THE EFFECTS OF DIFFERENCE COMBINATIONS OF CRYOPROTECTANTS FOR THE VITRIFICATION OF *IN VITRO* MATURED BOVINE OOCYTES

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

Suranaree University of Technology

Academic Year 2017

ผลของสารไครโอโพรเทกแทนต์ต่อการแช่แข็งไข่โคด้วยวิธีวิทริฟิเกชั่น

นายวัชระ วิปัสสา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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ปีการศึกษา 2560

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

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วัชระ วิปัสสา : ผลของสารไครโอโพรเทกแทนต์ต่อการแช่แข็งไข่โคด้วยวิธีวิทริฟิเกชั่น (THE EFFECTS OF DIFFERENCE COMBINATIONS OF CRYOPROTECTANTS FOR THE VITRIFICATION OF *IN VITRO* MATURED BOVINE OOCYTES) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.รังสรรค์ พาลพ่าย, 69 หน้า

การวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาความเป็นพิษและประสิทธิภาพในการแช่แข็งของสารไครโอโพรเทกแทนต์ต่ออัตราการรอดชีวิตและการพัฒนาเป็นตัวอ่อนระยะต่างๆของไข่โคภายหลังแช่แข็งด้วยวิธีวิทริฟิเกชั่น ในการศึกษานี้สารไครโอโพรเทกแทนต์ ได้แก่ เอทิลีนไกลคอล (EG) โพรพิลีนไกลคอล (PROH) และไดเมทิลซัลฟอกไซด์ (DMSO) ได้ถูกนำมาใช้ในอัตราส่วน 4% (v/v) ในน้ำยา equilibration (ES) และ 35% (v/v) ในน้ำยา vitrification โดยใช้แบบผสม ดังนี้ EG + DMSO, EG + PROH, PROH + DMSO และ EG + PROH + DMSO

การทดลองที่หนึ่ง เพื่อศึกษาความเป็นพิษของสารไครโอโพรเทกแทนต์ ไข่โคที่ผ่านการเลี้ยงให้สุกในหลอดทดลองถูกนำไปผ่านน้ำยาแช่แข็งที่มีส่วนผสมของสารไครโอโพรเทกแทนต์แตกต่างกัน 4 ชนิด เป็นเวลา 30 วินาที โดยไม่ผ่านการแช่แข็ง ผลปรากฏว่า อัตราการรอดชีวิต (93.53% – 94.86%) การพัฒนาเป็นตัวอ่อนระยะ cleavage (58.73% - 61.39%) และบลาสโตซีสต์ (22.65% - 22.82%) ในกลุ่มไข่โคที่ผ่านน้ำยาแช่งแข็งทั้ง 4 ชนิด ไม่มีความแตกต่างกันทางสถิติและเมื่อเปรียบเทียบกับไข่โคในกลุ่มควบคุม (95.53%, 62.08% และ 24.88% ตามลำดับ)

การทดลองที่สอง เพื่อศึกษาประสิทธิภาพในการแช่แข็งของสารไครโอโพรเทกแทนต์ไข่โคที่ผ่านการเลี้ยงให้สุกในหลอดทดลองถูกนำไปผ่านน้ำยาแช่แข็งที่มีส่วนผสมของสารไครโอ-โพรเทกแทนต์แตกต่างกัน 4 ชนิด เป็นเวลา 30 วินาที แล้วนำไปแช่แข็งด้วยวิธีวิทริฟิเกชั่นและเก็บไว้ในไนโตรเจนเหลว ผลปรากฏว่า อัตราการรอดชีวิตหลังทำละลายในกลุ่มไข่โคที่ผ่านการแช่งแข็งด้วยน้ำยาแช่แข็งทั้ง 4 ชนิด (89.56% - 92.37%) ให้ผลไม่แตกต่างกันทางสถิติเมื่อเปรียบเทียบกับไข่โคในกลุ่มควบคุมที่ไม่ผ่านการแช่แข็ง (95.96%) ภายหลังการเลี้ยงไข่โคแช่แข็งที่รอดชีวิตต่อในหลอดทดลอง พบว่า อัตราการพัฒนาเป็นตัวอ่อนระยะ cleavage (36.80% - 41.56%) และระยะบลาสโตซีสต์ (5.45% - 7.61%) ในกลุ่มไข่โคที่ผ่านการแช่งแข็งด้วยน้ำยาแช่แข็งทั้ง 4 ชนิด ไม่แตกต่างกันทางสถิติ แต่ให้ผลต่ำกว่าอย่างมีนัยสำคัญทางสถิติ ($P>0.05$) เมื่อเปรียบเทียบกับไข่โคในกลุ่มควบคุมที่ไม่ผ่านการแช่แข็ง (64.86% และ26.35% ตามลำดับ)

การทดลองที่สาม ไข่โคถูกนำไปเลี้ยงให้สุกในน้ำยาเลี้ยงที่เติมสาร IGF-1 ในปริมาณ 0, 50, 100 และ 200 นาโนกรัมต่อมิลลิลิตร ในน้ำยาเลี้ยงไข่สุก ภายหลังการแช่แข็งและทำละลายผลปรากฏว่า ไม่พบความแตกต่างทางสถิติในอัตราการรอดชีวิตของกลุ่มไข่โคที่ผ่านการเลี้ยงในน้ำยา

เลี้ยงไข่สุกที่เติมสาร IGF-1 ก่อนการแช่งแข็ง (92.55% - 95.11%) และไข่โคในกลุ่มควบคุมที่ไม่ผ่านการแช่แข็ง (95.96%) ภายหลังการปฏิสนธิในหลอดทดลอง ถึงแม้ว่าอัตราการพัฒนาเป็นตัวอ่อนระยะ cleavage ในกลุ่มไข่โคที่ผ่านการเลี้ยงในน้ำยาเลี้ยงไข่สุกที่เติมสาร IGF-1 ในปริมาณ 50 และ 100 นาโนกรัมต่อมิลลิลิตร (50.91% และ 53.15%) ก่อนการแช่งแข็งให้ผลสูงกว่ากลุ่มไข่โคแช่แข็งที่ไม่ได้เติมสาร IGF-1 (0 นาโนกรัมต่อมิลลิลิตร) ในน้ำยาเลี้ยงไข่สุกอย่างมีนัยสำคัญทางสถิติ ($P>0.05$) แต่เมื่อเปรียบเทียบกับไข่โคในกลุ่มควบคุมที่ไม่ผ่านการแช่แข็ง (63.51%) ปรากฏว่าให้ผลต่ำกว่าอย่างมีนัยสำคัญทางสถิติในส่วนอัตราการพัฒนาเป็นตัวอ่อนระยะบลาสโตซีสต์ในกลุ่มไข่โคที่ผ่านการเลี้ยงในน้ำยาเลี้ยงไข่สุกที่เติมสาร IGF-1 (7.19% - 8.24%) ให้ผลไม่แตกต่างกันทางสถิติเมื่อเปรียบเทียบในกลุ่มการเติมสาร IGF-1 ในน้ำยาเลี้ยงไข่สุกในปริมาณต่างๆ แต่ให้ผลต่ำกว่าอย่างมีนัยสำคัญทางสถิติ ($P>0.05$) เมื่อเปรียบเทียบกับไข่โคในกลุ่มควบคุมที่ไม่ผ่านการแช่แข็ง (25.11%)

ผลของการศึกษานี้แสดงให้เห็นว่า สามารถแช่แข็งไข่โคโดยใช้น้ำยาแช่แข็งที่มีส่วนผสมของสารไครโอโพรเทกแทนต์ ทั้ง เอทิลีนไกลคอล โพรพิลีนไกลคอล และไดเมทิลซัลฟอกไซด์ ซึ่งน้ำยาแช่แข็งเหล่านั้นไม่ก่อความเป็นพิษและให้ประสิทธิภาพการแช่แข็งที่เหมือนกันต่ออัตราการรอดชีวิตและการพัฒนาเป็นตัวอ่อนระยะต่างๆของการแข็งไข่โคด้วยวิธีวิทริฟิเคชั่น และถึงแม้การเติมสาร IGF-1 ในน้ำยาเลี้ยงไข่สุกจะช่วยเพิ่มอัตราการพัฒนาเป็นตัวอ่อนระยะ cleavage แต่ไม่มีผลต่อการพัฒนาเป็นตัวอ่อนระยะบลาสโตซีสต์ของการแข็งไข่โคด้วยวิธีวิทริฟิเคชั่น

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

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VAJARA WIPASSA : THE EFFECTS OF DIFFERENCE COMBINATIONS OF CRYOPROTECTANTS FOR THE VITRIFICATION OF *IN VITRO* MATURED BOVINE OOCYTES. THESIS ADVISOR: ASSOC. PROF. RANGSUN PARNPAI, Ph.D., 69 PP.

BOVINE OOCYTE/CRYOPROTECTANT/IGF-1/VITRIFICATION

The objectives of this study were to investigate the toxic effect and vitrification efficiency of permeating cryoprotectants (CPAs) on oocyte survival and embryo development of *in vitro* matured (IVM) bovine oocytes after vitrification. In this study, three CPAs including ethylene glycol (EG), dimethyl-sulfoxide (DMSO) and propylene glycol (PROH) were added in a equilibration solution (ES) and a vitrification solution (VS) in total 4% (v/v) and 35% (v/v) of 4 kinds of combinations (EG + DMSO, EG + PROH, PROH + DMSO and EG + PROH + DMSO).

In experiment I, the toxic effect of these solutions was investigated. IVM bovine oocytes were exposed to 4 kinds of CPA combinations for15 min in ES and 30 sec in VS without cooling. There was no difference on survival (93.53% – 94.86%), cleavage (58.73% - 61.39%) and blastocyst rates (22.65% - 22.82%) in CPA exposed groups compared to the fresh control group (95.53%, 62.08%, and 24.88%, respectively).

In experiment II, the vitrification efficiency of these solutions was investigated. IVM bovine oocytes were vitrified with the above 4 kinds of CPAs combinations by using Cryotop. After warming, the survival rate in the 4 vitrified groups (89.56% - 92.37%) was not significantly different compared to the fresh control (95.96%).

The rates of cleavage (36.80% - 41.56%) and blastocyst (5.45% - 7.61%) in the 4 vitrified groups were significantly lower (P > 0.05) than those of the fresh control group (64.86% and 26.35%, respectively). But, these solutious made no statistically significant difference in the blastocyst rate among the 4 vitrified groups (P>0.05), EG+DMSO (7.61%), EG + PROH (6.74%), EG + PROH + DMSO (6.11%), and PROH + DMSO (5.45%), respectively.

In experiment III, bovine oocytes were IVM supplemented with 0, 50, 100 and 200 ng/mL IGF-1. Following vitrification and warming, the oocyte survival rates were not significantly different among the IGF-1 supplemented groups (92.55% - 95.11%) and fresh control (96.33%). The cleavage rate in the 50 and 100 ng/mL IGF-1 groups (50.91% and 53.15%) were significantly higher (p < 0.05) than those in 0 ng/mL IGF-1 group (42.73%) but there was significantly lower than that in the fresh control group (63.51%). The blastocyst rate was not significantly different among IGF-1 supplemented groups (7.19% - 8.24%) but significantly lower than that in the fresh control group (25.11%). The number of ICM and TE cells was not significantly different among IGF-1 supplemented groups and fresh control group.

In conclusion, there was no difference on the toxic effect among those CPAs combination treatments. They had the same efficientcy on oocyte survival and embryo development in IVM bovine oocytes vitrification. Although, the supplementation of IGF-1 during IVM of bovine oocytes improved their total cleavage rate, there was no

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and deep appreciation to my thesis advisor, Professor Rangsun Parnpai for his excellent guidance, kind advice, motivation, support and encouragement throughout this study and for his outstanding review and invaluable comments on my thesis.

I am equally grateful to Dr. Yuanyuan Liang for reviewing my thesis.

I would like to thank ESRC members especially, Dr. Karnchana Panyawai, Miss Nucharin Sripanya and Miss Suthathip Jirandon for their helpful advices and material support during my practical works.

I owe my sincere appreciation to my family for their love, patience, sincere support and encouragement.

I am particularly indebted to Suranaree university of Technology for "External Grants and Scholarships for Graduate Students", which enabled me to undertake this study.

Vajara Wipassa

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LIST OF ABBREVIATIONS

µL	=	Microliter
µM	=	Micrometer
ART	=	Assisted reproductive technologies
BM	=	Base medium
BSA	=	Bovine serum albumin
CO_2	=	Carbon dioxide
COCs	=	Cumulus oocyte complex
CPA	=	Cryoprotectant
D	=	Day
DMSO	=	Dimethyl sulfoxide
DNA	=	Deoxyribonucleic acid
DPBS	=	Dulbecco's phosphate buffered saline
E_2	=	Estradiol
EG	=	Ethylene glycol
ES	=	Equilibration solution
FBS	=	Fetal bovine serum
FDA	=	Fluorescein diacetate
FSH	=	Follicle stimulating hormone
G	=	Gauge needle
GnRH	=	Gonadotropin-releasing hormone

LIST OF ABBREVIATIONS (Continued)

GV	=	Germinal vesicle
GVBD	=	Germinal vesicle breakdown
h	=	Hour
hCG	=	Human chorionic gonadotropin
ICM	=	Inner cell mass
ICSI	=	Intracytoplasmic sperm injection
IGF-1	=	Insulin-like growth factor 1
IVC	=	*In vitro* culture
IVF	=	*In vitro* fertilization
IVM	=	*In vitro* maturation
IVP	=	*In vitro* embryo production
kg	=	Kilogram
LH	=	Luteinizing hormone
LN_2	=	Liquid nitrogen
M	=	Molar
MAPK	=	Mitogen-activated protein kinase
mg	=	Milligram
MI	=	Metaphase I
MII	=	Metaphase II
min	=	Minute
mL	=	Milliliter

LIST OF ABBREVIATIONS (Continued)

mm	=	Millimeter
mOsm	=	Milliosmoles
MPF	=	Maturation promoting factor
mSOFaa	=	Modified synthetic oviduct fluid supplemented with amino acids
N_2	=	Nitrogen
NaCl	=	Sodium chloride
ng	=	Nanogram
No.	=	Number
O_2	=	Oxygen
^oC	=	Degree Celsius
Pa.s	=	Pascal second
PROH	=	Propylene glycol
PI	=	Propidium iodide
RNA	=	Ribonucleic acid
sec	=	Second
TALP	=	Tyrod's albumin lactate pyruvate
TCM	=	Tissue culture medium
TE	=	Trophectoderm
v/v	=	Volume / volume
VS	=	Vitrification solution

CHAPTER I

INTRODUCTION

1.1 Rationale and background

Oocyte cryopreservation technique has been proved an advancement of assisted reproductive technology (ART), allowing for long-term storage of oocytes for future use. This technique can be applied with advanced reproductive technologies such as *in vitro* embryo production (IVP) which is valuable tool for commercial livestock production and preserving genetic resource of endangered species. Metaphase II (MII) oocytes are traditionally preferred for cryopreservation. Oocytes in this stage have undergone several developmental paths including nuclear and cytoplasmic maturation, extrusion of the first polar body and arranging of the chromosome on MII spindle which ready for fertilization (Shaw *et al*., 2000; Trounson *et al*., 2001). Although the survival of oocytes after cryopreservation is relatively high but their fertilization and development competence are quiet low because of ice crystal that generated during chilling process can be damaged oocytes (Zhou and Li, 2009).

For prevention of ice crystal formation, vitrification appears to be the best alternative for oocyte cryopreservation by directly plunging oocytes in liquid nitrogen (LN_2) (Zhou and Li, 2009). The principles of vitrification compose of several factors including cooling and warming rate, concentration of CPAs added to the vitrification solution, and sample volume. CPAs influence the ability of oocytes to survive

vitrification. The permeating CPAs most commonly used for oocyte vitrification are ethylene glycol (EG), dimethyl sulfoxide (DMSO) and propylene glycol (PROH) which have high permeability into cell to prevent ice crystal formation. However, vitrification usually uses solution with high CPAs concentration that maybe induces individual CPA toxicity to oocyte structures and functions of organelles which affect ability to fertilize and continue embryonic development of oocytes. Therefore, the selection of suitable CPAs should be strategy used to overcome those adverse effects.

Permeating CPAs have been used to combine with each other in a mixture. The combination ofpermeating CPAs has the advantage of reducing each CPA concentration as well as reduce toxicity of individual CPAs, which were applied for cryopreservation of oocytes of several mammalian species such asmouse (Skuterud *et al*., 2005), cattle (Phongnimitr *et al*., 2013) and buffalo (Mahmoud *et al*., 2010; Liang *et al*., 2012). It has been observed that the cryoprotective effect of combination of permeating CPAs was greater than single CPA using (Somfai *et al*., 2013). In bovine oocytes vitrification, combination of EG and DMSO has been appears to be widely used (Vajta *et al*., 1998, Chain *et al*., 2004, Phongnimitr *et al*., 2013) which EG has high permeability and low toxicity. Moreover, Chian *et al*. (2004) reported that the replacement of combination of EG and DMSO with combination of EG and PROH was more efficient for bovine oocyte vitrification. It has been reported that PROH has more permeable than DMSO. The replacement of DMSO with PROH would decrease toxicity and increase permeability of vitrification solution. It indicates that combination of CPAs has advantages over only one CPAs and may improve the efficiency of the vitrification of bovine oocytes with another combination of CPAs.

The sensitivity of bovine oocytes to cooling has been reported as poor ability of embryo development (Yamada *et al*., 2007). Insulin-like Growth Factor 1 (IGF-1) is a growth factor which has major role in regulating reproductive ability resides in promoting proliferation, differentiation and inhibiting apoptosis. IGF-1 had been frequently used in inducing follicle growth as well as oocyte maturation (Spicer and Chamberlain, 1998).

Moreover, IGF-1 could be enhanced the expression of cold inducible RNA-binding protein (CIRP) to protect cell from damage at low temperature (Pan *et al*., 2015).

1.2 Research objectives

1.2.1 Investigate the toxic effects of different combinations of permeating CPAs, including EG + DMSO, EG + PROH, PROH + DMSO and EG + PROH + DMSO on oocyte survival, embryo development and IVF-derived blastocysts cell number, including trophectoderm (TE), inner cell mass (ICM) and total cell number.

1.2.2 Investigate the vitrification efficiency of different combinations of permeating CPAs, including EG + DMSO, EG + PROH, PROH + DMSO and EG + PROH + DMSO on oocyte survival, embryo development and IVF-derived blastocysts cell number.

1.2.3 Investigate the effects of IGF-1 supplementation in IVM medium on vitrified-warmed oocyte survival, embryo development and IVF-derived blastocysts cell number.

1.3 Research hypotheses

1.3.1 The combinations of permeating CPAs, including EG + DMSO, EG + PROH, PROH + DMSO and EG + PROH + DMSO have no toxic effects on the survival, embryo developmental rates and blastocysts cell number of IVM bovine oocytes.

1.3.2 The combinations of permeating CPAs, including EG + DMSO, EG + PROH, PROH + DMSO and EG + PROH + DMSO have efficiency in vitrification and suitable combinations of permeating CPAs could improve the survival, embryo developmental rates and blastocysts cell number of IVM bovine oocytes after vitrification.

1.3.3 IGF-1 supplementation in IVM medium could enhance the cryotolerance as well as development competent of IVM bovine oocytes after vitrification.

1.4 Scope and limitations of the study

The cryopreservation using vitrification method of bovine oocytes and *in vitro* production (IVP) of bovine embryos were used in this research. Bovine oocytes were matured *in vitro*. Then, IVM bovine oocytes were preserved using vitrification method with different vitrification solution and kept in LN_2 until warmed. After warming, vitrified-warmed oocytes were evaluated survival rate using FDA staining. The surviving oocytes were fertilized and cultured *in vitro* until develop to blastocyst stage. Then, obtained blastocysts were evaluated the TE, ICM and total cell number using differential staining.

1.5 Research methodology

1.5.1 Instrumentation

Ovaries were collected from local slaughterhouse at Praputtabath, Saraburi. All equipments and materials were provided by Embryo Technology and Stem Cell Research Center (ESRC) and Laboratory Service Unit, The Center for Scientific and Technological Equipment, Suranaree University of Technology.

1.5.2 Location of research

The experiments were carried out at ESRC, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

CHAPTER II

LITERATURE REVIEWS

2.1 Oocyte cryopreservation

The cryopreservation of female gamete is an important technology that is applied in both human and animal reproduction. Cryopreservation permits the long-term storage of cells and tissues such that acceptable numbers of cells are viable upon subsequent warming (Mazur, 1970). Oocyte cryopreservation in mammals has a series of potential applicationsaimed at the maintenance of biodiversity.

2.1.1 Oocyte cryopreservation in livestock

From a commercial point of view, in livestock farming there is a need for cryopreserving oocytes from domestic animals (e.g. cattle, pigs and sheep) with high economic value in order to enhance the development of improved breeding program. Additionally, producers could preserve valuable genetic blood lines from females and could market oocytes rather than embryos whose sire had already been chosen. In livestock, where large numbers of ovaries can be easily obtained from slaughterhouse, many oocytes can be collected, preserved and then used for IVP when re-warmed, thus diminishing seasonal variations or sanitary constraints. Also, oocyte cryopreservation could be used to rescue gametes when females die unexpectedly or accidentally (Checura and Siedel 2007; Ledda *et al*. 2007). Moreover, cryopreserved oocytes could be used in genetic recombination through fertilization and production of cross-bred animals. Oocyte banking also permits intermation exchange of oocytes,

avoiding animal transportation and its sanitary risks and injuries (Pereira and Marques 2008).

2.1.2 Oocyte structure and cryopreservation damage

The oocyte is one of the largest mammalian cells ranging from 80 to 120 µM in diameter depending on species.

Immature oocytes at the germinal vesicle (GV) stage are characterized by a large diploid nucleus (prophase I), a dense band of filamentous actin subjacent to the oolemma, and other organelles scattered throughout the ooplasm, including mitochondria, endoplasmic reticulum, and Golgi apparatus. The Golgi of the oocytes produces lysosome-like vesicles, the cortical granules that are randomly distributed throughout the cytoplasm. In GV oocytes, microtubules are not organized in the MII spindle. Therefore, in principle the cryopreservation of immature GV-stage oocytes bypasses the risk of chromosome aberrations because at this stage the chromatin is decondensed and protected by a nuclear envelope (Cooper *et al*., 1998; Isachenko *et al*., 1999).

The mature oocyte (metaphase of the second meiotic division, MII) is characterized by a large peripheral spindle apparatus with microtubules extending from pole to pole and from each pole to kinetochores of the chromosomes aligned along the metaphase plate. Abundant actin containing microfilaments are distributed in the perinuclear and cortical ooplasm, where they direct organelle distribution and polar body extrusion during meiosis (Shaw *et al*., 2000; Trounson *et al*., 2001). In MII oocytes cortical granules migrate to the periphery of the ooplasm just beneath the actin band, where they are ready to undergo exocytosis at the time of fertilization. However, the most characterized cytoskeletal damage caused by cryopreservation concerns

disruption of the sub-cortical actin network, microtubule depolymerization, abnormal spindle configuration and chromosome scattering (Rho *et al*., 2002; Mandelbaum *et al*., 2004). All these effects profoundly modify the oocyte architecture and function, giving rise to cytokinesis abnormalities with subsequent developmental failure. Nevertheless, several studies demonstrate that some cytoskeletal alterations may be reversible. Under specific conditions, spindle fibers may depolymerize and repolymerize after thawing, re-establishing the physical interactions with chromosomes, which can be repositioned at the equatorial plate (Coticchio *et al*., 2006; Larman *et al*. 2007).

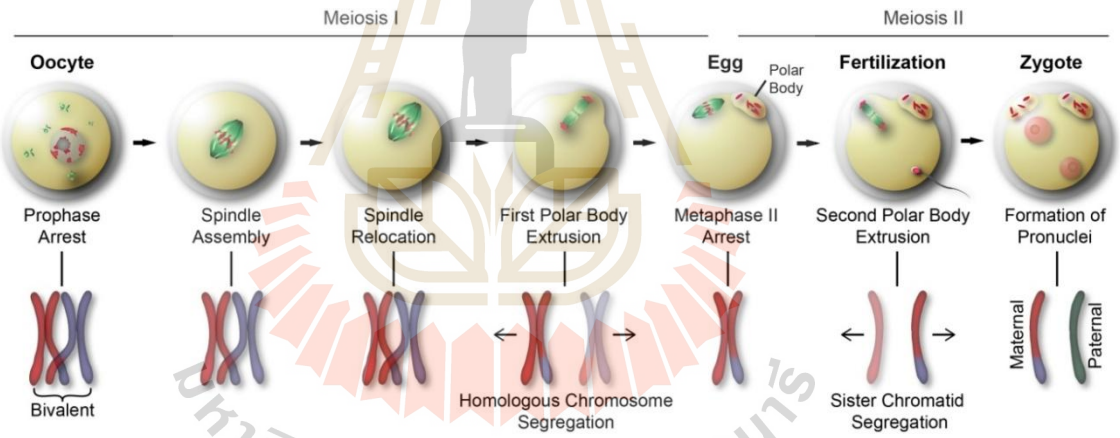


Figure 2.1 Maturation stages of oocytes (Yi *et al*. 2013).

However, oocyte cryopreservation remain problems because of it is very sensitive to chilling. Moreover, ice crystal formation is generated during the cryopreservation process. This phenomenon would contributors to destruction of the cell resulting in the poor re-viability following cryopreservation process. Disadvantages of oocyte cryopreservation can be summarized as follows, (Carroll *et al*., 1990; Aigner *et al*., 1992; Aman and Parks, 1994; Martino *et al*., 1996).

1. Their complex structure, oocytes are a low surface-to-volume ratio and rich in cytoplasmic lipid droplets, resulting in very sensitive to chilling and highly susceptible to intracellular ice formation.

2. The plasma membrane of oocytes has a low permeability, making the movement of CPAs and water slower.

3. The freezing and thawing process induces a premature cortical granule exocytosis, leading to zona pellucida hardening and preventing of sperm penetration.

4. The mature (MII) oocyte shows chromosome aligned in the meiotic spindle, which temperature and CPA sensitivity of the spindle and microtubules provoke cytoskeleton disorganization and chromosome and DNA abnormalities.

2.2 Methods of cryopreservation

The methods currently uses in oocyte cryopreservation are slow freezing and vitrification.

2.2.1 Slow freezing

Slow freezing is a classic method that preserved germplasm and embryos using programmable freezing machine to control the cooling rate. (Whittingham, 1971; Wilmut and Rowson, 1973). Following this method, oocyte is exposed to low concentration of permeating CPAs. The oocyte is then loaded into straws and cooled slowly to -7^{o}C and seeded to induce ice crystals at about 0.3 - 0.5^{o}C/min to -30^{o}C and finally, stored in LN_2. Slow freezing has many steps and the process takes about 2-3 h which is longer than vitrification. And critically, slow

freezing uses cooling rate and CPA at low level, it may result in ice crystals resulting in intracellular organelles damage (Figure 2.2).

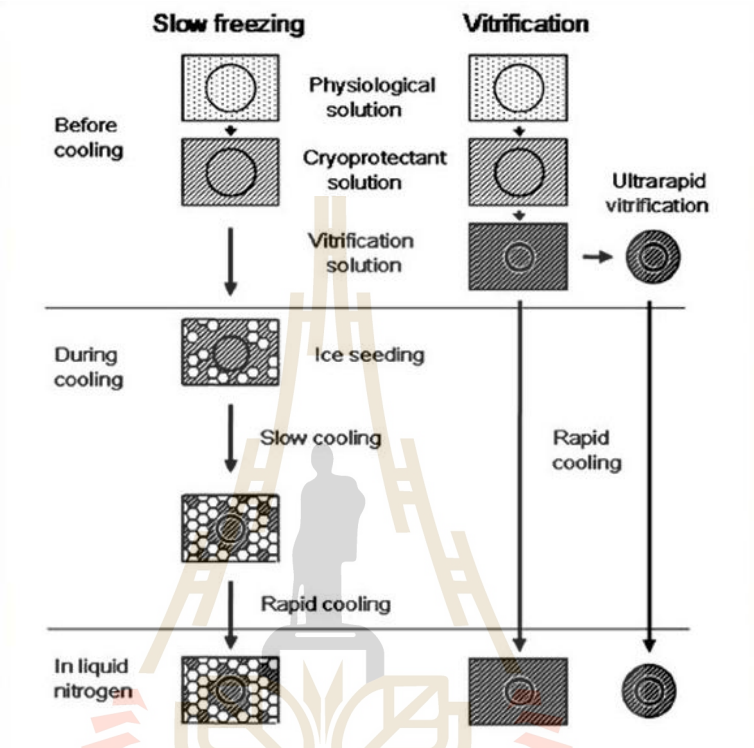


Figure 2.2 The schematic representation of an oocyte (circle) during slow freezing and vitrification. White hexagons represent ice crystals. The concentration of CPA is shown by the darkness of shading (Kasai 2002).

2.2.2 Vitrification

Vitrification refers to ice crystal-free solidification of aqueous solution. There is the direct conversion of liquid state into glass-like state by rapidly passing through the critical temperature of ice formation (Figure 2.3). The successful of vitrification depends on factors including, cooling and warming rates, cryoprotectant (CPA) solution and sample volume to optimize the cooling and warming rates. (Lyu *et al*., 2013; Yu *et al*., 2016).Rapid cooling rate (up to 25000^{o}C per min) by plunging the cell directly into LN_2 reaches the glass transition temperature more effectively but vitrifying a single drop (1 µL) of pure water requires a cooling rate of up to 10^{8o}C per min, which is impossible to achieve (Rall, 1987; Kohl *et al*., 2005; Arav, 2014). Therefore, vitrification usually uses solution with high concentration of CPA that lowers the ice formation temperature and increases the glass transition temperature as well as prevents intracellular ice crystal formation.

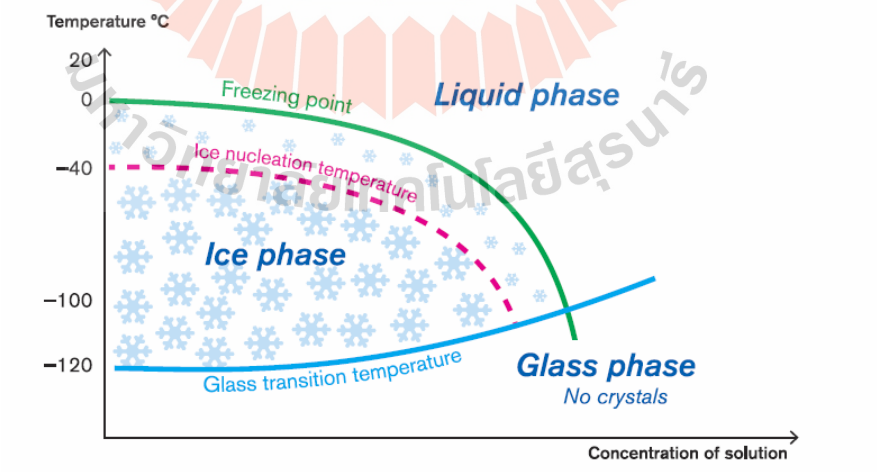


Figure 2.3 Transition phase of liquid.

In vitrification, oocytes are first equilibrated in a solution containing a low concentration of CPA for incubation in less toxic conditions, and then in vitrification solution (VS) for a short time to oocyte dehydration and CPA replacement. The oocytes in the vitrification solution are then loaded on the cryo-device in the form of a 2–3 μL droplet and plunged immediately into LN_2 for storage.

2.3 Cryoprotectant (CPA)

Vitrification solutions are aqueous solutions containing CPA that prepared in buffered media with a stable pH between 7.2-7.4. Dulbecco's phosphate buffered saline (PBS) and HEPES-buffered culture media such as TCM-199 have been used successfully.

2.3.1 CPA

Cryoprotectant (CPA) is chemical substances that used to avoid ice crystal formation. There are divided into two types including, permeating and non-permeating CPAs. Permeating CPAs are low molecules weight that have the ability to permeate the cell membrane, form hydrogen bonds with intracellular water molecules, lower the freezing temperature of the solution and consequently inhibit the formation of ice crystals by lead to the development of a solid glass like called vitrified state in which water is solidified, but not expanded (Watson, 1995; Jain and Paulson, 2006; Pereira and Marques, 2008). The most common of which include dimethyl sulfoxide (DMSO), propylene glycol, ethylene glycol and glycerol. In contrast, non-permeating CPAs are large molecule weight substance remain extracellular that act by drawing free water from the cell, and subsequently causing dehydration of the intracellular space. Non-permeating CPAs are used together with permeating CPAs to increase the

net concentration of permeating CPAs inside the cell and also prevent ice crystal formation (Jain and Paulson, 2006). Non-permeating cryoprotectants are commonly sugars, macromolecules and polymers.

The following CPAs are commonly used and considered to apply in this study;

2.3.1.1 Ethylene glycol (EG)

Ethylene glycol (EG) is an odorless, colorless, and syrupy. Its freezing point is −12.9°C, and its boiling point is 197.3°C. The EG have been used widely for the vitrification of oocyte and embryo because its characteristic features of low molecular weight, high permeability and low toxicity (Kuwayama *et al*., 2005).

2.3.1.2 Dimethyl sulfoxide (DMSO)

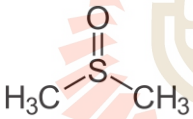
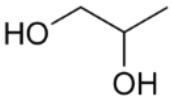
Dimethyl sulfoxide (DMSO) is a clear and colorless liquid. Its freezing point is 19°C, and its boiling point is 189°C. It has a distinctive property of skin irritation and penetrating the skin very readily. DMSO has been used as an important CPA for vitrification used to preserve organs, tissues, and cell (Mazur *et al*., 1972; Karlsson and Toner, 1996). It was initially used as a CPA for mouse and human embryo cryopreservation (Whittingham *et al*., 1972; Trounson and Mohr, 1983). In one study dimethyl sulfoxide treatment led to significantly better cleavage and development up to 4-cell stage in oocytes cryopreserved at the MII stage (Schellander *et. al*, 1994).

2.3.1.3 Propylene glycol (PROH)

Propylene glycol (PROH) is an organic compound, colorless, clear viscous liquid. Propylene glycol has properties similar to those of EG. Its freezing point is −59°C, and its boiling point is 188.2°C. PROH was one permeating CPA that used to successfully cryopreserve mammalian sperm as well as human

embryo. Also, PROH is the most commonly used permeating CPA in bovine embryo cryopreservation (Kuwayama and Nagai, 1994). In a recent study comparing the permeating speed of several CPAs through the mouse oocytes membrane, PROH and DMSO exhibited significantly better permeability than EG (Predo *et al*., 2005; Somfai *et al*., 2013)

Table 2.1 The properties of CPAs including DMSO, EG and PROH.

Chemicals	**Dimethyl sulfoxide (DMSO)**	**Ethylene glycol (EG)**	**Propylene glycol (PROH)**
Other names	-Methylsulfinylmethane - Methyl sulfoxide	- 1,2-Ethanediol - Ethylene alcohol - 1,2-Dihydroxyethane	- 1,2-Propanediol - α-Propylene glycol -Methyl ethyl glycol
Chemical formula	- C_2H_6OS 	- $C_2H_6O_2$ 	- $C_3H_8O_2$ 
Molar mass	78.13 g·mol^{-1}	62.07 g·mol^{-1}	76.10 g·mol^{-1}
Density	1.1004 g cm^{-1}	1.1132 g cm^{-1}	1.036 g cm^{-1}
Freezing point	19°C (66 F)	−12.9°C (8.8 F)	−59°C (−74.2 F)
Boiling point	189°C (372 F)	197.3°C (387.1 F)	188.2°C (370.8 F)
Solubility in water	Miscible	Miscible	Miscible

Source: https://en.wikipedia.org

2.4 CPA toxicity

The cryoprotective action of CPA is preventing ice crystal formation during freezing resulting in increasing of survival rate in cells. In vitrification, cells were exposed in vitrification solution that containing CPAs. The exposure to the CPAs solution would be dehydrate the cells and allow the CPAs to permeate sufficiently. However, the concentration of CPAs that used in vitrification is high that can damaging cell (Meryman, 1971).

2.4.1 Chemical toxicity

CPAs can damage to oocytes on the meiotic spindle, actin cytoskeleton, chromosomal arrangement, and ability to fertilize and continue embryonic development. Compounds such as DMSO and PROH can cause depolymerization of oocyte tubulin and malformation of the meiotic spindle (George *et al*., 1996; Vincent *et al*., 1989). DMSO causes disassembly of the spindle microtubules and movement of the pericentriolar material to oocytes (Trounson and Kirby, 1989). It has been found to be detrimental to the meiotic competence of immature porcine oocytes (Gupta *et al*., 2007). Extended exposure of oocytes to PROH also leads to degeneration and parthenogenetic activation (Van Der Elst *et al*., 1992). Additionally, the oocyte competence of oocyte preserved in PROH was reduced compared with oocytes in control and EG groups that result suggest a specific toxic effect of PROH on oocytes that subsequent detrimental effects during embryo development (Somfai *et al*., 2013).

CPAs, such as DMSO and EG leads to fusion of cortical granule release to oocyte plasma membrane resulting in zona pellucida hardening in oocytes, which significantly reduces fertilization (Ledda *et al*., 2007; Larman *et al*., 2006; Tian *et al*., 2007; Hwang *et al*., 2014; Kohaya *et al*., 2011). It has been shown high

concentrations of EG (10 – 40% v/v) increase Ca^{2+} that induces parthenogenetic activation (Takahashi *et al*., 2004). Also, in vitrification of bovine oocytes the reported by Magnusson *et al*, (2008), oocytes were exposure in solution containing 30 - 40% (v/v) of EG at several time interval affect the development potential of oocytes after vitrification. Therefore, it is important to selection of a suitable CPA with low adverse effect for oocyte.

2.4.2 Osmotic stress

One of the most important factors affecting cell survival during expose to CPAs is excessive cell swelling and osmotic stress in the oocyte (Prentice-Biensch *et al*., 2012). If cells are exposed in an isotonic solution, they will neither swell nor shrink since the concentrations in the intra- and extracellular fluid are the same. But if, cells are exposed in a hypertonic and hypotonic (anisotonic) solution, they will shrink or swell as a result of the movements of water across the plasma membrane which cause osmotic stress on cell. Osmotic stress resulting from exposure to high concentrations of CPA could lead oocytes to undergo dramatic volume changes during equilibration (Prentice-Biensch *et al*., 2012). Studies have been shown that volume fluctuations can affect the integrity of the plasma membrane and cytoskeletal organization of oocytes (Schellander et al., 1994; Agca et al., 1998) as well as these can induce adverse effects on oocyte survival and consequence embryo development.

In general, vitrification requires a more than one step procedure of moving cells through increasing concentrations of CPAs to minimize chemical toxicity and osmotic stress (Meryman, 1971). The first solution consists of low concentration to remove the majority of the water from the cell often referred to as an equilibrating solution. The follow solutions consist of higher concentrations with the

cells only exposed for a brief time to minimize the risk of toxicity (Ishimori *et al*, 1993; Zhou *et al*., 2014). Moreover, the combination of permeating CPA has been the way to reduce the concentration of each CPA as well as reduce their individual toxicity of CPAs is used.

2.5 Combination of permeating CPAs

Permeating CPAs can use by combine with each other in a mixture. It has been observed that the cryoprotective effect of combination of CPAs was greater than single CPA using (Somfai *et al*., 2013). Otherwise, combination of permeating CPA is the way to reduce the concentration of CPA as well as reduce their individual CPA toxicity that has reported in oocyte and embryo cryopreservation of several mammalian species.

Mahmoud *et al*., (2010) compared effects of different combination of CPAs which EG, DMSO and glycerol for vitrified immature buffalo oocyte that show highest was seen in oocytes vitrified in EG + DMSO when compared with oocytes vitrified in EG alone.

Cha *et al*., (2011) reported maturation rate of immature mouse oocyte vitrified after warming was greater in the EG + DMSO group than in the EG only group.

Somfai et al., (2013) reported the combination of EG and PROH for vitrified immature porcine oocytes resulted in oocyte survival was significantly higher than using EG only (which solution containing 35% (v/v) of total CPA). Moreover, a toxic effect on oocyte was observed in oocytes exposed in PROH which did not in combination of EG and PROH group when compared with those control groups.

Nohalez *et al*, (2015) examined two CPA combinations, EG + DMSO and EG + PROH which solution containing 16% (v/v) of each CPA for vitrified immature porcine oocytes. The results show high fertilization efficiency in EG + PROH (39.6%) and control (42.0%).

Zhou *et al*., (2015) was tested the effects of different several vitrification parameters that including CPA compositions to determine the most effective protocol for mouse oocyte vitrification. In experiment of toxic effect was found oocytes exposed to solution containing total 40% (v/v) combination of EG with DMSO and glycerol with PROH that exhibited greater survival, fertilization and blastocyst formation than those exposed to glycerol, EG, DMSO and PROH were used alone which solution containing 40% (v/v) of each CPA.

A study of Gajda *et al*. (2015) shows that CPA combinations of EG and DMSO improved the *in vitro* development of vitrified mature porcine oocytes

In cryopreservation of bovine oocytes, these has been a report that when a combination of CPAs was used, a higher blastocyst development was obtained compared to using only 40% (v/v) EG in bovine oocytes (Vajta *et al*., 1997). Combination of DMSO and EG appears to be one of the most combinations has been used (Chian *et al*., 2004). Another combination is EG and PROH, reported by Chain *et al*. (2004) that the *in vitro* matured bovine oocytes can be vitrified with mixture of the EG+PROH and EG+DMSO, moreover, it has been reported that a combination of EG+PROH gave superior results in blastocyst developmental rate of bovine oocyte vitrification compared with EG+DMSO.

2.6 Insulin-like growth factor 1 (IGF-1)

Insulin-like Growth Factor 1 (IGF-1) is a growth factor which is composed of polypeptides, receptors and binding proteins. IGF-1 had been frequently used in inducing oocyte maturation. The major role of IGF-1 in regulating reproductive ability resides in promoting proliferation and differentiation and inhibiting apoptosis of granulosa cells, promoting the synthesis of steroid hormones, and synergistically acting with gonadotropin to increase activity of aromatase which plays an important role in cell cycles. (Spicer and Chamberlain, 1998).

IGF-1 is required for the development and maturation of follicles during reproduction. At early stages of follicular development, from maturation of primordial follicles through to preantral phase, the regulation of initiation is dependent on presence of IGF-1, especially the parascrine IGF-1 (Walters *et al*., 2006). Studies suggest that IGF-1 is an important regulator in multiple biological processes, which is involved in many aspects of reproductive activity (Adashi, 1998).

Several research groups reported that addition of IGF-I to culture media stimulates the maturation of oocytes and development of murine (Harvey and Kaye, 1992), bovine (Herrler *et al*., 1993; Lorenzo *et al*., 1994; Lonergan *et al*., 1996; Matsui *et al*., 1997), porcine (Xia *et al*., 1994) and human (Lighten *et al*., 1998) embryos *in vitro*. IGF-1 has been shown to have additive positive effects on development competence.

Numerous studies have reported that IGF-1 enhanced the development and cryo-tolerance of oocytes. IGF-1 has been reported promoted increase rates of maturation rate and IVF in bovine oocytes (Herrler *et al*., 1992). Carneiro *et al*. (2001) studied a system for *in vitro* maturation in the presence of IGF-I, resulting in positive

effect on cytoplasmic maturation through migration of cortical granules of oocytes and increasing the rate of cleavage. That is involved in the compaction and formation of the blastocysts, inhibition of apoptosis, enhancement of endocytosis and probably protein turnover (Głabowski *et al*., 2005; Wasielak and Bogacki, 2007). Pan *et al*. (2015) reported the 100 ng/mL IGF-1 supplemented in IVM medium enhanced developmental competence of yak oocytes after vitrification. Moreover, that could be enhanced the expression of cold inducible RNA-binding protein (CIRP) which is involved in the activation of kinase signaling pathway to protect cell from damage at low temperature. There indicated that the development competence of mature yak oocyte after vitrification was also enhanced by 100 mg/mL IGF-1 (Pan *et al*., 2015).

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals and reagents

All chemical and reagent were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA), unless otherwise indicated.The medium used for IVM was TCM199 supplementd with 10% fetalbovine serum (FBS; Gibco BRL, Grand Island, NY, USA), 0.02 IU/mL FSH (Antrin, Kyoritsu Seiyaku, Tokyo, Japan), 50 IU/mL hCG (Chorulon, Intervet, Boxmeer, Netherlands) and 1 µg/mL estradiol-17β. The medium for embryo culture was modified synthetic oviduct fluid supplemented with amino acids and 0.3% fatty acid-free BSA (mSOF).

3.2 Oocyte collection and IVM

The bovine ovaries were collected from slaughterhouses and kept in saline solution (0.9% NaCl) during transportation to laboratory. Cumulus oocyte complexes (COCs) were aspirated from follicle of 2-8 mm diameter using 18 G needle connected with syringe. COCs with more than three layers cumulus cell and uniform cytoplasm wereselected to wash with Dulbecco' s Phosphate Buffered Saline (mDPBS) supplemented 10 mg/mL polyvinylpyrrolidone (PVP) and cultured with IVM medium, cover with mineral oil in plastic dishesat 38.5^{o}C under a humidified atmosphere of 5% CO_2in air for 20 h (Figure 3.1).

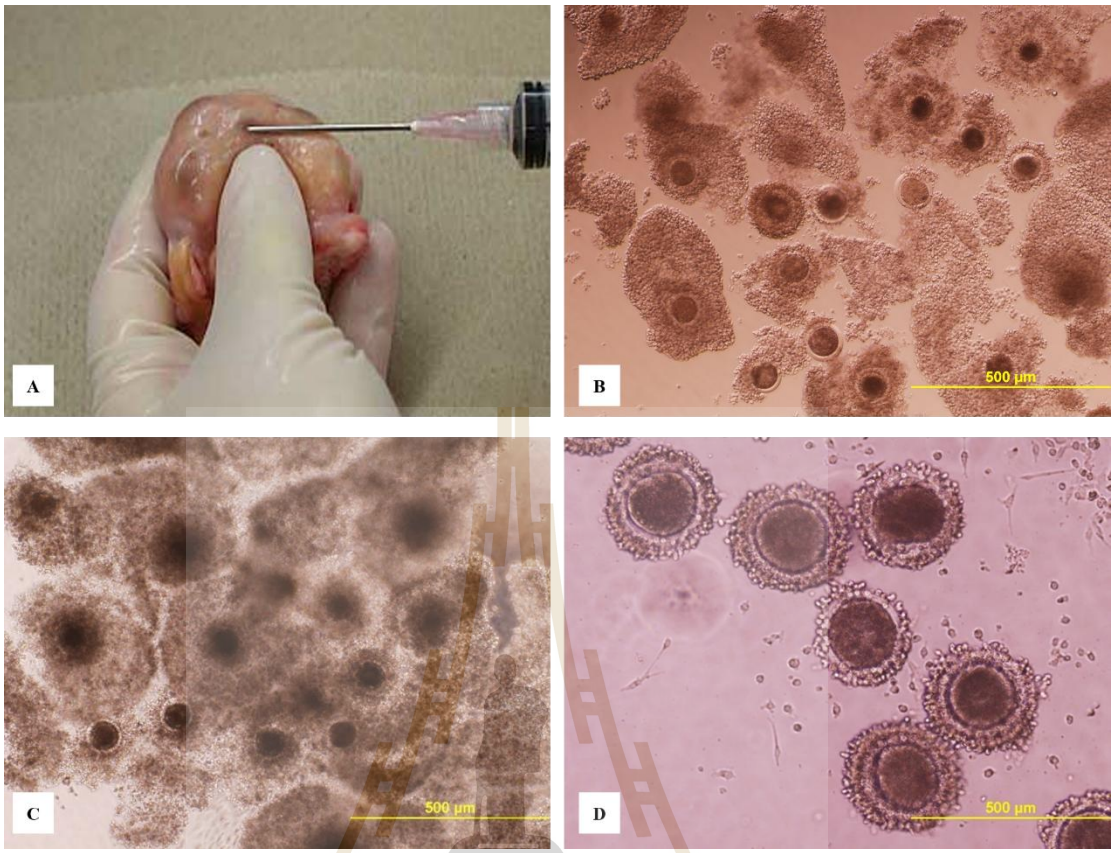


Figure 3.1 Oocyte collection and IVM. Bovine ovaries (A). COCs after aspirating (B). Oocytes with expanded cumulus cells after 20 h of IVM (C). Partially denuded oocytes after IVM (D) (magnification 40x).

3.3 Experimental groups

IVM bovine oocytes were separated into five groups (Table3. 1) for vitrification using different combination of permeating CPAs, EG (E9129, Sigma-Aldrich) + DMSO (D1435, Sigma-Aldrich), EG + PROH (P1009, Sigma-Aldrich), PROH + DMSO, EG + PROH+ DMSO and control group, respectively.

Table 3.1 Experimental groups.

Groups	Equilibration solution* (ES)		Vitrification solution** (VS)	
	CPAs %(v/v)	**Duration**	**CPAs %(v/v)**	**Duration**
1	2% EG + 2% DMSO	15 min	17.5% EG + 17.5% DMSO	30 sec
2	2% EG + 2% PROH	15 min	17.5% EG + 17.5% PROH	30 sec
3	2% PROH + 2% DMSO	15 min	17.5% PROH + 17.5% DMSO	30 sec
4	1.33% EG + 1.33% PROH + 1.33% DMSO	15 min	11.67% EG + 11.67% PROH + 11.67% DMSO	30 sec
5	Fresh control: oocytes were IVF and IVC without exposed to ES and VS.			

Base medium (BM): TCM199-Hepes + 20% FBS

* Equilibration solution (ES) consisted of BM supplemented with total 4% (v/v) permeating CPA combination.

** Vitrification solution (VS) consisted of BM supplemented with total 35% (v/v) permeating CPA combination 50 mg/mL PVP + 0.4 M sucrose.

3.4 Measurement of osmotic value and viscosity of vitrification solution

Osmotic value (mOsm/kg) of vitrification solutions were measured using a freezing point osmometer (Osmomat 3000 basic, Gonotec GmbH, Germany). And viscosity of vitrification solution (Pa. s) was measured using a modular compact rheometer (Rheometer MCR 502, Anton Paar GmbH, Austria) at 25°C.

Figure 3.2 Freezing point osmometer (Osmomat 3000 basic, Gonotec GmbH, Germany) (A). Rheometer (Rheometer MCR 502, Anton Paar GmbH, Austria) (B).

3.5 Measurement of oocytes volume changes following CPA exposure

The volume changes of oocytes exposed to VS were measured using micropipette perfusion technique according Agca *et al.*, 1998. Briefly, each oocyte was immobilized by negative pressure using holding pipette in 1 µL holding medium (TCM199-Hepes + 20% FBS) (Figure3.3). Oocyte was perfused with 100 µL of either VS by a preloaded syringe. Oocyte volume changes were observed and video-recorded before, during, and after perfusion using inverted microscope (Nikon, Inc., Garden City, NY) under 100x total magnification. Only oocytes that remained close to spheroid during the perfusion process were analyzed. Relative volumes of oocyte were calculated by cross sectional area (S) and converted into relative volume (V) with the equation V = S3/2.

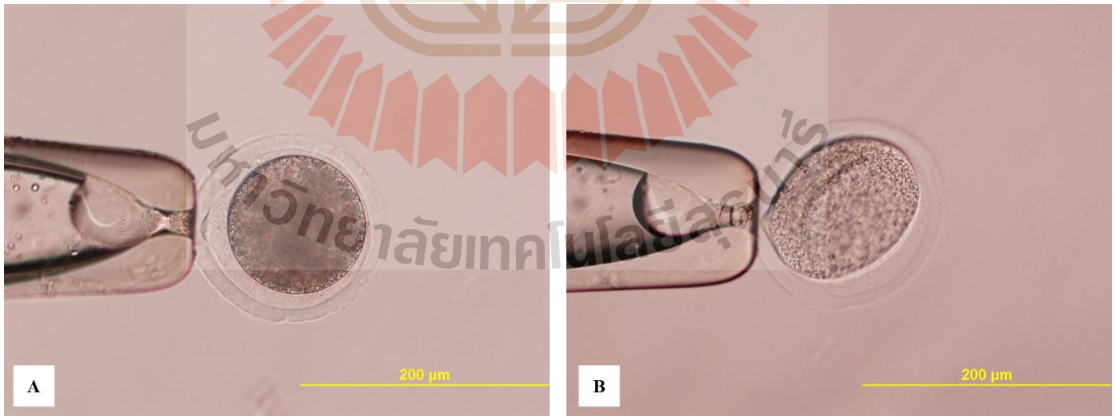


Figure 3.3 Micropipette perfusion techniques. Oocyte volume changes before (A) and after (B) perfusion (magnification 100x).

3.6 Oocyte vitrification and warming

IVM oocytes were partially denuded by gentle pipetting through a fine glass pipette using 0.1% (w/v) hyaluronidase. Oocytes containing two - three layers of cumulus cells were separated into five groups for vitrification using different combination of CPA (EG + DMSO, EG + PROH, PROH + DMSO, EG + PROH + DMSO and control group, respectively).

EG + DMSO group, oocytes were placed in 2% EG + 2% DMSO for 15 min. After that, they were exposed into 17.5% EG + 17.5% DMSO + 50 mg/mL PVP + 0.4 M sucrose for 30 sec.

EG + PROH group, oocytes were placed in 2% EG + 2% PROH for 15 min. After that, they were exposed into 17.5% EG + 17.5% PROH + 50 mg/mL PVP + 0.4 M sucrose for 30 sec.

PROH + DMSO group, oocytes were placed in 2% PROH + 2% DMSO for15 min. After that, they were exposed into 17.5% PROH + 17.5% DMSO + 50 mg/mL PVP + 0.4 M sucrose for 30 sec.

EG + PROH + DMSO group, oocytes were placed in 1.33% EG + 1.33% PROH + 1.33% DMSO for15 min. After that, they were exposed into 11.67% EG + 11.67% PROH + 11.67% DMSO + 50 mg/mL PVP + 0.4 M sucrose for 30 sec.

Then, within the last 30 sec, oocytes were placed on a Cryotop (Kitazato Ltd., Tokyo, Japan) and plunged directly into LN_2, storage of more than 24 h.

Vitrified oocytes were warmed by transferring Cryotop into warming solution consisted of 0.3 M sucrose in BM at 38^{o}Cfor 2 min. Then oocytes were consecutively transferred into droplets of BM supplemented with 0.15, 0.075, 0.0375 M sucrose for 1 min, respectively. They were washed three times in BM and returned into their

original droplets of maturation medium and incubated for an additional 2 h at 38.5ºC under a humidified atmosphere of 5% CO_2 in air.

3.7 FDA staining

Vitrified-warmed oocytes were evaluated survival rate by FDA staining. Oocytes were treated with 2.5 µg/mL FDA in PBS supplemented with 5 mg/mL bovine serum albumin (BSA) for 2 min in a dark room, and then washed 3 times in PBS supplemented with 5 mg/mL BSA and evaluated under an epifluorescence microscope with UV irradiation. Oocytes expressing bright green fluorescence were regarded as living and used in subsequent experiments (Figure 3.4).

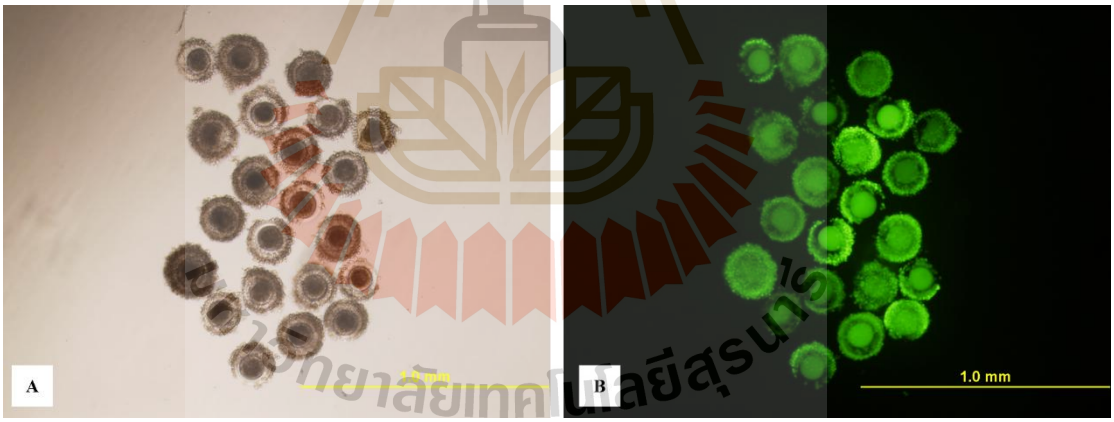


Figure 3.4 FDA stained vitrified bovine oocytes in bright field (A) and fluorescence image (B). Oocytes expressing bright green fluorescence were regarded as living. (magnification 40x).

3.8 *In vitro* fertilization (IVF)

Surviving oocytes were fertilized and cultured *in vitro.* Sperms used for IVF were prepared using swim-up method. The frozen bull semen was thawed in air for 10 sec and placed in 37°C in water bath for 30 seconds. The swim-up method was carried out by placing 100 µl of semen into the bottom of snap tube containing 2 mL Tyrod's albumin lactate pyruvate (TALP) medium and incubated at 38.5°C under a humidified atmosphere of 5% CO_2in air for 30 minutes to allow live spermatozoa to swim up. After that, upper phase of medium was transferred to a 15 mL tube and centrifuged at 2100 rpm for 5 minutes. The supernatant was removed, and sperm pellets were re-suspended in TALP medium. The solution was diluted to a final sperm concentration of 2×10^6 sperm/mL before IVF. In the IVF procedure, oocytes were co-incubated with spermatozoa by placed in 100 µL / droplets of TALP medium at 38. 5°C under a humidified atmosphere of 5% CO_2in air for 12 h. The day of IVF were considered as Day 0 (D0).

3.9 *In vitro*culture (IVC)

The presumptive zygotes (Figure 3. 5) were cultured in modified synthetic oviduct fluid supplemented with amino acids (mSOF) medium at 38. 5°C under a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 for 48 h. At D2, the cleavage rates were recorded using the day of IVF as D0. Then, embryos at the 8-cell stage were selected and co-cultured with bovine oviduct epithelial cellsin mSOFaa medium at 38.5°C under a humidified atmosphere of 5% CO_2in air until D7. Half of medium were replaced with fresh medium during the embryos development and their stages

and growth rates were recorded. The developments of the blastocyst (Figure 3. 5B) were noted on D6, D7 and D8.

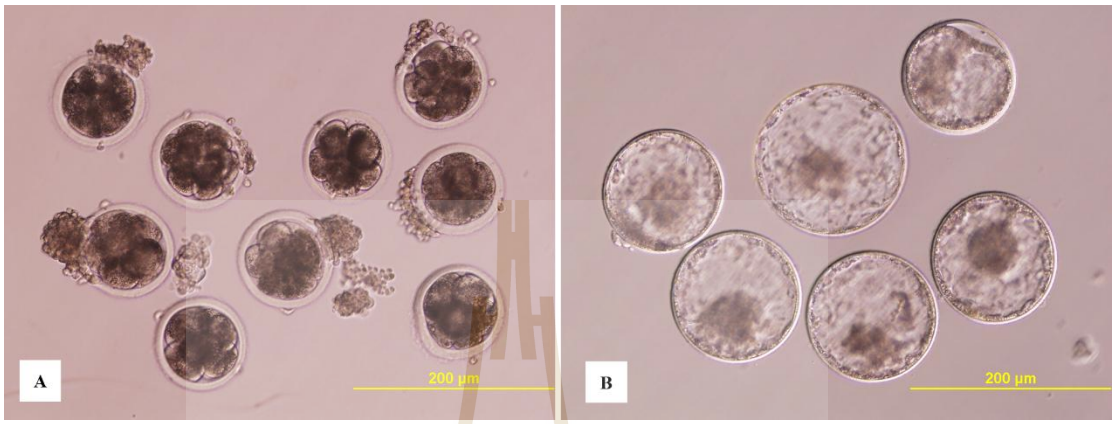


Figure 3.5 Embryo development of bovine oocytes at 8 cell (A) and blastocyst (B) stage after IVC for 2 and 7 days, respectively (magnification 100x)

3.10 Evaluation of blastocyst quality by differential staining

The TE, ICM and total cell number in blastocysts were evaluated using differential staining according to Sripanya *et al.* (2010). The blastocysts were treated with 0.1 mg/mL propidium iodide (PI) and 0.2% (v/v) Triton-X 100 in mDPBS for 30 sec to permeabilize the membrane. Then, the blastocysts were incubated with 25 ug/ mL Hoechst 33342 (Calbiochem, San Diego, CA, USA) dissolved in 99. 5% ethanol for 3 min. Then, the blastocysts were mounted on glass slide in glycerol droplets, flatted by cover slips and examined under UV light using an epifluorescence microscope. For these, The ICM appeared blue color of Hoechst and TE appeared pink or red color of both PI and Hoechst. (Figure 3.6)

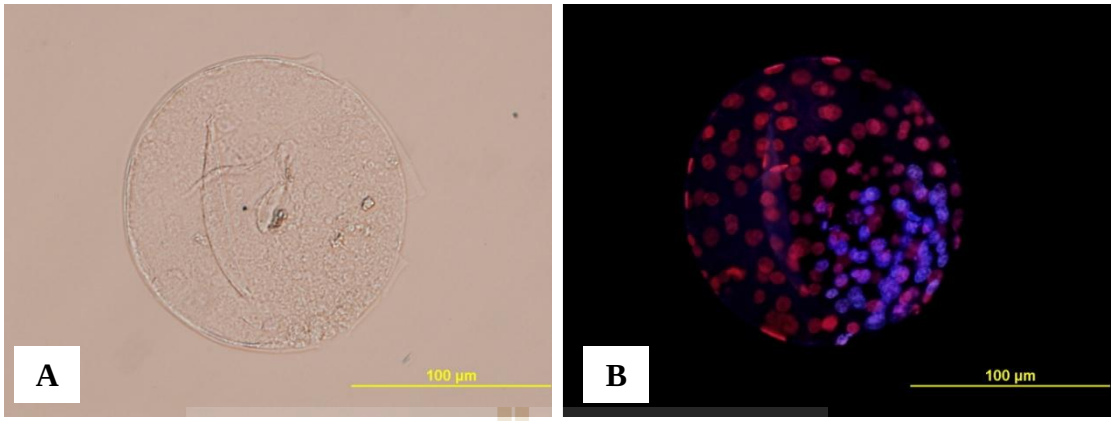


Figure 3.6 Differential stained bovine blastocyst in bright field (A) and fluorescence image (B). Blue color of Hoechst stained ICM and pink or red color of both PI and Hoechst stained TE (magnification 200x).

3.11 Experimental designs

Experiment 1 (Figure 3.7) was conducted to investigate the toxic effects of combination of permeating CPAs. IVM oocytes were separated in five groups (4 treatments and 1 control). Oocytes were exposed to four difference equilibration (ES) and vitrification (VS) solution without cooling. Then, oocytes were exposed to warming solution and evaluated for the survival rate by FDA staining. Surviving oocytes were subjected to IVF and IVC. Rates of development of oocytes to the cleavage, morula and blastocyst stage and total cell number of the obtained blastocysts were compared with those of oocytes that were not subjected to CPA solution (fresh control).

Experiment 2 (Figure 3.8) was performed to compare vitrification efficiency of the combination of permeating CPAs. Bovine IVM oocytes were separated into five groups (4 treatments and 1 control). Oocytes were exposed to four difference ES and

VS, and then plunged directly into LN_2. After warming, surviving oocytes were subjected to IVF and IVC. Rates of development of oocytes to the cleavage and blastocyst stage and total cell number of the obtained blastocysts were compared with those of oocytes that were not subjected to vitrification (fresh control).

Experiment 3 (Figure 3.9) was performed to investigate the effect of IGF-1 supplementation in IVM medium on survival rate and blastocyst developmental rates of IVM bovine oocytes after vitrification. Bovine oocytes were matured *in vitro* in IVM medium supplemented with 0, 50, 100 and 200 ng/mL IGF-1 before vitrification. Survival rate of vitrified oocytes were evaluated after warming, surviving oocytes were subjected to IVF and IVC. Rates of development of oocytes to the cleavage, morula and blastocyst stage and total cell number of the obtained blastocysts were compared with those of oocytes that were not subjected to IGF-1 supplementation and vitrification (fresh control).

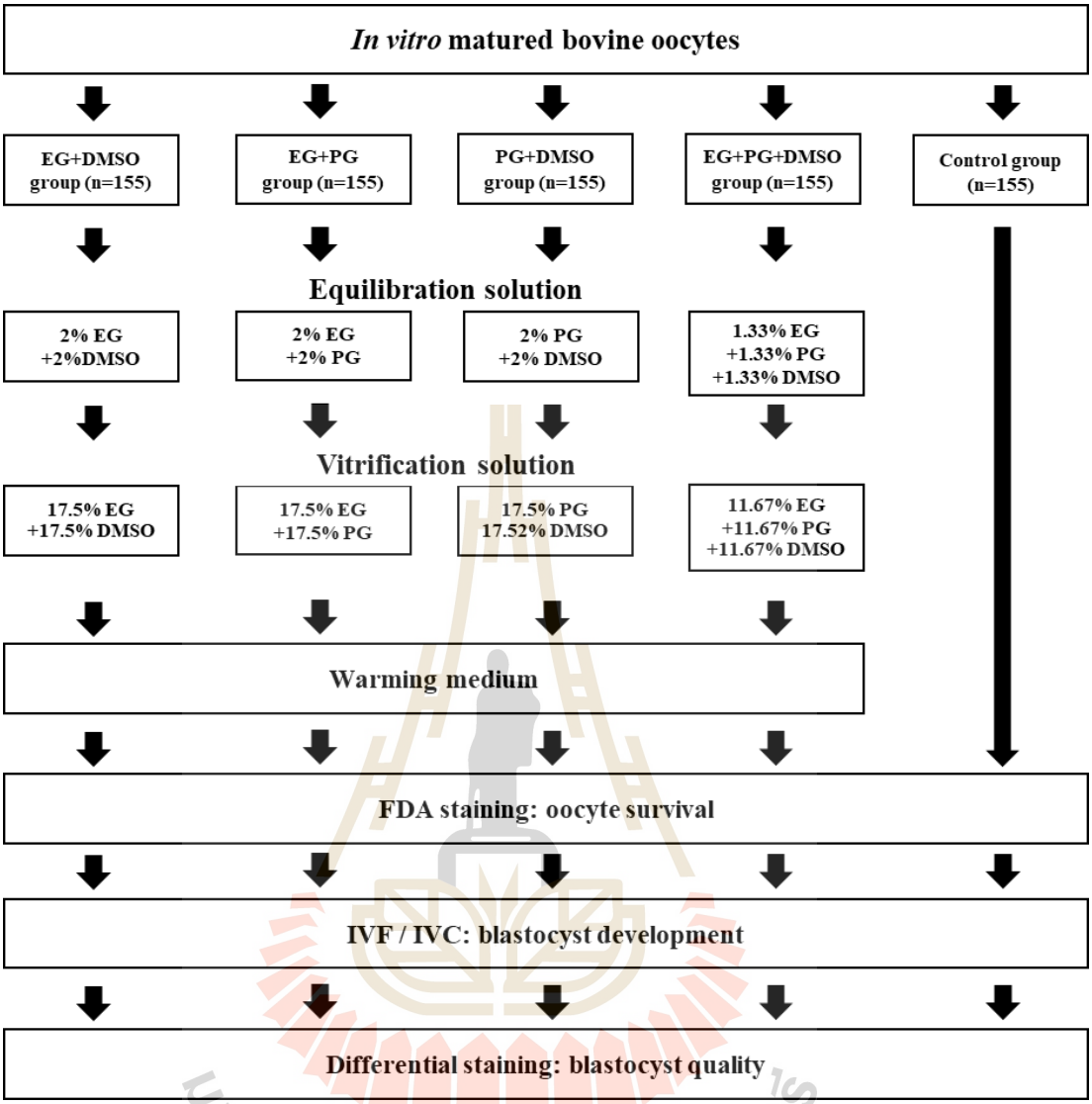


Figure 3.7 Experimental design of experiment 1. Investigate the toxic effects of different CPA combinations, including EG + DMSO, EG + PROH, PROH + DMSO and EG + PROH + DMSO on oocyte survival, embryo development and TE, ICM and total cell number in blastocysts of *in vitro* matured bovine oocytes.

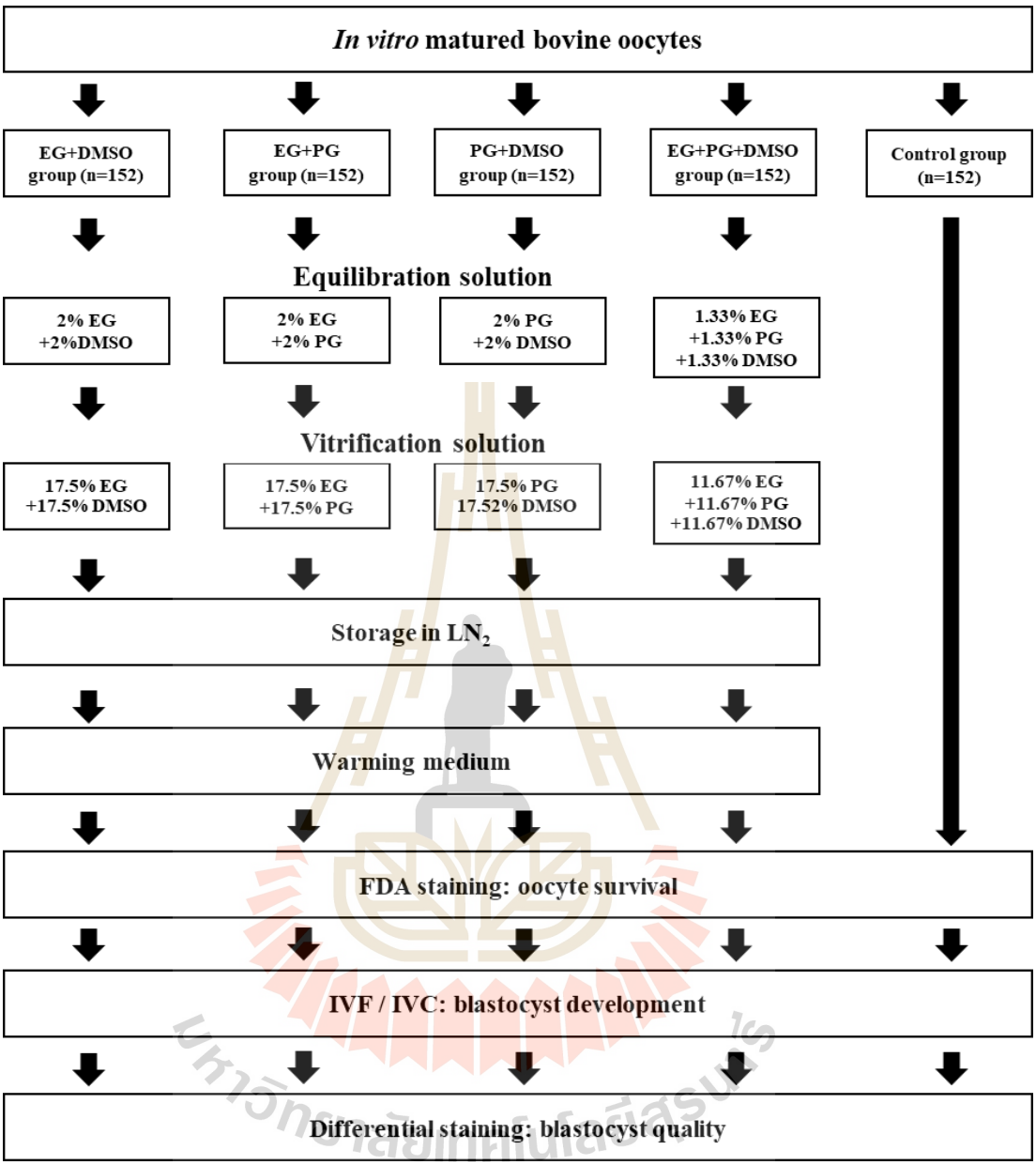


Figure 3.8 Experimental design of experiment 2. Investigate the vitrification efficiency of different CPA combinations, including EG + DMSO, EG + PROH, PROH + DMSO and EG + PROH + DMSO on oocyte survival, embryo development and TE, ICM and total cell number in blastocysts of *in vitro* matured bovine oocytes after vitrification.

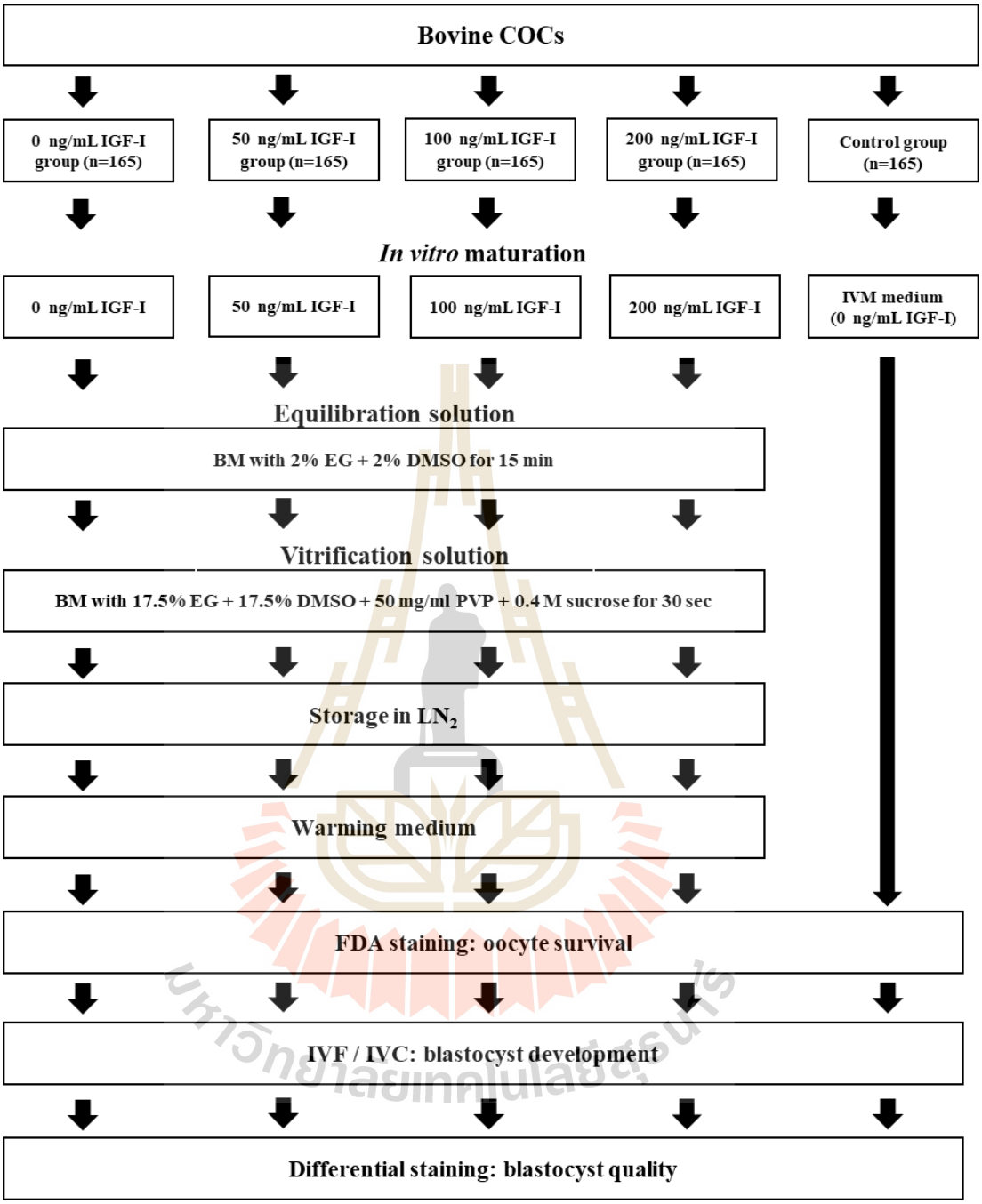


Figure 3.9 Experimental design of experiment 3. Investigate the effects of IGF-I supplemented in IVM medium on oocyte survival, embryo development and TE, ICM and total cell number in blastocysts of IVM bovine oocytes after vitrification.

3.12 Statistical analysis

The data of survival rates of vitrified-warmed oocytes, cleavage and blastocyst developmental rates of surviving oocytes after IVF were expressed as percentages (%) and analyzed by One Way Analysis of Variances (ANOVA) using Statistical Package for Social Sciences (SPSS®) Software. P value at < 0.05 was considered a statistically significant difference.

CHAPTER IV

RESULTS

4.1 The toxic effects of different CPA combinations exposure on *in vitro* matured bovine oocyte survival, embryo development and blastocystscell number.

Bovine oocytes were exposed to ES (4% CPAs combination) for 15 min and VS (35% CPAs combination) for 30 sec. As shown in Table 4.2, the survival rates of bovine oocytes were 93.23%, 92.42%, 91.33% and 92.04% when exposed to ES and VS containing EG + DMSO, EG + PROH, PROH + DMSO and EG + PROH + DMSO, respectively, which were not significantly different compared to fresh control (95.14%). Although the cleavage rate was not different among four treatments groups (62.44% , 62.90% , 60.22% and 62.08%), they were not significantly different compared to fresh control (63.85%). The rates of embryo develop to the 8-cell stage (42.65%, 42.12%, 40.81% and 43.73%) and morula (29.53%, 28.23%, 28.77% and 29.08%) were not different among the four treatment groups and they were not different compared to fresh control (8-cell; 45.38% and morula; 30.82%). The rates of embryos developing to the blastocyst stage were also not different among four treatment groups (23.11%, 22.63%, 21.93% and 23.33%) and they were not difference compared to fresh control (24.86%). And, as shown in Table 4.3, the total cell number in blastocysts was no different among four treatment group and fresh control.

Similarly, the numbers of ICM and TE cells were no different among four treatment groups and fresh control groups.

Within these experiments, the osmotic value and viscosity of each VS were measured (Table 4. 1) . VS containing PROH + DMSO (5860 mOsm) showed significantly lower ($P < 0.05$) osmotic value compared with solution containing EG + DMSO (6880 mOsm), EG + PROH (6680 mOsm) and EG + PROH + DMSO (7070 mOsm). In addition, osmotic values of vitrification solution containing single CPAs such as EG, PROH and DMSO were also measured. The results showed osmotic values of there were significantly different ($P < 0.05$) which VS containing EG (8110 mOsm) was highest osmotic values (PROH; 6980 mOsm and DMSO; 5520 mOsm).

Table 4.1 The osmotic value and viscosity of vitrification solution.

Vitrification solution*	**Osmotic value (mOsm/kg)**	**Viscosity (Pa.s)**
EG	8110 ± 0.14 [a]	0.103 ± 0.01 [a]
PROH	6980 ± 0.18 [b]	0.117 ± 0.01 [b]
DMSO	5520 ± 0.11 [c]	0.106 ± 0.01 [a]
EG + DMSO	6880 ± 0.21 [b]	0.107 ± 0.002 [ac]
EG + PROH	6680 ± 0.21 [b]	0.106 ± 0.002 [a]
PROH + DMSO	5860 ± 0.20 [c]	0.109 ± 0.001 [c]
EG + PROH + DMSO	7070 ± 0.23 [b]	0.107 ± 0.003 [ac]

*Vitrification solution consisted of BM supplemented with total 35% (v/v) CPA combination 50 mg/mL PVP + 0.4 M sucrose

[a,b,c] Means within columns with different superscripst differ ($P > 0.05$ ANOVA).

Figure 4.1 showed the relative volume of IVM bovine oocytes during exposure to VS at room temperature. As expected, the oocytes shrank on initial exposure to the VS and gradually re-expanded to their initial volume as CPA and water entered the oocyte. The changes in oocyte volume in presence of each VS were measured. At 10 sec after exposure, minimum oocytes volumes were 82% - 90% of their initial volume. At 30 sec after exposure, oocytes exposed to VS containing EG + PROH, EG + DMSO and EG + PROH + DMSO were returned to near their initial volume, where relative volume of oocytes exposed to PROH + DMSO was 90.20% of initial volume.

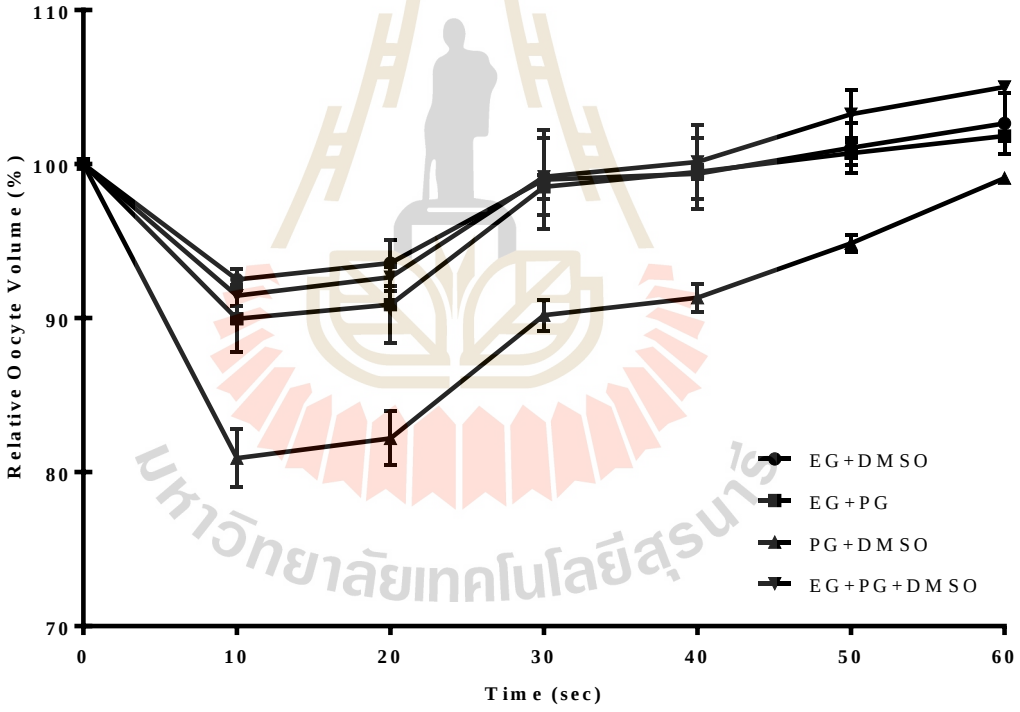


Figure 4.1 Volume changes of IVM bovine oocyte during exposure to VS containing different CPA.

4.2 The vitrification efficiency of different CPA combinationson *in vitro* matured bovine oocyte survival, embryo development and blastocysts cell number.

Vitrification efficiency of CPAs combination as: EG + DMSO, EG + PROH, PROH + DMSO and EG + PROH + DMSO were compared in this experiment. As shown in Table 4.4, the survival rate of oocytes were 91.46%, 92.32%, 91.36% and 92.44% respectively, when EG + DMSO, EG + PROH, PROH + DMSO and EG + PROH + DMSO were used as CPA. There was no difference when compared to fresh control (95.35%). The rates of cleavages were not different among four vitrified groups (41.83%, 40.08%, 38.16% and 39.98%) but there were significantly lower ($P < 0.05$) when compared to fresh control (64.91%). The rate of embryo develop to the 8-cell stage (20.23%, 18.60%, 17.15% and 18.25%) and morula (9.00%, 7.74%, 6.58% and 9.02%) were not different among four vitrified groups, but significantly lower than that in the fresh control group (8-cell; 44.77% and morula; 29.70%). Similarly, the rates of embryos development into the blastocyst were not significantly difference among four vitrified groups (7.14%, 6.38%, 4.62% and 5.75%), but significantly lower than that in the fresh control (26.31%). There was no significantly difference in the number of ICM, TE and total cell number in blastocysts amongthe control and vitrified groups (Table 4.5).

4.3 The effects of IGF-I supplementation in IVM medium on vitrified-warmed *in vitro* matured bovine oocyte survival, embryo development and blastocysts cell number.

Bovine oocytes were *in vitro* matured with IVM medium supplemented with 0, 50, 100 and 200 ng/mL IGF-1. Following vitrification and warming, results shown in Table 4. 6, the rate of survival oocyte were not different among the groups supplemented with 0, 50, 100 and 200 ng/mL IGF-1 (92.55%, 95.11%, 93.22% and 92.66%) and fresh control (96.33%). The rate of cleavage in the 50 and 100 ng/mL IGF-1 groups (50.91% and 53.15%) were significantly higher ($p < 0.05$) than those in 0 ng/mL IGF-1 group (42.73%). However, the rates of cleavage in all vitrified groups were significantly lower than that of fresh control (63.51%). The rate of embryo develops to the 8-cell stage (21.11% - 24.36%) and morula (9.10% - 11.14%) were not different among IGF-1 supplemented groups, but significantly lower than that of the fresh control (8-cell; 45.32% and morula; 34.60%). Similarly, the rates of embryos development into the blastocyst were not significantly different among IGF-1 supplemented groups (7.79%, 8.24%, 7.19% and 7.89%) but significantly lower than that in the fresh control (25.11%). The number of ICM and TE cells was no different among IGF-1 supplemented groups and fresh control groups (Table 4.7).

Table 4.2 Survival and embryo developmental rate of IVM bovine oocytes exposed to difference CPA combinations.

Groups	No. of oocytes	Survived (%)	Oocyte Cultured	Cleavage (%)	No. (%) embryo developed to		
					8-cell	Morula	Blastocysts
Fresh control	155	148 (95.14 ± 3.39)	148	94 (63.85 ± 2.54)	67 (45.38 ± 2.90)	46 (30.82 ± 3.55)	37 (24.86 ± 2.75)
EG + DMSO	155	144 (93.23 ± 4.74)	144	89 (62.44 ± 3.08)	61 (42.65 ± 3.37)	42 (29.53 ± 3.66)	34 (23.11 ± 2.92)
EG + PROH	155	143 (92.42 ± 4.49)	143	90 (62.90 ± 2.70)	60 (42.12 ± 3.73)	40 (28.23 ± 2.74)	32 (22.63 ± 1.93)
PROH + DMSO	155	142 (91.33 ± 4.22)	142	86 (60.22 ± 2.29)	58 (40.81 ± 2.11)	41 (28.77 ± 4.51)	31 (21.93 ± 1.04)
EG + PROH + DMSO	155	143 (92.04 ± 5.42)	143	88 (62.08 ± 2.50)	62 (43.73 ± 2.84)	42 (29.08 ± 4.75)	34 (23.33 ± 2.94)

Seven replications were performed. No statistical difference was obtained (P > 0.05 ANOVA).

Table 4.3 Cell number in blastocysts derived from IVM bovine oocytes exposed to difference CPA combinations.

Groups	No. of blastocysts	Mean number of cells in blastocysts				
		No. of TE	No. of ICM	Total cell	TE : ICM	ICM ratio
Fresh control	20	76.08 ± 4.21	32.44 ± 3.06	108.52 ± 4.04	2.35 ± 0.22	30.01 ± 2.63
EG + DMSO	20	68.80 ± 6.19	29.67 ± 4.11	98.47 ± 5.06	2.34 ± 0.33	30.00 ± 3.82
EG + PROH	20	70.53 ± 3.24	30.93 ± 2.09	101.47 ± 4.05	2.28 ± 0.16	30.51 ± 2.07
PROH + DMSO	20	64.73 ± 5.25	32.00 ± 3.09	96.73 ± 5.07	2.23 ± 0.22	31.14 ± 3.15
EG + PROH + DMSO	20	70.47 ± 3.25	28.80 ± 2.16	99.27 ± 3.08	2.45 ± 0.18	29.03 ± 2.04

Seven replications were performed. No statistical difference was obtained (P > 0.05 ANOVA).

Table 4.4 Survival and embryo developmental rate of IVM bovine oocytes vitrified using difference CPA combinations.

Groups	No. of oocytes	Survived (%)	Oocyte Cultured	Cleavage (%)	No. (%) embryo developed to		
					8-cell	Molura	Blastocysts
Fresh control	152	145 (95.35 ± 3.16)	145	94 (64.91 ± 3.11)[a]	65 (44.77 ± 1.92)[a]	43 (29.70 ± 3.05)[a]	38 (26.31 ± 2.25)[a]
EG + DMSO	152	139 (91.46 ± 2.90)	139	58 (41.83 ± 3.15)[b]	28 (20.23 ± 2.67)[b]	13 (9.00 ± 2.95)[b]	10 (7.14 ± 1.92)[b]
EG + PROH	152	140 (92.32 ± 4.11)	140	56 (40.08 ± 3.19)[b]	26 (18.60 ± 3.28)[b]	11 (7.74 ± 1.76)[b]	9 (6.38 ± 1.67)[b]
PROH + DMSO	152	139 (91.36 ± 4.52)	139	53 (38.16 ± 1.90)[b]	24 (17.15 ± 1.62)[b]	9 (6.58 ± 2.16)[b]	6 (4.62 ± 2.14)[b]
EG + PROH + DMSO	152	141 (92.44 ± 3.12)	141	56 (39.98 ± 2.86)[b]	26 (18.25 ± 1.98)[b]	13 (9.02 ± 2.02)[b]	8 (5.75 ± 1.49)[b]

Six replications were performed. [a,b] Means within columns with different superscript differ (P > 0.05 ANOVA).

Table 4.5 Cell number in blastocysts derived from IVM bovine oocytes vitrified using difference CPA combinations.

Groups	No. of blastocysts	Mean number of cells in blastocysts				
		No. of TE	No. of ICM	Total cell	TE : ICM	ICM ratio
Fresh control	20	73.80 ± 5.25	31.93 ± 6.09	105.73 ± 4.08	2.38 ± 0.48	30.22 ± 5.74
EG + DMSO	5	76.40 ± 4.07	28.80 ± 4.06	105.20 ± 3.06	2.67 ± 0.23	27.39 ± 3.41
EG + PROH	5	64.80 ± 6.09	32.00 ± 3.11	96.80 ± 5.19	2.02 ± 0.01	33.12 ± 3.18
PROH + DMSO	5	68.80 ± 3.24	32.00 ± 5.19	100.80 ± 3.16	2.17 ± 0.25	31.76 ± 4.54
EG + PROH + DMSO	5	74.40 ± 3.08	26.20 ± 5.04	100.60 ± 3.04	2.89 ± 0.44	26.05 ± 4.39

Five replications were performed. No statistical difference was obtained ($P > 0.05$ ANOVA).

Table 4.6 Survival and embryo developmental rate of bovine oocytes matured *in vitro* using IVM medium supplemented with IGF-1 after vitrification and warming.

Groups	No. of oocytes	Survived (%)	Oocyte cultured	Cleavage (%)	No. (%) embryo developed to		
					8-cell	Molura	Blastocysts
Fresh control	165	159 (96.33 ± 4.46)	159	101 (63.51 ± 3.11)[a]	72 (45.32 ± 2.59)[a]	55 (34.60 ± 2.13)[a]	40 (25.11 ± 2.28)[a]
0 ng/mL IGF-1	165	153 (92.55 ± 4.10)	153	65 (42.73 ± 3.11)[b]	34 (22.36 ± 2.04)[b]	16 (10.41 ± 2.61)[b]	12 (7.79 ± 2.10)[b]
50 ng/mL IGF-1	165	157 (95.11 ± 3.83)	157	80 (50.91 ± 3.63)[c]	38 (24.36 ± 2.28)[b]	17 (10.66 ± 2.88)[b]	13 (8.24 ± 2.57)[b]
100 ng/mL IGF-1	165	154 (93.22 ± 3.76)	154	82 (53.15 ± 2.13)[c]	38 (24.85 ± 2.30)[b]	14 (9.10 ± 3.87)[b]	11 (7.19 ± 1.75)[b]
200 ng/mL IGF-1	165	153 (92.66 ± 3.37)	153	70 (46.00 ± 3.32)[bc]	32 (21.11 ± 2.83)[b]	17 (11.14 ± 2.78)[b]	12 (7.89 ± 2.72)[b]

Six replications were performed. [a,b,c] Means within columns with different superscript differ (P > 0.05 ANOVA).

Table 4.7 Cell number in blastocysts derived from bovine oocytes matured *in vitro* using IVM medium supplemented with IGF-1 after vitrification and warming.

Groups	No. of blastocysts	Mean number of cells in blastocysts				
		No. of TE	No. of ICM	Total cell	TE : ICM	ICM ratio
Fresh control	20	74.84 ± 6.06	30.10 ± 4.81	106.75 ± 5.25	2.47 ± 0.31	28.16 ± 3.36
0 ng/mL IGF-1	7	75.80 ± 5.17	33.60 ± 4.26	103.80 ± 5.61	2.26 ± 0.13	31.66 ± 3.33
50 ng/mL IGF-1	8	78.00 ± 4.04	28.60 ± 4.12	106.60 ± 3.28	2.75 ± 0.25	26.84 ± 3.42
100 ng/mL IGF-1	6	73.20 ± 6.21	26.80 ± 3.12	101.60 ± 6.03	2.73 ± 0.08	26.44 ± 2.99
200 ng/mL IGF-1	7	73.60 ± 6.08	25.40 ± 5.02	97.00 ± 5.78	2.89 ± 0.36	25.55 ± 4.80

Six replications were performed No statistical difference was obtained ($P > 0.05$ ANOVA).

CHAPTER V

DISCUSSIONS AND CONCLUSIONS

5.1 Discussions

In the present studies, permeating CPAs as EG, PROH and DMSO were combined in mixture (EG + DMSO, EG + PROH, PROH + DMSO and EG + PROH + DMSO) at total 35% (v/v) to VS containing 25 mM Hepes-buffered TCM 199 supplemented with 50 mg/mL PVP and 0.4 M sucrose for IVM bovine oocytes vitrification. The toxic effect and vitrification efficiency of permeating CPA combinations on oocyte survival, embryo development and quality of blastocysts by evaluated the TE, ICM and total cell numbers in blastocysts were investigated. Within this experiment, the osmotic value and viscosity of each vitrification solution as well as relative oocyte volume changing during exposure to these solutions were measured.

Vitrification is the cryopreservation method that requires high concentration of permeating CPAs to prevent the ice crystals formation that resulting in high survival of oocytes after vitrification. During vitrification, oocytes exposed to CPA may result in chemical toxicity and extreme osmotic stress to oocytes (Agca *et al.*, 2000). To minimize those effects, CPAs are used as combination in a mixture. It has been reported that the cryoprotective effect of CPA combination was greater than single CPA using (Somfai *et al.*, 2013). Otherwise, combination of permeating CPA

is the way to reduce the concentration of CPA as well as reduce their individual CPA toxic effects. In experiment 1, our results showed IVM bovine oocytes exposed to VS containing those CPA combinations appeared similar in oocyte survival and embryo development rates as fresh control. Moreover, the quality of the resultant blastocysts was not affected in term of TE, ICM and total cell numbers.

Several reports showed that CPA interfered oocytes in organization of microtubules and structures of the cytoskeleton (Johnson and Pickering, 1987; Vincent *et al.*, 1989). Moreover, CPAs have been thought to cause premature cortical granule release resulting in zona hardening and reduced subsequent fertilization rates (Vincent *et al.*, 1990; Checura and Seidel, 2007). In the present studies, cleavage and blastocyst development following CPAs exposure did not differ from fresh controls. Similar results have been reported for bovine oocytes in solution control group without vitrification (Sripunya *et al.*, 2010; Punyawai *et al.*, 2015).

When exposed to VS, oocytes undergo suddenly volume changes due to osmotic pressure differences across the cell membrane and then return to slightly greater than their initial volume as the permeating CPA enters the cell (Figure 4.1). The extent of the initial shrinkage and the subsequent time course for oocytes to return to near their initial volume are directly related to osmotic values in this study. In the present study, we measured osmotic values of each VS which VS containing PROH + DMSO (5860 mOsm) showed significantly lower ($P < 0.05$) osmotic value compared with EG + DMSO (6880 mOsm), EG + PROH (6680 mOsm) and EG + PROH + DMSO (7070 mOsm). In addition, osmotic values of VS containing single CPAs such as EG, PROH and DMSO were also measured. The results showed osmotic values were significantly different ($P < 0.05$) which VS containing EG (8110 mOsm) was

significantly higher osmotic values compared with PROH; (6980 mOsm), DMSO (5520 mOsm) and EG-based CPA combination. This indicated that VS containing 35% (v/v) EG may cause osmotic stress on oocytes more than VS containing PROH and DMSO. However, combination of EG with PROH and DMSO can reduce osmotic values as well as reduce osmotic stress of VS on oocytes.

In a previous study, reduction in embryo development caused by exposure to hypertonic and hypotonic (anisotonic) solution was damage in oocytes. Avery low rate of 2 - 4 cell cleavage and blastocyst formation were observed after a 10 min exposure of GV and MII bovine oocytes to 4800 mOsm NaCl solution (Agca *et al*. 2000). However, in present study, the rates of oocyte survival and embryo development after a 30 sec exposure to vitrification solutions that have osmotic value around 5860 – 7070 mOsm were not significantly different from fresh control. Although several studies have reported that adverse effect of CPA solution of oocyte survival and embryo development competent (Agca *et al*. 2000), this study did not observe such differences among all treatment groups. Probably because of the beneficial effect of a viscocity-increasing compound (PVP) and a membrane-protective sugar (sucrose) that supplemented in vitrification solution act like membrane stabilizer and minimizing damage caused by osmotic stress (Saha *et al*., 1996; Dinnyés *et al*., 2000).

In experiment 2, vitrification efficiency of CPA combinations including EG + DMSO, EG + PROH, PROH + DMSO and EG + PROH + DMSO, was investigated. The oocytes showed similar survival rate among four vitrified groups (91.36% - 92.44%) and fresh control (95.35%) but embryo development into cleavage, 8-cell, morula and blastocyst of four vitrified groups were significantly lower ($P < 0.05$) compared to fresh control.

Bovine oocyte vitrification using single EG, DMSO or in combination with other CPAs has been reported to be successful (Vieira *et al.*, 2002). In a previous study, Chian *et al.* (2004) group vitrified matured bovine oocytes using combination of 15% EG + 15% PROH and 15% EG + 15% DMSO. Although, similar oocyte survival (97.6% vs 91.8%, respectively) were found after vitrification, rate of blastocyst in 15% EG + 15% PROH (7.4%) group shows significantly higher ($P < 0.05$) than that of 15% EG + 15% DMSO (1.7%), which was different from our results that showed similar blastocyst rate in oocytes vitrified with combination of 17.5% EG + 17.5% PROH (6.38%) and 17.5% EG + 17.5% DMSO (7.14%). The cause for these various results may be related to differences in vitrification and warming procedure, CPAs concentration or oocyte source. A recent study of Somfai *et al.* (2015) in immature porcine oocytes showed that vitrification using combination of 17.5% EG with 17.5% DMSO (51.1%) gave the lower oocyte survival rate than that vitrified in the 17.5% EG + 17.5% PROH (73.8%) combination. This contrast to Nohalez *et al.* (2015) who showed that oocyte viability of vitrified immature porcine oocytes in the 16% EG + 16% PROH (57.6%) was lower than 16% EG + 16% DMSO (67.0%) group. However, cleaved embryos and blastocysts from cultured oocytes among all vitrification groups that are not differ in both of two studies (Nohalez *et al.*, 2015; Somfai *et al.*, 2015). However, the difference of oocyte composition such as size and lipid content among porcine and bovine or the dissimilarities in the CPA concentration could give different results.

Our results demonstrated that vitrified oocytes were able to fertilize and reached 4.62% – 7.14% blastocyst rate which embryo developments of vitrified-warmed oocytes was future reduced compared with the fresh control group (26.31%).

Similar results were obtained by several studies showing lower blastocyst rate of bovine matured oocytes after vitrification. The vitrification of IVM bovine oocytes using 35% EG resulted in 8.7% – 9.8% (Sripunya *et al*., 2010) and 20.0% - 25.5% (Punyawai *et al.*, 2015) blastocyst development (Fresh control: 33.1% and 43.3%, respectively). In other report, IVM bovine oocytes vitrified in 20% EG + 20% DMSO showed 9.0% blastocyst rate (Fresh control: 26.6%) (Paul *et al.*, 2018). However, the vitrification of IVM bovine oocytes with multi-layered cumulus cells using 30% EG resulted in 35.8% blastocyst formation that was similar to those of the fresh control group (37.4%) (Ishii *et al*., 2018). The dissimilarities in the permeating CPA and their concentration, which was 35% CPA combination in present study and 30% EG in Ishii *et al*. (2018) could play a key role in difference in blastocyst development. Moreover, the difference in blastocyst development between the studies could be due to technical aspects of the vitrification procedure or the quality of the oocytes used.

Following results from experiment 1, oocytes exposed to VS containing different kinds of CPA combinations did not affect survival and embryo development rates. However, vitrification reduced embryo development rates of oocytes. The sensitivity of bovine oocytes to cooling has been reported as poor ability of embryo development after vitrification (Yamada *et al*., 2007; Sripunya *et al*., 2010; Punyawai *et al*., 2015; Paul *et al*., 2018).

Matured oocytes have meiotic spindles that are extremely sensitive to cooling which often results in tubulin depolymerization (Arav *et al.*, 1993; Martino *et al.*, 1996; Lim *et al*., 1999). Cryopreservation of oocytes results in several ultrastructural and morphological alterations including abnormal distribution of chromosomes, microtubules and actin microfilaments (Men *et al.*, 2003; Stachowiak *et al.*, 2009).

These morphological changes have been linked to failure in fertilization and embryo development (Succu *et al.*, 2007). The low cleavage and blastocyst rates in the vitrified groups indicate that cooling had detrimental effects on the bovine oocytes (Chian *et al*., 2004: Yamada *et al*., 2007; Sripunya *et al*., 2010; Punyawai *et al*., 2015; Paul *et al*., 2018).

In the present study, the effect of IGF-1 during IVM on survival and embryo development of IVM bovine oocyte after vitrification was determined. Oocytes were equilibrated with 2% (v/v) EG + 2% (v/v) DMSO in HM for 15 min and then transferred into a vitrification solution consisted of 17.5% (v/v) EG + 17.5% (v/v) DMSO supplemented with 50 mg/mL PVP and 0.4 M sucrose for 30 sec, and the effect of IGF-1 was compared. These results indicated that the cleavage of IVM oocytes after vitrification was improved by 50 and 100 ng/mL IGF-1. However, the embryo development was still lower ($P < 0.05$) than those fresh control. The total cell number did not differ significantly among fresh control groups, vitrified groups treated with and without IGF-1.

IGF-1 is the one kinds of growth factor which has positive effect on the development of embryos. This study suggested that embryos showed an improved cleavage rate after vitrification when maturation medium supplemented with IGF-1. These findings were also consistent with the effects of IGF-1 on matured yak oocytes after vitrification demonstrated that IGF-1 can improved embryo development and can enhance the expression of cold-inducible RNA-binding protein (CIRP) that play important roles in protect cells against adverse environmental stress as well as cryopreservation procedure and maintaining the development of oocytes and embryo (Pan *et al.*, 2015). IGF-1 concentrations of between 23 and 168 ng/mL have been

reported in bovine follicular fluid (Spicer *et al.*, 1998) , which indicates that the concentration used in this study was within the physiological range.

The results of this study demonstrated that IVM bovine oocytes can be vitrified successfully with the combination of EG, PROH and DMSO in both of two combinations and three combinations.

5.2 Conclusions

In conclusion, the combination of CPA including EG, PROH and DMSO showed same efficiency in vitrification of IVM bovine oocytes, it is possible to use either EG + DMSO, EG + PROH, PROH + DMSO or EG + PROH + DMSO using equilibration in at total 4% (v/v) CPA combination for 15 min and vitrification solution at total 35% (v/v) CPA combination for 30 sec. Although, the supplementation of 50 and 100 ng/mL IGF-1 during IVM of bovine oocytes improved their total cleavage rate, there was no effect on development to the blastocyst stage of IVM oocyte after vitrification.

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