การพัฒนาอุปกรณ์และปรับปรุงเทคนิคสำหรับการแช่แข็งไข่และตัวอ่อนโค โดยวิธี vitrification

นายอชิต คูมาร์ พอล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2557

DEVELOPMENT OF A NOVEL DEVICE AND IMPROVED TECHNIQUES FOR BOVINE OOCYTES AND EMBRYOS VITRIFICATION

Ashit Kumar Paul

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy in Biotechnology

Suranaree University of Technology

Academic Year 2014

DEVELOPMENT OF A NOVEL DEVICE AND IMPROVED TECHNIQUES FOR BOVINE OOCYTES AND EMBRYOS VITRIFICATION

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

_____
(Assoc. Prof. Dr. Montarop Yamabhai)
Chairperson

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

_____
(Assoc. Prof. Dr. Mariena Ketudat-Cairns)
Member

_____
(Assoc. Prof. Dr. Thevin Vongpralub)
Member

_____
(Dr. Siwat Sangsritavong)
Member

_____
(Prof. Dr. Sukit Limpijumnong)
Vice Rector for Academic Affairs and Innovation

_____
(Asst. Prof. Dr. Suwayd Ningsanond)
Dean of Institute of Agricultural Technology

อชิต คูมาร์ พอล : การพัฒนาอุปกรณ์และปรับปรุงเทคนิคสำหรับการแช่แข็งไข่และตัวอ่อนโคโดยวิธี vitrification (DEVELOPMENT OF A NOVEL DEVICE AND IMPROVED TECHNIQUES FOR BOVINE OOCYTES AND EMBRYOS VITRIFICATION) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.รังสรรค์ พาลพ่าย, 164 หน้า.

การผลิตตัวอ่อนในหลอดแก้ว และการแช่แข็งไข่ มีความสำคัญในการเก็บรักษาพันธุกรรมที่ดีไว้ จุดประสงค์ของการศึกษานี้เพื่อพัฒนาอุปกรณ์ และปรับปรุงเทคนิคสำหรับการแช่แข็งไข่สุกและตัวอ่อนโคระยะบลาสโตซีสโดยวิธี vitrification การศึกษานี้พบว่า Paper device ที่พัฒนาขึ้นมาให้ผลเทียบเท่า Cryotop device ที่เป็นอุปกรณ์มาตรฐาน อัตารอดของไข่หลังจากการแช่แข็ง และอัตราตัวอ่อนระยะบลาสโตซีสที่ผลิตได้ไม่แตกต่างกันในกลุ่มที่ใช้ Paper device และ Cryotop device ไข่โคแช่แข็งด้วยวิธี 2-ขั้นตอน สามารถมีชีวิตรอดได้แต่อัตราตัวอ่อนระยะบลาสโตซีส ที่ผลิตได้ต่ำกว่ากลุ่มไข่สดอย่างมีนัยสำคัญทางสถิติ ($p<0.01$) ดังนั้นจึงได้พัฒนาการทำ virification ไข่ โดยวิธี 3-ขั้นตอน จากการศึกษานี้พบว่าน้ำยาทำ vitrification โดยวิธี 3-ขั้นตอน ไม่ก่อให้เกิดผลเสียต่อการอยู่รอดของไข่ และการเจริญของตัวอ่อนถึงระยะบลาสโตซีส อีกทั้งยังพบว่าไข่ที่ผ่านการแช่แข็งด้วยวิธี 3-ขั้นตอน ให้ตัวอ่อนระยะบลาสโตซีสสูงกว่าไข่ที่แช่แข็งด้วยวิธี 2-ขั้นตอน แต่ต่ำกว่ากลุ่มไข่สดอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) การแช่แข็งตัวอ่อนระยะบลาสโตซีสด้วยวิธี 3-ขั้นตอน พบว่ามีอัตารอด และอัตราการฟักตัวของตัวอ่อนระยะบลาสโตซีสเกรด 2 สูงกว่าการแช่แข็งด้วยวิธี 2-ขั้นตอน อย่างมีนัยสำคัญทางสถิติ ($p<0.05$) แต่กับตัวอ่อนระยะบลาสโตซีสเกรด 1 พบว่าการแช่แข็งทั้ง 2 แบบ มีอัตารอด และอัตราการฟักตัวของตัวอ่อนระยะบลาสโตซีสไม่แตกต่างกัน

การทดลองอีกประการเป็นการศึกษาอัตราการฟักตัวของตัวอ่อนระยะบลาสโตซีส หลังจากการแช่แข็งไข่สุก และตัวอ่อนระยะบลาสโตซีสโดยวิธี vitrification 3-ขั้นตอน และใช้ Paper device ในการทดลองนี้ โดยแบ่งการทดลองออกเป็น 4 กลุ่ม ได้แก่ A) กลุ่มไข่สด และตัวอ่อนระยะบลาสโตซีสสด B) กลุ่มไข่สด และแช่แข็งตัวอ่อนระยะบลาสโตซีส C) กลุ่มไข่แช่แข็ง และตัวอ่อนระยะบลาสโตซีสสด และ D) กลุ่มที่แช่แข็งทั้งไข่ และตัวอ่อนระยะบลาสโตซีส ผลการทดลองพบว่าอัตราตัวอ่อนเจริญถึงระยะบลาสโตซีสในกลุ่ม A, B, C และ D เป็น 15.7, 16.5, 9.4 และ 9.4% ตามลำดับ ในกรณีของอัตราการฟักตัวของตัวอ่อนระยะบลาสโตซีสต่อจำนวนตัวอ่อนระยะบลาสโตซีสของกลุ่ม A, B, C และ D เป็น 73.3, 56.5, 66.6 และ 16.7% ตามลำดับ อัตราการฟักตัวของตัวอ่อนระยะบลาสโตซีสต่อจำนวนตัวอ่อนระยะบลาสโตซีสของกลุ่ม C และ D ไม่มีความแตกต่างจากกลุ่ม A อย่างไรก็ตาม อัตราการฟักตัวของตัวอ่อนระยะบลาสโตซีสต่อจำนวน

ของไข่ที่ทำ IVF ของกลุ่ม C (6.3%) ต่ำกว่ากลุ่ม A (12.1%) อย่างมีนัยสำคัญทางสถิติ ($p<0.05$) และสูงกว่ากลุ่ม D (1.6%) อย่างมีนัยสำคัญทางสถิติ ($p<0.05$) อัตราการฟักตัวของตัวอ่อนระยะบลาสโตซีสต่อจำนวนไข่ที่ทำ IVF ของกลุ่ม D (1.6%) ต่ำกว่ากลุ่ม A (12.1%) B (9.4%) และ C (6.3%) อย่างมีนัยสำคัญทางสถิติ ($p<0.05$) การทดลองนี้สรุปได้ว่าการแช่แข็งด้วยวิธี vitrification 3-ขั้นตอน โดยใช้ Paper device เหมาะสำหรับแช่แข็งไข่โคสุก และตัวอ่อนระยะบลาสโตซีส แต่ยังไม่เหมาะสำหรับการทำ double vitrification

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2557 ลายมือชื่ออาจารย์ที่ปรึกษา _____

ASHIT KUMAR PAUL : DEVELOPMENT OF A NOVEL DEVICE AND IMPROVED TECHNIQUES FOR BOVINE OOCYTES AND EMBRYOS VITRIFICATION. THESIS ADVISOR : ASSOC. PROF. RANGSUN PARNPAI, Ph.D., 164 PP.

BOVINE/*IN VITRO*/ VITRIFICATION/VITRIFICATION DEVICE/OOCYTE/ EMBRYO

The *in vitro* embryo production and vitrification of oocytes and embryos are crucial for preserving desired genetics. The aims of this study were to develop a novel device and improved techniques for bovine matured (MII) oocytes and blastocysts vitrification. This study found that the developed paper device gave similar results with modern standard Cryotop device. There was no difference in the rate of oocytes survival and blastocysts production after vitrification of oocytes using paper or Cryotop devices. Oocytes could survive from the 2-steps method but the blastocyst rate was significantly ($p<0.01$) lower than that of the fresh group. Therefore, the 3-steps technique for oocytes vitrification was developed. The results showed that the 3-steps vitrification solution was non-toxic for oocytes survival and embryo development to blastocyst stage. Additionally, the blastocyst rate in the 3-steps method of MII oocytes vitrifcation was higher than that of the 2-steps but was significantly ($p<0.05$) lower than those in the fresh group. In case of blastocysts vitrification, the 3-steps technique gave significantly higher survival as well as hatched blastocyst rates of grade-2 blastocysts than that of 2-steps ($p<0.05$), whereas for grade-1 blastocysts, it did not show any significant difference in both cases.

Another experiment was carried out to determine the blastocyst hatchability following MII oocytes and blastocyst vitrification using Paper device and 3-steps vitrification. This experiment was conducted into four groups; A) fresh MII oocytes and blastocysts, B) fresh MII oocytes with vitrified blastocysts, C) vitrified MII oocytes with fresh blastocysts and D) vitrified both MII oocytes and blastocysts. The results found that blastocyst production rates of group A, B, C and D were 15.7, 16.5, 9.4 and 9.4, respectively. In case of blastocysts hatchability rate, the blastocysts in group A, B, C and D were 73.3, 56.5, 66.6 and 16.7%, respectively. The blastocysts hatchability rates of blastocyst of group C and D were not different when compared with group A. However, blastocysts hatched rate of IVF oocytes in group C (6.3%) was significantly ($p<0.05$) lower than that of Group A (12.1%) and significantly ($p<0.05$) higher than that of group D (1.6%). The blastocyst hatched rate of IVF oocytes in group D (1.6%) was also significantly ($p<0.05$) lower than that of group A (12.1%), B (9.4%) and C (6.3%). This experiment can be concluded that 3-steps vitrification using paper device was suitable for bovine MII oocytes and blastocysts, but was not suitable for double vitrification.

School of Biotechnology Student's Signature _____

Academic Year 2014 Advisor's Signature _____

ACKNOWLEDGEMENTS

Firstly, I express my deep appreciation and sincere gratitude to my advisor Dr. Rangsun Parnpai, Associate Professor, School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology and co-advisor Dr. Takashi Nagai, Deputy Director, Food and Fertilizer Technology Center, Taipei, Taiwan for their potential planning, scholastic guidance, inspiration, sincere advice, valuable suggestion, wish criticism, fruitful discussions and encouragement throughout the entire period of this study and preparation of this thesis. Dr. Parnpai has become friendly to me, and without his constant motivation I would not be where I am today.

Secondly, I would like to express my heartfelt gratitude to Assoc. Prof. Dr. Montarop Yamabhai, Chair, School of Biotechnology, and Assoc. Prof. Dr. Mariena Ketudat-Cairns, School of Biotechnology who gave me the valuable suggestions and impressive encouragement during my study.

I am obliged to all my teachers and secretary Ms. Prangkaow Prukhet, School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Thailand for their dedication and useful instructions throughout my time as a Ph.D. student.

I am thankful to Dr. Yuan Yuan Liang, post-doctoral fellow who taught me many skills as well as help me to revise my manuscript and thesis. A debt of gratitude is given to all fellows, members of Embryo Technology and Stem Cell Research Center (ESRC).

Special recognition goes to Patuakhali Science and Technology University, Patuakhali, Bangladesh to provide scope and approve my study leave.

I would also like to express my deep feeling to my parents, Pabitra Kumar Paul and Sulochana Rani Paul, and my sister Parshati Paul and other cousins, for always pushing me to do my best and mental supporting me all these years in Thailand, even from miles and miles away. I love you all very much.

This research was funded by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission and Suranaree University of Technology.

Finally, my deepest thanks and gratitude goes to all of the committee members for their time to be a member in my thesis examination.

Ashit Kumar Paul

CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF TABLES (Continued)

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

µg	=	microgram
µl	=	microliter
AI	=	Artificial Insemination
BL	=	Blastocyst
BO	=	Brackett and Oliphant
COCs	=	Cumulus Oocytes Complexs
CPA	=	Cryoprotective Agent
D	=	Day
DMSO	=	Dimethylsulphooxide
EG	=	Ethylene Glycol
ET	=	Embryo Transfer
Exp-BL	=	Expanded Blastocyst
FDA	=	Fluorescein Diacetate
GV	=	Germinal Vesicle
GVBD	=	Germinal Vesicle Breakdown
h	=	Hour
Hed-BL, Hed	=	Hatched Blastocyst
Hing	=	Hatching
ICM	=	Inner Cell Mass
ICSI	=	Intracytoplasmic Sperm Injection
IVC	=	*In Vitro* Culture

LIST OF ABBREVIATIONS (Continued)

IVEP	=	*In Vitro* Embryo Production
IVF	=	*In Vitro* Fertilization
IVM	=	*In Vitro* Maturation
LN_2	=	Liquid Nitrogen
MII	=	Metaphase II
min	=	minute
ml	=	milliliter
mm	=	millimeter
ºC	=	Degree Celsius
PVP	=	Polyvinylproledone
sec	=	seconds
SCNT	=	Somatic Cell Nuclear Transfer
TALP	=	Tyrode's Albumin Lactate Pyruvate
TE	=	Trophectoderm
ZP	=	Zona Pellucida

CHAPTER I

INTRODUCTION

1.1 Introduction

When the biotechnological tools are applied for animal reproduction to improve or speed up the genetic merit according to people desire, then it is called 'Animal Reproductive Biotechnology'. In animals, biotechnology techniques are being used frequently to improve genetics and for pharmaceutical or industrial applications. The different reproductive biotechnological tools are commonly known as 'Assisted Reproductive Technology (ART)'. ART in animal includes *in vitro* fertilization (IVF), intra-cytoplasmic sperm injection (ICSI), artificial insemination (AI), somatic cell nuclear transfer (SCNT), multiple ovulation and embryo transfer (MOET), cryopreservation, embryo transfer (ET). All of these assisted techniques are unique for animal reproduction. However, among those, the IVF is accepted as one of the popular tool to obtain rapid desired genetics. On the other hand, cryopreservation is one of the best tools to preserve desired oocytes or embryos with suitable genetics. Therefore, in this study, we focused on the bovine *in vitro* embryo production (IVEP) and cryopreservation.

The IVEP could play a central role in dairy and beef production systems. Genetic pre-selection and crossbreeding schemes can be optimized through strategies involving use of IVEP. The rate of selection for quantitative traits can be increased by exploitation of IVF technologies to improve the precision and intensify of selection as well as to reduce the generation interval (Hansen and Block, 2004). Production of

embryos *in vitro* also has the unique potentiality to bypass the infertility caused by ovulation and fertilization failure, early embryonic death and other causes (Royal et al., 2000). Although the IVEP in bovine is still a new technology at the commercial level. The IVF began at early 1930's in rabbit, while these attempts at IVF were not successful, subsequent research in the late 1950's led to the birth of IVF rabbit pups. In the late 1960's, work with human oocytes led to the first baby born in 1978, named Louise Brown which was the world's first IVF animal born (Figure 1.1). Since then production of IVF embryos have become a common treatment for infertility in human. In terms of IVF in domestic animals, the first IVF calf (Figure 1.1), lambs and pigs were born in the early 1980's and the live births of foal was reported at 1990's. However, the production of bovine IVF embryos has been the most successful among the domestic animal species.

Figure 1.1 The first IVF baby born, Louise Brown, in 1978 (left panel) and first IVF calf born, Virgil (right panel).

Recently, researchers are trying to adopt suitable technique for preserving oocytes and embryos to use at desired time. For these circumstances, cryobiology is now an interesting subject in the IVEP system. Previously people thought and believed that very low temperatures would only exert negative effects on cells and tissues. The history of cryobiology dates back to the late 1600's. Henry Power froze a jar of vinegar cells in salt water and after thawing, he found that they were still as active as they were prior to freezing. Power was the first to theorize that cold did not have so-called "killing properties" like what heat has influenced (Sittig, 1963). Robert Boyle wrote a monograph entitled "New Experiments and Observations Touching Cold" in 1683 in which he described the effects of freezing on living animals (Parkes, 1960). Another pioneer of cryobiology was Lazzaro Spallanzani who conducted extensive studies on tissues of several species and their reaction to low temperatures in the late 1700s (Sittig, 1963). In 1940, Luyet and Gehenio published a book entitled "Life and Death at Low Temperatures" in which they discussed the basic components for the study of cryobiology (Luyet and Gehenio, 1940). In the late 1940's, Christopher Polge and his colleagues at the University of Cambridge accidentally discovered the protective capabilities of glycerol when they used bottles of chemicals that had been inadvertently mislabeled. These accidental findings enabled them to successfully cryopreserve spermatozoa of chickens (Polge et al., 1949). Discovery of the ability of glycerol to protect cells against freezing damage led to the derivation of the science of low temperature biology. In 1951, the first calf was born by artificial insemination with frozen-thawed bovine spermatozoa (Stewart, 1951). In the 1960s, Peter Mazur conducted extensive experiments to model the responses of microorganisms when subjected to low temperatures and freezing. These early studies

resulted in the development of the discipline that is now known as cryobiology (Mazur, 1963, 2004).

Successful cryopreservation of mammalian cells dependens on several variables. These variables include the type of cell itself, the solution in which the cell is suspended and whether or not the solution contains a cryoprotective agents (CPAs), the rate at which the cell is cooled to low subzero temperatures, the minimum subzero temperature to which the cell is cooled, the rate at which the cell is warmed, and the conditions under which the CPA is removed from the cell. Depending on the suspending solution, different types of cells exhibit different optimum cooling rates that may vary from a low rate of ~0.2ºC/min to a high of 1,000ºC/min. Under certain conditions, cells may even survive after being cooled at rates >100,000ºC/min. Examples of such large molecular weight compounds used as supplements include polyvinylpyrrolidone (PVP), hydroxyethyl starch (HES), Ficoll, and polyethylene glycol (PEG). Although these compounds usually will not protect cells against freezing damage when used alone in solution, they will often enhance the protective effect of other low molecular weight CPAs. For example, Leibo and Oda (1993) found that survival of mouse embryos after freezing could be improved by using a mixture of PVP and ethylene glycol (EG) (Watson, 1990, Watson, 1995; Holt, 2000a, Holt, 2000b; Leibo and Bradley, 1999).

Whittingham et al. (1972) were the first to report the successful cryopreservation of embryos of any animal species, including the births of mice from frozen-thawed embryos. They froze almost 1,000 mouse embryos in LN_2 at -196°C and transferred hundreds of these cryopreserved embryos into recipient foster mothers, producing hundreds of normal full-term fetuses and dozens of live mouse

pups. After this first demonstration, embryo cryopreservation was applied to other species and it developed into a standard procedure for cattle producers in the 80s. A human pregnancy was first reported by Trounson and Mohr (1983), although it spontaneously aborted. The first births in humans from frozen embryos were described elaborately by Zeilmaker et al. (1984) which allowed embryo cryopreservation to become commonplace in the human field. Over the years, various protocols have been established for the cryopreservation of embryos and oocytes. Rall (1992) first reported vitrification mouse embryo. Although the cryopreservation of cleavage-stage embryos is now a standard procedure, the mammalian oocyte has proven to be much more difficult to cryopreserve successfully. Early attempts with mouse oocytes used the conventional cryopreservation protocols for embryos, but these resulted in only 6 to 14% of these oocytes developing into fetuses or offspring after IVF (Parkening et al., 1976; Whittingham, 1977; Glenister et al., 1987; Schroeder et al., 1990). Cattle oocytes cryopreserved by slow-cooling exhibited low fertilization rates after IVF and fewer than 13% developed to 2-cell embryos (Schellander et al., 1988; Lim et al., 1991). Nevertheless, offspring have been produced from oocytes that have been successfully cryopreserved in mice (Parkening et al., 1976; Whittingham, 1977), rabbits (Al-Hasani et al., 1989), cattle (Fuku et al., 1992; Otoi et al., 1992) and humans (Chen, 1988). Successful cryopreservation depends, at least in part, on the modality of freeze-thaw technique.

The idea of vitrification or achieving a glass-like state was first delivered in 1860, and then again in 1937 described by Luyet (1937). It was not until or unless about 50 years later, Rall and Fahy (1985) first achieved in vitrification and pointed out that idea and designated ‘vitrification’ as a potential alternative to

slow-freezing/cooling. On the other hand, the latest approach to achieve minimum volume of vitrification solution (VS) is a novel vitrification method using the Cryotop® device (Kitazato BioPharma, Fujinimiya, Japan). Kuwayama and Kato (2000) characterized that Cryotop device is consists of a fine, transparent polypropylene film attached to a plastic handle and is equipped with a cover straw, in which oocytes/embryos can be loaded in a very small volume (<0.1µl) of VS. But the ingredients and concentration of CPAs are also remarkable for successful the hypothesis. The Cryotop method has been efficaciously used to cryopreserve the bovine (Chian et al., 2004), bubaline (Gaspirini et al., 2007) and ovine oocytes (Succu et al., 2007), in addition to embryos in bovine and bubaline species (Laowtammathron et al., 2005). Though, the recent development of the *in vitro* culture (IVC) system for human embryos (Gardner et al., 2000; Iwayama et al., 2008) has resulted in effective production of expanded blastocyst (Exp-BL). The Exp-BL is probably more sensitive to cryopreservation than early BLs because detrimental ice crystal formation may occur in the blastocoel cavity due to insufficient dehydration. In connection with, post warming survival rate over and above hatching rate may also influenced by the day of development of embryo as an Exp-BL. Nevertheless, it is not clear which day as well as stage of embryo is suitable for vitrification. There is no specific report regarding the vitrification and post warming of Exp-BL at diverse culture period and its relationship to hatched rate. Therefore we were designed our experiment in attention to assess the progressive developmental potentiality of graded expanded embryos at Day7 and Day8 culture period subsequent Cryotop vitrification.

In recent years, cryopreservation of mammalian oocytes has become much more successful for a number of reasons. However, these latest accomplishments are

most likely due to viewing the differences between oocytes and embryos, rather than their similarities. Oocytes are difficult to cryopreserve because of their large size, low surface area to volume ratio, high water content and low hydraulic conductivity (Leibo, 1980). This has led to increased investigation of the vitrification of oocytes, as an alternative to cryopreservation by slow-cooling methods. When fully optimized, cryopreservation of oocytes will have multiple applications. In livestock, producers could preserve valuable genetic blood lines from females and could market oocytes rather than embryos whose sire has already been chosen. There are so many devices i.e. cryotop (Kuwayama and kato, 2000), Cryoloop (Mukaida, 2001), Microgrid (Cho et al., 2002), Hemistraw (Vanderzwalmen et al., 2003), Cryotip (Kuwayama et al., 2005), Cryoleaf (Chian et al., 2005), Rapid-I (Larman and Gardner, 2010) Hollow fiber (Matsunari et al., 2012) effectively used in different animal oocytes and embryos vitrification. However, all of these devices are not always available, expensive and difficult for handling during vitrification as well as warming. Therefore, it is crucial to develop a new device or container to serve those problematic issues. Recently, there have been numerous publications on bovine oocyte and embryo vitrification. Some of these manuscripts clearly show improved results in terms of survival and clinical pregnancy rate with the use of vitrification. The current trend of vitrification procedures involve exposure of oocytes or embryos suspended in very low to high concentrations of CPAs for short periods of time, followed by rapid cooling of liquid nitrogen. The osmolarity of the vitrification solution is very high which helps the cells for rapidly dehydration as well as submersion into liquid nitrogen quickly solidifiers the cell so that remaining intracellular water does not have time to form damaging crystals. In case of slow cooling similar action occurs; however, the cells are

dehydrated over a longer period of time and then plunged into liquid nitrogen at much lower temperature. Hotamisligil et al. (1996) and Mukaida et al. (1998) reported that the vitrification has been allowed improved survival and pregnancy rates compared with slow cooling, despite posing a potentially greater risk because of the possible toxicity of the highly concentrated CPAs used and relatively high exposure temperature. There are several new hypothesizes or ideas associated with vitrification have since become prevalent in the literature. These include: (i) high concentrations of CPAs are toxic, and exposure to the final solution with the highest concentration should be reduced to 60 sec or less (Shaw et al., 1992; Hunter et al., 1995; Hong et al., 1999; Chung et al., 2000; Wu et al., 2001; Yoon et al., 2003); and (ii) the faster cooling rate, the better survival (Stachecki et al., 2008). However, the both hypothesizes might be suitable for good quality blastocysts and just only FDA survival of oocytes vitrification. But the post warming oocytes survival to development of embryo as blastocyst stage, and the hatchability of vitrified medium quality embryos are still questionable.

There are two key constraints of vitrification; (i) the toxic effect of CPAs which damages the micro-organelles of oocytes and cells of embryos, and (ii) the exposure high concentrations of sucrose to immediately zero sucrose of warming solution is very frantic for oocytes and embryos. So that acclimatization of oocytes or embryos before plunge to the liquid nitrogen is obligatory. Therefore, we hypothesized that the rapid stepwise (not more than 30 sec in each) exposure to final concentrations of solution as well as stepwise warming the vitrified oocytes or embryos will be less toxic and stressful. Concerning this hypothesis, this research was designed to find out the alternative ideal device as well as a good technique for

oocytes cryopreservation. In this study we also identified the correlationship between the oocytes survival rate and cleavage rate. Therefore the overall objectives of this study are being mentioned as follows:

1. To assess the progressive developmental potential of graded expand embryos at Day7 and Day8 culture period subsequent Cryotop vitrification.

2. To develop a novel device and improve technique for bovine oocytes and embryos vitrificaation.

3. To assess the hatchability of blastocysts following double vitrification of oocytes at MII stage and embryos blastocyst stage by using paper device.

1.2 References

Al-Hasani, S., Krisch, J., Diedrich, K., Blanke, S., van der Ven, H., and Krebs, D. (1989). Successful embryo transfer of cryopreserved and in-vitro fertilized rabbit oocytes. **Hum. Reprod.** 4:77-79.

Chen, C. (1988). Pregnancies after human oocyte cryopreservation. **Ann. N.Y. Acad. Sci.** 541:541-549.

Chian, R. C., Kuwayama, M., 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.

Chian, R. C., Son, W. Y., Huang, J. Y., Cui, S. J., Buckett, W. M., and Tan, S. L. (2005). High survival rates and pregnancies of human oocytes following vitrification: preliminary report. **Ferti. Steril.** 84:S36.

Cho, H. J., Son, W. Y., Yoon, S. H., Lee, S. W., and Lim, J. H. (2002). An improved protocol for dilution of cryoprotectants from vitrified human blastocysts. **Hum. Reprod.** 17:2419-2422.

Chung, H. M., Hong, S. W., Lim, J. M., Lee, S. H., Cha, W. T., Ko, J. J., Han, S. Y., Choi, D. H., and Cha, K. Y. (2000). In vitro blastocyst formation of human oocytes obtained from unstimulated and stimulated cycles after vitrification at various maturational stages. **Fertil. Steril.** 73:545–551.

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

Gardner, D. K., Lane, M., Stevens, J., Schlenker, T., and Schoolcraft, W. B. (2000). Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. **Fertil. Steril.** 73:1155–1158.

Gasparini, 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.

Glenister, P. H., Wood, M. J., Kriby, C., and Whittingham, D. G. (1987). Incidence of chromosomal anomalies in first-cleavage mouse embryos obtained from frozen-thawed oocytes fertilized *in vitro*. **Gamete Res.** 16:205-216.

Hansen, P. J., and Block, J. (2004). Towards an embryocentric world: the current and potential uses of embryo technologies in dairy production. **Reprod. Fertil. Dev.** 16:1-14.

Holt, W. V. (2000a). Basic aspects of frozen storage of semen. **Anim. Reprod. Sci.** 62:3-22.

Holt, W. V. (2000b). Fundamental aspects of sperm cryobiology: the importance of species and individual differences. **Theriogenology** 53:47-58.

Hotamisligil, S., Toner, M., and Powers, R. D. (1996). Changes in membrane integrity, cytoskeletal structure, and developmental potential of murine oocytes after vitrification in ethylene glycol. **Biol. Reprod.** 55:161-168.

Huang, C. C., Lee, T. H., Chen, S. U., Chen, H. H., Cheng, T. C., Liu, C. H., Yang, Y. S., and Lee, M. S. (2005). Successful pregnancy following blastocyst cryopreservation using super-cooling ultra-rapid vitrification. **Hum. Reprod.** 20:122-128.

Hunter, J. E., Fuller, B. J., Bernard, A., Jackson, A., and Shaw, R. W. (1995). Vitrification of human oocytes following minimal exposure to cryoprotectants; initial studies on fertilization and embryonic development. **Hum. Reprod.** 10:1184-1188.

Inaba, Y., Alkawa, T., Hirai, T., Hashiyada, Y., Yamanouchi, T., Misumi, K., Ohtake, M., Somfai, T., Kobayashi, S., Saito, N., Matoba, S., Konishi, K., and Imai, K. (2011). In-straw cryoprotectant dilution for bovine embryos vitrified using Cryotop. **J. Reprod. Dev.** 57:437-443.

Iwayama, H., Korekane, M., Hara, T., Hirai, Y., Tokonami, K., Kimura, H., and Yamashita, M. (2008). Clinical application of a microwell system to in vitro culture of human preimplantation embryos. **J. Mammal Ova Res.** 5:167-171.

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

Kuwayama, M., Vajta, G., Ieda, S., and Kato, O. (2005). Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. **Reprod. Biomedi. Online** 11:608–614.

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

Larman, M. G., and Gardner, D. K. (2010). Vitrifying mouse oocytes and embryos with supercooled air. **Hum. Reprod.** 25:265.

Leibo, S. P. (1980). Water permeability and its activation energy of fertilized and unfertilized mouse ova. **J. Memb. Biol.** 53:179-188.

Leibo, S. P., and Bradley, L. (1999). Comparative cryobiology of mammalian spermatozoa. **The Male Gamete.** C. Gagnon, ed. Cache River Press, Vienna, IL. P. 501-516.

Leibo, S. P., and Oda, K. (1993). High survival of mouse zygotes and embryos cooled rapidly or slowly in ethylene glycol plus polyvinylpyrrolidone. **CryoLetters** 14:133-144.

Lim, J. M., Fukui, Y., and Ono, H. (1991). The post-thaw developmental capacity of frozen bovine oocytes following in vitro maturation and fertilization. **Theriogenology** 35:1225-1235.

Luyet, B. J., and Gehenio, P. M. (1940). Life and Death at Low Temperatures. **Biodynamica,** Normandy, MO.

Luyet, B. (1937). The vitrification of organic colloids and protoplasm, **Biodynamica.** 1:1-14.

Matsunari, H., Maehara, M., Nakano, K., Ikezawa, Y., Hagiwara, Y., Sasayama, N., Shirasu, A., Ohta, H., Takahashi, M., and Nagashima, H. (2012). Hollow fiber

vitrification: A novel method for vitrifying multiple embryos in a single device. **J. Reprod Dev.** 58:599-608.

Mazur, P. (1963). Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. **J. Gen. Physiol.** 47:347-369.

Mazur, P. (2004). Principles of cryobiology. **Life in the Frozen State.** Fuller, B. J., Lane. N. and Benson, E. E. eds. CRC Press, Boca Raton, FL. P. 3-65.

Mukaida, T., Nakamura, S., Tomiyama, T., Wada, S., Kasai, M., and Takahashi, K. (2001). Successful birth after transfer of vitrified human blastocysts using a cryoloop container-less technique. **Fertil. Steril.** 76:618-620.

Mukaida, T., Wada, S., Takahashi, K., Pedro, P. B., An, T. Z. and Kasai, M. (1998). Vitrification of human embryos based on the assessment of suitable conditions for 8-cell mouse embryos. **Hum. Reprod.** 13:2874-2879.

Otoi, T., Tachikawa, S., Kondo, S., and Suzuki, T. 1992. Developmental capacity of bovine oocytes cryopreserved after maturation in vitro and of frozen-thawed embryos derived from frozen mature oocytes. **Theriogenology** 38:711-719.

Parkening, T. A., Tsunoda, Y., and Chang, M. C. (1976). Effects of various low temperatures, cryoprotective agents and cooling rates on the survival, fertilizability and development of frozen-thawed mouse eggs. **J. Exp. Zool.** 197:369-374.

Parkes, A. S. (1960). Marshall's Physiology of Reproduction. 3rd ed. Longmans, London.

Polge, C., Smith, A. U., and Parkes, A. S. (1949). Revival of spermatozoa after vitrification and dehydration at low temperatures. **Nature** 164:666.

Rall, W. F. (1992). Cryopreservation of oocytes and embryos: methods and applications. **Anim. Sci. Reprod.** 28:237-245.

Rall, W. F., Fahy, G. M. (1985). Ice-free cryopreservation of mouse embryos at -196°C by vitrification. **Nature** 313:573-575.

Royal, M. D., Darwash, A. O., Flint, A. P. F., Webb, R., Wooliams, J. A. and Lamming, G. E. (2000). Declining fertility in dairy cattle: changes in traditional and endocrine parameters of fertility. **Anim. Sci.** 70:487-501.

Schellander, K., Brackett, B. G., Fuhrer, F., and Schleger, W. (1988). IVF of frozen-thawed cattle oocytes. **Proceedings of the 11th International Conference on: Animal Reproduction and Artificial Insemination,** Dublin, Ireland. P. 349.

Shaw, P. W., Bernard, A. G., Fuller, B. J., Hunter, J. H. and Shaw, R. W. (1992). Vitrification of mouse oocytes using short cryoprotectant exposure: effects of varying exposure times on survival. **Mol. Reprod. Dev.** 33:210-214.

Sittig, M. (1963). Cryogenics: Research and Applications. D. Van Nostrand Company, Inc., New York.

Stachecki, J. J., Garrisi, J., Sabino, S., Caetano, J. P. J., Wiemer, K. E. and Cohen, J. (2008). A new safe, simple and successful vitrification method for bovine and human blastocysts. **Reprod. BioMedi. Online** 17:360-367.

Stewart, D. L. (1951). Storage of bull spermatozoa at low temperatures. **Vet. Rec.** 63:65-66.

Succu, S., Leoni, G. G., Bebbere, D., Berlinguer, F., Mossa, F., Bogliolo, L., Madeddu, M., Ladda, S., and Naitana, S. (2007). Vitrification devices affect structural and molecular status of in vitro matured ovine oocytes. **Mol. Reprod. Dev.** 74:1337-1344.

Trounson, A., and Mohr, L. (1983). Human pregnancy following cryopreservation, thawing and transfer of an eight-cell embryo. **Nature** 305:707-709.

Vajta, G., and Kuwayama, M. (2006). Improving cryopreservation systems. **Theriogenology** 65:236-244.

Vanderzwalmen, P., Bertin, G., Debauche, C., Standaert, V., Bollen, N., van Roosendaal, E., Vandervorst, M., Schoysman, R., and Zech, N. (2003). Vitrification of human blastocysts with the Hemi-Straw carrier: application of assisted hatching after thawing. **Hum. Reprod.** 18:1504-1511.

Watson, P. F. (1990). Artificial insemination and preservation of semen. **Marshall's Physiology of Reproduction.** Lamming, G. E. ed. Churchill Livingstone, New York. P. 747.

Watson, P. F. (1995). Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. **Reprod. Fertil. Dev.** 7:871-891.

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

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

Wu, J., Zhang, L., and Wang, X. (2001). In vitro maturation, fertilization and embryo development after ultrarapid freezing of immature human oocytes. **Reproduction** 121:389-393.

Yoon, T. K., Kim, T. J., Park, S. E., Hong, S. W., Ko, J. J., Chung, H. M., and Cha, K. Y. (2003). Live births after vitrification of oocytes in a stimulated in vitro fertilization-embryo transfer program. **Fertil. Steril.** 79:1323-1326.

Zeilmaker, G. H., Alberda, A. T., van Gent, I., Rijkmans, C. M., and Drogendijk, A. C. (1984). Two pregnancies following transfer of intact frozen-thawed embryos. **Fertil. Steril.** 42:293-296.

CHAPTER II

LITERATURE REVIEWS

2.1 *In vitro* embryo production (IVEP)

The IVEP is the sequential combination of *in vitro* maturation (IVM), fertilization (IVF) of oocytes and culture (IVC) of embryos. In IVEP process, the oocytes are the '*raw materials'*. The capability to produce large number of embryos from donors of high genetic merit has been considered as potential value in disseminating genetic improvement and replaced the herd. The applications of IVF technology have included efforts to: (1) upgrade the reproductive and genetic performance of animals; (2) overcome infertility of valuable high yielding animals; (3) to produce transgenic and cloned animals; (4) provide a source of sexed embryos; (5) for twin production in beef cattle; and (6) at this molecular level, the technique is used to elucidate events related to maturation, fertilization of oocytes and development of embryos, these events are difficult to study under natural conditions in living animals.

However, IVEP is intensely influenced by the events occurring during oocyte maturation, fertilization and the subsequent development of the presumptive zygotes after fertilization of oocytes. Therefore, improving the efficiency and identifying the sources of variations between IVF systems or among different laboratories are more important when routinely producing blastocysts from individuals of high genetic merits. Also, the development of specific culture regimes capable of supporting IVM,

IVF and IVC to the blastocyst stage is highly desirable. In addition, survival rate of *in vitro* produced embryos after freezing and thawing, and following some of the more advanced manipulation, is less than that of embryos produced *in vivo*, indicating that the techniques used to produce embryos *in vitro* still require considerable improvement.

2.1.1 Factors affecting *in vitro* maturation (IVM)

IVM is the most important and first step of IVEP. There is a constant need to emphasize the fact that affects oocyte maturation. It includes:

2.1.1.1 Ovary storage, temperature and time limits

The time interval between animal slaughter house and laboratory where oocytes will be aspirated from the ovaries and the temperature at which the ovaries should be stored are important considerations. Oocytes are aspirated within 2-3 h of animal slaughter; ovaries are usually stored at about 30-35ºC. In cattle**,** the follicular size might be an important circumstance. Pavlok et al. (1993) and Lonergan et al. (1994) revealed when follicles were pooled according to its size, it was shown that large follicles (≥10 mm diameter) encompass oocytes with a higher potential to become embryos. Several studies described that the fate of individual oocytes depending on the exact follicular size, and it confirmed an increased competence with follicle size, i.e. bovine oocyte complexes isolated from ovaries carrying follicles of 2-5 mm in diameter showed lower rates of maturation and blastocyst formation than those from ovaries carrying follicles of >10 mm in diameter (Gandolfi et al., 1997; Kubota and Yang, 1998). Likewise, Blondin et al. (1997) instituted a significantly

more oocyte developed from larger follicles (≥10 mm) than from medium follicles. These indicated that large follicles (≥6 mm diameter) provide the oocyte with a microenvironment which improves its quality (Lonergan, 1992). Dramatic changes in oocyte nuclei, especially nucleoli, are known to occur as the bovine follicle grows from 1-20 mm. These changes may have a crucial effect on the developmental potential of the oocytes. However, it is recognized that a very stable form of RNA accumulates in the oocytes and that it is translated during maturation, fertilization and early embryonic development; such RNA accumulation may be influenced by the nature of the follicular growth (Sirard et al., 1992). Furthermore, According to Assay et al. (1992) who compared the follicular environment and structure of oocytes originating from the dominant follicles (DF) with those of the two largest subordinate follicles observed a few days after ovulation. The DF is characterized by an estrogen-dominated environment, healthy cumulus cells functionally coupled to the oocyte. Subordinate follicles are characterized by a progesterone-dominated environment, usually with degenerated cumulus cells, meiotic activation and other features of atresia.

However, other studies suggested that follicular size may not the only important criterion; since some bovine oocytes originated from large follicles failed to produce embryos, while some medium sized follicular oocytes already have this capacity (Hyttel et al., 1997).

2.1.1.2 Oocytes retrieval

The recovery of large number of cumulus oocytes complex with high developmental competence is a key potentiality for successful embryo

production. There are different method are to collect the COCs. Generally by using 18-G needle connecting with 10 ml syringe are used to collect COCs are collected. The other method of retrieval includes slicing of ovary (Carolan et al., 1994), slicing after aspiration (Takagi et al., 1992) and transvaginal ultrasound-guide oocytes pick up (OPU; Pieterse et al., 1991). Among of these OPU is an important technique for oocyte retrieval in living previously genetically selected highly valuable donor cow. The success of OPU is measured in part by the recovery rate of oocytes, expressed as a percentage of the number of follicles punctured. This recovery rate in turn, is influence by numerous factors such as aspiration vacuum, hormonal pre-treatment of animals, puncture frequency, stage of the estrous cycle and the experience of the operator. However, aspiration by using syringe and needle are still now popular technique to get large number of oocytes with easiest and cheap ways, although OPU is important to get desirable genetic merits.

2.1.1.3 Quality of COCs

Good quality COCs selection in the laboratory is fundamental for successful embryo production. It is proved that the presence of an intact complement of cumulus cell layers surrounding the oocyte and a homogenous compact cytoplasm have been the best indicators of an immature oocyte which enable to undergo maturation and embryonic development (Hazeleger and Stubbings, 1992). The role of the cumulus cells is to provide nutrients through gap junction communication to the oocytes during its growth.

2.1.1.4 Culture media

IVM medium can be divided into simple and complex. Simple media are usually bicarbonate-buffered systems containing physiological saline with pyruvate, lactate and glucose, and they differ in their ion concentration and in the concentrations of the energy sources. Complex media contains in addition to the basic components of simple media, amino acids, vitamins and purines. Most the IVF laboratories routinely use M-199 as the basic IVM medium in cattle. Reactive oxygen species (ROS) and the decreased in the intracellular glutathione content of bovine oocytes. In addition beta (β)-mercaptoethanol to TCM-199 medium increased intracellular glutathione levels of bovine oocytes cultured individually, which improved maturation rate the and blastocyst rate throughout IVEP (Mizushima and Fukui, 2001). Concerning with the energy source, Hashimoto et al. (2000) showed that excessive glucose in the media used for oocyte maturation impairs the development of bovine oocytes to the blastocyst stage, possibly due to the increase of ROS and the decreased in the intracelluar glutathion content of bovine oocytes.

2.1.2 Factors affecting *in vitro* fertilization (IVF)

Fertilization is a complex and sensitive process, which results in the formation of gametes, the restoration of the somatic chromosome number and the start of the development of a new individual. Success of IVF depends on appropriate preparation of sperm and oocyte, as well as culture conditions that are favorable to the metabolic activity of the male and female gametes (Xu and King, 1990). The first report of IVM and IVF of cattle oocytes was that of Iritani and Niwa (1977) in Japan. The IVF influencing factors were discussing as below.

2.1.2.1 Sperm preparation and medium

Fertilization is the critical series of events in which sperms are activated, capacitated and express the acrosome reaction to reach the oocytes and cross the ZP. If any problem among these series of sperm preparation, it will influence the rate of fertilization. At the same time the composition of medium and protocol also trigger the success rate. The method that used for sperm treatment and separation includes Percoll density gradient, swim-up, sephadex, glass wool etc. The 100% motility of sperm is the remarkable potentiality of each method. Brackett and Oliphant (BO) medium and Tyrode's Albumin Lactate Pyruvate (TALP) medium are widely used. However, swim-up procedure resulted in more oocytes being penetrated than did by using Percoll method. Increasing number of sperm concentrations during IVF could eliminate this problem (Parrish et al., 1995). Avery and Greve (1995) suggested that these adverse effects of Percoll is not due to Percoll particle, but may be described to the effect of unbound PVP in the Percoll. For that reason, the presence of PVP stopped bull sperm motility. Therefore, in our study we used swim up technique to select the only progressive motile spermatozoa.

Several studies indicated that the capacitation of bull sperm by heparin probably reflects the *in vivo* mechanism (Parrish et al., 1989). Heparin dosage and incubation period for sperm capacitation are imperative factors influencing bovine IVF and subsequent embryo development (Vlaenderen et al., 1991). Heparin induces changes in the calmodulin (CaM) binding properties of sperm proteins and induces a reduction in Ca^{+} concentrations during capacitation (Leclerc et al., 1992). Parrish et al. (1999) found that capacitation of bovine sperm with heparin requires extracellular calcium, the maximal kinetics of heparin-induced capacitation occurs

when extracellular calcium exceeds 10 μM. Changes in calcium triggre subsequent increase in cAMP, pH and tyrosine phosphorylation, that are known to be essential for capacitation (Parrish et al., 1995). Niwa and Ohgoda (1988) reported a synergistic effect of 20 μg/ml heparin and 10 nM caffeine in their capacitation treatment of frozen-thawed bull sperm. It was evident that the optimum dose of the agent was 100 μg/ml (Shehata, 1998). Other studies suggested that the fertilization rate might be rapidly improved by adding heparin to the IVF medium at values varied between 0.5-5.0 μg/ml (Shamsuddin et al., 1992). Preincubation period of 15 min was found to be satisfactory. Miller et al. (1987) stated that heparin promotes capacitation processes by mechanisms seems to depend on sperm capability for absorption of seminal proteins at time of ejaculation, which increases the ability of spermatozoa to bind heparin through its sulfate residue, a basic requirement for triggering the heparin capacitation-promoting effect.

2.1.2.2 Removal of cumulus cells

Partial denudations of expanded cumulus cells help the attachment of sperms to the oocytes. The technique of denudation involves either by mechanical stripping with suitably diameter of micropipettes/Pasteur pipette or using of enzyme preparations such as 0.2% hyaluornidase or by vortex. The diameter of Pasteur pipette and skill of denudation influence to make a quality of oocytes.

2.1.2.3 Co-incubation system and time

Spermatozoa are added to the droplets at a concentration of approximately 2-3×10^6 spermatozoa/ml. The standard conditions for co-culture of

spermatozoa and oocytes are 12-16 h at 38.5-39˚C in an atmosphere of 5% CO_2 in air (Fukui et al., 1990). The optimal sperm-oocyte incubation time is required for achieving maximum fertilization rates after IVM-IVF. It is clear that the medium employed in IVF systems must be capable of providing the secondary oocyte and the capacitated sperm with the conditions, which will permit sperm penetration to occur readily.

2.1.3 Factors affecting IVC of embryos

The IVC of embryos requires an appropriate environment, so that the early embryos can undergo several cleavage divisions to enable it to reach the blastocyst stage of development. In case of co-culture system, oviduct epithelial cells are diversely used for culture of 8-cells stage to the morulae or blastocyst stage (Parnpai et al., 1999). However, owing to the loss of embryos in the oviduct (disappearance of the agar chips) and the impracticability of using live animals, the preferred and superior method is to use an in vitro system for embryo culture. The advantages of using the in vitro system for embryo culture are: (i) to study, in much greater detail, embryonic development, the requirements for embryonic development when maternal-embryonic transition in protein synthesis takes place; (ii) certain genes are switched on or off; and (iii) the use of very specific developmental stages for cloning and production of transgenic animals. However, cleavage rate and embryo development could be affected by many factors related to compositions of media, system of culture (single or co-culture), culturing skill, incubation environment etc.

2.1.4 Other factors

Buffering system and osmolarity, water quality, temperature and gas phase, effect of light, oxygen toxicity and miscellaneous factors are directly or indirectly influence the success of IVEP system.

2.2 Cryopreservation of oocytes and embryos

The word cryopreservation is derived from the Greek word 'kryos', which means 'cold'. According to the source of Wikipedia, cryopreservation or cryoconservation is a process where cells, whole tissues, or any other substances susceptible to damage caused by chemical reactivity or time, are preserved by cooling to sub-zero temperatures. At low temperatures, any enzymatic or chemical activity which might cause damage to the material in question is effectively stopped. Cryopreservation methods seek to reach low temperatures without causing additional damage caused by the formation of ice during freezing. Traditional cryopreservation has relied on coating the material to be frozen with a class of molecules termed cryoprotectants. However, new methods are constantly being investigated due to the inherent toxicity of many cryoprotectants.

In another words, Cryopreservation is a method of preserving biological tissue or cells at extremely low temperatures. Although there are many applications of cryopreservation, the most common uses of the process are to preserve sperm, embryos, eggs, and plant seeds. Cryopreservation occurs when tissue is frozen, generally in liquid nitrogen, until it reaches a temperature range of at least -148 degrees Fahrenheit (-100ºC). At these temperatures, tissue is preserved in a state similar to that of suspended animation. The metabolic processes of biological tissue slow down dramatically in the extreme cold, which in turn slows down the natural

decaying process. The cryopreservation process is not just simply freezing the tissue to be preserved. During the freezing process, liquids present in cells expand as they solidify. This expansion would cause the destruction of the cells and would negate any benefit of freezing biological tissue. Cryopreservation usually involves replacing the liquid in the tissue with a solution similar to anti-freeze that can withstand the extreme cold temperatures.

2.2.1 Fundamentals of cryopreservation

2.2.1.1 Principle variables

Cryopreservation encompasses five critical but obligatory steps: (1) exposure of cells or tissues to cryoprotectants, (2) cooling specimens to temperatures below 0°C, (3) storage at the "glass" transition temperature of water below -130°C, (4) warming and thawing (5) and finally, dilution and removal of cryoprotectants prior to incubation (Luyet and Rapatz, 1970; Mazur, 1988; Mazur 2004; Leibo, 1986; Leibo 2004b; Lebio and Songsasen, 2002). There are numerous protocols that have been used to cryopreserve cells and tissues of many types. However, there is no universal protocol to preserve all type of cells or tissues due to species and material differences. Therefore, it is claimed for researcher to develop a standard and universal protocol to preserve all categories of cells or tissues.

The causes for cellular damage and death from cryopreservation are not entirely understood. During the cryopreservation process, cells experience several changes in their milieu: water is removed from the solution in the form of ice; consequently solutes become more concentrated and can precipitate; the cell responds osmotically by losing water. These processes can also be caused by changes in

temperature, except for the precipitation of solutes. Researchers have debated whether changes in temperature, several solution effects or both are the cause of cellular damage and death during cryopreservation (Mazur, 1970; Karow and Critser, 1997; Fuller et al., 2004).

2.2.1.2 Cooling rates

Cooling rate is one of the principal determinants of cell survival during cryopreservation. Cooling too slowly may kill cells by exposing them to concentrated solutions, whereas cooling them too quickly can cause cell death by ice crystal formation. Mazur (Mazur, 1965; Mazur, 1970; Mazur et al., 1972; Leibo and Mazur, 1971) proposed that cell survival in respect to cooling rate can be plotted as a bell-shaped curve. Essentially, cell survival is low at low cooling rates, increases to a maximum at an optimal cooling rate, and finally declines at high cooling rates. Each type of cell has its own optimal cooling rate. CPA permeability also changes with changes in temperature (Mazur, 1977). When cells in suspension are cooled to subzero temperatures, ice crystals first form in the extracellular solution and the cell cytoplasm supercool. As the cell cytoplasm is cooled to lower temperatures (below -10°C or -15°C), ice crystals may form abruptly in the cytoplasm itself, a phenomenon referred to as intracellular nucleation. This is often, but not inevitably lethal to the cells. If cells that have frozen intracellularly are warmed very rapidly, the cells may be "rescued" from this damage (Mazur, 1970). In contrast, when cells are cryopreserved by vitrification, they are cooled in such high concentrations of CPA solution and at such high cooling rates that intracellular ice crystals do not form.

2.2.1.3 Warming rates

The warming rate is also very important for successful cryopreservation of mammalian cells. The optimum warming rate for a given type of cell is highly dependent on the optimum cooling rate that preceded it. Early investigators assumed that rapid warming of mammalian cells after cryopreservation was always better because cells had shorter times to re-crystalize and were exposed for less time to CPAs. However, the first investigations of mouse embryo freezing by Whittingham et al. (1972) proved that there are exceptions to this rule. Their study showed that embryos cryopreserved by slow-cooling had greater post-thaw survival when they were warmed slowly. In fact, they reported that embryo survival was dependent on a slow warming rate. They concluded that poor survival using faster warming rates is most likely due to osmotic effects.

The most common method for warming of oocytes after they have been vitrified is a rapid and direct method. Usually oocytes are placed into warming solutions at 20-37°C. After warming, oocytes must rehydrate and CPAs used for vitrification must be removed. This is also done quickly but the debate on whether step-wise dilution is necessary still remains.

2.2.1.4 Cryoprotective agents (CPAs)

The advantages and disadvantages of different CPAs were showed in Table 2.1. When mammalian cells suspended in dilute saline solutions are cooled and frozen, they are inevitably damaged and destroyed. As mentioned above, however, it was accidentally discovered in 1949 that fowl spermatozoa would survive freezing when suspended in a solution of 10% glycerol plus albumin (Polge et al.,

1949). Later, calves were born from artificial insemination with frozen-thawed sperm (Stewart, 1951; Polge and Rowson, 1952). These were the first explicit demonstrations that mammalian cells can be successfully frozen with the addition of a supplement to the culture medium. Since those first discoveries, it has been found that many low molecular weight compounds protect cells against freezing damage. These compounds, now referred to as CPAs, are all miscible with water in all proportions. These compounds are also non-toxic to cells and have low molecular weights that allow them to permeate the cell membrane. Cryoprotective additives are added to solutions used to freeze cells to lower the freezing point. In Table 2.2, some of the common injuries observed after cryopreservation which may also lead to cell death (Shaw et al., 2000a). CPAs also make the cell membrane more elastic by affecting lipids within the cell membrane.

Many compounds act as CPAs to protect cells against freezing damage. Examples of CPAs are methanol, ethylene glycol (EG), dimethylsulfoxide (DMSO), propylene glycol (1-2, propanediol) and glycerol. Leibo et al. (1970) reported that <2% of mouse marrow cells survive when cooled at rates varying from 0.3-600°C/min in a salt solution. However, they found that when glycerol was added to the solution, more than 70% of the cells survived cryopreservation. Glycerol was the only compound used as a CPA until the protective effects of DMSO were discovered by Lovelock and Bishop in 1959 (Mazur, 1970). Since then, many other compounds have been reported to act as CPAs. When cells are first exposed to multimolar solutions of CPAs, the cells contract by water loss. As the CPA permeates the cells, water re-enters and the cell returns to its initial isotonic volume.

Table 2.1 Example of cryopreservation agents (CPAs) and its advantages and disadvantages.

Name of CPAs	Advantages	Disadvantages
Dimethylsulfoxide (DMSO)	- Readily permeable. - Reduce ice formation and thereby prevent cell death during the freezing process. (Pegg, 2007). - High boiling point, 189°C (372°F), DMSO evaporates slowly at normal atmospheric pressure. - Anti-inflammatory roles prevent the cellular damage.	- High concentration fetal for cells. - Produce an explosive reaction when exposed to acyl chlorides; at a low temperature. - Denatured the serum in the media.
Ethylene glycol (EG)	- Useful desiccant due to its high boiling point and affinity for water. - Use to depress the temperature. - Permeable CPAs. - Prevent the inner ice crystal formation.	- Single use is toxic for cells. - High permeating rate - Damaged the cellular micro-organelles.

Table 2.1 (Continued).

Name of CPAs	Advantages	Disadvantages
Propylene glycol	- Readily permeable. - Non-corrosive. - Very low volatility. - Very low toxicity. - Able to lower the melting point of water.	- High concentration is hazardous for cells.
Glycerol	- Prevent the irritability of medium. - Help cells as soothing effect. - Dissolved in water to reduce damage by ice crystals. - Markedly used for semen freezing	- Need high concentration for using as a CPAs.
Polyvinylproledone (PVP)	- Confer water solubility. - Relatively high viscosity. - Less toxic for cells.	- Rarely used as CPAs. - Damages the cells and takes more time for thawing because of its high viscosity.

Table 2.2 Factors associated with cooling and cryopreservation that contribute to cellular injury and death in biological systems Shaw et al. (2000a).

System	Way of damage
All	- Intracellular ice formation, extracellular ice formation, apoptosis, toxicity, calcium imbalance, free radical, ATP levels, general metabolism, fertilization failure, cleavage failure, internal change of pH
Membrane	- Rupture, leakage, fusion, microvilli, phase transition
Chromosomes	- Loss/gain, polyspermy, polygny (failure to extrude polar body), tertraploidy
DNA	- Apoptosis, fusion, rearrangements
Cytoskeleton	- Microtubules dissolve, actin
Proteins/Enzymes	- Dehydration, loss of function
Ultrastructure	- Microvilli mitochondria, vesicles, cortical granules, zona pellucida
Zona pellucida	- Hardening, fracture
Lipids	- Free radicals

Cryoprotective additives have different rates of diffusion into the cell. For example, the rate of diffusion for propylene glycol into oocytes is relatively fast (5-7 min) compared to the rate of diffusion for DMSO (20-30 min) or glycerol (>60 min) (Jackowski et al., 1980; Renard and Babinet, 1984).

2.2.1.5 Supplements to CPA Solutions

Supplements to CPA solutions are compounds of various sizes that by themselves do not protect the cell from freezing damage. There are several classes of compounds that have been used as supplements to CPA solutions, including saccharides, other large molecular weight compounds and proteins. Various saccharides have been used as supplements. These include mono-, di- and trisaccharides that have molecular weights ranging from ~180-540. Examples of monosaccharides include fructose, glucose, and galactose. Examples of disaccharides include sucrose, trehalose and lactose, and an example of a trisaccharide is raffinose. Trehalose has been reported to be very effective as a CPA supplement for vitrification of oocytes by several groups (Dinnyes et al., 2000; Lj et al., 2002; Begin et al., 2003). Arav et al. (1993) showed that bovine oocytes exposed to trehalose had a higher rate of survival than oocytes exposed to sucrose and they also reported a very high rate of fertilization after exposure to 0.25 M trehalose (70%). There are other large molecular weight compounds that are often added to CPA solutions as supplements. Several reports have shown that PVP is not successful for cryopreserving embryos and that the compound is highly toxic to embryos (Wilmut and Rowson, 1973; Fahy et al., 1984). Polyethylene glycol has also been used to supplement CPA solutions (Rall and Fahy, 1985). Large proteins can also be used as supplements to CPA solutions.

Examples include bovine serum albumin (BSA) or fetal bovine serum (FBS). Another less common type of CPA supplement is thermal hysteresis proteins (THPs). Arctic fish live in icy polar waters that drop to temperatures of -1.8°C. DeVries and Wohlschlag (1969) isolated glycoproteins from the serum of these fish, and found that the glycoproteins changed the freezing point without changing the melting point, and named these glycoproteins THPs. In another study, THPs were again isolated from fish and used for the cryopreservation of pig oocytes (Rubinsky et al., 1991). These authors reported that 80% of the oocytes in the THP group were membrane-intact and 27% of these oocytes went on to mature in vitro. This was significant since previous studies of porcine oocyte cryopreservation found that no oocytes survived vitrification upon warming (Arav et al., 1990).

2.2.1.6 Viscosity of the medium

This is defined by the concentration and behavior of various CPAs and other additives during vitrification. The higher the concentration of CPAs, the higher the glass transition temperature (Tg), thus, lowering the chance of ice nucleation and crystallization. Different CPAs and other additives have different toxicity, penetration rate, and Tg. The combination of different CPAs is often used to increase viscosity, increase Tg, and reduce the level of toxicity. In the cattle industry, so as to avoid handling of the post-warmed embryos and allow direct transfer, EG is often used as the permeating CPA because of its high penetration rate (Saha et al., 1996).

2.2.1.7 Dehydration

Dehydration of cells is crucial in cryopreservation. Damage to cells most likely occurs between 15°C and -90°C. If cells are not properly dehydrated, intracellular ice formation occurs when temperature is lowered below 0ºC (Ruffing et al., 1993).

2.3 Slow freezing

Controlled-rate and slow freezing, also known as slow programmable freezing (SPF), is a set of well-established techniques developed during the early 1970s which enabled the first human embryo frozen birth Zoe Layland during 1984. Since then, machines that freeze biological samples using programmable sequences, or controlled rates, have been used all over the world for human, animal and cell biology – 'freezing down' a sample to better preserve it for eventual thawing, before it is frozen, or cryopreserved, in liquid nitrogen. Such machines were used for freezing oocytes, skin, blood products, embryo, sperm, stem cells and general tissue preservation in hospitals, veterinary practices and research laboratories around the world. As an example, the number of live births from frozen embryos 'slow frozen' is estimated at some 300,000-400,000 or 20% of the estimated 3 million IVF births.

Lethal intracellular freezing can be avoided if cooling is slow enough to permit sufficient water to leave the cell during progressive freezing of the extracellular fluid. That rate differs between cells of differing size and water permeability: a typical cooling rate of about 1°C/min is appropriate for many mammalian cells after treatment with cryoprotectants such as glycerol or dimethyl sulphoxide, but the rate is not a universal optimum. The 1°C/min rate can be achieved

by using devices such as a rate-controlled freezer or a benchtop portable freezing container such as CoolCell®.

Several independent studies have provided evidence that frozen embryos stored using slow-freezing techniques may in some ways be 'better' than fresh in IVF. The studies were presented at the American Society for Reproductive Medicine conference in San Francisco, USA, 2008. The studies indicate that using frozen embryos rather than fresh embryos reduced the risk of stillbirth and premature delivery though the exact reasons are still being explored.

2.4 Vitrification

Researchers helped introduce vitrification to reproductive cryopreservation in the mid-1980s (Fahy and Rall, 1980). As of 2000, researchers claim vitrification provides the benefits of cryopreservation without damage due to ice crystal formation. For clinical cryopreservation, vitrification usually requires the addition of cryoprotectants prior to cooling. The cryoprotectants act like antifreeze: they decrease the freezing point. They also increase the viscosity. Instead of crystallizing, the syrupy solution becomes an amorphous ice it vitrifies. Rather than a phase change from liquid to solid by crystallization, the amorphous state is like a "solid liquid", and the transformation is over a small temperature range described as the "glass transition" temperature.

Vitrification of water is promoted by rapid cooling, and can be achieved without cryoprotectants by an extremely rapid decrease of temperature (megakelvins per second). The rate that is required to attain glassy state in pure water was considered to be impossible until 2005.

Two conditions usually required to allow vitrification are an increase of the viscosity and a decrease of the freezing temperature. Many solutes do both, but larger molecules generally have larger effect, particularly on viscosity. Rapid cooling also promotes vitrification.

For established methods of cryopreservation, the solute must penetrate the cell membrane in order to achieve increased viscosity and decrease freezing temperature inside the cell. Sugars do not readily permeate through the membrane. Those solutes that do, such as dimethylsulfoxide, a common cryoprotectant, are often toxic in intense concentration. One of the difficult compromises of vitrifying cryopreservation concerns limiting the damage produced by the cryoprotectant itself due to cryoprotectant toxicity. Mixtures of cryoprotectants and the use of ice blockers have enabled the Twenty-First Century Medicine company to vitrify a rabbit kidney to -135°C with their proprietary vitrification mixture. Upon rewarming, the kidney was transplanted successfully into a rabbit, with complete functionality and viability, able to sustain the rabbit indefinitely as the sole functioning kidney.

Although these methods are drastically different, both can produce successful results in mammalian cell cryopreservation. Success depends on choosing the optimal method for each cell type. Slow-cooling involves use of low concentrations (1-2 M) of CPAs added to the cell culture medium, and rather low cooling rates (0.1-1.0°C/min), achieved by use of a programmable freezing machine, in most cases. Cells are dehydrated during the slow-cooling process. Damage to cells during this process may occur due to osmotic shock, ice crystal formation, or toxicity of CPAs (Rall, 1992).

Table 2.3 The comparison of advantages and disadvantages between slow freezing and vitrification.

Points	Slow freezing	Vitrification
Cryoprotectives	Low concentration (about 10)	High concentration (about 40)
Output/ results	Stable	Unstable
Equipment	Need expensive equipment	No need expensive equipment
Time requirement	Long time (2-3 h)	Very short time (3-4 min)
Rate of cooling	Slow (0.3-1°C/min)	High (2,000-25,000°C/min)
Volume of media	Need little bit higher	Smaller
Chilling injury	Low	High
Cold shock	Very low	High
Oocytes quality	Required moderate to good	Always best need best quality
Chance of damage	Low after warming	High after warming
Technical skill	No need high skilled technician	Need high skilled technician
Timing	Sometimes can be overlooked	Must be maintained
Preferences	Less preferable	More preferable

In 1937, Luyet first described the use of vitrification for the preservation of tissues (Luyet, 1937). The method of vitrification involves the use of high concentrations (5-7 M) of CPAs and ultra-rapid cooling rates (2,000-25,000°C/min). Rall (1987) noted that theoretically even water can be vitrified using a cooling rate of 107°C/sec. The cells are dehydrated by exposure to high concentrations of CPAs prior to cryopreservation which also increases the viscosity of the solution. Cells are suspended in a CPA are plunged directly into LN_2, forming a glass-like suspension of cells. This technique completely eliminates intracellular ice formation (Porcu, 2001). However, cells may be damaged by exposure to such high concentrations of CPA. Nevertheless, vitrification has the advantage of being low-cost, since it eliminates the need for programmable freezing equipment, and it is quicker and easier to perform compared to slow-cooling.

2.4.1 Vitrification devices

According to the observations of Arav (1992), Arav et al. (2002), Yavin and Arav (2007), the smaller volume of solution is increased the probability of vitrification. Smaller volumes allow better heat transfer, thus facilitating higher cooling rates. So that, researcher tried to adapt their techniques to reduce sample volume as well as increase the viability of cells. Hence, the techniques can usually be divided into two categories, surface techniques (Table 2.4) and tubing techniques (Table 2.5). The pros and cons of different devices are discussed in Table 2.6.

Table 2.4 Surface system vitrification devices.

Name of device	Reference
Electron microscopic (EM) grid	Steponkus et al., 1990; Martino et al., 1996; Cho et al., 2002
Minimum drop size (MDS)	Arav, 1992; Arav and Zeron, 1997; Yavin and Arav, 2001
Cryotop	Hamawaki et al., 1999; Kuwayama and Kato, 2000
Cryoloop	Lane et al., 1999
Hemi-straw	Vanderzwalmen et al., 2000
Solid surface (SS)	Dinnyes et al., 2000
Nylon mesh	Matsumoto et al., 2001
Cryoleaf	Chian et al., 2005
Direct cover vitrification	Chen et al., 2006
Fiber plug	Muthukumar et al., 2008
Vitrification spatula	Tsang and Chow, 2009
Cryo-E	Petyim et al., 2009
Plastic blade	Sugiyama et al., 2010
Vitri-Inga	Almodin et al., 2010

Table 2.5 Tubing system vitrification devices.

Name of device	Reference
Plastic straw	Rall and Fahy, 1985
Open-pulled straw (OPS)	Vajta et al., 1997; Vajta et al., 1998
Closed pulled straw(CPS)	Chen et al., 2001
Flexipet-denuding pipette	Liebermann et al., 2002
Superfine OPS	Isachenko et al., 2003
CryoTip	Kuwayama et al., 2005
Pipette tip	Sun et al., 2008
High-security vitrification device	Camus et al., 2006
Sealed pulled straw	Yavin et al., 2009
Cryopette	Portmann et al., 2010
Rapid-i	Larman and Gardner, 2010
Hollow fiber	Matsunari et al, 2012
Cryopette	Portmann et al., 2010
Rapid-i	Larman and Gardner, 2010
Hollow fiber	Matsunari et al, 2012

Figure 2.1 Vitrification surface carrier system. (1) electron microscope grid, (2) minimum drop size, (3) Cryotop, (4) Cryoloop, (5) Hemi-straw, (6) Cryoleaf, (7) fiber plug, (8) direct cover vitrification, (9) vitrification spatula, (10) nylon mesh, (12) Vitri-inga. (Source: Saragusty and Arav, 2011).

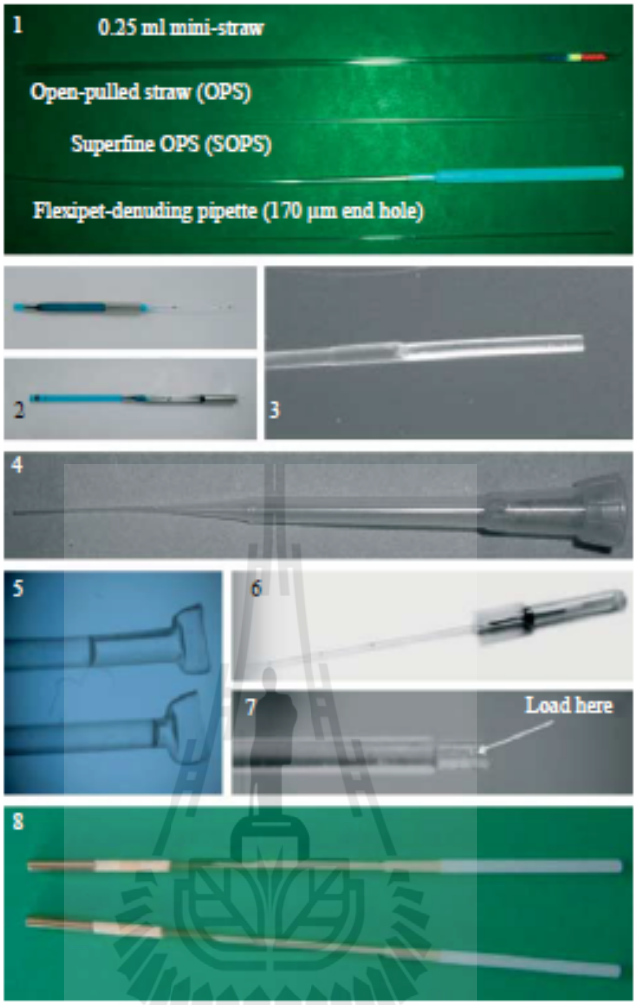

Figure 2.2 Vitrification tubing carrier systems. (1 top) plastic straw, (1,2nd from top) open-pulled straw, (1, 3rd from top) superfine open-pulled straw, (1 bottom) flexipet-denuding pipette, (2) Cryotip, (3) high-security vitrification device, (4) pipette tip, (50 sealed pulled straw, (6) Cryopette, (7) Rapid-I and (8) JY Straw. (Source: Saragusty and Arav, 2011).

Table 2.6 Vitrification methods and their pros and cons.

Vitrification Method	Pros	Cons
Cryotop (Kuwayama and Kato, 2000)	- Easily control the volume of medium. - Convenient to handle it.	- Expensive (about 40USD/device). - Over flow of sample with medium.
Cryotip (Kuwayama et al., 2005)	- Less chance of contamination with LN_2. - Convenient for thawing.	- Time required for loading, sealing, adjustment of the sleeve and plunging. - Expensive container.
Solid surface vitrification (SSV; Dinnyés et al., 2000)	- High cooling rate. - Comparatively cheap. - Large number of sample can be preserved with in short period.	- Requires extensive practice. - Oocytes sometime attached to the inner surface. - High chance of oocytes loss.
Cryoloop (Oberstein et al., 2001)	- High cooling rate. - Minimized the volume of solution due to small loop.	- Expensive (about 30USD/device). - Need more practice. - High possibility of loss of sample.

Table 2.6 (Continued).

Vitrification Method	Pros	Cons
Open Pulled straw (OPS; Vajta et al., 1998)	- Highest cooling rates. - Two sizes available (0.65 and 0.30mm inner diameter). - Easy loading process, "auto loading" by the capillary forces of OPS. - Easy to use at filed level.	- High chance of broken during thawing. - Need more practice. - Maintain of time sometime difficult. - Make it narrowing is difficult.
Hollow fiber vitrification (Matsunari et al., 2012)	- Large number of embryos can preserved at a time. - High cooling rate. - High embryo survival rate.	- Need expensive device. - High chance of mechanical damage of embryos during loading.
Microdrop (Papis et al., 2000)	- High cooling rate. - No need extra devices. - Can be storage by cryovial.	- High chance of oocytes/embryos loss. - Need more practice for shaking and dropping into LN_2.
Vitrification Spatula (Tsang and Chaw, 2009)	- Easy to loading. - Easy to handling.	- Expensive device. - Cooling rate comparatively low.

Table 2.6 (Continued).

Vitrification Method	Pros	Cons
Vitri-inga (Almodin et al., 2010)	- Cooling rate high. - Extensively used for mouse embryos vitrification.	- Expensive device. - High chance of broken of thin part during handling.
Cryoleaf (Chian et al., 2005)	- Rapid cooling rate. - No need more practice.	- Costly device. - Loading little bit difficult.
Hemi-straw (Vanderzwalmen et al., 2000)	- High cooling rate. - Stable results.	- Expensive. - Sample may loss during thawing. - It may break during handling.

2.4.2 Vitrification of oocytes

Oocytes can be stored either as metaphase II (MII) oocytes or as germinal vesicle (GV) stage oocytes. Oocytes at the MII stage of development have undergone several developmental paths including both nuclear and cytoplasmic maturation, extrusion of the first polar body and the arranging of the chromosomes on the MII spindle. The chromatin of these immature oocytes is still in the diplotene phase of prophase I. At this stage of development, GV oocytes do not have a spindle (Shaw et al., 1999).

In ovary, each fully grown oocyte undergoes maturation in response to gonadotropins. This process is completed after oocytes reach metaphase II (MII) stage. After oocytes begin to mature, their nuclei–germinal vesicles (GV) break down and chromosomes condense (germinal vesicle breakdown, GVBD). Chromosomes are then arranged in MI stage which is followed by anaphase I to telophase I transition and oocytes are arrested in MII, ready for fertilization (Trounson et al., 2001).

- **Cell cycle**

The cell cycle consists of four distinct phases: G_1 phase, S-phase (synthesis), G_2 phase (collectively known as interphase) and M-phase (mitosis). M phase is composed of two processes one is mitosis, in which the cell's chromosomes are divided between the two sisters' cells, and another one is cytokinesis, in which the cell's cytoplasm divides in half forming distinct cells. Activation of each phase is dependent on the proper development and completion of the previous one. Cells that have temporarily or reversibly stopped dividing is called G_0 phase. Completely grown mammalian oocytes are arrested at two points of maturation. The first point of arrest

is at the GV stage when oocytes are waiting for the gonadotropin signal or the release from an inhibitory follicular condition. The second point is at MII stage when oocytes are waiting for fertilization (Russell, 2001). The capacity to complete meiotic maturation is probably developed during oogenesis which is known as meiotic competence acquisition. This process of maturation is under the control of the maturation promoting factor (MPF). In immature oocytes, MPF is present in an inactive phosphorylated and cyclin B. This phosphorylation is controlled by Myt1 kinase. The dephosphorylation of MPF is induced by Cdc25 phosphatase. The activity of MPF reaches its peak in MI oocytes and then decreases during the anaphase to telophase transition. Then, high levels of MPF are again restored and oocytes are kept at this stage under the influence of a cytostatic factor (CSF; Smith, 2001) MPF is fully degraded when oocytes are fertilized (Nebreda and Ferby, 2000; Tunquist and Maller, 2003).

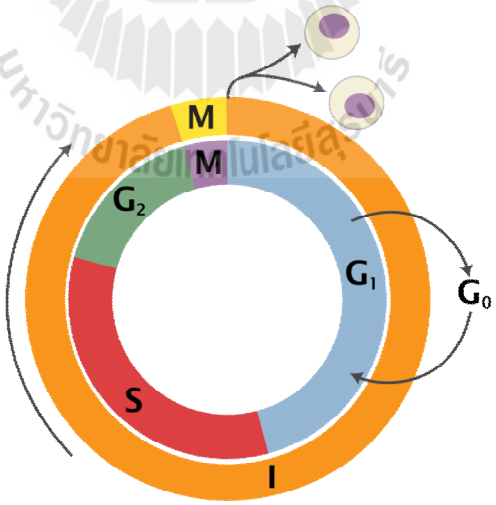

Figure 2.3 Schematic of the cell cycle. Outer ring: I = Interphase, M = Mitosis; inner ring: M = Mitosis, G_1 = Gap 1, G_2 = Gap 2, S = Synthesis; not in ring: G_0 = Gap 0/Resting (Cooper, 2000).

The main advantages of MII oocytes cryopreservation is for multipurpose use i.e. IVF, cloning, ICSI. In case of GV or GVBD stage also the similar advantages but the GV or GVBD stage is more sensitive and lower survival rate than MII stage. However, in case of embryonic stage whatever it is derived from IVF, ICSI or cloning, the main purpose of application is to embryo transfer (ET).

On the other hand, among the above mentioned stages, the different stages of oocytes are more sensitive to cryotolerence. And until now there is no standard method of oocytes cryopreservation. It is the researcher headache and big questions how to improve the technique of it. It is also difficult to find out the stages of oocytes after aspiration of oocytes from slaughter house derived ovary even it is also difficult to know in case of synchronization-OPU in live cattle. For IVF, we need matured oocytes (MII oocytes) otherwise we cannot success for IVF. In this consent, the oocytes maturation also a time dependent factor. Oocytes require 20-24 h to go at matured stage from pre mature stage (GV, GVBD, Anaphase, metaphase and Telophase). So that after 20-24 h culture of oocytes immediately after aspiration, we may confirm that the oocytes at MII stage and it is ready for IVF, ICSI or cloning.

In 1961, Smith reviewed the differences in cryopreservation survival between mammalian unfertilized oocytes and oocytes that had recently been fertilized noting that the fertilized ova had a much better chance of development in vitro than the unfertilized cells (Smith, 1961). Even though this difference between oocytes and embryos was recognized in the early days of oocyte cryopreservation research, the first mammalian oocyte cryopreservation protocols mimicked the equilibrium “slow-cooling” methods used for embryo cryopreservation (see Reviews by Rall, 1992; Fabbri et al., 1998, Fabbri et al., 2000; Paynter, 2000). Fabbri et al. (1998) described a

commonly used protocol for slow-cooling and rapid thawing of oocytes using DMSO and straws. Oocytes were frozen from 0ºC or 20ºC to -8ºC at a rate of 2ºC/min, then at a rate of 0.3ºC/min to -30ºC and finally at a rate of 50ºC/min to -150ºC. The straws were then transferred to LN_2. Thawing was performed at room temperature by washing oocytes through a series of decreasing concentrations of CPA solutions, with the final dilution containing sucrose. Live births have been reported using similar conventional slow-cooling methods in several species including those of mice (Parkening et al., 1976; Whittingham, 1977), rabbits (Al- Hasani et al., 1989), cattle (Fuku et al., 1992; Otoi et al., 1992) and humans (Chen, 1988). A list of births resulting from oocyte cryopreservation is listed in Table 2.7.

Table 2.7 First births resulting from the successful cryopreservation of mammalian oocytes.

Species	**Method**	**Reference**
Mouse	Slow-cooling	Parkening et al., 1976
	Vitrification	Kono et al., 1991
Rabbit	Slow-cooling	Al-Hasani et al., 1989; Vincent et al., 1989
Cattle	Slow-cooling	Fuku et al., 1992
	Vitrification by OPS	Vajta et al., 1998
Human	Slow-cooling	Chen, 1986
	Vitrification by OPS	Kuleshova et al., 1999
Horse	Vitrification by Cryoloop	Maclellan et al., 2002

The procedure of vitrification of embryos was first described by Rall and Fahy (Rall, 1987; Rall and Fahy, 1985) who showed that mouse embryos could be successfully cryopreserved by suspending them in concentrated solutions of CPAs and cooling the embryos rapidly in LN_2. Vitrification is defined as the solidification of a solution (glass formation) at extremely low temperatures (-196°C) without any internal ice formation (Vajta, 2000). Vitrified mouse embryos were subsequently shown to develop into normal offspring (Rall et al., 1987). This procedure has become increasingly important as an alternative method of cryopreservation, since many reports have suggested that vitrification, rather than conventional slow-cooling, might be a better method to cryopreserve mammalian oocytes. Research has found that slow-cooling of oocytes results in zona hardening, due to release of cortical granules, disruption of the chromosomes, and the loss in the ability to be fertilized (Carroll et al., 1990; Aigner et al., 1992).

Recently, studies have focused on vitrification as a method for cryopreservation of oocytes. Vitrification has also been used to cryopreserve oocytes of mice (Chen et al., 2000; Lane and Gardner, 2001; Nakagata, 1989), rats (Nakagata, 1992), goats (Begin et al, 2003), horses (Maclellan et al., 2002), humans (Kuleshova et al., 1999; Liebermann and Tucker, 2002; Yoon et al., 2000) and especially cattle (Dinnyes et al., 2000; Le Gal and Massip; 1999; Le Gal et al., 2000; Martino et al., 1996b; Matsumoto et al., 2001; Mavrides and Morroll, 2002; Otoi et al., 1998; Papis et al., 2000; Vajta et al., 1998; Vieira et al., 2002). Recently, bovine blastocysts have been produced by transfer of nuclei from adult fibroblasts into oocytes or cytoplasts that had been vitrified (Booth et al., 1999).

Injury of oocytes resulting from exposure to temperatures near 0ºC is a time-dependent phenomenon (Martino et al., 1996a). Therefore, various methods have

been derived to increase cooling and warming rates to “outrace” chilling injury. These include use of electron microscope grids as a carrier of oocytes (Martino et al., 1996b), placing oocytes into small diameter open pulled straws (Vajta et al., 1998), dropping 1-2 µl volumes of medium containing oocytes directly onto a cold surface at -150ºC (Dinnyes et al., 2000), or onto films of CPA within small nylon cryoloops (Lane et al., 1999). The rationale of all of these methods is that the oocytes are suspended in very small volumes of medium so that they can be cooled at extremely high rates. Martino et al. (1996b) found that bovine oocytes succumb to chilling damage very quickly. These researchers used electron microscope grids as a vitrification device, which allowed for faster cooling rates and compared this method to conventional straw freezing. After >20 h in culture post-warming, oocytes cryopreserved on grids had a 51-72% survival rate compared to 34% of the oocytes cryopreserved in straws. After IVF, 40% of the oocytes that had been cryopreserved on grids cleaved when placed into culture and 15% developed into blastocysts. The straw method resulted in a 3% cleavage rate and <1% blastocyst development. Rates of blastocyst formation of oocytes cryopreserved on grids, although significantly lower than controls, were significantly higher compared to that of oocytes cryopreserved in conventional straws.

Other groups have used open pulled straws (OPS), which hold oocytes in small amounts of solution for vitrification inside extremely thin hand-pulled straws. Vajta et al. (1998) had improved pregnancy rates with the OPS when compared to conventional straws. Three (3/14; 21%) normal bull calves were born after nonsurgical transfer of embryos resulting from IVF of previously cryopreserved oocytes. Isachenko et al. (2001) also found that the OPS method of vitrification, when

used to cryopreserve ovine GV-stage oocytes, resulted in a higher number of MII stage oocytes after culture than conventional straw freezing.

Dinnyes et al. (2000) developed a vitrification procedure that uses a precooled metal surface (solid-surface vitrification; SSV) and small amount of vitrification solutions dropped directly onto this metal surface. In their IVF experiment, they found a 58-62% cleavage rate of oocytes fertilized after IVF compared with 69% cleavage of controls. Of the vitrified group of oocytes, 11-19% of them developed into blastocysts on D9 compared with 33% of the fresh control group.

The cryoloop, first described as a device for embryo cryopreservation by Lane et al. (1999), is a small nylon loop with a film of medium formed within it, onto which oocytes or embryos are carefully pipetted; the loop itself is then plunged directly into LN_2. The cryoloop has been successfully used to cryopreserve oocytes and embryos of the mouse (Lane and Gardner, 2000), the hamster (Lane et al., 1999), cattle (Le Gal et al., 2000), human (Kuleshova et al, 1999) and more recently the horse (Maclellan et al., 2002).

Lane and Gardner (2001) compared the cryoloop method of vitrification with slow cooling using sodium-free medium, replacing sodium with choline, in mouse oocytes. Lovelock (1954) hypothesized that an increase in electrolyte concentration can cause cellular damage. During cryopreservation, cells are exposed to an increase in electrolyte concentration. Virtually all cell culture media contain sodium salts and, therefore, damage to cells can be attributed to solution effects during cryopreservation. Stachecki et al. (1998a, b) reported that sodium ions may contribute to osmotic shock in slow-freezing methods. They demonstrated that as the concentration of choline increased and sodium decreased in the freezing medium, higher survival and blastocyst rates were observed post-thaw. Lane and Gardner

(2001) used the cryoloop to vitrify mouse oocytes that they fertilized after puncturing the zona pellucida with a laser. They found a 99.3% survival rate using the cryoloop for vitrification compared to 80.9% survival rate using slow-freezing. The cryoloop resulted in 69.7% fertilization and 67.1% blastocyst rates after IVF post-warming. These results were significantly higher than the rates achieved with the slow-freezing methods, 39.7% and 25.9%, respectively. After transfer into foster recipient mice, the resultant embryos from IVF using vitrified-warmed oocytes developed into full-term fetuses and offspring. In the vitrified oocyte group, 52 of 92 (56.5%) blastocysts transferred resulted in viable fetuses, which was significantly higher than the slow-freezing group (11 of 42; 26.2%). Very few groups have cryopreserved equine oocytes (Hochi et al., 1994; Hochi et al., 1995, Hochi et al., 1996; Hurtt et al., 2000; Maclellan et al., 2001; Maclellan et al., 2002).

2.4.3 Vitrification of embryos

From zygote to blastocyst stage of embryo can be vitrified. In 1953, Smith reported the effects of low temperatures on rabbit zygotes and their subsequent development. Since this early effort, much progress has been made (Smith, 1953). Since the birth of the first live animals from cryopreserved embryos (Whittingham et al., 1972), births have now been reported in >20 mammalian species. For example, live young of all of the common lab animal species (mouse, rat, rabbit, hamster, guinea pig) and domestic animal species (sheep, goat, horse, cat, and dog) have been achieved. Exotic animals have also been successfully produced using previously cryopreserved embryos (antelope, baboon, and marmoset). Table 2.8 lists the first reports of live young produced from the transfer of frozen-thawed embryos. Whittingham et al. (1972) reported the first live births in the mouse following transfer

of frozen-warmed embryos into recipient females. In that study, an implantation rate of 65% (n=501) of the embryos transferred was observed. Forty-three percent of those implanted embryos resulted in live births. It was also noted that an increase in cooling rate (above 0.3°C/min) resulted in a decrease in post-warmed embryo survival.

Table 2.8 First births resulting from the successful cryopreservation of mammalian embryos (Leibo and Songsasen 2002).

Species	References
Mouse	Whittingham et al., 1972
Cow	Wilmut and Rowson, 1973
Rabbit	Bank and Maurer, 1974
Rat	Whittingham, 1975
Sheep	Willadsen et al., 1976
Goat	Bilton and Moore, 1976
Horse	Yamamoto et al., 1982
Human	Zeilmaker et al., 1984)
Antelope	Kramer et al., 198)
Baboon	Pope et al., 1984
Cynomolgus monkey	Balmaceda et al., 198)
Marmoset monkey	Summers et al., 1987
Cat	Dresser et al., 1988
Pig	Hayashi et al., 1989

The first live calf born from a frozen-warmed embryo was reported by Wilmut and Rowson (1973). That study involved slow-cooling of D-10 to D-13

bovine hatched blastocysts in DMSO in glass ampules. Nine embryos were transferred to five recipients, resulting in one healthy calf named “Frosty II”. The first calf produced by artificial insemination with frozen-thawed spermatozoa in the U.S. had been named “Frosty I” (Polge and Rowson, 1952). Vitrification has also been used for the cryopreservation of embryos. The first report was by Rall and Fahy (1985). Vitrification has been used widely to cryopreserve the embryos of different animals (Table 2.8).

2.5 Prospects of cryopreservation

As previously mentioned, MII oocytes are vulnerable to cryoinjury due to the delicate spindle they possess. In contrast, GV oocytes seem to be less susceptible to cryoinjury than MII oocytes, because they are slightly smaller, lack a zona pellucida and cortical granules and are still in a quiescent stage of development. These immature oocytes also have a longer period to recover from cryoinjury because they have to mature *in vitro* prior to insemination or other manipulations refer to Table 2.9 (Shaw et al., 1999).

Table 2.9 Factors that influence the sensitivity to cryoinjury and suitability for cryostorage (Shaw et al. 1999).

Material	Premordial oocyte	GV stage oocyte	MII stage oocyte
Availability	Abundant, always present	Scarce, only from antral follicles	Scarce, only at mid-cycle
Ease of collection	Easy, e.g. biopsy	Oocyte retrieval	Oocyte retrieval
Size	<50 μm	80-300 μm	80-300 μm
Nuclear status	Resting prophase I, Nuclear membrane	GV, has nuclear membrane	Resting MII, temperature sensitive spindle, no nuclear membrane
Zona	No	Yes	Yes
Cortical granules	No	Central	Peripheral
Intracellular lipid	Little	May be abundant	May be abundant
Metabolic rate	Low	Low	Low
Ratio of surface-volume	High	Low	Low

2.5.1 Oocyte structure and cryopreservation damage

Oocytes are more difficult to freeze than embryos due to the fact that they are large, delicate spherical cells with a low surface area to volume ratio and low hydraulic conductivity (Leibo, 1980). The oocyte is the largest cell in the body of most mammalian species (Wassarman, 1988). Approximately 80% of their volume is water; when they are cooled to subzero temperatures, they may undergo intracellular ice formation, the likelihood of which is dependent on the cooling rate (Leibo et al., 1978). The mature oocyte of most mammalian species contains a spindle that is usually arrested in the metaphase stage of the second meiotic division. This spindle is made up of microtubules connected to maternal chromosomes (Aigner et al., 1992). The microtubules of oocytes are vulnerable to CPAs and changes in temperature (Pickering et al., 1990; Van Blerkom and Davis, 1994). Exposure of oocytes to CPAs and changes in temperature may cause depolymerization of tubulin within the oocyte (Aman and Parks, 1994). Damage to the meiotic spindle may change the position of the chromosomes and thus limit the fertilization capabilities of the oocyte (Eroglu et al., 1998). Spindle damage is not only a concern with fertilizability, but also because of the potential for chromosomal abnormalities in the resultant embryo (Aman and Parks, 1994).

Furthermore, as first described for mouse oocytes by Magistrini and Szöllösi (1980), the microtubules and meiotic spindles of oocytes of all mammalian species undergo disassembly and disaggregation when oocytes are cooled to temperatures near 0°C for just a few minutes (reviewed in Parks and Ruffing, 1992; Vincent and Johnson, 1992). Observations of chilling injury have been made on oocytes of cattle (Martino et al., 1996a), monkeys (Songsasen et al., 2002) and

humans (Pickering et al., 1990; Zenzes et al., 2001). Oocytes are also damaged when exposed to various CPAs. Johnson and Pickering (1987) showed that brief exposure of oocytes to Me2SO resulted in the emergence of microtubular asters and longer exposure to Me2SO resulted in disassembly of the spindle and chromosome dispersal. Vincent et al. (1990) also found that DMSO affects microtubules and spindles in oocytes.

However, they concluded that this damage is temperature-dependent. They observed that at certain temperatures, DMSO cannot permeate the cell very rapidly and therefore, less CPA is inside the cell. Shaw and Trounson (1989) reported that propylene glycol causes parthenogenetic activation of mouse oocytes. Parthenogenic activation is the artificial activation of the oocyte. This activation occurs due to an increase in the concentration of Ca^{2+}, which normally comes from spermatozoa. The wave of Ca^{2+} causes the release of cortical granules within the oocyte and this in turn results in the resumption of meiosis and the formation of the second polar body (White and Yue, 1996). Gook et al. (1995) reported that human oocytes also undergo parthenogenesis after cryopreservation. They observed that 27% of fresh and 29% of aged oocytes that had been cryopreserved were parthenogenetically activated after thawing. Cortical granules are aligned under the oolemma of most mammalian oocytes. Zona hardening after fertilization or activation of the oocyte is caused by the release of these cortical granules (Wolf and Hamada, 1977; Gulyas and Yuan, 1985). Coritical granules are released to the outer edge of the cytoplasm during the zona reaction. This reaction is normally caused by exposure to sperm prior to fertilization and prevents polyspermy (Wassarman, 1988). Propylene glycol has been shown to cause premature cortical granule release (Schalkoff et al., 1989) and disruption of

cortical microfilaments (Vincent et al., 1992). Dimethylsulphoxide has been shown to cause zona hardening and a reduction of cortical granules in mouse oocytes (Vincent et al., 1990). This latter study found that exposure of oocytes to Me_2SO between 20 and 37°C had negative effects on the zona pellucida, fertilization rate and spindle organization. This study also indicated that the oocyte itself caused the hardening of the zona and not the zona. Zonae were isolated from the oocytes, but the isolated zonae were not affected by exposure to DMSO, thus proving that the oocyte itself, not the zona was responsible for the hardening effect. Caroll et al. (1990) reported that adverse effects to the zona pellucida due to cryopreservation can be bypassed by zona drilling. Zona drilling was first reported by Gordon and Talansky (1986) in which the mouse zona was drilled with acid Tyrode's solution through a fine micropipette. The zona can also be penetrated with enzymes such as trypsin or pronase (Gordon et al., 1986).

2.5.2 Reversal of microtubule damage and post-warming incubation periods

Magistrini and Szöllösi (1980) found that the spindles of mouse oocytes are damaged by being exposed to low temperatures. However, they also reported that this damage may in fact be reversible. After step-wise rewarming, a significant number of the oocytes contained normal spindles. Another study by Pickering et al. (1990) found that 25-50% of the disassembled spindles in human oocytes reverted back to normal appearance after 30 min incubation post-warming. Chen et al. (2000) developed a way to judge oocyte intracellular freezing damage using fluorescent stains in the mouse. Using this method they were able to decide which method of

vitrification and warming worked best based on morphological changes of the meiotic spindle and chromosomes of the oocyte. They hypothesized that after being warmed, longer incubation times of oocytes prior to their being inseminated or undergoing SCNT may increase cleavage and blastocyst rates (Chen et al., 2000; Chen et al., 2001). That study found that oocytes contained severe microtubule damage immediately after warming. After 1, 2 and 3 h of culture, the percentage of normal oocytes increased significantly. Leaving post-warmed oocytes in the incubator for 1-3 h prior to fertilization may promote spindle and chromosomal recovery. Also, the CPAs are still inside the cells post-warming. Allowing longer post-warm culture periods may permit complete expulsion of these CPAs.

2.5.3 Applications of oocyte cryopreservation

The inability to cryopreserve oocytes reliably poses major problems in the field of gamete and embryo biology of the large domestic species. Although full-term pregnancies and live calves have been derived by IVF of bovine oocytes that have been cryopreserved by being cooled slowly to low subzero temperatures, the rate of development of cryopreserved oocytes into blastocysts has been low, usually amounting to <5% of the cooled oocytes (see reviews in Hochi, 2003; Parks and Ruffing, 1992; Vajta, 2000). The ability to cryopreserve oocytes reliably would ease logistical problems associated with the use of abattoir-derived oocytes, since these must be collected from the ovaries and subjected to maturation and fertilization within a relatively short time.

Furthermore, long-term storage of oocytes would substantially alleviate constraints of time in the procedure of SCNT, since it would permit oocytes to be

stored in LN_2 and warmed to physiological temperatures to be used as required. In the case of oocytes to be enucleated for use as cytoplasts, it is possible that slight damage to the germinal vesicle or chromosomes resulting from cooling or exposure to CPAs might be tolerated, since the nuclear material itself is to be removed. For example, the nuclei of mouse zygotes subjected to vitrification were found to be more damaged than the cytoplasm (Kono and Tsunoda, 1988).

Oocyte cryopreservation is also crucial in the human reproduction field due to legal and ethical issues associated with embryo cryopreservation. This technique could also be useful for women wanting to cryopreserve their genetic material for use later in life and also women undergoing radio- or chemotherapy for treatment of various types of cancer.

2.6 References

Aigner, S., van er Elst, J., Siebzehnrubl, E., Wildt, L., Lang, N., and van Steirteghem, A. C. (1992). The influence of slow and ultra-rapid freeing on the organization of the meiotic spindle of the mouse oocyte. **Hum. Reprod.** 7:857-864.

Al-Hasani, S., Krisch, J., Diedrich, K., Blanke, S., van der Ven, H., and Krebs, D. (1989). Successful embryo transfer of cryopreserved and in-vitro fertilized rabbit oocytes. **Hum. Reprod.** 4:77-79.

Almodin, C. G., Minguetti-Camara, V. C., Paixao, C. L., and Pereira, P. C. (2010). Embryo development and gestation using fresh and vitrified oocytes. **Hum. Reprod.** 25:1192-1198.

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.

Arav, A. (1992). Vitrification of oocytes and embryos. **In New Trends in Embryo Transfer**, edn 1, Eds Lauria, A., and Gandolfi, F. Cambridge: Portland Press. PP. 255-264.

Arav, A., and Zeron, Y. (1997). Vitrification of bovine oocytes using modified minimum drop size technique (MDS) is effected by the composition and the concentration of the vitrification solution and by the cooling conditions. **Theriogenology** 47:341.

Arav, A., Rubinshky, B., and Bacci, M. L. (1990). The effect of volume, cooling rate and composition on vitrification of two cell mouse embryos and pig oocyte. **Cryobiology** 27:628-629.

Arav, A., Shehe, D., and Mattioli, M. (1993). Osmotic and cytotoxic study of vitrification of immature bovine oocytes. **J. Reprod. Fertil.** 99:353-358.

Arav, A., Yavin, S., Zeron, Y., Natan, D., Dekel, I., and Gacitua, H. (2002) New trends in gamete's cryopreservation. **Mol. Cellular Endocrino.** 187:77-81.

Assey, R. J., Hyttel, P., Purwantara, B., Greve, T., and Schmidt, M. (1992). Oocyte structure in dominant versus subordinate follicles during the first follicular wave in cattle. **Proc. 12th Inter. Cong. Anim. Reprod.** 1:303-305.

Avery, B., and Greve, T. (1995). Impact of Percoll on bovine spermatozoa for in vitro insemination. **Theriogenology** 44:871-878.

Balmaceda, J., Heitman, T., Garcia, A., Pauerstein, C., and Pool, R. (1986). Embryo cryopreservation in cynamologus monkey. **Fertil. Steril.** 45:403-406.

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

Begin, I., Bhatia, B., Baldassarre, H., Di Nnyes, A., and Keefer, C. L. (2003). Cryopreservation of goat oocytes and in vivo derived 2- to 4-cell embryos

using the cryoloop (CLV) and solid-surface vitrification (SSV) methods. **Theriogenology** 59:1839-1850.

Bhat, S. N., Sharma, A., and Bhat, S. V. (2005). Vitrification and glass transition of water: insights from spin probe ESR. **Phys. Rev. Letter** 95:235702.

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

Blondin, P., Guilbalt, L. A., and Sirard, M. A. (1997). The time interval between FSH-P administration and slaughter can influence the developmental competence of beef heifer oocytes. **Theriogenology** 48:803-813.

Booth, P. J., Vajta, G., Hoj, A., Holm, P., Jacobsen, 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.

Camus, A., Clairaz, P., Ersham, A., van Kappel, A. L., Savic, G., and Staub, C. (2006). Principe de la vitrification: cine´ tiques comparatives. The comparison of the process of five different vitrification devices. **Gyné cologie Obst. Fertil.** 34:737–745.

Carolan, C., Monaghan, P., Gallgher, M., and Gordon, I. (1994). Effect of recovery method on yield of bovine oocytes per ovary and their developmental competence after maturation, fertilization and culture in vitro. **Theriogenology** 41:1061-1068.

Caroll, J., Depypere, H., and Mathew, 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, C. (1988). Pregnancies after human oocyte cryopreservation. **Ann. N.Y. Acad. Sci.** 541:541-549.

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

Chen, S. U., Lien, Y. R., Cheng, Y. Y., Chen, H. F., Ho, H. N., and Yang, Y. S. (2001). Vitrification of mouse oocytes using closed pulled straws (CPS) achieves a high survival and preserves good patterns of meiotic spindles, compared with conventional straws, open pulled straws (OPS) and grids. **Hum. Reprod.** 16:2350-2356.

Chen, S. U., Lien, Y. R., Chen, H. F., Chao, K. H., Ho, H. N., and Yang, Y. S. (2000). Open pulled straws for vitrification of mature mouse oocytes preserve patterns of meiotic spindles and chromosomes better than conventional straws. **Hum. Reprod.** 15:2598-2603.

Chian, R. C., Son, W. Y., Huang, J. Y., Cui, S. J., Buckett, W. M., and Tan, S. L. (2005). High survival rates and pregnancies of human oocytes following vitrification: preliminary report. **Fertil. Steril.** 84:S36.

Cho, H. J., Son, W. Y., Yoon, S. H., Lee, S. W., and Lim, J. H. (2002). An improved protocol for dilution of cryoprotectants from vitrified human blastocysts. **Hum. Reprod.** 17:2419-2422.

DeVries, A. L., and Wohlschlag, D. E. (1969). Freezing resistance in some antartic fishes. **Science** 163:1074.

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.

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.

Eroglu, A., Toth, T. L., and Toner, M. (1998). Alterations of the cytoskeleton and polyploidy induced by cryopreservation of metaphase II mouse oocytes. **Fertil. Steril.** 69:944-957.

Fabbri, R., Porcu, E., Marsella, T., Primavera, M. R., Rocchetta, G., Ciotti, P. M., Magrini, O., Seracchioli, R., Venturoli, S., and Flamigni, C. (2000). Technical aspects of oocyte cryopreservation. **Mol. Cell Endo.** 169:39-42.

Fabbri, R., Porcu, E., Marsella, T., Primavera, M. R., Seracchioli, R., Ciotti, P. M., Magrini, O., Venturoli, S., and Flamigni, C. (1998). Oocyte cryopreservation. **Hum. Reprod.** 4:98-108.

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

Fahy, G. M., Wowk, B., Pagotan, R., Chang, A., Phan, J., Thomson, B., and Phan, L. (2009). Physical and biological aspects of renal vitrification. **Organogenesis** 5:167-175.

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

Fukui, Y., Sonoyama, T., Mochoizuki, H., and Ono, H. (1990). Effect of heparin dosage and sperm capacitation time on *in vitro* fertilization and cleavage of bovine oocytes matured in vitro. **Theriogenology** 34:575-591.

Fuller, B. J., Lane, N., and Benson, E. E. (2004). Life in the Frozen State. CRC Press, Boca Raton, FL.

Gandolfi, F., Luciano, A. M., Modina, S., Ponzini, A., Pocar, P., Armstrong, D. T., and Lauria, A. (1997). The in vitro developmental competence can be related to the morphology of the ovary. **Theriogenology** 48:1153-1160.

Gook, D., Osborn, S., and Johnston, W. (1995). Parthenogenetic activation of human oocytes following cryopreservation using 1,2-propanediol. **Hum. Reprod.** 10:793-798.

Gordon, J. W., and Talansky, B. E. (1986). Assisted fertilization by zona-drilling: a mouse model for the correction of oligospermia. **J. Exp. Zool.** 239:347-354.

Gulyas, B. J., and Yuan, L. C. (1985). Cortical reaction and zona hardening in mouse oocytes following exposure to ethanol. **J. Exp. Zool.** 233:269-276.

Hamawaki, A., Kuwayama, M., and Hamano, S. (1999). Minimum volume cooling method for bovine blastocyst vitrification. **Theriogenology** 51:165.

Hashimoto, S., Minami, N., Yamada, M., and Imai, H. (2000). Excessive concentration of glucose during in vitro maturation impairs the developmental competence of bovine oocytes after in vitro fertilization: revelance to intracellular reactive oxygen species and glutathion contents. **Mol. Reprod. Dev.** 56:520-526.

Hayashi, S., Kobayashi, K., Mizuno, J., Saitoh, K., and Hirano, S. (1989). Birth of piglets from frozen embryos. **Vet. Rec.** 125:43-44.

Hazelger, N. L., and Stubbings, R. B. (1992). Developmental potential of selected bovine oocyte cumulus complexes. **Theriogenology**. 37:219.

Hochi, S. (2003). Cryopreservation of follicular oocytes and preimplanatation embryos in cattle and horses. **J. Reprod. Dev.** 49:13-21.

Hochi, S., Fujimoto, T., Choi, Y., Braun, J., and Oguri, N. (1994). Cryopreservation of equine oocytes by 2-step freezing. **Theriogenology** 42:1085-1094.

Hochi, S., Kimura, K., Ito, K., and Hirabayshi. M. (1995). Viability of immature horse oocytes cryopreserved by vitrification. **Theriogenology** 43:236.

Hochi, S., Kozawa, M., Fjuimoto, T., Hondo, E., Yamada, J., and Oguri, N. (1996). In vitro maturation and transmission electron microscopic evaluation of horse oocytes after vitrification. **Cryobiology** 33:300-310.

Hurtt, A. E., Landim-Alvarenga, F., Seidel, G. E., and Squires, E. L. (2000). Vitrification of immature and mature equine and bovine oocytes in an ethylene glycol, ficoll and sucrose solution using open-pulled straws. **Theriogenology** 54:119-128.

Hyttel, P., Fair, T., Callsen, H., and Greve, T. (1997). Oocyte growth, capacitation and final maturation in cattle. **Theriogenology** 47:23-32.

Iritani, W. G., and Niwa, K. (1977). Capacitation of bull spermatozoa and in vitro of cattle follicular oocytes matured in culture. **J. Reprod. Fertil.** 50:119-121.

Isachenko, V., Folch, J., Isachenko, E., Nawroth, F., Krivokharchenko, A., Vajta, G., Dattena, M. and Alabart, J. L. (2003). Double vitrification of rat embryos at different developmental stages using an identical protocol. **Theriogenology** 60:445-452.

Jackowski, S. C., Leibo, S. P., and Mazur, P. (1980). Glycerol permeabilities of fertilized and unfertilized mouse ova. **J. Exp. Zool.** 212:329-341.

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

Karow, A. M. and Critser, J. K. (1997). Reproductive Tissue Banking. Academic Press, San Diego, CA.

Kono T., Kwon, O. Y., and Nakahara, T. (1991). Development of vitrified mouse oocytes after IVF. **Cryobiology**. 28:50-54.

Kramer, L. B., Dresser, B. L., Pope, C. E., Dahlhausen R. D., and Baker, R. D. (1983). The nonsurgical transfer of frozen-thawed eland (Tragelaphus oryx) embryos. **Annual Proceedings-American Assoc. of Zoo Veterinarians.** Hills Division, Riviana Foods, Topeka, KS. 104-105.

Kubota, C., and Yang, X. (1998). Cytoplasmic incompetence results in poor development of bovine oocytes from small follicles. **Theriogenology** 49:183.

Kuleshova, L., Gianaroli, L., Magli, C., Ferraretti, A., and Trounson, A. (1999). Birth following vitrification of a small number of human oocytes. **Hum. Reprod.** 14:3077-3079.

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

Kuwayama, M., Vajta, G., Ieda, S., and Kato, O. (2005). Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. **Reprod. Biomedi. Online** 11:608-614.

Lane, M., and Gardner. D. K. (2001). Vitrification of mouse oocytes using a nylon loop. **Mol. Reprod. Dev.** 58:342-347.

Lane, M., Forest, K. T., Lyons, E. A., and Bavister, B. D. (1999a). Live births following vitrification of hamster embryos using a novel containerless technique. **Theriogenology.** 51:167.

Larman, M. G., Sheehan, C. B., and Gardner, D. K. 2006. Calcium free vitrification reduces cryopreservation induced zona pellucida hardening and increased fertilization rates in mouse oocytes. **Reproduction** 131:53-61.

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

Le Gal, F., De Roover, R., Verhaeghe, B., Etienne, D. and Massip, A. (2000). Development of vitrified matured cattle oocytes after thawing and culture in vitro. **Vet. Rec.** 146:469-471.

Leclerc, P., Sirard, M. A., Chafoules, J. G., and Naitana, S. (1992). Decrease of concentrations during heparin-induced capacitation in bovine spermatozoa. **J. Reprod. Fertil.** 94:23-32.

Leibo, S. P., and Mazur, P. (1971). The role of cooling rates in low-temperature preservation. **Cryobiology**. 8:447-452.

Leibo, S. P., and Songsasen, N. (2002). Cryopresrevation of gametes and embryos of non- domestic animals. **Theriogenology.** 57:303-326.

Leibo, S. P., Farrant, J., Mazur, P., Hanna, M. G., and Smith, L.H. (1970). Effects of freezing on marrow stem cell suspensions: interactions of cooling and warming rates in the presence of PVP, sucrose, or glycerol. **Cryobiology** 6:315-332.

Leibo, S. P., McGrath, J. J., and Cravalho E. G. (1978). Microscopic observation of intracellular ice formation in unfertilized mouse ova as a function of cooling rate. **Cryobiology.** 15:257-271.

Leibo, S .P. (1980). Water permeability and its activation energy of fertilized and unfertilized mouse ova. **J. Memb. Biol.** 53:179-188.

Leibo, S. P. (1986). Cryobiology: preservation of mammalian embryos. **Basic Life Sci.** 37:251-272.

Leibo, S. P. (2004b). **Cryopresrvation of mammalian oocytes**. Preservation of Fertility. T. Tulandi and R.G. Gosden, eds. Taylor and Francis, London. 141-155.

Liebermann, J., and Tucker, M. J. (2002). Effect of carrier system on the yield of human oocytes and embryos as assessed by survival and developmental potential after vitrification. **Reproduction** 124:483-489.

Liebermann, J., Tucker, M., Graham, J., Han, T., Davis, A., and Levy, M. (2002). Blastocyst development after vitrification of multipronuclear zygotes using the felxipet denuding pipette. **Reprod. Biomedi. Online** 4:146-150.

Lj, X., Su, L., Li, Y., Ji, W., and Dinnyes, A. (2002). Vitrification of Yunnan Yellow Cattle oocytes: work in progress. **Theriogenology** 58:1253-1260.

Lonargan, P., Monghan, P., Rizoz, D., Boland, M. P., and Gordon, I. (1994). Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization and culture *in vitro*. **Mol. Reprod. Dev.** 37:48-53.

Lonergan, P. (1992). Studies on the in vitro maturation, fertilization and culture of bovine follicular oocytes. Ph. D. Thesis, University college, Dublin.

Lovelock, J. E. (1954). Biophysical aspects of the freezing and thawing of living cells. **Proc. R. Soc. Med.** 47:60-62.

Luyet, B. J. (1937). The vitrification of organic colloids and of protoplasm. **Biodynamica.** 1:1-14.

Luyet, B. J., and Rapatz, G. (1970). A review of basic research on the cryopreservation of red blood cells. **Cryobiology** 6:425-481.

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.

Maclellan, L. J., Lane, M., Sims, M. M., and. Squires, E. L. (2001). Effect of surcrose or trehalose on vitrification of equine oocytes 12 or 24 h after the onset of maturation, evaluated after ICSI. **Theriogenology**. 555:310.

Magistrini, M., and Szöllösi, D. (1980). Effects of cold and isopropyl-N-phenylcarbamate on the second meiotic spindle of mouse oocytes. **Europ. J. Cell. Biol.** 22:699-707.

Martino, A., Pollard, J. W., and Leibo, S. P. (1996a). Effect of chilling bovine oocytes on their developmental competence. **Mol. Reprod. Dev.** 45:503-512.

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.

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

Matsumoto, H., Jiang, J. Y., Tanaka, T., Sasada, H. and Sato, E. (2001). Vitrification of large quantities of immature bovine oocytes using nylon mesh. **Cryobiology** 42:139-144.

Matsunari, H., Maehara, M., Nakano, K., Ikezawa, Y., Hagiwara, Y., Sasayama, N., Shirasu, A., Ohta, H., Takahashi, M., and Nagashima, H. (2012). Hollow fiber vitrification: A novel method for vitrifying multiple embryos in a single device. **J. Reprod. Dev.** 58:599-608.

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.

Mazur, P. (1965). Causes of injury in frozen and thawed cells. **Fed. Proc.** 24:175-182.

Mazur, P. (1970). Cryobiology: The freezing of biological systems. **Science** 168:939-949.

Mazur, P. (1977). The role of intracellular freezing in the death of cells cooled at supra-optimal rates. **Cryobiology** 14:251-272.

Mazur, P. (1988). Stopping biological time. The freezing of living cells. **Ann. N.Y. Acad. Sci.** 541:514-531.

Mazur, P. (2004). Principles of cryobiology. Life in the Frozen State. Fuller, B. J., Lane, N., and Benson, E. E. eds. CRC Press, Boca Raton, FL. 3-65.

Mazur, P., Leibo, S. P., and Chu, E. H. Y. (1972). A two-factor hypothesis of freezing injury. Evidence from Chinese hamster tissue-culture cells. **Exp. Cell Res.** 71:345-355.

Miller, D. J., Bellin, M. E., First, N. L., and Ax, R. L. (1987). Competition of follicular fluid glycosaminoglycans and desulfated heparin for heparin binding sites on bull sperm. **J. Ani.l Sci.** 65:363.

Mizushima, S., and Fukui, Y. (2001). Freezabiliy and developmental capacity of bovine oocytes cultured individually in a chemically defined maturation medium. **Theriogenology** 55:1431-1445.

Muthukumar, K., Mangalaraj, A. M., Kamath, M. S. and George, K. (2008). Blastocyst cryopreservation: vitrification or slow freeze. **Fertil. Steril.** 90:S426–S427.

Nakagata, N. (1989). High survival rate of unfertilized mouse oocytes after vitrification. **J. Reprod. Fertil.** 87:479-483.

Nakagata, N. (1992). Cryopreservation of unfertile zed rat oocytes by ultrarapid freezing (in Japanese). **Exper. Anim.** 41:443-447.

Nebreda, A. R., and Ferby, I. (2000). Regulation of the meiotic cell cycle in oocytes. **Current Opinion of Cell Biol.** 12:666-675.

Niwa, K., and Ohgoda, O. (1988). Synergistic effect of caffeine and heparin on in vitro fertilization of cattle oocytes matured in culture. **Theriogenology** 30:733-741.

Otoi, T., Tachikawa, S., Kondo S., and Suzuki, T. (1992). Developmental capacity of bovine oocytes cryopreserved after maturation in vitro and of frozen-thawed embryos derived from frozen mature oocytes. **Theriogenology** 38:711-719.

Otoi, T., Yamamoto, K., Koyama, N., Tachikawa, S., and Suzuki, T. (1998). Cryopreservation of mature bovine oocytes by vitrification in straws. **Cryobiology** 37:77-85.

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

Parkening, T. A., Tsunoda, Y., and Chang, M. C. (1976). Effects of various low temperatures, cryoprotective agents and cooling rates on the survival, fertilizability and development of frozen-thawed mouse eggs. **J. Exp. Zool.** 197:369-374.

Parks, J. E. and Ruffing, N. A. (1992). Factors affecting low temperature survival of mammalian oocytes. **Theriogenology** 37:59-73.

Parnpai, R., Tasripoo, K., and Kamonpatana, M. (1999). Deveopment 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.

Parrish, J. J., Susko-Parrish, J. L., and First, N. L. (1989). Capacitation of bovine sperm by heparin: inhibitory effect of glucose and role of intracellular pH. **Biol. Reprod.** 41:683-699.

Parrish, J. J., Susko-Parrish, J. L., and Graham, J. K. (1999). In vitro capacitation of bovine spermatozoa: role of intracellular calcium. **Theriogenology** 51:461-472.

Parrish, K. A., and Susko-Parrish, J. L. (1995). Effect of bovine sperm separation by either swim-up or Percoll method on success of in vitro fertilization and early embryonic development. **Theriogenology** 44:859-869.

Pavlok, A., Kopecny, V., Lucas-Hahn, A. and Neimann, H. (1993). Transcriptional activity and nuclear ultrastructure of 8-cell bovine embryos developed by in vitro maturation and fertilization of oocytes from different growth categories of antral follicles. **Mol. Reprod. Dev.** 35:233-243.

Paynter, S. J. (2000). Current status of the cryopreservation of human unfertilized oocytes. **Hum. Reprod.** 6:449-456.

Pegg, D. E. (2007). Principles of Cryopreservation. In Day JG, Stacey GN. Cryopreservation and Freeze-Drying Protocols, Second Edition. **Methods in Mol. Biol.** 368:39-57.

Petyim, S., Makemahar, O., Kunathikom, S., Choavaratana, R., Laokirkkiat, P., and Penparkkul, K. (2009). The successful pregnancy and birth of a healthy baby after human blastocyst vitrification using Cryo-E, first case in Siriraj Hospital. **J. Medical Assoc. Thailand** 92:1116-1121.

Pickering, S. J., Braude, P. R., Johnson, M. H., Cant, A., and Currie, J. (1990). Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. **Fertil. Steril.** 54:102-108.

Pieterse, M. C., Vos, P. L. A. M., Kruip, T. A. M., Wurth, Y. A., Van Benden, T. H., Willemse, A. H. and Taverne, M. A. M. (1991). Transvaginal ultrasound guided follicular aspiration of bovine oocytes. **Theriogenology** 35:857-862.

Polge, C., and Rowson, L. E. A. (1952). Fertilizing capacity of bull spermatozoa after freezing at -79ºC. **Nature** 169:626-627.

Polge, C., Smith, A. U., and Parkes, A. S. (1949). Revival of spermatozoa after vitrification and dehydration at low temperatures. **Nature** 164:666.

Pope, C. E., Pope, V. Z., and Beck, L. R. (1984). Live birth following cryopreservation and transfer of a baboon embryo. **Fertil. Steril.** 42:143-145.

Porcu, E. (2001). Oocyte freezing. **Semin. Reprod. Medi.** 19:221-230.

Portmann, M., Nagy, Z. P., and Behr, B. (2010) Evaluation of blastocyst survival following vitrification/warming using two different closed carrier systems. **Hum. Reprod.** 25:i261.

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

Rall, W. F. (1992). Cryopreservation of oocytes and embryos: methods and applications. **Ani. Sci. Reprod.** 28:237-245.

Rall, W. F., and Fahy, G. M. (1985). Ice-free cryopr eservation of mouse embryos at -196ºC by vitrification. **Nature** 313:573-575.

Renard, J. P., and Babinet, C. (1984). High survival of mouse embryos after rapid freezing and thawing inside plastic straws with 1-2, propa nediol as cryoprotectant. **J. Exper. Zool.** 230:443-448.

Rubinsky, B., Arav, A., and De Vries, A. L. (1991). Cryopreservation of oocyte using directional cooling and antifreeze glycoproteins. **Cryoletters**. 12: 93-106.

Ruffing, N. A., Steponkus, P. L., Pitt, R. E., and Parks, J. E. (1993). Osmometric behaviour, hydraulic conductivity, and incidence of intracellular ice formation in bovine oocytes at different developmental stages. **Cryobiology** 30:562-580.

Russell, J. B. (2001). In vitro oocyte maturation. In: Wolf DP, Zelinski-Wooten M, editors. **Assisted Fertilization and Nuclear Transfer in Mammals**. Totowa: Humana Press, PP. 67-79.

Saha, S., Otoi, T., Takagi, M., Boediono, A., Sumantri, C. and Suzuki, T. (1996) Normal calves obtained after direct transfer of vitrified bovine embryos using ethylene glycol, trehalose, and polyvinylpyrrolidone. **Cryobiology** 33:291-299.

Shamsuddin, M., Larsson, B., Gustafsson, H., and Rodriguez-Martinez, H. (1992). Number of cells in the in vitro pre-implantation bovine embryos obtained from different methods of in vitro culture of IVM-IVF oocytes**. Proc. Nordic A.I. Virtanen Institute symposium on the In vitro Culture of Domestic Animal Embryos.** 30-31.

Shaw, J. M., Oranratnachai, A., and Trounson, A. O. (1999). Cryopreservation of oocytes and embryos. **Handbook of IVF.** 2nd Edition. Trounson, A.O., Gardner, D., eds. CRC press, Boca Raton, FL.

Shaw, J. M., Oranratncachai A., and Trounson, A. O. (2000a). Fundamental cryobiology of mammalian oocytes and ovarian tissue. Theriogenology. 53:59-72.

Shaw, J. M., Wood C., and Trouson A. O. (2000b). Transplantation and cryopreservation of ovarian tissue. **Handbook of IVF,** 3rd Edition. Trounson, A.O., Gardner, D., eds. CRC Press Boca Raton, FL.413.

Shaw, J. M., and Trounson, A. O. (1989). Parthenogenetic activation of unfertilized oocytes by exposure to 1,2-propanediol is influenced by temperature, oocyte age and cumulus removal. **Gamete Res**. 24:269-279.

Shehata, S. H. (1998). *In vitro* buffalo sperm capacitation with special attention to acrosome reaction and oocyte penetration test. **Assuit Vet. Med. J.** 38:56-72.

Sirard, M. A., Coenen, K., and Bilodeau, S. (1992). Effect of fresh or cultured follicular fractions on meiotic resumption in bovine oocytes. **Theriogenolgy** 37:39-57.

Smith, A. U. (1961). Biological Effects of Freezing and Supercooling The Williams and Wilkins Company, Baltimore.

Smith, G. D. (2001). Control of oocyte nuclear and cytoplasmic maturation. In: Wolf DP, Zelinski-Wooten M, editors. **Assisted Fertilization and Nuclear Transfer in Mammals** Totowa: Humana Press, PP. 53-65.

Songsasen, N., Yu, I., Ratterree, M. S., VandeVoort C. A., and Leibo, S. P. (2002). Effect of chilling on organization of tubulin and chromosomes in rhesus monkey oocytes. **Fertil. Steril.** 77:818-825.

Stachecki, J. J., Cohen, J., and Willadsen, S. (1998a). Detrimental effects of sodium during mouse oocyte cryopreservation. **Biol. Reprod.** 59:395-400.

Stachecki, J. J., Cohen, J., and Willadsen, S. M. (1998b). Cryopreservation of unfertilized mouse oocytes: the effect of replacing sodium with choline in the freezing medium. **Cryobiology** 37:346-354.

Steponkus, P. L., Myers, S. P., Lynch, D. V., Gardner, L., Bronshteyn, V., Leibo, S. P., Rall, W. F., Pitt, R. E., Lin, T. T., and MacIntyre, R. J. (1990) Cryopreservation of Drosophila melanogaster embryos. **Nature** 345:170-172.

Stewart, D. L. (1951). Storage of bull spermatozoa at low temperatures. **Vet. Rec.** 63:65-66.

Sugiyama, R., Nakagawa, K., Shirai, A., Sugiyama, R., Nishi, Y., Kuribayashi, Y., and Inoue, M. (2010). Clinical outcomes resulting from the transfer of vitrified human embryos using a new device for cryopreservation (plastic blade). **J. Assist. Reprod. Gene.** 27:161-167.

Summers, P. M., Shephard, A. M., Taylor, C. T., and Hearn, J. P. (1987). The effects of cryopreservation and transfer on embryonic development in the common marmoset monkey, Callithrix jacchus. **J. Reprod. Fertil.** 79:241-250.

Sun, X., Li, Z., Yi, Y., Chen, J., Leno, G. H., and Engelhardt, J. F. (2008). Efficient term development of vitrified ferret embryos using a novel pipette chamber technique. **Biol. Reprod.** 79:832-840.

Takagi, Y., Mori, K., Takahashi, T., Sugawara, S., and Masaki, J. (1992). Differences in development of bovine oocytes recovered by aspiration or by mincing. **J. Ani. Sci.** 70:1923-1227.

Thompson, M., Nemits, M., and Ehrhardt, R. (2011). Rate-controlled Cryopreservation and Thawing of Mammalian Cells. **Nat. Prot. Exch**. doi:10.1038/protex.2011.224.

Trounson, A., Anderiesz, C., and Jones, G. (2001). Maturation of human oocytes in vitro and their developmental competence. **Reproduction** 121:51-75.

Tsang, W. H., and Chow, K. L. (2009) Mouse embryo cryopreservation utilizing a novel high-capacity vitrification spatula. **BioTechniques** 46:550-552.

Tunquist, B. J., and Maller, J. L. (2003). Under arrest: Cytostatic factor (CSF)-mediated metaphase arrest in vertebrate eggs. **Genes Dev.** 17:683-710.

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

Vajta, G., Holm, P., Greve, T., and Callesen, H. (1997). Vitrification of porcine embryos using the open pulled straw (OPS) method. **Acta Veterinaria Scandinavica** 38:349-352.

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

Van Blerkom, J. and Davis, P. W. (1994). Cytogenetic, cellular, and developmental consequences of cryopreservation of immature and mature mouse and human oocytes. **Micryoscopy Res. Tech.** 27:165-193.

Vanderzwalmen, P., Bertin, G., Debauche, C., Standaart, V., and Schoysman, E. (2000). In vitro survival of metaphase II oocytes (MII) and blastocysts after vitrification in a hemi-straw (HS) system. **Fertil. Steril.** 74:S215–S216.

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.

Vincent, C. and Johnson, M. H. (1992). Cooling, cryoprotectants, and the cytoskeleton of the mammalian oocyte. **Oxford Rev. Reprod. Biol.** 14:73-100.

Vincent, C., Pickering, S. J., Johnson M. H., and Quick, S. J. (1990). Dimethylsulphoxide affects the organization of microfilaments in the mouse oocyte. **Mol. Reprod. Dev.** 26:227-235.

Vlaenderen, I, Van Soom, A., and Van Kruif, A. D. (1991). Bull specific effect on in vitro penetration, fertilization of bovine oocytes using various levels of heparin. **ARTA** 2:105-106.

Vutyavanich, T., Piromlertamorn, W., and Nunta, S. (2010). Rapid freezing versus slow programmable freezing of human spermatozoa. **Fertil. Steril**. 93:1921-1928.

Wassarman, P. (1988). The mammalian ovum. **The Physiology of Reproductio.** Knobil, E., and Neill, J. eds. Raven Press, New York. PP. 69.

White, K. L., and Yue, C. (1996). Intracellular receptors and agents that induce activation in bovine oocytes. **Theriogenology** 45:91-100.

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

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

Wilmut, I., and Rowson, L. E. A. (1973). Experiments in the low temperature preservation of cow embryos. **Vet. Rec.** 92:686-690.

Wolf, D. P., and Hamada, M. (1977). Induction of zonal and egg plasma membrane blocks to sperm penetration in mouse eggs with cortical granule exudates. **Biol. Reprod.** 17:350-354.

Xu, K. P., and King, W. A. (1990). Effect of oviductal cells and heparin on bovine sperm capacition in vitro. **Biol. Reprod.** 42:89.

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

Yavin, S., and Arav, A. (2001). Development of immature bovine oocytes vitrified by minimum drop size technique and a new vitrification apparatus (VIT-MASTER). **Cryobiology** 43:331.

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

Yavin, S., Aroyo, A., Roth, Z., and Arav, A. (2009). Embryo cryopreservation in the presence of low concentration of vitrification solution with sealed pulled straws in liquid nitrogen slush. **Hum. Reprod.** 24:797-804.

Yoon, T. K., Chung, H. M., Lim, J. M., Han, S. Y., Ko J. J., and Cha. K. Y. (2000). Pregnancy and delivery of healthy infants developed from vitrified oocytes in a stimulated IVF-embryo transfer program. **Fertil. Steril.** 74:180-181.

Zeilmaker, G. H., Alberda, A. T. van Gent, I., Rijkmans, C. M., and Drogendijk, A. C. (1984). Two pregnancies following transfer of intact frozen-thawed embryos. **Fertil. Steril.** 42:293-296.

Zenzes, M. T., Bielecki, R. Casper, R. F., and Leibo, 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

IN VITRO DEVELOPMENTAL POTENTIAL OF DAY 7 AND DAY 8 GRADED EXPANDED BOVINE BLASTOCYSTS SUBSEQUENT TO CRYOTOP VITRIFICATION

3.1 Abstract

Embryo cryopreservation is a promising area of study in the field of reproductive research. One of the issues that have recently arisen is the discovery the developmental ability of embryos after cryopreservation varies depending on the culture period of the embryo prior to its cyropreservation. Therefore, the present study was designed to explore the developmental potentiality of bovine embryos at D7 and D8, as well as their survivability and hatchability after Cryotop vitrification. The blastocyst rate at D8 (28.1%) was higher than that of D7 (19.1%). Grade 1 (G1) and grade 2 (G2) expanded blastocysts at D7 and D8 were vitrified by Cryotop device using 10% dimethylsulfoxide (DMSO), 10% ethylene glycol (EG) in TCM199+20%FBS (Base medium) as an equilibration solution for 1 min, and 20% DMSO, 20% EG and 0.5 M sucrose in base medium as a vitrification solution for 30 sec. Except for the G2 expanded blastocysts at D8, I found that the survival rate of vitrified G1 and G2 expanded blastocysts was not significantly different from the control group in both D7 and D8 but the D7 vitrified embryos showed superior rates of hatchability than those of D8. In a curve estimation of correlation regressions, the

hatched rate of D7 G1 expanded blastocysts at 48 h showed a strong correlation (R^2=0.914) with their survival rate. Therefore, I concluded that the D7 culture period is the most suitable for vitrification of IVF derived embryos.

3.2 Introduction

Cryopreservation in general and, in particular, the vitrification of oocytes and embryos (of different species of animals as well as humans) is an important field of research. Successful vitrification of mammalian embryos has numerous economic benefits, and can have a major impact on improving the efficiency of animal research, breeding and production. The first successful cryopreservation of bovine embryos was reported by Wilmut and Rowson (1973). Nowadays, the focus is on studying the vitrification of embryos using different devices and cryoprotective agents (CPAs) in order to increase their survival rate as well as the success rate of embryo transfer (ET) and ET related pregnancies. According to Laowtammathron et al. (2005), high survival rates after bovine embryo vitrification have been recently achieved using different devices and techniques. Electron microscopic (EM) grid (Steponkus et al., 1990; Martino et al., 1996; Cho et al., 2002), minimum drop size (MDS; Arav, 1992; Arav and Zeron, 1997; Yavin and Arav, 2001), Cryotop (Hamawaki et al., 1999; Kuwayama and Kato, 2000), Cryoloop (Lane et al., 1999), Hemi-straw (Vanderzwalmen et al., 2003), solid surface vitrification (SSV, Dinnyes et al., 2000), open pulled straw (OPS, Vajta et al., 1997, Vajta et al., 1998), closed pulled straw (CPS; Chen et al., 2001), Rapid-i (Larman and Gardner, 2010) and hollow fiber (Matsunari et al., 2012) are some of the devices and procedures that have been used in animal oocytes and embryos vitrification.

The survival rate of embryos is markedly dependent on their exposure to CPAs prior to their immersion in liquid nitrogen (Inaba et al., 2011). On the other hand, the latest approach to achieve minimum volume of vitrification solution (VS) is a novel vitrification method using the Cryotop device The Cryotop method has been efficaciously used to cryopreserve bovine (Chian et al., 2004), bubaline (Gaspirini et al., 2007) and ovine oocytes (Succu et al., 2007), in addition to embryos in bovine and bubaline species (Laowtammathron et al., 2005). But the composition and concentration of CPAs are also very important for successful vitrification.

Although successful cryopreservation depends, at least in part, on the modality of the freeze-thaw technique employed, the embryo's developmental ages (in terms of "days") as well as its blastocyst stage are also important issues. The survival and hatching rate of vitrified embryos has been found to vary in relation to their developmental day/age and blastocyst stage at the time of their vitrification (Machatkova et al., 2006). One possible reason for this may be that later stage (and thus more expanded) blastocysts are more sensitive to cryopreservatives than early stage blastocysts because detrimental ice crystal formation may occur in the larger, later stage, blastocoel cavity due to insufficient dehydration (Stachecki et al., 2008). Similarly, the post warming survival rate as well as the hatching rate might be also influenced by the day of development of embryos and their expanded blastocyst stage at the time of their vitrification. Vitrification of blastocysts involves their exposure to highly concentrated CPAs to prevent ice crystal formation (Rall and Fahy, 1985; Kim et al., 2012). As most CPAs are embryo-toxic, this can result in severe embryonic damage (Katkov, 2007). As a result, the concentration and duration of exposure to CPAs obviously is an important issue for successful vitrification (Kim et al., 2012).

However, to our knowledge, there is no specific report and/or published data regarding the vitrification and post warming of bovine expanded blastocysts at diverse culture periods and their correlationship with hatchability rates. Therefore, the aim of this study was to evaluate the extent to which the chronology of development affects the cryotolerance of *in vitro* derived embryos vitrified using the Cryotop method. In particular, this was evaluated by assessing the developmental speed of embryos in different days of culture (Experiment 1), their survival rates after vitrification and re-development into different grades and days of expanded blastocysts (Experiment 2) and the correlation between the post warming survival rate and the hatchability rate of the redeveloped embryos (Experiment 3).

3.3 Materials and methods

3.3.1 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 IU/ml FSH (Antrin, Kyoritsu Seiyaku, Tokyo, Japan), 50 IU/ml hCG (Chorulon, Intervet, Boxmeer, Netherlands) and 1 µg/ml estradiol-17β. The medium for embryo culture was modified synthetic oviduct fluid supplemented with amino acids and 0.3% fatty acid-free BSA (mSOF; Gardner et al., 1994).

3.3.2 Oocyte collection and *in vitro* maturation (IVM)

Bovine ovaries were obtained from slaughter houses and kept at room temperature in 0.9% NaCl for up to 4 h during their transport to the laboratory.

Cumulus-oocyte complexes (COCs) were collected from follicles 2-8 mm in diameter using an 18-gauge needle connected to a 10 ml syringe. A group of 20 COCs were 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 22-23 h.

3.3.3 *In vitro* fertilization (IVF) and culture (IVC)

After IVM, cumulus cells were partially removed from oocytes by gentle pipetting with a fine glass pipette with 0.1% hyarulonidase. After that a group of 10 oocytes was washed 3-4 times in Tyrode's Albumin Lactate Pyruvate (TALP) medium supplemented with 1 mmol caffeine, 100 µg/ml heparin, 20 mmol/l penecilamine, 10 mmol/l hypotaurine and 20 mmol/l ephinephrine. The sperm were prepared by swim up technique. One straw (0.25-ml straws, 25 $\times 10^6$ sperms/straw) was thawed at 39°C for 30 sec. One hundred µl of thawed semen was placed to the bottom of a snapped tube containing 2 ml of TALP and kept at a 45° angle in a humidified atmosphere of 5% CO_2 in air at 38.5°C for 30 min. Then the supernatum was collected in a 15 ml conical tube and centrifuged at 500×g for 5 min. After centrifuging, supernatum was discarded and 3 ml TALP was added for washing. After that it was centrifuged and sperm concentration was calculated by using a hemocytometer and adjusted to 2×10^6 /ml (Seneda et al., 2001). Finally, 10 oocytes washed 3 times in TALP medium and were kept in each 100 µl/drop on 60 mm dish and cover with mineral oil of sperm suspension and sperm-oocytes were co-incubated in a humidified atmosphere of 5% CO_2 in air at 38.5°C for 13-14 h. Then the presumptive zygotes were further cultured in mSOFaa medium (20 zygotes/100 µl) under a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C for 48 h. At

D2, the cleavage rates were recorded using the day of IVF as D0. Thereafter, embryos at the 8-cells stage were selected and co-cultured with bovine oviduct epithelial cells in mSOFaa medium under a humidified atmosphere of 5% CO_2 in air at 38.5°C, as reported previously (Parnpai et al., 1999) for 6 days. Half of the medium was replaced with fresh medium during the embryos development and their stages and growth rates were recorded. The development of the blastocyst was noted on D7 and D8 (the day of IVF was considered as D0).

3.3.4 Grading and vitrification of blastocysts

The blastocysts were graded according to the manual of The International Embryo Transfer Society, as to their development and the quality of their inner cell mass (ICM) and trophectoderm (TE). First, the blastocyst was graded into the categories of blastocyst (BL: the volume of blastocoel is more than 50% of the total volume) and expanded blastocyst (Exp-BL; the blastocoel occupies the whole volume inside the zona pellucida (ZP) and has started expansion, leading to a thinning of the ZP). After this the BLs and Exp-BLs stages were divided into G1 (ICM contains many small compacted cells and the TE appears clearly made of many small cells tightly attached and forming a single layer epithelium) and G2 (the cells in the ICM are still separated and poorly compacted and the TE is identifiable and contains few cells). The vitrification and warming of embryos by the Cryotop method was done according to the technique described by Liang et al. (2011). Briefly, a group of 1-3 Exp-BLs was washed in TCM199-Hepes + 20% FBS (base medium; BM) before being placed in BM containing 10% DMSO and 10% EG (equilibration solution) for 1 min, and then exposed in BM containing 20% DMSO, 20% EG and 0.5 M sucrose

(vitrification solution, VS) for 30 sec at 22-24°C. Finally within 30 sec, embryos with about 2 µl of VS were loaded onto Cryotop (Kitazato BioPharma, Fujinimiya, Japan) and plunged directly into liquid nitrogen (LN_2). The vitrified Cryotop was warmed by immersing directly into 2 ml of 0.5 M sucrose in BM at 38.5°C on a warm plate for 5 min, washed 5 times, and then put in BM for 5 min and washed again 5 times. Finally, the warmed blastocysts were washed 2 times in SOFaa medium and transferred to a culture dish and cultured for another 2 days under a humidified atmosphere of 5% CO_2 in air at 38.5°C.

3.3.5 Experimental design

Experiment 1: *The developmental ability of embryos at D7 and D8*

Embryos were cultured according to the co-culture technique. The development of the embryo was checked and graded carefully under an inverted microscope (IX71, Olympus, Tokyo, Japan) and the data was recorded. The numbers of G1 and G2 blastocysts, and expanded blastocysts, were noted at D7 and D8 to record the developmental speed of the blastocysts. A count was also done of the total number of developed blastocysts at D7 as well as at D8.

Experiment 2: *Assessment of the developmental speed of D7 and D8 Exp-BLs subsequent to Cryotop vitrification*

The G1 and G2 Exp-BLs at D7 and D8 were vitrified by the Cryotop method then warmed and checked. Their survivability was assessed after culture for 24 h. The re-expansion of the embryo within 24 h was used as an indicator of blastocyst survival. The hatching (Hing) and hatched blastocysts (Hed-BLs) were

documented at 24 h and 48 h after warming and culture. The survivability, Hing as well as Hed-BLs of G1 and G2 stage Exp-BLs were noted and compared with the fresh control group.

Experiment 3: *Estimation of the strength of the correlation between the embryo survival rate and the hatched rate*

The survival and hatched rates of G1 and G2 Exp-BLs at D7 and D8 periods in experiment 2 were calculated and compared to estimate the correlation regressions and strength of influence between them after a 48 h post warming culture.

3.3.6 Statistical analysis

The data for embryo development as well as their survival and re-developmental rates after vitrification and warming were expressed as percentages (%). The data was analyzed by ANOVA and the estimations of the correlation (R square value) between the embryo survival and hatched rates were done by curve estimation of regressions using SPSS® Software (version 16.0, SPSS Inc., Chicago, IL, USA). Differences were considered significant at a level of $p<0.01$ and $p<0.05$.

3.4 Results

3.4.1 Experiment 1: The developmental ability of embryos at D7 and D8

As shown in Figure 1.1, the average blastocyst production rate at D8 (28.2%) was higher than that of D7 (19.1%). The developmental frequency of G1 and G2 blastocysts at D7 (5.1 and 3.3%, respectively) was greater than at D8 (2.6 and

3.0%, respectively). In relation to Exp-BLs, the rate of development of both G1 and G2 Exp-BLs at D8 (11.4 and 11.0%, respectively) was also greater when compared to D7 (6.4 and 4.6%, respectively). The rates of blastocyst development in more advanced stages were found to be dependent on the duration of culture.

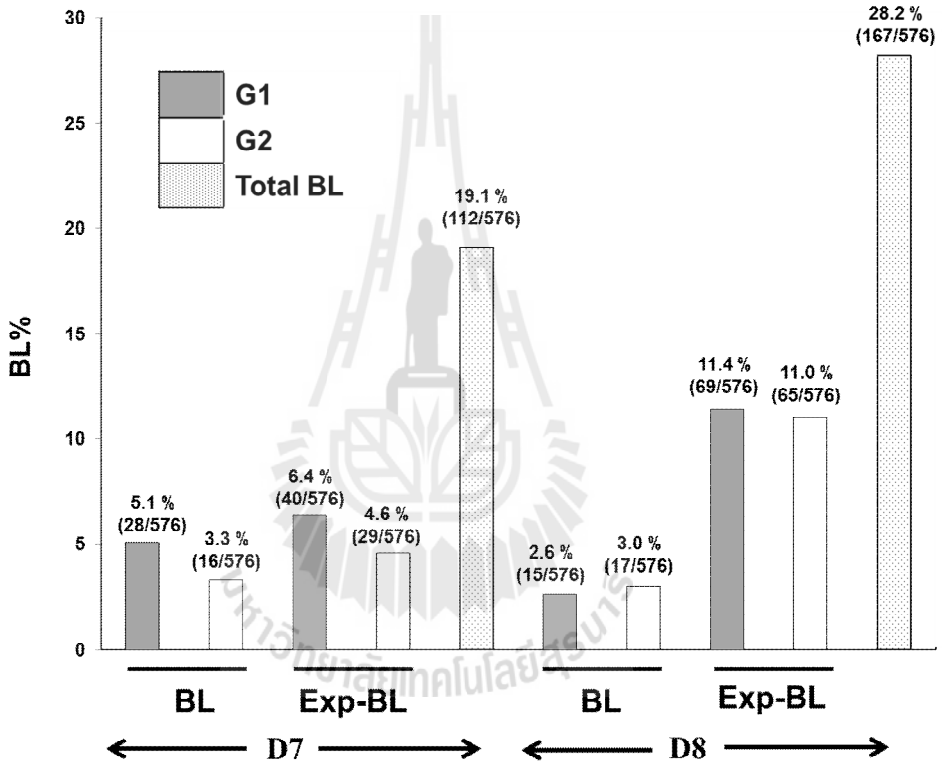

Figure 3.1 The developmental ability of embryos at D7 and D8 culture period. G1: Grade 1 blastocyst, G2: Grade 2 blastocyst, T BL: Total number of blastocysts, BL: Blastocyst, Exp-BL: Expanded blastocyst, D7: Data recorded at day 7, D8: Data recorded at day 8.

3.4.2 Experiment 2: Assessment of the progressive developmental potentiality of expanded blastocysts subsequent to Cryotop vitrification

The survival rates of G1 Exp-BLs in both D7 (97.1%) and D8 (85.7%) culture periods were not significantly (p>0.05) higher than those of the fresh blastocysts (100 and 100%) as shown in Table 1. After 24 h culture, both the fresh G1 and G2 Exp-BLs showed a significantly (p<0.05) higher tendency to develop into a Hing-BL stage than those of the vitrified groups at both D7 and D8. The hatchability rate of G1 Exp-BLs in both periods was lower than that of the fresh control group but did not show a significant variance at 24 h or 48 h post warming culture. On the other hand, the G2 Exp-BLs revealed a significantly (p<0.05) lower Hed-BL rate at 24 h and 48 h post warming re-culture compared with that of the fresh control which were vitrified in both D7 and D8.

Table 3.1 Post warming survivability and developmental potential of G1 and G2 expanded blastocysts subsequent to Cryotop vitrification at D7 and D8.

Day	Embryo types	Grade (G)	No. of embryos	Survival (%)	Hatching rate (%)			
					24 h		48 h	
					Hing	Hed	Hing	Hed
D7	Fresh	G1	35	35/35 (100)[a]	13/35 (37.1)[a]	20/35 (57.1)[a]	2/35 (5.7)[a]	33/35 (94.3)[a]
		G2	33	33/33 (100)[a]	18/33 (54.5)[a]	9/33 (27.3)[b]	10/33 (30.3)[b]	23/33 (69.7)[a]
	Vitrified	G1	35	34/35 (97.1)[a]	6/35 (17.1)[b]	17/35 (48.6)[a]	3/35 (8.6)[a]	29/35 (82.8)[a]
		G2	31	21/31 (67.7)[a]	6/31 (19.4)[b]	5/31 (16.1)[c]	5/31 (16.1)[c]	12/31 (38.7)[b]
D8	Fresh	G1	34	34/34 (100)[a]	21/34 (61.8)[a]	13/34 (38.2)[b]	6/34 (17.6)[bc]	29/34 (85.3)[a]
		G2	36	36/36 (100)[a]	19/36 (52.8)[a]	8/36 (22.2)[bc]	15/36 (41.7)[d]	19/36 (52.8)[a]
	Vitrified	G1	28	24/28 (85.7)[a]	6/28 (21.4)[b]	6/28 (21.4)[bc]	2/28 (7.1)[a]	20/28 (71.4)[a]
		G2	26	13/26 (50)[b]	2/26 (3.8)[c]	2/26 (7.7)[d]	6/26 (23.1)[b]	4/26 (15.4)[c]

Different letters ([a, b, c, d]) within a column represent significant differences (p<0.05).

Hing: Hatching, Hed: Hatched.

3.4.3 Experiment 3: Estimation of the strength of the correlationship between the blastocyst survival rate and the hatched rate

I found that the hatched rate of embryos at 48 h post warming culture showed a strong correlation to its corresponding survival rate as shown in Figure 3.2 and Figure 3.3. However, the G1 blastocysts at D7 showed a significantly (p<0.01) higher correlation between hatchability in relation to their survival rate.

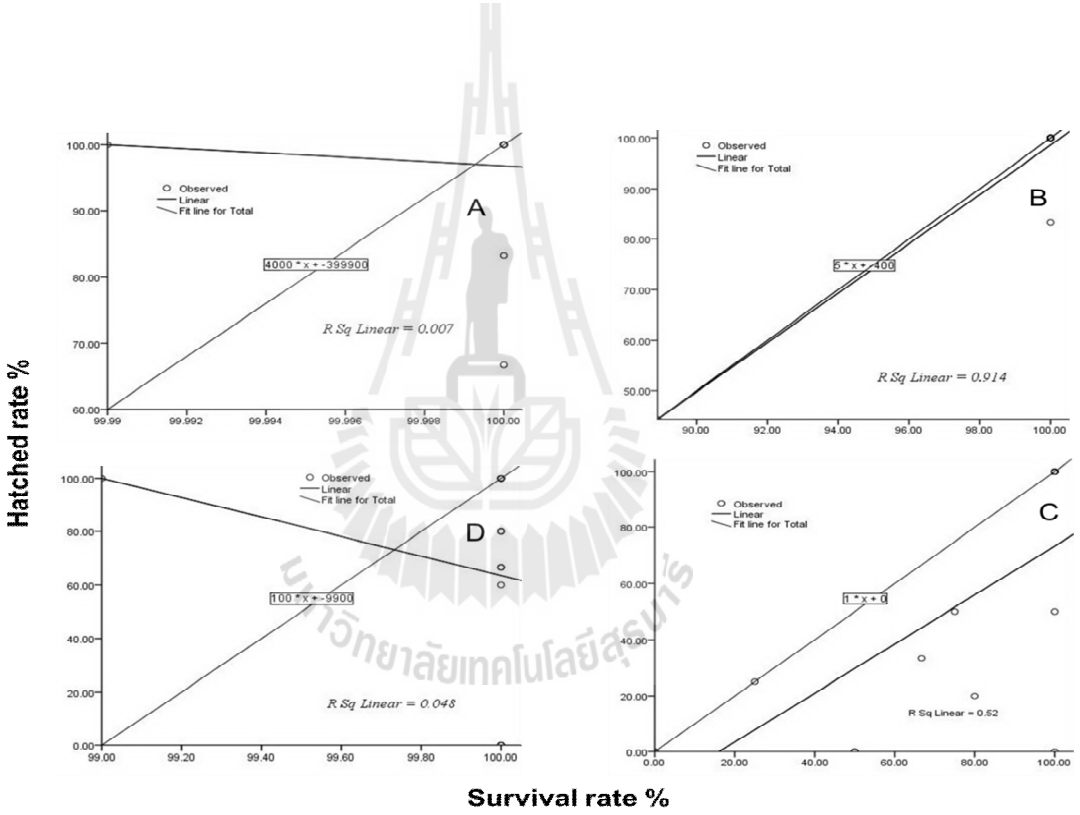

Figure 3.2 Curve estimation of correlation regressions between D7 expanded blastocysts survival and hatched rates. X-axis survival rate and Y-axis hatched rate; (A): Fresh control D7 G1 Exp-BLs, (B): Vitrified D7 G1 Exp-BLs, (C): Fresh control D7 G2 Exp-BLs and (D): Vitrified D7 G2 Exp-BLs.

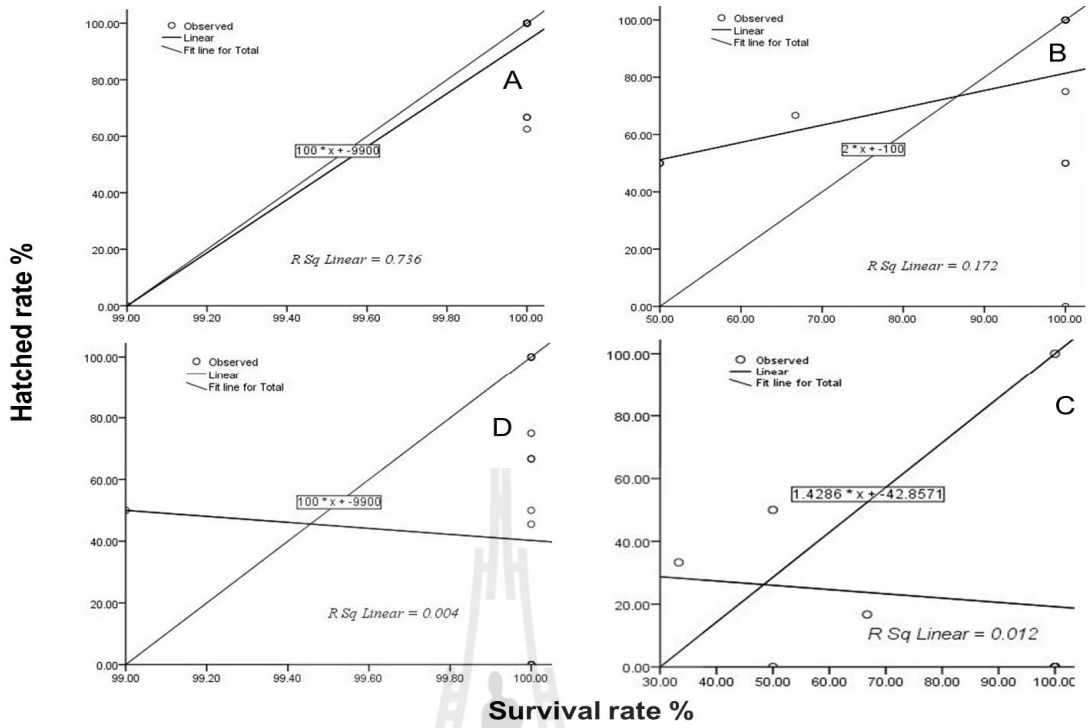

Figure 3.3 Curve estimation of correlation regressions between D8 expanded blastocysts survival and hatched rates. X-axis survival rate and Y-axis hatched rate; (A): Fresh control D8 G1 Exp-BLs, (B): Vitrified D8 G1 Exp-BLs, (C): Fresh control D8 G2 Exp-BLs and (D): Vitrified D8 G2 Exp-BLs.

3.5 Discussion

In this study, I demonstrated that the growth of good quality embryos at different culture periods was not similar. Currently, the study of vitrification techniques seems to be a primary focus among many embryologists. However, the success rate of vitrification, implantation subsequent to ET, as well as the pregnancies deriving from these procedures, are still subject to many variations and are open to question. All of these are primarily dependent on the day of culture period, the stage of the embryo, and the embryo's quality prior to vitrification. In this study I have

proven which stage of bovine blastocysts is most favorable and adaptable for vitrification, as well the best culture period for vitrification in terms of the subsequent survivability and hatchability of the embryos involved.

In investigating good quality embryo development at different cultural periods I found that the developmental potentiality of G1 and G2 blastocysts was not significantly different between D7 and D8 culture periods, but that the rate of expanded blastocyst advancement at D8 was higher than that of D7. Our results partially or fully agreed with other studies in embryo development (Machatkova et al., 2005; Niemann et al., 1986). The rate of blastocyst development has been found to vary markedly according to the length of the culture period (Machatkova et al., 2005). Niemann et al. (1986) has stated that the frequency rate of good quality embryo development may vary in relation to its age. Recently, Sugimura et al. (2012) stated that the quality of blastocysts is also related to their hatchability after 48 h of additional post warming culture of the expanded blastocysts.

In this study, I found that the survival rate of G1 Exp-BLs was significantly higher than that of G2 in both D7 and D8 culture periods; but the survivability of good quality embryos was not significantly different from the fresh group even between these vitrification periods. The survival rates of good quality Exp-BLs in our study were similar to the rates in the study by Inaba et al. (2011; 94.3%) who used only good quality embryos for different vitrification techniques. In contrast, our vitrification and warming methods showed almost double the survival rate than that of other studies that did not use only good quality embryos. Piereira et al. (2007;) used a medium of PBS supplemented with 15% FCS and PBS with 15% FCS and 5% gycerol for 10 min and then finally transferred to PBS supplemented with 10%

glycerol and 15% FCS for another 10 min and had a survival rate of 34.7%. Machatcova et al. (2006) used 10% glycerol in TCM 199 medium with 10% ECS and equilibrated for 5 min at room temperature and had a survival rate of 51.6%. Bruyere et al. (2012) used a different synthetic substitution (CRY03) of animal derived serum and found that it increased the embryo survival rate by inhibiting the fragility of the embryos. The authors Fahning and Garcia (1992), Balasubramanian et al. (1998) and Machatkova et al. (2006) studied factors involved in the post thaw survival of *in vitro* embryos demonstrated that the cryosurvival of an embryo depended on its morphology and developmental stage at the moment of freezing. Even though these modified procedures have provided some technological advantages, *in vitro* produced embryos generally showed higher viability when frozen by vitrification than that of slow freezing, as demonstrated by a lower occurrence of morphological changes after thawing (Moreira da Silva and Metelo, 2005). Our putative results coincided with the declaration of Massip et al. (1995) that, in addition to the morphology and developmental stage of embryos, the kinetics of embryo development and the age of embryos before freezing plays an important role in embryo survival after thawing. However, in contrast to these reports, I found out that the survival rate depends on the quality of embryos not on the culture period of vitrification or freezing. Nevertheless the blastocyst hatching rate did significantly depended on the culture period or age of the embryos subjected to vitrification. I also found that rapidly growing embryos were less likely to show the negative effect of CPAs than slow growing embryos.

In this study, the G1 Exp-BLs at D7 had a nearly similar rate of hatchability to the fresh embryos. In this study, I also investigated the correlation between the post warming embryo survival rate and the hatched rate of embryos by curve estimation of

correlation regression. I discovered that the hatched rates of G1 embryos at 48 h of the D7 culture periods showed a significant and strong relationship to these embryos' development and post warming survival rates.

However, my findings partially agreed with those of Vajta et al. (1995), Dinnyes et al. (1999) and Machatkova et al. (2006) that, in embryos of equal quality in terms of morphology, D7 embryos showed higher cryosurvival rates. But they did not consider the grade of blastocysts or differences between culture periods in their studies. They did mention, however, that the highest difference in cryosurvival rates was between D7 and D8 embryos at the early blastocyst stage. These differences disappeared when frozen-thawed embryos were at the blastocyst or expanded blastocyst stage. In our study we also found that vitrified-thawed, lower quality (G2) expanded blastocysts after 48 h culture showed about 40% hatchability at D7, which was significantly higher than that of D8. Saha and Suzuki (1997) proposed that D8 blastocysts have lower quality because of fewer inner cells. Results by Kong et al. (2000) and Mezzalira et al. (2004) suggest that D8 embryos have important differences in their hatching rates.

3.6 Conclusions

The results of this study demonstrated that the survival rate of G1 Exp-BLs showed no difference between D7 and D8 culture periods but that D7 embryos had a significantly higher hatchability rate subsequent to Cryotop vitrification. However, it is our observation that rapidly growing embryos are more tolerant of the vitrification process than slower growing embryos. Therefore, we suggest that the D7 Exp-BL is more suitable for cryopreservation and embryo transfer it demonstrates the strongest

correlation between survival and hatched rate. In my next study, I will attempt to further confirm these findings by using additional tests involving ET.

3.7 References

Arav, A., and Zeron, Y. (1997). Vitrification of bovine oocytes using modified minimum drop size technique (MDS) is effected by the composition and the concentration of the vitrification solution and by the cooling conditions. **Theriogenology** 47:341.

Arav, A. (1992). Vitrification of oocytes and embryos. In New Trends in Embryo Transfer, edn 1, Eds A Lauria & F Gandolfi. **Cambridge: Portland Press**. PP. 255-264.

Balasubramanian, S., Rho, G., and Leibo, S. (1998). Effect on development of chilling *in vitro* produced bovine embryos at various cleavage stages. **Theriogenology** 49:162.

Bruyere, P., Baudot, A., Guyader-Joly, C., Guerin, P., Louis, G., and Buff, S. (2012). Improved cryopreservation of in vitro produced bovine embryos using a chemically defined freezing medium. **Theriogenology** 78:1294-1302.

Chen, S. U., Lien, Y. R., Cheng, Y. Y., Chen, H. F., Ho, H. N., and Yang, Y. S. (2001). Vitrification of mouse oocytes using closed pulled straws (CPS) achieves a high survival and preserves good patterns of meiotic spindles, compared with conventional straws, open pulled straws (OPS) and grids. **Hum. Reprod.** 16:2350-2356.

Chian, R. C., Kuwayama, M., 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.

Cho, H. J., Son, W. Y., Yoon, S. H., Lee, S. W., and Lim, J. H. (2002). An improved protocol for dilution of cryoprotectants from vitrified human blastocysts. **Hum. Reprod.** 17:2419-2422.

Dinnyes, 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.

Dinnyes, A., Lonergan, P., Fair, T., Boland, M., and Yang, X. (1999). Timing of the first cleavage post-insemination affects cryosurvival of *in vitro* produced bovine embryos. **Mol. Reprod. Dev.** 53:318-324.

Fahning, M., and Garcia, M. (1992). Status of cryopreservation of embryos from domestic animals. **Cryobiology** 29:1-18.

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

Gasparini, 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.

Hamawaki, A., Kuwayama, M., and Hamano, S. (1999). Minimum volume cooling method for bovine blastocyst vitrification. **Theriogenology** 51:165.

Inaba, Y., Alkawa, T., Hirai, T., Hashiyada, Y., Yamanouchi, T., Misumi, K., Ohtake, M., Somfai, T., Kobayashi, S., Saito, N., Matoba, S., Konishi, K., and Imai, K. (2011). In-straw cryoprotectant dilution for bovine embryos vitrified using Cryotop. **J. Reprod. Dev.** 57:437-443.

Katkov, P. A. G. (2007). Influence of exposure to vitrification solutions on 2-cell mouse embryos: II. Osmotic effects or chemical toxicity? **Cryo Lett.** 28:407-427.

Kim, Y. M., Uhm, S. J., Gupta, M. K., Yang, J. S., Lim, J. G., Das, Z. C., Heo, Y. T., Chung, H. J., Kong, I. K., Kim, N. H., Lee, H. T., and Ko, D. H. (2012). Successful vitrifcation of bovine blastocysts on paper container. **Theriogenology** 78:1085-1093.

Kong, I. K., Lee, S. I., Im, Y. T., Cho, H. J., Ohh, H. J., Ohh, D. H., and Bae, I. H. (2000). Improvement of post thaw hatching rates of in vitro-produced bovine embryos vitrified by ultra-mini straw. **Theriogenology** 53:258.

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

Lane, M., Forest, K. T., Lyons, E. A., and Bavister, B. D. (1999). Live births following vitrification of hamster embryos using a novel container less technique. **Theriogenology** 51:167.

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

Larman, M. G., and Gardner, D. K. (2010). Vitrifying mouse oocytes and embryos with super-cooled air. **Hum. Reprod.** 25:i265.

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.

Machatkova, M., Hanzalova, K., Horakova, J., Reckova, Z., and Hulinska, P. (2006). Collection of oocytes from donors in the growth phase of follicular development can enhance the production of bovine embryos for cryopreservation. **Veterinarni Medicina** 51:232-238.

Machatkova, M., Horakova, J., Rybar, R., Hanzalova, K., and Rubes, J. (2005). Embryos produced in vitro from bulls carrying 16; 20 and 1; 29 Robertsonian translocations: efficiency and kinetics of oocyte fertilization and embryo development. **Zygote** 13:97-101.

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.

Massip, A., Mermillod, P., and Dinnyes, A. (1995). Morhology and biochemistry of in vitro produced bovine embryos: implications for their cryopreservation. **Hum. Reprod.** 10:3004-3011.

Matsunari, H., Maehara, M., Nakano, K., Ikezawa, Y., Hagiwara, Y., Sasayama, N., Shirasu, A., Ohta, H., Takahashi, M., and Nagashima, H. (2012). Hollow fiber vitrification: A novel method for vitrifying multiple embryos in a single device. **J. Reprod. Dev.** 58:599-608.

Mezzalira, A., Mezzalira, J. C., Moraes, A. N., Thaler Neto, A., Vieira, A. D., Barreta, M. H., and Damiani, J. (2004). Vitrification of bovine IVP embryos:

Age of embryos and exposure time to cryoprotectant influence viability. **Archiv. Vet. Sci.** 9:107-111.

Moreira da Silva, F., and Metelo, R. (2005). Relation between physical properties of the zona pellucida and viability of bovine embryos after slow-freezing and vitrification. **Reprod. Dom. Anim.** 40:205-209.

Niemann, H., Brem, G., Sacher, B., Smidt, D., and Kräusslich, H. (1986). An approach to successful freezing of demi-embryos derived from day-7 bovine embryos. **Theriogenology** 25:519-524.

Parnpai, R., Tasripoo, K. and Kamonpatana, M. (1999). Deveopment 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.

Pereira, R. M., Baptista, M. C., Vasques, M. I., Horta, A. E., Portugal, P. V., Bessa, R. J., Silva, J. C., Pereira, M. S., and Marques, C. C. (2007). Cryosurvival of bovine blastocyst is enhanced by culture with trans-10 cis-12 conjugated linoleic acid (10t, 12c CLA). **Anim. Reprod. Sci.** 98:293-301.

Rall, W. F., and Fahy, G. M. (1985). Ice-free cryopreservation of mouse embryos at K196 8C by vitrification. **Nature** 313:573-575.

Saha, S., and Suzuki, T. (1997). Vitrification of in vitro produced bovine embryos at different ages using one and three step addition of cryoprotective additives. **Reprod. Fertil. Dev.** 9:741-746.

Seneda, M. M., Esper, C. R., Garcia, J. M., Oliveira, J. A., and Vantini, R. (2001). Relationship between follicle size and ultrasound-guided transvaginal recovery. **Anim. Reprod. Sci.** 67:37-43.

Stachecki, J. J., Garrisi, J., Sabino, S., Caetano, J. P. J., Wiemer, K. E., and Cohen, J. (2008). A new safe, simple and successful vitrification method for bovine and human blastocysts. **Reprod. BioMedi. Online** 17:360-367.

Steponkus, P. L., Myers, S. P., Lynch, D. V., Gardner, L., Bronshteyn, V., Leibo, S. P., Rall, W. F., Pitt, R. E., Lin, T. T., and MacIntyre, R. J. (1990). Cryopreservation of Drosophila melanogaster embryos. **Nature** 345:170-172.

Succu, S., Leoni, G. G., Bebbere, D., Berlinguer, F., Mossa, F., Bogliolo, L., Madeddu, M., Ladda, S., and Naitana, S. (2007). Vitrification devices affect structural and molecular status of in vitro matured ovine oocytes. **Mol. Reprod. Dev.** 74:1337-1344.

Sugimura, S., Akai, T., Hashiyada, Y., Somfai, T., Inaba, Y., Hirayama, M., Yamanouchi, T., Matsuda, H., Kobayashi, S., Aikawa, Y., Ohtake, M., Kobayashi, E., Konishi, K., and Imai, K. (2012). Promising system for selecting healthy *in vitr*o fertilized embryos in cattle. **PLoS ONE** 7:e36627.

Vajta, G., Holm, P., Greve, T., and Callesen, H. (1995). Overall efficiency of in vitro embryo production and vitrification in catle. **Theriogenology** 43:683-689.

Vajta, G., Holm, P., Greve, T., and Callesen, H. (1997). Vitrification of porcine embryos using the open pulled straw (OPS) method. **Acta Veterinaria Scandinavica.** 38:349-352.

Vajta, G., Holm, P., Kuwayama, M., Booth, P. J., Jacobsen, 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.

Vanderzwalmen, P., Bertin, G., Debauche, C., Standaert, V., Bollen, N., van Roosendaal, E., Vandervorst, M., Schoysman, R., and Zech, N. (2003).

Vitrification of human blastocysts with the Hemi-Straw carrier: application of assisted hatching after thawing. **Hum. Reprod.** 18:1504-1511.

Wilmut, I., and Rowson, L. E. (1973). Experiments on the low temperature preservation of cow embryos. **Vet Res.** 26:686-690.

Yavin, S., and Arav, A. (2001). Development of immature bovine oocytes vitrified by minimum drop size technique and a new vitrification apparatus (VIT-MASTER). **Cryobiology** 43:331.

CHAPTER IV

PAPER DEVICE VITRIFICATION: A NOVEL DEVICE AND SUCCESSFUL TECHNIQUE FOR OOCYTES AND BLASTOCYSTS VITRIFICATION

4.1 Abstract

"*One oocyte-one embryo-one calf*" might be the current concept of ART. In this study, I hypothesized that the rapid stepwise (30 sec in each) exposure to final concentrations of solution as well as stepwise warming the vitrified oocytes or blastocysts will be less toxic and less stressful. The aim of present study was to introduce a novel device and an improved technique for oocytes and blastocysts vitrification. I found that the Paper device is the best alternative of modern standard Cryotop. There was no significant ($p>0.05$) different rate of oocytes survival and blastocysts production after vitrification of oocytes by using both devices. I also proved that the oocytes can survive in 2-steps method but the blastocyst rate was significantly ($p<0.01$) lower than that of fresh group. Therefore, we developed 3-steps technique for MII oocytes vitrification and warming. Moreover, I found that the 3-steps vitrification solution was non-toxic for oocytes survival and blastocyst development. The blastocyst rate in 3-steps method was higher than that of 2-steps but both are significantly ($p<0.05$) different from fresh group. In case of blastocysts vitrification, the 3-steps technique was shown significantly ($p<0.05$) higher survival

as well as hatched blastocyst rate of grade-2 blastocyst than that of 2-steps, whereas for grade-1blastocysts were not significantly different in both cases.

4.2 Introduction

There is a time of need, to find out an available, cheap, readily useable and more fruitful device, and an improved technique for oocytes and blastocysts vitrification. Vitrification, via rapid cooling, still seems to be one of the methods of choice which have already been occupied position in replacement of conventional or slow freezing cryopreservation in the field of reproduction. It is frequently quoted as being inexpensive, fast and simple (Stachecki et al., 2008). However, these are not important reasons to perform or adapt a technique. The only reasons that should matter of fact in an improved performance, resulting in higher survival and on an average increase in calving rates, as well as enhanced and ensured safety for the animal. For that, the chances of long term effects and short term technical concerns are abridged. Nonetheless, rapid freezing or vitrification has been the focus of research in recent years, based upon a growing number of reports in the literature. In many instances, it is now the preferred method of choice, obviously wealthy one. The idea of vitrification or achieving a glass-like state was first delivered in 1860, and then again in 1937 described by Luyet (1937). It was not until or unless about 50 years later in 1985 that Rall and Fahy (1985) pointed out that idea and designated ‘vitrification’ as a potential alternative to slow-freezing/cooling.

There are many devices i.e. electron microscopes (EM) grid (Steponkus et al., 1990; Martino et al., 1996; Cho et al., 2002), Cryotop (Hamawaki et al., 1999; Kuwayama and Kato 2000), Cryoloop (Lane et al., 1999), solid surface (SS; Dinnyes

et al., 2000), nylon mesh (Matsumoto et al., 2001), Cryoleaf (Chian et al., 2005), plastic blade (Sugiyama et al., 2010), and Vitri-Inga (Almodin et al., 2010). In the tubing system, it belongs to the plastic straw (Rall and Fahy, 1985), open pulled straw (OPS; Vajta et al., 1997; Vajta et al., 1998), closed pulled straw (CPS; Chen et al., 2001), Cryo-Tip (Kuwayama et al., 2005), Rapid-i (Larman and Gardner, 2010), hollow fiber (Matsunari et al, 2012) effectively used in different animal oocytes and embryos vitrification. However, all of these devices are expensive and not readily available at all. Therefore, it is crucial to develop a new device or container to solve, surely alternative one.

There are several new ideas associated with vitrification have since it become prevalent in the literature. These include: (i) high concentrations of cryoprotectants are toxic, and exposure to the final solution with the highest concentration should be reduced to 60s or less (Shaw et al., 1992; Hunter et al., 1995; Hong et al., 1999; Chung et al., 2000; Wu et al., 2001; Yoon et al., 2003); and (ii) the faster cooling rate, the better survival rate (Stachecki et al., 2008). However, both hypotheses might be suitable for good quality blastocysts and just only fluorescein diacetate (FDA) survival of oocytes vitrification. But the blastocysts rate after vtrfcation of MII oocytes is still very low and questionable. There are two key constraints of vitrification including (i) the toxic effect of CPAs which damages the micro-organelles of oocytes and cells of embryos, and (ii) the exposure high concentrations of sucrose to immediately zero sucrose of warming solution is very frantic for oocytes and embryos (Stachecki et al., 2008). So that acclimatization of oocytes or embryos before plunging into the liquid nitrogen is obligatory. Therefore, I hypothesized that the rapid stepwise (30 sec in each) exposure to final concentrations of solution as well

as stepwise warming the vitrified oocytes or embryos will be less toxic and less stressful. Concerning this hypothesis, this research was designed to find out the alternative standard device as well as an improved technique for oocytes and embryos vitrification. In this study we also demonstrated the correlation between the oocytes survival and cleavage rates, and blastocysts survival rate and hatched rate.

4.3 Materials and methods

4.3.1 Chemicals and media

All reagents were purchased from Sigma Chemical Company unless otherwise stated. The medium used for IVM was TCM199 supplemented with 10% FBS, 0.02 IU/ml FSH, 50 IU/ml hCG and 1 μg/ml estradiol-17β. The medium for embryo culture was mSOF supplemented with amino acids and 0.3% fatty acid-free BSA (Gardner et al., 1994). The sperm preparation medium was TALP supplemented with 1 mM caffeine, 100 μg/ml heparin, 20 mM/l penecilamine, 10 mM/l hypotaurine and 20 mM/l ephenephrine.

4.3.2 Preparation, characterization and sterilization of Paper device

The Paper devices were prepared by cutting from A4 size (21×29.7 cm) Paper commonly called tracing or drawing Paper. The shape and size of the Paper was illustrated in the Figure 4.1. The total length of the device was 27 mm whereas the wide of tip was 3 mm and the base was 12 mm, and total length from the base to tip was 15 mm. The Paper device is easily loaded into cryovial and can be stored in liquid nitrogen (LN_2). During loading of Paper device, I ensured that the tip is inside the cryovial otherwise it has the possibility of broken. After preparing it according to

the size and shape, it was sterilized by exposing it under the ultraviolet (UV) light for 30 min. I had written down the specification of oocytes or embryos at the base of the device during use.

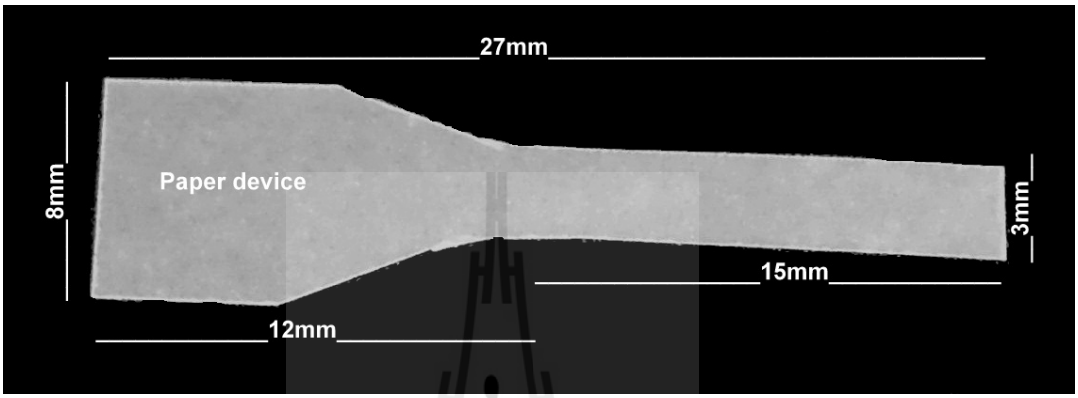

Figure 4.1 The Paper device; size and shape of device.

4.3.3 Oocytes aspiration and *in vitro* maturation (IVM)

Bovine ovaries were obtained from slaughter-houses and kept in 0.9% NaCl during transport to the laboratory within 4 h at room temperature. COCs were collected from follicles 2-8 mm in diameter using an 18-gauge needle connected to a 10 ml syringe. Group of 20 COCs were 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 22-23 h.

4.3.4 Oocytes or embryos vitrification and warming

The oocytes were vitrified by Cryotop or Paper devices (two methods):

4.3.4.1 Two (2) steps method

A group of 4-5 oocytes or 1-3 blastocysts were washed in TCM199-Hepes + 20% FBS as a base medium (BM) before being placed in BM containing 10% DMSO and 10% EG as a equilibration solution (ES) for 1 min, and then exposed in BM containing 20% DMSO, 20% EG and 0.5 M sucrose as a vitrification solution (VS) for 30 sec at room temperature (24-25°C). The oocytes/blastocysts were placed with about <1 µl VS into the vitrification device and then plunged directly into LN_2. The vitrified devices were warmed by immersing directly into 2 ml of 0.5 M sucrose in BM at 38.5°C on warm plate for 5 min following to BM for 5 min, and washed 5 times in each.

4.3.4.2 Three (3) steps method

A group of 4-5 oocytes or 1-3 blastocysts were washed in BM before being placed in BM containing 5% DMSO and 5% EG for 30 sec, then exposed in BM containing 12% DMSO, 12% EG and 0.25 M sucrose for 30 sec after that finally exposed in BM containing 20% DMSO, 20% EG and 0.5 M sucrose for 30 sec at room temperature (24-25°C). The oocytes or blastocysts were dropped with about <1 µl VS into the vitrification device and then plunged into LN_2. The vitrified devices were warmed by immersing directly into 2 ml of BM with 0.5 M sucrose for 2 min, transferred to BM with 0.25 M sucrose for 2 min then 0.125 M sucrose in BM for 2 min and finally washed with BM.

4.3.5 Evaluation of oocytes viability

Oocytes viability was evaluated by FDA staining according to the method previously described by Mohr and Trouson (1980). Briefly, oocytes were treated with 2.5 µg/ml FDA in PBS supplemented with 5 µg/ml BSA at 38.5ºC for 2 min in a dark room. Then it was washed 3 times in PBS supplemented with 5 µg/ml BSA and evaluated under a fluorescent microscope with UV irradiation using a U-MWIB# filter with an excitation wavelength of 460-495 nm and emission at 510 nm. Oocytes' expressing a bright green fluorescence was regarded as viable one and were used subsequently.

4.3.6 *In vitro* fertilization (IVF) and culture (IVC)

One straw of frozen semen (0.25-ml straws, 25 $\times 10^6$ sperms/straw) was thawed at 39°C for 30 sec. 100 µl of thawed semen was placed to the bottom of snapped tube containing 2 ml of TALP for sperm swim up for 30 min. Then the supernatum was collected in a 15 ml conical tube and centrifuged at 500×g for 5 min. After centrifuging, supernatum was discarded and 3 ml TALP was added for washing. After that it was centrifuged and sperm concentration was calculated by using a hemocytometer and adjusted to 2×10^6 /ml (Seneda et al. 2001). Finally, 10 oocytes were kept in each 100 µl drop of sperm suspension and sperm-oocytes were co-incubated in humidified atmosphere of 5% CO_2 in air at 38.5°C for 13-14 h. Then the presumptive zygotes were further cultured in mSOFaa medium (20 zygotes/100 µl) under a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C for 48 h. At D2, the cleavage rates were recorded the day of IVF was considered as D0. Thereafter, embryos at 8-cells stage were selected and co-cultured

with bovine oviduct epithelial cells in mSOFaa medium under a humidified atmosphere of 5% CO_2 in air at 38.5°C, as reported previously (Parnpai et al., 1999) for 6 days. Half of the medium was replaced with fresh medium and embryo development was recorded. The development of blastocyst stage was recorded on D7 and D8.

4.3.7 Grading and evaluation of survivability of blastocysts

The blastocysts were graded according to the manual of IETS, to its development and the quality of ICM and TE as described in chapter III. Again the blastocysts and expand blastocysts stage were divided into G1 and G2 as described in chapter III. The re-expansion or developing to a more advanced stage of blastocysts with a clearly visible ICM at 24 h of warming was indicated as survived blastocysts.

4.3.8 Evaluation of blastocysts by differential staining of ICM and TE cells

Differential staining of ICM and TE cells of pooled blastocysts were performed by the method previously described by Sripunya et al. (2010). Briefly, blastocysts were simultaneously treated with 0.1 mg/ml PI and 0.2% Triton X-100 dissolved in Dulbecco's phosphate buffered saline for 40-60 sec to permeable the membrane and stain the nuclei of TE cells. Then the embryo were treated with 25 µg/ml Hoechst 33342 dissolved in 99.5% ethanol for 5 min, mounted on glass slides in glycerol droplets, flattened by coverslips and examined under UV light with an excitation wavelength of 330-385 nm under a fluorescent microscope. The nuclei of TE cells were labeled by both PI and Hoechst appeared pink or red whereas the nuclei of ICM cells will be labeled only by Hoechst appeared blue. A digital image of

each embryo were taken, and the cell numbers of both cells types were counted using the NIH image J (v. 1.40) software (Abramoff et al., 2004). The numbers of ICM and TE cells were counted separately in embryos that had clearly distinguishable populations of red and blue nuclei.

4.3.9 Experimental design

Experiment 1: *Comparison of blastocysts development rate after oocytes vitrification by using Cryotop and Paper device*

In this experiment, oocytes were divided into three groups (i) fresh control: without vitrification oocytes, (ii) vitrification of oocytes by Cryotop device and (iii) vitrification of oocytes by Paper device. Oocytes were vitrified by Paper devices and Cryotop devices according to the 2-steps technique which was reported by Liang et al. (2011). After that, oocytes were fertilized and cultured *in vitro* for 8 days and the embryo development was recorded.

Experiment 2: *Toxicity test of 2-step and 3-step vitrification-warming solutions (VS-WS)*

In this experiment; oocytes were divided into three groups (i) fresh control: oocytes without exposure to any VS-WS, (ii) oocytes exposed to 2-step VS-WS and (iii) oocytes exposed to 3-step VS-WS. Oocytes were exposed to the 2-step and 3-step VS-WS according to the pre-hypothesized time table. Succeeding of exposure, viability of oocytes was evaluated by FDA staining. Survival oocytes were cultured in IVM medium for 2 h, followed by IVF and continue cultured for 8 days. Embryo development were recorded and compared with fresh control oocytes.

Experiment 3: *Oocytes vitrification and warming through 2-step and 3-step method by using Paper device*

In this experiment, oocytes were divided into three groups (i) fresh control: oocytes without vitrification, (ii) oocytes vitrified through 2-step VS-WS and (iii) oocytes vitrified through 3-step VS-WS. Oocytes were vitrified by Paper device and the survival rate was evaluated by FDA staining. After cultured in IVM medium for 2 h, oocytes were subjected to IVF. IVF derived embryos were compared the development with fresh control group.

Experiment 4: *Estimation the strength of correlation between the oocytes survival and cleavage rates subsequently V-W*

The oocytes FDA survival rate and cleavage rate of presumptive zygote in experiment 1, 2 and 3 were under taken separately in each experiment to estimate the correlation regressions and strength of influence among them.

Experiment 5: *Blastocyts vitrification through 2-steps and 3-steps method by using Paper device*

In this experiment, D8 blastocysts were divided into three groups (i) fresh control: embryos without vitrification, (ii) embryos vitrified through 2-step VS-WS and (iii) embryos vitrified through 3-step method. Paper device was used in this experiment. Embryo survival rate and hatchability were assessed, compared with fresh control group.

4.3.10 Statistical analysis

The data of oocytes survivability, embryo development after different treatment were expressed as percentage (%), and analyzed the data by one way Analysis of Variances (ANOVA) and the estimation of correlation regressions (R^2 value) between oocytes FDA survival rate and cleavage rate by curve estimation, using Statistical Package for Social Sciences (SPSS®) Software (version 16.0). Differences were considered significant at a level of $p<0.01$ and $p<0.05$.

4.4 Results

4.4.1 Experiment 1: Efficiency of oocytes vitrification by Cryotop and Paper devices

After 2-steps vitrification method, the oocytes survival rate by FDA staining, using Cryotop (89.2%) and Paper device (90.1%) were similar and not significantly ($p>0.05$) different from fresh group (99.1%; Table 4.1). In addition, Cryotop and paper devices showed the similar ZP broken rate which was significantly ($p<0.05$) higher than that of fresh control group. However, the embryonic developmental rate including cleavage, 8-cells, 16-cells, morula, D6 blastocyst, D7 blastocyst and D8 blastocyst between Cryotop (48.0, 23.0, 22.0, 18.0, 4.0, 9.0 and 9.0%, respectively) and Paper device (54.0, 22.6, 21.0, 17.0, 4.0, 9.0 and 9.0%, respectively) vitrification had shown the non-significantly ($p> 0.05$) different among of them but the significantly ($p<0.05$) different from fresh group (74.3, 45.9, 36.7, 31.2, 7.3, 21.2 and 26.6%, respectively). In case of quality blastocyst assessment, I found that the number of TE and ICM cells as well as the total cells number were not significantly ($p>0.05$) different from the fresh control group.

4.4.2 Experiment 2: Toxicity test of 2-steps and 3-steps vitrification-warming (V-W) solution

In this experiment, I compared the oocytes survival and embryo development rate after oocytes exposed into 2-steps and 3-steps V-W solution. I found that there was no toxic effect of solution for the oocytes survival rate as well as embryonic developmental rate in both groups. However, the cleavage, 8-cells, 16-cells, morula, D6 blastocyst, D7 blastocyst and D8 blastocyst rate in 3-steps solution (65.6, 38.7, 32.3, 29.0, 8.6, 12.9 and 13.9%, respectively) was higher than that of 2-steps (48.9, 25.0, 23.8, 20.5, 3.4, 6.8 and 9.0%, respectively) but both were significantly (p<0.05) different from fresh control (70.8, 43.8, 40.4, 35.9, 13.5, 19.1 and 28.1%, respectively) except that of cleavage, 8-cells, 16-cells, morula rate in 3-steps. Nevertheless the blastocyst qualities (ICM, TE and total cells number) in all groups were not significantly (p>0.05) different among each other.

4.4.3 Experiment 3: Oocytes V-W through 2-steps and 3-steps method by using Paper device

The oocytes survival rates were not significantly (p>0.05) different among the different treatment groups. The ZP broken rate in 2-steps (1.7%) vitrification was significantly (p<0.05) higher than that of fresh control group (0.0%) as well as 3-steps (0.7%) vitrification (Table 4.3). The cleavage rate in fresh control (70.2%) was significantly higher than that of 2-steps (42.3%), but did not show significantly higher when compared with 3-steps (58.2%). The blastocysts development potentiality at D6 (7.2%) in 3-steps vitrification were shown not significantly (p>0.05) different from fresh control (10.8%). However the D8 blastocysts rate in 2-steps vitrification was

lower than that of fresh control as well as 3-steps. Interestingly, the cell numbers of blastocyst after differential staining in different groups were not significantly (p>0.05) varied each other's but that numbers were comparatively lower in 2-steps than that of fresh control as well as 3-steps.

4.4.4 Experiment 4: Estimation the strength of correlation between the oocytes survival and cleavage rates subsequent V-W

I found there was a correlation between oocytes survival rate and cleavage rate in all circumstance of experimental groups. Figure 4.2 showed the paper device vitrification comparatively more correlation (R^2=0.15; p=0.38) than that of fresh (R^2=0.06) and Cryotop (R^2=0.12). Figure 4.3 showed that the instances of toxicity test of 2-steps and 3-steps VS have no significant correlation (p>0.05; R^2>1.00). Figure 4.4 was revealed that 3-steps oocytes vitrification method has significantly higher correlation (R^2=0.15; p=0.00) than that of 2-steps (R^2=0.001; p=0.94) vitrification method.

4.4.5 Experiment 5: Embryos V-W through 2-steps and 3-steps method by using Paper device

As shown in Table 4.4. I found that the survival and hatched rate of G1 blastocysts were not significantly different among 2-steps, 3-steps and fresh group, but 3-steps (86.7% and 73.3%) has shown more tendency of survival rate as well as hatched rate than that of 2-step (76.6% and 60.0%). However, in case of G2 blastocysts the survival rate and hatched rate in 3-steps (75.0% and 41.7%) vitrification technique was significantly (p<0.05) higher than that of 2-steps (36.0%

and 12.0%) whereas non-significantly (p>0.05) different from fresh (100% and 60.9%). The correlation regressions curve between post-warming survival rate and hatched rate shown nearly similar R^2 value (Figure 4.5). It revealed that there was a relationship among them.

Table 4.1 Comparison the efficiency of Cryotop device and Paper device for oocytes vitrification.

Group	No. oocytes IVM	No. (%) oocytes survived	No. (%) ZP broken	No. IVF	No. (%) CL	No. (%) Oocytes develop to						Differential staining of BL			
						8-C	16-C	Mo	No. (%) BL at day			No. BL evaluated	Number of cells		
									D6	D7	D8		TE	ICM	Total
Control	110	109 (99.1)	0[a] (0.0)	109	81[a] (74.3)	50[a] (45.9)	40[a] (36.7)	34[a] (31.2)	8[a] (7.3)	23[a] (21.2)	29[a] (26.6)	14	68.7	26.1	94.8
Cryotop	112	100 (89.2)	2[b] (1.8)	100	48[b] (48.0)	23[b] (23.0)	22[b] (22.0)	18[b] (18.0)	4[b] (4.0)	9[b] (9.0)	9[b] (9.0)	13	46.6	21.7	68.4
Paper	111	100 (90.1)	1[b] (0.9)	100	54[b] (54.0)	22[b] (22.0)	21[b] (21.0)	17[b] (17.0)	4[b] (4.0)	8[b] (8.0)	9[b] (9.0)	14	50.6	22.8	73.4

[a, b] within a column represent significant differences ($p<0.05$). ZP = Zona pellucida, CL = Cleavage, 8-C = 8 cells, 16-C = 16 cells, Mo = Morula, D = day, BL = Blastocyst, TE = Trophoectoderm, ICM = Inner cell mass.

Table 4.2 Toxicity test of 2-step and 3-step vitrification solution.

Group	No. oocytes IVM	No. (%) oocytes survived	No. oocytes IVF	No. (%) CL	No. (%) Oocytes develop to						Differential staining of BL			
					8-C	16-C	Mo	No. (%) BL at day			No. BL evaluated	Number of cells		
								D6	D7	D8		TE	IC	Total
Control	89	89 (100.0)	89	63[a] (70.8)	39[a] (43.8)	36[a] (40.4)	32[a] (35.9)	12[a] (13.5)	17[a] (19.1)	25[a] (28.1)	14	76	26	102
2-step	93	88 (94.6)	88	43[b] (48.9)	22[b] (25.0)	21[b] (23.8)	18[b] (20.5)	3[b] (3.4)	6[b] (6.8)	8[b] (9.0)	12	49.9	23.3	73.2
3-step	95	93 (97.8)	93	61[ab] (65.6)	36[ab] (38.7)	30[ab] (32.3)	27[ab] (29.0)	8[a] (8.6)	12[a] (12.9)	13[b] (13.9)	14	65.9	25.8	91.7

[a, b] within a column represent significant differences (p<0.05). CL = Cleavage, 8-C = 8 cells, 16-C = 16 cells, Mo = Morula, D = day, BL = Blastocyst, TE = Trophectoderm, ICM = Inner cell mass.

Table 4.3 Comparison of 2-steps and 3-steps method for oocytes vitrification by using Paper device and assessments the embryo quality.

Group	No. oocytes IVM	No. (%) survived	No. (%) ZP broken	No. oocytes IVF	No. (%) CL	No. (%) Oocytes develop to						Differential staining of BL			
						8-C	16-C	Mo	No. (%) BL at day			No. BL evaluated	Number of cells		
									D6	D7	D8		TE	ICM	Total
Control	114	114 (100)	0[a] (0.0)	114	80[a] (70.2)	47[a] (41.2)	40[a] (35.1)	36[a] (31.5)	12[a] (10.8)	20[a] (17.5)	30[a] (26.3)	12	72.3	23.9	96.2
2-step	114	104 (91.4)	2[b] (1.7)	104	44[b] (42.3)	18[b] (17.3)	16[b] (15.3)	13[b] (12.5)	4[b] (3.8)	7[b] (6.7)	9[b] (8.6)	11	46.3	20.8	67.1
3-step	118	110 (93.2)	1[b] (0.7)	110	64[ab] (58.2)	42[ab] (33.3)	32[ab] (29.1)	26[b] (23.6)	8[ab] (7.2)	11[b] (10.0)	15[b] (13.6)	12	52.3	22.4	74.7

[a, b] within a column represent significant differences (p<0.05). ZP = Zona pellucida, CL = Cleavage, 8-C = 8 cells, 16-C = 16 cells, Mo = Morula, D = day, BL = Blastocyst, TE = Trophoectoderm, ICM = Inner cell mass.

Table 4.4 Comparison of 2-steps and 3-steps method for blastocysts vitrification by using Paper device.

Groups	Grade	No. BL	No. (%) survival at 24 h	No. (%) hatched at 48 h
Fresh	G1	29	29 (100.0)[a]	25 (86.2)[a]
	G2	23	23 (100.0)[a]	14 (60.9)[a]
2-steps	G1	30	23 (76.6)[a]	18 (60.0)[a]
	G2	25	9 (36.0)[b]	3 (12.0)[b]
3-steps	G1	30	26 (86.7)[a]	22 (73.3)[a]
	G2	24	17 (75.0)[a]	10 (41.7)[a]

[a, b] within a column represent significant differences ($p<0.05$). G1 = grade 1, G2 = grade 2, BL = blastocyst.

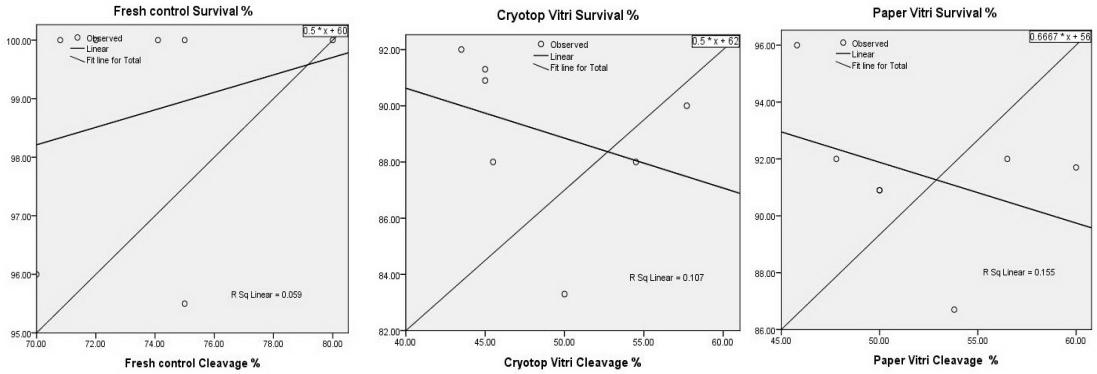

Figure 4.2 Curve estimation of correlation regression between survival and cleavage rates of Cryotop and Paper device vitrification.

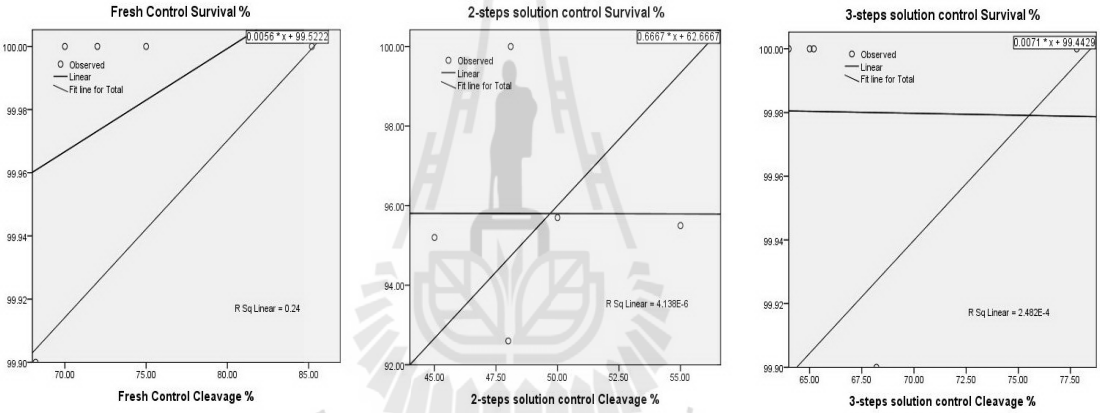

Figure 4.3 Curve estimation of correlation regression between survival and cleavage rates of 2-steps and 3-steps vitrification solution.

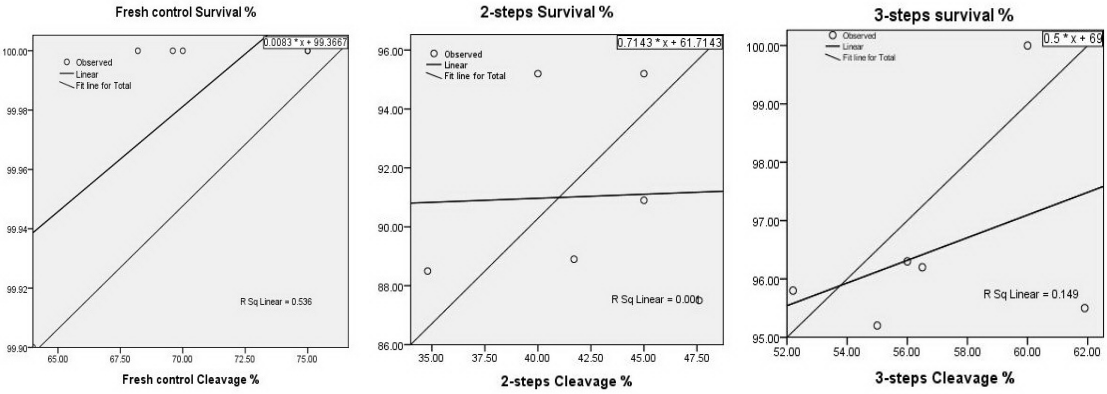

Figure 4.4 Curve estimation of correlation regression between survival and cleavage rates of 2-steps and 3-steps methods.

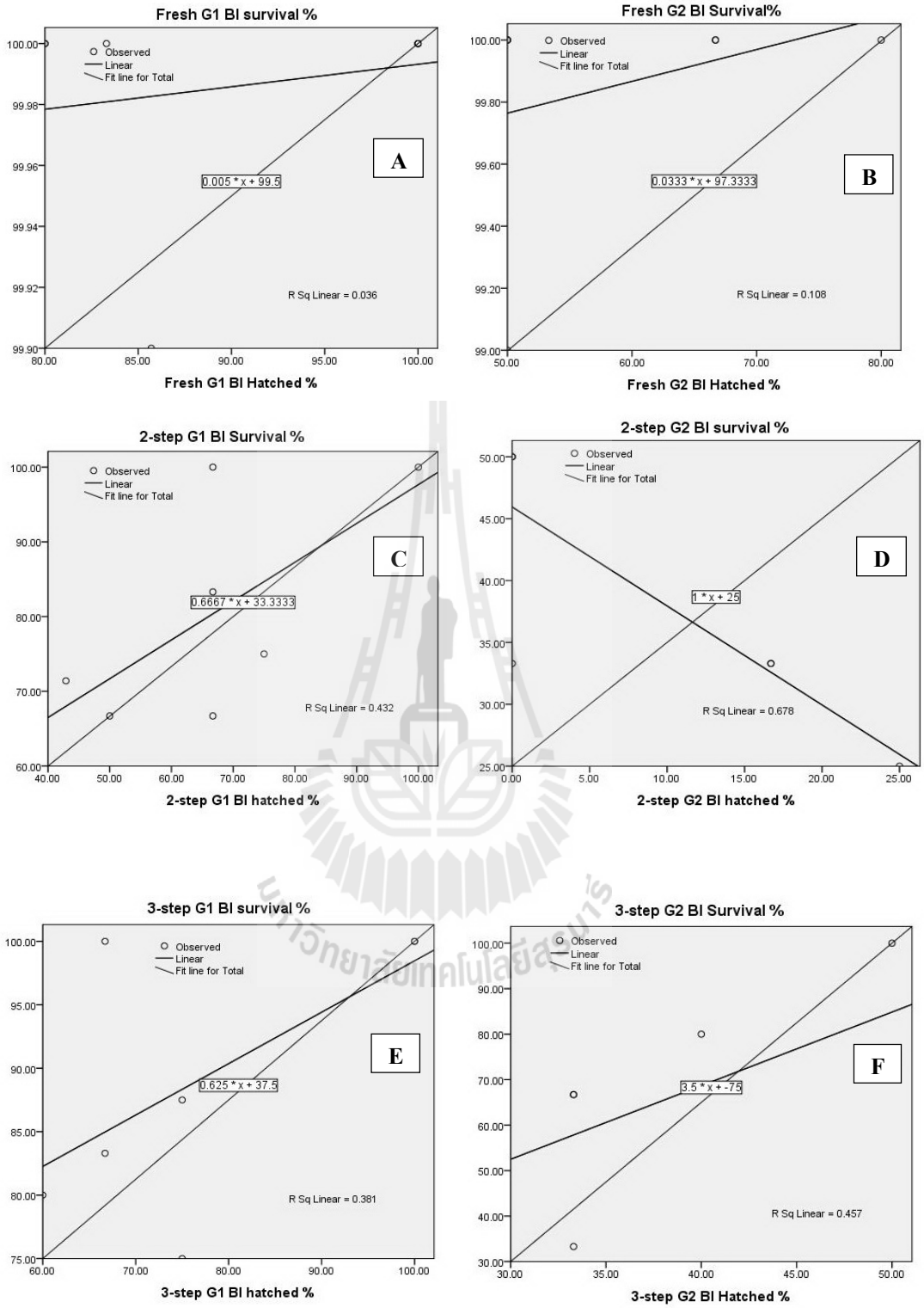

Figure 4.5 Correlation between blastocysts survival and hatched rates. A-B: fresh control; C-D: 2-steps method; E-F: 3-steps method.

4.5 Discussion

The cryopreservation of oocytes and embryos by vitrification, offers a feasible and often better approach to storage than slow freezing/cooling technique. Refinement of earlier methods has led to the use of different containers or devices and rapid cooling rate that coincide with a marked increase in oocytes as well as blastocysts survival rates. However, the cost of previously and recently developed devices or containers, is very high as well as not always available ubiquitously. On the other hand, recent reports revealed that the embryo developmental potential after vitrification of oocytes was not only few but also significantly low. Therefore, we were searching a novel, standard, cheap and readily available device as well as unique potential technique of oocytes and embryos vitrification to overcome the recent problem and researcher nuisance.

Recently, the Cryotop device is considered as a standard device for human oocytes and embryos vitrification which is firstly cited by the Kuwayama and Kato (2000). The efficiency of this device is definitely good, also standard one but costly, and has the chances of dropping of oocytes or embryos during vitrification due to overflow of solution. Therefore, I had investigated a device which would be efficient as like or more as Cryotop device. In this study, I introduced and characterized a new device, I called 'Paper' device which was prepared from tracing paper (at market also known as clear drawing paper; A4 size; 21×29.7 cm). In my study, I found that the rate of oocytes survival as well as the embryonic development was not significantly different among the Paper and Cryotop devices; however in case of embryonic development rate both of these devices were shown significantly lower than that of fresh control group. According to our hypothesis I proved that the Paper device is as

efficient as Cryotop device. Therefore, Paper device might be an alternative, novel and standard as Cryotop device.

Recent reports in bovine (Albarracin et al., 2005; Morato et al. 2008; Sripunya et al., 2010; Zhao et al., 2011) in swine (Somfai et al., 2007), regarding the oocytes vitrification and post-warming embryonic development rate in different animals were revealed very low. It might be due to use of different method and CPAs to improve the embryo development rate after oocytes vitrification, but the rate was still very low and questionable. Therefore, in this study, I hypothesized that the rapid step wise vitrification and warming technique would be less toxic and less stressful. Because of matured (MII) oocytes are very sensitive to cooling, which affect the metaphase spindle and spindle is susceptible to chilling injury (Shaw et al., 2000; Hwang and Hochi, 2014). Fuku et al. (1995), Sathananthan (1997) and Khalili et al. (2012) reported that the high concentrations of CPAs is inhibited and damaged the micro-organelles specially mitochondria and endoplasmic reticulum (Fuku et al., 1995; Sathananthan, 1997; Khalili et al., 2012). Therefore, most of the researcher had got good survival rate but not got good cleavage as well as blastocyst rate.

According to my hypothesis, I found that the 3-steps vitrification-warming solution had no toxic effect for MII oocytes survival as well as blastocyst rates. In contrast, the 2-steps method showed significantly lower than that of fresh control as well as 3-steps method. However, the quality of blastocysts was not affected by vitrification protocols in terms of ICM and TE cell numbers. Finally in experiment three, I vitrified MII oocytes by 2-steps and 3-steps techniques and shown solely the similar results as like experiment two. The cleavage rate and blastocyst rate in my study were higher than others report of vitrification (Martino et al., 1996; Chen et al.,

2003; Albarracin et al., 2005; Morato et al., 2008; Sripunya et al., 2010). Morato et al., (2008) compared the Cryotop and OPS for bovine oocytes vitrification and confirmed that vitrification solution had the negative effect on spindle and chromosome configuration as well as embryo development. On the other hand, Sripunya et al. (2010) had compared Cryotop and SSV methods for oocytes vitrification and embryo development. They found that Cryotop and SSV methods has similar efficacy which is agreed with our results. My results also showed that the Paper device vitrification as similar efficient as Cryotop device vitrification. In case of MII oocytes survival rate, both studies showed approximately similar results, but different for embryo development. It is indicated that the composition of vitrification and thawing solution as well as technique was different from our experiment. They used EG and polyvinyl pyrolidone as a CPAs whereas I have used DMSO and EG.

In agreement with others results, I also observed that the embryos derived from matured oocytes vitrification, had no effect on ICM and TE cells number, which were parallel to fresh group and not significantly different. It is still unclear and questionable also, why embryo quality is not varied. But some researchers from unpublished sources indicated that cryopreservation at MII stage doesn't affect the alive and growth blastocyst stage and also its quality, although Larman et al. (2006) pointed out that vitrification was delayed the expansion of blastocyst because of ZP hardening. In my study, I estimated the co-relation regression between oocytes survival rate and cleavage rate. The curve estimation of corelation in all experimental groups is shown not statistically significant different from each other. The "R square value" had shown a good relationship with smart formula ('y' value). As far as we knew that this study had first demonstrated the correlation between the oocytes

survival rate and cleavage rate after IVF. I found that the survival rate and cleavage rate proportionally related each other's. Therefore, oocytes survival is promising for *in vitro* cleavage.

As reducing multiple pregnancies becomes a greater focus for assisted reproduction treatment clinics, the transfer of a single embryo is warranted sometimes to maintain the pregnancy. In many cases, blastocyst transfer is the method of choice in order to achieve a high pregnancy rate with the transfer of only one embryo. As more clinics become proficient at culturing embryos up to the blastocyst stage, there is an increasing need to store the surplus blastocysts. Blastocysts are morphologically very different from a non-cavitating cleavage stage of embryo and their freezing has been presented different challenges. The main problem is that the blastocoel is made up mainly of water that can form ice crystals when the temperature is lowed, and thus cause damage to the ICM and TE.

To overcome this problem some researchers have tried collapsing the blastocoel either by pipetting the blastocyst in and out of a fine bore pipette or by rupturing it applying an intracytoplasmic sperm injection (ICSI) needle or similar device (Vanderzwalmen et al., 2002; Son et al., 2003; Hiraoka et al., 2004). Although these reports increased survival rates using these methods, the obvious drawback is that an additional procedural step is involved that is potentially damaging to the embryo. However, an optimal protocol would avoid such manipulations. Therefore I used the similar vitrification warming technique as like oocytes vitrification (2-steps and 3-steps) for different graded blastocysts vitrification. In case of G1 blastocysts, the survival rate and hatched blastocysts rate were not significantly different between these two techniques. While 3-steps protocol has shown statistically similar results of

G1 blastocyst compared with 2-steps but it were significantly increased the survival and hatchability of G2 blastocyst.

In this study, the survival rate of G1 expanded blastocyst was similar with the study of Inava et al. (2011) who used only G1 embryo for different vitrification technique. But our vitrification and warming method is shown almost double survival rate than that of the studies of Piereira et al. (2007) and Machatcova et al. (2006). Bruyere et al. (2012) used different synthetic substitution (CRY03) of animal derived serum and found that it increased the embryo survival rate through inhibit the fragility of embryo. Research on factors involved in post thaw survival of embryos demonstrated that the cryotolerence of an embryo was dependent on its morphology and the developmental stage at the moment of freezing (Fahning and Garcia 1992; Balasubramanian et al., 1998; Machatkova et al., 2006). This study is in agreement with the declaration of Massip et al. (1995) that, in addition to the morphology and developmental stage of embryos, the kinetics of embryo development and the age of embryos before freezing plays and important role in embryo survival after thawing.

The success of vitrification is not only depending on solution but also the skill and expertness of the researchers (Saragsty and Arav, 2011). They also mentioned that same technique used different persons might be varied the success rate. Yavin et al. (2009) reported that decreasing the vitrified volume and increasing the cooling rate allow a moderate decease in CPA concentration so as to minimize its toxic and osmotic hazardous effects. According to Saragsty and Arav (2011), three factors i.e. cooling rate, viscosities, volume are involved for probability of vitrification. The cooling rate and viscosity are proportionally but volume of solution is inversely proportional to the probability of vitrification. Hence the drop size has to be smaller.

Therefore in this study the volume of vitrification solution was <1 µl. I also found that the diameter of the pastuer pipette tip as well as the pipetting for equilibration and washing are also important influencing factors for preventing the mechanical injuries of oocytes and embryos.

4.6 Conclusions

This study have developed a new and ideal 'Paper' device as standard as Cryotop device, for MII oocytes and embryos vitrification. Recent studies demonstrated that the 3-steps protocol is non-toxic and more efficient than that of 2-steps method. The blastocyst development rate after oocytes vitrification is higher in 3-steps than that of 2-steps method. In case of blastocysts vitrification, 3-steps method is also showed higher survival and hatched rates than that of 2-steps method. Therefore, it might be declared that Paper device and 3-steps method is suitable and less stressful for MII oocytes and blastocysts vitrification. This finding, in particular opens a new avenue for ultra-rapid freezing. In future, more study on practical application of our technique by transferring vitrified embryo to the recipient is needed.

4.7 References

Abramoff, M. D., Magelhaes, P. J., and Ram, S. J. (2004). Image processing with image. **J. Biophot. Inter.** 11:36-42.

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

Almodin, C. G., Minguetti-Camara, V. C., Paixao, C. L., and Pereira, P. C. (2010). Embryo development and gestation using fresh and vitrified oocytes. **Hum. Reprod.** 25:1192–1198.

Arav, A., and Zeron, Y. (1997). Vitrification of bovine oocytes using modified minimum drop size technique (MDS) is effected by the composition and the concentration of the vitrification solution and by the cooling conditions, **Theriogenology** 47:341.

Arav, A. (1992). Vitrification of oocytes and embryos. In New Trends in Embryo Transfer, edn 1, Eds A Lauria & F Gandolfi. **Cambridge: Portland Press.** PP. 255-264.

Balasubramanian, S., Rho, G., and Leibo, S. (1998). Effect on development of chilling *in vitro* produced bovine embryos at various cleavage stages. **Theriogenology** 49:162.

Bruyere, P., Baudot, A., Guyader-Joly, C., Guerin, P., Louis, G., and Buff, S. (2012). Improved cryopreservation of *in vitro* produced bovine embryos using a chemically defined freezing medium. **Theriogenology** 78:1294-1302.

Camus, A., Clairaz, P., Ersham, A., Van Kappel, A. L., Savic, G., and Staub, C. (2006). Principe de la vitrification: cine´tiques comparatives. The comparison of the process of five different vitrification devices. **Gyne. Obst. Fertil.** 34:737-745.

Chen, S. U., Chien, C. L., Wu, M. Y., Chen, T. H., Lai, S. M., Lin, C. W., and Yang, Y. S. (2006). Novel direct cover vitrification for cryopreservation of ovarian tissues increases follicle viability and pregnancy capability in mice. **Hum. Reprod.** 21:2794-2800.

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

Chen, S. U., Lien, Y. R., Cheng, Y. Y., Chen, H. F., Ho, H. N., and Yang, Y. S. (2001). Vitrification of mouse oocytes using closed pulled straws (CPS) achieves a high survival and preserves good patterns of meiotic spindles, compared with conventional straws, open pulled straws (OPS) and grids. **Hum. Reprod.**16:2350-2356.

Chian, R. C., Son, W. Y., Huang, J. Y., Cui, S. J., Buckett, W. M., and Tan, S. L. (2005). High survival rates and pregnancies of human oocytes following vitrification: preliminary report. **Fertil. Steril.** 84:S36.

Cho, H. J., Son, W. Y., Yoon, S. H., Lee, S. W., and Lim, J. H. (2002). An improved protocol for dilution of cryoprotectants from vitrified human blastocysts. **Hum. Reprod.** 17:2419-2422.

Chung, H. M., Hong, S. W., Lim J. M., Lee, S. H., Cha, W. T., Ko. J. J., Han S. Y. Choi, D. H., and Cha, K. Y. (2000). *In vitro* blastocyst formation of human oocytes obtained from unstimulated and stimulated cycles after vitrification at various maturational stages. **Fertil. Steril.** 73:545-551.

Dinnyes, 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.

Fahning, M., and Garcia, M. (1992). Status of cryopreservation of embryos from domestic animals. **Cryobiology** 29:1-18.

Fuku, E., Xia, L., and Downey, B. R. (1995). Ultrastructural changes in bovine oocytes cryopreserved by vitrification. **Cryobiology** 2:139-156.

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

Hamawaki, A., Kuwayama, M., Hamano, S. (1999). Minimum volume cooling method for bovine blastocyst vitrification. **Theriogenology** 51:165.

Hiraoka, K., Hiraoka, K., Kinutani, M., and Kinutani, K. (2004). Blastocoele collapse by micropipetting prior to vitrification gives excellent survival and pregnancy outcomes for human day 5 and 6 expanded blastocysts. **Hum. Reprod.** 19:2884-2888.

Hong, S. W., Chung, H. M., Lim, J. M., Ko, J. J., Yoon, T. K., Yee, B., and Cha, K. Y., (1999). Improved human oocyte development after vitrification: a comparison of thawing methods. **Fertil. Steril.** 72:142-128.

Hotamisligil, S., Toner, M., and Powers, R. D. (1996). Changes in membrane integrity, cytoskeletal structure, and developmental potential of murine oocytes after vitrification in ethylene glycol. **Biol. Reprod.** 55:161-168.

Huang, C. C., Lee, T. H., Chen S. U., Chen, H. H., Chen, T. C., Liu, C. H., Yang, Y. S., and Lee, M. S. (2005). Successful pregnancy following blastocyst cryopreservation using super-cooling ultra-rapid vitrification. **Hum. Reprod.** 20:122-128.

Hunter, J. E., Fuller, B. J., Bernard A., Jackson, A., and Shaw, R. W. (1995). Vitrification of human oocytes following minimal exposure to cryoprotectants;

initial studies on fertilization and embryonic development. **Hum. Reprod.** 10:1184-1188.

Hwang, I. S., and Hochi, S. (2014). Recent progress in cryopreservation of bovine oocytes. **BioMed research Int.** 2014: Article ID 570647.

Inaba, Y., Alkawa, T., Hirai, T., Hashiyada, Y., Yamanouchi, T., Misumi, K., Ohtake, M., Somfai, T., Kobayashi, S., Saito, N., Matoba, S., Konishi, K., and Imai, K. (2011). In-straw cryoprotectant dilution for bovine embryos vitrified using Cryotop. **J. Reprod. Devel.** 57:437-443.

Isachenko, V., Alabart, J. L., Nawroth, F., Isachenko, E., Vajta, G., and Folch, J. (2001). The open pulled straw vitrification of ovine GV-oocytes: positive effect of rapid cooling or rapid thawing or both? **Cryo Letters** 22:157-162.

Khalili, M. A., Maione, M., Palmerini, M. G., Bianchai, S., Macchiarelli, G., and Nottola, S. A. (2012). Ultrastucture of human mature oocytes after vitrification. **Europ. J. Histochem.** 56:236-242.

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

Kuwayama, M., Vajta, G., Ieda, S., and Kato, O. (2005). Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. **Reprod. Biomedi. Online** 11:608-614.

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

Lane, M., Forest, K. T., Lyons, E. A., and Bavister, B. D. (1999). Live births following vitrification of hamster embryos using a novel containerless technique, **Theriogenology** 51:167.

Larman, M. G., and Gardner, D. K. (2010). Vitrifying mouse oocytes and embryos with super-cooled air. **Hum Reprod.** 25:i265.

Larman, M. G., Sheehan, C. B., and Gardner, D. K. (2006). Calcium free vitrification reduces cryopreservation induced zona pellucida hardening and increased fertilization rates in mouse oocytes. **Reproduction** 131:53-61.

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.

Liebermann, J., Tucker, M., Graham, J., Han, T., Davis, A., and Levy, M. (2002). Blastocyst development after vitrification of multipronuclear zygotes using the felxipet denuding pipette. **Reprod. Biomedi. Online** 4:146-150.

Luyet, B. (1937). The vitrification of organic colloids and protoplasm. **Biodynamica** 1:1-14.

Machatkova, M., Hanzalova, K., Horakova, J., Reckova, Z., and Hulinska, P. (2006). Collection of oocytes from donors in the growth phase of follicular development can enhance the production of bovine embryos for cryopreservation. **Vet. Medi.** 51:232-238.

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

Massip, A., Mermillod, P. and Dinnyes, A. (1995). Morhology and biochemistry of *in vitro* produced bovine embryos: implications for their cryopreservation. **Hum. Reprod.** 10:3004-3011.

Matsumoto, H., Jiang, J. Y., Tanaka, T., Sasada, H., and Sato, E. (2001). Vitrification of large quantities of immature bovine oocytes using nylon mesh. **Cryobiology** 42:139-144.

Matsunari, H., Maehara, M., Nakano, K., Ikezawa, Y., Hagiwara, Y., Sasayama, N., Shirasu, A., Ohta, H., Takahashi, M., and Nagashima, H. (2012). Hollow fiber vitrification: A novel method for vitrifying multiple embryos in a single device. **J. Reprod. Devel.** 58:599-608.

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

Morato, R., Izquierdo, D., Paramio, M. T., and Mogas, T. (2008). Cryotop versus ope-pulled straws (OPS) as carriers for the cryopreservation of bovine oocytes: Effect of spindle and chromosome configuration and embryo development. **Cryobiology** 57:137-141.

Mukaida, T., Nakamura, S., Tomiyama, T., Wada, S., Kasai, M., and Takahashi, K. (2001). Successful birth after transfer of vitrified human blastocysts using a cryoloop container-less technique. **Fertil. Steril**. 76:618-620.

Mukaida T., Wada, S., Takahashi K. Pedro, P. B., An, T. Z., and Kasai, M. (1998). Vitrification of human embryos based on the assessment of suitable conditions for 8-cell mouse embryos. **Hum. Reprod.** 13:2874-2879.

Muthukumar, K., Mangalaraj, A. M., Kamath, M. S., and George, K. (2008). Blastocyst cryopreservation: vitrification or slow freeze. **Fertil. Steril.** 90:S426–S427.

Pereira, R. M., Baptista, M. C., Vasques, M. I., Horta, A. E., Portugal, P. V., Bessa, R. J., Silva, J. C., Pereira, M. S., and Marques, C. C. (2007). Cryosurvival of

bovine blastocyst is enhanced by culture with trans-10 cis-12 conjugated linoleic acid (10t, 12c CLA). **Anim. Reprod. Sci.** 98:293-301.

Petyim S., Makemahar, O., Kunathikom, S., Choavaratana, R., Laokirkkiat, P., and Penparkkul, K. (2009). The successful pregnancy and birth of a healthy baby after human blastocyst vitrification using Cryo-E, first case in Siriraj Hospital. **J. Medi. Assoc. Thailand** 92:1116-1121.

Portmann, M., Nagy, Z. P., and Behr, B. (2010). Evaluation of blastocyst survival following vitrification/warming using two different closed carrier systems. **Hum. Reprod.** 25:i261.

Rall, W. F., and Fahy, G. M. (1985). Ice-free cryopreservation of mouse embryos at –196°C by vitrification. **Nature** 313:573-575.

Saragusty, J., and Arav, A. (2011). Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. **Reproduction** 141:1-19.

Sathananthan, A. H., (1997). Ultrastructure of human egg. **Hum. Cell** 10:21-38.

Seneda, M. M., Esper, C. R., Garcia, J. M., Oliveira, J. A., and Vantini, R. (2001). Relationship between follicle size and ultrasound-guided transvaginal recovery. **Anim. Reprod. Sci.** 67:37-43.

Shaw, J. M., Oranratnachai, A., and Trouson, A. O. (2000). Fundamental cryobiology of mammalian oocytes and ovarian tissue. **Theriogenology** 53:59-72.

Shaw, P. W., Bernard, A. G., Fuller, B. J., Hunter, J. H., and Shaw, R. W. (1992). Vitrification of mouse oocytes using short cryoprotectant exposure: effects of varying exposure times on survival. **Mol. Reprod. Dev.** 33:210-214.

Somfai, T., Ozawa, M., Noguchi, J., Kaneko, H., Karja, N. W. K., Farhudin, M., Dinnyes, A., Nagai, T., and Kikuchi, K. (2007). Developmental competence of

in vitro fertilized porcine oocytes after *in vitro* maturation and solid surface vitrification: Effect of cryopreservationon oocytes antioxydative system and cell cycle stage. **Cryobiology** 55:115-126.

Son, W. Y., Yoon, S. H., Yoon, H. J. Lee, S. M., and Lim, J. H. (2003). Pregnancy outcome following transfer of human blastocysts vitrified on electron microscopy grids after induced collapse of the blasocoele. **Hum. Reprod.** 18:137-139.

Sripunya, N., Somfai, T., Inaba, Y., Nagai, T., Imai, K., and Parnpai, R. (2010). A comparison of cryotop and solid surface vitrification methods for the cryopreservation of in vitro matured oocytes. **J. Reprod. Devel.** 56:176-181.

Stachecki, J. J., Garrisi, J., Sabino, S., Caetano, J. P. J., Wiemer, K. E., and Cohen, J. (2008). A new safe, simple and successful vitrification method for bovine and human blastocysts. **Reprod. BioMed. Online** 17:360-367.

Steponkus, P. L., Myers, S. P., Lynch, D. V., Gardner, L., Bronshteyn, V., Leibo, S. P., Rall, W. F., Pitt, R. E., Lin, T. T., and MacIntyre, R. J. (1990). Cryopreservation of Drosophila melanogaster embryos. **Nature** 345:170-172.

Sugiyama, R., Nakagawa, K., Shirai, A., Sugiyama, R., Nishi, Y., Kuribayashi, Y., and Inoue, M. (2010). Clinical outcomes resulting from the transfer of vitrified human embryos using a new device for cryopreservation (plastic blade). **J. Assist. Reprod. Gene.** 27:161-167.

Sun, X., Li, Z., Yi, Y., Chen, J., Leno, G. H., and Engelhardt, J. F. (2008). Efficient term development of vitrified ferret embryos using a novel pipette chamber technique. **Biol. Reprod.** 79:832-840.

Tsang, W. H., and Chow, K. L. (2009). Mouse embryo cryopreservation utilizing a novel high-capacity vitrification spatula. **BioTechniques** 46:550-552.

Vajta, G., Holm, P., Greve, T., and Callesen, H. (1997). Vitrification of porcine embryos using the open pulled straw (OPS) method. **Acta Veterinaria Scandinavica** 38:349-352.

Vajta, G., Holm, P., Kuwayama, M., Booth, P. J., Jacobsen, 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.

Vanderzwalmen, P., Bertin, G., Debauche, C., Standaert, V., Bollen, N., van Roosendaal, E., Vandervorst, M., Schoysman, R., and Zech, N. (2003). Vitrification of human blastocysts with the Hemi-Straw carrier: application of assisted hatching after thawing. **Hum. Reprod.** 18:1504-1511.

Vanderzwalmen, P., Bertin, G., Debauche, C., Standaert, V., van Roosendaal, E., Vandervorst, M., Bollen, N., Zech, H., Mukaida, T., Takahashi, K., and Schoysman, R. (2002). Births after vitrification at morula and blastocyst stages: effect of artificial reduction of the blastocoelic cavity before vitrification. **Hum Reprod.** 17:744-751.

Wu J., Zhang, L. and Wang, X. (2001). *In vitro* maturation, fertilization and embryo development after ultrarapid freezing of immature human oocytes. **Reproduction** 121:389-393.

Yavin, S., and Arav, A. (2001). Development of immature bovine oocytes vitrified by minimum drop size technique and a new vitrification apparatus (VIT-MASTER). **Cryobiology** 43:331.

Yavin, S., Aroyo, A., Roth, Z., and Arav, A. (2009). Embryo cryopreservation in the presence of low concentration of vitrification solution with sealed pulled straws in lipid nitrogen slush. **Hum. Reprod.** 24:797-804.

Yoon, T. K., Kim, T. J., Park, S. E. Hong, S. W., Ko, J. J., Chung, H. M.,and Cha, K. Y. (2003). Live births after vitrification of oocytes in a stimulated in vitro fertilization-embryo transfer program. **Fertil. Steril.** 79:1323-1326.

Zhao, X. M., Du, W. H., Wang, D., Hao, H. S., Liu, Y., Qin, T., and Zhu, H. B. (2011). Recovcery of Mitochondrial function and endogenous antioxidant systems in vitrified bovine oocytes during extended in vitro culture. **Mol. Reprod. Dev.** 78:942-950.

CHAPTER V

IN VITRO ASSESSMENT OF BLASTOCYSTS HATCHABILITY FOLLOWING MATURED OOCYTES AND BLASTOCYSTS VITRIFICATION IN BOVINE

5.1 Abstract

Oocyte and embryo vitrification is the best way to preserve genetics. This study was aimed to determine the blastocyst hatchability following matured oocytes (MII) and blastocyst vitrification. In order to prove the aim, experiment were conducted into four groups; (A) fresh MII and blastocysts, (B) fresh MII with vitrify blastocysts, (C) vitrify MII with fresh blastocysts and (D) vitrify both MII and blastocyst stage. In this study, there was no significant ($p>0.05$) difference in survival rate of oocytes after vitrification of MII stage and the blastocyst rate among the different groups. In case of the blastocyst quality in both fresh and vitrified group, there was no significant ($p>0.05$) difference. However, in case of group D, the blastocyst survival rate (56.7%) as well as hatched rate (16.7%) was led to significantly lower level that of control group. I also estimated that the oocytes survival rate in group C was significantly correlated with cleaved rate whereas the blastocysts hatchability rate in group A showed significantly correlated ($P<0.01$) with its survival rate. In group D, the strength of correlation between blastocysts survival and hatched rate was very lower than that of others. In conclusion, our data show that the twice vitrification has more damages of the microorganelles of the

oocytes and harden the zona pellucida which interfere the hatchability of blastocysts.

5.2 Introduction

Recently storage of oocytes and embryos is the time claim issue in the ART. There are two successful methods of cryopreservation including slow freezing and vitrification, used for animal cells cryopreservation. Out of two methods, vitrification is more widespread and quick method for cryostorage. Generally animal breeders or biotechnologists are intended to preserve or showing deepest sense to keep the animal cells, oocytes, sperms and embryos for preservation of the good genetics as well as the conservation of endangered species. Wilmut and Rowson (1973) first reported cryopreservation of bovine embryos. Recently, it is highly concerned that the vitrification of oocytes and embryos by using different device and CPAs to increase its survival rate (Matsunari et al., 2012). Successful vitrification of mammalian oocytes and embryos has numerous ethically applicable and economic benefits, which implies strongly positive impact for animal breeding podium.

Over the past decade, significant advancements have been made in ART such as AI, IVF, superovulation etc. to improve the genetic merit and infertility. However, the IVEP is the platform of genetic bank and useful application, to overcome the infertility problem, production of transgenic animals, embryo sexing and/or calf pre-selection. Despite these widely importance's, the embryo production and storage is still questionable at farmers leveled in comparing with AI. Oocytes cryopreservation combined with ART such as IVF is the fundamental strategic technology for gene banking of female germplasm (Ledda et al., 2007; Somfai et al., 2013). In recent studies, there are various attempts for oocyte and embryo vitrification

for future use of interest. However, to our knowledge, there is no study regarding the double vitrification at MII stage oocytes as well as blastocysts stage. As it is challenging task from collection of oocytes to production of embryos, it is promising fact to vitrify oocytes as well as embryos. But the question might be raised, why do we need double vitrification? It is known that IVF in any animal is the combination of many complicated steps and its ultimate output to get blastocyst is few. Finally, the collection of good genetic donor oocytes as well as the production of embryo to blastocyst stage is quite expensive and time bound absolutely.

We can storage the surplus oocytes by using standard protocol whereas the successful vitrified matured oocytes could be used for multidimensional purpose as it might be used for IVF or ICSI or cloning. Simultaneously, the surplus blastocyst, derived from IVF or ICSI or cloning, could be stored for future use when needed or demand is created. Therefore, the objective of our study was to assess the hatchability of blastocysts following double vitrification at MII and blastocyst stage by using Paper device. To achieve this goal I investigated the blastocysts development and hatchability following different treatments.

5.3 Materials and methods

5.3.1 Experimental design

I divided the experiment into four different groups as showed in Figure 5.1, (A) fresh MII and blastocysts, (B) fresh MII but vitrify blastocysts, (C) vitrify MII but fresh blastocysts and (D) vitrify MII and blastocyst stage.

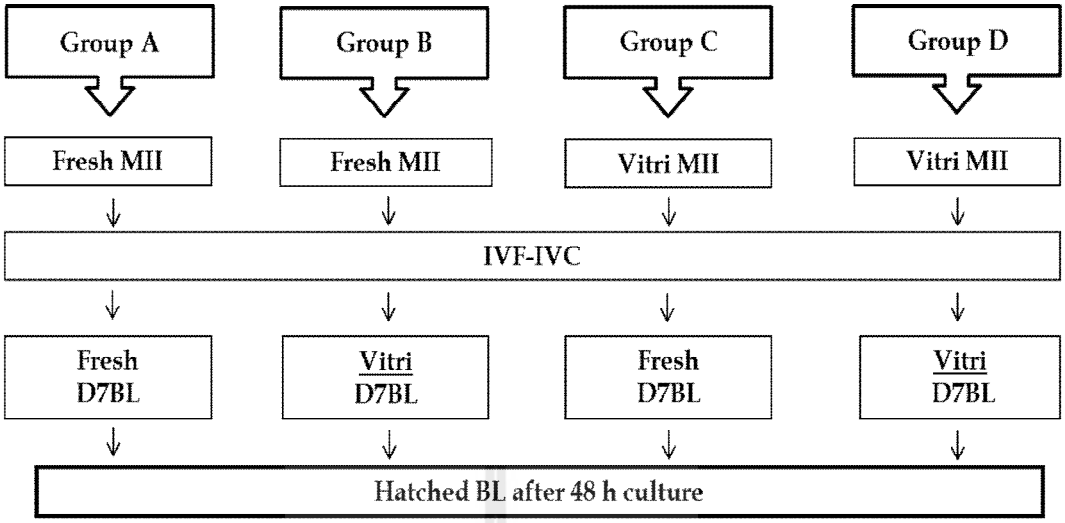

Figure 5.1 Design of experiment.

5.3.2 Chemicals and media

All reagents were purchased from Sigma Chemical Company unless otherwise stated. The medium was used for IVM was TCM199 supplemented with 10% FBS, 0.02 IU/ml FSH, 50 IU/ml hCG and 1 μg/ml estradiol-17β. The medium for embryo culture was mSOF supplemented with amino acids and 0.3% fatty acid-free BSA (Gardner et al., 1994).

5.3.3 Oocyte aspiration and IVM

Bovine ovaries were collected from slaughterhouses and kept in 0.9% NaCl and transported within 4 h at room temperature to the laboratory. For the follicles (2-8 mm in diameter), COCs were aspirated using a 18-gauge needle attached to a 5 ml syringe. Each of 20 COCs were cultured in 100 μl droplets of IVM medium TCM-199 covered with mineral oil in a humidified atmosphere of 5% CO_2 at 38.5°C for 22-23 h.

5.3.4 Oocytes and blastocysts vitrification and warming

A group of 4-5 oocytes or 2-3 blastocysts was washed in TCM199-Hepes + 20% FBS (base medium, BM) before being placed in BM containing 5% DMSO and 5% EG for 30 sec, then exposed in BM containing 12% DMSO, 12% EG and 0.25 M sucrose for 30 sec after that finally exposed in BM containing 20% DMSO, 20% EG and 0.5 M sucrose (VS) for 30 sec at room temperature (24-25°C). The oocytes or blastocysts were dropped with about 2 µl VS into the Paper device and then plunged into liquid nitrogen. The vitrified Paper devices were warmed by immersing directly into 2 ml of 0.5 M sucrose in BM for 2 min, then transferred to 0.25 M sucrose in BM for 2 min, then transferred to 0.125 M sucrose in BM for 2 min, finally washed with BM.

5.3.5 Evaluation of oocytes viability

Oocytes viability was evaluated by FDA staining as described previously (Mohr and Trouson, 1980). Briefly, oocytes were treated with 2.5 µg/ml FDA in PBS supplemented with 5 µg/ml BSA at 38.5ºC for 2 min in a dark room. Then they were washed three times in PBS supplemented with 5 µg/ml BSA and evaluated under a fluorescent microscope. It is equipped with UV irradiation using a U-MWIB# filter with an excitation wavelength of 460-495 nm and emission at 510 nm. Oocytes' expressing a bright green fluorescence was regarded as viable one and were used subsequently.

5.3.6 *In vitro* fertilization (IVF) and culture (IVC)

After IVM, cumulus cells were partially denuded from oocytes by gentle pipetting using a fine glass pipette with 0.1% hyarulonidase. After that, a group of 10 oocytes were washed 3-4 times in TALP medium supplemented with 1 mM caffeine, 100 µg/ml heparin, 20 mM/l penecilamine, 10 mM/l hypotaurine and 20 mM/l ephenephrine. The sperm were prepared by swim up technique. One straw (0.25-ml straws, 25 $\times 10^6$ sperms/straw) was thawed at 39°C for 30 sec. One hundred microliter of thawed semen was placed to the bottom of snapped tube containing 2 ml of TALP and kept 45° angle in humidified atmosphere of 5% CO_2 in air at 38.5°C for 30 min. Then the supernatum was collected in a 15 ml conical tube and centrifuged at 500×g for 5 min. After centrifuging, supernatum was discarded and 3 ml TALP was added for washing. After that it was centrifuged and sperm concentration was calculated by using a hemocytometer and adjusted to 2×10^6 /ml (Seneda et al. 2001). Finally, 10 oocytes were kept in each 100 µl drop of sperm suspension and sperm-oocytes were co-incubated in humidified atmosphere of 5% CO2 in air at 38.5°C for 13-14 h. Then the presumptive zygotes were further cultured in mSOFaa medium (20 zygotes/100µl) under a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C for 48 h. At D2, the cleavage rates were recorded in which the day of IVF was considered as D0. Thereafter, embryos at the 8-cells stage were selected and co-cultured with bovine oviduct epithelial cells in mSOFaa medium under a humidified atmosphere of 5% CO_2 in air at 38.5°C, as reported previously (Parnpai et al. 1999) for 6 days. Half of the medium was replaced with fresh medium during the embryo development was recorded. The development of blastocyst stage was recorded on D7 and D8.

5.3.7 Evaluation of blastocysts by differential staining of ICM and TE cells

Differential staining of ICM and TE cells of pooled blastocysts were performed as previously described (Sripunya et al., 2010). Briefly, blastocysts were simultaneously treated with 0.1 mg/ml propidium iodide (PI) and 0.2%Triton X-100 dissolved in Dulbecco's phosphate buffered saline for 40-60 sec to permeable the membrane and stain the nuclei of TE cells. Then the embryo were treated with 25 µg/ml Hoechst 33342 dissolved in 99.5% ethanol for 5 min, mounted on glass slides in glycerol droplets, flattened by coverslips and examined under UV light with an excitation wavelength of 330-385 nm under a fluorescent microscope. The nuclei of TE cells were labeled by both PI and Hoechst appeared pink or red whereas the nuclei of ICM cells will be labeled only by Hoechst appeared blue. A digital image of each embryo were taken, and the cell numbers of both cells types were counted using the NIH image J (v. 1.40) software (Abramoff et al., 2004). Total cell numbers were counted in all embryos. The numbers of ICM and TE cells were counted separately in embryos that had clearly distinguishable populations of red and blue nuclei.

5.3.8 Statistical analysis

The data of oocytes survivability, and embryo development after different treatment were expressed as percentage (%). For all statistical analyses, $p<0.01$ and $p<0.05$ were considered significant. For group/treatment comparisons, statistical analyses were done by one-way ANOVA using SPSS® Software. The estimation of correlation regression (Menard, 2000) between oocytes survival and cleaved as well as blastocysts survival and hatched rate, was done by curve estimation.

5.4 Results

To analyses the impact of following double vitrification of MII and blastocysts stages, four treatments was done. As shown in Table 5.1, vitrification of MII stage oocytes had no significant (p>0.05) variation for FDA survival rate. I also found that the embryonic developmental rate as 8 cells, morula and blastocysts stage in different groups was not significantly different among the experimental groups. I also noted that the blastocysts derived from both fresh and vitrified group of oocytes were of similar quality (Figure 5.2). The numbers of TE, ICM and total cells (TE+ICM) remained unchanged (p>0.05). These data suggest that there was no difference in blastocysts quality among the different group.

In an attempt to determine the rate of blastocyst production after vitrification, 3-steps vitrification was performed by using paper device. The rates of blastocyst production in group A, B, C and D were 15.7%, 16.5%, 9.4% and 9.4%, respectively. In case of hatchability of blastocysts in group A, B, C and D were 73.3, 56.5, 66.6 and 16.7%, respectively. The hatchability rate of group C and D were not significantly (p>0.05) different when compared with group A. Most importantly, the hatched rate in group D was significantly lower than that of other groups.

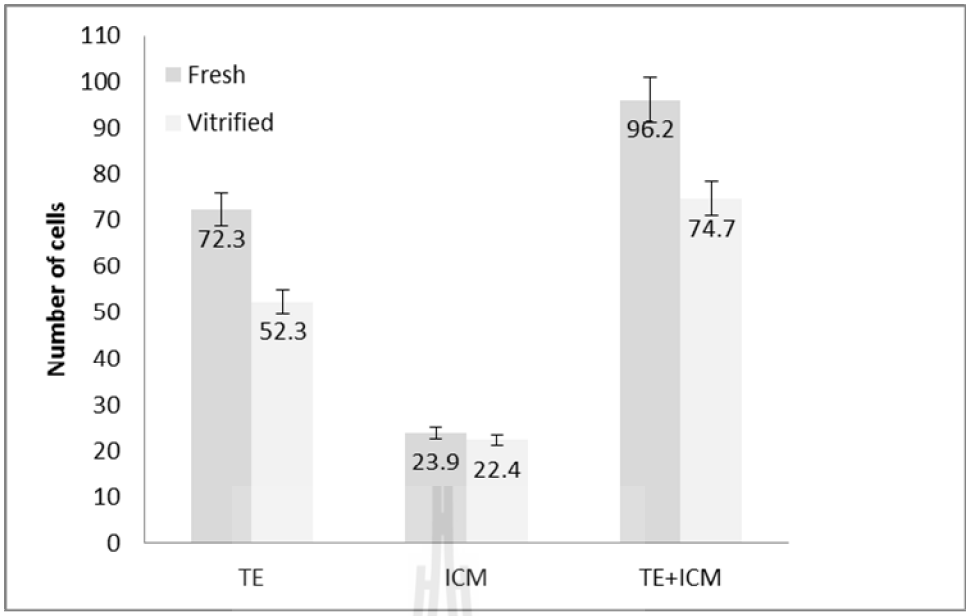

Figure 5.2 Differential staining of blastocyts. p>0.05; TE= Trophoectoderm; ICM= Inner cell mass.

Furthermore, to detect the degree of relationship, correlation regression of oocytes FDA survival and cleaved as well as blastocyst survival and hatched were analyzed within the same treatment group. The degree of co-relationship (R^2 value) between oocytes survival and cleaved in group A, B, C and D were 0.24, 0.36, 0.70 and 0.41, respectively (Table 5.2, Figure 5.3). The strength of correlation (R^2 value) in between post warming blastocyst survival and hatchability rate of group A, B, C and D were 0.81, 0.33, 0.10 and 0.03, respectively (Table 5.3, Figure 5.4). I calculated that the oocytes survival rate in group C was significantly correlated (R^2=0.70, F=9.47, P=0.04) with cleaved rate whereas the blastocysts hatchability rate in group A showed significantly correlated (R^2=0.81, F=16.57, P=0.01) with its survival rate. In group D, the strength of correlation between blastocysts survival and hatched rate was very lower (R^2=0.03) than that of other group.

Table 5.1 Comparison of blastocysts developmental rate among different grop.

Group	No. oocytes IVM	No. FDA survived (%)	No. oocytes IVF	No. cleaved (%)	No. (%) developed to			No. BL	No. (%) survived at 24 h	No. (%) hatched BL at 48 h	No. (%) hatched/# IVF oocytes
					8-C	Morula	D7 BL				
A	145	140 (96.5)[a]	140	85 (60.1)[a]	51 (36.4)[a]	37 (26.4)[a]	22 (15.7)[a]	22	22 (100.0)[a]	17 (77.3)[a]	17 (12.1)[a]
B	145	139 (95.8)[a]	139	81 (58.3)[a]	51 (36.7)[a]	38 (27.3)[a]	23 (16.5)[a]	23	18 (78.3)[a]	13 (56.5)[a]	13 (9.4)[ab]
C	146	127 (86.9)[a]	127	57 (44.9)[a]	32 (25.2)[a]	21 (16.5)[a]	12 (9.4)[a]	12	12 (100.0)[a]	8 (66.6)[a]	8 (6.3)[b]
D	146	127 (86.8)[a]	127	59 (46.5)[a]	34 (26.7)[a]	23 (18.1)[a]	12 (9.4)[a]	12	7 (56.7)[b]	2 (16.7)[b]	2 (1.6)[c]

Six replications, [a, b, c] within a column represent significant differences ($P < 0.05$). CL= Cleaved, 8-C = 8 cells, BL = Blastocyst

A: Fresh MII and Fresh BL.

B: Fresh MII and Vitrification BL.

C: Vitrification MII and Fresh BL.

D: Vitrification MII and Vitrification BL.

Table 5.2 Model summaries after curve estimation between oocytes survival and cleaved.

Group	Model summary			Parameter estimates	
	R square value	F value	P value	Constant	Coefficient
A	0.24	1.28	0.32	83.44	0.22
B	0.36	2.23	0.21	106.63	-0.181
C	0.70	9.47	0.04	73.79	0.300
D	0.41	2.74	0.17	70.89	0.36

A: Fresh MII and Fresh BL.

B: Fresh MII and Vitrification BL.

C: Vitrification MII and Fresh BL.

D: Vitrification MII and Vitrification BL.

Table 5.3 Model summaries after curve estimation between blastocyst survivals and hatched.

Group	Model summary			Parameter estimates	
	R square value	F value	P value	Constant	Coefficient
A	0.81	16.57	0.01	102.08	-0.03
B	0.33	1.98	0.23	53.49	0.45
C	0.10	0.44	0.54	99.50	0.01
D	0.03	0.12	0.74	63.18	-0.16

A: Fresh MII and Fresh BL.

B: Fresh MII and Vitrification BL.

C: Vitrification MII and Fresh BL.

D: Vitrification MII and Vitrification BL.

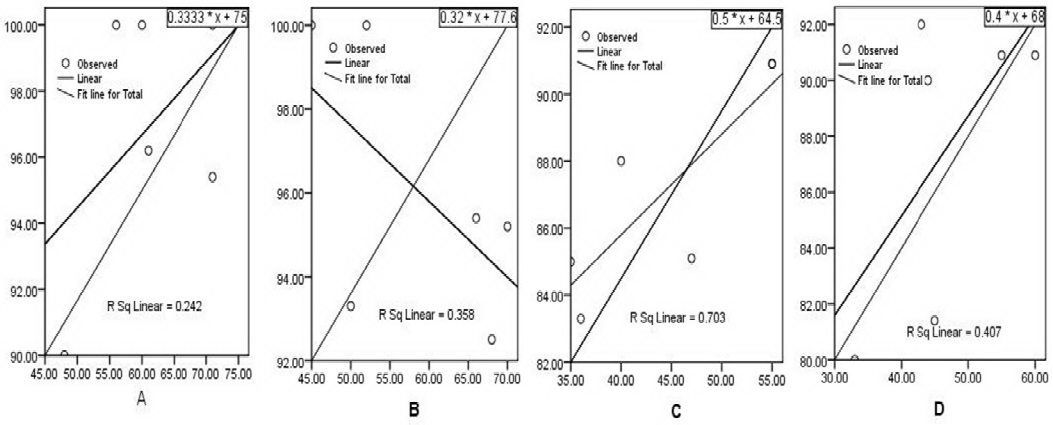

Figure 5.3 Correlation between oocytes survival rate and cleaved rates.

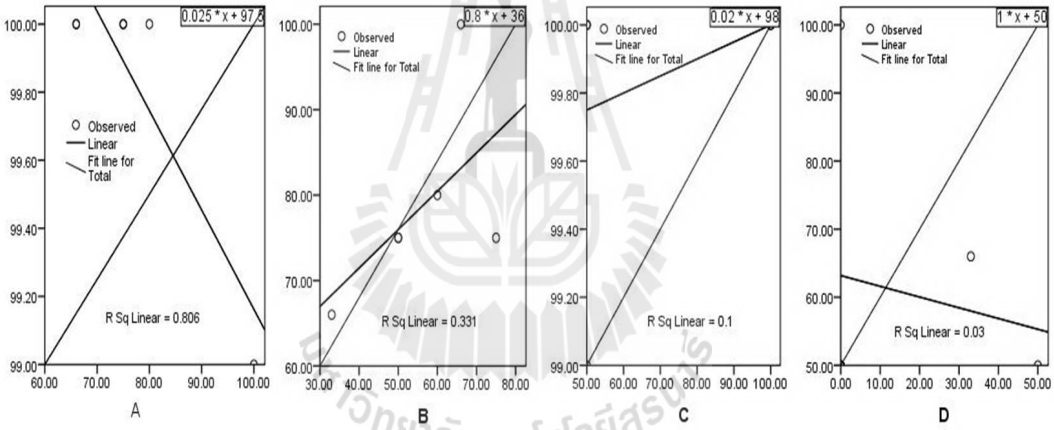

Figure 5.4 Correlation between blastocysts survival rate and hatched rates.

5.5 Discussion

This study was designed to explore the hatchability of blastocysts subsequent double vitrification at MII and blastocyst stage as well as their correlation. The collection of oocytes from live animals through ultrasound guided ovum pick up (OPU) or slaughter house to IVEP is a series of complicated and sophisticated steps which implies the high cost of embryo production. However, the quality of oocytes is a key factor affecting the success of bovine IVEP (Choi et al., 2013). It is proved and

indicated that each oocyte and embryo is very costly. Ghetler et al. (2005) mentioned that the ability to cryopreserve oocytes and embryos would have beneficial impact with overcoming the moral, ethical and legal issues. On the other hand, according to Phongnimitr et al. (2013), Liang et al. (2012), Sugimura et al. (2012) and Zhou et al. (2010), IVEP and cryopreservation of oocytes and embryos have worldwide research interest to preserve the good genetics. Therefore it is very clear that vitrification of oocytes has the both economic and genetic values.

In this study, I found that the oocytes vitrificantion and FDA survival rate remained unchanged with fresh and vitrified oocytes. These data are consistent with previous reports (Sripunya et al., 2010; Phongnimitr et al., 2013; Morato et al., 2008; Chian et al., 2004) indicating that they used different protocol and device for bovine oocytes vitrification. However, the technique, CPAs, concentration of CPAs and device or container for vitrification is obviously different. In agreement with others results, I also detected that the blastocysts derived from matured oocytes vitrification, had no effect on ICM and TE cells number, which were parallel to fresh group and not significantly different. It is still unclear and questionable also, why embryo quality is not varied. It is indicated that cryopreservation at MII stage is not affect the alive and growth blastocyst stage and also its quality, although vitrification was delayed the expansion of blastocyst as because of zona pellucida hardening (Larman et al., 2006).

This study also investigated the embryo survival and hatched rate was not significantly different among the group A, B and C except group D. Without the double vitrification group (group D), the survival rate of blastocyst in our study were similar with the study of Inaba et al. (2011) who used in-straw vitrification technique for bovine blastocyts vitrification. Our success rate was similar with

Laowtammathron et al. (2005) too who used linoleic acid-albumin in IVC medium and Ficoll supplementation to vitrification solution. However, survival rate in current study was almost double that of Piereira et al. (2007) and Machatcova et al. (2006), it might be due to the different method and different CPAs.

Bruyere et al. (2012) used different synthetic substitution (CRY03) of animal derived serum and found that it increased the embryo survival rate through inhibit the fragility of embryo. Recently, Sugimura et al. (2012) stated that the quality of blastocyst is also depending on its hatchability after 48 h additional post warming culture of expanded blastocyst.

In this experiment, we also estimated the correlation by curve estimation of correlation regressions between the oocytes FDA survival rate and cleavage rate. I found that there was corelation between them in each group. However, among of these groups, the group C showed significantly strong correlation. On the other hand, the correlation between blastocyst survival and hatched rate also had the positive relationship except the group A. But group A showed significantly higher correlation that that of others but that correlation was negative coefficient (-0.03). Interestingly, group D was also showed very lower correlation (R^2=0.03) with negative coefficient (-0.16). Finally, it can be interpreted that the variables in the model is required to fit the model.

However, double vitrification at different stage might influence the embryonic development and lead to low hatched rate. It is in agreement with the declaration of Ghetler et al. (2005) and Sugimura et al. (2012) that, the chilling is injurious for cell membrane integrity and intracellular damage of ultramicroscopic structure. It also interfere the blastocysts development and hatchability rate due to hardening of zona

pellucida. Therefore further study is needed to improve the blastocysts hatchability after twice vitrification at MII and blastocyst stage.

5.6 Conclusions

It might be concluded that double vitrification affect the hatchability of blastocysts. There was also no positive correlation between the blastocyst survival and hatchability. It is might be due to certain changes in membrane composition of oocytes and embryos that might occur during vitrification and finally zona harden may weaken the hatchability of blastocyst.

5.7 References

Abramoff, M. D., Magelhaes, P. J., and Ram, S. J. (2004). Image processing with image. **J. Biophot. Inter.** 11:36-42.

Bruyere, P., Baudot, A., Guyader-Joly, C., Guerin, P., Louis, G., and Buff, S. (2012). Improved cryopreservation of in vitro produced bovine embryos using a chemically defined freezing medium. **Theriogenology** 78:1294-1302.

Chian, R. C., Kuwayama, M., 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.

Choi, B. H., Bang, J., Jin, J. I., Kim, S. S., Jo, H. T., Deb, G. K., Ghanem, N., Cho, K. W., and Kong, K. I. (20130. Coculturing cumulus oocytes complexes with denuded oocytes alters zona pellucida ultratructure in in vitro matured bovine oocytes. **Theriogenology** 80:1117-1123.

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

Ghetler, Y., Yavin, S., Shagi, R., and Arav, A. (2005). The effect of chilling on membrane lipid phase transition in human oocytes and zygotes. **Hum. Reprod.** 20:3385-3389.

Inaba, Y., Alkawa, T., Hirai, T., Hashiyada, Y., Yamanouchi, T., Misumi, K., Ohtake, M., Somfai, T., Kobayashi, S., Saito, N., Matoba, S., Konishi, K., and Imai, K. (2011). In-straw cryoprotectant dilution for bovine embryos vitrified using Cryotop. **J Reprod Dev.** 57:437-443.

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

Larman, M. G., Sheehan, C. B., and Gardner, D. K. (2006). Calcium free vitrification reduces cryopreservation induced zona pellucida hardening and increased fertilization rates in mouse oocytes. **Reproduction** 131:53-61.

Ledda, S., Bogliolo, L., Succu, S., Ariu, F., Bebbere, D., Leoni, G. G., and Naitana, S. (2007). Oocyte cryo-preservation: oocyte assessment and strategies for improving survival. **Reprod. Fertil. Dev.** 19:13-23.

Liang, Y. Y., Rakwongrit, D., Phermthai, T., Somfai, T., Nagai, T., and Parnpai, R. (2012). Cryopreservation of immature buffalo oocytes: Effects of cytochalasin

B pretreatment on the efficiency of cryotop and solid surface vitrfication methods. **Anim Sci J.** 83:630-638.

Machatkova, M., Hanzalova, K., Horakova, J., Reckova, Z., and Hulinska, P. (2006). Collection of oocytes from donors in the growth phase of follicular development can enhance the production of bovine embryos for cryopreservation. **Vet. Medi.** 51:232-238.

Matsumoto, H., Jiang, J. Y., Tanaka, T., Sasada, H., and Sato, E. (2001). Vitrification of large quantities of immature bovine oocytes using nylon mesh. **Cryobiology** 42:139-144.

Matsunari, H., Maehara, M., Nakano, K., Ikezawa, Y., Hagiwara, Y., Sasayama, N., Shirasu, A., Ohta, H., Takahashi, M., and Nagashima, H. (2012). Hollow fiber vitrification: A novel method for vitrifying multiple embryos in a single device. **J. Reprod. Devel.** 58:599-608.

Menard, S. (2000). Applied Logistic Regression Analysis 2nd ed (Sage publications).

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

Morato, R., Izquierdo, D., Paramio, M. T., and Mogas, T. (2008). Cryotop versus open-pulled straws (OPS) as carriers for the cryopreservation of bovine oocytes: Effect of spindle and chromosome configuration and embryo development. **Cryobiology** 57:137-141.

Nagai, T. (2006). There will be no way to get funding for the project between Asian countries without application based on a strong will: The chance is floating in air. **The 3rd Annual conference of Asian Reproductive Biotechnology Society**. Hanoi, Vietnam, 111-116.

Parnpai, R., Tasripoo, K. and Kamonpatana, M. (1999). Deveopment 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.

Pereira, R. M., Baptista, M. C., Vasques, M. I., Horta, A. E., Portugal, P. V., Bessa, R. J., Silva, J. C., Pereira, M. S., and Marques, C. C. (2007). Cryosurvival of bovine blastocyst is enhanced by culture with trans-10 cis-12 conjugated linoleic acid (10t, 12c CLA). **Anim. Reprod. Sci.** 98:293-301.

Phongnimitr, T., Liang, Y. Y., Srirattana, K., Panyawai, K., Sripunya, N., Treetamipinich, C., and Parnpai, R. (2013). Effect of L-carinitine on maturation, cryo-tolerance and embryo developmental competence of bovine oocytes. **Anim Sci. J.** 84:719-725.

Seneda, M. M., Esper, C. R., Garcia, J. M., Oliveira, J. A., and Vantini, R. (2001). Relationship between follicle size and ultrasound-guided transvaginal recovery. **Anim. Reprod. Sci.** 67:37-43.

Somfai, T., Nakai, M., Tanihara, F., Noguchi, J., Kaneko, H., Kashiwazaki, N., Egerszegi, I., Nagai, T., and Kikuchi, K. (2013). Comparison of Ethylene Glycol and Propylene Glycol for the Vitrification of Immature Porcine Oocytes. **J. Reprod. Dev.** 59:378-384.

Sripunya, N., Somfai, T., Inaba, Y., Nagai, T., Imai, K., and Parnpai, R. (2010). A comparison of cryotop and solid surface vitrification methods for the cryopreservation of *in vitro* matured oocytes. **J. Reprod. Dev.** 56:176-181.

Sugimura, S., Akai, T., Hashiyada, Y., Somfai, T., Inaba, Y., Hirayama, M., Yamanouchi, T., Matsuda, H., Kobayashi, S., Aikawa, Y., Ohtake, M.,

Kobayashi, E., Konishi, K., and Imai, K. (2012). Promising system for selecting healthy *in vitr*o fertilized embryos in cattle. **PLoS ONE** 7:e36627.

Wilmut, I., and Rowson, L. E., (1973). Experiments on the low temperature preservation of cow embryos. **Vet. Res.** 26:686-690.

Zhou, X. L., Al Naib, A., Sun, D. W., and Lonergan, P. (2010). Bovine oocytes vitrification using cryotop method: Effect of cumulus cells and vitrification protocol on survival and subsequent development. **Cryobiology** 61:66-72.

CHAPTER VI

CONCLUSIONS

The results of experiment one demonstrated that the survival rate of G1 Exp-BLs showed no difference between D7 and D8 culture periods but that D7 embryo had a significantly higher hatchability rate subsequent to Cryotop vitrification. However, it is our observation that rapidly growing embryos are more tolerant to the vitrification process than slower growing embryos. Therefore, I suggest that the D7 Exp-BL is more suitable for cryopreservation and embryo transfer. It demonstrates the strongest co-relation between survival and hatched rate.

In experiment two, I have developed a new and ideal 'Paper' device as standard as Cryotop device, for oocytes and embryos vitrification. Our studies also demonstrated that the 3-steps protocol is non-toxic and more efficient than 2-steps method. The blastocyst development rate after oocytes vitrification is higher in 3-steps than that of 2-steps method. In case of blastocysts vitrification, 3-steps method also showed higher survival rate and hatched rate than that of 2-steps method. Therefore it might be declared that paper device and 3-steps method is suitable, confortable and less stressful for oocytes and embryos vitrification. This finding in particular opens a new avenue for ultra-rapid freezing.

In experiment three, it might be concluded that double vitrification affect the hatchability of blastocysts. There was also no positive co-relationship between the blastocyst survival and hatchability. It is might be due to certain changes in membrane

composition of oocytes and embryos that occured during vitrification and finally zona harden may weaken the hatchability of blastocyst. In future, more study on practical application of our technique is needed.

BIOGRAPHY

Mr. Ashit Kumar Paul was born on October 14, 1984 in Naogaon, Bangladesh. In 2008, Mr. Paul graduated in Doctor of Veterinary Medicine (D.V.M.) from Bangladesh Agricultural University, Mymensingh, Bangladesh. In 2010, he completed Master of Science (M.S.) in Theriogenology from the Department of Surgery and Obstetrics at the same university under the supervision of Professor Dr. Golam Shahi Alam. His research topic was "Factors that limit first service pregnancy rate in cows." Mr. Paul has been a lecturer at the Department of Medicine and Surgery, Faculty of Animal Science and Veterinary Medicine, Patuakhali Science and Technology University, Patuakhali, Bangladesh since 2011. In 2012, Mr. Paul started higher study to pursue Doctor of Philosophy (Ph.D.) at School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Thailand under the supervision of Associate Professor Dr. Rangsun Parnpai. Mr. Paul has been promoted to be Assistant Professor in 2013 and he has many articles and books published internationally. Part of this research has been published in The Thai Journal of Veterinary Medicine.

Mr. Paul's academic areas of interest include animal reproduction, animal biotechnology, cryopreservation and animal health.