EFFECTS OF VITRIFICATION TECHNIQUES ON THE SURVIVAL OF BUFFALO OOCYTES AND DEVELOPMENTAL POTENTIAL OF EMBRYO FOLLOWING INTRACYTOPLASMIC SPERM INJECTION

Yuanyuan Liang

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biotechnology

Suranaree University of Technology

Academic Year 2010

ผลของวิธีการแช่แข็งแบบแก้วต่ออัตรารอดของไข่กระบือและอัตราการเจริญของตัวอ่อนหลังจากฉีดอสุจิเข้าไปในไข่

นางสาวหยวนหยวน เหลียง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2553

EFFECTS OF VITRIFICATION TECHNIQUES ON THE SURVIVAL OF BUFFALO OOCYTES AND DEVELOPMENTAL POTENTIAL OF EMBRYO FOLLOWING INTRACYTOPLASMIC SPERM INJECTION

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

Thesis Examining Committee

_____
(Asst. Prof. Dr. Mariena Ketudat-Cairns)
Chairperson

_____
(Asst. Prof. Dr. Rangsun Parnpai)
Member (Thesis Advisor)

_____
(Dr. Siwat Sangsritavong)
Member

_____
(Dr. Tassanee Suteevum)
Member

_____
(Dr. Surintorn Boonanuntanasarn)
Member

_____
(Dr. Parinya Noisa)
Member

_____ _____

(Dr. Wut Dankitikul) (Asst. Prof. Dr. Suwayd Ningsanond)

Acting Vice Rector for Academic Affairs Dean of Institute of Agricultural Technology

หยวนหยวน เหลียง : ผลของวิธีการแช่แข็งแบบแก้วต่ออัตรารอดของไข่กระบือและอัตราการเจริญของตัวอ่อนหลังจากฉีดอสุจิเข้าไปในไข่ (EFFECTS OF VITRIFICATION TECHNIQUE ON THE SURVIVAL OF BUFFALO OOCYTES AND DEVELOPMENTAL POTENTIAL OF EMBRYO FOLLOWING INTRACYTOPLASMIC SPERM INJECTION) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.รังสรรค์ พาลพ่าย, 121 หน้า

การฉีดอสุจิเข้าสู่ไซโตพลาซึมของไข่ (อิ๊กซี่) เป็นเทคนิคการปฏิสนธิภายนอกร่างกายวิธีหนึ่ง โดยใช้อสุจิหนึ่งตัวฉีดเข้าไปในไข่หนึ่งใบโดยตรง ซึ่งลูกที่ได้จากเทคนิคอิ๊กซี่มีรายงานในสัตว์หลายสปีชีส์ในการทดลองแรกได้ทำการศึกษากระบวนการกระตุ้นของเทคนิคอิ๊กซี่ในกระบือ พบว่าอัตราการเกิด second polar body (PB) ปรากฏสูงที่สุดเมื่อไข่ได้ถูกกระตุ้น 3 ชั่วโมง และการเกิด second PB พบในกลุ่มที่ถูกกระตุ้นด้วย ethanol (EtOH) มากกว่ากลุ่ม Io อย่างมีนัยสำคัญ นอกจากนี้พบว่าวิธีการกระตุ้นโอโอไซต์กระบือที่ผ่านการทำอิ๊กซี่ที่ได้ประสิทธิภาพ คือ การกระตุ้นด้วย Io ร่วมกับ 6-dimethyl amino purine (6-DMAP) และ EtOH ร่วมกับ cycloheximide (CHX) ซึ่งให้อัตราการแบ่งตัวและการพัฒนาเป็นตัวอ่อนระยะบลาสโตซิสสูงที่สุด และวิธีการกระตุ้นด้วย EtOH ร่วมกับ CHX ถูกนำมาใช้กับไข่กระบือที่ผ่านการแช่แข็งแล้วนำมาทำอิ๊กซี่ ในการทดลองที่สองศึกษาผลของระยะเวลาการสัมผัสกับสารป้องกันการแข็งตัว cryoprotectant (CPA) ของโอโอไซต์และเทคนิคการแช่แข็งต่อการพัฒนาของตัวอ่อนภายนอกร่างกายหลังจากการกระตุ้นให้ไข่ที่ไม่ได้รับการปฏิสนธิแบ่งตัว parthenogenetic activation (PA) และอิ๊กซี่ โดยโอโอไซต์ที่ผ่านกระบวนการเลี้ยงให้เป็นไข่สุกภายนอกร่างกาย (IVM) สัมผัสกับ 10% DMSO ร่วมกับ 10% EG เป็นเวลา 1 นาที จากนั้นถูกแบ่งออกเป็น 3 กลุ่ม คือ สัมผัสกับ 20% DMSO + 20% EG + 0.5 M sucrose เป็นเวลา 30 วินาที 45 วินาที หรือ 60 วินาที (กลุ่ม 1 นาที + 30 วินาที กลุ่ม 1 นาที + 45 วินาที และกลุ่ม 1 นาที + 60 วินาที ตามลำดับ) ผลของอัตราการพัฒนาเป็นตัวอ่อนภายหลังการสัมผัสกับ CPA หรือการแช่แข็ง พบว่า กลุ่มที่สัมผัสกับสาร CPA เป็นระยะเวลา 1 นาที + 30 วินาที แล้วนำมาแช่แข็งด้วยวิธี microdrop สามารถได้ตัวอ่อนระยะบลาสโตซิสสูงสุดภายหลังจากการทำ PA และ อิ๊กซี่ และเมื่อย้อมด้วย FDA เพื่อดูการมีชีวิตรอดพบว่าการย้อมด้วย FDA ไม่ส่งผลเสียต่อการพัฒนาของตัวอ่อนภายนอกร่างกาย การทดลองที่สามศึกษาสารละลายสำหรับการแช่แข็ง 2 ประเภท คือ VA (10% DMSO + 10% EG เป็นเวลา 1 นาที และต่อด้วย 20% DMSO +20% EG + 0.5 M sucrose เป็นเวลา 30 วินาที) และ VB (4% EG เป็นเวลา 12-15 นาที และต่อด้วย 35% EG + 50 mg/ml PVP + 0.4 M trehalose เป็นเวลา 30 วินาที) ร่วมกับวิธีการแช่แข็ง 2 วิธี คือ microdrop และ cryotop ต่ออัตราการอยู่รอด การสร้าง pronuclear และการพัฒนาภายหลังการทำอิ๊กซี่ของโอโอไซต์กระบือระยะ MII

ที่ผ่านการแช่แข็งและทำละลาย พบว่า การมีชีวิตรอดของโอโอไซต์ที่แช่แข็งด้วยสารละลาย VA (ด้วยวิธีการแช่แข็งแบบ microdrop 93% และแบบ cryotop 97% ตามลำดับ) สูงกว่ากลุ่มที่แช่แข็งด้วยสารละลาย VB (ด้วยวิธีการแช่แข็งแบบ microdrop 79% และแบบ cryotop 81% ตามลำดับ) อย่างมีนัยสำคัญ แต่ต่ำกว่ากลุ่มควบคุม (100%) อย่างมีนัยสำคัญ สารละลาย VA ให้อัตราการรอดและการสร้าง pronuclear 2 อันของโอโอไซต์ที่ผ่านการแช่แข็งที่สูงกว่า ส่วนวิธีการแช่แข็งแบบ microdrop และ cryotop เป็นวิธีการที่เหมาะสมสำหรับการแช่แข็งโอโอไซต์กระบือ

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2553 ลายมือชื่ออาจารย์ที่ปรึกษา_____

YUANYUAN LIANG : EFFECTS OF VITRIFICATION TECHNIQUES ON THE SURVIVAL OF BUFFALO OOCYTES AND DEVELOPMENTAL POTENTIAL OF EMBRYO FOLLOWING INTRACYTOPLASMIC SPERM INJECTION. THESIS ADVISOR : ASST. PROF. RANGSUN PARNPAI, Ph.D., 121 PP.

BUFFALO/OOCYTES VITRIFICATION/EMBRYO/ INTRACYTOPLASMIC SPERM INJECTION

Intracytoplasmic sperm injection (ICSI) is an *in vitro* fertilization procedure in which a single sperm is injected directly into an egg. ICSI offsprings have been reported in many species. In the first experiment, activation protocol of buffalo ICSI was studied. The highest rate of second polar body (PB) extrusion occurred at 3 h of activation and the second PB extrusion in ethanol (EtOH) treated group was significantly higher than that in ionomycin (Io) treated group. The result indicated that buffalo ICSI oocytes were effectively activated by combination treatment of Io with 6-dimethyl amino purine (6-DMAP) and EtOH with cycloheximide (CHX) resulting in the highest cleavage and blastocyst formation rates, and EtOH + CHX activation protocol was used in the vitrified buffalo oocytes following ICSI. In the second experiment, the effects of exposure time of oocytes to cryoprotectant (CPA) and vitrification on their *in vitro* development after parthenogenetic activation (PA) or ICSI was examined. IVM oocytes were placed in 10% DMSO + 10% EG for 1 min and then exposed to 20% DMSO + 20% EG + 0.5 M sucrose for 30 s, 45 s or 60

s (1min+30s, 1min+45s and 1min+60s groups, respectively). The results of embryo developmental rates after CPA exposure or vitrification showed that 1min+30s CPA treatment regimen could yield the highest blastocyst formation rates after PA and ICSI for oocytes vitrified by the microdrop method. The fluorescein diacetate (FDA) staining for viability checking had no detrimental effect on the embryo development *in vitro*. In the third experiment, two kinds of vitrification solution VA (10% DMSO + 10% EG for 1 min, 20% DMSO + 20%EG + 0.5M sucrose for 30 sec) and VB (4% EG for 12-15 min, 35% EG+ 50 mg/ml PVP and 0.4 M trehalose for 30 sec) were examined with Microdrop and Cryotop methods on the survival rates, pronuclear formation, and developmental competence following ICSI of vitrified-warmed MII buffalo oocytes. The oocytes viability with VA solution (Microdrop: 93%, Cryotop: 97%, respectively) were significantly higher than that with VB solution (Microdrop: 79%, Cryotop: 81%), but significantly lower than control groups (100%). VA solution yielded higher survival rate and 2 pronuclei formation rates of vitrified oocytes. Thus, Cryotop and Microdrop are equally suitable methods for buffalo oocytes vitrification.

School of Biotechnology Student's Signature_____

Academic Year 2011 Advisor's Signature_____

ACKNOWLEDGEMENTS

This work was supported by the Thailand Research Fund, Suranaree University of Technology and Japan Society for the Promotion of Science - National Research Council of Thailand (JSPS- NRCT) scientific cooperation program.

Looking back on the whole process of my thesis writing, I experienced very mixed feeling. There were both excitement and hardship as I worked my way to the final draft.

My first thanks go to my supervisor, Assistant Prof. Dr. Rangsun Parnpai. When I first met him in China and talked to him about animal biotechnology, I felt he was good at research and I took him to my heart as my teacher. During my four years of study at Suranaree University of Technology (SUT), he has been very professional and rigorous, but also flexible. He has always been ready to help me with my studies and to offer me very quick and helpful comments. Without his help, the thesis would not have been finished in time. I am proud to have him as my advisor. I was sure, and will be sure in the future that I was right to choose to follow him.

I would like to express my heartfelt gratitude to Dr. Takashi Nagai and Dr. Tomas Somfai, who gave me very valuable suggestions that were very impressive during my thesis work and has proofread my thesis and made the wording of my thesis more appropriate. My appreciation is also to the instructors of the course work

at SUT. Their teaching enlightened me and gave me many ideas for the present research design.

I would like to express my heartfelt gratitude to the committee members for the defense of the present thesis, Asst. Prof. Dr. Mariena Ketudat-Cairns, Drs. Siwat Sangsritavong, Tassanee Suteevum, Surintorn Boonanuntanasarn, and Parinya Noisa. They all offered very useful suggestions that were very impressive and improved my thesis a lot.

My deepest thanks should go to my friends and colleagues in the Embryo Technology and Stem Cell Research Center for their helps. Because of them, I was very happy when I stayed at SUT. Special thanks should be given to Drs. Chanchao Lorthongpanich and Chuti Laowtammathron, Mr. Sumeth Imsoonthornruksa, Ms. Kanokwan Srirattana, Mr. Anawat Sangmalee, Ms. Nucharin Sripunya, Ms. Wanwisa Phewsoi, Ms. Kwanrudee Keawmungkun and Ms. Danna Ye, who offered me very professional advices and technical assistants.

Finally, I would like to express my feeling to my parents when I studied in Thailand. They supported my studies wholeheartedly. Without their sincere support and encouragement, I would not have been able to finish my studies in Thailand.

Yuanyuan Liang

CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF ABBREVIATIONS

BOEC	=	bovine oviductal epithelium cells
Ca^{2+}	=	calcium ion
CD	=	cytochalasin D
CG	=	cortical granule
CHX	=	cycloheximide
COCs	=	cumulus-oocyte complexes
CPA	=	cryoprotectant agents
CSF	=	cytostatic factor
DMSO	=	dimethyl sulfoxide
DTT	=	dithiothreitol
EG	=	ethylene glycol
EMG	=	electron microscope grids
EtOH	=	ethanol
FDA	=	fluorescein diacetate
GMP	=	glass micropipette
GV	=	germinal vesicle
ICM	=	inner cell mass
ICSI	=	intracytoplasmic sperm injection
Io	=	ionomycin
IP3	=	inositol 1,4,5-triphosphate

LIST OF ABBREVIATIONS (Continued)

LN_2	=	liquid nitrogen
MAPK	=	mitogen-activated protein kinases
MPF	=	maturation promoting factor
MII	=	metaphase II
MTOCs	=	microtubule organizing centers
OPS	=	open pull straw
PA	=	parthenogenetic activation
PB	=	polar body
PZD	=	partial zona dissection
PVP	=	polyvinylpyrrolidone
SUZI	=	subzonal sperm injection
TE	=	trophectoderm
ZP	=	zona pellucida
6-DMAP	=	6-dimethyl amino purine
mm	=	millimeter
mL	=	milliliter
µg	=	microgram
µL	=	microliter

CHAPTER I

INTRODUCTION

1.1 Introduction

Water buffalo is divided into two main types: river buffalo and swamp buffalo. The river buffaloes have 50 chromosomes while the swamp buffaloes have 48. They are fond of bathing in rivers, swamps, irrigation canals and artificial lakes. The swamp buffaloes (*Bubalus bubalis*), on the other hand, are mainly for draught power and meat production as it is known to have low milk yield. They are the predominant type of swamp buffalo in Asian countries. Of the estimated 40.277 million in the region, 22.416 million are found in China. Of the remaining number, they are distributed in Myanmar, Indonesia, Cambodia, Laos, Malaysia, Thailand and Vietnam.

However, the high costs of breeding and inherent reproductive problems such as silent oestrous signs (Esposito et al., 1992; Zicarelli et al., 1997), long calving interval, delayed age of puberty, low number of primordial follicles (Van et al., 1994) and low conception rates have limited propagation of buffalo. Although, the biotechnologies of reproduction have been used in this special, most of them are not as efficient as in bovine. Hence, it is imperative to study the factors necessary to improve the success rate of the application for reproductive biotechnologies in this

species. Intracytoplasmic sperm injection (ICSI) is an optimun procedure for producing the next generation swamp buffalo, that a single spermatozoon is injected directly into cytoplasm by passing the zona pellucida (ZP) and oolemma. Theoretically, the egg will be fertilized using only single sperm. This technique can make up for the deficiency of the frozen spermatozoa immotility after thawing. The activation of oocytes is necessary for the success of fertilization and subsequent *in vitro* development to pre-implantation embryos irrespective of their origin. Only ICSI alone seems to be sufficient to trigger the events of oocyte activation in some species such as hamsters, mice and humans (Perreault et al., 1988; Kimura and Yanagimachi, 1995; Tesarik and Sousa, 1995; Kuretake et al., 1996). In contrast, other species such as cattle (Rho et al., 1998) and pigs (Lee et al., 2003) require additional activation of the oocytes after ICSI to assure their development. From another perspective, activation treatment following ICSI increases the possibility of parthenogenetic embryo development. Activation procedures aimed at making buffalo oocytes suitable for ICSI differ from those aimed at producing parthenogenesis. So it is imperative to establish an efficiency activation protocol for buffalo ICSI in order to produce haploid activated oocytes.

Yet the limited number of buffalo oocytes made it difficult to assess the success rate of ICSI in this trial. From this problem, the topic of “How to store the oocytes for further used?” has been raised several years ago.

Cryopreservation is one of the significant breakthroughs in science because of its potent use in protecting animal genetic diversity, including species facing eminent danger toward extinction. Genetic materials that can be cryopreserved include semen, oocytes, embryos, and somatic cells. Oocyte cryopreservation can utilize the oocytes

resource for *in vitro* embryo production and safeguard the endangered species. The main problems with oocyte cryopreservation concerned with the survival and fertilization rates. Cryopreservation of mammalian oocytes has become more successful using vitrification as an alternative technique to cryopreservation compared with slow cooling methods in recent years (Chian et al., 2004; Vajta and Nagy, 2006). Vitrification is a process in which are formed a glasslike solidification of living cells without formation of ice crystals during cooling. Several factors have been proposed to be responsible for the low efficiency of the cryopreservation procedure, which include ice crystal formation that could lyse the plasma membrane, chromosomal abnormalities (Johnson and Pickering, 1987) due to meiotic spindles disorganization or disruption, and a high incidence of polyspermy (Glenister et al., 1987; Hyttel et al., 2000) when cryopreserved oocytes were fertilized *in vitro*. In addition, changes in the ZP that caused by the release of premature cortical granule (Vincent et al., 1990) have shown to induce lower fertility rates (Carroll et al., 1990), which resulted in lower the incidence of the sperm entry into the oocytes. This consequence of ZP hardening could be overcome by micromanipulation techniques such as ICSI (Carroll et al., 1990; Kazem et al., 1995; Karlsson et al., 1996., Porcu et al., 1997).

The objective of this study was to find the effective of activation treatments on buffalo ICSI oocytes for the research of vitrified buffalo oocytes. Furthermore, the effect of exposure time in vitrification solution and different vitrification solution were investigated on the post-thaw viability and the developmental competence of vitrified-warmed swamp buffalo oocytes that had been matured *in vitro* after parthenogenetic activation (PA) or ICSI.

1.2 References

Carroll, J., Depyere, H., and Matthews, C.D. (1990). Freeze-thaw-induced changes of the zona pellucida explains decreased rates of fertilization in frozen-thawed mouse oocytes. **J. Reprod. Fertil.** 90: 547-553.

Chian, R., Kuwayama, M., Tan, L., Tan, J., Kato, O., and Nagai, T. (2004). High survival rate of bovine oocytes matured *in vitro* following vitrification. **J. Reprod. Dev.** 50: 685-696.

Esposito, L., Campanile, G., Di, Palo, R., Boni, R., Di, Meo, C., and Zicarelli, L., (1992). Seasonal reproductive failure in buffaloes bred in Italy. In: **Proceedings of the Twelfth International Congress of Animal Reproduction, Hague, The Netherlands** 4: 2045-2047.

Glenister, PH., Wood, MJ., Kirby, C., and Whittinghan, DG. (1987). Incidence of chromosome anomalies in first-cleavage mouse embryos abtained from frozen-thawed oocytes fertilized *in vitro*. **Gamete. Res.** 16: 205-216.

Hyttel, P., Vajta, G., and Callesen, H. (2000). Vitrification of bovine oocytes with the open pulled straw method: Ultrastructural consequences. **Mol. Reprod. Dev.** 56: 80-88.

Johnson, MH., and Pickering, S.J. (1987). The effect of dimethylsulphoxide on the microtubular system of the mouse oocytes. **Development** 100: 313-324.

Karlsson, J.O., Eroglu, A., Toth, T.L., Cravalho, E.G., and Toner, M. (1996). Fertilization and development of mouse oocytes cryopreserved using theoretically optimized protocol. **Hum. Reprod.** 11: 1296-1305.

Kazem, R., Thompson, L.A., Srikantharajah, A., Laing, M.A., Hamilton, M.P., and Templeton, A. (1995). Cryopreservation of human oocytes and fertilization

by two techniques: In-vitro fertilization and intracytoplasmic sperm injection. **Hum. Reprod.** 10: 2650-2654.

Kimura, Y., and Yanagimachi, R. 1995. Intracytoplasmic sperm injection in the mouse. **Biol. Reprod.** 52: 709-720.

Kuretake, S., Kimura, Y., Hoshi, K., and Yanagimachi, R. (1996). Fertilization and development of mouse oocytes injected with isolated sperm head. **Biol. Reprod.** 55: 789-795.

Lee, J.W., Tian, X.C., and Yang, X. (2003). Failure of male pronucleus formation is the major cause of lack of fertilization and embryo development in pig oocytes subjected to intracytoplasmic sperm injection. **Biol. Reprod.** 68: 1341-1347.

Perreault, S.D., Barbee, R.R., Elstein, K.H., Zucher, R.M., and Keefer, C.L. (1988). Interspecies differences in the stability of mammalian sperm nuclei assessed *in vivo* by sperm microinjection and *in vitro* by flow cytometry. **Biol. Reprod.** 39: 157-167.

Porcu, E., Fabbri, R., and Seracchioli, R. (1997). Birth a healthy female after intracytoplasmic sperm injection of cryopreserved human oocytes. **Fertil. Steril.** 68: 724-726.

Rho, G.J., Kawarsky, S., Johnson, W.H., Kochhar, K., and Betteridge, K.J. (1998). Sperm and oocyte treatment to improve the formation of male and female pronuclei and subsequent development following intracytoplasmic sperm injection into bovine oocytes. **Biol. Reprod.** 59: 918-924.

Tesarik, J., Sousa, M., 1995. More than 90% fertilization rates after intracytoplasmic sperm injection and artificial induction of oocyte activation with calcium

ionophore. **Fertil. Steril.** 63: 343-349.

Zicarelli, L., Esposito, L., Campanile, G., Di Palo, R., and Armstrong, D.T. 1997. Effect of using vasectomized bulls in AI practice on the reproductive efficiency of Italian buffalo cows. **Anim. Reprod. Sci.** 47: 171-180.

Vajta, G., and Nagy, ZP. (2006). Are programmable freezers still needed in the embryo laboratory? Review on vitrification Reprod. Biomed. Online 12:779-796.

Van, Ty.L., Nguyen, B.X., Son, H.N., and Driancurt, M.A. (1994). Superovulation and ovarian follicular population of juvenile buffaloes and calves. **Anim. Reprod. Sci.** 35: 191-199.

Vincent, C., Pickering, S.J., and Johnson, M.H. (1990). The hardening effect of dimethylsulfoxide on the mouse zona pellucida requires the presence of an oocytes and is associated with a reduction in the number of cortical granules present. **J. Reprod. Fertil.** 89: 253-259.

CHAPTER II

REVIEW OF LITERATURES

2.1 Intracytoplasmic sperm injection (ICSI)

Of the micromanipulation technique developed in the twenty first century, which include partial zona dissection (PZD), subzonal sperm injection (SUZI) and ICSI. ICSI has been the major breakthrough in the field of assisted fertilization. The aim of this microassisted fertilization technique is to bypass the oocytes's natural barriers to fertilization.

Since the first ICSI applied in mammals was reported in hamsters (Uehara and Yanagimachi, 1976), several species such as mice (Kimura and Yanagimachi, 1995), humans (Palermo et al., 1992), sheeps (Catt et al., 1996), rabbits (Hosoi et al., 1998), horses (Cochran et al., 1998), cattle (Hamano et al., 1999), domestic cats (Gomez et al., 2000), and pigs (Martin, 2000) have been produced by the application of this technique. ICSI has helped the understanding of the early events of fertilization such as capacitation, acrosome reaction, the mechanisms of sperm oocytes interaction, sperm-induced oocytes activation, pronucleus formation and the control of the first cell cycle (Keskintepe et al., 1997). Dramatic advances in the treatment of severe male-factor infertility have been achieved by using ICSI, and at present, the technique of ICSI is being widely used in clinical applications for human infertility.

2.2 Activation of ICSI oocytes

Oocytes activation refers to the release of the meiotically arrested oocytes by the entry of sperm during fertilization. The activation of oocytes is necessary for the success of fertilization and further development from oocytes to embryos whether it is natural fertilization or artificial fertilization. It is known that for a portion of the sperm-injected human oocytes as well as all in bovine, and pig, ICSI alone is insufficient to activate oocytes. Artificial stimulations are needed.

Only ICSI alone seems to be sufficient to trigger the events of oocyte activation in some species such as hamsters, mice and humans (Perreault et al., 1988; Kimura and Yanagimachi, 1995; Tesarik and Sousa, 1995; Kuretake et al., 1996). In contrast, other species such as cattle (Rho et al., 1998) and pigs (Lee et al., 2003) required additional activation of the oocyte after sperm injection. Up until recently, no information has been done on buffalo oocyte activation following ICSI.

The events of oocytes activation include the release of cortical granules, accomplishment of meiosis, second polar body extrusion and pronucleus formation. High levels and activity of mitogen-activated protein kinases (MAPK), maturation promoting factor (MPF) and cytostatic factor (CSF) maintain the oocytes arrested at MII stage. MAPK are serine/threonine kinases that require phosphorylation to become fully activated. The MPF is composed of two subunits, cyclin B and $P34^{cdc2}$ kinase. The stabilization of MPF is sustained by high level of CSF, which is easily collapsed by repetitive elevation in intracellular calcium (Liu and Yang, 1999). Complete oocyte activation leading to the extrusion of the second polar body and the formation of pronuclear further requires the inactivation of MAPK, which happens several hours after MPF inactivation. In bovine, treatment of oocytes with a calcium ionophore alone

decrease cyclin B level within 1 h and cyclin B level rise again at 4 h to 15 h (Liu and Yang, 1999).

Sperm penetration of mammalian oocytes during fertilization initiates a series of signal transduction events that correspond to structural, morphological and biochemical changes. The first observed event at oocytes activation is the Ca^{2+} transients that occur at certain intervals, i.e. Ca^{2+} oscillations, the frequency of which varies from 5 to 30 min among species and individuals. Sperm penetration-triggered Ca^{2+} oscillations have been found in all mammalian species studied so far, including human.

The rise in Ca^{2+} concentration is followed by critical morphological changes in the oocytes. The first Ca^{2+} transient is the primary trigger that induces cortical granule (CG) exocytosis. Exocytosis of CG has been shown to cause structrural changes in the ZP (Ducibella, 1996). It is Ca^{2+} dependent and MPF and MAPK independent. Typically, membrane enclosing CGs fuses with the plasma membrane of the oocytes and releases enzymes. The released enzymes modify ZP structures and the fusion of membranes changes membrane potential and possibly membrane structures. Both of these events prevent subsequent penetration by additional sperm (polyspermy). Consequently, the oocytes undergo a series of pre-programmed processes: spindle elongation, chromosome separation, progression into anaphase and telophase II, and extrusion of a second polar body. If inadequately activated, oocytes will arrest at MII-anaphase II. If oocytes are fully activated, the chromosomes de-condense and pronuclei form, followed by DNA synthesis and the first mitosis of development (Swann and Ozil, 1994). In mammals, this can take 4-8 hours depending upon the species and the conditions of stimulation. Pronuclear formation has been commonly

used to assess the completeness of oocytes activation (Presicce and Yang, 1994; Soloy et al., 1997; Wang et al., 1998).

At the molecular level, the rise in Ca^{2+} is believed to be a trigger for MPF inactivation and probably indirect inactivation of MAPK as well. Following PA or fertilization in mammalian MII oocytes, MPF is inactivated through degradation of cyclin B and the low level of MPF is sustained by continuous degradation of newly synthesized cyclin B, and subsequently by phosphorylation of $P34^{cdc2}$. These molecular events are followed by MAPK inactivation through dephosphorylation of ERK2. Interestingly, evident differences in the kinetics of MPF and MAPK activities have been observed in bovine, ovine, and pocine oocytes following PA (Liu et al., 1998; Bogliolo et al., 2000). Oocytes undergoing "full" activation, defined by pronuclear formation, MAPK is inactivated. A decrease in MPF activity correlated with MII exit and a decrease in MAPK activity correlated with pronuclear formation. These correlations have been shown to be true regardless of the speed of nuclear progression. Inactivation of MAPK was independent of MPF inactivation and its low activity persisted throughout the pronuclear formation stage. In general, low MPF but high MAPK activity is indicative of partial oocyte activation, resulting in arrest at MII. However, the only reliable assessment for genuine full activation of oocytes is probably full term development, an impossible at present upon PA of oocytes.

2.2.1 Methods that induce Ca^{2+} oscillations

Many different agents or methods that elicit a rise in intracellular Ca^{2+} can induce some degree of PA and development.

Ethanol (EtOH) was first used for PA in mouse oocytes (Cuthbertson et al., 1981). Subsequently, treatments with 6-10% EtOH for 5-10 min have been extensively used

for activating oocytes of large domestic animals as well. Effective activation by EtOH treatment is oocytes age dependent. Newly arrested oocytes do not respond well to EtOH. The treatment induces the extrusion of the second polar body, but the oocytes do not enter interphase. Instead, it arrests again at metaphase and this is termed aborted activation (Kubiak, 1989). EtOH disrupts the organization of cytoskeletal elements and the resulting aborted activation may result in significant increases in the incidence of aneuploidy (Kubiak, 1989; O'Neill et al., 1989). The lack of a good activation response by EtOH treatment might be because it causes a single large rise in the intracellular Ca^{2+}, which appear to derive from both intracellular Ca^{2+} release and extracellular Ca^{2+} influx (Cuthbertson et al., 1981). EtOH has been used to activate buffalo oocytes (Parnpai and Tasripoo, 2003) by promoting the formation of inositol 1,4,5-triphosphate (IP3) at plasma membrane and inducing the extracellular calcium influx which would form a large intracellular calcium concentration (Ilyin and Parker, 1992).

Ca^{2+} ionophore: Similarly, Ca^{2+} ionophore A23187 or ionomycin (Io) also induces a single Ca^{2+} rise in mammalian oocytes. The single Ca^{2+} rise by ionophore A23187 is mostly derived from that released by internal stores (Vincent et al., 1992). In buffalo nuclear transfer, Io was used to induce repeated transient intracellular calcium rising (Saikhun et al., 2004).

Electrical stimulation: An appropriate electrical direct current (DC) pulse in the presence of Ca^{2+}-containing medium brings a transient Ca^{2+} increase in the cytosol and initiates oocytes activation in all species examined so far. Electrical pulse causes the formation of transient pores in the oocytes plasma membrane, and Ca^{2+} in the external medium enter the oocytes through these transient pores. A single electrical

pulse causes a single large Ca^{2+} transient (Sun et al., 1992; Collas et al., 1993).

2.2.2 Combination of chemical activation-targeting on MPF and MAPK

Although multiple Ca^{2+} transient can improve parthenogenetic development, the procedures are complicated and labor consuming. Multiple Ca^{2+} oscillations is believed to maintain low activity of MPF by degradation of cyclin B and/or phosphorylation of $P34^{cdc2}$ and inactivation of MAPK by ERKS dephosphorylation (Figure 2.1). Full activation of oocytes that leads to pronuclear formation requires Ca^{2+} oscillations and inactivation of MPF and MAPK activity. Thus, combination of a calcium rise and an inhibition of either protein synthesis with cycloheximide (CHX) or protein phosphorylation with 6-dimethyl amino purine (6-DMAP) can be an approach for fully activation of oocytes. The chemical 6-DMAP was shown to enhance the activation of young mouse and bovine oocytes (Susko-Parrish et al., 1994; Szollosi et al., 1993). The first Ca^{2+} increase is indispensable for full activation of oocytes, because inhibiting either protein synthesis with CHX or kinase activation with 6-DMAP alone fails to promote subsequent parthenogenetic development (Liu et al., 1998). CHX treatment does not change the intracellular Ca^{2+} profile (Bos-Mikich et al., 1995), but can cause instant decline in cyclin B levels which maintain low activity of MPF. Moreover, dephosphorylation of MAPK occurs several hours later when most oocytes reach pronuclear stage after CHX treatment (Liu and Yang, 1999). Different from CHX or IVF induced activation, 6-DMAP induces dephosphorylation of MAPK, and therefore earlier pronuclear development. Agents that increase intracellular Ca^{2+} or prevent protein synthesis/phosphorylation alone result in low cleavage and development rates.

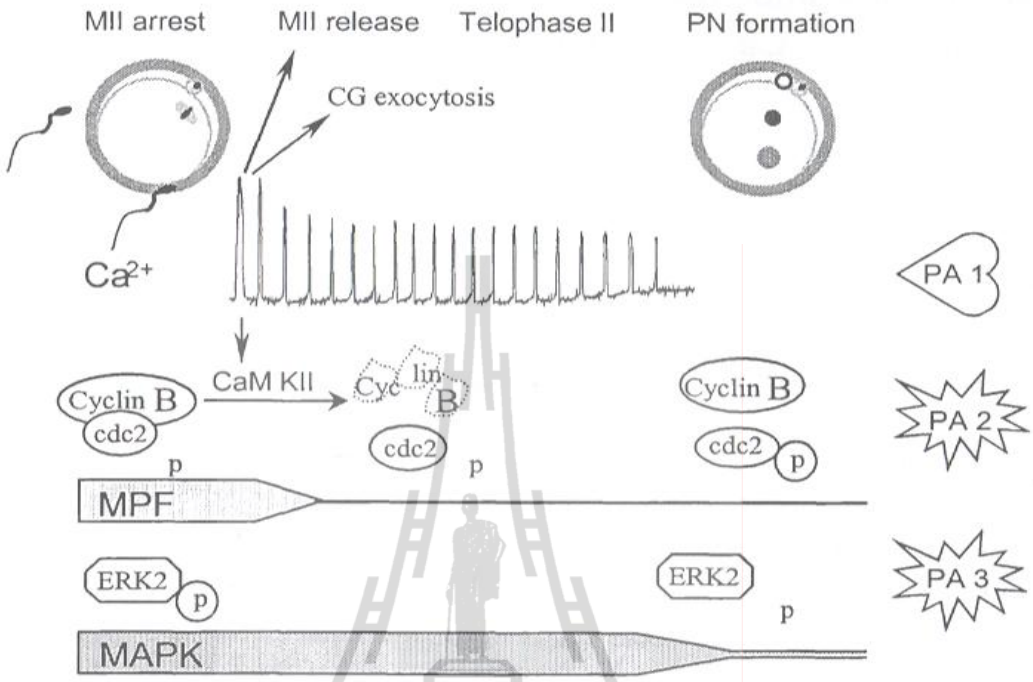

Figure 2.1 Activation of mammalian oocytes during fertilization and proposed methods for PA. Sperm penetration triggers a Ca^{2+} transient, followed by Ca^{2+} oscillations. The first Ca^{2+} wave is critical for both CG exocytosis and activation of CaMKII, which decomposes cyclin B through "cyclin degradation machinery" and resulting in inactivation of MPF. Decline in MPF activity is associated with oocytes meiotic release. Low MPF activity can be maintained by the lack of cyclin B or phosphorylation of cdc2. The subsequent Ca^{2+} oscillation are indispensable for continuous degradation of cyclin B and maintenance of the low MPF, which is associated with pronuclear (PN) formation. Cyclin B degradation is responsible for the instant inactivation of MPF activity. Low MPF activity can be maintained by either inhinition of continuous synthesis of cyclin B (CHX) or by dephosphorylation of ERK2 (6-DMAP). The balance of phosphate seems to play an important role in keeping MPF activity low and MAPK

inactivated.

Goto et al. (1990) reported the production of the first calf by ICSI using immobilized, killed cryopreserved bovine epididymal sperm. Oocytes were chemically activated post-injection with 50 μM Ca^{2+} ionophore (A23187). This was the first report to evaluate embryonic development of ICSI embryo up to the blastocyst stage, which only 1.8 % injected oocytes reached the blastocyst stage. The high inefficiency of the ICSI procedure in cattle led researchers to evaluate different chemical activation treatments and sperm pretreatments in order to increase pronuclear formation and blastocyst rates.

Hamano et al. (1999) reported the birth of 8 male and 2 female calves using flow cytometrically sex sorted sperm heads. Oocytes were activated for 5 minutes in 7% EtOH once before and twice after sperm injection (at 30 and 60 minutes). Cleavage and blastocyst rates were 46.6% and 6.9%, respectively. They obtained a 20.8% pregnancy rate (10/48) from the blastocysts nonsurgically transferred into recipient females.

Sperm pretreatments were evaluated by Wei and Fukui (1999) in pursue for an answer the low fertilization rates of ICSI oocytes in cattle. They investigated the effect of sperm type (dead, immotile or motile), sperm mechanical pretreatment (tail cutting or scoring) and sperm chemical pretreatment (heparin, heparin and caffeine, calcium ionophore (A23198) or dithiothreitol (DTT) on fertilization rates 20 hours after sperm injection. No significant difference was found when dead (21.8%), immotile (20.8%) or motile sperm (11.3%) without mechanical pretreatment were injected into oocytes. However, the rate of pronuclear formation with motile sperm tended to be lower than those with dead or immotile sperm. When motile sperm was

used, tail scoring (36.4%) yielded higher pronuclear formation than the control at 11.3%. However, no significant difference was found between tail scoring (36.4%) and tail cutting (22.8%). All chemical pretreatment agents used significantly increased the formation of the male pronucleus 20 hours post-injection. Pretreatment with calcium ionophore (A23187) (58.9%) or DTT (61.3%) resulted in higher fertilization rates than those pretreated with heparin alone (32.8%) or the control (23.2%). Fertilization rates were also higher with heparin and caffeine treatment (46.4%) than in the control.

The development of the piezo ICSI technique in the mouse by Kimura and Yanagimachi (1995) led to a dramatic increase in fertilization and blastocyst rates when compared with those of the conventional technique. With this novel technique, the ZP and oolema are penetrated by rapid vibrations produced at the pipette tip caused by longitudinal wavelength produced by a piezo actuator. One of the main advantage of the piezo technique is that it causes less deformation to the oocyte during injection. The oolema is easily broken without suction of the ooplasm and produces less ooplasm leakage increasing oocyte survival post sperm injection.

Katayose et al. (1999) were the first to apply this technique in cattle and reported a dramatic increase in fertilization rates with piezo ICSI (55%) when compared with the conventional technique (0.05%) when no exogenous chemical activation was used. The addition of 50 μM calcium ionophore (A23187) to activate the oocytes increased fertilization rates for the conventional technique to 15%, while no beneficial effect was found in the piezo ICSI group (58%).

In contrast, a year later Suttner et al. (2000) reported in cattle that no significant difference between the piezo and conventional ICSI techniques as assessed

by pronuclear formation, cleavage and blastocyst rates. They evaluated different chemical agents to activate sperm injected oocytes. Io followed by 6-DMAP produced the highest quantity of blastocysts when compared with calcium ionophore (A23187) and Io followed by CHX. This is in agreement with others where the highest quantities of blastocysts were produced by using Io followed by DMAP (Ock et al., 2003). However, the use of Io followed by DMAP was found to produce up to 67% chromosomal abnormalities as assessed by polyploidy and mixploidy (Ock et al., 2003). This high percentage of abnormal embryos was slightly reduced by allowing a 3 hours delay in DMAP treatment (46% mixoploid and polyploid). The occurrence of chromosomal abnormalities may be due to inhibition of extrusion of the second polar body, with some nuclei presumably reentering S-phase of the cell cycle without having passed through metaphase.

Horiuchi et al. (2002) produced 5 healthy calves from the transfer of 10 blastocysts activated with 7% EtOH 4 hours post-injection. They reported that ~60% of the sperm-injected oocytes were activated by the spermatozoon as assessed by second polar body extrusion 4 hours post-piezo ICSI. It was concluded that treating activated oocytes 4 hours post-injection with 7% EtOH for 5 minutes, increased subsequent embryo development. By treating activated oocytes with or without EtOH, they obtained 20% and 11.9% blastocyst rates, respectively. Moreover, they demonstrated that using killed sperm readily affects embryo development to the blastocyst stage when compared with motile sperm immobilized prior to injection. Only 0.8% of activated oocytes reached the blastocyst stage when killed sperm were used.

Fujinami et al. (2004) studied the effects of artificial activation with EtOH

on kinetics of MPF activity and development of bovine oocytes following ICSI. They reported that MPF activity decreases immediately after sperm injection but elevates temporarily at 6 hours post-injection. This temporary elevation of MPF was inhibited when sperm-injected oocytes were treated with EtOH 4 hours post-injection. They obtained 14 and 4% blastocyst rates when sperm-injected oocytes were treated with or without EtOH 4 hours after sperm injection, respectively.

Wei and Fukui (2002), using the piezo ICSI technique, were the first to report the birth of 3 calves from ICSI derived embryos without any exogenous oocyte activation. They felt that the poor outcome in bovine ICSI could be improved by making technical improvements. The oocyte ooplasm was made clearer by centrifugation, the sperm tail was cut leaving the midpiece intact and the amount of polyvinylpyrrolidone (PVP) was reduced from 10% to 4% to ensure proper delivery of sperm into the oocytes. With these adjustments, they reported 78.2, 71.8 and 22.7% fertilization, cleavage and blastocyst rates, respectively.

Subsequently, Galli et al. (2003) also concluded that exogenous oocyte activation did not improve the development of piezo ICSI embryos. They obtained 15, 6 and 17% blastocyst rates when sperm-injected oocytes were not treated or were treated with Io or Io followed by CHX, respectively. One healthy calf was produced by the nonsurgical transfer of 11 nontreated piezo ICSI blastocysts into 6 recipient females.

Rho et al. (2004) reported the use of frozen-thawed bovine oocytes for ICSI. Oocytes were cryopreserved by vitrification with copper electron microscope grid. They reported that blastocyst rates of 9.8% and 16.3% were significantly different when using frozen-thawed and fresh oocytes, respectively. They detected a

significant difference ($P<0.05$) between the treatment groups. Total cell counts on day 8 post-injection were 99±19 and 124±21 for frozen-thawed and fresh oocytes, respectively. It was concluded that frozen-thawed bovine oocytes were suitable for ICSI to produce transferable embryos.

In cattle, Keskintepe et al. (2002) reported cleavage and blastocyst rates for oocytes injected with lyophilized sperm and activated with Io followed by DMAP of 63.3% and 29.6%, respectively. No significant difference was detected with lyophilized sperm when compared with frozen-thawed sperm (72.4% and 34.2% cleavage and blastocyst rates, respectively).

The highest pregnancy and birth rates to date were reported for piezo ICSI bovine embryos treated with 7% EtOH 4 hours after sperm injection (Oikawa et al., 2005). This study compared activation protocols for blastocyst development. They reported 8.0, 29.4 and 40.1% blastocyst rates for sperm-injected oocytes nontreated, treated with 7% EtOH 4 hours post-injection or with Io followed by DMAP, respectively. Although the percentage of blastocysts was higher for Io followed by DMAP group. The subsequent pregnancy and birth rates were significantly higher for the EtOH-treated group. One (12.5%) of the 11 recipients that received blastocysts produced following the Io-DMAP activation became pregnant. However, blastocysts from the EtOH-treated group resulted in 10 of 17 (58.8%) pregnant recipient females. The birth rate per transferred embryo was 47.4% and 9.2% for the EtOH and Io -DMAP-treated piezo ICSI oocytes, respectively.

Additionally, Horiuchi (2006) reported that 24 calves were produced by the nonsurgical transfer of 61 ICSI-derived blastocysts activated with EtOH 4 hours after sperm injection. With the exception of the birth of a few calves from

sperm-injected oocytes without the need of an exogenous activation stimulus (Wei and Fukui, 2002; Galli et al., 2003), ICSI techniques have failed to produce expected rates of embryonic and fetal development (Malcuit et al., 2006).

From another perspective, activation treatment following ICSI increases the possibility of parthenogenesis development. Activation procedures aimed at making buffalo oocytes suitable for ICSI differ from those aimed at producing parthenogenesis; haploidy is required of the former, diploid of the latter. But the CHX or 6-DMAP immediately followed the calcium rise agents after ICSI would inhibit the extrusion of the second polar body and induce triploidy or haploidy embryos. Therefore, the selection of buffalo eggs by the presence of the second PB, then the additional activation treatment of CHX or 6-DMAP may decrease the parthenogenesis.

2.3 Oocytes cryopreservation technology

Since the first successful mouse 8-cell stage embryo cryopreservation was reported (Whittingham et al., 1972), hundreds of other studies designed to examine factors associated with cryosurvival of mammalian embryos and oocytes have followed for its efficient and reproducible. Then, Wilmut and Rowson (1973) were the first to show cattle embryos would survive after freezing. Following early experiments, the procedures were used in several mammalian species and several different protocols which were applied around the world.

Cryopreservation is an applied aspect of cryobiology which is the study of the effects of extremely low temperatures on biological systems. Oocyte cryopreservation not only can be sufficiently utilized the oocytes resource and safeguard the endangered species, but also is a viable solution for the ethical problems related to

embryo storage and can provide the oocytes for scientific research.

The main problems with oocyte cryopreservation are concerned with the survival rate and the fertilization rate. Most oocytes have been cryopreserved by either one of two procedures: (1) equilibrium freezing, often referred to as “slow freezing” and (2) non-equilibrium freezing, often referred to as “ultra-rapid cooling” or vitrification.

2.4 Conventional slow freezing method

Starting with the initial reports in the 1970s, cryobiological studies conducted over the past 40 years have led to the following understanding of the general principles that determine the survival of oocytes and embryos. To survive cryopreservation, oocytes and embryos are first suspended in a >10% solution of a cryoprotectants agents (CPA). Next, they are cooled at a controlled rate to subzero temperatures below -30 ºC and plunged into liquid nitrogen (LN_2) at -196 ºC for long-term storage. To restore their function, the cryopreserved oocytes then are warmed to physiological temperatures at a rate compatible with the rate at which they were initially cooled, and finally the CPA is removed.

Let us consider the cryobiology of oocytes and oocytes from the perspective of Mazur’s mathematical analysis (Mazur, 1963). When oocytes are to be frozen, they are first suspended in and allowed to equilibrate with a solution of CPA. These CPA are low molecular weight, non-electrolytes that are completely miscible with water. The most common CPA that have been used to successfully cryopreserve oocytes are dimethyl sulfoxide (DMSO) (MW = 78.2), glycerol (MW = 92.1), ethylene glycol (EG) (MW = 62.1), propylene glycol (MW = 76.1), and methanol (MW = 32.0).

Given differences in the molecular dimensions of these compounds as well as the properties of cell membranes, these CPA permeate cells at different rates, differences that are expressed in the form of permeability coefficients. These permeating compounds apparently protect cell structure by preventing intracellular ice crystal formation during freezing and thawing (Bowler and Fuller, 1987; Schneider, 1986). Moreover, different stages of oocytes development, from oocyte to zygote to the various cleavage stages, vary in their permeability to the same CPA. When oocytes solutions are cooled to subzero temperatures, regardless of whether cells are present or not, ice forms (either spontaneously or when seeded artificially) so that liquid water is effectively removed from the solution. A graphical representation of the changes that occur in solutions when they are frozen is often given on the form of a phase diagram.

The mechanism of slow freezing methods is to induce extracellular ice formation and prevent intracellular ice formation. Permeable CPA can go into the oocytes and dehydrate the oocytes. Slow freezing control the cooling speed at 0.3~0.5 ºC/min, so intracellular water can have enough time out of the oocytes. plunging at -30 ºC to -70 ºC by using cooling rate 0.3 ºC/min and storage in liquid nitrogen (-196 ºC). In order to survive cryopreservation, cells must remain undamaged and physiologically functional during the entire procedure. Therefore, appropriate supplement of each step is absolutely critical.

Slow freezing procedures can be highly damaging to oocytes of chill-sensitive species. They involve prolonged exposure to the temperature at most damage occurs. This may explain the protocols which minimize exposure to the temperature at most damage occurs, e.g., by using high equilibration temperature combined with rapid or

very rapid cooling rates can get good results for oocytes of chill sensitive species (Martino et al, 1996; Isachenko, 1997; Le Gall and Massip, 1999; Vajta et al., 1998).

This technique has been widely used in several animal species such as cattle (Booth et al., 1999), mice (Whittingham, 1977; Carroll et al., 1993), rabbits (Bank and Maurer, 1974), humans (Fugger et al., 1988), sheep (Willadsen et al., 1976), goats (Bilton and Moore, 1976), domestic cats (Dresser et al., 1988), horses (Yamamoto et al., 1982), swamp buffalos (Kasiraj et al., 1993) and hamsters (Lane et al., 1999).

2.5 Ultra-rapid freezing or vitrification method

Vitrification is a process that produces a glasslike solidification of living cells that completely avoids ice crystal formation during cooling. Equally important, the vitrification process completely avoids ice crystal formation in cryopreserved cells during warming to recover the cells for biological applications.

The vitrification methods use high concentrated CPA to balance the oocytes intracellular and extracellular concentration. It must control the balance time and temperature for the high CPA concentrations are toxic to cells. It control the volume of solution (carrier) and rapid cooling rate at 2000 ºC/min ~ 20000 ºC/min.

Vitrification may be regarded as a radical approach, as one of the main sources of injuries, ice crystal formation, is entirely eliminated. However, a negative consequence of this strategy is the increased probability of nearly all forms of injury except for those caused by ice crystal formation. To achieve vitrification of solutions, a radical increase of both the cooling rates and the concentration of CPA are required. With the extreme increase of cooling rate, vitrification can also be achieved in pure water, but the usual limits in embryology are far below this rate (Rall, 1987). The

balance required in vitrification is between (1) establishment of a safe system for maximal and reliable cooling (and warming) rates while avoiding consequent damage including fracture of the ZP or the cells, and (2) elimination or minimization of the toxic and osmotic effects of high CPA concentrations needed to obtain and maintain the glasslike solidification. Cell shrinkage caused by non-permeable cryoprotectants and the incomplete penetration of permeable components may cause a relative increase of intracellular.

2.5.1 Vitrification container systems

In vitrification techniques, the smallest possible volumes of vitrification solutions are used in specialized container or support device. To improve chances that the sample is surrounded with liquid and not vapor. The sample size should be minimized and the duration of any vapor coat is reduced, including increased of the cooling rate. Furthermore, to facilitate vitrification by even higher cooling rates, it is also necessary to minimize the volume of the vitrification solution. To minimize the volume of the vitrification solution, special carriers are used during the vitrification process.

Early vitrification experiments have been performed in the traditional vessels of cryopreservation, i.e. 0.25 mL plastic insemination straws or cryovials. These tools were not designed for the special purpose, had a thick wall and required a relatively large amount of solution for safe loading. Accordingly, the theoretically achievable cooling and warming rates were quite limited. The vitrification container systems are as follows.

Cryotop is the minimum volume procedures where the carrier tool, a thin film attached to a plastic holder is equipped with a protective plastic tube (Kuwayama

et al., 2005) (Figure 2.2). Oocytes are loaded on the film, the solution is almost entirely removed by aspiration (Figure 2.3), and the sample is immersed into LN_2. Subsequently, for safe storage, the plastic tube is pulled over the film. The method is easy to learn and perform, and the simple manipulation decreases the risk of inconsistency.

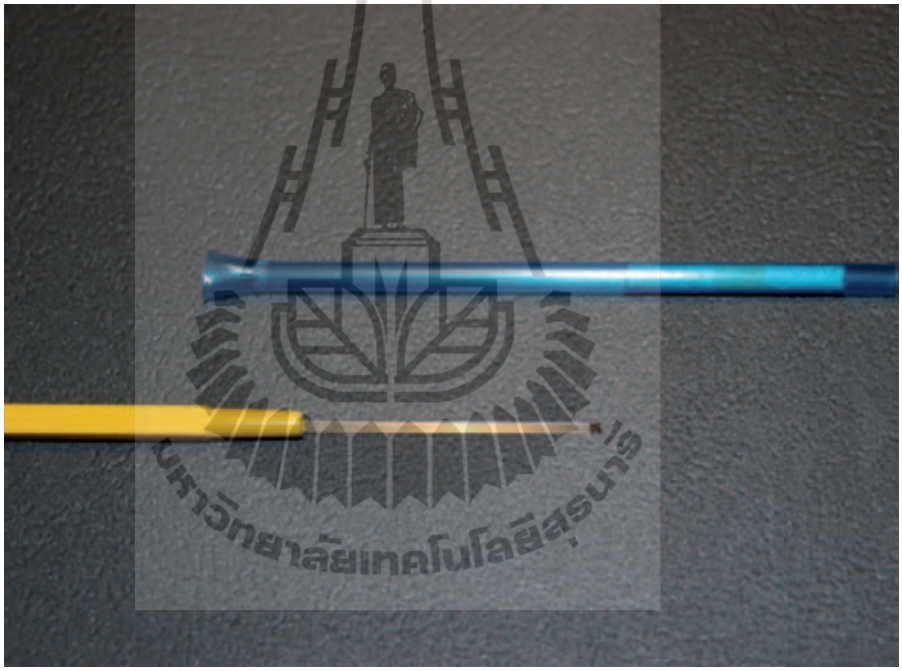

Figure 2.2 The Cryotop vitrification container. A narrow filmstrip is attached to a handle. After vitrification, a plastic tube is used to protect the filmstrip from mechanical damage during storage.

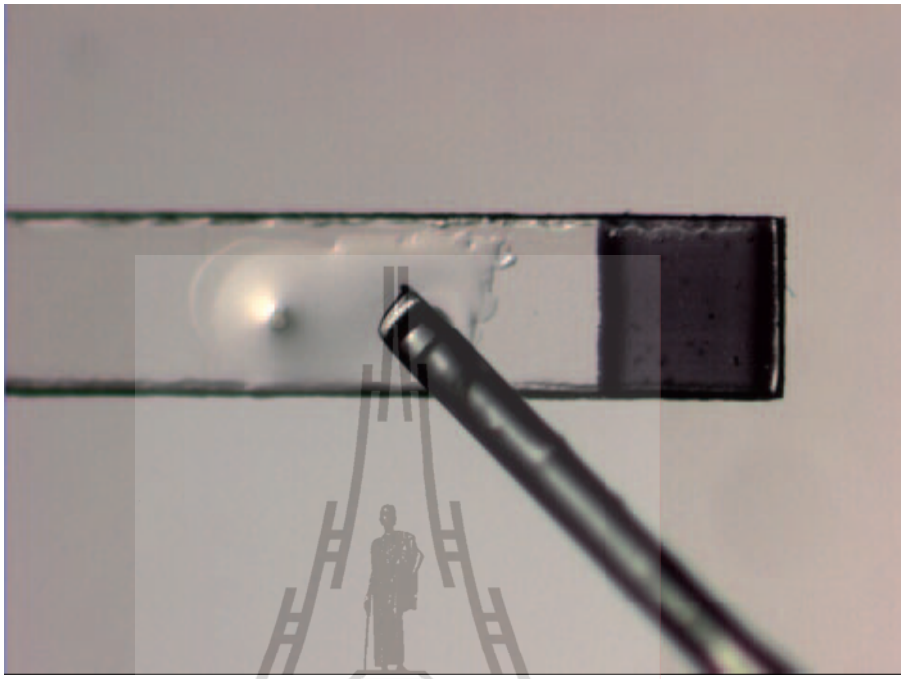

Figure 2.3 Loading of the Cryotop. The oocyte or embryo is loaded in a small drop onto the filmstrip, then the excess solution is removed with a capillary, leaving only a thin layer of cryoprotectant to cover the sample.

Cryoloop is composed with nylon loop and stainless steel stick, and the lid and cryovial would cover the cryoloop (Figure 2.4). A small nylon loop attached to a holder and equipped with a container. It has been used for cryopreservation in crystallography and is now widely used for oocyte and embryo cryopreservation (Lane et al., 1999). The solution film bridging the hole of the loop is strong enough to hold the oocyte or the embryo, and with this minimal solution volume, the achievable cooling rate may be extremely high. Using this tool, safe cryopreservation can be achieved even in the vapor of LN_2 (Larman et al., 2006).

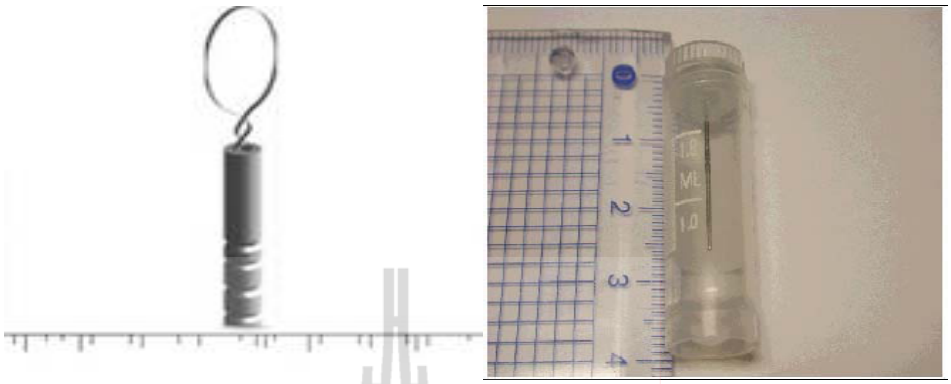

Figure 2.4 The Cryoloop vitrification container.

Open pull straw (OPS) was reported as a novel way of vitrification for bovine embryos as well as oocytes (Vajta et al., 1998). OPS is based on a very simple idea, and a very simple technique. Its unexpected additional benefits making the method safe and easy to use have only been discovered subsequently. The idea was to minimize the required volume of the sample by minimizing the diameter of the common insemination straw. Similar to glass capillary production, straws were warmed and pulled by hand, then cut at the thinnest point with a razor blade. As the result, the diameter and the wall thickness of the straw decreased to approximately half of the original. While the required amount of solution to form a safe column decreased from 5 μL to less than 1 μL, leading eventually to a 10-fold increase in the achievable cooling rate, thus allowing a 30% decrease in CPA concentration required for safe vitrification. The only problem, the floating of straws, can be easily resolved by inserting a standard plastic plug into the wide end. Moreover, the transparent walls allow microscopic follow-up during loading and expelling, and the glass-like solidification can be detected by naked eye. In animal embryology, the possibility of semi-direct transfer after in-straw dilution means a considerable benefit (Figure 2.5,

2.6).

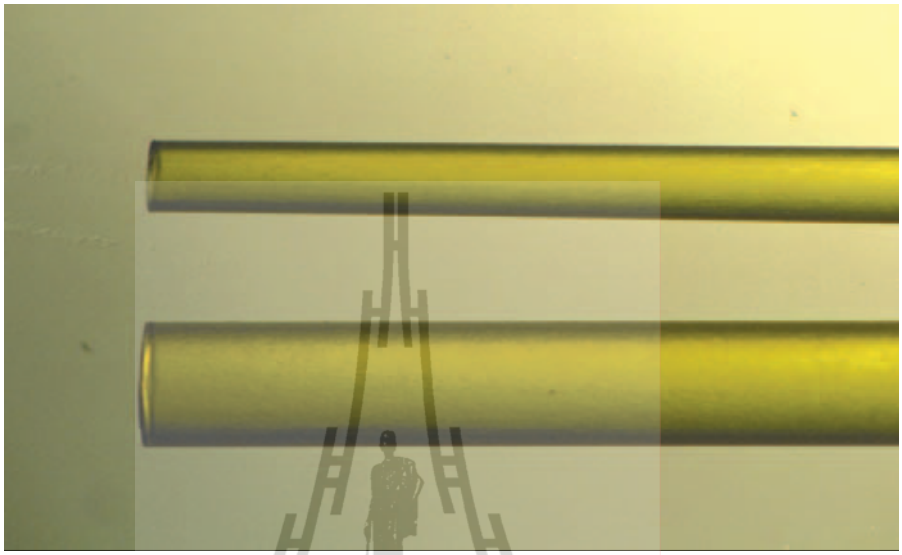

Figure 2.5 OPS with approximately 50% diameter of a standard 0.25 mL insemination straw.

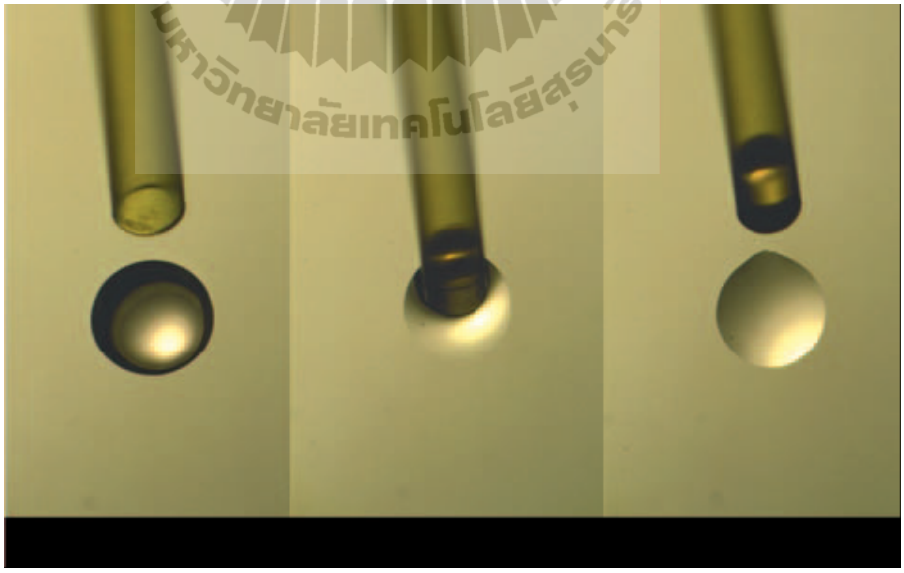

Figure 2.6 Loading of the OPS. Oocytes are placed into an approximate 1 µL droplet of cryoprotectant medium; the drop is touched with the OPS straw; as the result of the capillary effect, the medium with the oocytes enters the straw.

Glass micropipette (GMP) is nearly the same as OPS (Kong et al., 2000). The difference between OPS and GMP is GMP use glass capillary so it easy to broken but heavy than OPS. It carry smaller volume than OPS and the cooling speed is faster than OPS.

Microdrop is the earliest documented attempt used the simplest way, and dropped the sample without any container directly into the LN_2 (Papis et al., 2000). However, it was soon revealed that this simple solution is probably not the optimal one. To form a drop requires a relatively large amount of solution (approximately 2 μL). Moreover, once the drop reaches the LN_2, it will not sink immediately but remains floating and starts bizarre cruises on the surface for several seconds. The explanation of this phenomenon is quite simple: the LN_2 in the containers is usually just at its boiling point, at −196 ºC. Anything that is warmer and contacts it induces an extensive boiling and induces strong evaporation at its surface. The vapor functions as an insulating layer decreasing the cooling of the sample. Additionally, this vapor coat did not allow the sample to sink, decreasing the cooling rate even further.

Electron microscope grids (EMG) can also be used very successfully as oocyte containers during cooling and warming. With this method, oocytes were spread on an electron microscopy grid and then were preserved in LN_2 by rapid freezing (Figure 2.7). In 1996, Martino et al. use this method to preserve cattle oocytes, 60% oocytes morphology normal after warming, after IVF cleavage rate reach 29%~32% and blastocyst rate 10%~15%.

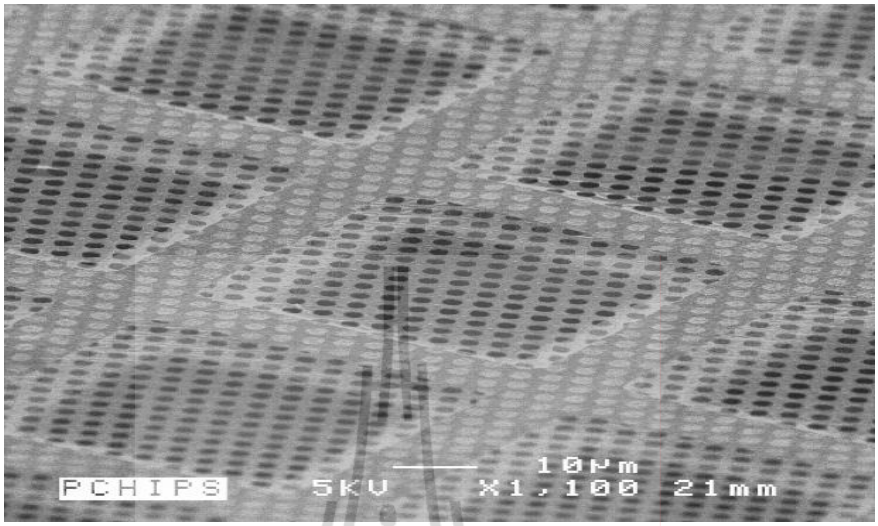

Figure 2.7 The EMG vitrification container.

It is important to realize that the final goal of both slow cooling and vitrification is the same: to induce a glasslike solidification inside cells to protect them from damage by ice crystals at all stages of cryopreservation.

2.5.2 Vitrification of different stage oocytes

At present, oocyte cryopreservation has been performed at two meiotic stages: metaphase II (MII) and germinal vesicle (GV). The cell cycle stage during meiosis appears to influence the survival of mammalian oocytes and affects the results of cryopreservation due to varying sensitivity to cooling procedures. MII oocytes remain the preferred stage for cryostorage owing to better membrane stability during being freezing, however, exposure to sub-physiological temperatures induces several form of damage to other structures determining disorganization of the spindles (Mandelbaum et al., 2004) which, in turn, provokes chromosomal aberrations (Sathananthan et al., 1998), increasing polyploidy, and fertilization impairment (Wood et al., 1992).

Conversely, GV stage oocytes would not be directly affected by the

problem posed by the meiotic spindle at the MII stage, as the genetic material remains confined within the nucleus. However, the number of experiments using immature oocytes is low, as data obtained to date, indicate that immature oocytes are more susceptible to cryoinjury and that the survival and developmental ability are greatly impaired in comparison to fresh oocytes. Structural modifications of the cytoskeleton, mitochondria, cortical granules and nucleoli have been described.

Different lines of evidence clearly indicate that the gap functional coupling between oocytes and cumulus cells fulfils an important role in the maturation process. It has been demonstrated that GV stage oocytes which have been stripped of cumulus cells show a lack of coordination between nuclear and cytoplasm maturation and undergo a lower development compared to cumulus-enclosed oocytes. The factors contributing to these deficiencies have not yet been fully elucidated. It has been reported that the absence of cumulus cells could provoke a possible shortcoming in protein synthesis and could reflect the levels of molecules involved in the regulation of meiotic and mitotic cell cycles. According to these indications, the success of immature oocyte cryopreservation could depend on the ability to preserve the structural and functional integrity cumulus–oocytes complexes as a whole. However, the need to maintain cumulus cells during cryopreservation of immature oocytes is still a matter for debate question, and may well be subject to species-specific variation .

Oocytes at MII stage are not only difficult to equilibrate with CPA, but they are also quite sensitive to physical/chemical insults because of their vulnerable spindle apparatus (Zenzes et al., 2001; Stachecki et al., 2004). Such damage can be caused to oocytes that are only exposed to vitrification solution before cooling (Fuku

et al., 1995).

Metaphase oocytes are collected as part of an existing practice in assisted reproduction. At this stage in development, oocytes have undergone nuclear and have begun cytoplasmic maturation, the first polar body has been extruded, and the chromosome chromatins are condensed and arranged on the MII spindle. A spindle apparatus is a dynamic conglomerate of microtubules, comprised of α- and β-tubulin and associated structural proteins, acting to coordinate cyto- and karyokinetic events essential for normal chromosome segregation. Microtubules persist in phases of elongation or shortening, where subunits are added or subtracted from tubule ends. As oocytes transition into metaphase, microtubules change from radial arrays to an organized barrel-shaped bipolar structure containing a blend of dense material at either pole known as microtubule organizing centers (MTOCs) (Breed et al., 1994). The main structural proteins comprising oocyte MTOCs include γ-tubulin and pericentrin (Combelles and Albertini, 2001). Numerous reports have demonstrated that exposure of oocytes to cooling (Aman and Parks, 1994), cryoprotectants (Vincent et al., 1989; Aigner et al., 1992), or the cryopreservation process (Aigner et al., 1992) can cause depolymerization and disorganization of spindle microtubules. This event could lead to chromosomal scattering and development of aneuploidy.

2.6 References

Aman, R., and Parks, J. (1994). Effects of cooling and rewarming on the meiotic spindle and chromosomes of *in vitro* matured bovine oocytes. **Biol. Reprod.** 50: 103-110.

Aigner, S., Van der Elst, J., Siebzehnrubl, E., Wildt, L., Lang, N., and Van

Steirteghem, A. (1992). The influence of slow and ultra-rapid freezing on the organization of the meiotic spindle of the mouse oocyte. **Hum. Reprod.** 7: 857-864.

Bank, H. and Maurer, R.R. (1974). Survival of frozen rabbit embryos. **Exp. Cell Res.** 89: 188-196.

Bilton, R.J., and Moore, N.W. (1976). *In vitro* culture, storage and transfer of goat embryos. **Aust. J. Biol. Sci.** 29: 125-129.

Bogliolo, L., Ledda, S., Leoni, G., Naitana, S., and Moor, R.M. (2000). Activity of maturation promoting factor and mitogen-activated protein kinase after parthenogenetic activation of ovine oocytes. **Cloning** 2: 185-196.

Booth, P.J., Vajta, G., HØj, A., Holm, P., Facobsen, H., Greve, T., and Callesen, H. (1999). Full-term development of nuclear transfer calves produced from open-pulled straw (OPS) vitrified cytoplasts: work in progress **Theriogenology** 51: 999-1006.

Bos-Mikich, A., Swann, K., and Whittingham, D.G. (1995). Calcium oscillations and protein synthesis inhibition synergistically activate mouse oocytes. **Mol. Reprod. Dev.** 41: 84-90.

Bowler, K. and Fuller, B.J. (1987). Temperature and animals cells. (Eds) Cambridge, UK: **Soc. Exp. Biol. Symp**. 41: 231-241.

Breed,W., Simerly, C., Navara, C., VanderBerg, J., and Schatten, G. (1994). Microtubule configurations in oocytes, zygotes, and early embryos of a marsupial, Mondelphis domestica. **Dev. Biol.** 164: 230-240.

Carroll, J., Wood, M.J., and Whittingham, D.G. (1993). Normal fertilization and development of frozen thawed mouse oocytes: Protective action of certain

macromolecules. **Biol. Reprod.** 48: 606-612.

Catt, S.L., Catt, J.W., Gomez, M.C., Maxwell, W.M.C., and Evans, G. (1996). The birth of a male lamb derived form an *in vitro* matured oocyte fertilized by intracytoplasmic injection of a single presumptive male sperm. **Vet. Rec.** 139: 494-549.

Cochran, R., Meintjes, M., Reggio, B.C., Hylan, D., Carter, J., Pinto, C., Paccamonti, D., and Godke, R.A. 1998. Live foals produced from sperm-injected oocytes derived from pregnant mares. **J. Vet. Sci.** 18: 736-740.

Collas, P., Fissore, R., Robl, J.M., Sullivan, E.J., and Barnes, F.L. (1993). Electrically induced calcium elevation, activation, and parthenogenetic development of bovine oocytes. **Mol. Reprod. Dev.** 34: 212-223.

Combelles, C., and Albertini, D. (2001). Microtubule patterning during meiotic maturation in mouse oocytes is determined by cell cycle specific sorting and redistribution of g-tubulin. **Dev. Biol.** 239: 281–294.

Cuthbertson, K.S., Whittingham, D.G., and Cobbold, P.H. (1981). Free Ca^{2+} increase in exponential phases during mouse oocytes activation. **Nature** 294: 754-757.

Dresser, B.L., Gelwicks, E.J., Wachs, K.B, and Keller, G.L. (1988). First successful transfer of cryopreserved feline (Felis catus) embryos resulting in live offspring. **J. Exp. Zool.** 246: 180-186.

Ducibella, T. (1996). The cortical reaction and development of activation competence in mammalian oocytes. **Hum. Reprod.Update.** 2: 29-42.

Fugger, E.P., Bustillo, M., Katz, P., Dorfmann, A.D., Bender, S.A., and Schulman, J.D. (1988). Embryonic development and pregnancy from fresh and

cryopreserved sibling pronucleate human zygotes. **Fertil. Steril.** 50: 273-278.

Fuku, E., Xia, L., and Downey, B.R. (1995). Ultrastructural changes in bovine oocytes cryopreserved by vitrifi cation. **Cryobiology** 32: 139-156.

Fujinami, N., Hosoi, Y., Kato, H., Matsumoto, K., Saeki, K., and Iritani, A. (2004). Activation with ethanol improves embryo development of ICSI-derived oocytes by regulation of kinetics of MPF activity. **J. Reprod. Develop.** 50: 171-178.

Galli, C., Vassiliev, I., Lagutina, I., Galli, A., and Lazzari, G. (2003). Bovine embryo development following ICSI: Effect of activation, sperm capacitation and pretreatment with dithiothreitol. **Theriogenology** 60: 1467-1480.

Gomez, M.C., Pope, C.E., Harris, R., Davis, A., Mikota, S., and Dresser, B.L. (2000). Births of kittens produced by intracytoplasmic sperm injection of domestic cat oocytes matured *in vitro*. **Reprod. Fertil. Dev.** 12: 423-433.

Goto, K., Kinoshita, A., Takuma, Y., and Ogawa, K. (1990). Fertilisation of bovine oocytes by the injection of immobilised, killed spermatozoa. **Vet. Rec.** 127: 517-520.

Hamano, K., Li, X., Funauchi, K., Furudate, M., and Yoshiaki, M. (1999). Gender preselection in cattle with intracytoplasmically injected, flow cytometrically sorted sperm heads. **Biol. Reprod.** 60: 1194-1197.

Horiuch, T., Emuta, C., Yamauchi, Y., Oikawa, T., Numabe, T., and Yanagimachi, R. (2002). Birth of normal calves after intracytoplasmic sperm injection of bovine oocytes: A methodological approach. **Theriogenology** 57: 1013-1024.

Horiuchi, T. (2006). Application study of intracytoplasmic sperm injection for golden hamster and cattle production. **J. Reprod. Develop.** 52: 13-21.

Hosoi, Y., Miyake, M., Utsumi, K., and Iritani, A. (1998). Development of rabbit oocytes after microinjection of spermatozoon. In: **Proceedings of the 11th International Congress of Animal Reproduction and Artificial Insemination. Dublin, Ireland,** Vol. 3, pp. 331-333.

Kasiraj, R., Misra, A.K., Rao, M.M., Jaiswal, R.S., and Rangareddi, N.S. (1993). Successful culmination of pregnancy and live birth following transfer of frozen-thawed buffalo embryos. **Theriogenology** 39: 1187-1192.

Katayose, H., Yanagida, K., Shinoki, T., Kawahara, T., Horiuchi, T., and Sato, A. (1999). Efficient injection of bull spermatozoa into oocytes using a Piezo-driven pipette. **Theriogenology** 52:1215-1224.

Keskintepe, L., Morton, P.C., Smith, S.E., Tucker, M.J., Simplicio, A.A., and Brackett, B.G. (1997). Caprine blastocyst formation following intracytoplasmic sperm injection and defined culture. **Zygote** 5: 261-265.

Keskintepe, L., Pacholczyk, G., Machnicka, A., Norris, K., Curuk, M.A., Khan, I., and Brackett, B.G. (2002). Bovine blastocyst development from oocytes injected with freezedried spermatozoa. **Biol. Reprod.** 67: 409-415.

Kimura, Y., and Yanagimachi, R. (1995). Intracytoplasmic sperm injection in the mouse. **Biol. Reprod.** 52: 709-720.

Kong, I.K., Lee, S.I., Cho, S.G., Cho, S.K., and Park, C.S. (2000). Comparison of open pulled straws (OPS) vs glass micropipette (GMP) vitrification in mouse blastocysts. **Theriogenology** 53: 1817-1826.

Kubiak, J.Z. (1989). Mouse oocytes gradually develop the capacity for activation

during the metaphase II arrest. **Dev.Biol.** 136: 537-545.

Kuretake, S., Kimura, Y., Hoshi, K., and Yanagimachi, R. (1996). Fertilization and development of mouse oocytes injected with isolated sperm head. **Biol. Reprod**. 55: 789-795.

Kuwayama, M., Vajta, G., Kato, O., and Leibo, S.P. (2005). Highly efficient vitrification method for cryopreservation of human oocytes. **Reprod. Biomed. Online.** 11: 300-308.

Ilyin, V., and Parker, I. (1992). Effects of alcohols on responses evoked by inositol trisphosphate in Xenopus oocytes. **J. Physiol.** 448: 339-354.

Isachenko, V. (1997). Vitrification of porcine immature oocytes: new aspect. Probl. **Cryobiology** 1-2: 100-110.

Lane, M., Schoolcraft, W.B., and Gardner, D.K. (1999). Vitrificaiton of mouse and human blastocysts using a novel cryoloop container-less technique. **Fertil. Steril.** 72: 1073-1078.

Larman, M.G., Sheehan, C.B., and Gardner, D.K. (2006). Vitrification of mouse pronuclear oocytes with no direct liquid nitrogen contact. **Reprod. Biomed. Online** 12: 66-69.

Le Gall, F., and Massip, A. (1999). Cryopreservation of cattle oocytes: effects of meiotic stage, cycloheximide treatment, and vitrification procedure. **Cryobiology** 38: 290-300.

Lee, J.W., Tian, X.C., and Yang, X. (2003). Failure of male pronucleus formation is the major cause of lack of fertilization and embryo development in pig oocytes subjected to intracytoplasmic sperm injection. **Biol. Reprod.** 68: 1341-1347.

Liu, L., Ju, J.C., and Yang, X. (1998). Differential inactivation of maturation-promoting factor and mitogen-activated protein kinase following parthenogenetic activation of bovine oocytes. **Biol. Reprod.** 59: 537-545.

Liu, L., and Yang, X. (1999). Interplay of maturation-promoting factor and mitogen-activated protein kinase inactivation during metaphase-to-interphase transition of activation bovine oocytes. **Biol. Reprod**. 61: 1-7.

Malcuit, C., Maserati, M., Takahashi, Y., Page, R., and Fissore, R.A. (2006). Intracytoplasmic sperm injection in the bovine induces abnormal [Ca^{2+}] responses and oocyte activation. **Reprod. Fertil. Develop.** 18: 39-51.

Martin, M.J. (2000). Development of *in vivo*-matured porcine oocytes following intracytoplasmic sperm injection. **Biol. Reprod.** 63: 109-112.

Mandelbaum, J., Anastasiou, O., Levy, R., Guerin, J.F., de Larouziere, V., and Antoine, J.M. (2004). Effects of cryopreservation on the meiotic spindle of human oocytes. **Eur. J. Obstet. Gynecol. Reprod. Biol.** 113S: S17-S23.

Martino, A., Songsasen, N., and Leibo, S.P. (1996). Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. **Biol. Reprod.** 24: 1059- 1069.

Mazur, P. (1963). Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. **J. Gen. Physiol.** 47: 347-69.

Ock, S.A., Bhak, J.S., Balasubramanian, S., Lee, H.J., Choe, S.Y., and Rho, G.J. (2003). Different activation treatments for successful development of bovine oocytes following intracytoplasmic sperm injection. **Zygote** 11: 69-76.

Oikawa, T., Takada, N., Kikuchi, T., Numabe, T., Takenaka, M., and Horiuchi, T. (2005). Evaluation of activation treatments for blastocyst production and

birth of viable calves following bovine intracytoplasmic sperm injection. **Anim. Reprod. Sci.** 86: 187-194.

O'Neill, G.T., McDougall, R.D., and Kaufman, M.H. (1989). Ultrastructural analysis of abnormalities in the morphology of the second meiotic spindle in ethanol-induced parthenogenones. **Gamete. Res.** 22: 285-299.

Palermo, G., Joris, H., Devroey, P., and Van Steirteghem, A.C. (1992). Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. **Lancet** 340: 17-18.

Papis, K., Shimizu, M., and Izaike, Y. (2000). Factors affecting the survivability of bovine oocytes vitrified in droplets. **Theriogenology** 15: 651-658.

Parnpai, R., and Tasripoo, K. (2003). Effect of different activation protocols on the development of cloned swamp buffalo embryos derived from granulosa cells. **Theriogenology** 59: 279.

Perreault, S.D., Barbee, R.R., Elstein, K.H., Zucher, R.M., and Keefer, C.L. (1988). Interspecies differences in the stability of mammalian sperm nuclei assessed *in vivo* by sperm microinjection and *in vitro* by flow cytometry. **Biol. Reprod.** 39: 157-167.

Presicce, G.A., and Yang, X. (1994). Parthenogenetic development of bovine oocytes matured *in vitro* for 24 h and activated by ethanol and cycloheximide. **Mol. Reprod. Dev.** 38: 380-385.

Rall, W.F. (1987). Factors affecting the survival of mouse embryos cryopreserved by vitrifi cation. **Cryobiology** 24: 387-402.

Rho, G.J., Kawarsky, S., Johnson, W.H., Kochhar, K., and Betteridge, K.J. (1998). Sperm and oocyte treatment to improve the formation of male and female

pronuclei and subsequent development following intracytoplasmic sperm injection into bovine oocytes. **Biol. Reprod.** 59: 918-924.

Rho, G.J., Lee, S.L., Kim, Y.S., Yeo, H.J., Ock, S.A., Balasubramanian, S., and Choe, S.Y. (2004). Intracytoplasmic sperm injection of frozen-thawed bovine oocytes and subsequent embryo development. **Mol. Reprod. Dev.** 68: 449-455.

Saikhun, J., Kitiyanant, N., Songtaveesin, C., Pavasuthipaisit, K., and Kitiyanant, Y. (2004). Development of swamp buffalo (*Bubalus bubalis*) embryo after parthenogenic activation and nuclear transfer using serum fed or starved fetal fibroblasts. **Reprod. Nutr. Dev.** 44: 65-78.

Sathananthan, A.H., Trouson, A., Freeman, L., and Brady, T. (1988). The effect of cooling human oocytes. **Hum. Reprod.** 3: 968-977.

Schneider, U. (1986). Cryobiological principles of embryo freezing. **J. IVF-ET** 3: 3-9.

Soloy, E., Kanaka, J., Viuff, D., Smith, S.D., Callesen, H., and Greve, T. (1997). Time course of pronuclear deoxyribonucleic acid synthesis in parthenogenetically activated bovine oocytes. **Biol. Reprod.** 57: 27–35.

Stachecki, J.J., Munne, S., and Cohen, J. (2004). Spindle organization after cryopreservation of mouse, human and bovine oocytes. **Reprod. Biomed.** Online 8: 664-672.

Sun, F.Z., Hoyland, J., Huang, X., Mason,W., and Moor, R.M. (1992). A comparison of intracellular changes in porcine eggs after fertilization and electroactivation. **Development** 115: 947-956.

Susko-Parrish, J.L., Leibfried-Rutledge, M.L., Northet, D.L., Schutzkus, V., and First,

N.L. (1994). Inhibition of protein kinases after induced calcium transient causes transition of bovine oocytes to embryonic cycles without meiotic completion. **Dev. Biol.** 166: 729-739.

Suttner, R., Zakhartchenko, V., Stojkovic, P., Muller, S., Alberio, R., Medjugorac, I., Brem, G., Wolf, E., and Stojkovic, M. (2000). Intracytoplasmic sperm injection in bovine: Effects of oocyte activation, sperm pretreatment and injection technique. **Theriogenology** 54:935-948.

Swann, K., and Ozil, J.P. (1994). Dynamics of the calcium signal that triggers mammalian egg activation. **Int. Rev. Cytol.** 152: 183-222.

Szollosi, M.S., Kuubiak, J.Z., Debey, P., de Pennart, H., Szollosi, D., and Maro, B. (1993). Inhibition of protein kinase by 6-dimethyllaminopurine accelerates the transition to interphase in activated mouse oocytes. **J. Cell. Sci.** 104: 861-872.

Tesarik, J., and Sousa, M. (1995). More than 90% fertilization rates after intracytoplasmic sperm injection and artificial induction of oocyte activation with calcium ionophore. **Fertil. Steril**. 63: 343-349.

Uehara, T., Yanagimachi, R. (1976). Microsurgical injection of spermatozoa into hamster eggs with subsequent transformation of sperm nuclei into male pronuclei. **Biol. Reprod.** 15: 467-470.

Vajta, G., Holm, P., Kuwayama, M., Booth, P.J.,Jacobsen, H., Greve, T., and Callesen, H. (1998). Open Pulled Straws (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. **Mol. Reprod. Dev.** 51: 53-58.

Vincent, C., Garnier, V., Heyman ,Y., and Renard, J. (1989). Solvent effects on cytoskeletal organization and in-vivo survival after freezing of rabbit oocytes.

J. Reprod. Fertil. 87: 809-820.

Vincent, C., Cheek, T.R., and Johnson, M.H. (1992). Cell cycle progression of parthenogenetically activated mouse oocytes to interphase is dependent on the level of internal calcium. **J. Cell. Sci.** 103: 389-396.

Wang, W.H., Abeydeera, L.R., Prather, R.S., and Day, B.N. (1998). Functional analysis of activation of porcine oocytes by spermatozoa, calcium ionphore, and electrical pulse. **Mol. Reprod. Dev.** 51: 346-353.

Wei, H., and Fukui, Y. (1999). Effects of bull, sperm type and sperm pretreatment on male pronuclear formation after intracytoplasmic sperm injection in cattle. **Reprod. Fertil. Develop.** 11: 59-65.

Wei, H., and Fukui, Y. (2002). Births of calves derived from embryos produced by intracytoplasmic sperm injection without exogenous oocyte activation. **Zygote** 10: 149-153.

Whittingham, D.G., Leibo, S.P., and Mazur, P. (1972). Survival of mouse embryos frozen to -196 ºC and -269 ºC. **Science** 178: 411-414.

Whittingham, D.C. (1977). Fertilization *in vitro* and development to term of unfertilized mouse oocytes previously stored at -196oC. **J. Reprod. Fertil.** 49: 89-94.

Willadsen, S.M., Polge, C., Rowson, L.E., and Moor, R.M. (1976). Deep freezing of sheep embryos. **J. Reprod. Fertil** 46: 151-154.

Wilmut, I., and Rowson, L.E.A. (1973). The successful low-temperature preservation of mouse and cow embryos. **J. Reprod. Fertil.** 33: 352-353.

Wood, M.J., Whittingham, D.G., and Lee, S.H. (1992). Fertilization failure of frozen mouse oocytes is not due to premature cortical granule release. **Biol. Reprod.**

46: 1187-1195.

Yamamoto, Y., Oguri, N., Tsutsumi, Y., and Hachinohe, Y. (1982). Experiments in the freezing and storage of equine embryos. **J. Reprod. Fertil. Suppl.** 32: 399-403.

Zenzes, M.T., Bielecki, R., Casper, R.F., Leibo, and S.P. (2001). Effects of chilling to 0°C on the morphology of meiotic spindles in human metaphase II oocytes. **Fertil. Steril.** 75: 769–777.

CHAPTER III

EFFECTS OF CHEMICAL ACTIVATION TREATMENT ON DEVELOPMENT OF SWAMP BUFFALO OOCYTES MATURED IN VITRO AND FERTILIZED BY INTRACYTOPLASMIC SPERM INJECTION

3.1 Abstract

The objective of this study was to optimize the activation protocol for buffalo oocytes after ICSI. The release of the second polar body (PB) at 3, 6 and 9 h after ICSI of *in vitro* matured oocytes activated either with 5 μM Io or 7% EtOH was preliminary examined. The highest rate of second PB extrusion occurred at 3 h of activation and the second PB extrusion in the EtOH treated group was significantly higher than in the Io treated group. Oocytes that extruded the second PB were selected and cultured either with 1.9 mM 6-DMAP for 3 h or with 10 μg/mL CHX for 5 h. Significantly higher rate of oocytes formed 2 pronuclei in EtOH combined with CHX (EtOH+CHX) (62%) group compared to those of Io+CHX (42%) and EtOH+6-DMAP (48%) groups ($P<0.01$) whereas Io+6-DMAP group showed intermediate value (58%). Significantly higher blastocyst formation rates were obtained in Io+6-DMAP (29%) and EtOH+CHX (24%) groups than in Io+CHX (6%) and EtOH+6-DMAP (17%) groups. Our results indicate that buffalo ICSI oocytes were effectively activated by combine treatmen of Io with 6-DMAP and EtOH with

CHX resulting in the highest cleavage and blastocyst formation rates.

3.2 Introduction

The importance of swamp buffalo (*Bubalus bubalis*) in many area of the world is equal to cattle in terms of meat and milk production, and labor, especially due to its resistance to hot climate, stress and diseases. Although traditional assisted reproduction technologies are currently utilized in this species, the efficiency of reproduction is somewhat less than that of cattle because of inherent reproductive problems such as silent oestrous signs (Esposito et al., 1992; Zicarelli et al., 1997), long calving interval, delayed age of puberty, and low number of primordial follicles (Van et al., 1994). As an assisted reproduction technology, artificial insemination using frozen spermatozoa has been established and applied for the genetic improvement of swamp buffalo (Muer et al., 1988; Shukla and Misra 2007). However, frozen buffalo spermatozoa sometimes show immobility after thawing (Muer et al., 1988) which may cause reduced fertility. To overcome this problem ICSI, the injection of a single spermatozoon directly into cytoplasm bypassing the ZP and oolemma is the optimum procedure. Since the egg will theoretically be fertilized using only a single sperm this method is considered a standard way to produce normal diploid embryos therefore it may be the preferable way to produce the next generation swamp buffalo. This technique has successfully been applied for buffalos (Lu et al., 2006). Although ICSI alone seems to be sufficient to trigger the events of oocyte activation in some species such as hamsters, mice and humans (Perreault et al., 1988; Kimura and Yanagimachi 1995; Tesarik and Sousa 1995; Kuretake et al., 1996) certain species such as cattle (Rho et al., 1998) and pigs (Lee et al., 2003) require

additional activation of the oocytes after ICSI to assure their development.

The events of oocyte activation including the release of cortical granules, accomplishment of meiosis, second PB extrusion and pronucleus formation require the inactivation of protein kinases such as MPF and MAPK (Sun and Nagai 2003; Liu and Yang 1999). During fertilization the activity of these kinases collapses by repetitive elevation (oscillations) of intracellular calcium triggered by the sperm (Ducibella and Fissore 2008). Such effect can be imitated artificially by certain drugs; In cattle for instance, treatment of oocytes with calcium ionophore alone decreased cyclin B (a subunit of MPF) level within 1 h and cyclin B level rose again 4 h to 15 h after the treatment (Liu and Yang 1999). EtOH was reported, for the first time, to be effective for the activation of *in vitro* matured bovine oocytes (Nagai 1987), and has been used to activate buffalo oocytes (Parnpai and Tasripoo 2003) by promoting the formation of IP3 at the plasma membrane and inducing the extracellular calcium influx which would lead to a high intracellular calcium concentration (Ilyin and Parker 1992). Another chemical that elevates Ca^{2+} in oocytes is Io that has been successfully used in buffalo nuclear transfer, to induce repeated transient intracellular calcium rising (Saikhun et al., 2004). Since full activation of oocytes leading to pronuclear formation requires the proper inactivation of both MPF and MAPK activities a combination of the calcium rise and the inhibition of MPF and MAPK activities either by protein synthesis inhibitors (such as CHX) or protein phosphorylation inhibitors (such as 6-DMAP) is considered a reasonable approach to obtain optimal activation of oocytes.

From another perspective, activation treatment following ICSI increases the possibility of parthenogenetic embryo development. Activation procedures aimed at

making buffalo oocytes suitable for ICSI differ from those aimed at producing parthenogenesis; haploidy is required for the former whereas diploid is required for the latter. Treatment with CHX or 6-DMAP following the calcium rise reagents after ICSI would immediately inhibit the extrusion of the second PB resulting in triploid or parthenogenetic diploid embryos (Liu et al., 1998). Therefore, the selection of buffalo oocytes by the presence of the second PB, together with the additional activation treatment with CHX or 6-DMAP may decrease the triploidy and parthenogenesis.

With the goal of producing diploid activated oocytes for ICSI, the objective of this study was to optimize the activation procedure. The effects of four combined activation treatments using EtOH, Io, CHX and 6-DMAP on second PB extrusion, pronuclear formation, cleavage, and subsequent development of buffalo ICSI oocytes were compared. The efficiency of EtOH and Io was examined preliminary by the release of second PB following ICSI.

3.3 Materials and methods

3.3.1 Oocyte collection and *in vitro* maturation

Abattoir-derived buffalo ovaries were transported to the laboratory within 4 h and kept in physiological saline (0.9% NaCl). Cumulus-oocyte complexes (COCs) were collected from the follicles 2–6 mm in diameter using a 21-gauge needle attached to a 10 mL syringe as described earlier (Muenthaisong et al., 2007) for buffalo ovaries. Each of 20 COCs was washed five times with PBS supplemented with 0.1% PVP, and three times with IVM medium, then cultured in 100 μL droplets of IVM medium covered with mineral oil in a humidified atmosphere of 5% CO_2 in air at 37 ºC for 21 h.

The *in vitro* matured COCs were mechanically denuded by repeated pipetting with a fine-tip pipette in 0.2% hyarulonidase and washed five times in the Emcare holding medium (ICP-Bio, Auckland, New Zealand). Oocytes with a visible first PB were defined to be MII (Figure 3.1A) and selected for the ICSI.

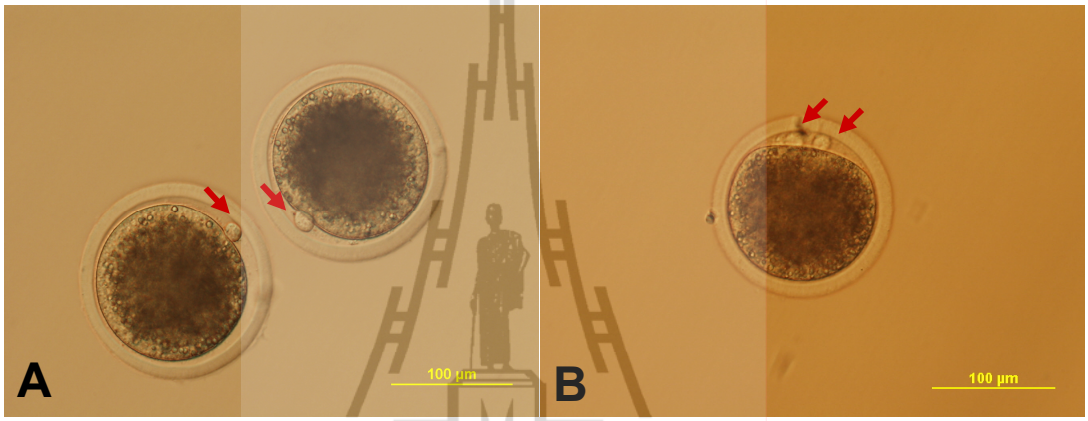

Figure 3.1 MII oocytes with a first PB (A). Activated oocytes with first and second PBs (B).

3.3.2 Sperm preparation for ICSI

A straw of frozen buffalo semen from the same bull was thawed at 37 ºC for 30 sec. Thawed spermatozoa were washed twice by centrifugation at 500 ×g for 7 min in Brackett and Oliphant medium (Brackett and Oliphant 1975) supplemented with 10 mM caffeine (caffeine-BO) as described previously (Suteevun et al., 2006). The sperm pellet was resuspended in caffeine-BO at a concentration of 8×10^6 sperm/mL.

3.3.3 ICSI procedures

Sperm injection was performed under an inverted microscope (IX71, Olympus, Tokyo, Japan) with a micromanipulator (M0188NE, Narishige Tokyo, Japan). The inner diameter of the sperm injection needle was 8-10 µm, and the inner

diameter of the holding pipette was 20 µm. Three droplets of solution were prepared on the lid of 60 mm culture dish and covered with paraffin oil. The first droplet was PVP solution (ICSI®, Vitrolife, Sweden) for washing the pipette, the second droplet was the sperm suspension with PVP solution (1:5) and the third droplet was Emcare medium for the ICSI procedure. Approximately 10 MII oocytes were placed in the droplet of Emcare medium. A single, motile buffalo spermatozoon was immobilized against the bottom of the second droplet (Figure 3.2 A). The tail of the sperm was loaded first with a minimum volume of the medium into the injection pipette, and then the injection pipette was moved to the droplet containing the oocytes. An oocyte was aspirated by the holding pipette at the 9 o'clock position, with the first PB being at either the 12 or 6 o'clock position. The immobilized sperm was injected into the ooplasm at 3 o'clock position (Figure 3.2 B). Sham-injection of oocytes was conducted by using the same procedure as sperm injection, except that no sperm was loaded in the injection pipette.

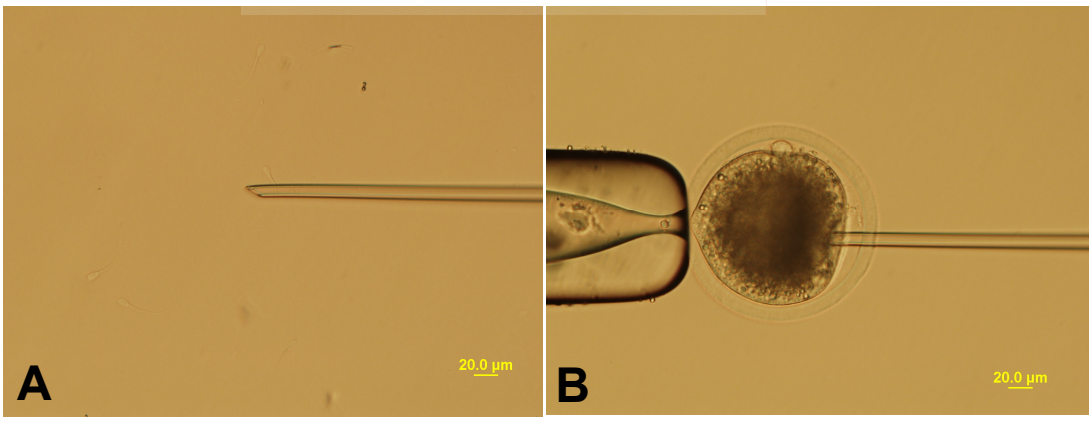

Figure 3.2 Spermatozoon immobilization (A). Sperm was injected into the ooplasm (B).

3.3.4 Activation of ICSI oocytes

Within 1 h of the injection, the injected oocytes (both ICSI and Sham

groups) were activated by exposure either to 7% EtOH in Emcare medium for 5 min or to 5 μM Io in Emcare medium for 5 min, and then subsequently cultured in TCM199 + 10% FCS to allow extrusion of the second PB (Figure 3.1 B). The completion of the second meiosis was determined by the extrusion of the second PB observed at 3, 6 and 9 h after activation, and the optimal starting time for the further chemical activation treatments was decided. Injected oocytes without activation treatment were treated as the control group.

The injected and activated oocytes which extruded the second PB at 3 h of activation were selected and transferred to mSOF medium supplemented with either 1.9 mM 6-DMAP or 10 μg/mL CHX and cultured for 3 and 5 h, respectively, at 38.5 ºC under humidified atmosphere of 5% CO_2 in air. The oocytes treated with combined activations were grouped as Io + 6-DMAP, Io + CHX, EtOH + 6-DMAP and EtOH + CHX groups.

3.3.5 Nuclear staining

The pronuclear formation status in oocytes was evaluated after 18 h of ICSI. Total 193 ICSI oocytes were mounted on glass slides, immersed in ethanol: acetic acid (3:1, w:v) for 24 h and stained with 1% (w/v) aceto-orcein for observation. As all the evaluated oocytes presented a second PB, the absence of an intact spermatozoon or male pronucleus was considered as ICSI failure. Male and female pronuclei were identified by their position relative to the remaining sperm tail or mid-piece and to the second PB of the oocytes.

3.3.6 *In vitro* embryo culture

After the combined activation treatment, presumptive zygotes were further cultured in mSOF medium (Gardner et al., 1994) supplemented with 3 mg/mL

fatty acid free BSA at 38.5 ºC under a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 for 2 days. The day of ICSI was considered Day 0. After that, 8-cell stage embryos were selected and co-cultured with buffalo cumulus cells in mSOF medium at 38.5 ºC under humidified atmosphere of 5% CO_2 in air for additional 5 days. The medium was changed daily and the development of embryos was recorded at the same time for medium changing.

3.3.7 Evaluation of blastocyst cell number

The Sham injected and ICSI blastocysts harvested from each treatment group at 7 days of IVC were counter-stained to distinguish total cell numbers. The blastocysts were fixed in 4% paraformaldehyde for 15 min, and then washed twice in PBS at room temperature. They were transferred to PBS containing 5 µg/mL Hoechst 33342 for 5 min. After being washed in PBS, they were transferred on a clean glass slide and covered with a cover slide. The total cell numbers were counted under a fluorescent microscope (IX71, Olympus, Tokyo, Japan) at 400× magnification.

3.3.8 Experimental design

Experiment 1. The timing and frequency of second PB extrusion was determined after stimulation by EtOH or Io. Oocytes after ICSI or sham-injection were treated either with 7% EtOH or 5µM Io or without activation (Control) and cultured for 9 h. The extrusion of second PB was recorded at 3 h, 6 h and 9 h after activation treatment.

Experiment 2. Pronuclear formation and second PB extrusion in ICSI oocytes was assessed after combined activation with Io or EtOH and CHX or 6-DMAP.

Experiment 3. *In vitro* embryo development and cell numbers in

blastocysts were investigated after combined activation with Io or EtOH and CHX or 6-DMAP of ICSI and sham-injected oocytes.

3.3.9 Statistical analysis

Statistical analysis of data was evaluated by Completely Randomized Design (CRD) with Statistical Analysis System (SAS Inst. INC., Cary, N.C., USA). Analysis of Variance (ANOVA) and Comparison of means by Duncan's Multiple Range Test (DMRT) were analyzed. Each treatment was replicated at least six times. The differences between groups were considered to be statistically significant at a probability value of 0.05 or less.

3.4 Results

3.4.1 Induction of the second PB release by EtOH or Io

As shown in Table 3.1, a total of 104 injected buffalo oocytes including sham injection group without activation (control group) did not extrude the second PB after culture. The highest second PB extrusion rate (68%) was obtained in the EtOH group after 3 h of ICSI among all the groups ($p<0.05$). When compared within the same treatment, most of the second PB extrusion occurred at 3 h after ICSI in both EtOH and Io groups. The number (%) of oocytes extruding the second PB at 3 h of activation out of total number of oocytes with the second PB within 9 h of activation was 73/87(84) and 50/66(76), respectively. There was no difference in the second PB extrusion rates between EtOH and Io groups in sham-injected oocytes and in both groups the most of the second PB extrusion occurred at 3 h of injection as well. These results show that based on the percentage of second PB extrusion EtOH was more effective for the activation of buffalo oocytes following ICSI than Io.

3.4.2 Pronuclear formation in ICSI oocytes

As shown in Table 3.2, a significantly higher proportion of ICSI oocytes extruded a second PB and had 2 pronuclei (2PB+2PN) in EtOH + CHX group (62%) than those in Io + CHX (42%) and EtOH + 6-DMAP (48%) groups; however, there was no difference from the Io+6-DMAP group (58%). The proportion of activated oocytes having a single pronucleus showed no difference among oocytes of the four activation treatments.

Table 3.1 The effect of exposure of ICSI and sham-injected oocytes to 7% EtOH or 5µM Io on the second PB extrusion in swamp buffalo.

Chemical treatments	Sperm injection	No. of oocytes examined	No. (%) of oocytes extruded the second PB			
			Times after activation			
			3 h	6 h	9 h	Total
5 µM Io	+	108	50 (46)^{b}	15 (14)^{a}	1(1)	66 (61)^{b}
	_	102	44 (43)^{b}	6 (6)^{ab}	0(0)	50 (49)^{b}
7% EtOH	+	107	73 (68)^{a}	13 (12)^{a}	1(1)	87 (81)^{a}
	_	101	54 (53)^{b}	5 (5)^{ab}	0(0)	59 (58)^{b}
Control*	+	52	0(0)^{c}	0(0)^{c}	0(0)^{c}	0(0)^{c}
	_	52	0(0)^{c}	0(0)^{c}	0(0)^{c}	0(0)^{c}

* Control means no activation

^{a,b} Means within columns with different superscripts significantly differ (P<0.05)

Table 3.2 Pronuclear formation after intracytoplasmic sperm injection of buffalo oocytes followed by various activation protocols.

Activation treatments	No. oocytes examined	No. (%) oocytes exhibiting			
		2PB+2PN[1]	2PB+1PN[2]	2PB+Sperm[3]	2PB[4]
Io + 6-DMAP	45	26(58)[ab]	16(36)[a]	1(2)	2(4)[b]
Io + CHX	50	21(42)[b]	21(42)[a]	2(4)	6(12)[a]
EtOH+6-DMAP	48	23(48)[b]	18(38)[a]	1(2)	6(13)[a]
EtOH + CHX	50	31(62)[a]	15(30)[a]	1(2)	3(6)[b]

[a,b] Means within columns with different superscripts differ ($P<0.05$)

[1] Oocytes extruded a second PB and had 2 pronuclei.

[2] Oocytes extruded a second PB and had 1 pronucleus.

[3] Oocytes extruded a second PB and had an intact sperm head.

[4] Oocytes extruded a second PB and had no pronucleus.

3.4.3 Effect of combined activation treatment after ICSI on embryonic development *in vitro*

As shown in Table 3.3, the cleavage rates of ICSI oocytes in Io + 6-DMAP, EtOH + 6-DMAP and EtOH + CHX groups (76%, 69% and 78%, respectively) were significantly higher than those in Io + CHX group (52%) and sham injected control group in all activation treatments. Significantly higher blastocyst formation rates were obtained in Io + 6-DMAP and EtOH + CHX groups (29 and 24% respectively) than those in Io + CHX and EtOH + 6-DMAP groups (6 and 17 %, respectively) and sham injected groups. The Io + CHX group showed the lowest blastocyst formation rate among all the groups. There was no difference in cleavage

and blastocyst formation rates between ICSI and sham injected oocytes in Io + CHX oocytes. Within the sham injected group, the cleavage rate of these four treatments was not different, however, the blastocysts formation rate in the Io + CHX group (4%) was significantly lower than that in Io + 6-DMAP (14%) and EtOH + CHX (12%) groups. There was no difference in blastocyst rates among sham injected oocytes in Io + 6-DMAP, EtOH + 6-DMAP and EtOH + CHX groups.

3.4.4 Cell numbers in Day 7 blastocysts derived from injected oocytes

As shown in Table 3.4, the cell number of blastocysts obtained from ICSI oocytes in EtOH + CHX group (88.9±23.0) was significantly higher than that in Io + CHX (53.8±14.9); however, it did not differ from those in Io + 6-DMAP (79.3±21.7) and EtOH + 6-DMAP (74.1±13.9) groups. Within each treatment group, the cell number of blastocysts in ICSI and sham injected oocytes was not different.

Table 3.3 *In vitro* development of buffalo embryos derived from ICSI followed by different activation treatments.

Activation treatments	Sperm injection	No. (%) of oocytes			No. (%) cleavage	No. (%) of embryos developed to		
		Injected	Extruded second PB	Cultured		8-cell stage	Morulae	Blastocysts
Io + 6-DMAP	+	187	84(45)^{b}	84	64(76)^{a}	47(56)^{a}	28(33)^{a}	24(29)^{a}
	-	102	44(43)^{b}	44	27(61)^{bc}	19(43)^{bc}	8(18)^{d}	6(14)^{b}
Io + CHX	+	170	84(49)^{b}	84	44(52)^{c}	19(23)^{d}	11(13)^{c}	5(6)^{c}
	-	102	46(45)^{b}	46	23(50)^{c}	10(22)^{d}	2(4)^{d}	2(4)^{c}
EtOH + 6-DMAP	+	132	85(64)^{a}	85	59(69)^{ab}	35(41)^{bc}	21(25)^{b}	14(17)^{b}
	-	101	54(53)^{b}	54	28(52)^{c}	18(33)^{cd}	8(15)^{d}	4(7)^{bc}
EtOH + CHX	+	125	83(66)^{a}	83	65(78)^{a}	40(48)^{ab}	27(33)^{ab}	20(24)^{a}
	-	97	49(51)^{b}	49	29(59)^{bc}	19(39)^{bc}	8(16)^{d}	6(12)^{b}

^{a,b,c} Means within columns with different superscripts differ (P<0.05)

Table 3.4 Cell numbers of blastocysts derived from injected oocytes followed by various activation treatments.

Activation treatments	Sperm injection	No. of embryos examined	Cell numbers (Mean ±S.D.)
Io + 6-DMAP	+	9	79.3±21.7[ab]
	-	6	64.3±22.7[bc]
Io + CHX	+	4	53.8±14.9[bc]
	-	2	38.5±9.2[c]
EtOH + 6-DMAP	+	7	74.1±13.9[ab]
	-	4	70.8±9.9[b]
EtOH + CHX	+	10	88.9±23.0[a]
	-	6	72.0±14.6[ab]

[a,b,c] Means within columns with different superscripts differ (P<0.05)

3.5 Discussion

In this study, we investigated the second PB extrusion, pronuclear formation, cleavage and development to the blastocyst stage of buffalo ICSI oocytes to determine the optimal activation protocol. To our knowledge this is the first paper to report high percentages of buffalo ICSI oocytes developing to the blastocyst stage by the combination of chemical activation treatments such as Io + 6-DMAP or EtOH + CHX. These two combinations supported the highest second PB extrusion and resulted in the highest blastocyst rates and cell numbers per embryo. The activation protocol combining Io with 6-DMAP has also been proven to get the best results in sheep (Shirazi et al., 2008), goats (Ongeri et al., 2001), and cattle (Rho et al., 1998).

Combining EtOH with CHX treatment was also reported to achieve high activation rates for both young and aging bovine oocytes (Presicce and Yang 1994a; Presicce and Yang 1994b).

In this study, without additional chemical activations, none of the oocytes extruded the second PB and developed, indicating that mere sperm injection was not sufficient to activate buffalo oocytes. In other words, an additional activation treatment of buffalo oocytes following ICSI was necessary for the oocytes to complete the meiosis, subsequent pronuclear formation and embryo development. The same necessity of additional activation treatments for the oocytes after ICSI to assure their development was reported for other species such as cattle (Rho et al., 1998) and pigs (Lee et al., 2003). In contrast, in hamsters (Uehara and Yanagimachi 1976), rabbits (Keefer 1989) and humans (Tesarik et al., 1994), the mechanical processes during ICSI, penetration of an ICSI needle through the ZP and cytoplasmic suction, were reported to be enough to activate oocytes. The reason why only mechanical activation and the existence of spermatozoon in the cytoplasm could not induce extrusion of the second PB of oocytes to complete the meiosis processes in cattle, pigs and swamp buffalo is not clear, which maybe related with the packaging of chromatin in spermatozoa that was reported to be more stable in cattle than in other species like human, mouse and hamster (Perreault et al., 1988).

A series of multiple intracellular calcium ion transient increases were exhibited in fertilized mammalian oocytes (Fissore et al., 1992; Fissore and Robl 1994; Miyazaki et al., 1986; Miyazaki et al., 1993). After sperm-oocyte fusion, these early calcium ion responses have important roles in the exocytosis of cortical granules and the resumption of meiotic arrest in mouse oocytes (Kline and Kline 1992). The

completion of oocyte meiosis was monitored by the rate of oocytes extruding the second PB after activation in this study, and it was found that EtOH treatment was more effective to activate buffalo ICSI oocytes than Io treatment. This could be due to the different ways of calcium mobilization induced by EtOH and Io. EtOH activates oocytes by promoting the rapid potentiation of IP3 mediated Ca^{2+} release through stimulation of IP3 formation at the plasma membrane, and then it induces a single calcium rise coming from both extracellular entry and intracellular stores (Shilina et al., 1993). On the other hand, Io induces a single Ca^{2+} rise in mammalian oocytes through the release of Ca^{2+} only from internal stores (Vincent et al., 1992). The current result was consistent with that for cattle in which EtOH was considered to be a more effective calcium rise reagent to induce the second PB extrusion from oocytes than Ca^{2+} ionophore IA23187 (Nakada and Mizuno 1998). In cattle, EtOH can induce a greater rise of Ca^{2+} and longer Ca^{2+} releasing duration than those observed at fertilization (Nakada and Mizuno 1998).

Oocytes exposed to a single chemical (e.g., calcium ionophore; EtOH) or electrical stimulus would induce a transient rise in intracellular calcium; however, this is inadequate for full activation of oocytes, resulting in the incomplete cortical granule formation (CG exocytosis) (Wang et al., 1998) and failures of pronuclear formation, mRNA recruitment and DNA synthesis (Schultz and Kopf 1995; Soloy et al., 1997; Susko-Parrish et al., 1994). Studies have demonstrated that the activation protocols with the best results were those that combined Ca^{2+} rise treatment and inhibition of MPF activity on ICSI and somatic cell nuclear transfer in bovine embryos (Rho et al., 1998; Bhak et al., 2006), except for the recent report that combined activation treatment with Io and EtOH yield higher blastocyst rate than Io combined with CHX

or 6-DMAP (Abdalla et al., 2009). Thus, combination of a stimulation for Ca^{2+} rise and an inhibition of either protein synthesis with CHX or protein phosphorylation with 6-DMAP can be a reasonable approach for the establishment of the optimal activation method for buffalo oocytes. Inhibition of protein synthesis can enhance oocyte activation by inducing temporal changes in MPF and MAPK. CHX not only inhibits the cyclin B synthesis and maintains low activity of MPF, but also inhibits the phosphorylation of MAPK after pronucleus formation in oocytes (Liu and Yang 1999). Also, dephosphorylation of MAPK can be induced by 6-DMAP leading to earlier pronuclear development (Susko-Parrish et al., 1994; Soloy et al., 1997; Leal and Liu 1998).

After receiving the first intracellular calcium rise, the MII oocytes continue to resume meiosis until the extrusion of the second PB. In this study, most of the second PB extrusions from the buffalo oocytes were detected after 3 h of calcium rise treatment. The reason why others did not extrude the second PB in these circumstances at that time is not clear. However, it might be due to disintegration of their meiotic spindle. When those showing delayed extrusion of the second PB after 6 and 9 h of EtOH or Io treatment were cultured in IVC medium with 6-DMAP or CHX, they had a significantly lower ability to develop to the blastocyst stage (unpublished data) compared with those of oocytes which extruded the second PB after 3 h of treatment. It is possible that the delayed extrusion of the second PB might be relevant to the ability of oocytes to resume meiosis. The oocytes that needed a longer time to finish meiosis might have started the cell cycle later than those which finished meiosis within 3 h of the treatment. This might be the one of the reasons why the oocytes with delayed extrusion of the second PB had lower development ability compared to those

which extruded the second PB within 3 h of the treatment. In cattle it was reported that 93% of oocytes extruded the second PB within 3 h of Io treatment following ICSI (Fulka et al., 1991; Susko-Parrish et al., 1994; Rho et al., 1998). The results in this study indicated that 3 h is the optimal time for the selection of oocytes which extruded the second PB after EtOH or Io treatment following ICSI in buffalo.

In this study the cleavage and blastocyst formation rates for ICSI oocytes were significantly higher than those for sham injected oocytes in EtOH + CHX and Io + 6-DMAP groups, suggesting that the spermatozoon could enhance oocyte activation and subsequent development in those groups. It has been reported that the sperm cell is a natural activator of oocytes (Bootman and Berridge 1995). In mice (Vitullo and Ozil 1992; Jones and Whittingham 1996; Lawrence et al., 1998), pigs (Sun et al., 1992), and cattle (Nakada and Mizuno 1998; Tosti et al., 2002), sperm penetration induces periodical and transient increases in the free intracellular calcium concentration for several hours. It also has been suggested that after fusion of sperm and oocyte, a sperm CSF is released into the oocyte, which generates the cytoplasmic calcium increase (Fissore et al., 1998; Kimura et al., 1998). However, our study has demonstrated, that a mere sperm injection or sham injection into IVM buffalo oocytes without any additional activation procedures resulted in no activation and that in EtOH + CHX and Io + 6-DMAP groups sperm injection combined with activation treatment resulted in higher activation and development than activation treatment alone. This suggests that the synergetic effect on oocyte activation of sperm and activation treatment is necessary to guarantee high rates of embryo development by ICSI in buffaloes. It is of interest that oocytes activated by the sham injection extruded the second PB and developed to blastocysts; however with a lower rates and

embryonic cell numbers compared to ICSI oocytes. Embryos generated from sham-injected oocytes are considered haploid, and the developmental potential of haploid embryos was reported to be much lower than that of diploids in mice (Tarkowski and Rossant 1976). In this study only the ICSI oocytes had the possibility to develop as diploids. The reduced cell number of blastocysts and lower rate of *in vitro* embryo development in sham injected oocytes suggest that such haploid embryos might have insufficient DNA to support themselves to develop into early stage embryos compared with diploids.

In conclusion, our study demonstrated that activation of the ICSI swamp buffalo oocytes with EtOH gave the highest 2 pronuclei formation, and the combination treatments with Io+6-DMAP and EtOH+CHX gave the highest cleavage and blastocyst formation rates.

3.6 References

Abdalla, H., Shimoda, M., Hirabayashi, M., and Hochi, S. (2009). A combined treatment of ionomycin with ethanol improves blastocyst development of bovine oocytes harvested from stored ovaries and microinjected with spermatozoa. **Theriogenology** 72: 453-460.

Bootman, M.D., and Berridge, M.J. (1995). The elementary principles of calcium signaling. **Cell** 83: 675-678.

Bhak, J.S., Lee, S.L., Ock, S.A., Mohana Kumar, B., Choe, S.Y., and Rho, G.J. (2006). Developmental rate and ploidy of embryos produced by nuclear transfer with different activation treatments in cattle. **Anim. Reprod. Sci.** 92: 37-47.

Brackett, B.G., and Oliphantm, G. (1975). Capacitation of rabbit spermatozoa *in vitro*.

Biol. Reprod. 12 :260-274.

Ducibella, T., and Fissore, R. (2008). The roles of Ca^{2+}, downstream protein kinases, and oscillatory signaling in regulating fertilization and the activation of development. **Dev. Biol.** 315: 257-279.

Esposito, L., Campanile, G., Di Palo, R., Boni, R., Di Meo, C., and Zicarelli, L. (1992). Seasonal reproductive failure in buffaloes bred in Italy. In: Dieleman SJ (ed), **Proceedings of the 12th International Congress in Animal Reproduction, Netherlands,** pp. 2045-2047.

Fissore, R.A., Dobrinsky, J.R., Balise, J.J., Duby, R.T., and Robl, J.M. (1992) Patterns of intracellular Ca^{2+} concentrations in fertilized bovine eggs. **Biol. Reprod.** 47: 960-969.

Fissore, R.A., and Robl, J.M. (1994). Mechanism of calcium oscillations in fertilized rabbit eggs. **Dev. Biol.** 166: 634-642.

Fissore, R.A., Gordo, A.C., and Wu, H. (1998). Activation of development in mammals: is there a role for a sperm cytosolic factor? **Theriogenology** 49: 43-52.

Fulka, J.Jr., Leibfried-Rutledge, M.L., and First, N.L. (1991). Effect of 6-dimethylaminopurine on germinal vesicle breakdown of bovine oocytes. **Mol. Reprod. Dev.** 29: 379-384.

Gardner, D.K., Lane, M., Spitzer, A., and Batt, A. (1994). Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage *in vitro* in the absence of serum and somatic cells: amino acids, vitamins, and culturing embryos in groups stimulate development. **Biol. Reprod.** 50: 390-400.

Jones, K.T., and Whittingham, D.G. (1996). comparison of sperm- and IP3-induced

Ca^{2+} release in activated and aging mouse oocytes. **Dev. Biol.** 178: 229-237.

Keefer, C.L. (1989). Fertilization by sperm injection in the rabbit. **Gamete. Res.** 22: 59-69.

Kline, D., and Kline, J.T. (1992). Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. **Dev. Biol.** 149: 80-89.

Kimura, Y., and Yanagimachi, R. (1995). Intracytoplasmic sperm injection in the mouse. **Biol. Reprod.** 52: 709-720.

Kimura, Y., Yanagimachi, R., Kuretake, S., Bortkiewitz, H., Peny, A.C.E., and Yanagimachi, H. (1998). Analysis of mouse oocytes activation suggests the involvement of sperm perinuclear material. **Biol. Reprod.** 58: 1407-1415.

Kuretake, S., Kimura, Y., Hoshi, K., and Yanagimachi, R. (1996). Fertilization and development of mouse oocytes injected with isolated sperm head. **Biol. Reprod.** 55: 789-795.

Ilyin, V., and Parker, I. (1992). Effects of alcohols on responses evoked by inositol trisphosphate in Xenopus oocytes. **J. Physiol**. 448: 339-354.

Lawrence, Y., Ozil, J.P., and Swann, K. (1998). The effects of a Ca^{2+} chelator and heavy-metal-ion chelators upon Ca^{2+} oscillations and activation at fertilization in mouse egg suggest a role for repetitive Ca^{2+} increases. **Biochem. J.** 335: 335-342.

Leal, C.L., and Liu, L. (1998). Differential effects of kinase inhibitor and electrical stimulus on activation and histone H1 kinase activaty in pig oocytes. **Anim. Reprod. Sci.** 52: 51-61.

Lee, J.W., Tian, X.C., and Yang, X. (2003). Failure of male pronucleus formation is

the major cause of lack of fertilization and embryo development in pig oocytes subjected to intracytoplasmic sperm injection. **Biol. Reprod.** 68: 1341-1347.

Liu, L., Ju, J.C., and Yang, X. (1998). Parthenogenetic development and protein patterns of newly matured bovine oocytes after chemical activation. **Mol. Reprod. Dev.** 49: 298-307.

Liu, L., and Yang, X. (1999). Interplay of maturation-promoting factor and mitogen-activated protein kinase inactivation during metaphase-to-interphase transition of activation bovine oocytes. **Biol. Reprod.** 61: 1-7.

Lu, Y.Q., Wang, W.L., Yang, H., Liang, X.W., Liang, Y.Y., Zhang, M., Lu, S.S., Kitiyanant, Y., and Lu, K.H. (2006) Flow-cytometric sorting the sperm and production of sex-preselected embryo in buffalo (*Bubalus bubalis*). In: Lu KH (ed), Central compilation and translation press. **Proceedings of the 5th Asian Buffalo Congress, China**, pp. 155-161.

Miyazaki, S., Hashimoto, N., Yoshimoto, Y., Kishimoto, T., Igusa, Y., and Hiramoto, Y. (1986). Temporal and spatial dynamics of the periodic increase in intracellular free calcium at fertilization of golden hamster eggs. **Dev. Biol.** 118: 259-267.

Miyazaki, S., Shirakawa, H., Nakada, K., and Honda, Y. (1993). Essential role of the inositol 1,4,5-trisphosphate receptor/Ca^{2+} release channel in Ca^{2+} waves and Ca^{2+} oscillations at fertilization of mammalian eggs. **Dev. Biol.** 158: 62-78.

Muenthaisong, S., Laowtammathro, C., Ketudat-Cairns, M., Parnpai, R., and Hochi, S. (2007). Quality analysis of buffalo blastocysts derived from oocytes vitrified before or after enucleation and reconstructed with somatic cell nuclei.

Theriogenology 67: 893-900.

Muer, S.K., Roy, S.B., Mohan, G., and Dhoble, R.L. and (1988). Cryogenic changes in seminal protein of cattle and buffalo. **Theriogenology** 30: 1005-1010.

Nagai, T. (1987). Parthenogenetic activation of cattle follicular oocytes in vitro with ethanol. **Gamete. Res.** 16: 243-249.

Nakada, K., and Mizuno, J. (1998). Intracellular calcium responses in bovine oocytes induced by spermatozoa and by reagents. **Theriogenology** 50: 269-282.

Ongeri, E.M., Bormamn, C.L., Butler, R.E., Melican, D., and Gavin, W.G. (2001). Development of goat embryos after *in vitro* fertilization and parthenogenetic activation by different methods. **Theriogenology** 55: 1933-1945.

Parnpai, R., and Tasripoo, K. (2003). Effect of different activation protocols on the development of cloned swamp buffalo embryos derived from granulosa cells. **Theriogenology** 59: 279.

Perreault, S.D., Barbee, R.R., Elstein, K.H., Zucher, R.M., and Keefer, C.L. (1988). Interspecies differences in the stability of mammalian sperm nuclei assessed *in vivo* by sperm microinjection and *in vitro* by flow cytometry. **Biol. Reprod.** 39: 157-167.

Presicce, G.A., and Yang, X. (1994a). Nuclear dynamics of parthenogenesis of bovine oocytes matured *in vitro* for 20 and 40 h and activated with combined ethanol and cycloheximide treatment. **Mol. Reprod. Dev.** 37: 61-68.

Presicce, G.A., and Yang, X. (1994b). Parthenogenetic development of bovine oocytes matured *in vitro* for 24 h and activated by ethanol and cycloheximide. **Mol. Reprod. Dev.** 38: 380-385.

Rho, G.J., Kawarsky, S., Johnson, W.H., Kochhar, K., and Betteridge, K.J. (1998).

Sperm and oocyte treatment to improve the formation of male and female pronuclei and subsequent development following intracytoplasmic sperm injection into bovine oocytes. **Biol. Reprod.** 59: 918-924.

Saikhun, J., Kitiyanant, N., Songtaveesin, C., Pavasuthipaisit, K., and Kitiyanant, Y. (2004). Development of swamp buffalo (*Bubalus bubalis*) embryo after parthenogenic activation and nuclear transfer using serum fed or starved fetal fibroblasts. **Reprod. Nutr. Dev.** 44: 65-78.

Schultz, R.M., and Kopf, G.S. (1995). Molecular basis of mammalian egg activation. **Curr. Top. Dev. Biol.** 30: 21-62.

Shilina, Y., Kaneda, M., Matsuyama, K., Tanaka, K., Hiroi, M., and Doi, K. (1993). Role of the extracellular Ca^{2+} on the intracellular Ca^{2+} changes in fertilized and activated mouse oocytes. **J. Reprod. Fertil.** 97: 143-150.

Shirazi, A., Ostad-Hosseini, S., Ahmadi, E., Heidari, B., and Shams-Esfandabadi, N. (2008). *In vitro* developmental competence of ICSI-derived activated ovine embryos. **Theriogenology** 71: 342-348.

Shukla, M.K., and Misra, A.K. (2007). Effect of Bradykinin on Murrah buffalo (*Bubalus bubalis*) semen cryopreservation. **Anim. Reprod. Sci.** 97: 175-179.

Soloy, E., Kanaka, J., Viuff, D., Smith, S.D., Callesen, H., and Greve, T. (1997). Time course of pronuclear deoxyribonucleic acid synthesis in parthenogenetically activated bovine oocytes. **Biol. Reprod.** 57: 27-35.

Sun, F.Z., Hoyland, J., Huang, X., Mason, W., and Moor, R.M. (1992). A comparison of intracellular changes in porcine eggs after fertilization and electroactivation. **Development** 115: 947-956.

Sun, Q.Y., and Nagai, T. (2003). Molecular mechanisms underlying pig oocyte

maturation and fertilization. **J. Reprod. Dev.** 49: 347-359.

Susko-Parrish, J.L., Leibfried-Rutledge, M.L., Northet, D.L., Schutzkus, V., and First, N.L. (1994). Inhibition of protein kinases after induced calcium transient causes transition of bovine oocytes to embryonic cycles without meiotic completion. **Dev. Biol.** 166: 729-739.

Suteevun, T., Parnpai, R., Smith, S.L., Chang, C.C., Muenthaisong, S., and Tian, X.C. (2006). Epigenetic characteristics of cloned and *in vitro*-fertilized swamp buffalo (*Bubalus bubalis*) embryos. **J. Anim. Sci.** 84: 2065-2071.

Tarkowski, A.K., and Rossant, J. (1976). Haploid mouse blastocysts developed from bisected zygotes. **Nature** 259: 663-665.

Tesarik, J., Sousa, M., and Testart, J. (1994). Human oocyte activation after intracytoplasmic sperm injection. **Hum. Reprod.** 9: 511-518.

Tesarik, J., and Sousa, M. (1995). More than 90% fertilization rates after intracytoplasmic sperm injection and artificial induction of oocyte activation with calcium ionophore. **Fertil. Steril.** 63: 343-349.

Tosti, E., Boni, R., and Cuomo, A. (2002). Fertilization and activation currents in bovine oocytes. **Reproduction** 124: 835-846.

Uehara, T., and Yanagimachi, R. (1976). Microsurgical injection of spermatozoa into hamster eggs with subsequent transformation of sperm nuclei into male pronuclei. **Biol. Reprod.** 15: 467-470.

Van Ty, L., Nguyen, B.X., Son, H.N., and Driancurt, M.A. (1994). Superovulation and ovarian follicular population of juvenile buffaloes and calves. **Anim. Reprod. Sci.** 35: 191-199.

Vitullo, A.D., and Ozil, J-P. (1992). Repetitive calcium stimuli drive meiotic

resumption and pronuclear development during mouse oocyte activation. **Dev. Biol.** 151: 128-136.

Vincent, C., Cheek, T.R., and Johnson, M.H. (1992). Cell cycle progression of parthenogenetically activated mouse oocytes to interphase is dependent on the level of internal calcium. **J. Cell. Sci.** 103: 389-396.

Wang, W.H., Abeydeera, L.R., Prather, R.S., and Day, B.N. (1998). Functional analysis of activation of porcine oocytes by spermatozoa, calcium ionophore, and electrical pulse. **Mol. Reprod. Dev.** 51: 346-353.

Zicarelli, L., Esposito, L., Campanile, G., Di Palo, R., and Armstrong, D.T. (1997). Effect of using vasectomized bulls in AI practice on the reproductive efficiency of Italian buffalo cows. **Anim. Reprod. Sci.** 47: 171-180.

CHAPTER IV

IN VITRO DEVELOPMENT OF VITRIFIED BUFFALO OOCYTES FOLLOWING PARTHENOGENETIC ACTIVATION AND INTRACYTOPLASMIC SPERM INJECTION

4.1 Abstract

The objective of this study was to investigate the potential of swamp buffalo oocytes vitrified-warmed at the metaphase of the second meiotic cell division (M-II) stage to develop to the blastocyst stage after PA or ICSI. In Experiment 1, we examined the effects of exposure time of oocytes to CPA on their *in vitro* development after PA. *In vitro* matured (IVM) oocytes were placed in 10% DMSO + 10% EG for 1 min and then exposed to 20% DMSO + 20% EG + 0.5 M sucrose for 30 s, 45 s or 60 s (1min+30s, 1min+45s and 1min+60s groups, respectively). The oocytes were then exposed to warming solution (TCM199 HEPES + 20% FBS and 0.5M sucrose) for 5 min and then washed in TCM199 HEPES + 20% FBS for 5 min. IVM oocytes without CPA treatments served as a control group. The viability assessed by fluorescein diacetate (FDA) staining was 100% in all groups. The developmental rates after PA to the blastocyst stage between 1min+30s (16%) and control (26%) groups did not differ significantly, but they were significantly higher than those in 1min+45s(10%) and 1min+60s (2%) groups. In Experiment 2, we examined the effect

of two CPA exposure times, 1min+30s and 1min+45s on the *in vitro* development after PA of oocytes vitrified by the microdrop method. The viabilities in vitrified 1min+30s, 1min+45s and the control (without CPA treatments) groups were not different (97%, 95% and 100%, respectively). The development of surviving oocytes to the blastocyst stage in the vitrified 1min+30s group (8%) was significantly higher than that in the vitrified 1min+45s group (4%) and significantly lower than those in control group (26%). In Experiment 3, we examined the effect of two CPA exposure times, 1min+30s and 1min+45s on *in vitro* development after ICSI of vitrified oocytes. Viabilities in vitrified oocytes among 1min+30s, 1min+45s and control groups were not different (96%, 91% and 100%, respectively). After ICSI, vitrified-warmed oocytes were activated and oocytes with the second PB were cultured for 7 days. The development of ICSI oocytes to the blastocyst stage in the vitrified 1min+30s group (11%) was significantly higher than that in the vitrified 1min+45s (7%) group and significantly lower than those in control group (23%). In conclusion, our study demonstrated that the 1min+30s CPA treatment regimen could yield the highest blastocyst formation rates after PA and ICSI for oocytes vitrified by the microdrop method.

4.2 Introduction

Nowadays, the buffalo is the major milk and meat producing farm animal in many developing countries. Buffalo oocytes obtained from slaughterhouse ovaries and matured *in vitro* are useful sources for reproductive procedures such as IVF and ICSI, in which mainly cryopreserved spermatozoa are used. Cryopreservation of oocytes is also very important in preserving female gametes for future use. Efficient

oocyte cryopreservation protocols will widen and improve the strategic implementation of reproductive technologies in the buffalo species.

Cryopreservation of mammalian oocytes has become more successful using vitrification as an alternative to cryopreservation compared with slow cooling methods, in recent years (Chian et al., 2004; Vajta et al., 2006) The vitrification process induces a glass-like solidification of living cells at low temperatures. The unique advantage of the vitrification process is the elimination of ice crystal formation, the most severe cause of cryoinjury. Insufficient cooling rates of oocytes were considered one of the principal obstacles in vitrification technology (Vajta 1997). Since the first report on buffalo oocyte vitrification by using French straw (Dhali et al., 2000), some reports have been published regarding the cryopreservation of buffalo oocytes (Wani et al., 2004; Gasparrini et al., 2007; Muenthaisong et al., 2007; Boonkusol et al., 2007; Sharma and Loganathasamy, 2007; Gautam et al., 2008; Mahmoud et al., 2010). In 2004, the first successful production of a buffalo blastocyst derived from IVM and IVF of vitrified-warmed oocytes was reported (Wani et al., 2004). In order to overcome the problem of insufficient cooling rates several methods have been proposed using very small amounts of solution. Some improved vitrification methods have been successfully used for oocyte cryopreservation, including cryotop (Kuwayama and Kato, 2000), cryoloop (Lane et al., 1999), OPS (Vajta et al., 1998), glass micropipette (Hochi et al., 1994), microdrop (Papis et al., 2000), electron microscope grids (Martino et al., 1996) and solid surface vitrification (Dinnyés et al., 2000).

Among the various methods of vitrification, the microdrop method is considered easy and inexpensive, as it excludes the use of any specialized devices to

introduce oocytes into liquid nitrogen. The microdrop method is the simplest way of vitrification by dropping oocyte - containing solutions directly into liquid nitrogen. This method was first proposed for mouse embryos (Landa and Tepla, 1990), and then successfully applied to bovine embryos, zygotes and oocytes (Riha et al., 1991; Yang and Leibo, 1999; Papis et al., 1999; Boonkusol et al., 2007). After cryopreservation or treatment with CPA, structural changes in the ZP have been shown to reduce fertilization rates (Carroll et al., 1990; Vincent et al., 1990). Although the mechanism of ZP hardening of the cryopreserved oocytes is unclear, it seems to be caused by the premature release of cortical granules that induce the zona reaction (Vincent et al., 1990) resulting in lower incidences of sperm penetration into oocytes. This consequence of ZP hardening could be overcome by micromanipulation techniques such as ICSI (Carroll et al., 1990; Kazem et al., 1995; Karlsson et al., 1996; Porcu et al., 1997; Mavrides and Morroll, 2002).

Permeation of a certain amount of CPA into the oocytes is essential for cryosurvival of oocytes. The amount of CPAs within the cell increase with the duration of exposure (Yavin and Arav, 2007); however, the toxicity of the CPA must be considered, with an optimum exposure time being favorable for this purpose. Nevertheless the optimal CPA treatment regimen for the vitrification of IVM buffalo oocytes has not been determined. The aim of this study was to investigate the effect of exposure time in vitrification solution on the post-thaw viability and the developmental competence of IVM vitrified-warmed swamp buffalo oocytes after PA or ICSI. Differential cell staining was applied to assess the qualitative aspects of blastocysts that were derived from fresh or vitrified-warmed oocytes.

4.3 Materials and methods

4.3.1 Experimental design

Experiment 1 was performed to test the toxicity of vitrification solution. After treatment with the equilibration solution for 1 min, IVM oocytes at M-II were exposed to vitrification solution for 30, 45 or 60 s (1min+30s, 1min+45s and 1min+60s groups, respectively) and then transferred to warming solution. Surviving oocytes selected by the fluorescein diacetate (FDA) test were then subjected to PA and then *in vitro* cultured. The development of oocytes exposed to the vitrification solution was compared with CPA-untreated but FDA stained oocytes (FDA-exposed). To test the possible side effects of FDA staining, the oocytes without CPA and FDA treatments were also activated and cultured (control).

Experiment 2 was performed to assess the developmental ability of vitrified-thawed oocytes induced by PA. On the basis of the results from Experiment 1, only the 1 min+30 s and 1 min+45 s CPA treatment regimens were used to vitrify M-II oocytes (1min+30s and 1min+45s groups, respectively). Vitrified oocytes were stored in liquid nitrogen containers for 7 to 14 days. After warming, all surviving (FDA positive) oocytes were subjected to PA and their *in vitro* developments were compared to those of activated FDA-exposed and control oocytes.

Experiment 3 was performed to assess the development of ICSI embryos generated from vitrified-thawed M-II oocytes. Oocytes vitrified by the 1 min+30 s and 1 min+45 s treatment regimens (1min+30s and 1min+45s groups, respectively) were warmed and all of the surviving (FDA positive) oocytes were subjected to ICSI. Their *in vitro* developments were compared to those of FDA-exposed and control oocytes.

4.3.2 Chemicals and media

All reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated. The medium used for IVM was TCM199 supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA), 0.02 AU/mL FSH (Antrin, Denka Pharmaceutical, Tokyo, Japan), 50 iu/mL hCG (Chorulon, Intervet, Boxmeer, Netherlands) and 1 μg/mL estradiol-17β. The Emcare holding medium (EHM, ICP Bio, Auckland, New Zealand) was used as the basal medium throughout the process of ICSI and PA. The medium for embryo culture was modified synthetic oviduct fluid supplemented with amino acids and 0.3% fatty acid-free BSA (mSOF) (Gardner et al., 1994).

4.3.3 Oocyte collection and *in vitro* maturation

Buffalo ovaries were obtained from slaughterhouses and kept in 0.9% NaCl during transport to the laboratory within 4 h at room temperature. COCs were collected from follicles 2 to 8 mm in diameter using a 21-gauge needle attached to a 10 mL syringe. Each of 20 COCs was cultured in 100 μL droplets of IVM medium covered with mineral oil in a humidified atmosphere of 5% CO_2 in air at 38.5 °C for 21 h. After IVM culture, cumulus cells were removed from oocytes by gentle pipetting with a fine glass pipette in EHM supplemented with 0.1% hyarulonidase, and the oocytes were subsequently washed 3 times in EHM. Oocytes with a visible first PB were defined to be M-II oocytes and selected for the subsequent experiments.

4.3.4 Vitrification and warming

The M-II oocytes were vitrified-warmed by using the microdrop method. Groups of five oocytes were washed in TCM199-Hepes + 20% FBS before being placed in TCM199-Hepes + 20% FBS containing 10% DMSO and 10% EG for 1 min,

and then exposed in TCM199-Hepes + 20% FBS containing 20% DMSO, 20% EG and 0.5 M sucrose for 30, 45 or 60 s (1min+30s, 1min+45s or 60 s 1min+60s groups, respectively) at 22 to 24 °C. The oocytes were then directly dropped with about 2 µL vitrification solution into liquid nitrogen. For storage, vitrified microdrops were placed in a pre-cooled cryovial filled with liquid nitrogen using a pre-cooled forceps and kept for one to two weeks. The vitrified microdrops were warmed by immersing directly into 3 mL of 0.5 M sucrose in TCM199-Hepes + 20% FBS at 38.5 °C for 5 min, transferred to TCM199-Hepes + 20% FBS for 5 min, and then kept in the TCM199-Hepes + 20% FBS under a humidified atmosphere of 5% CO_2 in air at 38.5 °C for 1 h. Some oocytes were treated with CPA and washing solution without the cooling and warming process ("CPA-exposed" group).

4.3.5 Evaluation of oocyte viability

Oocyte viability was evaluated by FDA staining according to the method previously described by Mohr and Trounson (Mohr and Trounson, 1980). Briefly, oocytes were treated with 2.5 µg/mL FDA in PBS supplemented with 5 mg/mL BSA at 38.5 ºC for 2 min in a dark room and then they were washed three times in PBS supplemented with 5 mg/mL BSA and evaluated under a fluorescent microscope (IX71, Olympus, Tokyo, Japan) with UV irradiation using a U-MWIB3 filter with an excitation wavelength of 460 to 495 nm and emission at 510 nm. Oocytes expressing a bright green fluorescence were regarded as living ones and were used subsequently (Figure 4.1).

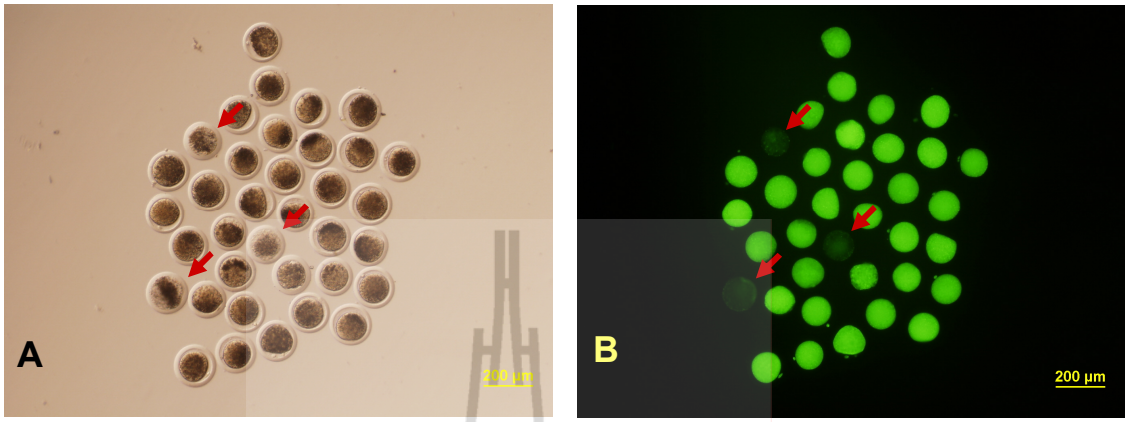

Figure4.1 FDA stained MII vitrified buffalo oocytes in bright field (A) and fluorescence image (B). Oocytes expressing a bright green fluorescence were regarded as living one, arrow indicated the dead oocytes following vitrification and FDA staining (magnification 200×).

4.3.6 PA of oocytes

The M-II oocytes were subjected to the PA treatment as previously described (Laowtammathron et al., 2005). Briefly, the oocytes were first treated with 7% ethanol in the EHM for 5 min at room temperature, and then incubated with 10 µg/mL CHX and 1.25 µg/mL cytochalasin D (CD) in mSOF medium + 10 % FBS under a humidified atmosphere of 5% CO_2 in air at 38.5 °C for 5 h.

4.3.7 ICSI and oocytes activation

Frozen spermatozoa were thawed at 37°C, and semen was placed in the bottom of snap –cap centrifuge tube containing 1 mL Brackett and Oliphant (BO) medium (Brackett and Oliphant, 1975) supplemented with 10 mM caffeine (caffeine-BO) for sperm swim up for 30 min. The top of 700 µL of medium was then collected and placed in a conical centrifuge tube. ICSI was performed according to the method of previous reported (Liang et al., 2010). Briefly, 1 µL sperm suspension was

transferred into oil-covered 5 μL 10% PVP solution and a single, motile buffalo spermatozoon was immobilized against the bottom of the dish, loaded tail first with a minimum volume of medium into the injection pipette and then injected into the cytoplasm of a buffalo oocyte. Within 1 h of the injection, the injected oocytes were activated by exposure to 7% ethanol in EHM for 5 min, and then subsequently cultured in TCM199 + 10% FBS for 3 h to allow extrusion of the second PB. Injected and activated oocytes which extruded the second PB were selected and transferred to mSOF medium supplemented with 10 μg/mL CHX and cultured for 5 h at 38.5 °C under humidified atmosphere of 5% CO_2 in air.

4.3.8 *In vitro* culture

The PA and ICSI oocytes were further cultured in mSOF medium (20 embryos/100 μL) under a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5 °C for 2 days. Thereafter, embryos at the 8-cell stage were selected and co-cultured with bovine oviductal epithelium cells in mSOF medium under a humidified atmosphere of 5% CO_2 in air at 38.5 °C for 5 days, as previously reported (Parnpai et al., 1999). Half of the medium was replaced with fresh medium every day and the embryo development was recorded at the same time as medium changing. The cleavage rates were recorded on Day 2 (the day of PA or ICSI was considered as Day 0), and the development of embryos to blastocyst stage was recorded on Day 7. In ICSI experiment, the rates of cleavage and blastocyst formation were calculated out of fertilized oocytes that extruded the second PB after ICSI.

4.3.9 Differential cell staining

The blastocysts harvested on Day 7 were stained to distinguish cells of the inner cell mass (ICM) and trophectoderm (TE), as previously reported (Suteevun et al.,

2006). Briefly, the ZP was removed from each blastocyst by exposure to 0.5% protease. After washing with mDPBS supplemented with 0.1% PVP, the zona-free blastocysts were incubated in 100 µL of 10% rabbit anti-buffalo spleenocyte antibodies for 45 min, and then transferred into a 100 µL mixture of 10% guinea pig complement, 10 µg/mL propidium iodide and 10 µg/mL Hoechst 33258 for 45 min. The blatocysts were mounted on glass slides with glycerol and covered with a cover slide. The ICM (blue) and TE cells (red) were counted under a fluorescent microscope at 330 to 380 nm, allowing determination of the total number of cells for blastocysts and the percentage of ICM cells based on the total number of cells (Figure 4.2).

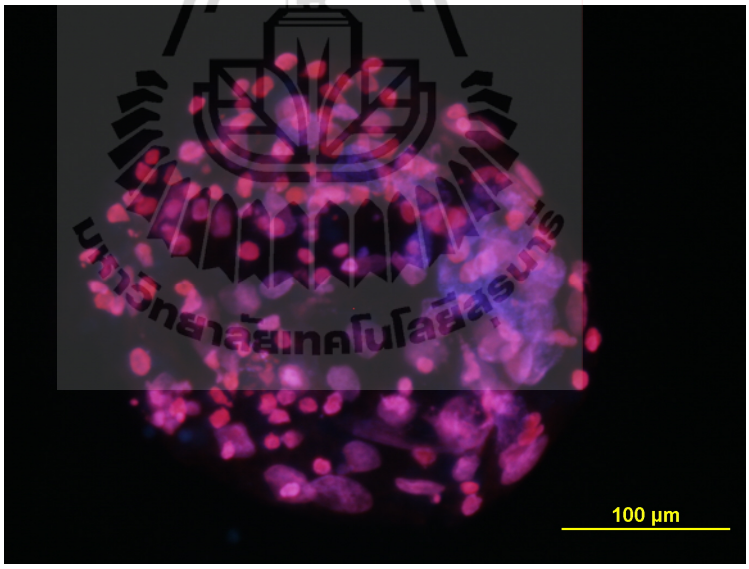

Figure 4.2 Fluorescence image of a blastocyst. Red represents Propidium iodide stain of trophectoderm cells and blue represents Hoechst stained inner cell mass cells (magnification 100×).

4.3.10 Statistical analysis

The experiments were replicated at least three times in each treatment group. The data for blastocysts cell numbers were expressed as mean±SD. As regards

the comparison of oocytes survival rate and embryo development among control groups and vitrified groups, one-way ANOVA and CRD using the Statistical Analysis Systems software (SAS Inst. INC.,Cary, N.C.,USA) were analyzed in order to determine whether difference between these test groups existed at a statistically significant level. Analysis of Variance (ANOVA) and Comparison of means by Duncan's Multiple Range Test (DMRT) were analyzed.

4.4 Results

4.4.1 Parthenogenetic development of CPA-treated oocytes.

Survival and *in vitro* development of buffalo M-II oocytes following CPA-exposure and PA treatment are shown in Table 4.1. The viability of oocytes determined by the FDA test in all groups of CPA treatment and the control were 100%. The cleavage rate and embryo development in the 1 min+30 s and 1min+45 s groups were significantly higher than in the 1 min+60 s group, but lower than in the FDA-exposed and control groups. The blastocyst formation rate did not significantly differ among the 1 min+30 s (16%), FDA-exposed (22%) and control groups (26%), but they were significantly higher than those of the 1 min+45 s (10%) and 1 min+60 s (2%) groups. The number of total and ICM cells were similar among the 1 min+30 s, 1 min+45 s, FDA-exposed and control groups and were higher than those in the 1 min+60 s group.

4.4.2 Parthenogenetic development of vitrified oocytes.

In vitro development of oocytes following vitrification and PA treatment is shown in Table 4.2. The oocyte viability rates among the vitrified 1 min+30 s (97%), 1 min+45 s (95%) and the control groups (100%) did not differ. The cleavage and

blastocyst rates were influenced when the oocytes were subjected to vitrification. The development of PA oocytes to the blastocyst stage in the vitrified 1 min+30 s group (8%) was significantly higher than that in the vitrified 1 min+45 s group (4%) but significantly lower than those in the FDA-exposed (24%) and control groups (26%). The total cell numbers of blastocysts in the 1 min+30 s (71.6±18.3) and 1 min+45 s (69.9±19.6) groups were not significantly different from that in the FDA-exposed (78.5±24.6) and control (80.0±23.1) groups. There was no difference in the ICM cell numbers among the four groups.

4.4.3 *In vitro* development following vitrification and ICSI.

In vitro development of buffalo M-II oocytes following vitrification and ICSI treatment are shown in Table 4.3. The oocytes viability rates among the vitrified 1 min+30 s (96%), 1 min+45 s (91%) and control (100%) groups did not differ. After activation of ICSI oocytes by ethanol, the second PB extrusion rate in the 1 min+30 s (43%) group was significantly higher than that in the 1 min+45 s group (35%) but significantly lower than those in the FDA-exposed (56%) and control groups (59%). The cleavage and blastocyst formation rates in the 1 min+30 s group (67 and 11%, respectively) were also significantly higher than those in the vitrified 1 min+45 s group (50 and 7%, respectively) and significantly lower than those in the FDA-exposed (86% and 21%, respectively) and the control groups (86% and 23%, respectively). There was no significant difference in total cell numbers among the four groups. However, the TE cell numbers of both control groups were significantly higher than those of vitrified groups.

Table 4.1 *In vitro* development of buffalo M-II* oocytes after exposure to CPA and the quality of blastocysts following PA.

Treatment groups	No. oocytes	FDA viability (%)	Cleavage (%)	No. (%) of oocytes developed to				No. of BL examined	Mean (±S.D.) blastocyst cell No.		
				8-C	16-C	Mor	BL		Total cell	TE cell	ICM cell
1min+30s	111	111(100)	97(87)^{b}	64(58)^{ab}	36(32)^{b}	25(23)^{b}	18(16)^{ab}	8	68.2±21.4^{ab}	48.1±6.3	19.6±8.4^{ab}
1min+45s	111	111(100)	92(83)^{b}	59(53)^{b}	31(28)^{b}	20(18)^{b}	11(10)^{bc}	5	70.7±17.5^{ab}	49.5±7.1	19.1±7.3^{ab}
1min＋60s	111	111(100)	76(68)^{c}	42(38)^{c}	17(15)^{c}	8(7)^{c}	2(2)^{c}	2	62.0±9.0^{b}	48.5±7.5	13.5±1.5^{b}
FDA-exposed	96	96(100)	85(89)^{ab}	64(67)^{ab}	43(45)^{a}	33(34)^{a}	21(22)^{a}	8	76.6±19.5^{a}	55.2±5.1	20.1±7.5^{a}
Control	96	-	90(94)^{a}	66(69)^{a}	46(48)^{a}	35(36)^{a}	25(26)^{a}	8	77.1±17.8^{a}	56.0±6.4	20.9±6.7^{a}

* Metaphase of the second meiotic cell division.

^{a,b,c} Means within columns with different superscripts differ (P<0.01).

Treatment groups: 1min+30s = 1 min equilibration followed by 30 sec of exposure to vitrification solution;

1min+45s = 1 min equilibration followed by 45 sec of exposure to vitrification solution;

1min+60s = 1 min equilibration followed by 60 sec of exposure to vitrification solution.

Abbreviations: 8-C = 8-cell stage; 16-C = 16-cell stage; Mor = morula; BL = blastocyst; TE = trophectoderm; ICM = inner cell mass.

Table 4.2 *In vitro* development of buffalo M-II* oocytes and quality of blastocysts following vitrification and PA.

Treatment groups	No. oocytes	FDA viability (%)	Cleavage (%)	No. (%) of oocytes developed to				No. of BL examined	Mean (±S.D.) blastocyst cell No.		
				8-C	16-C	Mor	BL		Total cell	TE cell	ICM cell
1min+30s	130	126(97)[ab]	84(67)[b]	42(33)[b]	24(19)[b]	15(12)[b]	10(8)[b]	9	71.6±18.3	49.1±4.7	21.4±9.5
1min+45s	132	126(95)[b]	58(46)[c]	23(18)[c]	10(8)[c]	7(6)[c]	5(4)[c]	5	69.9±19.6	48.2±4.3	20.2±5.8
FDA-exposed	118	118(100)[a]	105(89)[a]	81(69)[a]	53(45)[a]	41(35)[a]	28(24)[a]	10	78.5±24.6	59.0±6.1	22.7±4.2
Control	117	-	110(94)[a]	82(70)[a]	56(48)[a]	43(37)[a]	31(26)[a]	10	80.0±23.1	59.2±5.3	23.8±6.0

* Metaphase of the second meiotic cell division.

[a,b,c] Means within columns with different superscripts differ ($P<0.01$).

Treatment groups: 1min+30s = 1 min equilibration followed by 30 sec of exposure to vitrification solution;

1min+45s = 1 min equilibration followed by 45 sec of exposure to vitrification solution;

Abbreviations: 8-C = 8-cell stage; 16-C = 16-cell stage; Mor = morula; BL = blastocyst; TE = trophectoderm; ICM = inner cell mass.

Table 4.3 *In vitro* development of buffalo M-II* oocytes and quality of blastocysts following vitrification and ICSI.

Treatment groups	No. oocytes	FDA viability (%)	ICSI success (%)	2nd P B (%)	Cleavage (%)	No. (%) of oocytes developed to				No. of BL examined	Mean (±S.D.) blastocyst cell No.		
						8-C	16-C	Mor	BL		Total cell	TE cell	ICM cell
1min+30s	147	141(96)^{ab}	141(100)	61(43)^{b}	41(67)^{b}	24(39)^{b}	17(28)^{b}	9(15)^{b}	7(11)^{b}	5	99.1±11.2	61.0±9.1^{b}	31.2±6.7
1min+45s	146	133(91)^{b}	132(99)	46(35)^{c}	23(50)^{c}	14(30)^{b}	8(17)^{c}	5(11)^{b}	3(7)^{c}	3	94.8±14.7	60.2±5.8^{b}	30.5±9.6
FDA-exposed	126	126(100)^{a}	126(100)	70(56)^{a}	60(86)^{a}	47(67)^{a}	31(44)^{a}	23(33)^{a}	15(21)^{a}	9	101.7±12.3	70.0±6.8^{a}	32.2±4.5
Control	130	-	130(100)	77(59)^{a}	66(86)^{a}	50(65)^{a}	34(44)^{a}	24(31)^{a}	18(23)^{a}	9	104.2±10.6	69.2±8.6^{a}	35.5±3.1

* Metaphase of the second meiotic cell division.

^{a,b,c} Means within columns with different superscripts differ (P<0.01).

Treatment groups: 1min+30s = 1 min equilibration followed by 30 sec of exposure to vitrification solution;

1min+45s = 1 min equilibration followed by 45 sec of exposure to vitrification solution;

Abbreviations: 8-C = 8-cell stage; 16-C = 16-cell stage; Mor = morula; BL = blastocyst; TE = trophectoderm; ICM = inner cell mass.

Rates of cleavage, 8-C, 16-C, Mor and BL were calculated out of fertilized (2nd P B) oocytes.

4.5 Discussion

The major finding of this study is that buffalo M-II oocytes could be successfully cryopreserved by vitrification with microdrop method which was verified by their development to the blastocyst stage after PA and ICSI. Vitrification is a simple, rapid and cost-effective method of cryopreserving mammalian cells. Using this method, cryopreserved cells are less likely to experience solution effects and intracellular ice formation (Fahy et al., 1984). However, a negative consequence of this method is believed to be the increased probability of different kinds of stresses other than ice crystal formation such as osmotic injury, toxic effect of cryoprotectants, concentrated intracellular electrolytes or zona hardening (Vajta 2000). To achieve vitrification of solutions, radical increases of cooling rates are required.

The microdrop method involves dropping the oocyte-containing solution directly into liquid nitrogen. The success of this method is due to elimination of the insulation effect of the container wall. Warming of the oocytes is equally rapid when vitrified samples are directly dropped into a warm solution (Papis et al., 2000). In the present study more than 90% of the vitrified-warmed oocytes following microdrop method had a normal esterase enzyme activity as determined by FDA staining, proving that microdrop method was effective for buffalo M-II oocytes cryopreservation. One essential factor in cryosurvival is the permeation of a certain amount of CPA into the oocyte, increasing with the duration of exposure. However, the toxicity of the CPA must be considered, with an optimum exposure time being favorable for this purpose.

In the present study, we investigated the effects of different exposure time to equilibration/vitrification solutions. The survival and developmental rates in the

1min+30 s group were higher than those in 1 min+45 s or 1 min+60 s groups. Toxicity testing of different exposure time to equilibration/vitrification solutions showed that the embryo developmental rate decreased with increasing exposure time. Parthenogenetic development of buffalo M-II oocytes after CPA treatment in the 1 min+30 s group did not differ significantly from control groups, suggesting that exposure of oocytes to equilibration solution for 1 min and vitrification solution for 30 s without cooling and warming processes did not affect the embryo development. However, increasing the exposure time to vitrification solution to 60 s resulted in a reduction in the embryo developmental rate, probably due to excessive permeation of the CPA into the oocyte causing a higher toxicity level in the 1min+60s treatment. In mouse, bovine and rabbit oocytes, exposure to CPA can result in microtubule disorganization (Johnson and Pickering, 1987; Williams et al., 1992; Vincent et al., 1989). The very short exposure time to a high concentration of EG before cooling may reduce its toxic effects.

This study has demonstrated that vitrification applied on buffalo oocytes decreased second PB formation, cleavage and blastocyst rates when compared with fresh oocytes. For successful ICSI in bovine and buffalo species, applying activation to oocytes is considered to be a key factor (Keefer et al., 1990). The second PB formation is often taken to indicate the ability of oocytes to accomplish meiosis. Our study revealed that the vitrification and warming procedure could reduce the ability of oocytes to accomplish the second meiosis division, which might be caused by the altered spindle formation. A possible reason may be damage to the meiotic spindle, as has been frequently observed in cryopreserved oocytes (Chen et al., 2003). Freezing injury would lead to spindle disorganization, microtubules loss or clumping and

premature cortical granules which can cause zona hardening (Carroll et al., 1990; Aman and Parks, 1994). The ICSI into frozen-thawed bovine oocytes enhanced the pronucleus formation, cleavage and blastocyst development compared with IVF (Rho et al., 2004), suggesting that the release of cortical granules from vitrified oocytes led to zona-hardening and low fertilization rates (Hochi et al., 1998). In our study, cleavage and blastocyst rates in the ICSI control groups were significantly higher than those of vitrified groups. The rate of development to the blastocyst stage in the 1min+30s group was significantly higher than that in the 1min+45s group and significantly lower than those in control groups, but did not differ between the FDA-exposed and control groups. Similar observations were made for the total cell numbers of Day 7 blastocysts. This indicated that the vitrification procedure resulted in retarded embryonic development and that 30 s exposure to the vitrification solution was the most effective regimen for buffalo M-II oocyte vitrification. For bovine oocytes, 25-35 s for the final equilibration seem to be a general agreement about the rather optimal protocol (Vajta et al., 1998; Papis et al., 2000; Martino et al., 1996) which probably derived from some early experiements (Papis et al., 1995). Our research is also similar to recent studies of buffalo oocytes vitrification on 30 s of final equilibration for the same cryoprotectants and OPS vitrification method (Sharma et al., 2010).

In our study, oocyte viability was determined by FDA which selectively stains cells with normal esterase enzyme activity and oolemma integrity. We compared the control and FDA-exposed oocytes treated with FDA to determine the toxicity of the FDA staining procedure. Our results showed no difference in the second PB extrusion rates, embryo development and blastocyst cell numbers between FDA treated and

untreated control oocytes. This indicates that FDA staining does not have any negative effects upon embryo development *in vitro* and thus can be safely used for viability testing of oocytes.

To the best of our knowledge, the present study is the first report of ICSI-derived blastocysts from buffalo oocytes vitrified at the M-II stage by microdrop method. In conclusion, microdrop, a simple, economical and rapid procedure, allowed M-II buffalo oocytes to be vitrified, fertilized by ICSI and to develop to the blastocysts stage. The results of this study demonstrated that buffalo oocytes vitrified by the microdrop method with the 1 min+30 s regimen could yield reasonable blastocyst formation rates after ICSI. Furthermore, the FDA staining for viability checking had no detrimental effect on the embryo development *in vitro*.

4.6 References

Aman, R.R., and Parks, J.E. (1994). Effects of cooling and rewarming on the meiotic spindle and chromosomes of *in vitro*-matured bovine oocytes. **Biol. Reprod.** 50: 103-110.

Boonkusol, D., Faisaikarm, T., Dinnyes, A., and Kitiyanant, Y. (2007). Effects of vitrification procedures on subsequent development and ultrastructure of *in vitro*-matured swamp buffalo (*Bubalus bubalis*) oocytes. **Reprod. Fertil. Dev**. 19:383-391.

Brackett, B.G., and Oliphant, G. (1975). Capacitation of rabbit spermatozoa *in vitro*. **Biol. Reprod.** 12: 260-274.

Carroll, J., Depyere, H., and Matthews, C.D. (1990). Freeze-thaw-induced changes of the zona pellucida explains decreased rates of fertilization in frozen-thawed

mouse oocytes. **J. Reprod. Fertil.** 90: 547-553.

Chen, S.U., Lien, Y.R., Chao, K.H., Ho, H.N., Yang, Y.S., and Lee, T.Y. (2003). Effects of cryopreservation on meiotic spindles of oocytes and its dynamics after thawing: clinical implications in oocyte freezing – a review article. **Mol. Cell. Endocrinol.** 202: 101-107.

Chian, R., Kuwayama, M., Tan, L, Tan, J., Kato, O., and Nagai, T. (2004). High survival rate of bovine oocytes matured *in vitro* following vitrification. **J. Reprod. Dev.** 50: 685-696.

Dhali, A., Manik, R.S., Das, S.K., Singla, S.K., and Palta, P. (2000). Vitrification of buffalo (*Bubalus bulalis*) oocytes. **Theriogenology** 53: 1295-1303.

Dinnyés, A., Dai, Y., Jiang, S., and Yang, X. (2000). High developmental rates of vitrified bovine oocytes following parthenogenetic activation, *in vitro* fertilization, and somatic cell nuclear transfer. **Biol. Reprod.** 63: 513-518.

Fahy, G.M., McFralane, D.R., Angell, C.A., and Meryman, H.T. (1984). Vitrification as an approach to cryopreservation. **Cryobiology** 21: 407-426.

Gardner, D.K., Lane, M., Spitzer, A., and Batt, P.A. (1994). Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage *in vitro* in the absence of serum and somatic cells: amino acids, vitamins, and culturing embryos in groups stimulate development. **Biol. Reprod.** 50: 390-400.

Gasparrini, B., Attanasio, L., De Rosa, A., Monaco, E., Di Palo, R., and Campanile, G. (2007). Cryopreservation of *in vitro* matured buffalo (*Bubalus bubalis*) oocytes by minimum volumes vitrification methods. **Anim. Reprod. Sci.** 98: 335-342.

Gautam, S.K., Verma, V., Palta, P., Chauhan, M.S., and Manik, R.S. (2008). Effect of type of cryoprotectant on morphology and developmental competence of *in vitro*-matured buffalo (*Bubalus bubalis*) oocytes subjected to slow freezing or vitrification. **Reprod. Fertil. Dev.** 20: 490-496.

Hochi, S., Fujimoto, T., Braun, J., and Oguri, N. (1994). Pregnancies following transfer of equine embryos cryopreserved by vitrification. **Theriogenology** 42: 483-488.

Hochi, S., Ito, K., Hirabayashi, M., Ueda, M., Kimura, K., and Hanada, A. (1998). Effect of nuclear stages during IVM on the survival of vitrified-warmed bovine oocytes. **Theriogenology** 49: 787-796.

Johnson, M.H., and Pickering, S.J. (1987). Effects of dimethylsulphoxide on the microtubular system of mouse oocytes. **Development** 100: 313-324.

Karlsson, J.O., Eroglu, A., Toth, T.L., Cravalho, E.G., and Toner, M. (1996). Fertilization and development of mouse oocytes cryopreserved using theoretically optimized protocol. **Hum. Reprod.** 11: 1296-1305.

Kazem, R., Thompson, L.A., Srikantharajah, A., Laing, M.A., Hamilton, M.P., and Templeton, A. (1995). Cryopreservation of human oocytes and fertilization by two techniques: In-vitro fertilization and intracytoplasmic sperm injection. **Hum. Reprod.** 10: 2650-2654.

Keefer, C.L., Younis, A.I., and Brackett, B.G. (1990). Cleavage development of bovine oocytes fertilized by sperm injection. **Mol. Reprod. Dev.** 25: 281-285.

Kuwayama, M., and Kato, O. (2000). All-round vitrification method for human oocytes and embryos. **J. Assist. Reprod. Genet.** 17: 477 (abstract).

Landa, V., and Tepla, O. (1990). Cryopreservation of mouse 8-cell embryos in microdrops. **Folia. Biologica (Praha)** 6: 153-158.

Lane, M., Bavister, B.D., Lyons, E.A., and Forest, K.T. (1999). Containerless vitrification of mammalian oocytes and embryos. **Nat. Biotechnol.** 17: 1234-1236.

Laowtammathron, C., Lorthonpanich, C., Ketudat-Cairns, M., Hochi, S., and Parnpai, R. (2005). Factors affecting cryosurvival of nuclear-transferred bovine and swamp buffalo blastocysts: effects of hatching stage, linoleic acid-albumin in IVC medium and Ficoll supplementation to vitrification solution. **Theriogenology** 64: 1185-1196.

Liang, Y.Y., Ye, D.N., Laowtammathron, C., Phermthai, T., Nagai, T., Somfai, T., and Parnpai, R. (2010). Effect of chemical activation treatment on development of swamp buffalo (*Bubalus bubalis*) oocytes matured *in vitro* and fertilized by intracytoplasmic sperm injection. **Reprod. Dom. Anim.** 46: e67-73.

Mahmoud, K.G., Scholkamy, T.H., Ahmed, Y.F., Seidel Jr, G.E., and Nawito, M.F. (2010). Effect of different combinations of cryoprotectants on *in vitro* maturation of immature buffalo (*Bubalus bubalis*) oocytes vitrified by straw and open-pulled straw methods. **Reprod. Dom. Anim.** 45: 565-571.

Martino, A.N., Songsasen, S., and Leibo, S.P. (1996). Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. **Biol. Reprod.** 54:1059-1069.

Mavrides, A., and Morroll, D. (2002). Cryopreservation of bovine oocytes: is cryoloop vitrification the future to preserving the female gamete? **Reprod. Nutr. Dev.** 42: 73–80.

Mohr, L.R., and Trounson, A.O. (1980). The use of fluorescein diacetate to assess embryo viability in the mouse. **J. Reprod. Fertil.** 58:189-196.

Muenthaisong, S., Laowtammathron, C., Ketudat-Cairns, M., Parnpai, R., and Hochi, S. (2007). Quality analysis of buffalo blastocysts derived from oocytes vitrified before or after enucleation and reconstructed with somatic cell nuclei. **Theriogenology** 67: 893-900.

Papis, K., Avery, B., Holm, P., Callesen, H., and Greve, T. (1995). The effect of vitrification solution, equilibration time, and direct dilution method on survivability of equilibrated or vitrified bovine *in vitro* matured oocytes. **Theriogenology** 43: 293.

Papis, K., Shimizu, M., and Izaike, Y. (1999). A highly efficient modified vitrification method for Day 3 *in vitro* produced bovine embryos. **Cryo. Letters.** 20: 203-206.

Papis, K., Shimizu, M., and Izaike, Y. (2000). Factors affecting the survivability of bovine oocytes vitrified in droplets. **Theriogenology** 54: 651-658.

Parnpai, R., Tasripoo, K., and Kamonpatana, M. (1999). Development of cloned swamp buffalo embryos derived from fetal fibroblasts: comparison *in vitro* cultured with or without buffalo and cattle oviductal epithelial cells. **Buffalo. J.** 15: 371-384.

Porcu, E., Fabbri, R., and Seracchioli, R. (1997). Birth a healthy female after intracytoplasmic sperm injection of cryopreserved human oocytes. **Fertil. Steril.** 68: 724-726.

Rho, G.J., Lee, S.L., Kim, Y.S., Yeo, H.J., Ock, S.A., Balasubramanian, S., and Choe, S.Y. (2004). Intracytoplasmic sperm injection of frozen-thawed bovine

oocytes and subsequent embryo development. **Mol. Reprod. Dev.** 68: 449-455.

Riha, J., Landa, V., Kneiss, J., Matus, J., Jindra, J., and Kloucek, Z. (1991). Vitrification of cattle embryos by direct dropping into liquid nitrogen and embryo survival after nonsurgical transfer. **Zivoc. Vyroba.** 36: 113-119.

Sharma, G.T., and Loganathasamy, K. (2007). Effect of meiotic stages during *in vitro* maturation on the survival of vitrified-warmed buffalo oocytes. **Vet. Res. Commun.** 31: 881-893.

Sharma, G.T., Dubey, P.K., and Chandra, V. (2010). Morphological changes, DNA damage and developmental competence of *in vitro* matured, vitrified-thawed buffalo (*Bubalus bubalis*) oocytes: A comparative study of two cryoprotectants and two cryodevices. **Cryobiology** 60: 315-321.

Suteevun, T., Smith, S.L., Muenthaisong, S., Yang, X., Parnpai, R., and Tian, X.C. (2006). Anomalous mRNA levels of chromatin remodeling genes in swamp buffalo (*Bubalus bubalis*) cloned embryos. **Theriogenology** 65: 1704-1715.

Vajta, G. (1997). Vitrification of bovine oocytes and embryos. **Embryo Transfer Newsletter** 15: 12-18.

Vajta, G., Holm, P., Kuwayama, M., Booth, P.J., Jacobson, H., Greve, T., and Callesen, H. (1998). Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. **Mol. Reprod. Dev.** 51: 53-58.

Vajta, G. (2000). Vitrification of the oocytes and embryos of domestic animals. **Anim. Reprod. Sci.** 60-61: 357-364

Vajta, G., and Nagy, Z.P. (2006). Are programmable freezers still needed in the embryo laboratory? Review on vitrification. **Reprod. Online** 12: 779-796.

Vincent, C., Garnier, V., Heyman, Y., Renard, J.P. (1989). Solvent effects on cytoskeletal organization and in-vivo survival after freezing of rabbit oocytes. **J. Reprod. Fertil.** 87: 809–820.

Vincent, C., Pickering, SJ., and Johnson, M.H. (1990). The hardening effect of dimethylsulfoxide on the mouse zona pellucida requires the presence of an oocytes and is associated with a reduction in the number of cortical granules present. **J. Reprod. Fertil.** 89: 253-259.

Wani, N.A., Maurya, S.N., Misra, A.K., Saxena, V.B., and Lakhchaura, B.D. (2004). Effect of cryoprotectants and their concentration on *in vitro* development of vitrified- warmed immature oocytes in buffalo (Bubalus bulalis). **Theriogenology** 61: 831-842.

Williams, M.R., Richardson, R.R., and Parks, J.E. (1992). Effects of cryoprotective agents (CPAs) on the cytoskeleton and meiotic spindle apparatus of bovine oocytes. **Cryobiology** 29: 757.

Yang, B.S., and Leibo, S.P. (1999). Viability of *in vitro*-derived bovine zygotes cryopreserved in microdrops. **Theriogenology** 51: 178 (Abstract)

Yavin, S., and Arav, A. (2007). Measurement of essential physical properties of vitrification solution. **Theriogenology** 67: 81-89.

CHAPTER V

SURVIVAL RATES OF MATURED BUFFALO OOCYTES AFTER VITRIFICATION BY MICRODROP AND CRYOTOP AND SUBSEQUENT EMBRYOS DEVELOPMENT AFTER INTRACYTOPLASMIC SPERM INJECTION

5.1 Abstract

In the present study, effects of two kinds of vitrification solution with Microdrop and Cryotop were examined on the survival rates, pronuclear formation, and developmental competence following ICSI of vitrified-warmed MII buffalo oocytes. IVM oocytes were randomly separated and vitrified by 1) Microdrop with VA solution (10% DMSO + 10% EG for 1 min, 20% DMSO + 20%EG + 0.5M sucrose for 30 sec) (VA +Microdrop), 2) Cryotop with VA solution (VA+Cryotop), 3) Microdrop with VB solution (4% EG for 12-15 min, 35% EG+ 50 mg/mL PVP and 0.4 M trehalose for 30 sec) (VB +Microdrop), 4) Cryotop with VB solution (VB+Cryotop). Fresh groups were compared with FDA-exposed and control oocytes. The survival oocytes that evaluated by FDA staining were subjected to ICSI. After warming the recovery rates were similar among vitrified groups. The oocytes viability with VA solution (Microdrop: 93%, Cryotop: 97%, respectively) were significantly

higher than that with VB solution (Microdrop: 79%, Cryotop: 81%, respectively), but significantly lower than control groups (100%). Higher rate of oocytes formed 2 pronuclei in VA+Cryotop (20%) and VA+Microdrop (16%) groups compared to those in VB+Cryotop (11%) and VB+Microdrop (7%) groups, whereas FDA-exposed (46%) and control (48%) showed significantly higher rates than all the vitrified groups. The second polar body extrusion rate and the embryo developmental competence in FDA-exposed and control groups were significantly higher than all the vitrified groups, but no significantly difference was found among vitrified groups. There is no difference between control and fresh control group in second polar body extrusion and embryo development. In conclusion, VA solution can yield higher survival rate and 2 pronuclei formation rates of vitrified oocytes and Cryotop and Microdrop are equally suitable methods for buffalo oocytes vitrification. The FDA staining for viability checking had no detrimental effect on the *in vitro* embryo development.

5.2 Introduction

Cryopreservation of oocytes has great importance in buffalo for the efficient oocytes cryopreservation protocols will widen and improve the strategic implementation of reproductive technologies in the buffalo species.

Oocytes cryopreservation is still an open challenge in most mammalian species, due to the extreme sensitivity of gametes to chilling injuries. Vitrification is the process that induces a glass-like solidification of living cells during freezing. There are many species successfully cryopreserved by vitrification, such as bovine (Vieira et al., 2002), mouse (Wood et al., 1993), equine (Maclellan et al., 2002) and human oocytes (Katayama et al., 2003).The unique advantage of the vitrification

process is elimination of ice crystal formation, the most dangerous cause of cryoinjury. Many factors affected the efficacy of vitrification, such as cryoprotectant concentration, exposure time, container and temperature. There are some improved vitrification methods succeed in mammalian oocytes cryopreservation such as Cryotop (Kuwayama and Kato, 2000), Cryoloop (Oberstein et al., 2001), OPS (open pulled straw) (Vajta et al., 1998), GMP (glass micropipette) (Kong et al., 2000), Microdrop (Papis et al., 2000), and EMG (electron microscope grids) (Martino et al., 1996). Among the various methods of vitrification, Microdrop vitrification was easy and convenient, as it excluded the use of any specialized device using minimum volumes of vitrification to introduce oocytes into liquid nitrogen. One of the most successful ultra-rapid vitrification technique is the Cryotop vitrification that has resulted in excellent survival and developmental rates with human and bovine MII oocytes (Kuwayama et al., 2005) when cryopreserved oocytes were fertilized *in vitro*. Cryoprotectants influence the ability of buffalo oocytes to survive cryopreservation (Wani et al., 2004). Several studies demonstrated that EG would be the ideal cryoprotectant (Shaw et al. 1997; Cetin and Bastan 2006), because it penetrates membranes faster than glycerol (Cha et al. 2000) and is less toxic than other permeable cryoprotectants (Martino et al. 1996; Cha et al. 2000; Dinnyes et al. 2000). Moreover, cryoprotectant mixtures may have some advantages over solutions containing only one permeable cryoprotectant (Vajta et al. 1998; Chian et al. 2004).

In addition, change in the ZP has shown to induce lower fertility rates (Carroll et al., 1990; Vincent et al., 1990). These structural changes causing lower fertility could be overcome by micromanipulation technique such as ICSI (Carroll et al., 1990; Kazem et al., 1995; Karlsson et al., 1996; Porcu et al., 1997).

The aims of this study were to investigate the effects of two vitrification procedures Microdrop and Cryotop and two cryoprotectant procedures on the post-thaw viability, pronuclear formation and embryos developmental competence of vitrified buffalo MII oocytes subjected to ICSI.

5.3 Materials and methods

5.3.1 Oocyte collection and *in vitro* maturation

Buffalo ovaries were obtained from a slaughterhouse and transported to the laboratory within 4 h in physiological saline (0.9% NaCl). Cumulus-oocyte complexes (COCs) were collected from the follicles 2–8 mm in diameter and were matured *in vitro* for 22 h. The medium for IVM was TCM 199 supplemented with 10% fetal bovine serum, 0.02 AU/mL FSH(Antrin, Denka Pharmaceutical, Tokyo, Japan), 50 iu/mL hCG (Chorulon, Intervet, Boxmeer, Netherlands) and 1µg/mL estradiol-17ß. After maturation, cumulus cells were gently removed by pipetting, and the oocytes with a visible first polar body were selected for the following experiments. The Emcare holding medium (EHM, ICP Bio, Auckland, New Zealand) was used as the basal medium throughout the process of ICSI. The medium for embryo culture was modified synthetic oviduct fluid supplemented with amino acids and 0.3% fatty acid-free BSA (mSOF) (Gardner et al., 1994).

5.3.2 Vitrification and warming

The M-II oocytes were vitrified-warmed by either the Cryotop minimum volume cooling procedure, originally reported by Kuwayama and Kato (Kuwayama and Kato, 2000) or Microdrop vitrification method (Papis et al., 2000).

Two different cryoptotectant systems were used to vitrify buffalo MII

oocytes. Oocytes vitrified by VA method was described as following. *In vitro* matured oocytes were placed in TCM199-Hepes + 20% FBS (basic medium; BM) + 10% dimethylsulfoxide (DMSO) + 10% ethylene glycol (EG) for 1 min and then exposed in BM + 20% DMSO + 20% EG + 0.5 M sucrose for 30 sec. Finally, a group of 5 oocytes were directly dropped with about 2 μL vitrification solution into liquid nitrogen or placed on a sheet of each Cryotop in a small volume of the vitrification solution (<1μL). For storage, vitrified oocytes were kept in liquid nitrogen for 1-2 weeks. The vitrified Microdrops or Cryotops were warmed by directly immersing into 3 mL of 0.5 M sucrose in BM for 5 min, and then transferred to the BM for 5 min. Then they were kept in the BM under a humidified atmosphere of 5% CO_2 in air at 38.5 °C for 1 h.

VB method was originally described by Dinnyes (Dinnyés et al., 2000). Briefly, the oocytes were treated with an equilibration medium which consisted of 4% (v/v) EG in BM for 12 to 15 min at 38.5 °C followed by 35% (v/v) EG, 50 mg/mL PVP and 0.4 M trehalose for 30 s. Vitrified oocytes were warmed by transferring Microdrops/Cryotops into a warming solution (0.3 M trehalose in BM) at 38.5 ºC. One to 2 min later, the oocytes were consecutively transferred for 1-min into each 500-μL droplets of BM supplemented with 0.15 M, 0.075 M and 0.0375 M trehalose, respectively. And then they were cultured under a humidified atmosphere of 5% CO_2 in air at 38.5 °C for 1 h.

5.3.3 Experimental design

Oocytes vitrified by VA system using Microdrop (VA+Microdrop) and Cryotop (VA+Cryotop) methods were compared with oocytes vitrified by VB system using Microdrop (VB+Microdrop) and Cryotop (VB+Cryotop) methods. The

development of oocytes followed vitrification treatments were compared with vitrification-untreated but FDA stained oocytes (FDA-exposed). To text the possible side effects of FDA staining, the oocytes without vitrification and FDA treatments were also activated and cultured (control).

5.3.4 Evaluation of oocyte viability

Oocyte viability was evaluated by FDA staining according to the method previously described by Mohr and Trounson (Mohr and Trounson, 1980). Briefly, oocytes were treated with 2.5 µg/mL FDA in PBS supplemented with 5 mg/mL BSA at 38.5 ºC for 2 min in a dark room and then they were washed three times in PBS supplemented with 5 mg/mL BSA and evaluated under a fluorescent microscope (IX71, Olympus, Tokyo, Japan) with UV irradiation using a U-MWIB3 filter with excitation wavelength of 460–495 nm and emission at 510 nm. Oocytes expressing a bright green fluorescence were regarded as living ones and were used subsequently.

5.3.5 ICSI

Straw of frozen spermatozoa were thawed at 37 ºC for 30 sec. Thawed spermatozoa were gently placed to the bottom of the 1mL BO medium (Brackett and Oliphant, 1975), supplemented with 10 mM caffeine (caffeine-BO) in a centrifuge tube for sperm swim up for 30 min and then the supernatant was collected and centrifuged at 500×g for 5 min. The sperm pellet was washed twice with 1mL of caffeine-BO by centrifugation at 500×g for 5 min. The sperm pellet was resuspended in the caffeine-BO at concentration of 8×10^6 sperm/mL. ICSI was performed as previously described by Liang et al. (2010). Briefly, three droplets of media covered with mineral oil were prepared on the lid of 60 mm culture dish for the ICSI procedure; the first droplet was PVP solution (ICSI®, Vitrolife, Gothenburg, Sweden)

for washing the pipette, the second droplet was the sperm suspension diluted with PVP solution (1:5), and the third droplet was EHM for the ICSI procedure. Approximately 10 MII oocytes were placed in the droplet of EHM. A single, motile buffalo spermatozoon was immobilized against the bottom of the PVP droplet, loaded tail first with a minimum volume of medium into the injection pipette and then injected into the cytoplasm of a buffalo oocyte. Within 1 h of the injection, the injected oocytes were activated by exposure to 7% ethanol in EHM for 5 min, and then subsequently cultured in TCM199 + 10% FBS for 3 h to allow extrusion of the second PB. With the purpose of producing haploid activated oocytes for ICSI, the injected and activated oocytes which extruded the second PB were selected and transferred to mSOF medium supplemented with 10 µg/mL CHX and cultured for 5 h at 38.5 ºC under humidified atmosphere of 5% CO_2 in air.

5.3.6 Assessment of pronuclear formation after ICSI

The status of ICSI-derived pronuclear formation was assessed after 18 h of ICSI. Oocytes that extruded the second PB were mounted on glass slides and fixed with acetic alcohol (acetic acid: ethanol; 1:3) for 48 h and stained with 1% (w/v) aceto-orcein for observation under a phase-contrast microscope. The absences of a spermatozoon or pronucleus which only present 2 PB were considered as ICSI failure. The presences of male and female pronuclei were indentified as normal fertilization.

5.3.7 *In vitro* culture

The presumptive zygotes were cultured in the mSOF medium under a humidified atmosphere of 5% CO_2, 5% O_2, 90% N_2 at 38.5 ºC for 2 days. Thereafter, embryos at the 8-cell stage were selected and co-cultured with bovine oviductal epithelium cells (BOEC) under a humidified atmosphere of 5% CO_2 in air at 38.5 ºC

for 5 days (Parnpai et al., 1999). Half of the medium was replaced with fresh medium every day and the embryo development was recorded at the same time of medium changing. The cleavage rates were recorded on Day 2 (the day of ICSI was considered as Day 0), the development of embryos to blastocyst stage was recorded on Day 7.

5.3.8 Differential cell staining

The blastocysts harvested on Day 7 were stained to distinguish cells of the inner cell mass (ICM) and trophectoderm (TE), as previously reported (Suteevun et al., 2006). Briefly, the ZP of blastocysts was removed by exposure to 0.5% protease. After washing with mDPBS supplemented with 0.1% PVP, the zona-free blastocysts were incubated in 100 µL of 10% rabbit anti-buffalo spleenocyte antibodies for 45 min, and then transferred into a 100 µL mixture of 10% guinea pig complement, 10 µg/mL propidium iodide and 10 µg/mL Hoechst 33258 for 45 min. The blatocysts were mounted on glass slides with glycerol and covered with cover slide. The ICM (blue) and TE cells (red) were counted under a fluorescent microscope at 330–380 nm, allowing determination of the total number of cells for blastocysts and the percentage of ICM cells based on the total number of blastocysts.

5.3.9 Statistical analysis

Experiments were replicated at least three times in each treatment group. Data were analyzed by ANOVA using the statistical analysis systems. The differences between groups were considered to be statistically significant when $P < 0.05$.

5.4 Results

5.4.1 Effect of vitrification methods on recovery and viability of oocytes

Oocytes were vitrified either by Microdrop or Cryotop with VA or VB

solution (Figure 5.1). After warming, the recovery rate between Microdrop and Cryotop in VA solution (96 and 100%, respectively) was no difference, which was also found in VB solution for Microdrop and Cryotop (98 and 100%, respectively). Among these groups, there was no difference in the oocytes recovery rate when compared with control groups.

The viability of oocytes examined by FDA staining was illustrated in Figure 5.2. The proportion of live oocytes vitrified with VA solution was significantly higher than VB solution but lower than fresh control group (93-97%, 79-81% and 100% respectively). And the results of oocytes survival rate were nearly indentical for both Microdrop and Cryotop in same vitrification solution.

5.4.2 Pronuclear formation after ICSI of surviving oocytes

As shown in Table 5.1, the proportion of normal fertilization as judged by appearance of 2PN formation was significantly higher in VA+Microdrop (16%) and VA+Cryotop (20%) groups than in VB+Microdrop (7%) and VB+Cryotop (11%) groups among vitrification treatment groups. Control + FDA and Control (46% and 48%, respectively) showed significant higher 2PN formation rates than other treatment groups.

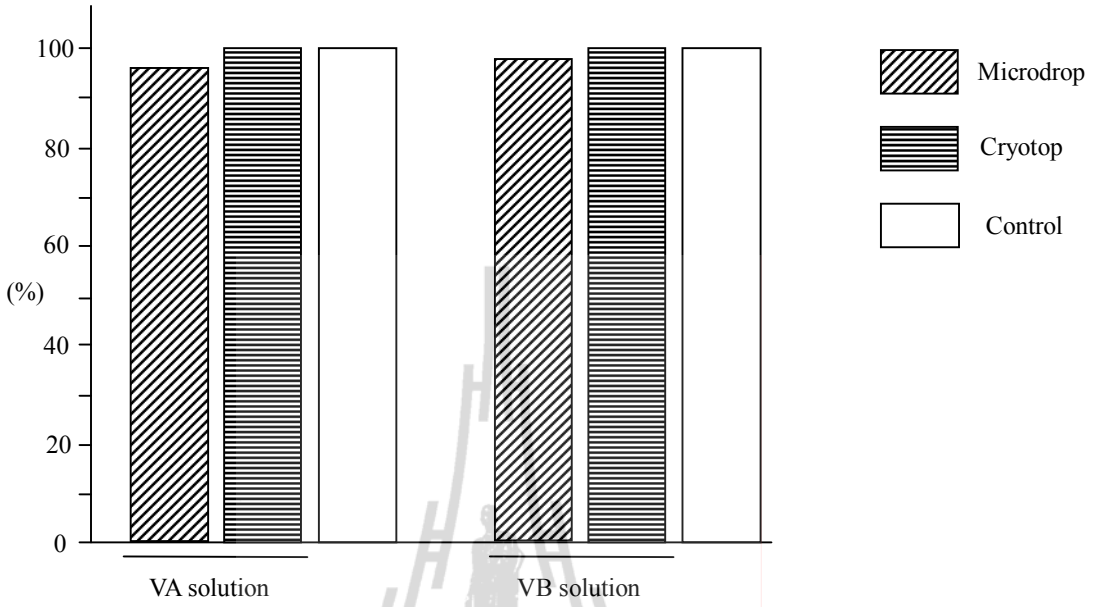

Figure 5.1 Recovery rate of vitrified-warmed buffalo MII oocyte.

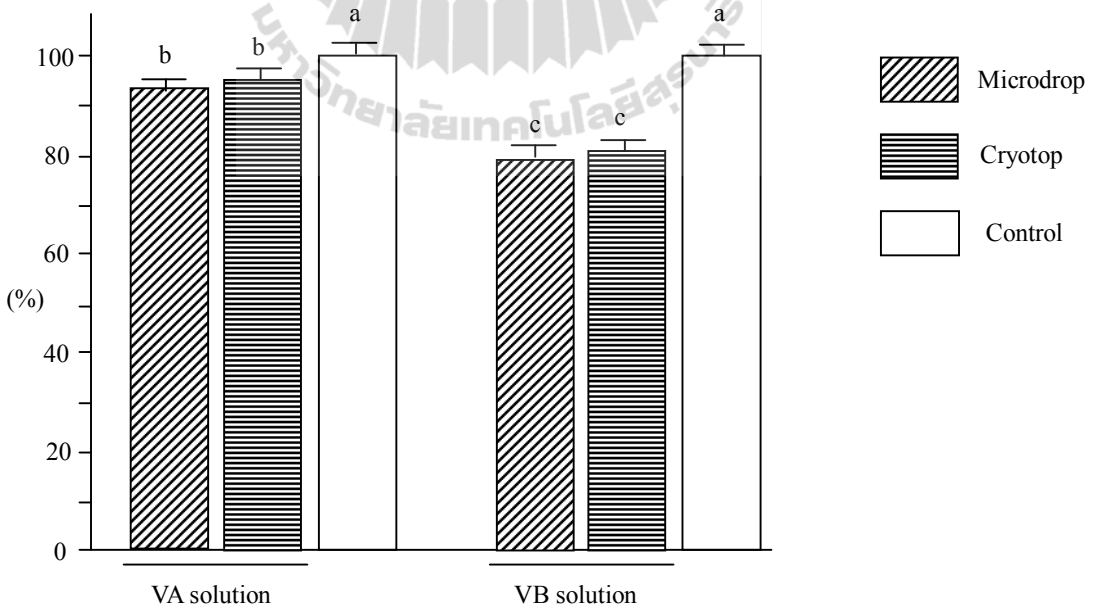

Figure 5.2 Survival rates of vitrified-warmed buffalo MII oocytes stained by FDA.

$^{a, b, c}$ Means within columns with different superscripts differ (P<0.05).

Table 5.1 Pronuclear formation at 18 h after ICSI.

Treatments	No. oocytes examined	No. (%) oocytes exhibiting			
		2PB+2PN[1]	2PB+1PN[2]	2PB+Sperm[3]	2PB[4]
VA+Microdrop	37	6(16)^{b}	15(41)^{a}	12(34)^{ab}	4(11)^{ab}
VA+Cryotop	40	8(20)^{b}	17(43)^{a}	9(23)^{b}	6(15)^{ab}
VB+Microdrop	30	2(7)^{bc}	10(33)^{ab}	13(43)^{a}	6(20)^{a}
VB+Cryotop	35	4(11)^{bc}	12(34)^{ab}	14(40)^{a}	5(14)^{ab}
FDA-exposed	41	19(46)^{a}	8(20)^{b}	9(22)^{b}	6(15)^{ab}
Control	44	21(48)^{a}	11(25)^{b}	7(16)^{b}	6(14)^{ab}

$^{a, b}$ Means within columns with different superscripts differ (P<0.05)

[1] Oocytes extruded 2 PB and had 2 pronuclei.

[2] Oocytes extruded 2 PB and had 1 pronucleus.

[3] Oocytes extruded 2 PB and had an intact sperm head.

[4] Oocytes extruded 2 PB and had no pronucleus.

5.4.3 Effect of vitrification methods on ICSI results

After ICSI, there was no difference in the second polar body formation rate between FDA-exposed (56%) and control (57%) but they were significantly higher than those of VA+Microdrop (39%), VB+ Microdrop (37%), VA+Cryotop (43%) and VB+Cryotop (40%). The second polar body formation between Microdrop and Cryotop were no significant difference in VA or VB. The cleavage rate of FDA-exposed (82%) and control (83%) was no significant difference, but they were significantly higher than those of vitrified treatments (65%-72%). The blastocyst rate of FDA-exposed (21%) and control (22%) was no significant difference, but they

were significantly higher than those of VA+Microdrop (8%), VB+ Microdrop (5%), VA+Cryotop (10%) and VB+Cryotop (11%). The blastocyst formation rate of vitrified treatment did not significantly different except VB+ Microdrop (5%) lower than other treatments (Table 5.2).

5.4.4 Cell number in day 7 ICSI-derived blastocyst from vitrified oocytes

The cell number of ICSI-derived blastocyst from vitrified oocytes was showed in Table 5.3. According to the blastocyst quality, there was no significantly difference among the vitrified groups and control groups, except that of VB+Microdrop treatment, which was significantly lower than that of control groups in the total cell number and ICM cell number.

Table 5.2 Development of ICSI derived embryos from vitrified-warmed buffalo MII oocytes.

Treatments	No. of ICSI	ICSI success	Second polar body	Cleavage rate	No. (%) of oocytes developed to			
					8-C	16-C	Mor	BL
VA+Microdrop	127	124(98)	48(39)^{b}	31(65)^{b}	16(33)^{b}	11(23)^{b}	5(10)^{b}	4(8)^{b}
VA+Cryotop	140	139(99)	60(43)^{b}	40(67)^{b}	23(38)^{b}	15(25)^{b}	9(15)^{b}	6(10)^{b}
VB+Microdrop	111	110(99)	41(37)^{b}	27(66)^{b}	15(37)^{b}	9(22)^{b}	3(7)^{b}	2(5)^{bc}
VB+Cryotop	117	116(99)	46(40)^{b}	33(72)^{ab}	20(43)^{b}	11(24)^{b}	7(15)^{b}	5(11)^{b}
FDA-exposed	111	111(100)	62(56)^{a}	51(82)^{a}	39(63)^{a}	25(40)^{a}	19(31)^{a}	13(21)^{a}
Fresh Control	115	115(100)	65(57)^{a}	54(83)^{a}	41(63)^{a}	25(38)^{a}	18(28)^{a}	14(22)^{a}

^{a,b,c} Means within columns with different superscripts differ (P<0.01).

Abbreviations: 8-C = 8-cell stage; 16-C = 16-cell stage; Mor = morula; BL = blastocyst

Table 5.3 Quality analysis of the vitrified-ICSI derived buffalo blastocyst.

Treatments	No. of embryos examined	Blastocyst cell numbers (Mean ± S.D)		
		Total cell	TE cell	ICM cell
VA+Microdrop	4	90.5 ± 12.0 [ab]	58.6 ± 7.1	30.7 ± 5.2 [a]
VA+Cryotop	6	92.6 ± 9.8 [ab]	56.0 ±10.2	32.6 ± 8.4 [a]
VB+Microdrop	2	72.0 ± 13.0 [b]	47.5 ± 8.5	24.5 ± 4.5 [b]
VB+Cryotop	5	87.5 ± 11.2 [ab]	53.2 ± 7.3	28.6 ± 7.1 [ab]
FDA-exposed	8	96.8 ± 14.3 [a]	62.5 ± 6.7	30.1 ± 8.5 [a]
Control	8	99.7 ± 9.1 [a]	63.0± 8.2	30.9 ± 6.4 [a]

[a,b] Means within columns with different superscripts differ (P<0.01).

5.5 Discussion

The major finding of this study is that buffalo MII oocytes could be cryopresered by vitrification with Microdrop and Cryotop methods and the oocytes were suitable for ICSI produce.

The recovery rate is the number of remaining oocytes after expulsion of solution from the container during warming. Its analysis is important because cells must tolerate a sequence of volumetric contractions and expansions because of different concentrations of solutions, and they can be lost or rupture during this process. Moreover, the recovery rate can be influenced by the container and vitrification solution as the oocytes might be lost during the last step of vitrification by sticking on the transfer pipette. After vitrification and warming of buffalo MII oocytes, 96-100% recovery rate was taken from both VA and VB solution, which demonstrated that this two vitrification solution and two containers did not affect the

oocytes recovery rate. The viability of vitrified oocytes after warming in VA solution was significantly higher than in VB solution, but the same vitrification solution Microdrop and Cryotop arrive the indentical survival rate. Our study revealed that VA method has the higher cryopreservation efficacy than VB system. It is important to consider the nature and concentration of the CPA for preservation of oocytes in any cryopreservation protocol. DMSO, glycerol, or EG alone or in combination protects the cells and tissues from freezing damage. Moreover, the cooling rate and freezing method are also important factors to consider in preventing cryoinjuries of cells.

Cryotop is a minimum volume procedure where the carrier tool, a thin film attached to a plastic holder is equipped with a protective plastic tube (Kuwayama et al., 2005). Oocytes are loaded on the film, the solution is almost entirely removed by aspiration, and the sample is immersed into liquid nitrogen. Subsequently, for safe storage, the plastic tube is pulled over the film. The method is easy to learn and perform, the cooling and warming rates are higher than those achievable with OPS, and the simple manipulation decreases the risk of inconsistency.

Microdrop method involves dropping an oocytes-containing solution directly into liquid nitrogen, the success of this method is due to elimination of the insulation effect of the container wall. Warming of the oocytes is equally rapid when vitrified sample are directly dropped into a warm solution (Paris et al., 2000). One essential factor in cryosurvival is permeation of a certain amount of cryoprotectants into the oocyte, which increase with the duration of exposure. However, the toxicity of the cryoprotectant must be avoided, with an optimum exposure time being favorable for this purpose.

In the result of fertilization status study, cleavage rates increased compared

with that of normal 2 pronuclear formation rates in the vitrified and control groups. This result indicated that not only vitrified oocytes but also un-vitrified oocytes could be parthenogenetically activated following ICSI. In general, oocytes activation and sperm decondensation was considered to be the main problems on ICSI in bovine (Keefer et al., 1990) and buffalo (Liang et al., 2010). The use of protein phosphorylation and synthesis inhibitors played important roles in bovine oocytes activation and further development, but also resulted in oocytes parthenogenetic activation. Many factors might be considered to be the cause of oocytes parthenogenetic activation, such as ICSI procedure, activation reagents (Liang et al., 2010), cryoprotectant exposure, vitrification-warming procedure (Tian et al., 2007). In buffalo ICSI (Liang et al., 2010), lower 2 pronuclear formation rates (42-62%) were compared with the following cleavage rates (52-78%), indicating the induction of parthenogenetic activation from ICSI, which corroborates our study. In addition, the mechanism of ZP hardening in cryopreserved oocytes remained unclear, but responsible for premature cortical granule exocytosis (Vincent et al., 1990; Fuku et al., 1992). The parthenogenetic activation from cryopreserved procedure might be the induction of calcium release following the cortical granule exocytosis (Wang et al., 1997).

In the present study, we investigated the effect of VA, VB methods and Microdrop, Cryotop technique on the oocyte viability after vitrified-warmed and embryo development following ICSI. This study has demonstrated that ICSI into frozen-thawed oocytes decreased the rates of second PB formation, cleavage and blastocyst development when compared with the fresh oocytes. The second PB formation was judged as the ability of oocytes accomplishing meiosis. Our study

revealed that frozen-thawed procedure could reduce the oocytes ability to accomplish the second meiosis.

Cryotop method that containing the most less vitrification solution, which can greatly increase the cooling speed had been applied to pronuclear-stage rabbit zygotes (Hochi et al., 2004), pre-hatching stage porcine embryos (Esaki et al., 2004), germinal vesicle-stage whale COCs (Iwayama et al., 2004), denuded M-II bovine oocytes (Chian et al., 2004), SCNTderived bovine and swamp buffalo blastocysts (Laowtammathron et al., 2005) and denuded M-II oocytes and IVF-derived blastocysts in humans (Kuwayama et al., 2000; Katayama et al., 2003), was successfully extended to both denuded M-II and enucleated swamp buffalo oocytes in the present study. On the other way, the success of Microdrop method is due to elimination of the insulation effect of the container wall. Warming of the oocytes is equally rapid when vitrified samples are directly dropped into a warm solution (Papis et al., 2000). Our results indicated that Cryotop and Microdrop are equally suitable methods for buffalo oocytes vitrification.

The higher survival rate were obtained from VA solution which using the CPA combination of EG+DMSO. This result may be due to oocytes membrane permeability differences in different cryoprotectants. The use of two permeable cryoprotectants rather than a single cryoprotectant enables the use of lower concentration of each cryoprotectant in the vitrification solution thereby reducing cryoprotectant toxicity (Sharma et al., 2010). Albarracyn et al., (2005) reported that oocytes exposed only to 20% EG and 20% DMSO showed a similar appearance to the control. 11.5% morula and 4.3% blastocyst development following oocytes vitrification by using 20% EG + 20% DMSO was reported in buffalo (Gautam et al.,

2008). Bovine (Vieira et al., 2007) and buffalo (Manjunatha et al., 2008) blastocyst vitrified in 20% EG + 20% DMSO shown a better hatching rate than those vitrified in 40% EG + 17.1% sucrose + 0.1 % polyvinyl alcohol. And similar reports also indicated that EG with DMSO when used as a cryoprotectant mixture may have some advantages over solutions having single penetrating cryoprotectants (Vajta et al., 1999).

In bovine, oocytes have higher permeability to DMSO than to EG (Agca et al., 1998), this may account for VA working better than VB according to the oocyte viability in our study. The reduction of the survival and embryo development could be resulting from possible multifactorial causes, including toxic effects of the CPA, ultrastructural damage to the oocytes, and deleterious effects on the chromosomes and other cytoplasmic structure (Johnson and Pickering 1987). Although the cryopreservation of bovine oocytes remains a challenge, some of the most encouraging results among domestic animals have been obtained in the bovine, where offspring have been born from immature and mature vitrified oocytes following IVF and culture (Papis et al., 2000; Vieira et al., 2002). Bovine oocytes are much more cryostable than porcine oocytes due to less lipid contents and intracellular lipid droplets and vesicles, thus porcine oocytes are much more difficult to successfully cryopreserve than bovine (Isachenko et al., 2001).

In the study, we found that development following FDA- exposed oocytes was not different compared with control oocytes in second PB extrusion rates, 2 pronuclei formation rates, embryo development rates and blastocyst cell numbers, but this two groups shown a significantly higher rates than any other 4 vitrified groups. This finding suggests that, despite our efforts to improve the vitrification method, the

cooling/thawing procedure and cryoprotectants negatively affected oocytes developmental competence, and FDA staining does not have any negative effects upon *in vitro* embryo development thus can be safely used for viability testing of oocytes.

In conclusion, from the higher survival rate of FDA stained oocytes, 2 pronucleal formation rates and ICM number among the vitrified groups, our study indicated that VA solution can work better than VB solution. Within the same solution, the comparison of Microdrop and Cryotop methods did not show the significant different. The development competence of frozen-thawed *in vitro* matured buffalo oocytes was significantly lower than fresh control oocytes, and the FDA staining for viability checking had no detrimental effect on the *in vitro* embryo development.

5.6 References

Agca, Y., Liu, J., Peter, A.T., Critser, E.S., and Critser, J.K. (1998). Effect of developmental stage on bovine oocyte plasma membrane water and cryoprotectant permeability characteristics. **Mol. Reprod. Dev.** 49: 408-415.

Albarracyn, J., Morato, R., Rojas, C., and Mogas, T. (2005). Effects of vitrification in open pulled straws on the cytology of *in vitro* matured prepubertal and adult bovine oocytes. **Theriogenology** 63: 890-901.

Brackett, B.G., and Oliphant, G. (1975). Capacitation of rabbit spermatozoa *in vitro*. **Biol. Reprod.** 12: 260-274.

Carroll, J., Depyere, H., and Matthews, C.D. (1990). Freeze-thaw-induced changes of the zona pellucida explains decreased rates of fertilization in frozen-thawed mouse oocytes. **J. Reprod. Fertil.** 90: 547-553.

Cetin, Y., and Bastan, A. (2006). Cryopreservation of immature bovine oocytes by vitrification in straws. **Anim. Reprod. Sci.** 92: 29-36.

Cha, K.Y., Chung, H.M., Lim, J.M., Ko, J.J., Han, S.Y., Choi, D.H., and Yoon, T.K. (2000). Freezing immature oocytes. **Mol. Cell. Endocrinol** 169: 43-47.

Chian, R.C., Kuwayama, M., Tan, L., Tan, J., Kato, O., and Nagai, T. (2004). High survival rate of bovine oocytes matured *in vitro* following vitrification. **J. Reprod. Dev.** 50: 685-696.

Dinnyés, A., Dai, Y., Jiang, S., and Yang, X. (2000). High developmental rates of vitrified bovine oocytes following parthenogenetic activation, *in vitro* fertilization, and somatic cell nuclear transfer. **Biol. Reprod.** 63: 513-518.

Esaki, R., Ueda, H., Kurome, M., Hirakawa, K., Tomii, R., and Yoshioka, H. (2004). Cryopreservation of porcine embryos derived from *in vitro* matured oocytes. **Biol. Reprod.** 71: 432-437.

Fuku, E., Kojima, T., Shioya, Y., Marcus, G.J., and Downey, B.R. (1992). *In vitro* ferilization and development of frozen-thawed bovine oocytes. **Cryobiology** 29: 485-492.

Gardner, D.K., Lane, M., Spitzer, A., and Batt, PA. (1994). Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage *in vitro* in the absence of serum and somatic cells: amino acids, vitamins, and culturing embryos in groups stimulate development. **Biol. Reprod.** 50: 390-400.

Gautam, S.K., Verma, V., Palta, P., Chauhan, M.S., and Manik, R.S. (2008). Effect of type of cryoprotectant on morphology and developmental competence of *in vitro*-matured buffalo (*Bubalus bubalis*) oocytes subjected to slow freezing or

vitrification. **Reprod. Fertil. Dev.** 20: 490-496.

Hochi, S., Terao, T., Kamei, M., Kato, M., Hirabayashi, M., and Hirao, M. (2004). Successful vitrification of pronuclear-stage rabbit zygotes by minimum volume cooling procedure. **Theriogenology** 61: 267-75.

Iwayama, H., Hochi, S., Kato, M., Hirabayashi, M., Kuwayama, M., and Ishikawa, H. (2004). Effects of cryodevice type and donors' sexual maturity on vitrification of minke whale (*Balaenoptera bonaerensis*) oocytes at germinal vesicle stage. **Zygote** 12: 333-338.

Isachenko, V., Isachenko, E., and Michelmann, H. W. (2001). "Lipolysis and ultrastructural changes of intracellular lipid vesicles after cooling of bovine and porcine GV-oocytes" **Anatomia, Histologia, Embryologia,** 30: 333-338.

Johnson, M.H., and Pickering, S.J. (1987). The effect of dimethylsulphoxide on the microtubular system of the mouse oocytes. **Development** 100: 313-324.

Karlsson, J.O., Eroglu, A., Toth, T.L., Cravalho, E.G., and Toner, M. (1996). Fertilization and development of mouse oocytes cryopreserved using theoretically optimized protocol. **Hum. Reprod.** 11: 1296-1305.

Katayama, K.P., Stehlik, J.B.S.,Kuwayama, M., Kato, O.M.D., and Stehlik, E.B.S. (2003). High survival rate of vitrified human oocytes results in clinical pregnancy. **Fertil. Steril.** 80: 223-224.

Kazem, R., Thompson, L.A., Srikantharajah, A., Laing, M.A., Hamilton, M.P., and Templeton, A. (1995). Cryopreservation of human oocytes and fertilization by two techniques: In-vitro fertilization and intracytoplasmic sperm injection. **Hum. Peprod.** 10: 2650-2654.

Keefer, C.L., Younis, A.I., and Brackett, B.G. (1990). Cleavage development of

bovine oocytes fertilized by sperm injection. **Mol. Reprod. Dev.** 25: 281-285.

Kuwayama, M., and Kato, O. (2000). All-round vitrification method for human oocytes and embryos. **J. Assist. Reprod. Genet.** 17: 477 (abstract).

Kuwayama, M., Vajta, G., Kato, O., and Leibo, S.P. (2005). Highly efficient vitrification method for cryopreservation of human oocytes. **Reprod. Biomed.** 11: 300-308.

Laowtammathron, C., Lorthonpanich, C., Ketudat-Cairns, M., Hochi, S., and Parnpai, R. (2005). Factors affecting cryosurvival of nuclear- transferred bovine and swamp buffalo blastocysts: effects of hatching stage, linoleic acid-albumin in IVC medium and Ficoll supplementation to vitrification solution. **Theriogenology** 64: 1185-1196.

Liang, Y.Y., Ye, D.N., Laowtammathron, C., Phermthai, T., Nagai, T., Somfai, T., and Parnpai, R. (2010). Effect of chemical activation treatment on development of swamp buffalo (*Bubalus bubalis*) oocytes matured *in vitro* and fertilized by intracytoplasmic sperm injection. **Reprod. Dom. Anim.** 46: e67-73.

Maclellan, L.J., Carnevale, E.M., Coutinho da Silva, M.A., Scoggin, C.F., Bruemmer, J.E., and Squires, E.L. (2002). Pregnancies from vitrified equine oocytes collected from super-stimulated and non-stimulated mares. **Theriogenology** 58: 911-919.

Manjunatha, B.M., Gupta, P.S., Ravindra, J.P., Devaraj, M., and Nandi, S. (2008). *In vitro* embryo development and blastocyst hatching rates following vitrification of river buffalo embryos produced from oocytes recovered from slaughterhouse ovaries of live animals by ovum pick-up. **Anim. Reprod. Sci.**

104: 419-426.

Martino, A., Songsasen, N., and Leibo, S.P. (1996). Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. **Biol. Reprod.** 54: 1059-1069.

Mohr, L.R., and Trounson, A.O. (1980). The use of fluorescein diacetate to assess embryo viability in the mouse. **J. Reprod. Fertil.** 58: 189-196.

Papis, K., Shimizu, M., and Izaike, Y. (2000). Factors affecting the survivability of bovine oocytes vitrified in droplets. **Theriogenology** 54: 651-658.

Parnpai, R., Tasripoo, K., and Kamonpatana, M. (1999). Development of cloned swamp buffalo embryos derived from fetal fibroblasts: comparison *in vitro* cultured with or without buffalo and cattle oviductal epithelial cells. **Buffalo J.** 15: 371-384.

Porcu, E., Fabbri, R., and Seracchioli, R. (1997). Birth a healthy female after intracytoplasmic sperm injection of cryopreserved human oocytes. **Fertil. Steril.** 68: 724-726.

Sharma, G.T., Dubey, P.K., and Chandra, V. (2010). Morphological changes, DNA damage and developmental competence of *in vitro* matured, vitrified-thawed buffalo (*Bubalus bubalis*) oocytes: A comparative study of two cryoprotectants and two cryodevices. **Cryobiology** 60: 315-21.

Shaw, J.M., Kuleshova, L.L., Mac Farlane, D.R., and Trounson, A.O. (1997). Vitrification properties of solutions of ethylene glycol in saline containing PVP, ficoll or dextran. **Cryobiology** 35: 219-229.

Suteevun, T., Smith, S.L., Muenthaisong, S., Yang, X., Parnpai, R., and Tian, X.C. (2006). Anomalous mRNA levels of chromatin remodeling genes in swamp

buffalo (*Bubalus bubalis*) cloned embryos. **Theriogenology** 65: 1

Tian, S.J., Yan, C.L., Yang, H.X., Zhou, G.B., Yang, Z.Q., and Zhu, S.E. (2007). Vitrification solution containing DMSO and EG can induce parthenogenetic activation of *in vitro* matured ovine oocytes and decrease sperm penetration. **Anim. Reprod. Sci.** 101: 365-371.

Vajta, G., Holm, P., Kuwayama, M., Booth, P.J., Jacobsen, H., and Greve, T. (1998). Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. **Mol. Reprod. Dev.** 51: 53-58.

Vajta, G., Rindom, N., Peura, T.T., Holm, P., Greve, T., and Callesen, H. (1999). The effect of media, serum and temperature on *in vitro* survival of bovine blastocysts after open pulled straw (OPS) vitrification. **Theriogenology** 52: 939-948.

Vieira, A.D., Mezzalira, A., Barbieri, D.P., Lehmkuhl, R.C., Rubin, M.I., and Vajta, G. (2002). Calves born after open pulled straw vitrification of immature bovine oocytes. **Cryobiology** 45: 91-94.

Vieira, A.D., Forell, F., Feltrin, C., and Rodrigues, J.L. (2007). In-straw cryoprotectant dilution of IVP bvine blastocysts vitrified in hand-pulled glass micropipettes. **Anim. Reprod. Sci.** 99: 377-383.

Vincent, C., Pickering, SJ., and Johnson, M.H. (1990). The hardening effect of dimethylsulfoxide on the mouse zona pellucida requires the presence of an oocytes and is associated with a reduction in the number of cortical granules present. **J. Reprod. Fertil.** 89: 253-259.

Wang, W., Hosoe, M., Li, R., and Shioya, Y. (1997). Development of the competence of bovine oocytes to release cortical granules and block polyspermy after

meiotic maturation. **Dev. Growth. Differ.** 39: 607-615.

Wani, N.A., Maurya, S.N., Misra, A.K., Saxena, V.B., and Lakhchaura, B.D. (2004). Effect of cryoprotectants and their concentration on *in vitro* development of vitrified-warmed immature oocytes in buffalo (*Bubalus bubalis*). **Theriogenology** 61: 831-842.

Wood, M.J., Barros, C., Candy, C.J., Carroll, J., Melendez, J., and Whittingham, D.J. (1993). High rates of survival and fertilization of mouse and hamster oocytes after vitrification in dimethyl sulfoxide. **Biol. Reprod.** 49: 489-495.

CHAPTER VI

OVERALL CONCLUSIONS

In recent years, increased environmental pollution and over hunting have contributed to the loss of species. Cryopreservation is seen as an important tool in assisted reproductive biotechnology because of its ability to preserve animal genetic resources for an indefinite period of time. The two main types of cryopreservation are equilibrium (Slow cooling) and non-equilibrium freezing procedures (Vitrification).

The primary objectives of this study were to develop a successful protocol for vitrification of swamp buffalo oocytes and ICSI. Developing a successful activation protocol will enhance the success rate of rare buffalo oocytes ICSI. And the development of a protocol to cryopreservation of swamp buffalo oocytes will allow the propagation of valuable female gametes for future generation.

Activation of the ICSI swamp buffalo oocytes with EtOH gave the highest 2 pronuclei formation, and the combination treatments with Io+6-DMAP and EtOH+CHX gave the highest cleavage and blastocyst formation rates.

Our results showed no difference in the second PB extrusion rates, embryo development and blastocyst cell numbers between FDA treated and untreated control oocytes. This indicates the FDA staining for viability checking had no detrimental effect on the embryo development *in vitro*, thus can be safely used for viability testing of oocytes.

buffalo oocytes vitrified by the microdrop method with the 1 min+30 s regimen

could yield reasonable blastocyst formation rates after ICSI. Furthermore, from the higher survival rate of FDA stained oocytes, 2 pronucleal formation rates and ICM number among the vitrified groups, our study indicated that VA solution can work better than VB solution. Within the same solution, the comparison of Microdrop and Cryotop methods did not show the significant different. The development competence of frozen-thawed *in vitro* matured buffalo oocytes was significantly lower than fresh control oocytes,

BIOGRAPHY

Miss Yuanyuan Liang was born on December 16, 1979 in Guangxi, China. In 2002, she graduated from Guangxi University, China with Bachelor's degree (B.Sc.) of Animal Husbandry. In 2005, she received a Master degree (M.Sc.) in Agricultural from Animal Reproduction Institute, Guangxi University, Nanning, Guangxi, China. Her research topic was the preliminary study of buffalo ICSI with sorted sperm. In 2005-2007, she started work at Guangxi Buffalo Research Institute on buffalo somatic cell nuclear transfer. In May 2007, she started study Ph.D. at School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology. Parts of this study had been presented as poster presentation in the 9th World Buffalo Congress on April 25-28, 2010 at Buenos Aires, Argentina and 35th Annual Conference of the International Embryo Transfer Society at San Diego, California, USA, January 3-7, 2009. Some parts of this study have been published in journals as follows:

Liang, Y.Y., Ye, D.N., Laowtammathron, C., Phermthai, T., Nagai, T., Somfai, T. and Parnpai, R. (2011). Effects of chemical activation treatment on development of swamp buffalo (*Bubalus bubalis*) oocytes matured in vitro and fertilized by intracytoplasmic sperm injection. **Reprod. Dom. Anim.** 46: e67–73.

Liang, Y.Y., Phermthai, T., Nagai, T., Somfai, T. and Parnpai, R. (2011). *In vitro* development of vitrified buffalo oocytes following parthenogenetic activation and intracytoplasmic sperm injection. **Theriogenology** 75: 1652–1660.