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CRYOPRESERVATION, CULTURE AND XENOTRANSPLANTATION OF DOMESTIC CAT OVARIAN TISSUES IN NUDE MICE

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Theriogenology
Department of Obstetrics Gynaecology and Reproduction
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Field of Study	Theriogenology
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การประยุกต์ใช้เทคนิคการเก็บรักษาความสมบูรณ์พันธุ์ของสัตว์ป่าที่ตายกะทันหันในภาคสนามนั้นถือเป็นความท้าทายในการเก็บรักษาพันธุกรรมหรือเซลล์สืบพันธุ์ที่มีคุณค่าของสัตว์ป่าหายากเหล่านั้น การเก็บรักษาอวัยวะที่สร้างเซลล์สืบพันธุ์ร่วมกับการใช้เทคโนโลยีชีวภาพทางระบบสืบพันธุ์นั้น ถือว่าเป็นเครื่องมือที่มีประสิทธิภาพในการนำเอาเซลล์สืบพันธุ์จากอวัยวะเหล่านั้นออกมาทำการเจริญจนถึงระยะที่สามารถนำไปปฏิสนธิได้ การเก็บรักษาชิ้นเนื้อรังไข่แช่แข็งนั้นก็ถือว่าเป็นวิธีการเก็บรักษาความสมบูรณ์พันธุ์เพียงวิธีเดียวที่สามารถใช้ได้ในสัตว์เพศเมียที่ยังไม่ถึงวัยเจริญพันธุ์รวมถึงสัตว์ที่ตายกะทันหันในภาคสนามอีกด้วย วิทยานิพนธ์ฉบับนี้มีวัตถุประสงค์เพื่อศึกษาประสิทธิภาพของการแช่แข็ง การเพาะเลี้ยง และการปลูกถ่ายเนื้อเยื่อวิวิธพันธุ์รังไข่แมวบ้านเพื่อเป็นตัวแทนของสัตว์ป่าตระกูลแมว จากการศึกษาผลของการเพิ่มซูโครสลงในน้ำยาแช่แข็งชิ้นเนื้อในความเข้มข้นต่างๆ (0, 0.1, 0.3 โมลาร์) ต่อค่าการมีชีวิตรอดของเซลล์, ลักษณะทางจุลกายวิภาคและความสมบูรณ์ของดีเอ็นเอ รวมทั้งการแสดงออกของโปรตีนแกปจังค์ชัน คอนเนกซิน 43 พบว่าเนื้อเยื่อรังไข่ที่แช่แข็งแบบช้าที่มีซูโครส 0.1 หรือ 0.3 โมลาร์อยู่ในน้ำยาแช่แข็งมีฟอลลิเคิลที่มีชีวิตรอดและมีลักษณะปกติเป็นสัดส่วนที่มากกว่าเนื้อเยื่อแช่แข็งที่ไม่มีซูโครสในน้ำยาแช่แข็ง และมีฟอลลิเคิลที่มีความสมบูรณ์ของดีเอ็นเอสูงกว่าอีกด้วย ($P < 0.05$) การเปรียบเทียบวิธีการแช่แข็งเนื้อเยื่อรังไข่ระหว่างการแช่แข็งแบบช้าและแบบวิทริฟิเคชั่นพบว่า กลุ่มที่ใช้การแช่แข็งแบบช้านั้นมีฟอลลิเคิลที่มีชีวิต, ลักษณะปกติ, และมีความสมบูรณ์ของดีเอ็นเออยู่ในเนื้อเยื่อรังไข่มากกว่าเนื้อเยื่อที่ผ่านการแช่แข็งแบบวิทริฟิเคชั่น ($P < 0.05$) จากนั้นได้ศึกษาผลของการใช้สาร FCCP หรือ carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone ซึ่งเป็นสารไมโตคอนเดรียลอันคูปลิงซึ่งสามารถลดกิจกรรมของไมโตคอนเดรียโดยให้ชิ้นเนื้อสัมผัสกับสารนี้ก่อนจะนำไปแช่แข็ง พบว่าชิ้นเนื้อรังไข่ที่สัมผัสกับสารนี้ในความเข้มข้น 200 นาโนโมลาร์ เป็นเวลา 120 นาทีนั้นสามารถคงความมีชีวิตรอดและลักษณะของฟอลลิเคิลที่ปกติรวมทั้งดัชนีการเพิ่มจำนวนเซลล์ (Ki-67) ได้ในการเลี้ยงชิ้นเนื้อรังไข่นานถึง 7 วัน อย่างไรก็ตามไม่พบผลกระทบเชิงบวกต่อคุณภาพหลังการแช่แข็งและละลายของเนื้อเยื่อรังไข่ การศึกษาผลของปลูกถ่ายเนื้อเยื่อวิวิธพันธุ์รังไข่แมวบ้านแช่แข็งในหนูไร้ขนที่มีภูมิคุ้มกันบกพร่องเป็นเวลา 15 วันต่อการเปลี่ยนแปลงทางจุลกายวิภาคของเนื้อเยื่อรังไข่ พบว่าเนื้อเยื่อรังไข่แช่แข็งหลังการปลูกถ่ายนั้นมีร้อยละของไพรมอเดียลฟอลลิเคิลที่มีลักษณะทางจุลกายวิภาคที่ปกติลดน้อยลงเหลือ 1.8 ± 2.6% ($P < 0.05$) จาก 57.9 ± 11.8% หลังการละลายชิ้นเนื้อแต่ยังไม่ปลูกถ่าย นอกจากนั้นยังพบว่าในไพรมารีฟอลลิเคิลนั้นก็มีร้อยละของฟอลลิเคิลที่มีลักษณะทางจุลกายวิภาคปกติลดเหลือ 2.8 ± 3.4% ($P < 0.05$) จาก 54.1 ± 11.7% ด้วยเช่นกัน นอกจากนี้พบว่าการปลูกถ่ายเนื้อเยื่อช่วยให้ร้อยละของพรีแอนทรัลฟอลลิเคิลที่เจริญนั้นเพิ่มมากขึ้นในเนื้อเยื่อที่ไม่ผ่านการแช่แข็ง แต่ในเนื้อเยื่อรังไข่แช่แข็งก็ไม่พบการเพิ่มขึ้นแต่อย่างใด การแช่แข็ง การเพาะเลี้ยง และการปลูกถ่ายเนื้อเยื่อวิวิธพันธุ์รังไข่แมวบ้านมีความเป็นไปได้ที่จะใช้เป็นวิธีการเก็บรักษาเซลล์สืบพันธุ์เพศเมียและสามารถเจริญพรีแอนทรัลในเนื้อเยื่อรังไข่สัตว์ตระกูลแมวได้

ภาควิชา	สูติศาสตร์-เธนุเวชวิทยาและวิทยาการสืบพันธุ์	ลายมือชื่อนิสิต .....
สาขาวิชา	วิทยาการสืบพันธุ์สัตว์	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
ปีการศึกษา	2558	ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORDS: DOMESTIC CAT / OVARIAN TISSUE / CRYOPRESERVATION / IN VITRO TISSUE CULTURE / CARBONYL CYANIDE 4-(TRIFLUOROMETHOXY) PHENYLHYDRAZONE (FCCP) / XENOTRANSPLANTATION

NAE TANPRADIT: CRYOPRESERVATION, CULTURE AND XENOTRANSPLANTATION OF DOMESTIC CAT OVARIAN TISSUES IN NUDE MICE. ADVISOR: ASSOC. PROF. DR. KAYWALEE CHATDARONG, DVM, MSc, PhD, CO-ADVISOR: DR. PIERRE COMIZZOLI, DVM, PhD, 108 pp.

Fertility preservation of endangered wild animals that decease unexpectedly is concerned as a challenge for preserving the high valuable genetic materials within the gonads to be future restore into offspring using ARTs. Ovarian tissue cryopreservation is the option for fertility preservation in prepubertal animals or animals that decease unexpectedly. The present thesis aims to investigate the potential of ovarian tissue cryopreservation, *in vitro* culture and xenotransplantation of domestic cat as a model for other wild felid species. Firstly, different sucrose supplementations (0 M, 0.1 M and 0.3 M) in the standard freezing medium were investigated for the effects on the follicular viability, follicle morphology, DNA integrity and gap-junction protein (Cx43) expression. Ovarian tissue slow frozen using 0.1 or 0.3 M sucrose showed better follicular viability, morphology, and fewer DNA damaged follicles than the control group ($P < 0.05$). Next, the cryopreservation methods were compared between slow-freezing and vitrification protocols. Slow-freezing groups showed a better quality of follicular viability, morphology, and less DNA damage follicles than vitrification group ($P < 0.05$). To achieved a better quality of ovarian tissue obtained from the fields, FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone), a mitochondrial uncoupling agent, have been studied for pre-exposing of this substance before *in vitro* culture of ovarian tissue in order to lower the mitochondrial activity. Ovarian tissues incubated with 200 nM FCCP for 120 min showed a protective effect on the follicular viability, morphology, and proliferation index (Ki-67) after *in vitro* tissue culture up to 7 days. However, this beneficial effect of FCCP pre-exposure, was absence after tissue freezing and thawing. Xenotransplantation of the cryopreserved ovarian tissue into the immunodeficient animal (nude mouse) was studied for histological changes within the grafted ovarian tissue after 15 days of transplantation. Percentage of morphologically normal primordial follicle of the cryopreserved graft was lower (1.8 ± 2.6%; $P < 0.05$) than the cryopreserved tissue before transplantation (57.9 ± 11.8%). Additionally, the normal primary follicle in the cryopreserved graft was also lower (2.8 ± 3.4; $P < 0.05$) than the cryopreserved tissue before transplantation (54.1 ± 11.7). Xenotransplantation of ovarian tissue still has potential to increase percentage of growing preantral follicles within the fresh tissue but not in cryopreserved tissue. Ovarian tissue cryopreservation and further follicle development i.e, *in vitro* ovarian tissue culture and xenotransplantation of ovarian tissue into nude mouse suggest the possibility to systematically preserve female gamete and develop the preantral follicle in feline species.

Department:	Obstetrics Gynaecology and Reproduction	Student's Signature	.....
		Advisor's Signature	.....
Field of Study:	Theriogenology	Co-Advisor's Signature	.....
Academic Year:	2015		

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During my study, I experienced the urgent needs of the conservation efforts both locally and globally. We cannot argue that earth's creatures are on the brink of a sixth mass extinction. However, if we all work together to protect threatened species and their habitats now, this catastrophe could be prevented or at least delayed until we find another way to live. If you are reading this, it's not too late to step out and do something good for the world we all live in.

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°C	degree Celsius
µm	micrometer
µM	micromolar
ABC	avidin-biotinylated complex
AKT	protein kinase B
AMH	antimüllerian hormone
AMPK	5′-adenosine monophosphate-activated protein kinase
ATP	adenosine triphosphate
ARTs	assisted reproductive technologies
BMP	Bone morphogenetic protein
CAM	calcein acetoxymethyl ester
CL	corpus luteum
CPAs	cryoprotectants
Csp2	Caspase 2
Cx43	connexin 43
DAB	3,3 diaminobenzidine
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DND1	dead end homolog 1
eCG	equine chorionic gonadotropin
EG	ethylene glycol
EGF	epidermal growth factor
ER	estrogen receptor
EthD-1	ethidium homodimer-1

FCCP	carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone
FIGα	factor in the germ line alpha
FOXO3	forkhead box O3
FSH	follicle stimulating hormone
FSHR	follicle stimulating hormone receptor
g	gram
GDF9	growth and differentiation factor 9
GENMOD	generalized linear model
GL	grey level
GLM	general linear model
GRBs	genetic resource banks
H&E	hematoxylin and eosin
h	hour/ hours
hCG	human chorionic gonadotropin
IGF1	Insulin-like growth factor 1
ITS	insulin transferrin selenium
IUCN	International Union for Conservation of Nature and Natural Resources
IVM	*in vitro* maturation
KITL	KIT ligand
LHR	luteinizing hormone receptor
M	molar
mM	millimolar
min	minute/ minutes
mm	millimeter
mTORC1	mammalian target of rapamycin complex 1
NANOS3	nanos homolog 3
NGF	nerve growth factor
nM	nanomolar
NPAR1WAY	nonparametric one-way analysis of variance

Oct-4	octamer-binding transcription factor 4
OD	optical density
PI3K	phosphatidylinositol 3 kinase
PROH	propylene glycol
PTEN	Phosphatase and tensin homolog
R123	Rhodamine123
rhFSH	human recombinant follicle stimulating hormone
ROD	relative optical density
ROS	reactive oxygen species
SD	standard deviation
SEM	standard error of the mean
TBP2	TATA-binding protein 2
TdT	terminal deoxynucleotidyl transferase
TGF	Transforming growth factor
TSC	tumor suppressor tuberous sclerosis complex
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
UNIVARIATE	univariate analysis
v:v	volume per volume
W	Watt/ Watts
wt/vol	weight by volume

CHAPTER I

Introduction

1.1 Importance and rationale

Declining of the forest area in Thailand from 43.21% to 33.6% during the last four decades associated with accelerated rate of natural habitat destruction and poaching resulted in decreasing of wild felid populations (Bristol-Gould and Woodruff, 2006; National Economic and Social Development Board, 2011). Most of the total of 37 species in the Felidae family are classified as threatened or endangered (Wilson and Reeder, 2005; IUCN, 2011). In natural habitat of Thailand, nine species of wild felids are recognized; three species are categorized as endangered (*Panthera tigris*; tiger, *Prionailurus planiceps*; flat-headed cat, and *Prionailurus viverrinus*; fishing cat), two species that are categorized as vulnerable (*Pardofelis marmorata*; marbled cat and *Neofelis nebulosa*; clouded leopard), two species are categorized as near threatened (*Pardofelis temminckii*; asiatic golden cat and *Panthera pardus*; leopard) and the other two species are listed as least concern (*Felis chaus*; jungle cat, *Prionailurus bengalensis*; leopard cat).

Conservation programs in natural habitat seemed to be insufficient for the propagation of small populations as well as for the maintenance of genetic diversity (Wildt et al., 2010; Holt et al., 2014). Moreover, captive breeding programs conveyed in zoos, breeding centers and other fence protected areas are increasingly important. Assisted reproductive techniques (ARTs) have been applied to many of rare species to support the breeding programs with the highest goal to sustain the gene diversity to prevent inbreeding in these species which may cause diseases, congenital defects, susceptibility to infections and reduce the ability of individuals to reproduce (Holt et al., 2014).

The concept of genetic resource banks (GRBs) as a safeguard for endangered species diversity has been raised extensively in the recent years (Santos et al., 2010; Comizzoli and Holt, 2014; Comizzoli, 2015). The GRBs stored biological materials to

further obtain offspring using ARTs such as artificial insemination, *in vitro* fertilization and embryo transfer. Aside semen banking in males, cryopreservation of embryos, oocytes and ovarian tissues provide potential tools to back up the female endangered species. However, to study fertility preservation, domestic cat (*Felis catus*) can be a good model for all other endangered felids that share the same family and also in human (Comizzoli et al., 2010).

To retrieve the oocytes from live animals, the major constraints are the small amount of oocyte that can be collected in each reproductive cycle. Moreover, reproductive pattern of many endangered animals have not been properly understood (Comizzoli et al., 2010). Though superovulation by gonadotropins or exogenous hormones is utilized to obtain a lot of fertilizable follicles, the results are unpredictable and varied among species of endangered felid (Pelican et al., 2006).

When animals decease unexpectedly or undergo sterilization for medical reasons, successful rescue and preservation of their gametes is the last option to restore their fertility. Cat ovaries presented a large pool of preantral follicles, however only 2% of all follicles leave the resting stage of primordial follicles and start growing (Jewgenow and Paris, 2006; Carrijo et al., 2010). Therefore, female gamete recovery from ovarian tissue is advantageous over retrieval of mature oocyte in the aspect of ease of tissue obtaining, age and cycle independence, ovulation induction independence, numbers of eggs per collection and susceptibility to cryopreservation. The ultimate goal of ovarian tissue cryopreservation is to obtain viable follicles within the thawed tissues aiming at growing them to the fertilizable stage. Many studies investigated the two methods of cryopreservation; slow-freezing and vitrification, however the results are still in controversy (Lima et al., 2006; Comizzoli et al., 2009; Carrijo et al., 2010; Amorim et al., 2011; Luvoni et al., 2012).

Slow-freezing using a passive cooling container has been studied in mouse ovarian tissue in order to replace the controlled rate programmable freezing machine (Cleary et al., 2001b) which is hardly obtained in remote areas. In previous study, after cryopreservation, follicles were cultured *in vitro* or transplantation. Ovarian tissue culture has been already successful in mice with healthy offspring by 2-step culturing system (Eppig et al., 1996; O'Brien et al., 2003). However, this method of culture has

not been optimized and thrived in carnivores yet, however *in vitro* culture is still a good method for assessing the developmental competence of the cryopreserved tissue (Green and Shikanov, 2016).

The duration between ovary excision and cryopreservation causes the cellular damages due to the high cellular activity and oxidative stress. Reducing metabolic activity of the cells could be the option for prolonging the viable cells in a short period by applying the cells to metabolic disrupting agent like carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), which is an uncoupler of oxidative phosphorylation in mitochondria can decrease metabolism of the ovarian cells during this prolonged transportation duration either prior to *in vitro* culture or *in vivo* growing of follicles.

Xenotransplantation, transplantation of tissue from one species to another has been utilized to obtain viable female gametes. Immunodeficient mice are often used as recipients. The combination of cryopreservation and xenotransplantation seems to be a promising procedure to reproduce valued animals in the future. Currently, follicular development within ovarian tissue xenotransplants in the immunodeficient mouse recipients has been studied in many species such as the humans, rats, cows, sheep, cats, marmosets, monkeys, elephants, wallabies and wombats. Not only survival of the grafted follicles and stromal cells but also the follicular development to antral follicles after graft harvested has been currently achieved (Aubard, 2003; Santos et al., 2010). To date, the live offspring has been produced by xenografting mouse ovarian tissue in nude rats (Snow et al., 2002). Little is known for folliculogenesis in the cats, assuming that it may require a longer period of follicular development than that in the mice. So far, there are only few studies of domestic cat ovarian tissue xenotransplantation (Gosden et al., 1994b; Bosch et al., 2004; Fassbender et al., 2007)

Taken together, to achieve the success of ovarian tissue cryopreservation, the techniques including gonad transportation, tissue collection and pretreatment, cryopreservation, and development of the gametes within the tissue both *in vitro* and *in vitro* have to be clarified to give a better understanding and translate these techniques to the endangered animals for conservation purposes.

1.2 Keywords

Domestic cat, Ovarian tissue, Cryopreservation, *in vitro* tissue culture, Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), Xenotransplantation

1.3 Literature review

1.3.1 Fundamental cryopreservation

Cryopreservation or storage of specimen to subzero temperatures is divided according to rates of cooling to slow cooling (equilibrium cooling) and vitrification (rapid cooling or non-equilibrium cooling) (Shaw and Jones, 2003). Both of them share the common schemes as: (1) addition of cryoprotective agents (CPAs), (2) cooling (usually to -196°C in liquid nitrogen), (3) warming, and (4) removal of CPAs. Samples subject to freezing may receive damages during the cooling or warming procedure. This is recognized as cryoinjury. There are three major cryoinjuries which are: excessive cell dehydration from osmotic stress, intracellular ice formation, and CPA toxicity.

Osmotic equilibrium is the balance of concentration of any solution inside and outside of the cell. When water moves into or out of the cell, it leads to the changes in the concentration of the solution outside the cell and permeability of the membrane. There are two patterns of the moves of the water through the lipid bilayer membrane, passive diffusion and active transport via intramembranous proteins which can transport water up to 100 fold than passive transport (Verkman et al., 1996). Furthermore, when temperature changes, the permeability of the cell membrane changes also (Elmoazzen et al., 2002). Increased temperature leads to higher permeability. Swelling or shrinkage of cell due to osmotic dissimilarity between inside and outside of the cell is defined as osmotic stress. If the swelling or shrinkage of the cell is at the high level, it might lead to major or permanent injury of the cell or even cell death.

The cooling rate is one of the critical factors that influences cell survival while cryopreservation, because it controls the transport of water across the cell membrane.

As a result, by controlling the osmolality of the surrounding fluid, the cooling rate also influences the rate at which water is moved out of the cells during cooling and into the cells during warming (Pegg, 2007). Post-thaw survival curve versus cooling rate presented in the inverted U shape (Figure 1).

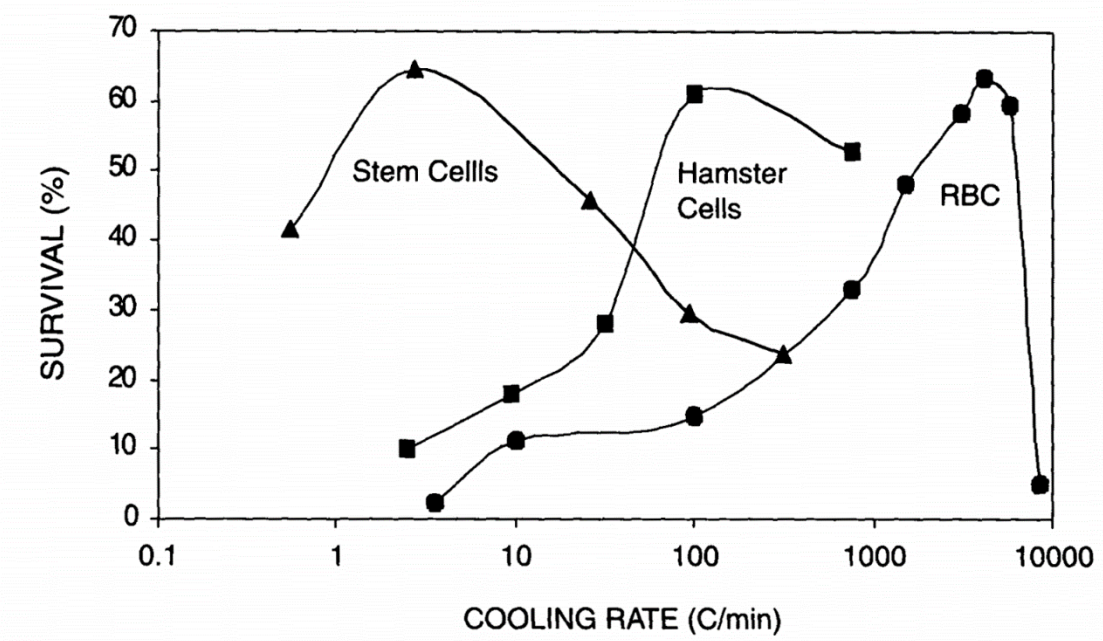

Figure 1. The effect of cooling rate on the survival of various types of cells following freezing. This figure is reprinted with permission from Gao and Critser (2000).

CPAs are chemical substances which modify the formation of ice crystal in the cryopreservation process and control dehydration of the cells during cooling process. There are two types of CPAs: non-permeable and permeable CPAs. Non-permeable cryoprotectants do not cross the cell membrane while permeable CPAs do. Non-permeable CPAs are commonly large polymers which can solute in water and increase osmolality of the solution. Disaccharides such as sucrose, glucose, fructose, sorbitol and trehalose are the most frequently used non-permeable CPAs. Other CPAs are macromolecules such as polyvinilpyrrolidone (PVP), polyvinyl alcohol and Ficoll. The objective of addition of the macromolecules is to dehydrate the cell, protect the osmotic stress and reduce the toxicity of the permeable CPAs.

The other type of CPA is the permeable CPA. The permeable CPAs are small molecules with hydrophilic attribute. CPA permeation rate and dilution depends on species, cell type, developmental stage of the gametes or embryo, temperature and hydrostatic pressure (Shaw and Jones, 2003). The addition of permeable CPAs was aimed to lower the freezing point of the cryopreservation by replacing some of bound water molecules with the CPAs. The permeable CPAs also stabilize cytoplasmic and cell membrane proteins (Gao and Critser, 2000). When the CPAs cross the membrane into the cytoplasm, the concentration of the electrolytes is lowered because the amount of ice crystal formation at a given temperature is diminished (Pegg, 2007). The permeable CPAs which commonly used are dimethylsulphoxide (DMSO), propylene glycol (PROH), ethylene glycol (EG), and glycerol (Shaw and Jones, 2003).

Slow-freezing or equilibrium cooling is a procedure which balances the rate of water loss from the cell and the rate at which the extracellular water incorporated into an ice crystal. Intracellular ice formation is a cause of cryodamage that could be avoided by the optimal slow-freezing procedure. This intracellular ice formation is reduced by initiation of extracellular ice formation (seeding) at high subzero temperature (usually about -5 to -9 °C). After seeding, the extracellular ice draws water out of the cell until little amount of free water left in the cell results in only minimal non-lethal ice crystal formation left inside the cell (Shaw et al., 2000). The extracellular ice formation growth rate depends on the freezing rate. The optimal freezing rate can equilibrate between the dehydrate rate of the water from the cell and the extracellular ice formation rate. For human oocytes and embryos, the optimal cooling rate is around 0.3-1°C/min and for mouse oocytes, the rate is lower than 1°C/min (Shaw and Jones, 2003; Mazur et al., 2005).

The rapid non-equilibrium cooling procedure known as vitrification can eliminate both intra- and extracellular ice formation by exposing the samples with high concentration of CPAs. These CPAs strongly interact with the water, preventing water molecules from ice crystal formation. Vitrification protocol is also different from the slow-freezing in two major aspects: 1) most dehydration and CPAs permeation take

place before cooling procedure begins and 2) cooling ramp is performed in only one step, typically decrease the temperature from more than 0°C to very low subzero temperature in single quick step by plunging the sample into liquid nitrogen or using liquid nitrogen slush. Normally, the cooling rate is around 200 to 20,000°C/min depends on the container, volume, thermal conductivity, solution composition (Shaw and Jones, 2003; Yavin and Arav, 2007). The concept of vitrification is to pass the sample through the critical temperature zone (for instance, the oocyte critical range is 15 to -5°C) where the cells are most sensitive from chilling injury (Liebermann et al., 2002). This results in no ice crystal formation in the successful vitrification processes as mentioned above. Instead, the solution and sample are solidified in a vitreous (glass-like) state throughout cooling and warming stages (Shaw and Jones, 2003). To improve the vitrification protocol, there are three major factors to be concerned: cooling rate, viscosity of the medium, and sample volume (Saragusty and Arav, 2011). Many modifications of vitrification protocol have been developed to fix these three factors such as open pull straw (Vajta et al., 1998), Cryotop (Hamawaki et al., 1999), solid surface (Dinnyes et al., 2000), novel needle immersion methods (Wang et al., 2008; Saragusty and Arav, 2011). Vitrification has recently been considered the method of choice for the whole organ cryopreservation (Fahy et al., 2006). Taken together, vitrification uses less equipment, fewer amounts of liquid nitrogen, is simple to perform and applicable to many biological systems.

1.3.2 Ovarian tissue cryopreservation

Ovarian tissue cryopreservation has long been studied in laboratory animals to maintain the genetic of the specialized animal breed. Successful cryopreservation of mouse ovaries with 1.4 M of DMSO and orthotopic transplantation of both fresh and cryopreserved ovaries were evaluated. This technique was proven successful when return in reproductive cycle was found both fresh and cryopreserved ovarian tissues after orthotopic transplantation (Harp et al., 1994).

Sucrose concentration was also found influencing the quality of cryopreserved human oocytes. Many studies found beneficial effects of high sucrose concentration

as non-permeable CPA on quality of thawed oocytes (Chen et al., 2004; Coticchio et al., 2006; Bianchi et al., 2007; Marsella et al., 2008). It was suggested that high concentration of sucrose promoted the survival of the cryopreserved immature oocyte significantly and also increased the further matured oocytes proportion (Chen et al., 2004). Increasing in sucrose concentration generates an osmotic gradient across the cell membrane that dehydrates the cell sufficiently prior and during cryopreservation process.

In clinical aspects, cryopreservation and transplantation of ovarian tissue are the methods of fertility preservation and restoration that have been recently studied because they are considered the option for the fertility preservation in premature ovarian failure (POF) patients. Chemo- and radiotherapy treatments in the female patients resulted in detrimental effects on the ovaries. Their follicle pools are destroyed, leading to POF and loss of fertility (Larsen et al., 2003; Angarita et al., 2016). Moreover, benign diseases such as hematologic and auto immune diseases that become life-threatening need treatment with gonadotoxic agents or radiation result in ovarian function interruption. Though embryo and oocyte cryopreservation are the standard for infertility treatment, the time required for ovarian stimulation before egg collection excludes these options in some cases. (Donnez et al., 2006; Angarita et al., 2016). Embryo cryopreservation requires the patient to be puberty, have a male partner or use donor sperm, and can response to ovarian stimulation, which is not possible when the chemotherapy has to be done instantaneously. Therefore, ovarian tissue cryopreservation is the only option available for prepubertal patients, and for anyone who cannot postpone the initiation of chemotherapy. In endangered animals, the concept of ovarian tissue cryopreservation is also applied to establish the GRBs. The ovarian tissue obtained from surgical remove or post-mortem can be used to preserve the valuable genetic before using ARTs (Comizzoli and Wildt, 2013).

1.3.3 Gap junction protein connexin 43 in the ovary

Gap junctions are the intercellular membrane channels which allow direct communication between adjacent cells sharing small molecules. Gap junction also

plays important roles in various physiological processes. In developing follicles, gap junctions couple the oocytes and their neighboring follicular cells into a functional syncytium. The gap junctions that are crucial in the folliculogenesis and oogenesis consist of a protein called connexin (Cx) 43 (Gershon et al., 2008). Reports of ovarian gap junctions indicated that Cx43 was indispensable for folliculogenesis in various species (Kidder and Mhawi, 2002; Gershon et al., 2008). Since cytoplasmic maturation of the oocyte cannot be completed without essential molecules produced by the follicle cells, Cx43, main gap junction protein among granulosa cells is critical in follicular development process. Cell communication among granulosa cells was impaired in cryopreserved ovarian tissue that had been transplanted subcutaneously (Lee et al., 2008). The impairment was suggested to be the cause of either poor development of follicles or increase in apoptosis within grafts.

1.3.4 Preantral follicular development and regulation

Mammalian female gamete, oocyte and follicle, development starts with the shortly after pregnancy and terminates with the ovulation of the fertilizable metaphase II oocyte. This long process of oocyte development is dependent on, and is cooperating with the follicular granulosa cells. Additionally, it requires various complex regulatory mechanisms associated with both extrinsic factors (endocrine) and intrinsic signaling pathways in the follicle stage-linked pattern (Figure 2). For the intrinsic pathway, various peptides locally produced from oocyte, granulosa cell, theca cell, and ovarian stroma appear to be the follicle growth activators or suppressors via the autocrine and paracrine effects (Kidder and Mhawi, 2002). In addition, effective communication between the different follicular cell types is completed with homologous and heterologous gap junctional contacts (Picton et al., 2007). Furthermore, follicle activator supplementation such as insulin-transferrin-selenium (ITS) which commonly used in many follicle culture systems also expressed beneficial effects on preantral follicle survival and growth (Demeestere et al., 2005).

Transition of primordial follicle to primary follicle is gonadotropin-independent stage and majorly influenced by intraovarian factors. It was suggested from the previous

studies in animal models that several members of the transforming growth factor-beta (TGF-beta) superfamily, such as BMP-4 and BMP-7 (expressed on ovarian stromal cells and/or theca cells) and GDF-9 (expressed on oocytes) play a critical role on the transition of primordial follicles (Oktem and Urman, 2010). In contrast, some inhibitory signals maintained primordial follicle in the dormant stage. These signals are tumor suppressor tuberous sclerosis complex I (TSC-I), phosphatase and tensin homolog deleted on chromosome 10 (PTEN), FOXO3, and AMH (Sánchez and Smitz, 2012).

Further development of primary follicle to early antral stage needs different factors to complete the processes that the major morphological changes in this stage are the proliferation and transformation of granulosa cells of single-layered primary into multi-layer of secondary follicles. In this stage, an increase in oocyte diameter, formation of basal lamina, zona pellucida, and theca cell layer are characterized. Follicular stimulating hormone plays an important role in this gonadotropin dependent period. Although, FSHR is found first expressed in this stage, the role of FSH is still unclear. In previous study, FSH has been shown to enhance the survival of ovarian graft transplanted into immune-deficient mice (Oktay et al., 1998). However, the study of McGee et al. (2001) suggested that FSH may have a permissive role, or synergized positive effect with other intra-ovarian regulators in serum, rather than being essential in preantral follicle growth itself. Preantral follicle development is a complex and regulated process. Factors and signaling that regulating early follicle development and survival seem to be different in smaller follicles than in more differentiated antral and preovulatory follicles. The factors involved in this gonadotropin dependent stage are, for instance, GDF-9, IGF1, BMP6, BMP15, activing, inhibin, Thyroxine (Figure 2). These factors are still needed an Increased understanding about the growing follicles regulation of growing follicles.

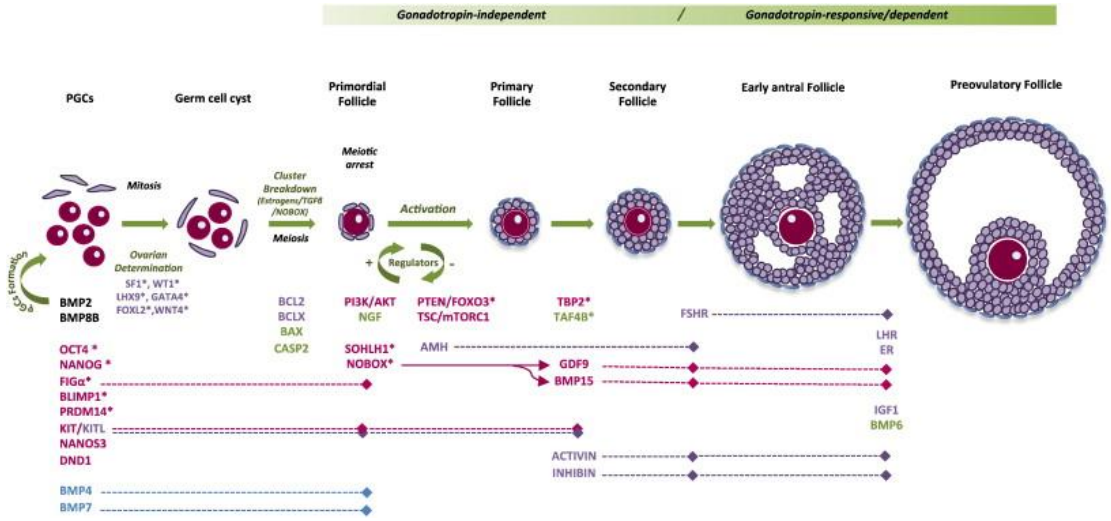

Figure 2. Schematic representation of the factors involved in primordial germ cell (PGC) formation, folliculogenesis and oogenesis. Some key growth regulators of somatic and oocyte origin are demonstrated. Ovarian factors produced by theca/stromal cells (in blue), somatic/granulosa cells (in purple), germ cells (in red) or in both germ cell and granulosa cell (green), participate and regulate oocyte and follicle development at each of the defined stages throughout folliculogenesis. Transcription factors involved are indicated with an asterisk (*). Proteins from the extra embryonic ectoderm that participate in PGC formation are indicated in black. This figure is reprinted with permission from Sánchez and Smitz (2012).

. 1.3.4 Ovarian tissue *in vitro* culture and isolated follicle culture

Live births from mouse oocyte were obtained by culturing preantral follicle totally *in vitro* (Eppig et al., 1996). This demonstrated the feasibility of *in vitro* growing of oocyte from primordial follicle stage to fertilizable stage by using 2-step culture system. Briefly, the newborn mouse ovaries were cultured in organ culture system for 8 days and then isolated the oocyte-granulosa cell complexes were further culture *in vitro* for an additional 14 days. This study gave rise to the following studies about in vitro growth of primordial follicles in other species (Wandji et al., 1996; Wandji et al., 1997). Subsequent study of improved the culture system successfully produced 72 pups from 1160 transferred embryos (O'Brien et al., 2003). However, these techniques work well only in mice since the mice follicles provided a uniform population of

primordial follicles within ovaries during the first few days after birth. Furthermore, the size of the mouse ovary is suitable for organ culture and enzymatic follicle isolation. Unlike mouse, in other mammals, formation of follicles occurs during fetus over a number of weeks and the ovaries are too large to use organ-culture system.

Furthermore, ovarian stroma is too tough to undergo enzymatic isolation without damage to oocytes (Wandji et al., 1996). Therefore, *in vitro* culture of ovarian tissue was developed for culturing only cortical part of the ovary (Smitz et al., 2010).

Ovarian tissue culture in bovine and baboon resulted in activation of the follicle from primordial stage to primary stage within 2 days of culture (Braw-Tal and Yossefi, 1997; Wandji et al., 1997). The fetal ovarian cortex was used because of its small size which can easily be cultured, secondly, the stroma is not tough compared to adult ovary and the fetal ovary has not yet under massive follicular depletion like adult ovary.

Human primordial follicles cultured in loosened cortical pieces were developed to secondary follicle stage in 6 days (Telfer et al., 2008) (Figure 3). Moreover, these grown follicles have potential to develop the antrum if isolated and cultured singly for further 4 days after tissue culture. Activin A and FSH were also found to be the key to the follicular development in the second step. This study showed the complete *in vitro* growth of human preantral follicle to antral follicle for the first time in a total period of 10 days. This accelerate growth rate might affect the subsequent development or fertilization process that still need to be clarified.

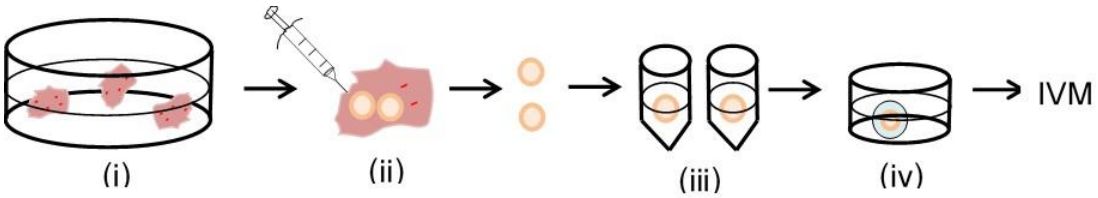

Figure 3. Flow chart of in vitro follicle culture steps including (i) ovarian cortical strips culture (ii) removal of preantral follicles (iii) individually culture and monitored for oocyte/follicle health marker (iv) finalize the follicular growth by removing the

oocyte granulosa cell complexes for placement in alginate bead or membrane then further in vitro maturation (IVM) the oocyte (Telfer et al., 2008).

Importance of various hormones and growth factors such as FSH, LH, insulin, serum, epidermal growth factor (EGF), insulin transferrin selenium (ITS), antimüllerian hormone (AMH), growth and differentiation factor 9 (GDF9), and activin A were tested in the culture of fresh and cryopreserved human ovarian tissue. FSH, insulin activin A and GDF 9 were proved to be the follicular development and survival promotor *in vitro*. In contrast, AMH inhibited primordial follicular activation (Smitz et al., 2010).

1.3.6 Pre-exposure of metabolic uncoupling agents

The time lapse between the ovarian excision and the cryopreservation generally causes cellular damages due to ischemia and oxidative stress. Pre-exposure of the tissue or cells by a mild uncoupling of the mitochondrial membrane potential has been found to involved in reactive oxygen species (ROS) production. The weak acid protonophore, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), provides reversible uncoupling proton motive force in a dose-dependent pattern (Scott and Nicholls, 1980). Proton motive force in electron transport chain is required for ATP synthase to produce ATP through the oxidative phosphorylation process in mitochondria. When FCCP is added to the cells or mitochondria, FCCP could depolarize the mitochondrial membrane potential by pumping the proton through the inner mitochondrial membrane, this is called 'membrane depolarization.' Without a proton gradient, ATP synthesis through the F1/F0-ATPsynthase is interrupted. This phenomenon results in lower metabolic activity and may exert the beneficial effect on the tissue that has to be lowered the metabolic activity such as in neuronal cells or cardiac cells that injured from any exitotoxic stimuli (Brennan et al., 2006a; Brennan et al., 2006c; Weisova et al., 2012).

1.3.7 Transplantation of fresh ovarian tissue

The concept of ovarian tissue transplantation is to resume the folliculogenesis with in the excised ovarian tissue. Techniques of transplantation have been studied in

many aspects in order to promote the graft survival and folliculogenesis resumption to obtain the fertilizable oocyte to produce the offspring.

Study on ovarian tissue transplantation was first carried out in the mouse 50 years ago. The fresh whole and hemi-ovary were grafted to the other mice in the same strain. Approximately, 65% of oocytes were lost after transplanted due to surgical trauma and ischemic period. Fertility was proven after transplanted. However, the grafted mice had smaller litter size than the control non-grafted intact group (Mussett and Parrott, 1961).

The other animal model that was frequently used in transplantation studies was a rat. The fresh ovarian tissue subcutaneously autotransplanted in the ovariectomized female rats was found 90% reproductive cycle restoration (Harris and Eakin, 1949). Moreover, follicles and corpora lutea were found within the graft. There are plenty of rat ovarian tissue both whole ovary and ovarian tissue fragment studies afterwards (Sugimoto et al., 2000; Sapmaz et al., 2003; Dorsch et al., 2004; Barros et al., 2008). Apart from experiments in the rodents, the ruminants are the species which have been extensively studied for ovarian tissue transplantation due to similar tissue structure as well as rodents) Gosden et al., 1994a; Arav et al., (2005.

Ovarian tissue can be transplanted back to the original site (orthotopic) or to alternative sites of the body like back muscles or subcutaneously (heterotopic). For each site, considerations such as ease of transplantation procedure, convenient access for oocyte monitoring and collection also the volume of space for transplantation must be taken. Endocrinological study and folliculogenesis were investigated in the heterotopic subcutaneous ovarian transplantation in the sheep. FSH level after transplantation was 4-fold higher than in the non-grafted sheep although some resulted in ovulation failure. Non-grafted sheep also had a lower level of E2 and inhibin compared to the grafted sheep that may cause by small antral follicle depletion after transplantation (Campbell et al., 2000). Apart from heterotopic subcutaneous grafting, orthotopic autotransplantation was used as a model for investigating hormonal influence on follicular development. The accelerated follicular growth rate in ovarian

transplants compared to the normal growth rate in intact ovary was found and suggested to be the result of high levels of LH and FSH.

In 2004, the first offspring of the monkey was obtained after ovarian tissue transplantation and the ovary remained fully function after heterotopic autotransplantation in subcutaneous pockets of forearm and abdomen region in oophorectomized recipient (Lee et al., 2004). Abdominal grafts showed the best follicular development. Vaginal bleeding and reproductive cycle were restored after 5 months. Follicular retrieval was done when 4 mm follicle was observed. Oocytes were fertilized by intracytoplasmic sperm injection (ICSI) and the embryos were transferred to the surrogate mother with resulting in a live birth. It was suggested the arm and abdomen as preferable sites due to the ease of access and monitoring of the graft. In more recent study, retroperitoneal iliac fossa, omentum and subcutaneous tissue of the back were evaluated for the transplantation site of ovarian cortex. MII oocytes have been collected after FSH stimulation from both retroperitoneal and omental transplants. Four out of 5 embryos could develop to morula after ICSI. Noteworthy, mature oocytes could be collected from omental grafts about 2.5 years after transplantation (Igarashi et al., 2010).

Clinical fresh ovarian tissue allotransplantation, transplantation among the same species, has been performed by orthotransplanting the fresh ovarian tissue into ovarian medulla of the recipients in each pair of monozygotic twins (Silber et al., 2008). The one sibling of each pair was undergone spontaneous POF and the other sister was the ovarian tissue donor. After transplantation, menstruations were resuming after 65-100 days with the majority of subsequent cycles in the normal range of duration. Moreover, 5 out of 8 recipients became pregnant. Moreover, ovarian tissue transplantation between non-identical Human leukocyte antigen (HLA)-compatible sisters has been recently published. Allotransplantation without immunosuppressive treatment was performed and the first pregnancy and live birth was obtained after *in vitro* fertilization of oocyte retrieved from the grafts (Silber et al., 2008). From the

studies mentioned above, transplantation of fresh ovarian tissue has potential to restore the fertility of the ovarian tissue donor in various species.

1.3.8 Transplantation of cryopreserved ovarian tissue

Cryopreserved ovarian tissue transplantation is a method of fertility restoration that has been applied in human by autotransplantation (Donnez et al., 2004). However, using this method of fertility preservation in endangered species has to be reconsidered about the tissue recipient. Xenotransplantation, a transplantation of tissue from one species to another, is selected to be the option for these species by transplanting the tissue into the immunodeficient recipient.

Both orthotopic and heterotopic transplantation of frozen ovarian tissue in ovariectomized adult mice using a slow cooling protocol with 1.5 M of DMSO to be the freezing procedure resulted in ovarian cyclicity restoration. In addition, follicles of all developmental stages were found after 2 to 8 weeks of transplantation (Cox et al., 1996). Cryopreserved ovarian tissue xenotransplantation had potential to produce live pups but it had a smaller litter size then fresh tissue did (Gunasena et al., 1997; Sztein et al., 1998). The feasible of vitrified bovine ovarian tissue using EG and DMSO as permeable CPAs showed the high proportion of oocyte survived after warming (Kagawa et al., 2007; Kagawa et al., 2009). The tissue was subcutaneously grafted in the neck or orthotopically to oophorectomized cow. Estrus cycle was restored after 2 months in both groups. Normal histological finding and 95% of viable preantral follicles were found in the cryopreserved grafts compared to fresh group. Their results are encouraging concerning the vitrification method for ovarian cortex vitrification.

Live births were obtained from both fresh and cryopreserved orthotopic autotransplantation in the sheep (Gosden et al., 1994a). The tissues were cryopreserved using the programmable freezer and stored for 3 months. Long term transplantation was found that longevity of the grafts was at least 22 months. However, very few primordial remained in the transplant. FSH and LH basal levels of the graft

group were more than the control group and normal cyclical pattern was presented in transplantation host (Baird et al., 1999).

Ischemic duration after transplantation was the major factor that caused follicular loss. It was investigated by xenotransplanting the cryopreserved ovarian cortical fragments under kidney capsule without vascular anastomosis. Follicular depletion after fresh tissue grafting was around 65% as compared to fresh non-grafted ovarian tissue. While follicular loss of the frozen-thawed slices was around 72%. Therefore, ischemic period rather than cryopreservation process is the major factor of massive follicular depletion after transplantation (Aubard et al., 1999; Baird et al., 1999). Angiogenesis of the ovarian transplant takes time at least 2-3 days after xenotransplantation and normal local oxygen pressure takes 10 days. After this quite long period, many follicles within the tissue may not survive through this critical duration (Martinez-Madrid et al., 2009; Van Eyck et al., 2009). Cryopreserved human ovarian tissue xenotransplanted in immunosuppressive models was able to develop antral follicles with normal histological morphology of the follicles within the grafts (Gook et al., 2001; Maltaris et al., 2007). Furthermore, primordial follicles, vascular and ovarian stroma ultrastructure within the autonomic transplant were found normal after examined by transmission electron microscope. Asynchrony development between oocyte and granulosa cells in the secondary follicles in nude mouse xenotransplantation was demonstrated also (Camboni et al., 2008). This compromising event may come from xenotransplantation. Moreover, abnormal nuclear and cytoplasmic maturation were revealed in similar xenotransplantation approach (Kim et al., 2004).

1.3.9 Ovarian tissue transplantation in domestic cats

The first domestic cat ovarian tissue transplantation was reported a century ago. The cat ovaries were grafted to the other cat. Vascular anastomosis and circulation re-establishment post-transplantation were investigated. However, the function of the transplanted ovaries after surgery was not reported (Carrel and Guthrie, 1906). The concept of xenotransplantation is to mimic the *in vivo* environment of the ovary to

reinitiate the follicular development after transplantation. The effects of gonadotropin treatments were investigated by intraperitoneal injection of human recombinant follicle stimulating hormone (rhFSH) at surgery and on 3 consecutive days, equine chorionic gonadotropin (eCG) in 62 days posttransplantation and hCG 88 h after eCG then the graft was recovered and examined histologically. Vascularization occurred with all developmental stages of follicles within the grafts. However, follicular numbers after graft harvesting were approximately 90% reduced when compared to the fresh control graft. Treatment of gonadotropins can trigger the luteinization signs in granulosa cells of the antral follicles with no ovulation (Bosch et al., 2004). Graft monitoring is an important process of transplantation especially for ovarian tissue so that the antral formation can be early detected and oocytes are picked up at optimal timing. High-resolution (10-22 MHz) transcutaneous ultrasonography was an efficient monitoring tool for the follicular development within mouse ovarian tissues transplanted to nude rats (Fassbender et al., 2007). Ultrasonography-assisted graft monitoring has been shown to detect early antral follicles from the size of 0.4 mm onwards. From the results of these studies, the ovarian tissue xenotransplantation in immunodeficient animal models gave the promising results of antral formation in the grafts (Bosch et al., 2004; Fassbender et al., 2007).

1.4 Thesis objectives

1.4.1 To optimize a freezing protocol for cat ovarian tissue cryopreservation

1.4.2 To reduce the cellular metabolism by FCCP pre-exposure to cat ovarian tissue prior to cryopreservation

1.4.3 To demonstrate *in vivo* development of domestic cat ovarian tissue using xenotransplantation into the nude mice in short period

The diagram of the thesis study design was shown in figure 4.

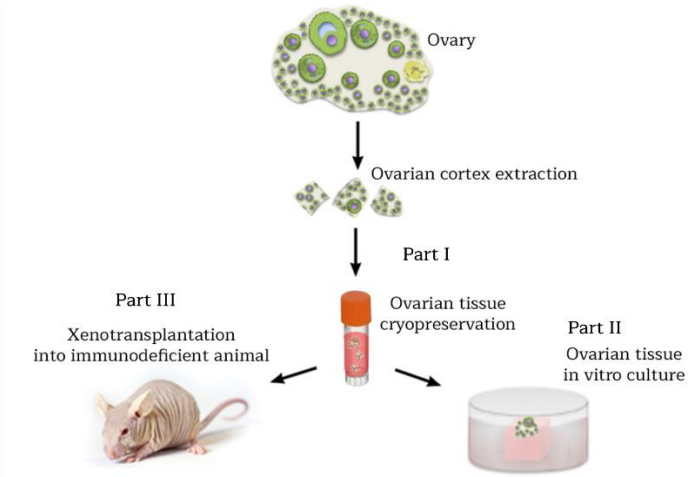

Figure 4. Schematic diagram of the study design in this thesis.

1.5 Thesis hypothesis

1.5.1 Ovarian tissue vitrification method can provide better preantral follicular survival, apoptosis and gap junction protein expression than slow-freezing using a passive cooling container method after cryopreservation.

1.5.2 FCCP preconditioning of ovarian tissue can provide better follicular viability, morphologically normal follicle and proliferation follicle after cryopreservation

1.5.3 Subcutaneously transplantation of domestic cat ovarian tissue in the nude mice can promote the development of domestic cat preantral follicles within the tissue.

1.6 Research merits

1.6.1 The knowledge in the effects of sucrose on domestic cat ovarian tissue cryopreservation and the cryopreservation methods provides additional information on the female fertility preservation in domestic cat.

1.6.2 The knowledge of pre-exposure of metabolic disruption agent, FCCP, with ovarian tissue before *in vitro* culture and cryopreservation which finally may succeed in the better integrity of ovarian tissue after treatments.

1.6.3 Xenotransplantation of cryopreserved ovarian tissue recovered from excised ovary conduct to the feasibility of translation of gamete rescue and follicle development *in vivo* from the domestic animal model to endangered wildlife species.

CHAPTER II

Positive impact of sucrose supplementation during slow freezing of cat ovarian tissues on cellular viability, follicle morphology, and DNA integrity

2.1 Abstract

The objectives of the study were to 1) examine and optimize the impact of sucrose during slow-freezing and 2) compare the results of two freezing methods (slow-freezing and vitrification) on cellular viability (germinal and stromal cells), follicle morphology, DNA integrity and gap-junction protein expression (connexin 43; Cx 43). Different sucrose supplementations (0 M, 0.1 M and 0.3 M) in standard freezing medium were compared before and after slow freezing. Ovarian tissue slow frozen using 0.1 M (4.0 ± 0.4) or 0.3 M (3.9 ± 0.5) sucrose yielded better follicular viability (the viability numbers of positive follicle in ovarian tisse per 0.0625 mm^2) than the group without sucrose (1.9 ± 0.2) ($P < 0.05$). Morphologically normal primordial follicles were higher in the sucrose treated groups (0.1 M, 47.4 % and 0.3 M, 43.5%) than the group without sucrose (0 M, 33.8 %) ($P < 0.05$). Moreover, less apoptotic primordial follicles were found in both sucrose groups (0.1M, 1.2% and 0.3M, 1.9 %) than the group without sucrose (7.7%) ($P < 0.05$). However, their Cx 43 expression showed no difference among the groups of different sucrose concentrations. In terms of the freezing methods used, vitrified ovarian tissues had fewer viable follicles (3.2 ± 0.6) than the slow-freezing method (4.6 ± 0.6) ($P < 0.05$). In addition, the slow freezing resulted in more post-thawed morphologically normal primordial follicles (38.8 % vs. 28.3 %, $P < 0.05$) and less apoptotic primordial follicles (3.8 % vs. 8.9 %, $P < 0.05$) than vitrification. The Cx 43 expression showed no difference between slow freezing and vitrification. The present study demonstrated the positive effects of sucrose supplementation and slow freezing method on the follicular viability, follicular histological appearances of follicles and apoptosis of the follicles and stromal cells in cat ovarian tissue.

2.2 Introduction

Assisted reproductive technologies (ARTs) associated with cryopreservation of germplasms play an important role in conservation of endangered species. However, the success of ARTs in female endangered animals is restricted by the limited number of mature oocytes that can be collected after hormonal stimulation or during post-mortem gamete rescue (Pelican et al., 2006; Jewgenow et al., 2011). Female fertility preservation by ovarian tissue cryopreservation therefore is a powerful strategy that has been explored in mammals since ovarian cortex contains over 99% primordial and preantral follicles of all follicle stages (Jewgenow et al., 1997; Jewgenow and Paris, 2006; Santos et al., 2010). Interestingly, only human live births have been reported from frozen-thawed ovarian pieces using a slow-freezing method (Donnez et al., 2013). However, many factors that affect the success of cryopreservation have yet to be elucidated to minimize the tissue damage after freezing and thawing in different species. Indeed, the follicle survival is a prerequisite for the success of tissue xenotransplantation or follicle culture *in vitro*.

Non-permeable cryoprotective agents (CPAs) are usually macromolecules or saccharides that do not readily cross the cytoplasmic membrane, but instead generate cell dehydration by osmotic pressure which is beneficial to the cell during cryopreservation. Specifically, sucrose is one of the non-permeable CPAs that has been widely used and reported as a membrane protective agent by diminishing the intracellular ice formation during cryopreservation (Fabbri et al., 2001). Many studies also found beneficial dehydration and membrane stabilization effects of high sucrose concentration on the viability or histological appearances of post-thawed immature oocytes in ovarian tissue (Chen et al., 2004; Coticchio et al., 2006; Bianchi et al., 2007; Marsella et al., 2008). In felines, few recent studies have used sucrose for ovarian tissue cryopreservation (Wiedemann et al., 2012; Wiedemann et al., 2013). However, the effect of sucrose as a non-permeable CPA has not been clarified in the domestic cat as in other species.

Previous reports revealed that cryopreservation using conventional slow-freezing procedures led to ovarian stromal cell, granulosa cell and theca cell damage (Siebzehnrubl et al., 2000; Fabbri et al., 2006; Camboni et al., 2008; Amorim et al., 2012). This may be the result of intracellular ice formation, which damages cells that have various cryotolerance as well as the cellular interactions, which could be damaged during freeze-thawing processes. DNA damage is another critical factor, leading to dysfunctional DNA replication and subsequent cellular apoptosis (Maffei et al., 2014). Cryodamaged oocytes within the follicles leads to failure of follicular growth through a loss of intact interactions with the surrounding cells including stromal cells (Carabatsos et al., 2000; Siebzehnrubl et al., 2000; Matzuk et al., 2002; Rodgers et al., 2003; Luyckx et al., 2013a). Thus, the monitoring of gap junction protein and apoptosis of the stromal cells in the cryopreserved ovarian tissues is critical. Lastly, compared to slow-freezing procedure, vitrification is a promising alternative to minimized ice nucleation. In cats, only a few ovarian tissue cryopreservation studies have been performed (Bosch et al., 2004; Lima et al., 2006; Luvoni et al., 2012; Wiedemann et al., 2013). Optimal protocol and CPA concentration used in cat ovarian tissue cryopreservation have not been concluded. The objectives of the study were to 1) optimize the concentration and exposure duration of sucrose as a non-permeable CPA during slow freezing and 2) compare effects of slow freezing and vitrification on follicular viability, apoptosis and granulosa cell interaction with oocyte in domestic cats.

2.3 Materials and methods

All chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise stated.

The animal use protocol was approved by the Chulalongkorn University Animal Care and Use Committee at the faculty of Veterinary Science, approval number 12310061.

2.3.1 Collection of ovarian tissue

Ovaries were obtained from adult, non-pregnant domestic cats after routine ovariohysterectomy conducted either at Chulalongkorn University Animal Teaching Hospital or public animal-spaying services. The cats were proven healthy by clinical examination and blood check according to our standard procedure prior to anesthesia. Follicles of more than 1 mm in diameter and visible corpora lutea were removed prior to tissue processing. Within 4 h, ovaries were transported to the laboratory in a holding medium containing 1% (v:v) fetal calf serum (FCS, Invitrogen, VA, USA), 100 IU/mL penicillin and 100 µg/mL streptomycin in Leibovitz's L-15 medium at room temperature. The ovarian cortex was removed from the outmost part of the ovaries and cut into small fragments of 2x2x0.5 mm^3 in a holding medium.

2.3.2 Experimental design

In Experiment 1, sucrose of different concentrations (0, 0.1 or 0.3 M) was added to the freezing medium. Ovarian cortical tissues (n = 21 pairs of ovaries) were frozen using the slow-freezing protocol. In Experiment 2, two cryopreservation protocols (slow freezing with 0.1 M sucrose and vitrification) were tested. Ovarian cortical tissues (n = 23) were allocated to each freezing protocol. After thawing and warming, preantral follicles were assessed for viability, histological morphology, apoptosis and immunohistochemistry expression of connexin 43.

2.3.3 Cryopreservation protocols

2.3.3.1 Slow freezing and thawing

Slow freezing was performed according to the previous reports (Cleary et al., 2001b; Martinez-Madrid et al., 2004b). Briefly, after excision, ovarian cortical fragments were placed in L-15 medium supplemented with 1.5 M dimethylsulphoxide (DMSO; Fluka Chemie GmbH, Buchs, Spain) and sucrose (0, 0.1 or 0.3 M in Experiment 1 and 0.1 M in Experiment 2) for 15 min at 4 °C. Next, the tissues were pre-cooled in cryovials

(1.6 mL cryogenic vial; Corning, NY, USA) and remained at 4 °C for a further 15 min. Afterwards, the cryovials were transferred to a passive cooling container (Coolcell; Biocision, Larkspur, CA, USA) to achieve the -1 °C/min cooling rate, then transferred to -80 °C freezer. After 24 h, the cryovials were taken out of the freezer and immersed directly in liquid nitrogen. Thawing was performed by immersing the vials in a 37 °C water bath for 3 min. The tissues were then placed into a thawing medium, containing L-15 with 0.75 M DMSO and 0.25 M sucrose, at room temperature for 10 min; they were then transferred into a medium containing L-15 with 0.25 M sucrose for 10 min, and finally they were placed in the holding medium.

2.3.3.2 Vitrification and warming

The vitrification procedure was modified from the previous report (Thuwanut and Chatdarong, 2012a). In brief, ovarian cortical fragments were suspended in an equilibration medium, consisting of L-15 supplemented with 20% (v:v) FCS, 0.96 M DMSO, and 1.21 M ethylene glycol (EG; Aldrich, Sigma-Aldrich Chemie GmbH, Steinheim, Germany), at room temperature for 10 min. Next, the tissues were transferred to a vitrification medium consisting of L-15 supplemented with 20% FCS, 1.92 M DMSO, 2.42 M EG and 0.5 M sucrose, kept at 4 °C for 30 min and then dropped into liquid nitrogen. The vitrified tissues were kept in cryovials in a liquid nitrogen tank until evaluation.

Prior to assessment, vitrified ovarian fragments were warmed by maintaining cryogenic tubes at room temperature for 30 s; they were then transferred into warming solution consisting of L-15 supplemented with 1 M sucrose and 20% (v:v) FCS at 37 °C for 10 min, and kept in a holding medium.

2.3.4 Assessment of preantral follicle viability

Assessment of preantral follicle viability was performed in two forms: within ovarian fragments and isolated form. The viability of preantral follicles within ovarian fragments was evaluated by staining with 50 µg/ mL neutral red (NR) in a holding medium at 37 °C for 2 h (Milenkovic et al., 2012) in four-well culture plates (Nunc,

Roskilde, Denmark) and analyzed within 20 min after staining. Viable follicles stained red were counted under a microscope (CX31; Olympus, Tokyo, Japan) using a calibrated squared grid reticule (Olympus) at magnification X200 (Chambers et al., 2010). Numbers of viable follicles in ten random microscopic reticule areas of 0.0625 mm^2 were counted.

The viability of isolated follicles was assessed by triple staining, using calcein acetoxymethyl ester (CAM; Invitrogen), ethidium homodimer-1 (EthD-1; Invitrogen) and Hoechst 33342 (Invitrogen). Cortical fragments were repeatedly sliced with surgical blades in a holding medium. Isolated preantral follicles were selected and transferred into a mixture of 2 µM CAM, 5 µM EthD-1 and 2 µM Hoechst 33342 in a 500 µL holding medium for 15 min in the dark. Stained follicles were examined under a fluorescence microscope (BX51; Olympus, Tokyo, Japan) at X400 magnification. Emitted fluorescence signals of Hoechst 33342 (blue), CAM (green), and EthD-1 (red) were collected at 350 nm, 485 nm, and 528 nm, respectively. Granulosa cells and oocytes stained green with CAM were defined as live while they were defined as dead when stained red with EthD-1. Live follicles were classified into three categories according to the percentage of damaged granulosa cells: A) live follicles, follicles with the oocyte and all the granulosa cells viable; B) minimally damaged live follicles, follicles with viable oocytes and up to 50% granulosa cells dead; C) moderately damaged follicles, follicles with viable oocytes and > 50% granulosa cells stained green. Dead follicles were classified into grade D which was follicle with dead oocyte or all granulosa cells stained red (Martinez-Madrid et al., 2004b).

2.3.5 Histological analysis

Ovarian fragments were fixed in 4% (wt/vol) paraformaldehyde for 24 h, and then 70% ethyl alcohol. After fixation and dehydration, they were embedded in paraffin, serially sectioned (4-µm thick), and stained with hematoxylin/eosin (H&E). Follicular morphology was examined under a light microscope (BX51; Olympus, Tokyo, Japan) at X400 magnification. Only follicles with presence of oocyte nucleus were counted. Evaluation of follicle quality was based on the integrity of the basement

membrane, presence or absence of pyknotic bodies, and integrity of the oocyte. Preantral follicles were classified as morphologically normal or atretic follicles. Stages of preantral follicles were classified as primordial, primary and secondary follicle, in accordance with the previous study (Gougeon, 1986). Primordial follicles were characterized by one layer of flattened granulosa cells around the oocyte, primary follicles by one layer of cuboidal granulosa cells, and secondary follicles by two or more layers of granulosa cells.

2.3.6 Evaluation of apoptosis

Apoptosis of primordial follicles and stromal cells were evaluated by TUNEL assay (Manee-in and Srisuwatanasagul, 2012). DNA fragmentation was labelled at the terminal end of fragmented nucleic acids (ApopTag® Peroxidase *In Situ* Apoptosis Detection Kit; Millipore, Billerica, MA, USA). In brief, after routine histopathological tissue preparation, sections were treated in a microwave oven (1 x 3 min at 500W and 1 x 3 min at 300W) in citrate buffer to retrieve the antigen. Endogenous peroxidase activity was inhibited by incubating the tissues with 3% (v:v) hydrogen peroxide for 10 min then treated with an equilibration buffer for 10 s at room temperature. Afterwards, they were treated with a terminal deoxynucleotidyl transferase (TdT) enzyme in a humidified chamber at 37 ℃ for 60 min. Detection was performed using antidegoxigenin conjugate by incubating the tissue for 30 min at room temperature, followed by incubation with 3,3 diaminobenzidine (DAB) for 3 min. Next, the sections were counterstained with Mayer's hematoxylin to detect non-stained follicles. PBS was used instead of TdT solution in negative controls. Primordial follicles with at least one positive brown staining of DAB (Vector Laboratories Inc., Burlingame, CA, USA) in the granulosa cell, theca cell or luteal cell were classified as apoptotic follicles. Positive primordial follicles in ten randomized fields of 0.0625 mm^2 were calculated as a percentage of apoptosis. Stromal cell apoptosis was calculated as the number of positive cells per area of 0.0625 mm^2.

2.3.7 Immunohistological assessment for connexin 43

Immunohistochemistry detection for gap junction protein connexin 43 was adapted from the previous study (Durlej et al., 2011b). In brief, after 4 µm-thick sections were deparaffinized and rehydrated, the antigens were retrieved by the microwave method (3 x 5 min, at 750W) in citrate buffer. Endogenous peroxidase activity was blocked by 3% (v:v) hydrogen peroxide incubation for 30 min. Normal horse serum was used to prevent non-specific binding of the antibody. Subsequently, sections were incubated with a rabbit polyclonal antibody against Cx 43 (Cell signaling technology; Danvers, MA, USA) for 24 h at 4 °C. Sections were incubated with a biotinylated secondary antibody: 1:100 horse anti-rabbit IgG (Vector Laboratories Inc.) for 30 min at room temperature, and 1:100 avidin-biotinylated complex (ABC; Vector Laboratories Inc.) for 30 min at room temperature. The antibody reaction was visualized using DAB incubation for 5 min, and counterstained with Mayer's hematoxylin. Sections incubated with rabbit IgG of the Cx 43 antibody were used as negative controls. Cat cardiac muscle sections were included in every batch of staining to be positive controls. The area of brown staining of DAB reaction between granulosa cells was considered the immunopositive area of Cx43 expression. All sections were photographed under a light microscope (BX51, Olympus) and microscope camera (DP73, Olympus) at X200 magnification using the same condition of exposure and ISO. The intensity of immunopositive areas was assessed using image analysis software (ImageJ; National Institute of Health, Bethesda, MD, USA) and expressed as relative optical density (ROD). ROD data of the positive area were obtained from mean of 10 different measurements in each tissue slides and were calculated using the formula of ROD = $OD_{specimen}$/ $OD_{background}$ = log (GL_{blank}/ $GL_{specimen}$)/ log (GL_{blank}/ $GL_{background}$), where GL is the gray level for the stained area (specimen) and unstained area (background), and blank is the gray level measured after the slide was not in the light path (Durlej et al., 2011a).

2.3.8 Statistical analysis

Data were analyzed using SAS version 9.0 (SAS® version 9.0; SAS Institute Inc., Cary, NC, USA). Least-square means were obtained from each treatment. Effects of

sucrose concentration (0, 0.1 and 0.3 M) and method of cryopreservation (slow-freezing and vitrification) were tested using ANOVA (GLM procedure). Controls were obtained by least significant difference test. Ovarian follicular viability, stromal cell apoptosis, and ROD were expressed as mean ± SEM. Dependent variables (isolated follicular viability, morphologically normal follicles, and apoptotic primordial follicles) were evaluated using the Univariate procedure for normality test. Skewness and Kolmogorov-Smirnov D statistic were used to test the normal distribution of the residual of the parameters. Isolated follicle viability, follicular morphology, follicular apoptosis and CL apoptosis were expressed as a percentage and analyzed using generalized linear model (GENMOD) procedure. The values with $P < 0.05$ were considered to be statistically significant.

2.4 Results

The sucrose concentration and cryopreservation method affected all parameters including ovarian follicular viability, isolated follicular viability, morphologically normal follicles, primordial follicles apoptosis, stromal cell apoptosis and Cx 43 expression ($P < 0.05$).

2.4.1 Impacts of sucrose exposure on the ovarian tissue qualities

Follicular viability within the tissues assessed by NR staining revealed that follicles from the fresh group (7.7 ± 0.7 positive follicles) had more positive follicles than the frozen-thawed groups. Moreover, exposure to sucrose was beneficial to the follicular survival (1.9 ± 0.2, 4.0 ± 0.4 and 3.9 ± 0.5 follicles in 0 M, 0.1 M, and 0.3 M sucrose groups, respectively, $P < 0.05$). Isolated follicle viability was assessed by triple staining of CAM, EthD-1 and Hoechst graded follicles into 4 grades. Fresh tissues yielded more grade A follicles than all of the cryopreservation groups, and the group of no sucrose had a lower viability than the others ($P < 0.05$, Table 1). There were no significant differences of percentage of grade B (minimally damaged) and grade C (moderately damaged) follicles in any of the groups. However, the no-sucrose group yielded more grade D (dead) follicles than the other groups (Table 1).

Table 1 Viability of isolated follicles with different grades in ovarian tissue after freezing and thawing using a different concentration of sucrose as non-permeable CPA compared with fresh control assessed by Hoechst33342/CAM/EthD-1 staining. Grade A = viable oocyte with all intact granulosa cells, B = viable oocyte with < 10% dead granulosa cells, C = viable oocytes with > 50% dead granulosa cells and D = dead oocyte or all dead granulosa cells.

Treatment	n	Follicles in each grade (%)			
		Grade A	Grade B	Grade C	Grade D
Fresh	511	27.6 [a]	31.3	23.5	17.6 [a]
0-M sucrose	253	9.1 [b]	26.1	23.3	41.5 [b]
0.1-M sucrose	288	20.1 [c]	25.1	26.0	28.8 [c]
0.3-M sucrose	301	18.9 [c]	27.6	26.6	26.9 [c]

[a,b,c] Different superscripts indicate significant difference in the same column ($P < 0.05$). Grade A = viable oocyte with all intact granulosa cells, B = viable oocyte with 50% or less dead granulosa cells, C = viable oocytes with greater than 50% dead granulosa cells, and D = dead oocyte or all dead granulosa cells.

The ovarian tissue cryopreservation using 0.1 M and 0.3 M sucrose could prevent the incidence of abnormal follicle appearance better than the no-sucrose group could (47.4 % morphologically normal follicles out of total follicles in 0.1 M and 43.5% in 0.3 M groups vs 33.8% in no sucrose group; $P < 0.05$). However, in both primary and secondary follicles, though the percentages of atretic follicles in 0 M sucrose group tended to be lower than the 0.1 M ($P = 0.08$) group, no significant difference was shown among cryopreservation groups (Figure 5). Apoptosis detection by TUNEL was assessed in primordial follicles and ovarian stromal cells. None of the negative control slices showed TUNEL-positive signals. Follicles within the fragment that underwent freezing without sucrose had more apoptotic primordial follicles (7.7%) than fresh (1.5%) or 0.1 M and 0.3 M sucrose (1.2% and 1.9%, respectively) ($P < 0.05$). Stromal cell apoptosis pattern showed the same trends in percentage. Cryopreservation without sucrose had a higher stromal cell apoptosis (21.9 ± 3.2 positive cells per area of 0.0625 mm^2) than the fresh, 0.1 M and 0.3 M groups (11.5 ±

1.3, 13.7 ± 2.3 and 17.9 ±2.8 positive cells, respectively, $P < 0.05$). When compared with the ROD of Cx 43 expression, all cryopreservation groups had less ROD than fresh groups did, and showed no difference among the cryopreservation groups. However, 0.1 M sucrose group tended to have more ROD than the 0 M sucrose group ($P = 0.08$, Figure 6).

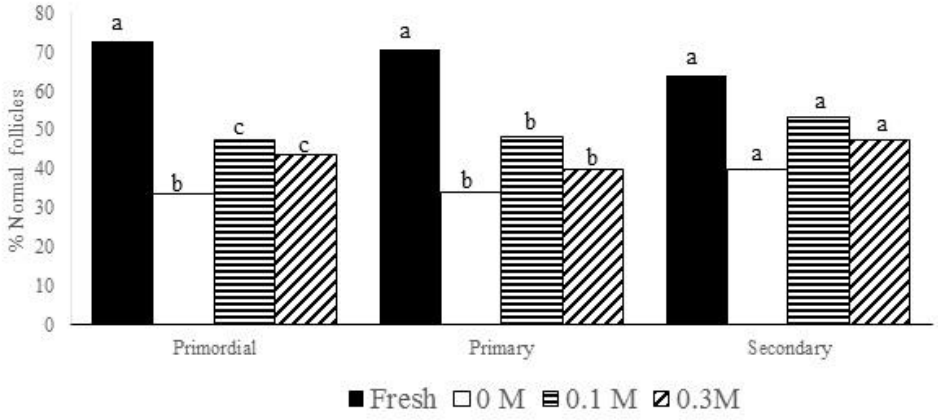

Figure 5. Percentage of morphologically normal follicles in cat ovarian tissue after frozen-thawed with different concentration of sucrose assessed by H&E staining. Different letters (a,b,c) indicate significant difference among the same stage of follicles ($P < 0.05$).

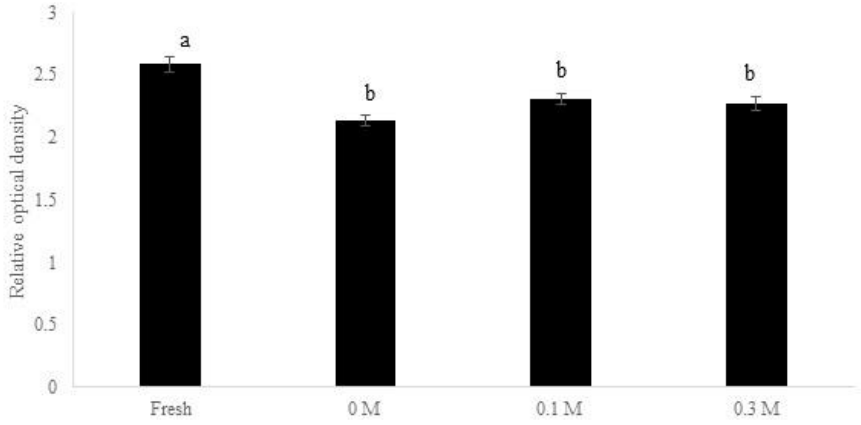

Figure 6. Intensity staining of Cx43 expression in a relative optical density (ROD) of diaminobenzidine (DAB) brown reaction of Cx43 expression of ovarian granulosa cells after cryopreservation by various concentrations of sucrose as a non-*permeable CPA.* Data were presented as mean ± SEM (n=21). Different letters above the bars indicate significant differences among treatment groups ($P < 0.05$).

2.4.2 Effects of cryopreservation methods on the ovarian tissue qualities

Ovarian tissue follicle viability from fresh tissues had more NR positive follicles than both slow-freezing and vitrification groups did, but no difference between cryopreservation groups (n=23; 7.4 ± 0.7, 4.6 ± 0.6 and 3.2 ± 0.6 positive follicles, respectively). Follicles isolated from vitrification tissue had lower percentages of grade A and B follicles than did either of the other two groups. On the other hand, vitrified tissues yielded a higher percentage of dead follicles than the other groups (Table 2).

In this study, fresh tissues had approximately two times higher percentages of normal morphological appearance follicles than cryopreservation groups ($P < 0.05$). When comparing among the cryopreserved groups, the slow-freezing procedure could maintain the primary follicle appearance (38.8%) better than vitrification ($P < 0.05$). However, there was no difference between the two procedures in primary and secondary follicles (Figure 7). Apoptotic primordial follicles were significantly higher in the vitrification group (8.9%) than in the fresh (2.7%) or the slow-freezing group (3.8%) ($P < 0.05$). Stromal cell apoptosis was found to be higher in cryopreservation groups, slow freezing (22.5 ± 2.4 positive cells per area of 0.0625 mm^2) and vitrification (28.3 ± 2.5 cells), than in the fresh group (13.5 ± 1.9 cells) ($P < 0.05$). The expression of Cx 43 in both cryopreservation groups were found to be significantly lower than in fresh groups but showed no difference between the two procedures ($P < 0.05$; Figure 8, 9).

Table 2 Viability of isolated follicles with different grades in ovarian tissue after freezing and thawing using slow-freezing and vitrification procedures, compared with fresh control assessed by Hoechst33342/CAM/EthD-1 staining. Follicles were graded by the criteria as in Table 1.

Treatments	*n*	Follicles in each grade (%)			
		Grade A	Grade B	Grade C	Grade D
Fresh	555	28.5 [a]	33.1 [a]	24.0 [a]	14.4 [a]
Slow freezing	501	24.8 [a]	28.9 [a]	21.4 [b]	24.9 [b]
Vitrification	467	17.1 [b]	21.6 [b]	27.9 [a]	33.4 [c]

[a,b,c] Different superscripts indicate significant difference in the same column ($P < 0.05$).

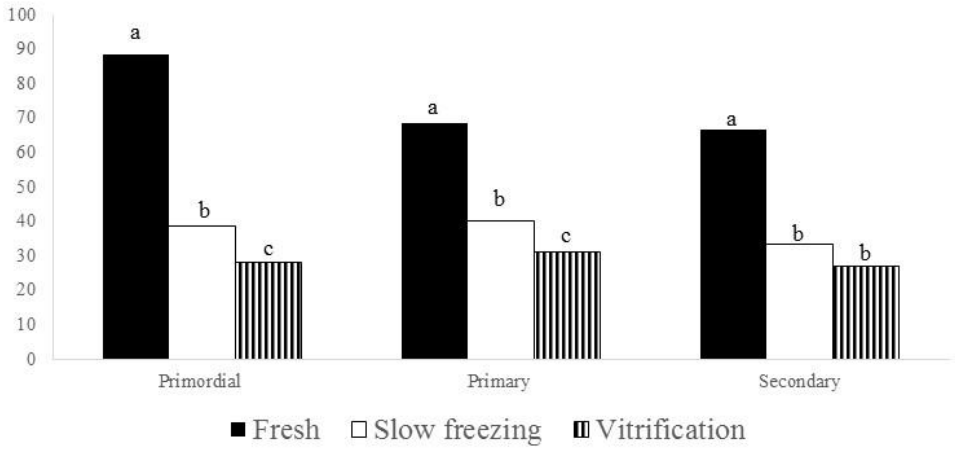

Figure 7. Percentages of morphologically normal follicles data after cryopreservation with slow-freezing (0.1 M sucrose) and vitrification assessed by H&E staining, presented as percentage and number of morphologically normal follicle per all follicles observed in each group. Different letters above the bars indicate significant differences among the same type of follicles ($P < 0.05$).

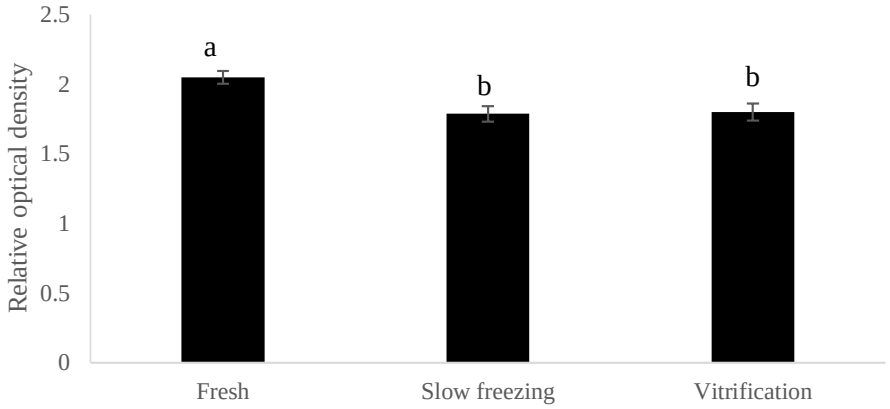

Figure 8. Comparison of intensity of Cx 43 staining, expressed as relative optical density of 3,30-diaminobenzidine brown reaction of Cx 43 expression of ovarian granulosa cells, between slow freezing (0.1-M sucrose) and vitrification. Data are presented as mean ± standard error of the mean (n = 23). Different letters above the bars indicate significant differences among treatment groups ($P < 0.05$)

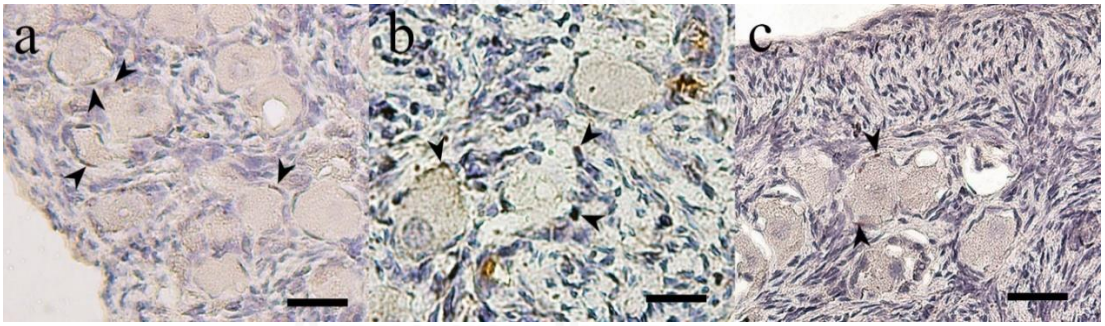

Figure 9. Cx 43 positive staining indicated by brown reaction of DAB (arrow head) in granulosa cells of primordial follicles of (a) fresh, (b) slow-freezing and (c) vitrified ovarian tissues. Black bars indicate the length of 20 μm.

2.5 Discussion

The present study demonstrated the positive effects of sucrose supplementation as well as the advantage of slow-freezing over vitrification of the cat ovarian tissues. We used ovaries from domestic cats because this could be a suitable model for application in other felids and human biomedical model (Comizzoli and Wildt, 2012). Cryopreservation of ovarian tissue led to a decrease in viable follicles detected by NR staining, CAM/EthD-1/Hoechst staining, and histological morphology.

The three types of viability assay were performed to predict the possibility of further applications of cryopreserved ovarian tissue. The NR staining and histological morphology implied functional and structural integrities that were important for tissue culture or transplantation. The CAM/EthD-1/Hoechst staining of the isolated follicles was beneficial for further *in vitro* follicular growth (Santos et al., 2010). Exposure to sucrose was beneficial to the follicle survival evaluated by all three methods. However, the protective effect of sucrose as a non-permeable CPA was not cumulative, because increase of sucrose concentration to 0.3 M did not show superior results over the concentration of 0.1 M. The studies of human ovarian tissue, oocyte and embryo cryopreservation demonstrated that increasing the sucrose concentration from 0.2 M to 0.3 M compromised intact oocytes and follicular cells assessed by histological and ultrastructural morphology (Marsella et al., 2008). Sucrose toxicity was suggested as the cause of reduced follicular survival when human ovarian tissue underwent slow-freezing (Fabbri et al., 2010). In contrast, the higher sucrose concentration (0.2 to 0.3 M) resulted in a greater embryo survival rate (Bianchi et al., 2007; Edgar et al., 2009). Similar results were observed in the cryopreservation of immature oocytes, using 0.1 M and 0.2 M sucrose (Chen et al., 2004). In this study, the follicles from the 0.3 M group had a slightly higher percentage of grade B and grade C follicles than the 0.1 M group did, these being follicles with live oocytes and some dead granulosa cells. The cryodamage to follicular cells rather than oocytes was also presented in the other studies (Siebzehnrubl et al., 2000; Eyden et al., 2004; Rodrigues et al., 2004; Navarro-Costa et al., 2005). In this study, the oocytes of fresh specimens appeared intact, but all of the cryopreservation groups had less morphologically normal follicles. In addition, the group with no sucrose yielded the lowest number of morphologically normal follicles. However, the difference of morphologically normal follicles was not seen in the primary and secondary follicles. This may be due to the lesser number of follicles assessed than the primordial follicles. In experiment 2, both slow freezing and vitrification were able to preserve the follicular morphology; however, the slow-freezing procedure could maintain more morphologically normal follicles than vitrification could. However, the previous study in domestic cats found no effect of vitrification on follicular morphology, but the meiosis resumption rate and

cytoskeleton integrities were much lower than the fresh control (Milenkovic et al., 2012).

In the present study, the cryopreservation apparently impaired the DNA integrity of the follicles, as demonstrated by the higher amount of TUNEL-positive follicular cells in the fresh ovarian tissues than in the frozen-thawed ones. Histologically, the 0.1 M sucrose group tended to protect the morphology of the follicular cells slightly better than the 0.3 M ($P > 0.05$) group. Moreover, the 0.1 M sucrose group also protected the stromal cells better than the 0.3 M group. These results showed the same pattern of cellular protection of sucrose in cryopreservation medium. Sucrose clearly prevented stromal cells from undergoing apoptosis after thawing. The stromal cell integrity was the crucial factor affecting follicular growth (Fujihara et al., 2012a; Milenkovic et al., 2012). In experiment 2, the percentage of apoptosis primordial follicle was increased after vitrification than did the slow-freezing. However, even the stromal cell apoptosis showed the increasing apoptotic cells like the primordial follicle apoptosis, they were considered not different after analysis (22.5 vs 28.3 cells, respectively). This may be due to the inequity of permeability property of different kinds of cells in the ovarian tissues after treatment with different methods of cryopreservation. Stromal cell apoptosis data were not different between the vitrification and slow-freezing group, which was in accordance with the previous studies in humans (Amorim et al., 2012; Herraiz et al., 2014). Interestingly, the percentage of TUNEL-positive thawed primordial follicles was around 3.7-8.9%, which was the minority of the follicular pool. The result was in accordance with previous studies, in that freezing and thawing induced minor apoptosis in oocytes and ovarian stromal cells (Fauque et al., 2007). Though there was an increase of apoptotic areas after ovarian tissue thawing, the previous report on humans suggested that this was not significant due to the minimal amount of apoptotic marker positive areas (Martinez-Madrid et al., 2007).

In the present study, the intensity of Cx 43 was also affected by cryopreservation, with the tendency that sucrose supplementation could protect

cryodamage to gap junction protein, Cx43, across follicular cells (P = 0.086). The follicles are comprised of oocyte and granulosa cells, which have hormonal and metabolic linkage to each other, especially after follicular growth initiation (Gosden, 2002). Cx 43 is a major gap junction protein that is found between granulosa cells and plays crucial roles in germ line development, postnatal folliculogenesis (Juneja et al., 1999; Lee et al., 2008) and cross-communication, among others (Boland and Gosden, 1994; Grazul-Bilska et al., 1997). Gap junctions have a hexameric structure, or connexon, which is composed of gap junctional proteins known as connexins. The cryopreservation in this study likely altered the connexin structure, resulting in the decrease of Cx 43 expression. The previous studies of Cx 43 in cryopreserved ovaries in other species also showed the lower Cx 43 expression in cryopreserved ovarian tissue, which is associated with further poor follicular development (Boland and Gosden, 1994; Grazul-Bilska et al., 1997; Luciano et al., 2009). Moreover, the present study found that vitrification and slow-freezing damaged the gap junction protein at the same level. This contrasts with the study of the cryopreservation of the germinal vesicle oocyte, which showed that vitrification damaged intact communications in cumulus-oocyte complexes to a larger extent than the slow-freezing method did (Luciano et al., 2009).

In conclusion, we have shown for the first time that 0.1 or 0.3 M sucrose supplementation in domestic cat ovarian tissue cryopreservation could be beneficial to follicular viability, histological morphology and prevention of apoptosis. However, increasing the concentration to 0.3 M did not show greater beneficial effects. In addition, the slow-freezing procedure seemed better than vitrification in terms of follicular viability, histological appearances and apoptosis assessment. Cryopreservation protocols lessened the expression of Cx 43 but did not show differences among the cryopreservation groups. Further studies are required to investigate the application of the cryopreserved tissues, such as *in vitro* culture of the ovarian tissue or xenotransplantation.

CHAPTER III

Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) pre-exposure ensures follicle integrity during *in vitro* culture but not during cryopreservation in the domestic cat model

3.1 Abstract

Temporary and reversible down-regulation of metabolism may improve the survival of cells and tissues exposed to non-physiological conditions during transport, *in vitro* culture, and cryopreservation. The present study aimed at: 1) optimizing the concentration and duration of FCCP (Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone – a mitochondrial uncoupling agent) exposure for biopsies of domestic cat ovarian tissue and 2) investigating the effects of FCCP pre-exposure on follicle integrity after tissue culture (up to 7 days), and on follicle integrity after a 2-day culture followed by cryopreservation. Biopsies of cat ovarian tissue were first treated with FCCP with various concentrations (0, 10, 40, 200 nM) for 10 or 120 min to determine the most suitable pre-exposure conditions. Then, tissues were pre-exposed with 200 nM FCCP for 120 min before culture and cryopreservation. Follicular viability (Calcein assay), mitochondrial membrane potential (Rhodamine 123 fluorescence, follicular morphology (histology), preantral follicle proliferation (Ki-67 immunostaining), and follicular density were evaluated for treatment and control groups. Ovarian tissues incubated with 200 nM FCCP for 120 min led to the lowest R123 fluorescence (1.17 ± 0.09; $P < 0.05$) compared to the control group (1.30 ± 0.12) and maintained a constant percentage of viable follicles (75.3 ± 7.8%) as the control group (71.8 ± 11.7%; $P > 0.05$). After 2 days of *in vitro* culture, percentage of viability (78.8 ± 8.9%) in the same pre-exposure conditions was higher ($P < 0.05$) than in the control (61.2 ± 12.0%). and the percentage of morphologically normal follicles (57.6 ± 17.3%) was similar to the fresh tissue (57.6 ± 17.3%; $P > 0.05$). Interestingly, percentage of proliferation follicle and follicular density were unaltered by the FCCP exposure. However, based on the same indicators, the FCCP-treated tissue fragments did not show a better follicle

integrity after freezing and thawing. In conclusion, domestic cat ovarian tissue pre-exposed to 200 nM FCCP during 120 min protects and enhances the follicle integrity during *in vitro* culture. However, FCCP does not appear to exert a beneficial effect during cryopreservation in the ovarian tissue.

3.2 Introduction

Ovarian tissue cryopreservation is a critical approach to ensure the fertility preservation in animal models as well as in human patients undergoing chemo- or radiotherapy for cancer treatment (Silber, 2012; Donnez et al., 2013). These options are particularly interesting in endangered species conservation to bank ovarian tissue containing an abundant amount of preantral and primordial follicles for individuals undergoing surgical ovarian removal or dying unexpectedly (Jewgenow and Paris, 2006; Wiedemann et al., 2012; Donnez et al., 2013). For this type of studies, the domestic cat has been shown as a good biomedical model for non-domestic cats and human (Comizzoli et al., 2010; Songsasen et al., 2012b; Wiedemann et al., 2012). In association with the freezing techniques, culture of the ovarian tissue *in vitro* is a good approach to reanimate the tissue and grow more oocytes to an advanced stage (Songsasen et al., 2012a; Wiedemann et al., 2013; Higuchi et al., 2015). However, the duration between the ovarian tissue collection and cryopreservation is detrimental to the viability and developmental competence of the gametes within the gonads due to ischemia and oxidative processes (Cleary et al., 2001a; Thuwanut and Chatdarong, 2012b; Wiedemann et al., 2012). Damages to the ovarian cell integrity during this critical period of time are mainly due to the cellular respiration in mitochondria still occurring despite the ischemic environment (Cleary et al., 2001a; Li and Jackson, 2002; Evecen et al., 2009). Reversibly reducing the metabolic activity of the cells (using a metabolic disrupting agent) could be the option for prolonging the tissue integrity. In addition, some studies demonstrated that the cell survival can be increased after incubation with metabolic quiescence agent and cryopreservation in mouse, rat, and human cells (Brennan et al., 2006d; Menze et al., 2010; Weisová et al., 2012). Mitochondria is a key organelle of the cell essential for the survival and proliferation of cells due to the role

in electron transport chain in aerobic cellular respiration. In a normal situation of electron transport chain, hydrogen ions are transported out from the mitochondrial matrix into the intermembrane space. This generates the potential energy gradients across the inner mitochondrial membrane. Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), is a commonly used proton ionophore for mitochondrial oxidative phosphorylation inhibitor. It is an uncoupling agent which disturbs the process of ATP synthesis by transporting hydrogen ions through a mitochondrial membrane before it provides the proton motive force to complete the ATP synthesis process (Brennan et al., 2006d). Application of low dosage of FCCP pre-exposure to the cells in order to mild uncoupling the mitochondrial membrane potential prior to inducing the stress environment has already been shown to be beneficial effects on human neuronal cell viability (Weisová et al., 2012). The objective of the present study was to investigate the effects of FCCP pre-exposure on follicle integrity after tissue culture (up to 7 days), and on follicle integrity after a 2-day culture followed by cryopreservation.

3.3 Materials and methods

All chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise stated.

3.3.1 Collection of ovarian tissue

Ovaries were obtained from domestic cats (8-week to 2-year old) after routine ovariohysterectomy at local veterinary clinics and transported in phosphate buffer solution at 4°C to the laboratory within 12 h of excision. Ovarian cortical fragments were removed from the outermost part of the ovaries using surgical blades and curved scissors (Tanpradit et al., 2015). Large antral follicles (diameter > 1 mm) and luteal tissue areas were excluded. Cortical tissues were cut into small fragments in the area of 1 x 1 mm^2 and approximately 200 µm thickness in a holding medium consisting of Eagle's MEM supplemented with 2 mM L-glutamine, 10 µg/mL, insulin, 5.5 µg/mL

transferrin, 5.0 µg/mL sodium selenite, 0.3% (wt/vol) bovine serum albumin (BSA), 10 mM HEPES, 100 µg/mL penicillin G sodium, and 100 µg/mL streptomycin sulfate.

3.3.2 Ovarian tissue culture

Ovarian tissue culture was performed as previously reported (Fujihara et al., 2014). Briefly, ovarian cortical fragments were transferred into 4-well cell culture plates (Nunc, Roskilde, Denmark) containing a cube hexahedron of 1.5% agarose gel (Bio-rad, Hercules, CA, USA) sized approximately 7 x 7 x 7 mm^3 soaked in a culture medium incubated at 38.5°C in 5% CO_2 in humidified air. The culture medium was Eagle's MEM supplemented with 5.5 µg/ml insulin, 5.5 µg/ml transferrin, 6.7 ng/ml selenium, 2 mM L-glutamine, 100 µg/ml penicillin G sodium, 100 µg/ml streptomycin sulfate, 0.05 mM ascorbic acid, 10 ng/ml porcine FSH (Folltropin-V, Bioniche Animal Health, Belleville, Canada), and 0.1% (wt/vol) polyvinyl alcohol. Half of the volume of culture media was changed every 48 h until the day of examination.

3.3.3 Cryopreservation of the ovarian tissue with slow freezing method and thawing

Cryopreservation was performed according to previous reports (Cleary et al., 2001a; Martinez-Madrid et al., 2004a; Tanpradit et al., 2015). Ovarian cortical fragments were transferred from holding medium to cryopreservation medium contained with L-15 medium supplemented with 1.5 M DMSO (Fluka Chemie GmbH, Buchs, Spain) and 0.1 M sucrose for 5 min at 4 °C. After incubation with cryopreservation medium, tissue fragments were transferred into precooled cryovials (1.6-mL cryogenic vial; Corning, NY, USA) and remained at 4 °C for a further 10 minutes. Then, the cryovials were moved to a passive cooling container (CoolCell; BioCision, Larkspur, CA, USA) to achieve the -1 °C/min cooling rate and placed in an -80 °C freezer. After 24 h, the cryovials were taken out of the freezer and immersed directly in liquid nitrogen.

Thawing was performed by immersing the vials in a 37 °C water bath for 3 minutes. The thawed fragments were then rapidly placed into a first thawing medium, containing L-15 medium with 0.75 M DMSO and 0.25 M sucrose at room temperature for 10

minutes and further transferred to a second thawing medium containing L-15 with 0.25-M sucrose for 10 minutes.

3.3.4 Follicular viability assessment

The viability of tissue follicles was assessed using calcein acetoxymethyl ester (CAM; Invitrogen, Life Technologies, Carlsbad, CA, USA)/ ethidium homodimer-1 (EthD-1; Invitrogen) (Martinez-Madrid et al., 2004b). The staining was performed by transferring ovarian cortical fragments into a mixture of 2 μM CAM and 5 μM EthD-1 in the holding medium for 15 min in the dark. Stained follicles were examined under a fluorescence microscope (BX41; Olympus, Tokyo, Japan) at X400 magnification. Preantral follicle with the oocyte and granulosa cells stained green by calcein was considered viable follicle and follicle that has red staining of EthD-1stained in the oocyte or granulosa cells were considered non-viable follicle. For each ovarian fragment, 10 random high power fields were observed and percentage of viable follicles was recorded.

3.3.5 Histological analysis and classification

Ovarian tissue fragments were fixed in 4% paraformaldehyde and transferred to 70% ethanol after 24 h. Tissue fragments were dehydrated in a graded series of ethanol solutions, and embedded in a paraffin block. Each cortical piece was serially dissected into 4-μM sections and stained with hematoxylin and eosin. Tissues were examined and photographed under a microscope (BX41, Olympus) and microscope camera (SPOT RT3, Diagnostic Instruments, Inc., Sterling Heights, MI, USA). Follicles containing oocytes with a visible nucleus were observed and counted.

Classification of follicle morphology was performed according to the previous study (Fujihara et al., 2014). Preantral follicles within the tissue with structural intact oocyte and granulosa cells were classified as normal. Abnormal follicles were characterized as the pyknotic, fragmented, or shrunken nucleus of the oocyte and/or granulosa cells. The number of normal follicles divided by total follicles within the sections was determining as a percentage of morphologically normal follicles.

Follicle density was determined by a number of preantral follicles per area of 1 mm^2 of ovarian tissue section determining under the microscope imaging program (SPOT 4.0, Diagnostic Instruments, Inc., Sterling Heights, MI).

3.3.6 Mitochondrial membrane potential assessment

Mitochondrial membrane potential was assessed according to the previous studies (Han et al., 2009; Cottet-Rousselle et al., 2011; Perry et al., 2011). In brief, the tissue fragments were incubated with holding medium supplemented with 10 µg/mL rhodamine123 (R123; Invitrogen), a cell-permeable cationic dye that preferentially enters mitochondria based on a highly negative mitochondrial membrane potential. As a result, this phenomenon reflected the mitochondrial activity within the cell. In brief, the tissue fragments were stained in 37 °C for 15 min and then washed out with holding medium. Tissues then were examined and photographed under a fluorescence microscope (BX41, Olympus) and microscope camera (SPOT RT3, Diagnostic Instruments Inc.) at X200 magnification with the exposure of excitation signal of 485 nm and collected the emission signal at 520 nm in 25 °C. All of the parameters of the microscope camera settings were unchanged throughout the experiments. The intensity of R123 staining was determined using image analysis software (ImageJ; National Institute of Health, Bethesda, MD, USA). Regions of interest for each fragment were drawn according to the area of the tissue fragment to measure the fluorescence intensity. The intensity of the fluorescence signal was expressed as relative optical density (ROD) of tissue R123 fluorescence emission and the background of the media with R123, calculated using the formula of ROD = $OD_{specimen}$/ $OD_{background}$ = log (GL_{blank}/ $GL_{specimen}$)/ log (GL_{blank}/ $GL_{background}$), where GL is the mean gray level for the stained area (specimen) and background area, and blank is the gray level measured after the slide was removed. All GLs were obtained using dark background mode for fluorescence images. Parameters involved to fluorescence intensity, such as gain signal, and pinhole size, were maintained at constant for all measurements performed. The tissue with high intensity of R123 ROD indicates the high mitochondrial membrane potential in the stained cells (Gan et al., 2011).

3.3.7 Proliferation assessment

The protocol of preantral follicle proliferation immunohistochemistry using Ki-67 proliferation marker was adapted from the previous study (Kenngott et al., 2013). After histological tissue preparation, sections were treated for antigen retrieval in an autoclave machine (121 °C, 1.2 kg/cm^2) for 30 min in citrate buffer (pH = 6.0). Endogenous peroxidase activity was inhibited by incubating the tissue slides with 3% (v/v) hydrogen peroxide in methanol for 10 min, then treated with normal horse serum for 30 min at room temperature. Tissue slides were incubated with monoclonal mouse anti-human Ki-67 antigen clone MIB-1 (DAKO, Glostrup, Denmark) in a humidified chamber at room temperature for 180 min. Afterwards, the slides were treated with anti-mouse made in horse secondary antibody at room temperature for 30 min. Detection was performed using avidin-biotin complex (Vector, Vector Laboratories Inc., Burlingame, CA, USA) by incubating the tissue for 60 min at room temperature, followed by incubation with ImmPACT DAB (Vector) for 2 min at room temperature. Next, the sections were counterstained with Mayer's hematoxylin to detect non-stained follicles. PBS was used instead of Ki-67 antibody in negative controls. Preantral follicle with positive brown staining of DAB in granulosa cell nucleus was classified as a proliferating follicle.

3.3.8 Experimental design

3.3.8.1 Short term effect of FCCP incubation with domestic cat ovarian tissue

For each of twelve replicates, pooled ovarian tissue fragments were obtained from two ovaries. Six tissue fragments were allocated to each experimental group. Tissue fragments were incubated in the culture medium supplemented with FCCP in the concentrations of 10, 40, and 200 nM in 4-well plates incubated at 38.5°C in 5% CO2 in humidified air. All groups were tested for viability and potential membrane after 10 min and 120 min of incubation. The groups incubated with culture medium without FCCP were used as controls.

3.3.8.2 Long term effect of FCCP pre-exposure after ovarian tissue culture up to 7 days

Two ovaries were pooled and extracted for ovarian cortical fragments in each replicate. The experiment was performed for twelve replicates. For each of the experimental group, ten cortical fragments were pre-exposed with FCCP (concentration and time according to the first experiment) before *in vitro* cultured. The ovarian tissue fragments were allocated to the experimental groups of various concentrations of FCCP (0, 10, 40, or 200 nM) for 2 or 7 days. When reaching the endpoint, the ovarian fragments were examined for follicular viability, mitochondrial membrane potential, histological analysis and proliferation marker immunohistochemistry. The groups pre-exposed with culture medium without FCCP were used as controls.

3.3.8.3 Cryopreservation of domestic cat ovarian tissue after pre-exposure with FCCP

This final experiment was performed for sixteen replicates. Twenty ovarian cortical fragments were allocated in each experimental group. The fragments were pre-exposed in the optimal conditions defined in the second experiment. At the end of culture, all tissues were washed out with the holding medium for 15 min before cryopreservation. The tissues were stored in liquid nitrogen. The thawed ovarian fragments were assessed for follicular viability, mitochondrial activity, histological analysis and proliferation marker. The cryopreserved control group were the group of fresh tissues that were undergone freeze-thawing right after ovarian cortical fragment extraction and the culture control is the group that culture for 2 days without FCCP pre-exposure before cryopreservation.

3.3.9 Statistical Analysis

The percentage of follicular viability, the percentage of morphologically normal follicles, follicular density, the percentage of the positive Ki-67 marker, and mitochondrial membrane potential data are expressed as mean ± SD. Kolmogorov-Smirnov test was performed to evaluate the normal distribution of each dataset. The normal distribution data were evaluated using general linear mixed models and the data that did not pass the normal distribution test were evaluated by Kruskal-Wallis analysis of variance. Dependent variables included the percentage of follicular viability,

percentage of morphologically normal follicles, follicle density, percentage of the positive Ki-67 marker, and mitochondrial membrane potential. The effect of treatments (concentrations and incubation durations of FCCP in experiment 1, concentrations and culture durations of FCCP and AICAR in experiment 2, and FCCP culture duration prior to cryopreservation in experiment 3) were included in the statistical models as fixed effects. The animal identities and tissue number nested within the animal ID were included in the models as random effects. Significant differences were considered when $P < 0.05$.

3.4 Results

3.4.1 Immediate effect of FCCP incubation on mitochondrial membrane potential and viability of the cortical tissue

The intensity of R123 fluorescence was lower ($P < 0.05$) with 10-min incubation with 5 and 200 nM (1.26 ± 0.9 and 1.21 ± 0.08, respectively;) than the control group (1.34 ± 0.11, Figure 10a) whereas it was unchanged ($P > 0.05$) with 40 nM (1.28 ± 0.09; Figure 10a). The follicular viability after 10 min incubation with 10 nM FCCP group was lower (64.0 ±7.3; $P < 0.05$) than the control group (77.9 ± 8.4; Figure 10b). However, the viability was not different ($P > 0.05$) after a 10 min exposure to 40 and 200 nM (65.7 ± 24.79 and 76.6 ± 13.7, respectively; Figure 10b). After 120 min of incubation, the intensity of the R123 fluorescence was lower with 200 nM (1.17 ± 0.09; $P < 0.05$) than the control group (1.30 ± 0.12; Figure 10a, 11). Interestingly, the follicular viability was not modified by any of the 120 min exposures (62.2 ± 9.8, 74.1 ± 8.7, 75.3 ± 7.8 and 71.8 ± 11.7 in 10 nM, 40 nM, 200 nM and control, respectively, Figure 10b). Based on those results, the 120-min exposure was chosen to be explored further.

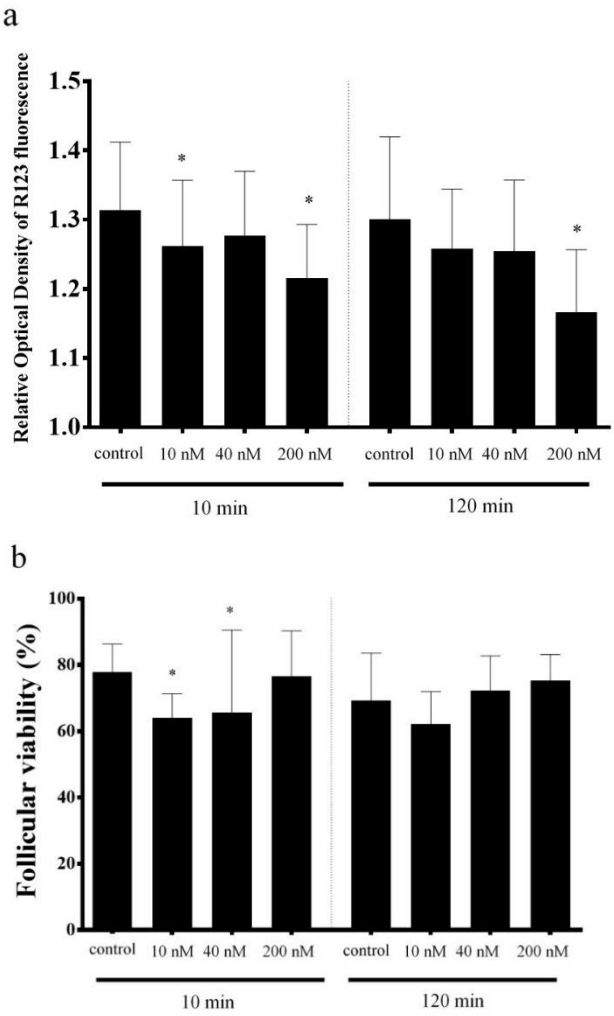

Figure 10. The impact of incubation with 0 (control), 10, 40 or 200 nM FCCP for 10 or 120 min on a) the mitochondrial activities (relative optical density of Rhodamine 123 fluorescence) and b) Percentage of viable follicles in domestic cat ovarian cortical tissues. Asterisks above the bar indicate significant difference to the control group within the same incubation duration ($P < 0.05$). Values are expressed as mean ± SD.

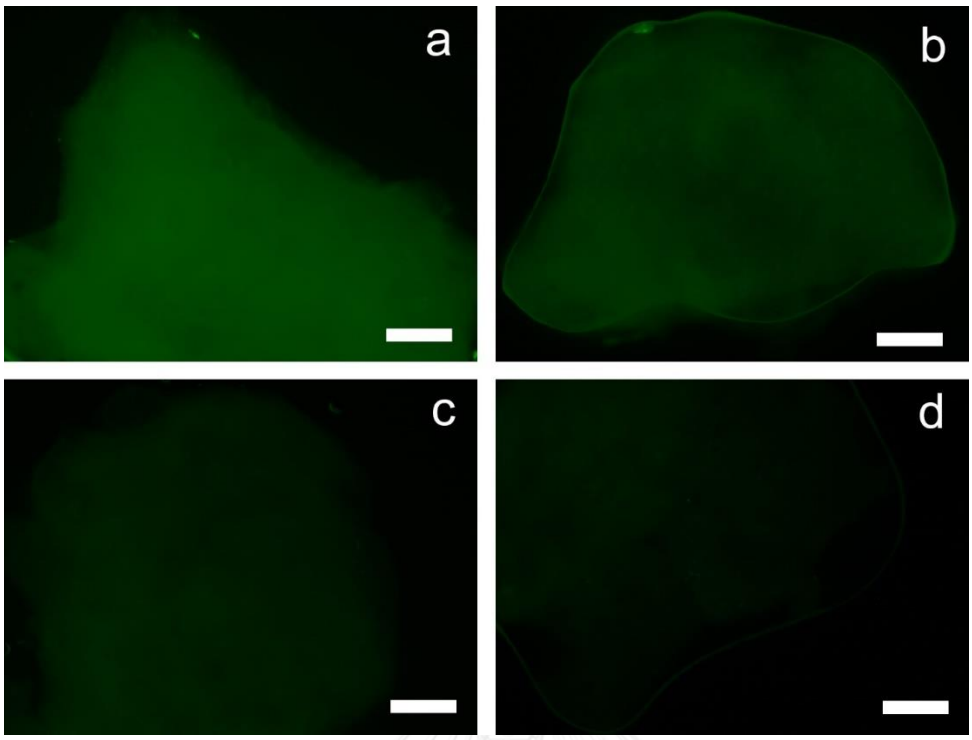

Figure 11. Fluorescence of Rhodamine 123 in ovarian tissues after 120 min incubation in (a) 0 nM (control), (b) 10 nM, (c) 40 nM, or (d) 200 nM FCCP. Bar = 100 μm.

3.4.2 Reversible and beneficial effects of FCCP exposure on the subsequent follicle integrity during *in vitro* culture of cortical tissue

The fresh tissue had more relative optical density of R123 fluorescence staining (1.37 ± 0.06; $P < 0.05$) than all other cultured groups (Figure 12a). After 2 days of culture with 120-min FCCP pre-exposure in various concentration, the intensity of the R123 fluorescence was constant (1.29 ± 0.08, 1.29 ± 0.07, and 1.27 ± 0.07 for 10 nM, 40 nM, and 200 nM, respectively) and not different ($P > 0.05$) from the 2-day control group (1.31 ± 0.01; Figure 12a). Follicular viability percentage of all treatment groups, 10 nM, 40 nM, and 200 nM FCCP, were higher (74.9 ± 6.0, 71.56 ± 7.1, and 78.8 ± 8.9, respectively; $P < 0.05$; Figure 12b) than 2-day control group (61.2 ± 12.0) but still lowered than fresh tissues. For the percentages of morphologically normal follicles, only tissues cultured with 200 nM FCCP was able to maintain morphologically normal follicles for 2 days (57.6 ± 17.3; $P = 0.1877$) compared with fresh tissues (70.2 ± 7.1; Figure 12c). Nevertheless, the groups of 10 nM, 40 nM and control had lower normal

follicles percentages (53.6 ± 8.5, 51.9 ± 15.5, and 48.4 ± 11.5, respectively; $P < 0.05$) than the fresh group. Although, percentages of morphologically normal preantral follicles in 2-day treatment groups were not different among all treatment groups (Figure 12c).

After 7 days of culture, the intensities of the R123 fluorescence of the tissue fragments in the groups pre-exposed with 10 and 40 nM FCCP for 120 min were not different (1.24 ± 0.07 and 1.25 ± 0.06, respectively; $P > 0.05$) from the 7-day control group (1.27 ± 0.03; Figure 12a). The group of 200 nM FCCP incubation, however, had less optical density of R123 fluorescence than the control group (1.22 ± 0.03; $P < 0.05$; Fig. 12a). Follicular viability of all of the FCCP-treated groups were higher (54.2 ± 31.8%, 63.9 ± 17.4%, and 53.8 ± 22.1% in the group of 10 nM, 40 nM, and 200 nM FCCP treatment, respectively; $P < 0.05$) than control group (21.7 ± 21.3%; Figure 12b) but all of them still lowered than the fresh uncultured groups. Morphologically normal follicle was not different among groups of 7-day culture (Figure 12c). However, all treatment groups were tended to be more (34.7 ± 20.8, 25.8 ± 11.1, 33.92 ± 17.3 in 10 nM, 40 nM, and 200 nM FCCP groups, respectively; $P < 0.1$) than control group (13.2 ± 7.4). Furthermore, all 7-days culture groups had lower percentages of morphologically normal follicle than the groups of the same concentration of FCCP in the 2-day culture duration (Figure 12c, 13).

Percentages of positive proliferation marker of all 2-day FCCP-treated groups (ranging from 13.0-16.8; $P > 0.05$) were not different from the fresh group (18.8 ± 5.0). Anyway, the 2-day control group had lower proliferative follicles (8.0 ± 3.4; $P < 0.05$) than the fresh group but not lower than other 2-day treatment groups. In the 7-day period, all groups share the same level of follicular proliferation except the group of 10 nM FCCP that had a lower percentage of proliferation follicles (1.7 ± 1.4; $P < 0.05$) than the control group (4.8 ± 1.5). Moreover, this 7-day 10 nM FCCP treatment group was the only group that has lower proliferation follicles than the 2-day groups of the same FCCP concentration (15.6 ± 4.8; $P < 0.05$; Fig. 12d). Notably, the 7-day culture of

200 nM FCCP group was the only group in this culture duration that had the same level of proliferation (8.8 ± 7.7; as the fresh group (P = 0.058).

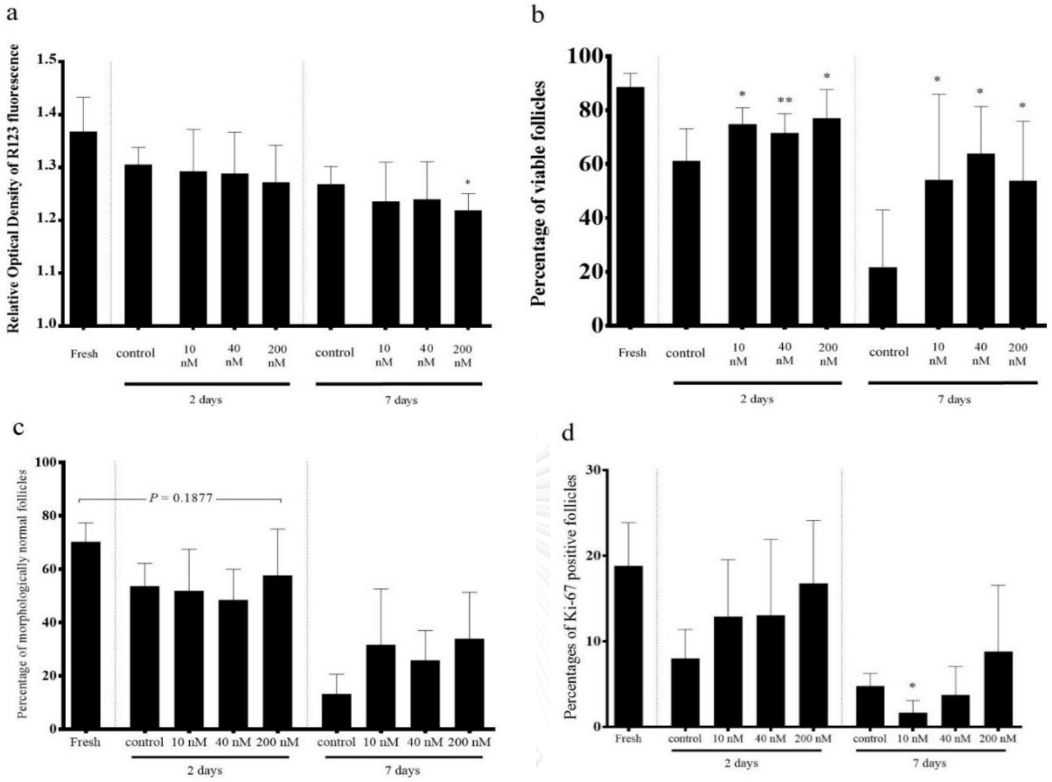

Figure 12. Impact of 120-min pre-exposure to 0 (control), 10, 40 or 200 nM FCCP on (a) mitochondrial activity (relative optical density of Rhodamine 123 fluorescence), (b) percentage of viable follicles, (c) percentages of morphologically normal follicles, and (d) percentage of Ki-67 positive follicles in domestic cat ovarian cortical tissues after 2 or 7 days of in vitro culture. Asterisks above bars indicate significant difference to the control group within the same incubation duration ($P < 0.05$). Values are expressed as mean ± SD.

Follicular density in 2-day culture groups (ranging from 19.1-23.5 follicles per mm^2) were not different from in the fresh group (27.9 ± 10.0; $P > 0.05$). However, the only group in the 7-day culture groups that had the same level of follicular density as the fresh group was the group of 200 nM (14.8 ±10.1 follicles per mm^2; P = 0.0864). When comparing among the same culture duration, there were no differences among groups in the same culture duration both 2- or 7- day duration. When comparing

between 2- and 7-day culture of the same FCCP concentration, only 200 nM FCCP group showed no difference follicular density (19.1 ± 17.3 and 14.8 ± 10.1 in 2- and 7-day culture; $P > 0.05$).

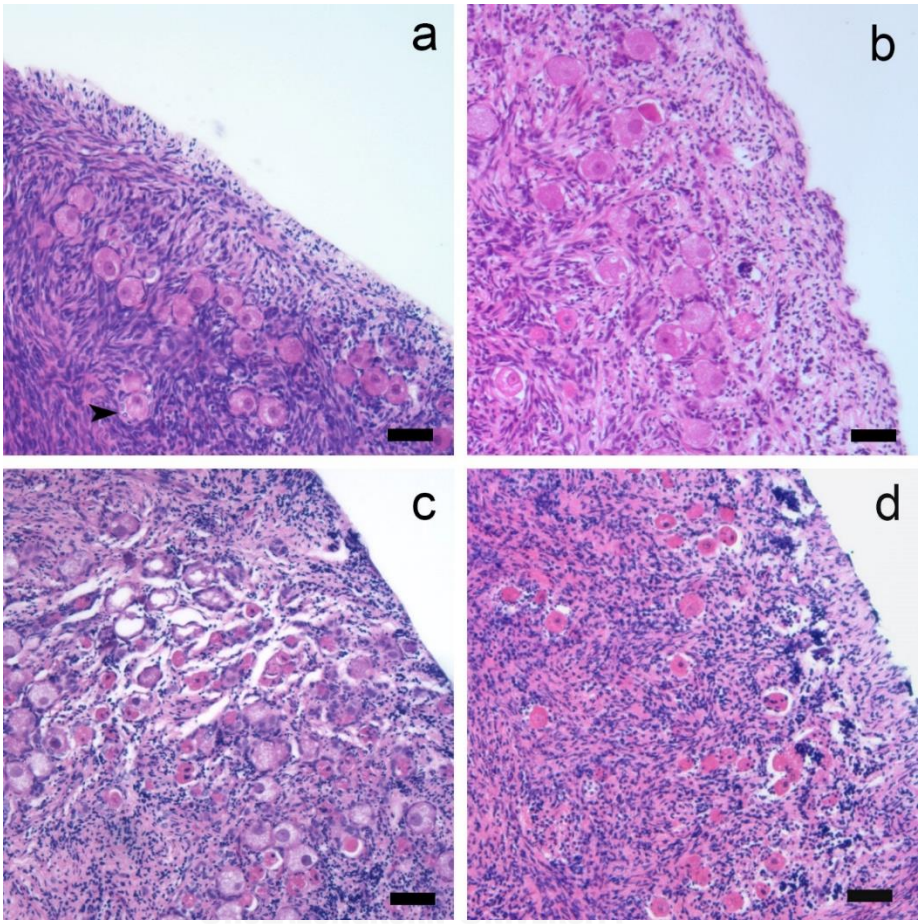

Figure 13. Histological sections of ovarian cortex from (a) fresh tissue, (b) control group after 2 days of culture, (c) control group after 7 days of culture, and (d) tissue pre-exposed to 200 nM of FCCP for 120 min and cultured for 7 days. Primary follicles are identified with an arrow head. Bar = 50 µm.

3.4.3 Effect of incubation with 200 nM FCCP on the survival of cortical tissue during cryopreservation

According to the first and the second experiment, the condition of 200 nM FCCP pre-exposure and the culture duration of 2 days was the most suitable for cortical fragment treatment before cryopreservation considering the mitochondrial activity,

follicle viability, and follicle morphology. Cryopreservation of domestic cat ovarian tissue without pre-exposure with FCCP decreased the fluorescence of R123 staining after thawing in the cryopreserved control group (1.21 ± 0.06; $P < 0.05$) than it was in fresh tissue (1.35 ± 0.08; Table 3). However, after cultured for 2 days, the optical density of R123 staining was not different from cryopreserved control (1.26 ± 0.04 and 1.23 ± 0.07 in culture control and 200 nM FCCP groups; $P > 0.05$; Table 3).

The viability of the follicles within the tissues pre-exposed with 200 nM FCCP and cultured for 2 days before cryopreservation was lower (60.6 ± 12.5; $P < 0.05$) than the group without FCCP (69.3 ± 12.5) and cryopreserved control (73.9 ± 10.2). Moreover, these two latter groups had the same level of percentages of viable follicles ($P > 0.05$; Table 3).

Proliferation marker of Ki-67 expression was decreased from 25.0 ± 9.2% in fresh tissues group into 6.4 – 11.0 % in all groups after cryopreservation ($P > 0.05$; Table 3).

Percentage of morphologically normal follicle of the FCCP treatment group (42.4 ± 10.5) was not changed from the cultured group without FCCP pre-exposure (44.8 ± 10.9; $P < 0.05$). However, the normal follicles of these two groups were still lower than the cryopreserved group which had not cultured before cryopreserved and fresh group (63.8 ± 12.4 and 78.8 ± 9.6, respectively; $P < 0.05$; Table 3).

Follicular density was decline in FCCP-treated group (26.4 ± 14.8; $P < 0.05$) when compared with fresh tissue (44.2 ± 23.8). Moreover, the follicular density of both cryopreserved control and culture control (13.1 ± 1.5 and 18.8 ± 11.7; $P > 0.05$) were not different from the FCCP-treated group and lower than fresh group likewise (Table 3).

Table 3 Mitochondrial activity (Relative optical density of Rhodamine 123 staining), follicular viability, cell proliferation, follicle morphology, and follicular density of domestic cat ovarian cortical tissues in the group of fresh tissue, cryopreserved tissues, and tissue pre-exposed to 200 nM FCCP for 120 min and 2 days of culture prior to cryopreservation and thawing. Values are expressed as mean ± SD.

	Fresh	Cryopreserved control	2-day culture control	FCCP pre-exposure+2-day culture
Rhodamine 123 fluorescence	1.32 ± 0.03 [a]	1.22 ± 0.04 [b]	1.26 ± 0.04 [b]	1.23 ± 0.07 [b]
Viability (%)	81.8 ± 7.5 [a]	73.9 ± 10.2 [b]	69.3 ± 12.5 [b]	60.6 ± 12.5 [c]
Proliferation marker (%)	25.0 ± 9.2 [a]	7.9 ± 5.9 [b]	11.0 ± 5.4 [b]	6.4 ± 3.1 [b]
Morphologically normal follicle (%)	78.8 ± 9.6 [a]	63.8 ± 12.4 [a]	44.8 ± 10.9 [b]	42.4 ± 10.5 [b]
Follicle density (follicle number/mm^2)	44.2 ± 23.8 [a]	13.1 ± 1.5 [b]	18.8 ± 11.7 [b]	26.4 ± 14.8 [b]

[a,b,c] Within the same row, data with the different superscripts are significantly different ($P < 0.05$).

3.5 Discussion

FCCP exposure for 120 min was better than 10 min incubation and the R123 fluorescence that reflect the mitochondrial membrane potential was lowered in 200 nM incubation than the control group did in both duration of incubation. In the second experiment of tissue culture after pre-exposure with FCCP, it showed that 200 nM FCCP pre-exposure and the culture duration of 2 days seemed to be the most suitable for treatment condition for lowering the metabolism of the cell before cryopreservation. Thus we selected this condition to be tested in the last part. However, the condition of 200 nM FCCP treatment did not show a beneficial effect over the non-treatment or cryopreserved control groups.

Regulation of the metabolic activity of the cells or tissues has been widely examined especially in the cells, tissues, and organs to minimize the effects of external stress stimuli (Weisová et al., 2012; Kenwood et al., 2014; Quarrie et al., 2014). These studies have the common concept of minimizing the metabolism of the cell through

the process of the cellular ATP synthesis. The ATP synthesis process majorly occurs in the mitochondria via the oxidative phosphorylation. The reactive species, however, is generated in this process also. The excessive molecules of reactive species are able to deteriorate the cells and cause DNA damages. This reactive species, can be generated during the time of ovarian excision, transportation, culture or even the cryopreservation procedure depended on the damage of the ovarian tissue (Otala et al., 2002; Fabbri et al., 2015).

The technique of mild mitochondrial uncoupling was also studied *in vivo* in the animal model consumption to increase the longevity of the mice. It was found that 2,4-dinitrophenol, the other proton ionophore was able to lower serological glucose, triglyceride and insulin levels in serum, decrease of reactive oxygen species levels and tissue DNA oxidation (Caldeira da Silva et al., 2008).

Studies of female fertility preservation have been proved that it could be the option for collecting the female gametes to be used in the future. Ovarian tissue cryopreservation is one of the options of female fertility cryopreservation methods that has potential for later application of the follicles both *in vivo* and *in vitro* or both of them combined (Wang et al., 2011; Imbert et al., 2014; Meirow et al., 2015). The cryopreservation method in this study was slow-freezing procedure applied from the previous study (Tanpradit et al., 2015). In addition, the slow-freezing procedure is considered the procedure that resulted in more reports of live birth in human ovarian tissue cryopreservation and reimplantation than the vitrification protocol (Donnez et al., 2013; Kawamura et al., 2013; Suzuki et al., 2015).

In the first part of this study, we investigated the concentration and duration of the FCCP in pre-exposure the ovarian cortical tissue fragments by assessing the changes in mitochondrial membrane potential and follicular viability. The FCCP treated groups can lower the relative optical density of R123 fluorescence that reflected the mitochondrial membrane potential within 10 min of incubation in the group of 10 nM and 200 nM. However, in the group of 40 nM did not show the significant difference with the control group. After 120 min of incubation, the only group that showed the

lower fluorescence was the group of 200 nM FCCP and there was no difference between the 10-min group and 120-min group in this concentration. The effects of FCCP have been studied that it can initially depolarize the mitochondrial membrane potential of the within a minute (Perry et al., 2011; Weisová et al., 2012). Moreover, the effect of 100 nM FCCP on transient depolarization of the mitochondrial membrane potential can linger on for 30 min (Weisová et al., 2012). Effect of depolarization of the higher concentration of FCCP can be detected on Calu-6 cells even treated for 72 h (Han et al., 2009). Depolarization of the mitochondrial membrane potential with the low dosage (100 nM) of the FCCP causes the 'mild uncoupling' of the mitochondrial membrane. This phenomenon was shown to provide the protective effect to the cell against external excitotoxic stimuli (Weisová et al., 2012). Although, this protective effect is required the activity of 5′-adenosine monophosphate-activated protein kinase (AMPK), which is the energy sensing pathway to counter the energetic stress response. However, this has not yet studied in the present study. The higher dose of the FCCP treatment i.e. 1 μM or 5-fold of the maximum concentration used in present study caused the full and irreversible depolarization of the mitochondrial membrane potential and resulted in the sharp increase in the overall cell death after 24 h (Brennan et al., 2006b). Follicular viability assessment by CAM/EthD-1 staining showed the lower percentage of viability in the 10 nM FCCP group than in the control group after 10 min incubation. This may be because of the addition of the very low concentration of the FCCP that can cause the hyperpolarization in the rapid effect to the cell (Perry et al., 2011). However, the average percentage of follicular viability in the groups of 10 nM FCCP incubation were 82% of the percentage of the viable follicle in the 10-min control group (Fig. 8b). In contrast, the viability of the 200 nM FCCP group in 10 min duration was maintained in the very similar percentage of the control group (P = 0.862) and fresh group (P = 0.577). The previous study showed the stimulation of neurons with 100 nM FCCP for 30 min did not induce a significant level of injury compare with the sham control group (Weisová et al., 2012). All FCCP-treated group had no difference in viability after longer incubation, but the viability in the 120-min control group had lower viability percentage than the 10-min control group. This might not show the clear protective effects of the FCCP in the immediate effect over the

120 min duration, but it had the potential to maintain the viability of the follicles with in the ovarian tissue after tissue pre-exposure.

In the second experiment, *in vitro* culture of ovarian tissue was performed in order to observe the effect of the FCCP pre-exposure of the domestic cat ovarian tissues. We were not able to distinguish the subtle changes in mitochondrial membrane potential in all treatment groups compare with the control groups (Fig. 10a) except the group of 200 nM in the 7-day culture period that had lowered fluorescence intensity than the 7-day control. Moreover, all culture groups had lower R123 fluorescence intensity than the fresh one. However, on the second day, there was a protective effect on preantral follicular viability all FCCP treatment groups. The viability of the 2-day cultured follicle within the tissue in the present study was comparable to the previous study of 3-day domestic cat ovarian tissue culture in the similar condition (Fujihara et al., 2012b). The effect of FCCP treatment was also found in the 7-day follicular viability. There was sharply increase in the treatment groups, but the only different fluorescence intensity level was found in only 200 nM group. This may due to the sensitivity of the R123 that could not detect the slight change of the mitochondrial membrane potential within the tissue or maybe the effect of the low concentrations on the mitochondrial membrane potential did not exist in this duration, but the viability in treatment groups that kept higher than the control group might be the latent accumulative effect of the better viability observed in the second day.

All of the 2-day culture groups except the group of 200 nM FCCP showed fewer percentages of normal follicle within the tissue. However, no different was found among the groups in the same culture period. This is quite the same pattern when comparing with the follicular viability determined using CAM/EthD-1 fluorescence staining. This might be due to different criteria of these two techniques. In many studies of ovarian tissue culture, the follicular viability examination by membrane integrity and esterase enzyme activity using CAM/EthD-1 staining were found higher than the histologic normal appearance of the follicle (Fujihara et al., 2012b; Fujihara et al., 2014). This may raise the issue that the FCCP could not protect the morphology of the

cultured tissues during *in vitro* culture. The major changes of the follicular appearance in this study were the eosinophilic shift of the cytoplasm and shrunken nucleus. The apoptosis examination should be performed to assess the DNA damage after culture. However, there were reports on the increase of the apoptotic cell after exposure to the FCCP also (Han and Park, 2011; Mlejnek and Dolezel, 2015). The follicular density of all of the cultured tissues was not different to the fresh tissues'. However, the 200 nM has a protective effect on follicular density after 7 days of culture. This observation is similar to the previous study of ovine ovarian tissue cultured with an anti-apototic agent that was found the preservation of normal primordial follicle density after 6 days of culture (Henry et al., 2016). This was suggested that a short period of 2 days was not enough for assessment for the follicular density (Sanfilippo et al., 2013; Henry et al., 2016).

Positive proliferation marker percentages in all of the FCCP treatment groups were not different from the control groups, but it seemed to be lower than the fresh control. However, there was the report suggested that FCCP depolymerized microtubules through the disruption of the mitochondrial proton gradient resulted in increasing the intracellular pH and decreasing the stability of microtubules by impairing the binding of microtubule-associated proteins as well as disrupted the centriole orientation (Maro et al., 1982; Alieva and Vorobjev, 1994).

From the first and second experiments, the condition of 200 nM FCCP pre-exposure for 120 min and culture for 2 days was chosen as condition for treating the tissue before cryopreservation in the third experiment due to the lower R123 found in the 120 min incubation, the viability maintenance both in the immediate effect and after 2-day culture, and the percentage of morphologically normal follicles after culture.

The intensity of the R123 fluorescence of the cryopreservation of the ovarian tissue was lowered even the cryopreserved control group. This was in accordance with the previous study of cryopreserved reproductive cells that had lower mitochondrial membrane potential than the untreated ones after thawing (Jones et al., 2004; Lei et

al., 2014; Yeste et al., 2015). The intensity was increased in all of the culture control group, but this was still lowered than the fresh tissues'. The R123 fluorescence intensity in the 200 nM FCCP pre-exposure group was not different from the culture control group after thawing. This might be because of the lower viable cell within the cryopreserved tissue and the mitochondrial membrane potential might not be recoverable after cryopreservation (Jones et al., 2004; Yeste et al., 2015). The recovery of the mitochondrial membrane potential after washing out has been demonstrated in the previous study (Weisová et al., 2012).

Follicular viability is the primary goal of the ovarian tissue cryopreservation. The viability of the cryopreserved control was lowered after thawing. Moreover, the FCCP treatment group had even lower follicular viability than cryopreservation and culture controls. The percentages of the viable follicle in the present study were similar to the grade A, B, and C (viable follicles) accumulated in the previous study of the domestic cat ovarian tissue cryopreservation using the same method (Tanpradit et al., 2015). This pattern of follicular viability was not in the same pattern of variation of R123 intensity or the morphologically normal follicle.

Percentages of positive proliferative follicles were decreased in all cryopreserved tissues. There still no difference among the groups of cryopreservation. This might due to the impairment of the microtubule found in the *in vitro* culture or cryopreserved tissues in previous studies (Kim et al., 2005). However, there was the report that had increased follicular proliferation after cryopreservation (Isachenko et al., 2013). Mild mitochondrial uncoupling has been shown to be detrimental to the proliferation in the cell culture in the previous study that aimed to induce the arrest of the cell cycle to minimize the proliferation of the pathologic cells (Guimaraes et al., 2012; Mlejnek and Dolezel, 2015). This was in according to the FCCP treatment group that has only 6.4 proliferative preantral follicles per 1 mm^2 of the tissue in the present study.

The follicular density of all of the cryopreserved tissues was found lower than the fresh group as described in the previous study (Milenkovic et al., 2012; Luyckx et

al., 2013b). The cryopreservation process could damage the follicle within the tissue and this parameter may need to be assessed in the long culture period after cryopreservation to see the different between experimental groups (Henry et al., 2016).

The excessive reactive oxygen species that should be lowered by the mitochondrial uncoupling agent after cellular stress or ovary transportation was found in the previous study that the uncoupling itself does not decrease reactive oxygen species production after ischemia-reperfusion situation (Quarrie et al., 2014). It was suggested that increasing of proton leakage using proton ionophore was shown to reduce the production of reactive oxygen species only in normal mitochondria. If the ischemia-reperfusion had been occurred before, increasing of proton leakage would express no effect on reducing the formation of reactive oxygen species molecule. This might apply to the phenomenon observed in the present study after cryopreservation that the FCCP treatment did not show a beneficial effect over the control groups. Other alternatives to reduce the reactive oxygen species in the ovarian tissue culture have been proven to be the beneficial effect recently such as insulin, anti-apoptotic drugs, or superoxide dismutase supplementation (Kim et al., 2015; Aguiar et al., 2016; Henry et al., 2016). Although there are some beneficial effects of FCCP pre-exposure treatment in ovarian tissue in present studies, there will be many aspects to be elucidated such as the AMPK pathway that proved to play a critical role in the mild uncoupling process of the mitochondria, ATP production, reactive oxygen species quantification (Perry et al., 2011; Weisová et al., 2012). The technique of mild mitochondrial uncoupling was also studied *in vivo* in the animal model consumption to increase the longevity of the mice. It was found that 2,4-dinitrophenol, the other proton ionophore was able to lower serological glucose, triglyceride and insulin levels in serum, decrease a reactive oxygen species levels and tissue DNA oxidation (Caldeira da Silva et al., 2008). Thus, it might be possible to treat the animal or human before collecting the ovary for cryopreservation also. In addition, the more precise measurement of the mitochondrial membrane potential should be studied. The techniques of mitochondrial isolation and the usage of electrode sensitive to tetraphenyl phosphonium are able to increase the sensitivity in the shorter timeframe

(Kamo et al., 1979). However, in this study, we use the R123 staining due to the application of the tissue culture that will be more suitable to do the tissue staining to measure the overall mitochondrial membrane potential changes in the whole tissue. In addition, other parameters should be measured to confirm the functional integrity of the ovarian tissue fragment after FCCP pre-exposure like the ovarian cell apoptosis, ATP production, or oxygen consumption assay.

Taken together, the application of FCCP to the domestic cat ovarian tissue was first to demonstrate in this study Domestic cat ovarian tissue pre-exposed to 200 nM FCCP during 120 min protects and enhances the follicle integrity during *in vitro* culture. However, FCCP does not appear to exert a beneficial effect during cryopreservation in the ovarian tissue. This technique is beneficial for pre-treatment of ovarian tissue before *in vitro* culture. However, further evaluation of apoptosis and *in vitro* development of the follicle after cryopreservation is necessary to confirm the potential of this technology.

CHAPTER IV

Xenotransplantation of cryopreserved cat ovarian tissue to nude mice

4.1 Abstract

Transplantation of cryopreserved ovarian tissue has been recently popular in patients undergone chemo- or radiotherapy in the pelvic area and resulted in live births in many studies. However, to translate this technique into the animal for conservation purpose, the ovary obtained from highly valuable genetic animals, xenotransplantation of the cryostorage ovarian tissue into the immunodeficient animal model can served as a model for gamete retrieval. Domestic cat was chosen to be a model for other endangered members in the same family. This study aimed to study the effects of xenotransplantation of cryopreserved domestic cat ovarian tissue into nude mice on the histological morphology of the follicles within the retrieved tissue. Female nude mice (n=8) were selected to be the hosts of adult domestic cat ovarian tissue. Both fresh and cryopreserved ovarian tissues were transplanted at the subcutaneous pocket of the same animal. Vaginal cytology, follicular morphology, follicular density, and percentage of growing preantral follicle were investigated after graft retrieval at 15 days post-transplantation. All mice were survived after transplantation until graft retrieval. However, percentage of morphologically normal follicle was obviously decreased in all preantral follicular stages. The percentage of growing preantral follicle was higher after transplantation (45.8 ± 23.8 and 11.0 ± 2.3 in transplanted tissue and fresh tissue, respectively; $P < 0.05$). In summary, our findings showed the possibility of domestic cat ovarian tissue transplantation in nude mouse especially in fresh tissue. Although, cryopreserved ovarian tissue cannot tolerate to the short-term transplantation as fresh tissue does. This approach still needs further investigation to optimize the transplantation technique in terms of cell proliferation, apoptosis and neovascularization.

4.2 Introduction

Over all of the Felidae family, 25 species are currently considered vulnerable to endangered. Besides *in situ* conservation, assisted reproductive techniques (ARTs) applied to wild species are also importance for reproduce the population and reintroduce them to the wild (Comizzoli et al., 2010). Domestic cat was used as a model for wild cat in the ovarian tissue transplantation to the nude mice in this study due to the genetically relation and the ease of ovary collection. Ovarian tissue cryopreservation is particularly interesting in endangered species conservation to bank ovarian tissue containing an abundant amount of preantral and primordial follicles for individuals undergoing surgical ovarian removal or dying unexpectedly (Jewgenow and Paris, 2006; Wiedemann et al., 2012). After warming the tissue, two major applications of the tissue have been widely studied: *in vitro* culture and *in vivo* transplantation of the tissue. Xenotransplantation, transplantation of tissue from one species to another, can be applied to ovarian tissue of the deceased animal to be grafted into immunodeficient recipient in order to obtain the viable female gametes within the graft later. A combination of female germplasm cryopreservation and xenotransplantation could be the promise to reproduce the valued animals in the future (Paris et al., 2004; Wiedemann et al., 2012; Arregui et al., 2014). Heterotopic ovarian transplantation, the transplantation of the ovary or ovarian cortex in the site other than original site of the ovary such as subcutaneous, back muscle or abdominal wall, was currently considered a promising alternative option for xenotransplantation. This technique showed antral follicle formation, less apoptosis than orthotopic transplantation, and even live births were delivered after *in vitro* fertilization of the oocytes retrieved from the grafts (Fassbender et al., 2007; Stern et al., 2014; Damasio et al., 2016). In present study, subcutaneous was selected to be the site of transplantation because of the ease of access to the transplantation site and to observe the morphology of the graft (Schubert et al., 2008; Youm et al., 2015). Moreover, subcutaneous ovarian tissue transplantation restored long-term ovarian function (> 3 years) and showed a potential to restore fertility in human also (Kim et al., 2009). Although there are many studies about domestic cat ovarian tissue

xenotransplantation, there is no single study of the technique of subcutaneously transplantation in nude mice (Bosch et al., 2004; Fassbender et al., 2007; Wiedemann et al., 2012). This study aimed to study the effects of xenotransplantation of cryopreserved domestic cat ovarian tissue into nude mice on the histological morphology of the follicles within the retrieved tissue.

4.3 Materials and Methods

All chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated.

4.3.1 Animals

Female Balb/c nude mice (8-week-old, n=8; National Laboratory Animal Center, Nakhon Pathom, Thailand) were selected as the hosts for xenotransplantation. Nude mice were selected to be the graft host in this study due to the partially immunocompromised (T-cell depleted) attribute of this animal model that impairs the cell mediated immunity in order to maintain the graft in the host animal. The mice were separately housed in individually ventilated cages supply with filtered air. All mice were freely accessed to food pellet and reverse osmosis water. The light cycle of the room was set as 12L:12D. The temperature was maintained at 24 ± 2 °C throughout the experiment. All animal procedures were approved by the Chulalongkorn University Animal Care and Use Committee by the protocol No. 1573002.

4.3.2 Source of the ovaries

Ovaries were obtained from two cycling 1-year-old female cats by routine ovariohysterectomy performed at the Small Animal Teaching Hospital, Faculty of Veterinary Science, Chulalongkorn University. Both ovaries were transported to the laboratory within 30 min in the holding medium of Leibovitz's L-15 medium with 100 µg/mL penicillin G sodium, and 100 µg/mL streptomycin sulfate. Ovarian cortical fragments were removed from the outermost part of the ovaries using surgical blades

and scissors. Large antral follicles (diameter > 1 mm) and luteal tissue areas were discarded. Cortical tissues were cut into small fragments in the area of 1 x 1 mm^2 and approximately 200 µm thickness in a holding medium with 10 mM HEPES.

4.3.3 Ovarian tissue cryopreservation

Cryopreservation of the tissue used in transplantation was performed 1 week before surgery using slow freezing method (Tanpradit et al., 2015). Briefly, ovarian cortical fragments were placed in cryopreservation medium consisted of L-15 medium supplemented with 1.5 M DMSO (Fluka Chemie GmbH, Buchs, Spain) and 0.1 M sucrose 10 min at 4 ℃. After incubated with cryopreservation medium, tissue fragments were transferred into precooled cryovials (1.6-mL cryogenic vial; Corning, NY, USA) and remained at 4 ℃ for a further 10 min. Then, the cryovials were moved to a passive cooling container (CoolCell; BioCision, Larkspur, CA, USA) to achieve the -1 ℃/min cooling rate and then placed in an -80 ℃ freezer. After 24 h, the cryovials were taken out of the freezer and immediately immersed in liquid nitrogen. Thawing was performed by immersing the vials in a 37 ℃ water bath for 3 min. The tissues were then rapidly placed into a first thawing medium, containing L-15 medium with 0.75 M DMSO and 0.25 M sucrose, at room temperature for 10 min; they were then transferred to a second thawing medium containing L-15 with 0.25-M sucrose for 10 min, and finally, the thawed fragments were placed in the holding medium and washed with holding medium 3 times before beginning of transplantation within an hour. Duration after thawing to the last surgical transplantation procedure was less than 4 h.

4.3.4 Transplantation procedure

After acclimatization for 2 weeks, nude mice were prepared for surgical transplantation of ovarian tissue by injection with analgesics (10 mg/kg BW of carprofen, Rimadyl®, Liboratorios Pfizer, São Paolo, Brazil) and antibiotics (5 mg/kg enrofloxacin, Baytril®, Bayer Korea, Ansan, Korea). In brief, nude mice were general anesthetized by isofurane until animals reached surgical pane. For transplantation, small dorsal incisions (approximately 0.3 mm length) were made in the skin of the mouse approximately 10 mm away from the midline of the lumbar area both left

and right side. Transplantation was performed by placing three ovarian tissue fragments using watchmaker's forceps into the back muscle approximately 2 mm away from the margin of the incision. Afterwards, the tissue fragments were covered by 0.05 mL of Matrigel® to maintain the tissue in the extracellular matrix. All surgical procedures were performed in aseptic condition. Fresh tissues will be transplanted on the left side of the incision line and cryopreserved domestic cat ovarian tissue will be transplanted on the right side of the same animal. After surgery, all mice were injected with carprofen and enrofloxacin for 5 consecutive days. All mice were intraperitoneally injected with 1.25 IU of porcine FSH (pFSH; Folltropin V, Bioniche, Canada) for 3 consecutive days post-transplantation. N-Acetylcysteine (Acetin; L.B.S. labs, Bangkok, 150 mg/kg) was intraperitoneally injected daily from the day of transplantation. Transplanted grafts were collected at the fifteenth day post-transplantation to be further examined. After euthanasia at the designed endpoint, transplants were removed by cutting the graft and adjacent tissue off the mice.

4.3.5 Vaginal cytology examination

Vaginal cytology was performed in all mice daily since 7 days pre-transplantation and every 2 days after surgery. The method of vaginal cytology procedure and interpretation was previously described (Cora et al., 2015).

4.3.6 Histological processing and examination

Fresh control fragments and frozen control fragments were randomly taken from a pool of fresh tissue fragment and thawed fragments before transplantation, respectively. Fresh transplanted and frozen transplanted were taken from the grafts that collected after 15 days of transplantation. All of the tissue fragments were fixed in 4% paraformaldehyde for 24 h and stored in 70% ethanol until paraffin embedding. Each fragment was sectioned at 5 µm thickness and 40 µm apart and stained with hematoxylin and eosin. Follicular morphology was examined under a light microscope (BX51; Olympus, Tokyo, Japan) at X200 magnification. Only follicles with the presence of oocyte nucleus were counted. Evaluation of preantral follicle viability was based

on the integrity of the basement membrane, presence or absence of pyknotic bodies, and integrity of the oocyte and graded as viable or atretic follicles. Stages of preantral follicles were classified as a primordial, primary and secondary follicle. Primordial follicles were characterized by one layer of flattened granulosa cells around the oocyte, primary follicles by one layer of cuboidal granulosa cells, and secondary follicles by two or more layers of granulosa cells. Follicular density was calculated from the amount of all follicles per tissue area of 1 mm^2. Growing follicle ratios were calculated from the sum of the amounts of primary and secondary follicles per a number of total follicles.

4.3.7 Statistical analysis

The percentage of morphologically normal follicle, follicular density, and growing follicle ratio data are presented as mean ± SD. Kolmogorov-Smirnov test was performed to evaluate the normal distribution of each dataset. Dependent variables included follicular viability, were evaluated using the UNIVARIATE procedure for a normality test. Skewness and the Kolmogorov–Smirnov statistic were used to test the normal distribution of the residual of the parameters. Data of preantral follicle viability and follicular density were analyzed using Kruskal -Wallis test by NPAR1WAY procedure. Growing follicle data were analyzed using a general linear model (GLM) procedure to test for the differences of least square means. Significant differences were considered when $P < 0.05$.

4.4 Results

All mice were survived after transplantation until the experimental endpoint. Vaginal cytology showed the normal cycles of mice reproductive pattern around 4-5 days throughout the experiment. No sign of systemic infection was found in all animals throughout the experiment. Half of the nude mice (4 out of 8 mice) had easily visible small (2-5 mm diameter) skin swellings at the transplantation sites of both fresh and cryopreserved tissues after transplanted for 5 days through the graft recovery (Figure 14). Grafted ovarian tissue fragments (21 and 22 pieces of fresh and cryopreserved grafts, respectively) were recovered after transplanted for 15 days in the host mice.

Two-thirds of the grafts from the fresh tissues were attached to the skin and/or back muscle of the mice (14 out of the 21 tissues). Whereas the 12 of 22 cryopreserved tissue pieces of the grafts attached to the adjacent to the mice tissue. Some blood vessels invading the grafts were visible by the time of graft collection (4 fresh grafts and 2 cryopreserved graft). No fluid-filled structure was found in all collected grafts. Histological observation demonstrated a severe inflammatory response in the fresh grafts as abundant white blood cells infiltration (Figure 15). Some grafted tissue fragments had abundant red blood cell infiltration (Figure 16), 12 of 21 fresh fragments and 11 of 22 cryopreserved fragments.

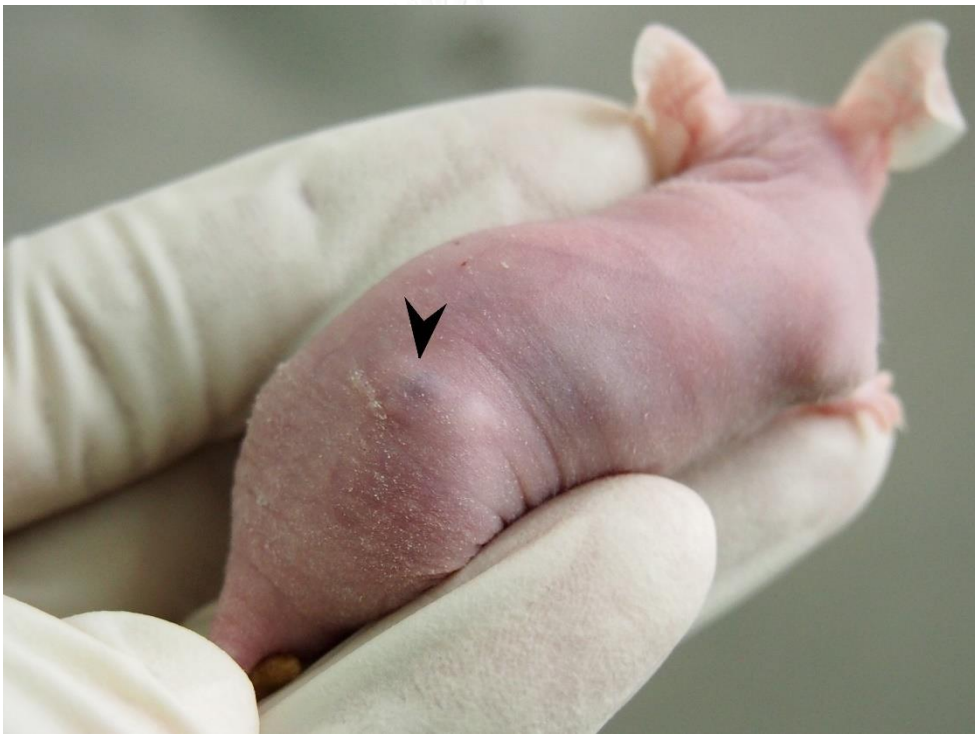

Figure 14. Small dark skin swelling was able to observed at the transplantation site of cryopreserved fragments of the mouse.

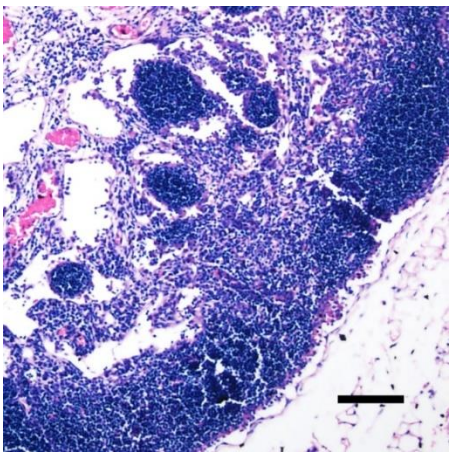

Figure 15. White blood cell severely infiltration in one of the non-cryopreserved tissue graft. Back bar on the bottom right indicates the actual of 100 μm.

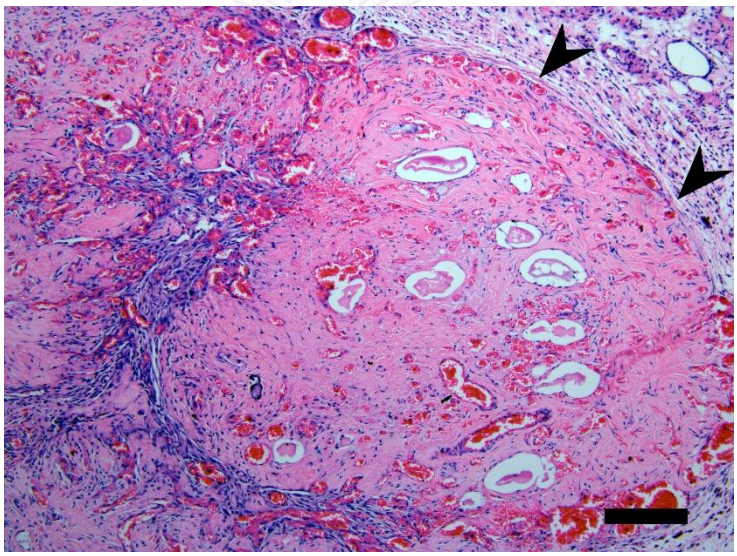

Figure 16. Red blood cell infiltration in the area that used to be follicles and filled in the area adjacent to the graft observed in one of the cryopreserved tissue graft. Black arrow head indicates the graft area boundary. Black bar on the bottom right indicates the actual length of 100 μm.

The percentage of normal preantral follicles was sharply reduced in all stages after transplantation (Table 4). Notably, for primordial and primary, the grafts from fresh tissues had more normal follicles than the cryopreserved tissues. Though the secondary follicles were not different between fresh and cryopreserved transplants. In all preantral stages, normal preantral follicles of the cryopreservation groups were lower than the fresh groups ($P < 0.05$, Table 4).

Table 4 Percentages of morphologically normal preantral follicle in each stage within the fresh or cryopreserved ovarian tissue fragments both before and after transplantation. Data are presented as mean ± SD.

Stage	Before transplantation		After transplantation	
	Fresh	Cryopreserved	Fresh	Cryopreserved
Primordial	88.9 ± 3.7 [a]	57.9 ± 11.8 [b]	15.9 ± 10.4 [c]	1.8 ± 2.6 [d]
Primary	77.0 ± 23.5 [a]	54.1 ± 11.7 [b]	13.4 ± 8.8 [c]	2.8 ± 3.4 [d]
Secondary	55.5 ± 46.4 [a]	14.7 ± 11.2 [b]	1.3 ± 3.3 [c]	3.2 ± 3.1 [c]

[a, b, c, d] Different superscript letters indicate significant differences among the groups in the same row.

Primordial follicular density in untreated grafts post-transplantation was lower (23.5 ± 11.1 follicles/mm^2; $P < 0.05$) than fresh ovarian tissue group (58.4 ± 20.7 follicles/mm^2, Table 5). However, this pattern of density reduction did not occur in the cryopreserved tissue after transplantation. Primary follicle density was higher in untreated transplanted tissues (30.1 ± 15.8 follicles/mm^2; $P < 0.05$) than fresh tissue (6.5 ± 4.0 follicles/mm^2, Table 5). However, there was no difference of secondary follicles between untreated transplants and fresh tissues. Additionally, no difference was found in all cryopreserved tissue between pre- and post-transplantation (Table 5).

Table 5 Follicular density of the preantral follicle in each stage within the fresh or cryopreserved ovarian tissue fragments both before and after transplantation. Data are presented as mean ± SD.

Stage	Before transplantation		After transplantation	
	Fresh	Cryopreserved	Fresh	Cryopreserved
Primordial	58.4 ± 20.7 [a]	55.9 ± 21.7 [a]	23.5 ± 11.1 [b]	21.5 ± 13.4 [ab]
Primary	6.5 ± 4.0 [a]	23.9 ± 7.2 [ab]	30.1 ± 15.8 [b]	10.4 ± 12.6 [a]
Secondary	1.0 ± 0.2 [a]	4.6 ± 2.4 [b]	4.3 ± 6.2 [ab]	3.46 ± 6.19 [ab]

[a, b] Different superscript letters indicate significant differences among the groups in the same row.

Majority of preantral follicles found in ovarian tissue were primordial follicles (n=5104, 69.1%) followed by primary follicles (n=1861, 25.2%), and secondary follicle (n=425, 5.7%). However, the distribution of growing preantral follicles was uneven in each treatment group. In fresh tissue, growing follicle percentage was increased after transplantation (11.0 ± 2.3 % and 34.8 ± 7.7 %; $P < 0.05$, Table 6). In cryopreserved tissues, however, did not show the significant difference between pre- and post-transplantation growing follicle percentages.

Table 6 Percentages of growing preantral follicle per total amount of preantral

	Before transplantation		After transplantation	
	Fresh	Cryopreserved	Fresh	Cryopreserved
Growing preantral follicle (%)	11.0 ± 2.3 [a]	34.8 ± 7.7 [b]	45.8 ± 23.8 [b]	45.1 ± 33.2 [b]

[a,b] Different superscripts indicate significant difference among the groups .

4.5 Discussion

This is the first study describing the xenotransplantation of frozen–thawed domestic cat ovarian tissue in nude mice. Cryopreserved ovarian tissues from domestic cat were transplanted into subcutaneous pocket of nude mice to investigate the possibility of follicle development *in vivo* after thawing. Percentages of morphologically normal preantral follicles of all stages within the retrieved graft were sharply decreased from that before transplantation. However, the percentage of growing preantral follicles were increased in the untreated tissue after transplantation.

The percentage of morphologically normal preantral follicles was decreased after transplantation whether the tissue was cryopreserved or not. This is the normal pattern found in the transplantation of the ovarian tissue due to the ischemic period after transplantation. However, there were many approaches to minimize damages from this period such as the supplementation of the growth factors that promotes neovascularization like vascular endothelial growth factor (VEGF), reducing the oxidative stress by precondition or injection of N-acetylcysteine, melatonin, vitamin C, or vitamin E (Abir et al., 2011; Shikanov et al., 2011; Friedman et al., 2012; Mahmoodi

et al., 2015). In the present study, N-acetylcysteine was chosen to be the antioxidant. Nevertheless, it cannot compensate the injury after transplantation.

The percentage of normal follicle after cryopreservation in the present study were similar to the previous study in the domestic cat ovarian tissue cryopreservation using the same method of slow-freezing in chapter II and III (Tanpradit et al., 2015). The normal primordial follicles in the untreated grafts post-transplantation were approximately 18% of the amount in the fresh tissue. In contrast of the normal follicles in the cryopreserved tissue were only 3% of those before transplantation. This pattern is also found in the primary follicle (24% vs 4%) but not in the secondary follicle which has very low percentage of normal follicle in both untreated and cryopreserved groups. The primordial follicle was found to be resistant to the hypoxic environments in the previous study (Youm et al., 2015). This was in accordance with the previous studies on cryopreservation that early stage follicles were more tolerance to cryopreservation than the advanced stage follicles (Gosden et al., 2002). This might be due to the less metabolic activity, less volume, and less nutrient consumption in this type of follicles that made them more tolerated to a stressful situation. However, more exact evaluations should be performed additionally to verify the results.

Several studies reported that the absence of antral and secondary follicles in ovarian tissue transplants could activate the primordial follicles and accelerate their growth because of the lack of inhibitory factors released by the advanced growing follicles (Abir et al., 2011). This was in the same as in the study of xenotransplantation of domestic cat ovarian tissue into NOD SCID mice that a large antral formation in the graft required 67 days of transplantation including 5 days of graft stimulation by eCG and hCG and in another study of ovarian tissue transplantation in nude rat that was able to detect the antral formation in the graft as early as 6 weeks post-transplantation (Fassbender et al., 2007). However, the duration of normal folliculogenesis from primordial to antral follicle was still unknown but this acceleration in follicular development after *in vivo* or *in vitro* culture of ovarian tissue was evidenced by previous studies of other species. In the present study, we found more percentage of

growing preantral follicle after transplantation of fresh tissue but no antral formation was found in any graft retrieved in a shorter period of 15 days. The cryopreserved tissue had more growing follicles than fresh tissue after thawing in the present study. This is in the same manner of the previous study both in the cats and bovines (Castro et al., 2014; Tanpradit et al., 2015)

Nowadays, many immunodeficient animal models and techniques for transplantation have been developed. The effective site for ovarian tissue transplantation has been studied for various sites. Each site has their own advantages and disadvantages. In the present study, subcutaneous pocket at the back was chosen due to the large space of the transplantation site that can be reached easily and the visibility of the grafts after transplantation in the nude mice. The back muscle, abdominal fat pad, kidney capsule and subcutaneous pocket have been investigated simultaneously for the outcome of ovarian tissue grafting (Youm et al., 2015). It was found that subcutaneous site had drawbacks on the relatively poor vascularization when compare with the other internal body sites. Moreover, the stressful and fluctuating environment in the subcutaneous site may lead to higher apoptosis follicles because it was closed to the skin and ambient environment. In addition, some studies suggested that the spacious pocket in the subcutaneous space allowed the graft to move and leaded to the poorer and delayed vascularization than other sites (Torrents et al., 2003; Dath et al., 2010). In the present study, about two-thirds of the grafts were attached to the muscle layer or skin of the mice. This was likely because of the Matrigel, a gelatinous protein mixture that resembles the complex extracellular environment found in many tissues injected at the time of transplantation. Matrigel was widely used in cell culture as the basement membrane-like substance that enables the 3D cell culture conditions and cellular differentiation. However, even the tissue was fixed by this strategy, vascularization was scarcely found invading the grafts (4/21 and 2/22 of fresh and cryopreserved tissue graft, respectively) even this mixture was suggested to promote capillary-like structure and angiogenic activity of vascular endothelial cells *in vitro* (Francescone et al., 2011; Khoo et al., 2011). However, there was a study using Matrigel incorporated with human fibroblast to promote *in vivo*

neovascularization in mice (Guerreiro et al., 2012). This technique should be further investigated for subcutaneous transplantation in the future. Despite a low vascularization in this transplantation site, we found abundant red and white blood cells infiltrated and surrounded the grafted tissue (Figure 13, 14). Moreover, the concept of Matrigel or other fibrin matrices was extensively studied to develop an 'artificial ovary' for propagating the female gamete (Luyckx et al., 2014; Soares et al., 2015). Further study of angiogenesis marker expression and apoptosis of the follicles and stroma are needed.

The immunodeficient murine model in this study was a nude mouse. It was shown that this animal model could support early follicular growth and preserved some quiescent follicles after short-term frozen-thawed human ovarian tissue transplantation (Dath et al., 2010). However, in this present study, we found numerous red blood cells and lymphocytes infiltration in the retrieved grafts. Despite Balb/c nude mice lacks a thymus that makes them unable to produce T-cells, they still have B-cells and NK-cells and may have T-cell leakage as they aged. This means they are still capable of producing antibodies, and cytokines and rejecting the non-self antigen in some cases even they are immunodeficiency. This was unlike the previous studies of the domestic cat, ovarian tissue xenotransplantation was performed in other hosts such as NOD SCID mice or nude rat which resulted in antral formation within the graft after transplantation for a longer period (Abir et al., 2011).

In summary, although the percentage of morphologically normal follicle were reduced after 15 days of ovarian tissue transplantation both in fresh and cryopreserved cat ovarian tissues, the remaining follicles, however, could retain their developmental competence within the tissue transplanted into the subcutaneous pocket of the nude mouse. Additionally, the massive follicular loss and inflammatory cell infiltration clearly warrant additional investigation to a technical improvement.

CHAPTER V

General discussion and conclusion

This thesis demonstrated the female gamete preservation and the development of the preantral follicles within the domestic cat ovarian tissue both *in vitro* culture and *in vivo* xenotransplantation.

5.1 Ovarian tissue cryopreservation

Ovarian tissue cryopreservation represents one of the most important advances in the field of reproductive biology (Frydman and Grynberg, 2016). In human, this technique restores the potential of being a genetic mother back from either radical treatment or the diseases themselves. These two reasons impair the limited follicular pool in the ovaries of the patients. The ovarian tissue cryopreservation technique is also important for conservation purpose. When female animal died unexpectedly, ovarian tissue cryopreservation is the only safeguard for the female gamete within the ovary to further grow to the fertilizable oocyte and produce a new offspring posthumously. However, female fertility preservation is still at the pioneering level and is thus often considered an experimental approach. In chapter II, we demonstrated the beneficial effects of sucrose supplementation and slow-freezing method on the follicular viability, histologic appearances of follicles, and follicular and stromal cell apoptosis in cat ovarian tissues. We also established the immunohistochemical procedure to evaluate the gap junction protein, Cx43, that plays important roles in various physiological processes including follicular development. The intensity of gap junction protein Cx43 was compromised by cryopreservation, with the tendency that sucrose supplementation could protect cryodamage to the gap junction protein across follicular cells. We also found that sucrose supplementation improved qualities of the ovarian tissue in many aspects which was in agreement with previous studies of fertility preservation in human (Coticchio et al., 2006; Bianchi et al., 2007; Marsella et al., 2008). Moreover, our study was the first to compare the two methods of cryopreservation in the cat ovarian tissue. The previous studies of cat ovarian tissue cryopreservation

before the present study, performed only one method of cryopreservation either slow-freezing or vitrification but the efficacy of these two methods have not yet been directly compared before (Jewgenow et al., 1998; Lima et al., 2006; Luvoni et al., 2012). Therefore, the findings from chapter II could establish the practical ovarian tissue cryopreservation protocol to be utilized in our research unit. In addition, this successful model of ovarian tissue cryopreservation in the domestic cats has been translated to a wild felid, the cheetah (Wongbandue et al., 2013). The cheetah ovarian follicles obtained from cryopreserved tissues can be grown *in vitro* for 7 days. The study in chapter II was further investigated in the next two chapters showing the applications of the thawed ovarian tissue to grow the pool of preantral follicles within.

5.2 Effects of FCCP on *in vitro* ovarian tissue culture

The concept of uncoupling the mitochondrial membrane potential before undergone the stress environment like cryopreservation or *in vitro* culture was tested in chapter III. Low concentrations of FCCP was chosen to be the proton ionophore which uncouples the oxidative phosphorylation and lower the metabolic activity in this study. We optimized the concentration and duration of the FCCP pre-exposure in the first part. Then the effects of FCCP pre-exposure with ovarian tissue on the integrity of the follicles after *in vitro* tissue culture were examined. In the last part of chapter III. Despite the FCCP pre-exposure had no beneficial effect after ovarian tissue cryopreservation as we hypothesized, the pre-exposure condition of 200 nM FCCP 120 min provided protective effects on the follicular viability and follicular morphology of the tissue cultured *in vitro.* This substance should be used for pre-treatment of ovarian tissue before *in vitro* culture in the further study.

5.3 Xenotransplantation of the cryopreserved ovarian tissue

The final study about the xenotransplantation of domestic cat ovarian tissue into nude mice for 15 days revealed that even massive follicular loss has occurred, there was a few proportion of preantral follicle that survived the ischemic period and developed inside the grafted tissue. Even though there were many studies about

ovarian tissue cryopreservation and transplantation, this present study in chapter IV gave more information about using the intact female nude mouse model for being a host for ovarian tissue and the possibility of using Matrigel with the subcutaneous transplantation. To date, this is the first study of domestic cat ovarian tissue xenotransplantation using Matrigel as a tissue fixator in subcutaneous transplantation. since the promising results of isolated ovarian cells embedded in alginate-Matrigel or artificial ovary study (Vanacker et al., 2012).

5.4 Conclusion

Ovarian tissue cryopreservation of the domestic cat as a model for other wild felids is a promising tool for female gamete preservation on conservation basis. To achieve the goal of fertility preservation, the preserved gamete needs to be grown to the fertilizable stages and further undergone ARTs manipulation to conceive the live births. The present study indicates the beneficial effects of sucrose in slow-freezing cryopreservation on follicle survival. After thawing, we investigated the application of the frozen-thawed tissue both *in vitro* and *in vivo.* The study of a low dosage of FCCP, a mitochondrial inhibitor, pre-exposure before *in vitro* tissue culture showed the protective effects on follicular viability and morphology up to 7 days. However, it does not exert these protective effects after tissue cryopreservation. Finally, despite subcutaneous xenotransplantation of the cryopreserved domestic cat ovarian tissue into nude mice shown the significant loss of preantral follicles after cryopreservation and transplantation for 15 days, it still has potential to develop growing preantral follicles within the graft. Taken together, the techniques of ovarian tissue cryopreservation and *ex situ* follicle development both *in vitro* culture and *in vivo* xenotransplantation suggested the possibility to rescue female gamete and develop the preantral follicle pool in feline species.

5.5 Research limitations

In chapter II, even though the apoptosis and Cx43 gap junction protein can be detected by immunohistochemistry, immunofluorescence was suggested in terms of

easier co-localization observation. However, the tissue sections were fixed in aldehyde-base fixative, thus the autofluorescence after staining was interfered the histological analysis and this technique has to be discarded and using only the immunohistochemistry staining instead. In Chapter III, the sources of the ovaries were far from the laboratory and it had to be collected at night and transported in 4 °C in normal saline before further processing in the next morning. This period might consider as the ischemic period after ovaries were excised from the animal. However, all of the ovary samples in that study were processed at the same condition of transportation. Additionally, this duration resembles the situation of the gamete rescue in the field. In the last experiment, the serum estradiol and progesterone cannot be detected due to the minimum volume of serum sample requirement in the hormonal analysis in the laboratory, so the vaginal cytology has to be the indirect tool for observing the hormonal change in the host. However, we used the intact adult mice and all of them shown a normal reproductive pattern throughout the study. The size of the 8-week mice is quite small (some are less than 20g at the day of transplantation) and this resulted in the prolonged transplantation operation time. However, all mice were survived until the day of graft retrieval.

5.6 Suggestions for further investigations

Other non-permeable CPAs like trehalose should be considered as an alternative or combined with sucrose due to properties of this disaccharides in other cryopreservation studies. Other novel techniques of vitrification that have been studied to be a potential cryopreservation method like Ovarian Tissue Cryosystem method (Carvalho et al., 2013) or solid-state vitrification should be further examined to increase the efficiency of vitrification protocol. Cellular apoptosis should be investigated in the tissue that has been pre-exposed with FCCP due to some previous studies that suggested the DNA damages after depolarization of mitochondria for a long time. More accurate mitochondrial membrane analysis like mitochondrial isolation and the usage of electrode sensitive to tetraphenyl phosphonium are able to increase the sensitivity of the test in a second scale. ATP production should be performed to confirm the

decrease in ATP production after FCCP exposure also. Proliferative marker examination such as Ki-67 should be performed in the last transplantation study to ensure and localize the proliferation in the graft tissue. Vascularization marker staining like CD34 (vascular endothelial cell marker) and quantification should be done to assess the possibility of vascularization after tissue transplantation. Moreover, tissue oxygenation should be further investigated in a new model of transplantation also.

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APPENDIX

Publications

1. Tanpradit N, Comizzoli P, Srisuwatanasagul S and Chatdarong K 2015. Positive impact of sucrose supplementation during slow freezing of cat ovarian tissues on cellular viability, follicle morphology, and DNA integrity. Theriogenology. 83: 1553-1561.

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