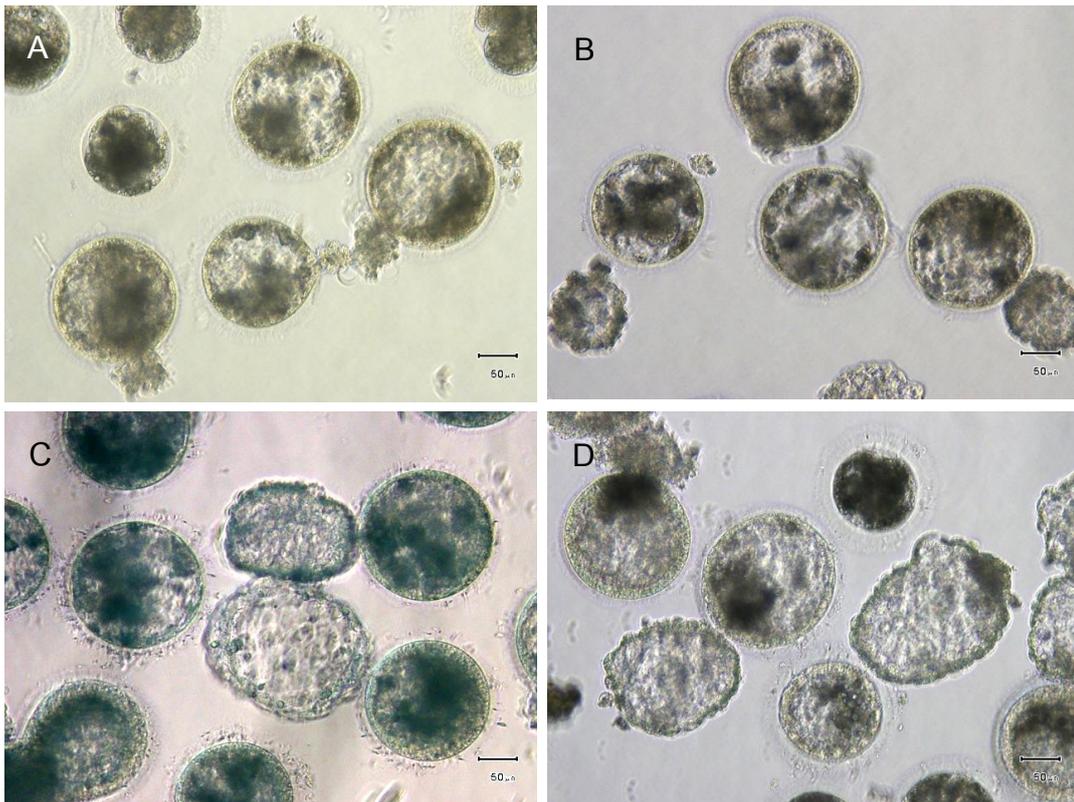
Figure 25 Day 8 blastocysts of fresh ICSI (A), frozen ICSI (B), fresh IVF (C) and frozen IVF (D) groups under light microscope. Bars = 50 μm.
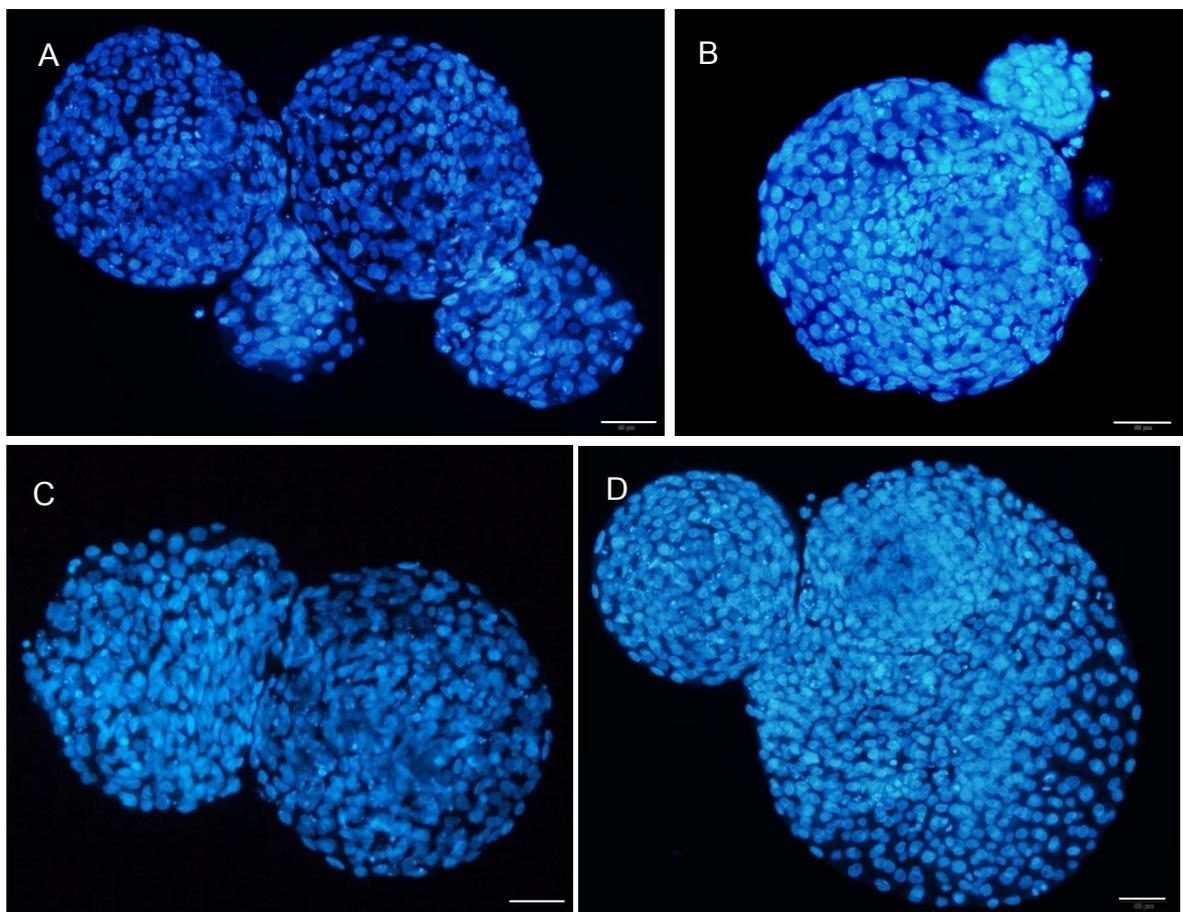
Figure 26 DAPI stained (A) Fresh ICSI-, (B) Frozen ICSI- , (C) Fresh IVF- and (D) Frozen IVF- hatching blastocysts on Day 8 of IVC. Bar = 50 μm.

5.4.2 Experiment II: *In vivo* development of frozen-thawed embryos derived from ICSI using spermatozoa from cryopreserved testicular tissues

Three out of semen recipients were pregnant (approximately 42.8 %). One pregnant queen aborted a fetus on Day 49 of gestation (**Figure 27A**), though the other two recipients carried their pregnancy to term. Fetal heart beats were detected by ultrasonography on Day 62 of gestation in both pregnant queens. One kitten from another queen died *in utero* (no heart beat was detected) at 65th day of gestation and was delivered by caesarean section (**Figure 27B**). Two healthy kittens (both male) were delivered from a queen (approximately 14.3 % birth rate) on Day 64 of gestation (**Figure 28**). Both kitten are growing normally and got weight ~1.5 kg at 4-month age.
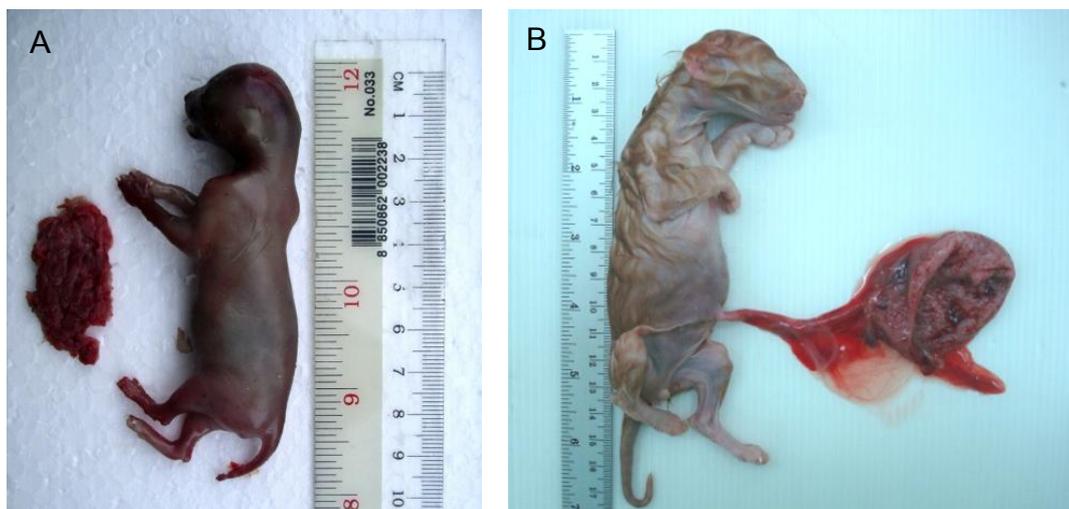
Figure 27 (A) a fetus aborted on Da 49 of gestation and (B) a kitten died *in uterine* on Day 65 of gestation.

Figure 28 Two healthy kittens were delivered from a queen on Day 64 of gestation

5.5   Discussion

Our results demonstrated for the first time that live kittens can be produced after the transfer of frozen/ thawed embryos fertilized by ICSI with frozen testicular spermatozoa. However, frozen-thawed cleaved ICSI-embryos developed to the morula and blastocyst stage *in vitro* at lower proportions than that of non-frozen embryos.

In our previous study, domestic cat testicular tissues cryopreserved by 2-step freezing with 5 % (v/v) glycerol could retain sperm membrane integrity, DNA integrity, and fertilizing ability, and ICSI derived embryos could develop to blastocyst stage *in vitro* (Buarpung et al., 2013). This freezing technique was therefore used in this study. However, we did not employ any oocyte activation in this study to avoid parthenogenesis as exhibited in another report (Buarpung et al., 2012). We found that the percentages of cleavage, morula and blastocyst fornations retrieved from ICSI with cryopreserved testicular spermatozoa was obviously lower than IVF with frozen ejaculated spermatozoa. It is possible that the calcium oscillation after ICSI begins lately when compared with IVF (Nagy et al., 1994) resulted in delayed embryo development. Another reason is the immaturity of cat testicular sperm centrosome which contributed to short sperm aster formation, resulted in delayed first cleavage, slow developmental rate, and reduction of morula and blastocyst formation (Comizzoli et al., 2006b). In addition, the ICSI procedures (Griffiths et al., 2000) and reactive oxygen species (ROS) secreted by ICSI embryos (Lee et al., 2012) may have a negative effect on the embryo developmental competence. However, we presumed that the techniques for fertilization did not influence on blastocyst quality as our data and the previous report (Piotrowska-Nitsche and Chan, 2013) revealed that the cell numbers of blastocysts retrieved either by IVF or ICSI were comparable.

Zona pellucida (ZP) damage occured after freezing and thawing contributed to developmental competence and embryonic degeneration (Cohen et al., 1986; Moreira da Silva and Metelo, 2005). Our study found that the number of ZP damage was low (5-8 %) and was comparable between IVF and ICSI embryos. This finding suggested that ZP damage did not impact by the techniques for embryo production and confirmed an earlier study stating that ICSI did not make the embryos more susceptible to ZP damage (Van Den Abbeel and Van Steirteghem, 2000). A higher number of degenerated embryos (on Day 8 of culture) were obtained from frozen-thawed embryos compared with fresh embryos, irrespective of fertilization methods. This could be due to embryonic cytoskeleton such as mitotic spindle, an essential structure for embryonic development,

was affected by freezing and thawing process (Sathananthan et al., 1988; Tharasanit et al., 2005).

Our result is in agreement with the others indicating that there was no difference between development to morula and blastocyst stage of frozen–thawed and fresh cat IVM/IVF embryos (Gómez et al., 2003). Although it was not clear that why *in vitro* development and the blastocyst number of frozen ICSI-embryos were lower than that of fresh ICSI-embryos, and almost a half of frozen-thawed ICSI embryos degenerated during *in vitro* culture, it is possible that ICSI procedure such as ooplasm aspiration, meiotic injury during injection and media injection may decrease cryotolerance of ICSI-oocyte cytoskeleton. Dissimilar results was reported in by other authors who showed that freezing procedure did not impact the cell number of blastocysts developed from frozen cleaved embryos (Uechi et al., 1997), and the fertilization methods including IVF and ICSI did not affect on survival of thawed human embryos (Kowalik et al., 1998), however, the latter report did not observe survival rate after prolong culture.

Although the reports in many species indicated that embryo developmental stages affect the embryo viability after freezing and thawing (Cocero et al., 1996; Leibo et al., 1996) and early cleavage stage embryos are sensitive to cryopreservation (Freitas et al., 1994; Garcia-Garcia et al., 2006), a previous study in cat demonstrated that the age of embryos at cryopreservation did not affect on *in vitro* development to blastocyst stage (Gómez et al., 2003). We assumed that early cleaved embryos represented a valuable source for freezing due to the number of morula and blastocyst developed from cleaved embryos produced by ICSI with testicular spermatozoa were low (Buarpung et al., 2013). In addition, the transfer of cleaved embryos can significantly increase the delivery rate per replacement (Van der Elst et al., 1997; Ziebe et al., 1998) and lower risk of miscarriage was recently reported after transfer of thawed cleaved embryos when compared with thawed blastocysts (Wang et al., 2011).

In this present study, we treated cat ICSI embryos with forskolin to reduce intracellular lipid content before freezing because of there were several reports mentioned that high lipid content in embryos produced *in vitro* can increase sensitivity to freezing and thawing (Nagashima et al. 1994; Abe et al. 2002). Moreover, our previous

study demonstrated that frozen cat embryos prior treated with forskolin increased cryoresistance and improved in vitro development to the morula and blastocyst stage (Tharasanit and Techakumphu, 2011).

Our study supported the results of a previous report in which cryopreserved testicular spermatozoa are able to fertilize oocytes after ICSI and result in embryonic development, and establish pregnancies after transfer of such embryos (Fischer et al., 1996; Hovatta et al., 1996; Gil-Salom et al., 2000; Fukunaga et al. 2001). However, spontaneous abortion and foetal death following transfer of frozen–thawed embryos may be due to embryonic damage during the freezing and thawing processes (Edgar et al. 2000) which affected on chromosome (Iwarsson et al., 1999; Salumets et al., 2004) or at the molecular level (Stinshoff et al., 2011) of *in vitro* produced embryos. Due to the number of blastocyst (20.6 %) developed from frozen ICSI-embryos was low, we transferred at least 14 embryos into each oviduct to increase the chance of pregnancy. However, pregnancies seemed not related to the number of transferred embryos. Although the blastomere number in transferred embryos is the most considerable parameter for prediction further embryonic development (Guerif et al., 2002), we could not determine that which stage of embryos (among 2- to 8- cell stage) is the best for cryopreservation and transfer because we assembled any stage together.

5.6　Conclusions

Cryopreserved testicular spermatozoa of domestic cat retained their ability to fertilize oocytes after ICSI. We confirmed that embryos can be frozen with 10 % (v/v) ethylene glycol by controlled slow freezing. Frozen–thawed ICSI cleaved embryos maintained the potential to develop to the blastocyst stage in vitro and to full-term kittens *in vivo*. This study demonstrated that cryopreservation program using cryopreserved testicular tissue as a source of spermatozoa combined with embryo freezing and transfer of embryos into cat recipients is feasible. However, improvement of all these techniques is a prerequisite for preserving the genetic potential of endangered species.

CHAPTER VI

GENERAL DISCUSSION AND CONCLUSIONS

Gamete preservation associated with assisted reproductive technologies (ARTs) is an advantageous technique which has been used for overcoming human infertility, and offspring production in farm and laboratory animals. These techniques can also be beneficial tools for genetic preservation and management of several endangered species (Wildt et al., 2010), including elimination of inbreeding-related problems which are arisen from the decrease of animal population. Development of ARTs and preservation techniques before application in rare species is essential because available gametes from particular wild or rare animals are limited. The domestic cat has therefore been used as the important model for endangered wild felids.

Indeed, ejaculated spermatozoa are routinely used for ARTs in felid species. However, testes will be the final source of spermatozoa from dead and castrated animals. Testes contain spermatozoa in several stages of development (including spermatogonia, spermatocytes, round spermatids, elongating spermatids and mature spermatids) which can be used for embryo production via intracytoplasmic sperm injection (ICSI). The kitten born were reported after transfer of embryos produced by ICSI with ejaculated and epididymal spermatozoa (Pope et al., 1998, 2012), while successful result from ICSI with cat testicular spermatozoa was still limited only *in vitro* blastocyst development (Comizzoli et al., 2006a, b). However, the studies on testicular sperm/tissue preservation and also the births of kitten after transfer of embryos produced by ICSI with testicular spermatozoa (Chapter V) indicated convincingly the potential of these technologies (Buarpung et al., 2012, 2013; Tharasanit et al., 2012).

Spermatozoa within the body of animals degenerated quickly after death (Kishikawa et al., 1999) and the time elapsing between death and sperm recovery affected sperm quality (Santiago-Moreno et al., 2006). Likewise, a high temperature urges the decomposition of testes results in declination of sperm quality. At room temperature, rat testicular cells were degraded as early as 12 h (Bryant and Boekelheide, 2007), though mouse epididymal spermatozoa remained their membrane

integrity, DNA integrity and fertilizing ability until 24 h postmortem (Songsasen et al., 1998). Our preliminary study demonstrated that domestic cat testes could be stored at room temperature up to 6 h after castration without negatively effects on sperm membrane integrity, although compromised membrane integrity could be observed after 24 h of storage (Thuwanut and Chatdarong, 2012). Cold storage of gamete tissue is routinely used for transportation because it can maintain the sperm viability by reduction of post-mortem change and cell metabolism (Kaabi et al., 2003; Karunakaran et al., 2007). A better motility of spermatozoa was achieved when the epididymide of mice (Songsasen et al., 1998; Kishikawa et al., 1999), dog (Stilley et al., 2000), ram (Kaabi et al., 2003) and deer (Hishinuma et al., 2003) were stored at 4 - 5 °C compared with room temperature. Our research found that cat testicular spermatozoa could be cold stored up to 7 days either in intact testes (Buarpung et al., 2012; Chapter II) or sperm suspension (Chapter III). Although the sperm viability decreased over the time, 7-day chilled spermatozoa could fertilize oocytes and yielded blastocyst development. Nevertheless, we did not evaluate testicular sperm quality if the testes were kept for longer than 7 days because this 'short-term' cold storage would be sufficient for transport the testicular tissues to nearby laboratory that has the facilities for testicular freezing or further embryo production.

Several factors have been demonstrated to involve sperm quality during cold storage such as storage temperature, type of preserving media and additives (Althouse et al., 1998; Van Thuan et al., 2005; Li et al., 2011; Sariözkan et al., 2012). DPBS was preferably used as a preserving medium in our studies because this basic salt solution can be easily prepared and stored compared to complex culture media. It was also reported to maintain the membrane integrity of testicular cells for at least 6 days of cold storage (Yang and Honaramooz, 2010). Spermatozoa could be preserved as whole testes or suspension in DPBS, although membrane and DNA integrity of spermatozoa seemed to be better preserved within intact testes than in suspension. It is possible that testicular parenchyma may protect testicular spermatozoa from direct exposure to rapid cooling which cause sperm injury. Furthermore, the testis contains several proteins actively involved with antioxidative mechanisms such as superoxide dismutase (SOD),

glutathione reductase (GSR), glutathione peroxidase (GPX), glutathione S-transferase (GST) (Bauché et al., 1994; Gu and Hecht, 1996) which may reduce the harmful effects of oxidative stress on germ cells during cold storage. The membrane integrity of isolated spermatozoa was improved when the spermatozoa were maintained in HEPES-buffered M199 (HM), a culture medium commonly used for *in vitro* maturation of oocytes, supplemented with bovine serum albumin (BSA). Although, the study in mouse revealed that HEPES did not improve sperm survival (Sato and Ishikawa, 2004), spermatozoa stored in HM might acquire energy supply from pyruvate which is a substrate for adenosine triphosphate (ATP) production within sperm cell (Hereng et al., 2011). In addition, antioxidant properties of pyruvate might protect plasma membrane from oxidative stress injury during cold storage resulting in the enhancement of sperm motility as reported in bovine (Bilodeau et al., 2002) and stallion (Bruemmert et al., 2002).

In Chapter III, we found that increase of BSA concentration significantly improved membrane integrity of testicular spermatozoa. It was possibly due to BSA was absorbed into sperm cell membrane and increase stability of membrane structure (Blank et al., 1976). BSA could be an energy source for spermatozoa during storage resulted in prolong sperm viability (Hossain et al., 2007). Furthermore, BSA could act as an antioxidant to protect spermatozoa from destruction by free radicals produced by oxidative stress during preservation (Uysal and Bucak, 2007). Although BSA supplementation yielded the protective property on sperm membrane integrity, high concentration of BSA could adversely affect on spermatozoa from osmotic stress (Nang et al., 2012; Osman et al., 2012). However, the osmolarity of our preserving medium seemed not to impact the plasma membrane integrity. It might be possible that cat spermatozoa were less susceptible to osmotic shock (Pukazhenthi et al., 2000). However, the effect of BSA concentration on DNA integrity of cat spermatozoa should be further examined.

Cold storage did not impaired fertilizing ability of spermatozoa after ICSI. This resulted was supported by the previous studies that ICSI by cold stored and fresh epididymal cat spermatozoa yield the similar cleavage rate (Ringleb et al., 2011). Furthermore, embryos derived from ICSI with cold stored (6 m) mouse spermatozoa

could develop *in vivo* to full term offspring (Li et al., 2011). Although DNA integrity of spermatozoa was significantly decreased after 7-day cold storage (Chapter III), these spermatozoa could fertilize *in vitro* matured oocytes leading to the comparable *in vitro* embryo development with fresh testicular spermatozoa. This could be due to the oocytes can repair damaged DNA of spermatozoa occurred less than a critical level as reported in human (Ahmadi and Ng, 1999). However, ICSI using spermatozoa with damaged DNA beyond that level will lead to fragmentation and low developmental competence of embryos.

The quality of testicular cells and spermatozoa can be affected by several factors during the freezing process such as types of CPA (Keros et al., 2005; Jezek et al., 2001), freezing techniques (Keros et al., 2007) and tissue size (Crabbé et al., 1999). Our studies found that the types of CPAs and freezing techniques influenced the quality of testicular spermatozoa in terms of sperm plasma membrane integrity, but not DNA integrity (Chapter IV). In this thesis, glycerol was found to be the best CPA for plasma membrane integrity of frozen-thawed testicular spermatozoa compared with EG, PrOH and DMSO. It was therefore assumed that the equilibration period and the concentration of glycerol used in this study might be appropriate for this size ($2 \times 3 \times 5 \text{ mm}^3$) of testicular tissue, whereas the concentration and equilibration time for the other lower molecular weight CPAs should be decreased to reduce the CPAs toxicity. Two-step freezing with glycerol (the method developed for semen freezing) was superior to the controlled slow freezing, although the sperm quality was not significantly different between the two freezing techniques. It was likely that the cooling rate used for controlled freezing was not suitable for this CPAs and further study about freezing rate was necessary. In addition, two-step freezing is more convenient than controlled slow freezing which is time comsuming and requires the expensive equipments.

The developmental competence of embryos produced by ICSI using testicular spermatozoa were significantly poorer than the results obtained from conventional *in vitro* fertilization (IVF) using ejaculated spermatozoa (Sananmuang et al., 2011; Chapter V). The possible reasons for this delayed development were that calcium oscillation after ICSI begins lately when compared with IVF (Nagy et al., 1994) and/or testicular

spermatozoa had immature centrosome which contributed to short sperm aster formation resulted in delayed first cleavage (Comizzoli et al., 2006b), rather than the detrimental effect of cold storage or freezing on spermatozoa. Furthermore, our results (Chapter IV) that cleavage rates and the developmental capability of ICSI embryos were not different between frozen and fresh testicular sperm ICSI (Buarpung et al., 2013) supported the finding in bull (Hara et al., 2011) that freezing procedure did not impair the function of microtubule-organizing center (MTOC) which originated by sperm centrosome. Poor fertilization after ICSI may be due to oocyte activation insufficiency (Flaherty et al., 1995a) or failure of male-pronucleus (Lee et al., 2003), as found in our studies that most of non-cleaved ICSI oocytes arrested at MII stage containing intact or decondensing sperm head. Due to testicular spermatozoa are immotile, we therefore did not immobilize spermatozoa by touching the pipette at sperm tail before injection, as suggested by the previous report (Vanderzwalmen et al., 1996). This may be a reason for failure of oocyte activation and sperm decondensation, because touching of sperm tail induces the destabilization in the plasma membrane with the release of an activating factor 'oscillin' into the ooplasm (Dozortsev et al, 1995; Parrington et al., 1996). In addition, depolymerized membrane is necessary for ooplasmic enzymes to reach the sperm nucleus in order to start chromatin decondensation (Tesarik et al., 1994).

Fertilization, cleavage and blastocyst rates were improved by additional oocyte activation treatment with electric stimulation (Hwang et al., 2000), calcium ionophore (Goto et al., 1990; Chen and Seidel, 1997; Wang et al., 2010), ethanol (Hamano et al., 1999; Emuta and Horiuchi, 2001), ionomycin plus 6-dimethylaminopurine (DMAP) (Rho et al., 1998; Chung et al., 2000; Suttner et al., 2000; Ock et al., 2003) or strontium (Loren and Lacham-Kaplan, 2006). These activators can induce intracellular calcium spikes, but the results were different in each species. Several chemical agents such as ethanol (Comizzoli et al., 2006a, b) and calcium ionophore (Ahmady et al., 2007) have been used for oocyte activation after testicular sperm injection. However, artificial activation can produce parthenogenesis, a form of reproduction in which the ovum develops into a new individual without fertilization, as reports in cat (Buarpung et al., 2012) and buffalo (Chankitisakul et al., 2012). In horse, the absence of oocyte activation was often

accompanied by failure of sperm decondensation (Tremoleda et al., 2003). The chemical agents such as Triton X-100 and dithiothreitol (DTT) have been used to remove the cytoplasmic membrane and reduce disulfide bonds of sperm head before microinjection, respectively, to improve sperm decondensation, although pronucleus formation and development to the blastocyst stage *in vitro* were not increased (Nakai et al., 2006). This observation is in accordance with previous report in hamster that reduction of disulfide bond is essential for sperm nuclear decondensation but not sufficient for pronuclear formation (Perreault et al., 1984). Our preliminary study indicated that the incidence of parthenogenetic embryos was remarkably low when ICSI was performed without any oocyte activation. We therefore did not employ any oocyte activation in the experiments in Chapter III to V to avoid parthenogenesis and found that testicular spermatozoa of domestic cat could fertilize matured oocytes after ICSI without oocyte activation by artificial stimuli. This similar result reported by Pope et al. (1998) which oocytes were injected with ejaculated spermatozoa. However, percentage of cleavage was lower than that of ICSI following oocyte activation (Comizzoli et al., 2006a).

Our observation that spermatozoa underwent gamete activation before oocytes confirmed the fact that the sperm-born oocyte activating factor is necessary for oocyte activation (Tesarik et al., 1994; Jones, 2005; Malcuit et al., 2006). Furthermore, our data agree with previous reports that male and female pronucleus formation occurred in an asynchronous manner (Gómez et al., 1998b; Bourgain et al., 1998; Flaherty et al., 1995b). The finding that male and female pronuclear formation occurred by 6 h after ICSI using cryopreserved testicular spermatozoa was similar to the results obtained from ICSI with cat ejaculated spermatozoa (Jin et al., 2012). By contrast, the transition between the first observed pronuclear and cleavage stages appeared to be delayed after ICSI with testicular spermatozoa. Although the reason of this remains unclear, the immaturity of testicular sperm centrosome, expressed by the distinctly short microtubular aster formation, can delay the cleavage and also slow the developmental rate (Comizzoli et al., 2006b).

Two major techniques have been used for embryonic freezing are controlled slow freezing and vitrification (Pereira and Marques, 2008). Both techniques have been used and successful deliveries were achieved after embryo transfers in domestic cats (Pope et al., 1994, 2012; Gómez et al., 2003). We preferred controlled freezing to avoid harmful effect from high concentration of cryoprotectant on early cleaved embryo (Uechi et al, 1999; Chi et al., 2002). Although several reports revealed superior result of vitrification for cleavage embryos, e.g., in human (Rezazadeh Valojerdi et al., 2009; Son et al., 2009; Lin et al., 2010) and tiger (Crichton et al., 2003), a recent report indicated that an optimal slow cooling rate can provide the high survival rate of early embryos as well as vitrification (Edgar and Gook, 2012). In addition, vitrification can cause more severe cellular alterations and reduced embryonic viability compared to slow freezing (Coutinho et al., 2007). However, it is difficult to indicate the optimal protocol for cat embryo freezing, especially ICSI embryos which the freezing protocol has not been reported. We proved that the protocols for freezing and thawing used in Chapter V could be applied for both IVF and ICSI cat embryos even if further development is required. Several cryoprotectants have been used for embryonic freezing in domestic animal, e.g., propylene glycol (PROH), ethylene glycol (EG), glycerol and dimethyl sulfoxide (DMSO). PROH (Pope et al., 1994, 2012; Gómez et al., 2003) and EG (Karja et al., 2006) were employed for slow rate freezing in domestic cat. In this study, the cryoprotective efficiency of 10 % (v/v) EG for early-cleaved cat embryos using slow freezing was confirmed by low percentage of zona pellucida damage, developmental potential to blastocyst stage of thawed embryos and kitten births.

As the previous study in cat demonstrated that the age of embryos at cryopreservation did not affect on *in vitro* development to blastocyst stage (Gómez_et al., 2003). We assumed that early cleaved embryos represented a valuable source for freezing due to the number of morula and blastocyst developed from cleaved embryos produced by ICSI with testicular spermatozoa were low (Buarpung et al., 2013). The transfer of cleaved embryos may increase the chance for *in vitro* embryo development and the delivery rate as reports in human (Van der Elst et al., 1997; Ziebe et al., 1998). The number of degenerated frozen-thawed embryos on Day 8 of IVC was significantly

higher in ICSI group than that of IVF. It is possible that ooplasm aspiration, meiotic injury during injection and media injected with spermatozoa may decrease cryotolerance of ICSI-oocyte cytoskeletons contributed to increasing of cryosensitivity of this structure within subsequent embryos. After thawing, if cytoskeleton did not efficiently sustain embryonic mitosis, developmental arrest and degeneration would occur finally. The observation that frozen-thawed embryos derived from ICSI with frozen testicular spermatozoa reached to morula and blastocyst stages at lower percentages than fresh embryos may be due to either reducing of glucose uptake in cryopreserved embryos resulted in delayed embryonic development (Uechi et al., 1997) and/or due to embryonic cytoskeleton, an essential structure for embryonic development, was affected by freezing and thawing process as report in horse (Tharasanit et al., 2005) and mouse (Sathananthan et al., 1988). However, our result is in agreement with another indicating that freezing procedure did not impact the blastomere number of blastocyst developed from frozen cleaved embryos (Uechi et al., 1997).

Our result supported the previous reports that spermatozoa from cryopreserved testicular tissue by two-step freezing with glycerol can be used for successful pregnancy after ICSI (Fischer et al., 1996; Hovatta et al., 1996; Gil-Salom et al., 2000). We transferred at least 14 embryos into each oviduct to increase the chance of pregnancy because the number of blastocyst (from ICSI using frozen testicular spermatozoa) developed *in vitro* was low (~21.3 %). However, pregnancies seemed not related to the number of corpora lutea and number of transferred embryos, but rather influenced by embryonic quality. Although the blastomere number in transferred embryos is the most considerable parameter for prediction further embryonic development (Guerif et al., 2002), we could not determine which stage of embryos (among 2- to 8- cell stage) is the best for cryopreservation and transfer because we assembled any stage together. Spontaneous abortion and still birth after transfer of frozen embryos may likely be caused by the damage of embryos occurred during the freezing and thawing procedures (Edgar et al., 2000) which affected on chromosome (Iwarsson et al., 1999; Salumets et al., 2004) or at the molecular level (Stinshoff et al., 2011) of *in vitro* produced embryos.

We justified that testicular tissue preservation for subsequence embryo production and cryopreservation is practicable. It is a beneficial technique for endangered-species propagation from testes of dead and castrated animals. However, development of these technologies is needed before realistic application for rare species.

Testicular tissue preservation and embryo production: The challenges

Although semen freezing and banking is routinely performed in the zoo, the major benefit of testicular tissue is that it enables us to collect spermatozoa post mortem or castration. In addition, optimal storage of testicular tissue can prolong the reproductive efficiency of that individual. However, ICSI is still very low efficiency and more complex than conventional *in vitro* fertilization (IVF) and artificial insemination (AI). As we assumed in this thesis that the low outcomes after ICSI with testicular spermatozoa may be due to immaturity of testicular centrosome, lack of oocyte activation and failure of sperm decondensation. The next directions for this work should be focused on the improvement of ICSI results. Several techniques have been used to enhance the ICSI outcomes such as sperm selection and treatment before ICSI (Ebner et al., 2011; Delaroche et al., 2013; Chankitisakul et al., 2013), replacement of centrosome from ejaculated spermatozoa (Comizzoli et al., 2006b) and oocyte activation after ICSI (Borges et al., 2009; Kim et al., 2012) . However, these protocols still require the development for felid species. Besides testicular tissue from adult animal, testicular tissue from prepubertal male animals can also be used for embryo production via ICSI. Grafting testicular tissue from immature individuals to immunodeficient mice resulted in sperm production in several species such as cat (Snedaker et al., 2004), ferret (Gourdon and Travis, 2011), bison (Abbasi and Honaramooz, 2011) and deer (Abbasi and Honaramooz, 2012), The births of offspring have been reported after ICSI with spermatozoa from testicular xenografts of pig, rabbit and mouse (Shinohara et al., 2002; Nakai et al, 2010). Nevertheless, improvement of the techniques for transplantation of cat testicular tissue and germ cell is still needed (Snedaker et al., 2004; Mota et al., 2012; Silva et al., 2012). For testicular tissue and embryo cryopreservation, further study on the efficiency of vitrification is being of interest because vitrification requires less time

and is more practicable in field. It also does not require the extensive laboratory equipments commonly used for programmed slow freezing. Vitrification prevents ice crystal formation by exposure of the cells or tissues to very high concentrations of cryoprotectant (CPA) before cooling by rapid freezing rates. This approach can cause the compromising of cells viability after thawing. In domestic cat, vitrified testicular tissue yielded a lower percentage of sperm membrane integrity when compared with 2-step freezing (Thuwanut and Chatdarong, 2012). To overcome the negative effects of vitrification on the cells or tissues, the proper media, equilibration conditions and CPAs concentration as well as the cooling techniques should be investigated (Otsuka et al., 2002; Kasai and Mukaida, 2004; Fuller and Paynter, 2004; Pegg, 2007; Desai et al., 2007).

Conclusions

Storage of testicular tissue at low temperature is of importance because a high temperature adversely affects on most of testicular cells results in failure of subsequent processes especially embryo production. The success of testicular tissue preservation, either cold storage or cryopreservation, would encourage the collection and storage of testicular tissue from wild species. It would support the genome banking for future embryo production as well as transportation these genetic materials between different areas to conserve genetic diversity, especially wild and zoo animals which are confronting to the inbreeding problems caused by restricted population.

Gamete preservation, ARTs and embryo cryopreservation are synergistic techniques. The ability to rescue gametes from rare species and to subsequently produce viable embryos holds tremendous potential to increase their population size. Appropriate handling of gametes and embryos would increase the success of species conservation. The result of this research indicate that testicular tissue can be the valuable sperm source for embryo production, although ICSI is still very low efficiency than conventional *in vitro* fertilization (IVF) and artificial insemination

(AI). Cold storage and cryopreservation of feline testicular tissue/spermatozoa are feasible and the preserved testicular spermatozoa exhibit adequate function to fertilize oocytes *in vitro*. DNA integrity of domestic cat seemed to tolerate cold storage and cryopreservation, while sperm plasma membrane is impacted by preserving durations or freezing methods. Cat testicular spermatozoa can be cold stored within basic medium such as DPBS and more complex culture medium such as HEPES-M199 (HM), routinely used for oocyte maturation and embryo culture. However, HM preserve membrane integrity of isolated testicular spermatozoa better than DPBS. BSA can improve sperm membrane integrity during cold storage. Basic media supplemented with BSA can also be applied for sperm cold storage in case that egg yolk based extender is not available or during the outbreak of avian influenza virus. Our study demonstrate that isolated spermatozoa cold stored within HM with 1.6 % BSA for 1 week could fertilize cat oocytes, and yielded the *in vitro* embryo development at the similar proportions to fresh-sperm injection. The study on the extended period for cold storage (more than 1 week) may be useful, although this period is enough for transportation and short-term preservation before subsequent embryo production. We suggest that the cryopreservation is preferred for longer period. A 2-step freezing technique was applied from freezing technique commonly used for semen freezing. It is easy and practicable in every laboratory, while controlled slow freezing is time consuming and require the expensive equipments. In this research, we found that testicular tissue cryopreservation by 2-step freezing with glycerol maintained the best sperm membrane integrity compared with the other cryoprotectants (ethylene glycol, propanediol and dimethyl sulphoxide) at 5% (v/v) concentration. This parameter was comparable to that of spermatozoa from non frozen tissues. Cryopreservation did not have harmful effects on sperm DNA integrity irrespective of cryoprotectants and freezing techniques. Spermatozoa from cryopreserved testicular tissues can fertilize cat oocytes without

external oocyte activation. Although male and female activation occurred independently after ICSI with cryopreserved testicular spermatozoa, the *in vitro* development and the blastocyst cell number of embryos produced by ICSI with cryopreserved testicular spermatozoa were comparable to non-crypreserved control. Since the morula and blastocyst rates achieved from ICSI with testicular spermatozoa were low irrespective to whether preserved or fresh testicular spermatozoa were used for ICSI. Embryo freezing at cleavage stage would increase the opportunity for *in vitro* and *in vivo* embryo development. Cleavage-stage embryos produced by ICSI with cryopreserved testicular spermatozoa retained there *in vitro* and *in vivo* developmental competence after freezing and thawing. In addition, this is the first report of the births of kittens after transfer of frozen-thawed embryos produced by ICSI with sperm recovered from cryopreserved testicular tissues. The studies in this thesis will be the fundamental knowledge for development of gamete preservation and embryo production for endangered wild felid conservation.

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APPENDICES

APPENDIX A

Stock solution

DAPI	0.1	mg/ml PBS
EGF	25	µg/ml Milli-Q water + 0.1 %BSA
FSH	100	IU/ml diluents
Gentamicin	50	mg/ml
hCG	300	IU/ml diluent
Heparin	10	mg/ml
Hoechst	10	mg/ml PBS
Penicillin	10^5	IU/ml
Sodium pyruvate	1	Molar
Streptomycin	0.1	g/ml
rhFSH	100	IU/ml diluent

Freezing extender for testicular tissue (prepared according to Axnér et al., 2004)

Extender I: *pH 6.4-6.5, Osmolarity 800-900 mOsm/kg*

Tris	2.4	% (w/v)
Citric acid	1.4	% (w/v)
Glucose	0.8	% (w/v)
Sodium benzylpenicillin	0.06	% (w/v)
Streptomycin sulphate in distilled water	0.1	% (w/v)
Egg yolk	20	% (v/v)
CPA*	3	% (v/v)

Extender II: *pH 6.4-6.5, Osmolarity 1,350-1,700 mOsm/kg*

Tris	2.4	% (w/v)
Citric acid	1.4	% (w/v)
Glucose	0.8	% (w/v)
Sodium benzylpenicillin	0.06	% (w/v)
Streptomycin sulphate in distilled water	0.1	% (w/v)
Egg yolk	20	% (v/v)
Equex STM paste	1	% (v/v)
CPA*	7	% (v/v)

Thawing medium: *pH 6.4-6.5, Osmolarity 250-260 mOsm/kg*

Tris	2.4	% (w/v)
Citric acid	1.4	% (w/v)
Glucose	0.8	% (w/v)
Sodium benzylpenicillin	0.06	% (w/v)
Streptomycin sulphate in distilled water	0.1	% (w/v)

*CPAs including glycerol, EG, DMSO and PrOH

Medium preparation

All media were prepared at room temperature. Oocyte holding medium was adjusted to pH 7-7.4, though the others were adjusted to pH 7.5-7.6. The osmolarity of cultured medium was *280-290 mOsm/kg*. The medium was sterilized, after pH and osmolarity adjustment, by filtered through 0.2 microns filter before storage.

Oocyte holding medium *(Store at 4 °C)*

Milli-Q water	1	L
HEPES	5.985	g/L
Medium M-199 (M3769: Sigma)	1	bottle/L
Bovine serum albumin	1	g/L
L-glutamine	0.292	g/L
1M Sodium pyruvate	1	ml/L
Penicillin	10^5	IU/L
Streptomycin	0.1	mg/L
Phenol red	500	µL/L

NaHCO$_3$ bufferred M-199 *(Store at 4 °C)*

Milli-Q water	1	L
Medium M-199	1	bottle/L
NaHCO$_3$	2.11	g/L

Oocyte maturation medium (prepare in NaHCO$_3$ buffered M-199) *(Store at 4 °C)*

L-glutamine	0.292	mg/ml
Bovine serum albumin	4	mg/ml
1M Sodium pyruvate	1	µL/ml
Penicillin	100	IU/ml
Streptomycin	100	µg/ml
rhFSH	0.05	IU/ml
EGF	25	ng/ml

In vitro **fertilization medium** (prepared in Tyrode's salt solution) *(Store at 4 ^{o}C)*

IVF X 100 stock	1	% (v/v)
NEAA	1	% (v/v)
Bovine serum albumin	6	mg/ml
Penicillin	100	IU/ml
Streptomycin	100	µg/ml
Heparin	30	µg/ml

** IVF X 100 stock: 100 mM L-glutamine, 36.3 mM Sodium Pyruvate and 110.89 mM Calcium Lactate were solubilized in 10 ml Tyrode's salt solution (T2397: Sigma)

Synthetic oviductal fluid medium (SOF) – IVC I *(Store at 4 ^{o}C)*

NaCl	107.6	mM
KCl	7.1	mM
$NaHCO_3$	25.0	mM
Na Lactate (60 % w/w)	5.3	mM
$MgCl_2.6H_2O$	0.5	mM
$CaCl_2.2H_2O$	1.7	mM
KH_2PO_4	1.2	mM
MEM NEAA	1	% (v/v)
BME EAA	2	% (v/v)
Glucose	1.5	mM
Penicillin	10^5	IU/L
Gentamicin	50	mg/L
Sodium Pyruvate	0.3	mM
Ala-Gln	2.0	mM
Bovine serum albumin	4.0	g/L
Phenol red	500	µL/L

Synthetic oviductal fluid medium (SOF) – IVC II *(Store at 4 ^{o}C)*

NaCl	107.6	mM
KCl	7.1	mM
NaHCO$_3$	25.0	mM
Na Lactate (60 % w/w)	5.3	mM
MgCl$_2$.6H$_2$O	0.5	mM
CaCl$_2$.2H$_2$O	1.7	mM
KH$_2$PO$_4$	1.2	mM
MEM NEAA	1	% (v/v)
BME EAA	2	% (v/v)
Glucose	1.5	mM
Penicillin	10^5	IU/L
Gentamicin	50	mg/L
Sodium Pyruvate	0.3	mM
Ala-Gln	2.0	mM
FCS	10	% (v/v)
Phenol red	500	µL/L

Synthetic oviductal fluid medium (SOF) – HEPES *(Store at -80 ^{o}C)*

NaCl	107.6	mM
KCl	7.1	mM
KH_2PO_4	1.2	mM
$MgCl_2.6H_2O$	0.5	mM
$NaHCO_3$	25.0	mM
$CaCl_2.2H_2O$	1.7	mM
Na Lactate (60 % w/w)	5.3	mM
Ala-Gln	2.0	mM
HEPES	25.0	mM
Glucose	1.5	mM
Sodium Pyruvate	0.3	mM
Bovine serum albumin	4.0	g/L
MEM NEAA	1	% (v/v)
BME EAA	2	% (v/v)
Penicillin	10^5	IU/L
Gentamicin	50	mg/L

Freezing medium for embryos (prepare in SOF-HEPES) *(Store at 4 ^{o}C)*

FCS	20	% (v/v)
Trehalose	0.125	M

Thawing solution for embryos (prepare in SOF-HEPES) *(Store at 4 ^{o}C)*

FCS	20	% (v/v)
Trehalose	0.25	M

APPENDIX B

Paper publications

1. Tharasanit, T., Manee-In, S., Buarpung, S., Chatdarong, K., Lohachit, C. and Techakumphu, M. 2011. Successful pregnancy following transfer of feline embryos derived from vitrified immature cat oocytes using 'stepwise' cryoprotectant exposure technique. Theriogenology. 76(8):1442-1449.

2. Buarpung, S., Tharasanit, T., Comizzoli, P. and Techakumphu, M. 2012. Effects of cold storage on plasma membrane, DNA integrity and fertilizing ability of feline testicular spermatozoa. Anim Reprod Sci. 131 (3-4): 219–227.

3. Tharasanit, T., Buarpung, S., Manee-In, S., Thongkittidilok, C., Tiptanavattana, N., Comizzoli, P. and Techakumphu, M. 2012. Birth of kittens after the transfer of frozen-thawed embryos produced by intracytoplasmic sperm injection with spermatozoa collected from cryopreserved testicular tissue. Reprod Domest Anim. 47 Suppl 6: 305-308.

4. Buarpung, S., Tharasanit, T., Comizzoli, P. and Techakumphu, M. 2013. Feline spermatozoa from fresh and cryopreserved testicular tissues have comparable ability to fertilize matured oocytes and sustain the embryo development after intracytoplasmic sperm injection. Theriogenology. 79 (1):149-158.

Submitted paper

Buarpung, S., Tharasanit, T., Thongkittidilok, C., Comizzoli, P. and Techakumphu, M. Spermatozoa isolated from cat testes retain their structural integrity as well as a full developmental potential after refrigeration for up to 7 days. (Submitted to Animal Reproduction Science)

Paper in preparation

Buarpung, S., Tharasanit, T., Manee-In, S., Thongkittidilok, C., Tiptanavattana, N., Comizzoli, P. and Techakumphu, M. *In vitro* and *in vivo* development of frozen embryos produced by ICSI with cryopreserved testicular spermatozoa

Proceeding and poster presentations

1. Buarpung S., Tharasanit T. and Techakumphu M. Testicular sperm extraction and cold storage of testicular tissue in domestic cat. The 15th Congress of the Federation of Asian Veterinary Associations, Thailand. 27 - 30 October 2008.

2. Buarpung S., Tharasanit T. and Techakumphu M. Viability and mitochondrial activity of testicular sperm after cold storage in domestic cat. The fifth annual meeting of the Asia Reproductive Biotechnology Society, Kunming, Yunnan Province, China. 27 November - 1 December 2008.

3. Buarpung S., Tharasanit T. and Techakumphu M. Viability and DNA integrity of chilled testicular sperm in domestic cat. The sixth annual meeting of the Asia Reproductive Biotechnology Society, Seam Reap City, Cambodia. 16 - 20 November 2009.

4. Buarpung S., Tharasanit T. and Techakumphu M. Viability and DNA integrity and fertilizability of cat testicular sperm after 7 Day-cold storage. The 13th Association of Institutions for Tropical Veterinary Medicine Conference, Bangkok. 23 - 26 August 2010.

5. Buarpung S., Tharasanit T. and Techakumphu M. Successful fertilization following intracytoplasmic injection of cat testicular sperm after cold storage. The 7th Annual conference of the Asian Reproductive Biotechnology Society, Kuala Lumpur, Malaysia. 8 - 10 November 2010.

6. Techakumphu M., Buarpung S. and Tharasanit T. The effect of freezing techniques on quality of cat testicular sperm. The 37th Annual Conference of the IETS. Orlando, Florida, USA. 8 - 12 January 2011.

7. Buarpung S., Tharasanit T. and Techakumphu M. *In vitro* embryo development after intracytoplasmic sperm injection (ICSI) with sperm derived from frozen-thawed cat testicular tissue. The 1st Joint Symposium of Thai and Japanese Societies for Animal Reproduction. Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. 29 - 30 September 2011.

8. Buarpung S., Tharasanit T., Thongkittidilok C., Comizzoli P. and Techakumphu M. *In vitro* development of feline embryos after intracytoplasmic sperm injection with 7 day refrigerated testicular sperm. The 17th International Congress on Animal Reproduction. Vancauver, British Columbia, Canada. 29 July - 2 August 2012.

Oral presentations

1. Testicular tissue storage in domestic cats: Model application in wild felid. The 1st Wildlife ARTs Workshop 2010; Eld's Deer Model. Khao Kiew Open Zoo, Chonburi Province, Thailand. 26 - 31 March 2010.

2. Preservation of cat testicular tissue: A Model for Endangered Species Conservation. RGJ Seminar Series LXXX "Innovations for Animal Health and Production II". Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. 20 April 2011.

3. Kittens born following transfer of frozen-thawed embryos produced by intracytoplasmic sperm injection using spermatozoa recovered from cryopreserved testicular tissue. The 7th International Symposium on Canine and Feline Reproduction. Whistler, British Columbia, Canada. 26 - 29 July 2012.

4. Preservation of Testicular Spermatozoa and Embryo Production by Intracytoplasmic Sperm Injection in Domestic Cats. The 14th RGJ-PhD congress. Jomtien Palm Beach Hotel and Resort, Pattaya, Thailand. 5 - 7 April 2013.

VITAE

Miss Sirirak Buarpung was born on April 25th 1981 in Bangkok province, Thailand. She graduated with Degree of Doctor of Veterinary Medicine (D.V.M.) with the 2nd honour from Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand in 2004. She worked as a small animal veterinarian at a private animal hospital for 3 years before enrolled a Master program at the Department of Obstetrics Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand in 2007. In 2008, she transformed to a Ph.D. program as she received a scholarship from the Royal Golden Jubilee PhD program of Thailand Research Fund. She is interested in gamete preservation and embryo production by assisted reproductive techniques (ARTs) for wild animal conservation, especially felid species.