VITRIFICATION OF MOUSE MATURED OOCYTES AND EMBRYOS AT VARIOUS STAGES: COMPARISON OF CRYOTOP AND HEMI STRAW CLOSED SYSTEM METHODS

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การทำ vitrification ไข่สุก และตัวอ่อนหนูถีบจักรระยะต่างๆ: เปรียบเทียบการใช้วิธี Cryotop และ hemi straw closed system

นายอนิรุธ อารีเอื้อ

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

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อนิรุธ อารีเอื้อ : การทำ vitrification ไข่สุก และตัวอ่อนหนูถีบจักรระยะต่างๆ: เปรียบเทียบการใช้วิธี Cryotop และ hemi straw closed system (VITRIFICATION OF MOUSE MATURED OOCYTES AND EMBRYOS AT VARIOUS STAGES: COMPARISON OF CRYOTOP AND HEMI STRAW CLOSED SYSTEM METHODS) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.รังสรรค์ พาลพ่าย, 107 หน้า.

การแช่แข็ง เป็นการรักษาสภาพของเซลล์ให้คงสภาพได้ยาวนานขึ้น โดยวิธีแช่แข็งแบบเยื่อแก้ว (vitrification) ถูกใช้เป็นวิธีหลักในการศึกษาการแช่แข็งในปัจจุบัน ซึ่งการศึกษานี้ในการทดลองที่หนึ่ง ตัวอ่อนหนูถีบจักรถูกผลิตจากไข่แช่แข็งด้วยวิธีฉีดอสุจิเข้าไปในไซโตพลาสซึมของไข่ โดยใช้เครื่องเปียโซ โดยเปรียบเทียบวิธีการแช่แข็ง ด้วยการใช้ Cryotop กับ hemi straw closed system (HS-CS) เปรียบเทียบกับกลุ่มที่ไม่ได้แช่แข็ง โดยสองวิธีนี้ใช้น้ำยาแช่แข็งและน้ำยาละลายความเข้มข้นสูตรเดียวกัน คือ น้ำยาปรับความสมดุลที่มี 7.5% EG + 7.5% DMSO + 20% FBS นาน 5 นาที ตามด้วย แช่ในน้ำยาแช่แข็งที่มี 15% EG + 15% DMSO + 20% FBS + 0.6 M ซูโครส นาน 30 วินาที และน้ำยาละลายที่ความเข้มข้นต่างกันคือ 1.0 M, 0.5 M, 0.25 M และ 0 M ซูโครสและ 20% FBS ในสารละลายของ D-PBS นานสารละลายละ 5 นาที ซึ่งจากอัตราการรอดที่สูงหลังการละลายไข่แช่แข็ง และอัตราการพัฒนาไปเป็นตัวอ่อนระยะเอ็กซ์แพนบลาสโตซีส ในกลุ่ม HS-CS พบว่ามีความแตกต่างทางสถิติจากกลุ่ม Cryotop (อัตราการรอดคือ 98.6% และ 96.9% และอัตราการพัฒนาไปเป็นตัวอ่อนระยะเอ็กซ์แพนบลาสโตซีสคือ 29.1% กับ 27.3% ของแต่ละกลุ่มเรียงตามลำดับ โดยค่านัยสำคัญน้อยกว่าร้อยละห้า) ยิ่งไปกว่านั้น เมื่อพิจารณาอัตราส่วนของมวลเซลล์ภายในต่อโทรเฟคโตเดิร์ม ของทั้งสองกลุ่มแช่แข็ง พบว่ามีค่าที่ต่ำกว่าแต่ไม่มีความแตกต่างทางสถิติจากกลุ่มควบคุมที่ไม่ได้แช่แข็ง (0.42 $\pm$0.10, 0.41$\pm$0.11 และ 0.45$\pm$0.11 ของแต่ละกลุ่มเรียงตามลำดับ โดยค่านัยสำคัญมากกว่าร้อยละห้า) ซึ่งจากผลการทดลองนี้บ่งชี้ได้ว่า การแช่แข็งแบบใช้ HS-CS และวิธีการใช้ Cryotop มีประสิทธิภาพในการแช่แข็งไข่ได้ดี ส่วนในการทดลองที่สอง ตัวอ่อนหนูถีบจักร จากหลายๆระยะ เช่น สองเซลล์ สี่เซลล์ แปดเซลล์ มอรูล่า และบลาสโตซีส ช่วงต้นที่ผลิตด้วยวิธีฉีดอสุจิเข้าไปในไซโตพลาสซึมของไข่โดยใช้เครื่องเปียโซ ถูกนำไปแช่แข็ง และทำการเปรียบเทียบวิธีระหว่างการใช้ Cryotop, HS-CS กับ กลุ่มควบคุมที่ไม่ได้แช่แข็ง ซึ่งผลจากการทดลองที่สองนี้บ่งชี้ว่าวิธี HS-CS มีประสิทธิภาพเทียบเท่าวิธี Cryotop เมื่อพิจารณาที่อัตราการรอดชีวิตของตัวอ่อนและพัฒนาการของตัวอ่อนหลังการละลาย ยิ่งไปกว่านั้นที่ระยะเอ็กซ์แพนบลาสโตซีส จากกลุ่มแช่แข็งทั้งสองวิธี พบว่ามีความแตกต่างทางสถิติกับกลุ่มควบคุมที่ไม่มีการแช่แข็ง เมื่อพิจารณาที่จำนวนเซลล์ทั้งหมดและอัตราส่วนของมวลเซลล์ภายในต่อโทรเฟคโตเดิร์ม

ดังนั้นจึงกล่าวโดยสรุปได้ว่า การแช่แข็งแบบ HS-CS มีประสิทธิภาพสำหรับแช่แข็งไข่และตัวอ่อนของหนูถีบจักร ซึ่งเตรียมอุปกรณ์ใช้ได้เอง ราคาไม่แพง และสามารถลดหรือกำจัดปัจจัยเสี่ยงต่อการติดเชื้อ ดังนั้น วิธีนี้จึงมีความเป็นไปได้ที่จะนำไปประยุกต์ใช้ ในการแช่แข็งไข่และตัวอ่อนของมนุษย์

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

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ANIRUTH AREE-UEA : VITRIFICATION OF MOUSE MATURED OOCYTES AND EMBRYOS AT VARIOUS STAGES: COMPARISON OF CRYOTOP AND HEMI STRAW CLOSED SYSTEM METHODS. THESIS ADVISOR : ASSOC. PROF. RANGSUN PARNPAI, Ph.D., 107 PP.

OOCYTES AND EMBRYOS VITRIFICATION/CRYOTOP/HEMI STRAW CLOSED SYSTEM/PIEZO MICROMANIPULATOR/INTRACYTOPLASMIC SPERM INJECTION.

Cryopreservation plays a key role in long-term cell preservation. A process called vitrification is a method used as a milestone in cryopreservation technique. In the first experiment, mouse blastocysts were produced from vitrified oocytes following intracytoplasmic sperm injection (ICSI) by using piezo micromanipulator. While, two vitrification methods, Cryotop and hemi straw closed system (HS-CS) were investigated using the same freezing (equilibration medium, 7.5% EG + 7.5% DMSO + 20% FBS for 5 min and vitrification medium, 15% EG + 15% DMSO + 20% FBS + 0.6 M sucrose for 30 sec) and thawing media (D-PBS solutions supplemented with 1.0 M, 0.5 M, 0.25 M and 0 M sucrose + 20% FBS for 5 min each period). Survival rate after frozen-thawed oocytes, and the expanded blastocyst formation rate in the HS-CS group was not significantly different with the Cryotop group (Survival rates: 98.6% vs 96.9%, and the expanded blastocyst formation rates: 29.1% vs 27.3%, respectively; $P>0.05$). Furthermore, the ICM/TE ratio from both vitrified groups were not significant difference but lower than the non-vitrified group. (0.42 ± 0.10 vs 0.41 ± 0.11 vs 0.45 ± 0.11, respectively; $P>0.05$). The result indicated that HS-CS method is effective to vitrify oocytes as well as Cryotop method were effective

to vitrify oocytes. In the second experiment, various stages of embryos, such as 2-cell, 4-cell, 8-cell, morula and early blastocyst stage derived from piezo-ICSI method, were vitrified. The HS-CS method wsa compared with the Cryotop method and the non-vitrified group. The results indicate that the HS-CS method is as effective as the Cryotop method in both embryo survival and embryo development after warming. Furthermore, the expanded blastocysts rate which developed after vitrification-warming were not significantly different from the non-vitrified embryos in terms of cell counts and ICM/TE ratios, suggesting their functional competence.

From the results, it can be concluded that the HS-CS method is an effective method for mouse embryo vitrification, because it is simple to operate, inexpensive to establish and also eliminates or minimizes the risk of contamination. This method would potentially be applicable to human oocyte and embryo cryopreservation.

School of Biotechnology Student's Signature _____

Academic Year 2011 Advisor's Signature _____

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Aniruth Aree-uea

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LIST OF ABBREVIATIONS

ART	=	Assisted reproductive technologies
BF	=	Bright field
COCs	=	Cumulus-Oocytes-Complexes
CPA	=	Cryoprotectant agents
CZB-H	=	ChatotZiomekBavister-Hepes
D-PBS	=	Dulbecco's phosphate-buffered saline
DMSO	=	Dimethyl sulfoxide
EG	=	Ethylene glycol
EMG	=	Electron-microscope grid
ES	=	Embryonic stem
FBS	=	Fetal bovine serum
GC	=	Glycerol
hCG	=	Human chorionic gonadotropin
HS-CS	=	Hemi-straw closed-system
Hg	=	Mercury
ICM	=	Inner cell mass
ICR mice	=	Institute cancer research mice
ICSI	=	Intracytoplasmic sperm injection
IU	=	International Unit
LN_2	=	Liquid nitrogen
MII	=	Metaphase II
MDS	=	Minimum drop size

LIST OF ABBREVIATIONS (Continued)

NT	=	Nuclear transfer
OPS	=	Open pulled straws
PZD	=	Partial zona dissection
PMSG	=	Pregnant mare serum gonadotropin
PROH	=	Propylene glycol or 1,2 propandiol
PVP	=	Polyvinylpyrrolidone
Ros-Drill	=	Rotationally oscillation drill
SAS	=	Statistical analysis software
SN_2	=	Slush nitrogen
SSV	=	Solid surface vitrification
TC	=	Total cells
TE	=	Trophectoderm
UV	=	Ultraviolet
°C	=	Degree Celsius
Ø	=	Diameter
h	=	Hour
M	=	Molar
min	=	Minute
mL	=	Milliliter
mm	=	Millimeter
s	=	Second
µl	=	Microliter

LIST OF ABBREVIATIONS (Continued)

µm	=	Micrometer or micron
Vs	=	Versus

CHAPTER I

INTRODUCTION

1.1 Background

Cryopreservation plays a key role in long-term cell preservation, while keeping the cells or tissue "alive". Vitrification is a process by which the cells are frozen rapidly without the formation of intracellular ice crystals and represent a milestone in cryopreservation techniques. In vitrification, certain membrane permeable reagents are being used to aid the process without changing the integrity of the cells. Disruption of intracellular organelles, as well as formation of intracellular ice crystal, is a major hazard of the freezing process, which may result in loss of cell viability.

The cryopreservation of gametes (sperm and ova), zygotes and embryos has become widely popular for various medical and social conditions. This technique may be used when IVF cycle complications arise (i.e., ovarian hyper-stimulation syndrome or failure to obtain sperm) or to achieve synchronization in oocyte-donation cycles. Cryopreservation of oocytes is not only limited to infertile patients it may be used as a fertility preservation strategy for women who need to undergo sterilizing therapies for cancer (chemotherapy, radiation) or those who prefer to electively delay childbearing to an older (but more appropriate) age (Jain and Paulson, 2006). There are many techniques for the cryopreservation of mammalian oocytes, which differ not only in the procedure but also in their success rates. The "Gold standard" of cryopreservation of mammalian oocytes is the "Slow Freezing Method", which has been successfully

achieved in the mouse (Whittingham, 1977), human (Chen, 1986), rabbit (Al-hasani et al., 1989) and cattle (Fuku et al., 1992). More recently, vitrification has emerged as an alternative to the traditional slow freezing methods as it avoids the chilling injury and ice crystals formation (Rall et al., 1985 and Gupta et al., 2007). The first birth from vitrified human oocytes was reported in 1999, using open pulled straws (OPS) to hold the oocytes (Kuleshova et al., 1999). Later in 2003, the vitrified oocytes were loaded on an electron-microscope grid (Yoon et al., 2003). In 2005 the use of the Cryoleaf was reported (Chain et al., 2005). In 2005, the both of Cryoloop (Saki and Dezfuly, 2005) and Cryotop (Kuwayama et al., 2005) were reported and showed high survival rates after warming. More specifically, the Cryotop method which used a combination of ethylene glycol (EG) and sucrose as cryoprotectants, showed a 90.8% survival rate, 89.6% fertilization rate, a pregnancy rate of 41.4% per transfer and a live-birth and ongoing-pregnancy rate per transfer of 10/29 (34.5%). Based on these study which showed very high survival rates and healthy babies (Katayama et al., 2003 and Lucena et al., 2006), Cryotop vitrification method is deemed as a "new benchmark" for mammalian oocyte cryopreservation.

The rapid cooling rates of vitrification are achieved by placing the oocytes in small volumes of media containing cryoprotectants and exposing them directly to liquid nitrogen (LN_2). Several open-carrier systems have been used, including open pulled straws (Kuleshova et al., 1999), electron-microscope grids (EMG) (Yoon et al., 2003), Cryoloops (Saki and Dezfuly, 2005) and Cryotop (Kuwayama et al., 2005). These carrier systems require direct contact between the oocyte-containing solution and LN_2. Although LN_2 typically shows very low microbial counts, during storage and transfer of frozen samples, it may be contaminated with microorganism such as fungal

spores, yeasts, bacteria and viruses (Fountain et al., 1997 and Morris et al., 2005). It has been shown that the hepatitis B virus may be transmitted to recipients of bone marrow tissue stored in contaminated LN_2 (Tedder et al., 1995). This highlighted the potential risk of disease transmission through contaminated LN_2 or metal surfaces of the instruments used during freezing and storage. This hazard of infectious contamination can be eliminated by sterilization of LN_2 (Parmegiani et al., 2010) or using a completely closed system (sealed straws) to hold the oocytes (Bielanski et al., 2000).

In 2009, Vutyavanich and colleagues reported a modified, low cost, closed-system solid surface vitrification (SSV) by using hemi-straws (ø 0.25 mm.), loaded with 2-cell mouse embryos, inserted into a pre-cooled outer straw (ø 0.50 mm.), which was sealed at both ends and directly immersed into LN_2 (Vutyavanich et al., 2009). Embryos in the closed-SSV group showed significantly higher survival rate after freezing/thawing compared with the standard slow freezing group (95.7% and 77.6%, respectively), higher cleavage rate (90.5% and 70.7%, respectively) and higher blastocyst formation rate (79.3% and 58.6%, respectively, Chi-square test, $p<0.001$). In addition, the development of embryos with the closed-SSV was not significantly different from non-frozen controls (further cleavage of 90.5% and 96.5%, and blastocyst formation rate of 79.3% and 89.6%, respectively; $p\geq0.05$).

Intracytoplasmic sperm injection (ICSI) has revolutionized in clinical treatment of male-factor infertility. For training and study of mammalian embryo development, a genetically tractable mouse model is widely used. However, mouse metaphase II (MII) oocytes are quite sensitive and ICSI with conventional micro-pipettes generally damages the oocytes membrane (Yoshida and Perry, 2007).

This problem can be solved with piezo micromanipulation, with which the piezo-electric effectively (crystal deformation in response to an externally applied voltage) propels a microinjection needle tip forward in a precise and rapid movement. The piezo micromanipulation enhanced the penetration of membranes and matrices, and offered a successful method for mouse ICSI (Kimura and Yanagimachi, 1995, Yoshida and Perry, 2007).

The objective of this study was to compare the developmental potential of mouse oocytes vitrified by Cryotop and hemi straw closed system (HS-CS) methods followed by ICSI with piezo micromanipulator. The oocytes survival rate, embryo development and total cell count of the expanded blastocyst were investigated in each group.

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CHAPTER II

REVIEW OF LITERATURES

2.1 Piezo micromanipulation technique

The first successful fertilization of a mammalian oocyte by ICSI was reported in golden hamsters more than 35 years ago (Uehara and Yanagimachi, 1976), since then it has been successfully applied in other species including human (Palermo et al., 1992), mice (Kimura and Yanagimachi, 1995), sheep (Catt et al, 1996), rabbit (Hosoi et al, 1998), horse (Cochran et al, 1998), cattle (Hamano et al, 1999), domestic cat (Gomez et al., 2000), and pig (martin, 2000).

However, though the conventional ICSI has acquired high success rates in humans, cattle, pigs and rabbits, it resulted in irreversible damage and cell death in mouse oocytes. This problem can be overcome with piezo micromanipulator, which induces crystal deformation in response to an externally applied voltage and propels the needle tip forward in a precise and rapid movement. It appeared that piezo micromanipulation enhances the penetration of membranes and matrices, resulting in high success rates after mouse ICSI (Kimura and Yanagimachi, 1995; Yoshida and Perry, 2007).

The first piezo-ICSI was successful applied in the mouse using piezo-driven micropipette by compared with conventional ICSI method. Eighty percent of sperm-injected oocytes survived, and approximately 70% of them developed into blastocysts in vitro (Kimura and Yanagimachi, 1995). With this novel technique, the zona

pellucida and oolemma are penetrated by rapid vibrations produced at the pipette tip caused by longitudinal wavelength produced by piezo actuator. One of the main advantages of the piezo technique is that it causes less deformation to the cytoplasm of oocyte during injection. The oolemma is easily broken without suction of the ooplasm and produced less ooplasm leakage increasing oocyte survival after sperm injection (Yoshida and Perry, 2007).

Katayose et al. (1999) reported the first piezo-ICSI applied in cattle and results showed a dramatic increase in fertilization rates with piezo-ICSI (55%) when compared with conventional technique (0.05%) without additional chemical activation. The addition of 50 μM calcium ionophore to activate the oocytes increased fertilization rates in conventional-ICSI group to 15%, while no beneficial effect was found in piezo-ICSI group (58%).

In addition, Galli et al. (2003) concluded that exogenous oocyte activation did not improve the development of Piezo-ICSI embryos. They obtained 15, 6 and 17% blastocyst rates when sperm-injected oocytes were not treated, treated with Ionomycin and Ionomycin followed by Cycloheximide, respectively.

During fertilization of some mammalian oocytes, the sperm-induced oscillations in the free calcium are sufficient for early activation of the oocyte and subsequent embryo development (Whitaker and Patel, 1990; Kline and Kline, 1992). This is the conventional ICSI for hamster (Uehara and Yanagimachi, 1976), human (Palermo et al., 1992) and mice (Kimura and Yanagimachi, 1995). In contrast, in other species such as cattle (Rho et al., 1998), pigs (Lee et al., 2003), and buffalo (Liang et al., 2011) additional activation of the oocyte is required after sperm injection. However, in cattle, piezo-ICSI technique or piezo-ICSI with oocytes activation are

used to increase the fertilization rates, blastocyst formation rates (Katayose et al., 1999; Galli et al., 2003; Wei and Fukui, 2002) and pregnancy rates (Oikawa et al., 2005). However, for good of piezo micromanipulator control, mercury (Hg) was used to fill in small volume inside the injection pipette which can significantly improved the success rate of piezo ICSI (Huang et al., 1996; Katayose et al., 1999; Kimura et al., 1998). However, elemental mercury is a cumulative neurotoxin and should be handled with gloves and be disposed of with care according to local institutional guidelines. It should be pointed out that mercury should be avoided in human ICSI (Yoshida and Perry, 2007); therefore, fluorinert, an electronic liquid FC-77 is a thermally stable, fully-fluorinated liquid that has long been used as a heat transfer fluid in a variety of industries. Fluorinert is used instead mercury because, fluorinert is non-toxic, it is not as efficient for piezo ICSI as is mercury (Yoshida and Perry, 2007).

Ergenc and colleagues (2008) have demonstrated a method for mouse ICSI without using mercury: rotationally oscillation drill or "Ros-Drill" technique. It used a high speed rotation injector pipette to produce a rotational oscillation at the pipette tip as it drills through the cell membrane. Athough they could produce offsprings with this technique, the efficiency of the Ros-Drill technique was lower than the piezo technique in terms of survival rate (81.9% vs. 95.5%, respectively), 2-cell formation rate (87.4% vs 97.2%, respectively) and blastocyst formation rate (56.6% vs. 73.8%, respectively)

Piezo micromanipulation has been applied in cloning of mice (Wakayama et al., 1998) and pigs (Onishi et al., 2000), frozen and freeze-dried sperm injections (Wakayama et al., 1998, Wakayama and Yanagimachi, 1998), and nuclear transfer of embryonic stem (ES) cells (Munsie et al., 2000). Piezo-micromanipulation was

utilized to generate transgenic offsprings (Perry et al., 1999) and has been extended to the delivery of artificial chromosome transgenes (Perry et al., 2001; Moreira et al., 2004). Piezo has been employed for RNA interference (RNAi) in MII oocytes (Shoji et al., 2006); it facilitates blastocyst injection with ES cells (Kawase et al., 2001) and the manipulation of gamete precursors (Tanemura et al., 1997). It appeared that piezo plays an important role in the renaissance of stem cell biology (Kanatsu et al., 2004).

2.2 Cryopreservation technology

Cryopreservation is a technology for the long term preservation of mammalian cells, gametes and zygotes or embryos that has improved greatly over the past 30 years (Bernard and Fuller, 1996; Walters and Graves, 1997). The temperature for storage of mammalian cells is the temperature of LN_2 or -196ºC, a temperature where there is no active cellular metabolism. The critical steps of cryopreservation in terms of cellular damage are the cooling to -196°C and the subsequent thawing to 37°C. This is because water in the cell solidifies in form of ice crystals when it is cooled to below its freezing point. Since ice is less dense than liquid water, the ice crystals occupy a greater volume than the liquid water from which they were formed. This volume expansion causes pressure and damage to intracellular organelles. As water transitions from liquid to ice, any solutes in the liquid phase are excluded from the solid. For avoidance of ice crystals formation therefore is one of the goals of successful cryopreservation. As the temperature drops and the solid form proliferates, the concentration of electrolytes and other solutes in the remaining unfrozen solution rises dramatically, resulting in lowering of the freezing temperature of the solution (Lovelock, 1954). However, these high solutes concentrations can be quite toxic to intracellular proteins and other cellular components in the form of osmotic injury.

Thus the aim of cryopreservation is to avoid both ice crystals formation and the rise in intracellular osmolarity. Similarly, during thawing the solid ice melts and releases free water, resulting in decreasing osmolarity of the cellular solution. When the rewarming process is slow, there is also a danger of free water recrystallizing, thus causing further cellular damage. On the other hand, when the rewarming is too rapid, a sudden drop in the extracellular osmotic pressure may lead to a rapid shift of free water into the cell, leading to swelling and cellular damage (Mazur, 1980). This phenomenon is sometimes referred to as osmotic shock and its avoidance is a third major goal of a successful cryopreservation.

In summary, successful cryopreservation needs to address these three injury mechanisms: ice-crystals formation, solution effects and osmotic shock. The use of cryoprotectants is an important measure to avoid cellular damage during cryopreservation.

2.2.1 Slow freezing method

Slow programmable freezing was initially the standard method of embryo cryopreservation in most ART centers world-wide. This method uses low initial cryoprotectant concentrations, which are associated with lower toxicity, at temperatures in which there is still active cellular metabolism. Because cell metabolism is thought to decrease by approximately 50% for every 10°C decrease in temperature, toxicity is limited by having concentrations of cryoprotectants and other solutes increase only after the cell has been cooled to temperatures at which metabolism is quite slow. The temperature then is lowered gradually (about 2°C/min) to the seeding temperature at - 6°C. During this time, all of the solution remains liquid. At a temperature of - 6°C, ice crystals may be induced in the solution by introducing a seed, a small crystal of ice that allows other water molecules to undergo

crystallization. Seeding commonly is accomplished by touching the outside of the cryopreservation vessel (conventional straw) with a very cold instrument (pre-cooled forceps), which then induces a small ice crystal to form in the area at which the cryostraw is touched. Therefore, seeding is performed in an area distant from the oocyte, so that the ice crystal grows gradually toward the oocyte. After equilibration, the temperature is decreased gradually to a final temperature of - 32°C. During this time, the ice crystal gradually propagates in the extracellular medium, thus further increasing the concentration of cryoprotectants, particularly in the intracellular space, which is further dehydrated through the use of nonpermeating cryoprotectants. The very slow rate of cooling (0.33°C/min) allows gradual diffusion into the oocyte of additional permeating cryoprotectant, while maintaining equilibrium with the extracellular space. At the final temperature of - 32°C, the metabolic rate of the oocyte is quite slow, further limiting the toxicity of the increasing concentrations of the cryoprotectants. The freezing vessel now is plunged into LN_2, and the remaining nonsolidified solution is converted to a solid state at - 196°C.

During thawing, rapid transition of temperature is preferred to prevent re-crystallization of water with the potential for ice-crystal damage. Here, caution must be taken to avoid osmotic shock from the permeating cryoprotectant, which is now in very high concentrations in the intracellular space. Therefore, additional nonpermeating cryoprotectant is used. As the permeating cryoprotectant gradually diffuses out of the oocyte, the concentration of the nonpermeating cryoprotectant gradually is decreased, until the oocyte is returned to standard culture medium.

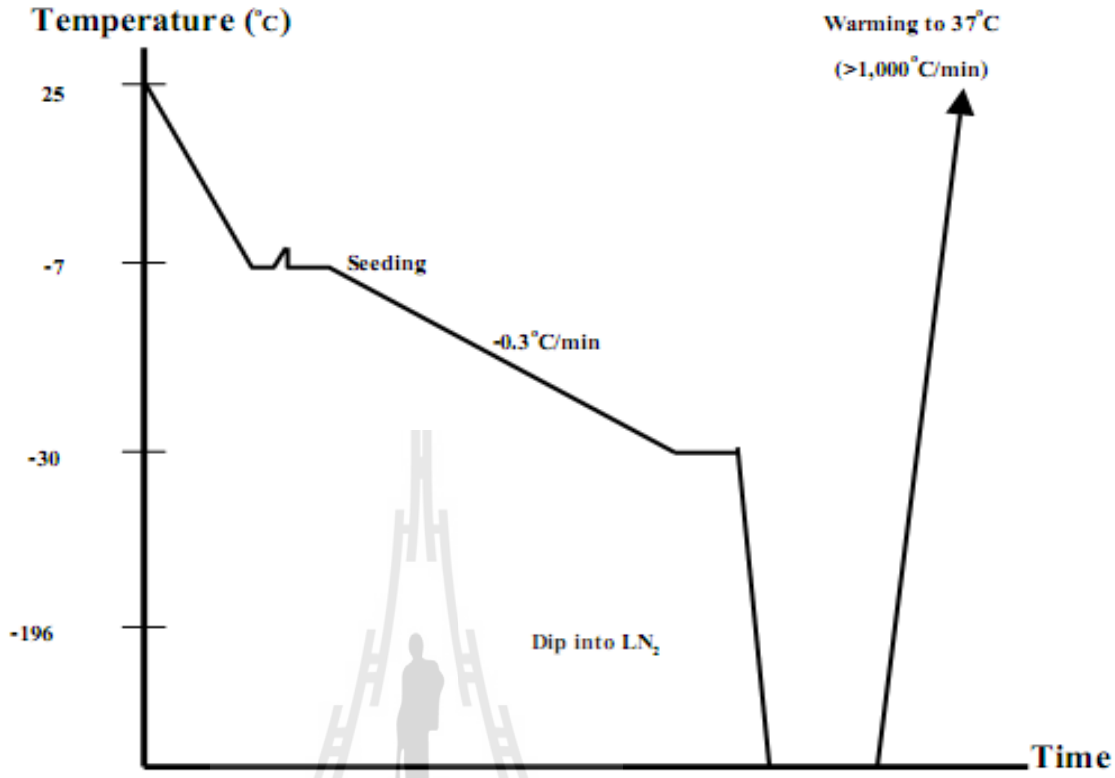

Figure 2.1 The cryopreservation procedure by slow freezing.

2.2.2 Ultra rapid freezing or vitrification

Cryopreservation of mammalian oocytes had become successful using the slow freezing method, which has been the "Gold standard" until recent years. More recently studies have demonstrated that vitrification is an alternative to traditional slow freezing methods, which avoids the chilling injury and ice crystal formation (Rall et al., 1985 and Gupta et al., 2007). During vitrification, permeating cryoprotectants are added at a high concentration, while the cell is still at room temperature. Because the toxicity of this high concentration of permeating cryoprotectant is substantial, the oocyte cannot be kept at this temperature for a long period. Therefore, a very short time is allowed for equilibration, after which the oocytes are plunged directly into liquid nitrogen.To further protect against ice-crystals

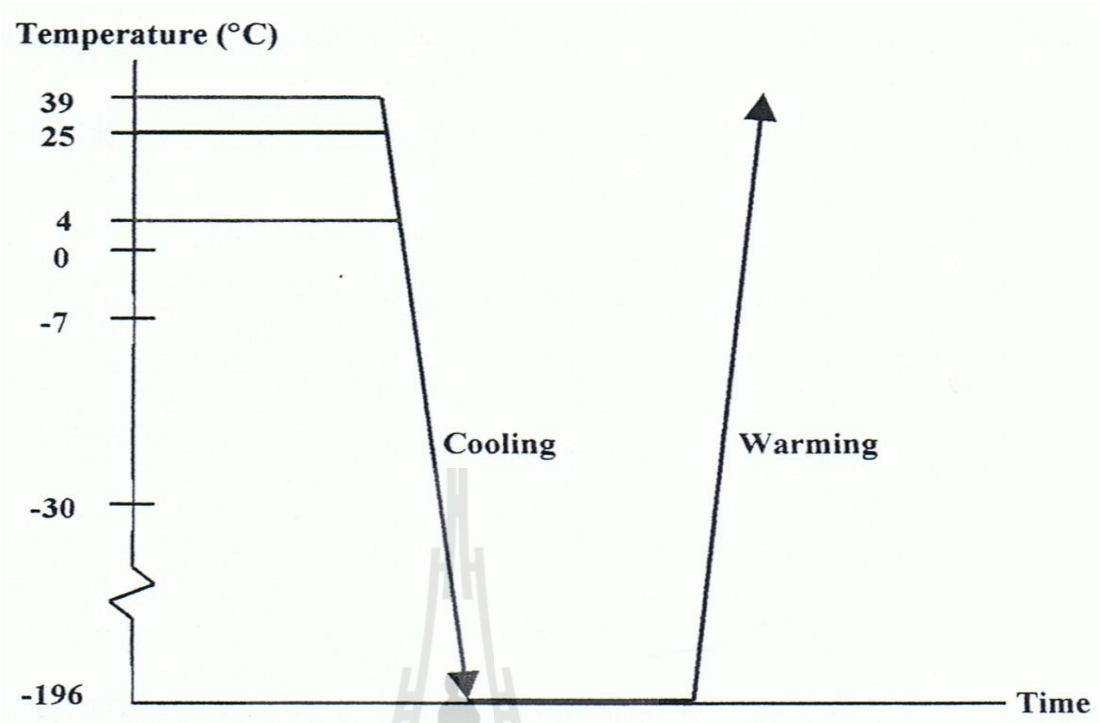

Figure 2.2 Cooling and warming rates of vitrification method.

formation, an extremely rapid rate of cooling is used. Vitrification does not necessarily require high cryoprotectant concentrations, because even pure water can be vitrified if the cooling rate is high enough (- 10^{7}°C/s) (Rall, 1987) and with moderately concentrated cryoprotectant solutions, vitrification can also be achieved with a moderate or even slow cooling rates (Fahy et al., 2004). Since increasing the cooling rate may permit decreasing the concentration of cryoprotectants and minimizing their potential toxic and osmotic effects, a minimum volume vitrification method is used. Minimizing the volume of the vitrification solution (<1µL) containing the oocytes or embryos not only offers the obvious benefit of increasing both cooling and warming rates, but also decreases the chance of ice crystals formation in the small sample. The minimum drop size (MDS) method was developed by Arav (Arav, 1992) and Arav et al. (Arav et al., 1993 and 1993). Droplets of 0.1–0.5 µL were prepared on

glass coverslip strips and immersed in liquid nitrogen or nitrogen slush. This method allows up to a 50% reduction in the concentration of cryoprotectants needed, compared to traditional protocols, while preserving safe ice- and fracture-free vitrification of material (Arav et al., 2002).

The first birth from vitrified human oocytes was reported in 1999, using open pulled straws (OPS) to hold the oocytes (Kuleshova et al., 1999). OPS is based on a very simple idea, and a very simple technique. Its unexpected additional benefits making the method safe and easy to use have only been discovered subsequently. The idea was to minimize the required volume of the sample by minimizing the diameter of the common insemination straw. When straws were warmed and pulled by hand, then cut at the thinnest point with scissors. While the required amount of solution to form a safe column decreased from 5 µL to less than 1 µL, leading eventually to a 10-fold increase in the achievable cooling rate, thus allowing a 30% decrease in CPA concentration required for safe vitrification.

Later in 2003, the vitrified oocytes were loaded on an electron-microscope grid (EMG) with very successfully as oocyte containers during cooling and warming. With this method, oocytes were spread on an EMG and then were preserved in LN_2 by rapid freezing (Yoon et al., 2003). In 2005 the use of the Cryoleaf (Chain et al., 2005) and the Cryoloop were reported. While, the Cryoloop is composed with nylon loop and stainless steel stick, and the lid and cryovial would cover the cryoloop. A small nylon loop attached to a holder and equipped with container. It has been used for cryopreservation in crystallography and is now used widely for oocyte and embryo cryopreservation (Saki and Dezfuly, 2005). The solution film bridging the hole of the loop is strong enough to hold the oocyte or the embryo, and with this

minimal solution volume, the achievable cooling rate may be extremely high. Using this tool, safe cryopreservation can be achieved even in the vapor of LN_2 (Larman et al., 2006). The Cryotop showed high survival rates after warming. More specifically, the Cryotop method which used a combination of ethylene glycol (EG) and sucrose as cryoprotectants, showed a 90.8% survival rate, 89.6% fertilization rate, a pregnancy rate of 41.4% per transfer and a live-birth and ongoing-pregnancy rate per transfer of 10/29 (34.5%) (Kuwayama et al., 2005). It is noteworthy that in the Cryotop method, a combination of EG and dimethylsulfhoxide (DMSO) was used as cryoprotectants, whith a two-step equilibration and supplementation with sucrose in the final concentration. EG, as a highly permeable cryoprotectant with moderate toxicity, is regarded as a standard component of most successful vitrification solutions (Kasai and Mukaida, 2004). Its successful combination with DMSO was first reported by Ishimori et al. (Ishimori et al., 1992; 1992; and 1993) and DMSO also appears to increase the permeability of EG (Vicente and Garcia-Ximenez, 1994). Cryotop vitrification has also been successfully used for cryopreservation of immature and in vitro matured horse oocytes (Bogliolo et al., 2006), bovine (Chian et al., 2004; and Kelly et al., 2006), ovine (Succu et al., 2006) and buffalo oocytes (Gasparrini et al., 2006), rabbit zygotes (Hochi et al., 2004), in vitro produced and in vivo derived porcine embryos (Esaki et al., 2004; Ushijima et al., 2004; and Hiruma et al., 2006), porcine blastocysts produced by parthenogenetic activation or somatic cell nuclear transfer from delipidated in vitro matured oocytes (Du et al., 2006), and bovine and buffalo embryos (Laowtammathron et al., 2005). It is suggested that the Cryotop vitrification method represents a “new benchmark” for mammalian oocyte and embryo cryopreservation.

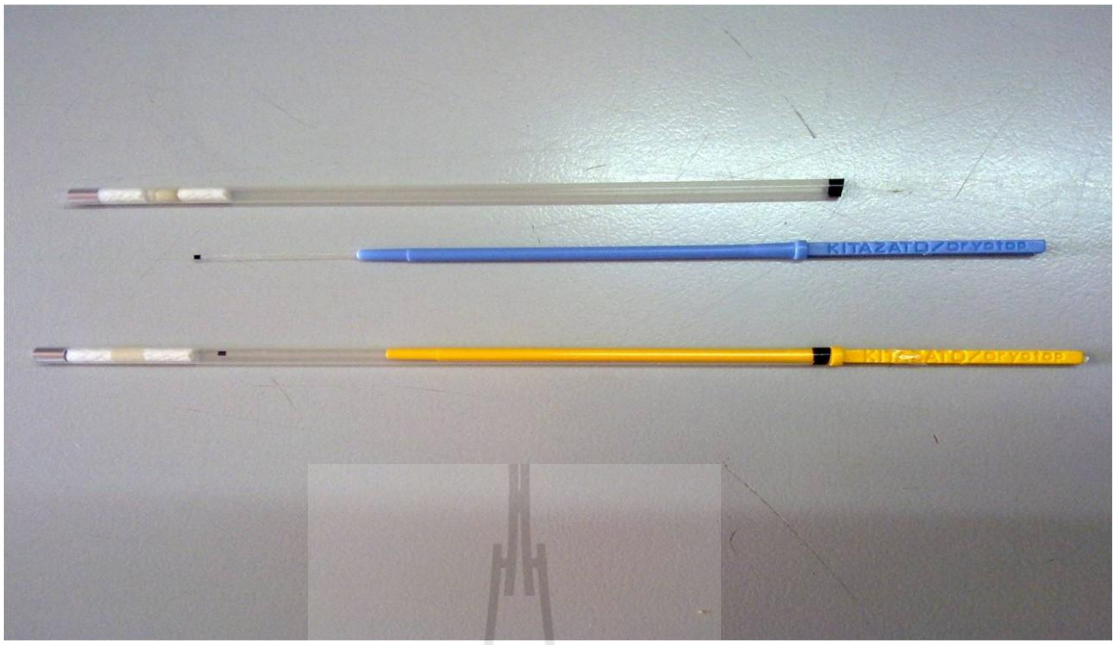

Figure 2.3 The Cryotop strips and plastic caps were designed by Kuwayama et al., (2005).

2.2.3 Permeating cryoprotectant

Permeating cryoprotectants are small molecules that have ability to permeate cell membranes and achieve adequate concentrations in the cytoplasm. Their hydrogen bonds with water molecules prevent ice crystallization by lowering the freezing point. At adequate concentrations they inhibit the formation of ice crystals and lead to the development of a solid or glasslike state. This is the so-called vitrified state in which water solidifies without crystals formation or expansion. However, the permeating cryoprotectants may also have a toxic effect at high enough concentrations. This requires that the cell be exposed to this solution either for a very short period of time, if the cryoprotectant is used at room temperature (vitrification techniques) or at very low temperatures, in which the metabolic rate of the cell is very low (slow-freezing protocols). The permeating cryoprotectants also protect the cell

from the solution effects, as free water solidifies immediately to ice, the remaining solution will contain progressively higher concentrations of both cryoprotectant and electrolytes.

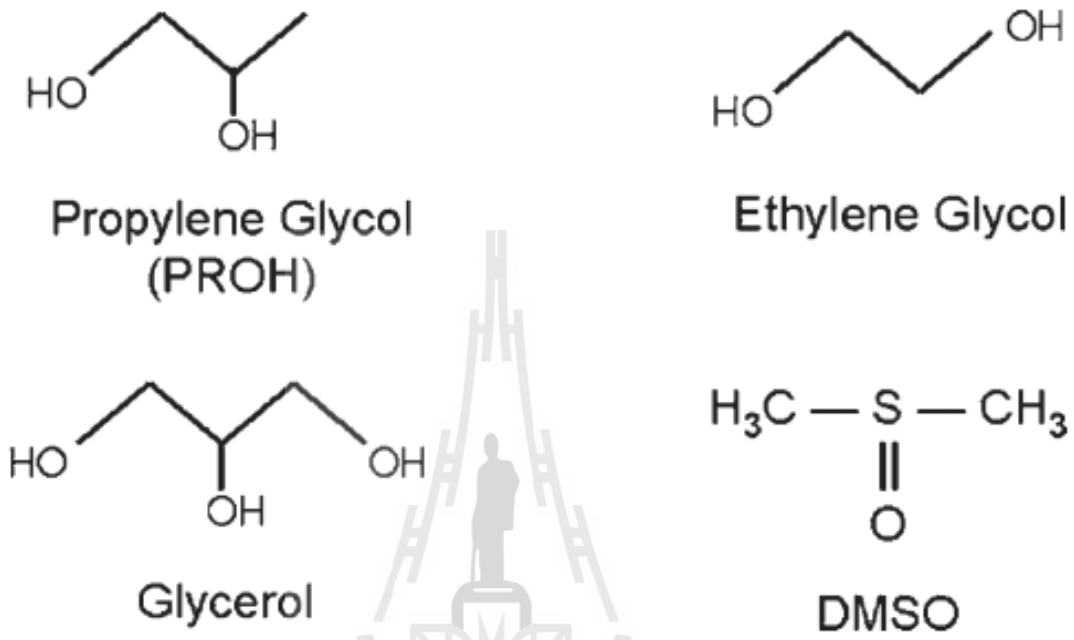

Figure 2.4 Chemical structure of commonly used permeating cryoprotectants (Jain and Paulson, 2006).

2.2.4 Non-permeating cryoprotectant

Non-permeating cryoprotectants remain in the extracellular space and act by drawing free water from within the cell, thus dehydrating the intracellular space such as Glucose, Sucrose, Trehalose, Ficoll. When they are used in combination with a permeating cryoprotectant, the net concentration of the permeating cryoprotectant is increased in the intracellular space. This facilitates the permeating cryoprotectant action in preventing ice-crystals formation. Furthermore, during thawing, the water generated by the melting ice rapidly decreases the extracellular osmotic pressure. Osmotic shock may occur if the intracellular cryoprotectant cannot

diffuse out quickly enough to prevent excessive influx of free water and the swelling, or even rupture of the cell. Therefore, freezing and thawing protocols commonly use a high concentration of non-permeating cryoprotectant during the thawing phase.

2.2.5 Effect of cryopreservation

Disruption of the meiotic spindle

The microtubular spindle apparatus is a dynamic structure that facilitates the segregation of chromosomes into daughter cells, during mitosis and into the first and second polar bodies during meiosis of the oocyte. It must function correctly to achieve accurate chromosome segregation and avoid aneuploidy (Stachecki et al., 2004). Although several investigators have raised concerns about possible embryonic aneuploidy after observing disruption of the spindle apparatus during oocyte cooling (Al-Hasani et al., 1987; Sathananthan et al., 1988; Pickering et al., 1990; Bernard et al., 1992; Almeida and Bolton, 1995), Gook et al., showed no increase in the number of abnormal or stray chromosomes in previously cryopreserved oocytes (Gook et al., 1994). Fluorescence in situ hybridization was used to show that the incidence of chromosomal abnormalities in human embryos that were obtained from cryopreserved oocytes was no different than that of control embryos (Cobo et al., 2001).

Zona pellucida hardening

There was evidence that cryopreservation induces zona pellucida hardening through premature cortical-granule release, which then acts as a barrier to fertilization (Schalkoff et al., 1995; Pickering et al., 1991; Matson et al., 1997; Ghetler et al., 2006). However, vitrifying oocytes in calcium-free media overcame the zona hardening, increased subsequent fertilization, and did not adversely affect embryonic

development to the blastocyst stage. Further studies on calcium physiology with other cryoprotectants ultimately may eliminate zona hardening and the requirement for ICSI.

2.2.6 Biosafety issues

Several open-carrier systems of cryoprservation had been used, including open pulled straws (Kuleshova et al., 1999), electron-microscope grids (Yoon et al., 2003), Cryoloops (Saki and Dezfuly, 2005) and the Cryotop (Kuwayama et al., 2005; Kuwayama, 2007). Typically in open systems there is a direct contact between the LN_2 and the solution containing the oocytes or embryos. Thus, there is a hypothetical risk of disease transmission if the cell were directly plunged into a contaminated LN_2 (Bielanski et al., 2000; Bielanski et al., 2003). While liquid nitrogen typically produces very low microbial counts, during storage and transfer it can become contaminated with microorganism such as fungal spores, yeasts, bacteria and viruses (Fountain et al., 1997; Morris et al., 2005). It has been shown that the hepatitis B virus can be transmitted to recipients of bone marrow tissue stored in contaminated liquid nitrogen (Tedder et al., 1995). To reduce or eliminate the risk of disease transmission through contaminated LN_2, one can filter the liquid nitrogen (McBurnie and Bardo, 2002) or use ultraviolet (UV) radiation for sterilization (Parmegiani et al., 2010). However, both these methods of LN_2 sterilization are quite expensive and require special machinery. Another approach is to use complete closed container system (sealed straws) for cryopreservation, which eliminate the direct contact of vitrified oocytes with the liquid nitrogen (Bielanski et al., 2000).

Vutyavanich et al. has reported a modified, home-made, closed-system solid surface vitrification (SSV) by using a hemi-straw (ø 0.25 mm.) loaded

with 2-cell embryos, which was inserted into a pre-cooled outer straw (ø 0.50 mm.). The outer straw was then sealed at both ends before it was directly immersed into the liquid nitrogen (Vutyavanich et al., 2009). This method showed high rates of post-thawing oocytes survival, further cleavage and blastocyst formation. It was concluded that the potential risk of cross-contamination during cryopreservation could be greatly reduced or eliminated by this method. In addition, this method was less costly, as all materials and instruments were readily made or modified.

2.2.7 Applications of cryopreservation technology

Fertility preservation for cancer patients

Since chemotherapy, especially alkylating agents, and ionizing radiotherapy can damage oocytes through induction of apoptotic pathways (Meirow and Nugent, 2001; Lobo, 2005), these treatments may lead to irreversible ovarian failure. Oocyte cryopreservation is offered before commencing such gonadotoxic therapies (for cancer and/or bone marrow suppression) in women of reproductive ages. However, the time required to stimulate the ovaries and perform oocyte retrieval may pose a problem for women and their oncologists, who are eager to commence cancer treatment.

Delayed childbearing

In present-day society, many women delay family plans and childbearing until they fulfill their professional career objectives. Obviously, the age-related decline in female fertility, also known as the “Biological clock”, may result in a large number of these women who will face difficulty conceiving at an older age. These women while still fertile may elect to cryopreserve their own oocytes to be used at an older age.

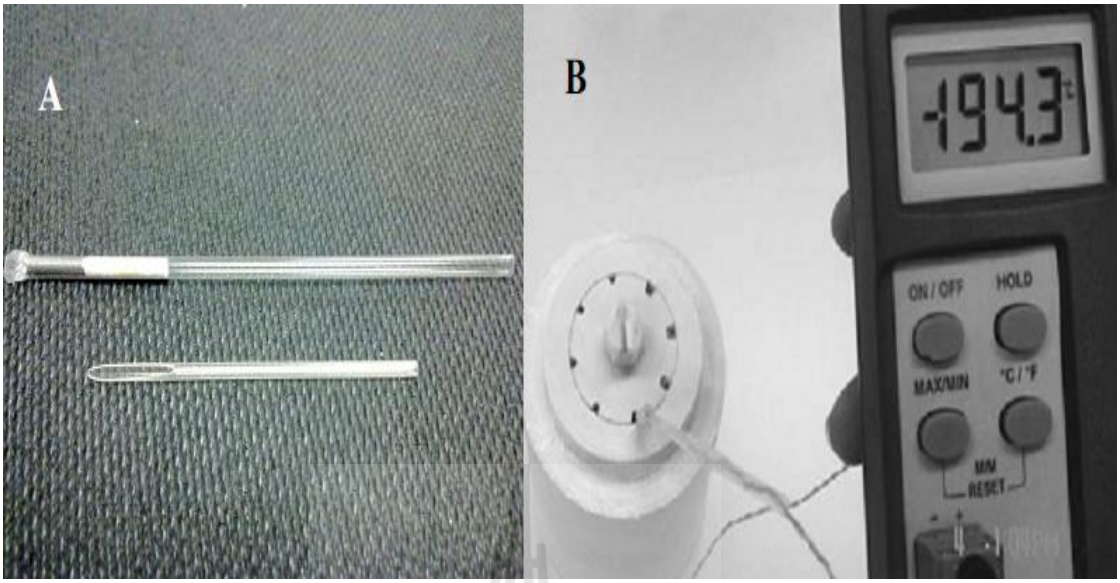

Figure 2.5 HS-CS method. (A) The hemi straw and outer straw (B) The cooling temperature at -194.3°C of equilibrated aluminum cylinders after put in LN_2 for 15-20 min (Vutyavanich et al., 2009).

Ethical opposition to embryo cryopreservation

Oocyte cryopreservation offers an alternative for couples who are opposed to cryopreserving embryos. This strategy also is being used in some countries in which embryo cryopreservation is illegal (Manna and Nardo, 2005). Furthermore, in one cycle of treatment, singleton pregnancy was required by fertilizing fewer oocytes and transferring fewer fresh embryos. Therefore, oocyte cryopreservation was offered to prevent the high rate of twin or multiple pregnancies.

Oocytes donation

Current limitations on traditional oocyte donation include donor availabili-ty, cost, need to synchronize donor and recipient schedules, travel requirements, and the inability to quarantine oocytes. The recipient can obtain oocytes without waiting for donor availability and can plan embryo transfer at a convenient

time. Other benefits of this application include the potential for shipping cryopreserved oocytes to allow recipients to stay under the care of their own physicians.

Table 2.1 Comparison of slow freezing method with various vitrification methods.

Details	Slow freezing	Vitrification (Cryotop)	Vitrification (HS-CS)
Permeable CPAs	PROH	EG and DMSO	EG and DMSO
Non-permeable CPAs	Sucrose	Sucrose	Sucrose
CPAs concentration	Low (~ 1.5 M)	High (~ 6.0-9.0 M)	High (~ 6.0-9.0 M)
Duration to expose CPAs	Long time	Very short time	Very short time
Duration of freezing process	Long time (~ 1.5-2.0 h)	Short time (~ 5-10 min)	Short time (~ 5-10 min)
Volume of solution was applied	More (~ 250 µL)	Less (< 1 µL)	Less (< 1 µL)
Dehydration	Occur while reduction of temperature	Occur before reduction of temperature	Occur before reduction of temperature
Seeding	Yes	No	No
Cooling rate	Slow	Very very fast	Very fast

Table 2.1 (Continued).

Details	Slow freezing	Vitrification (Cryotop)	Vitrification (HS-CS)
Effect of cryopreservations			
* **Chilling injury**	Yes	No	No
* **Solution effect**	Yes	No	No
* **Ice crystals formation**			
Outer cellular	Present	Absent	Absent
Intra cellular	Very less or absent	Absent	Absent
* **Fracture or cracking**	Very less or absent	Present	Present
* **Osmotic shock**	Present	Present	Present
Cotamination risks	Very less or absent	Present	Very less or absent
Machines, epuipments	Expensive	Inexpensive	Inexpensive
Cost-effectiveness	Good	Better	Better

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CHAPTER III

BLASTOCYST PRODUCTION FROM VITRIFIED OOCYTES FOLLOWING ICSI BY PIEZO MICROMANIPULATOR IN MICE: COMPARISON OF CRYOTOP AND HEMI STRAW CLOSED SYSTEM METHODS

3.1 Abstract

The objective of this study was to compare the production of mouse blastocysts from oocytes vitrified by Cryotop and HS-CS methods, followed by ICSI with piezo micromanipulator after thawing. Oocytes from 6-8 weeks old female ICR mice were collected for vitrification by the Cryotop method (group I)) and HS-CS method (group II), using the same freezing (equilibration medium, 7.5% EG + 7.5% DMSO and vitrification medium, 15% EG + 15% DMSO + 0.6 M sucrose), and thawing mediums (solutions of 1.0 M, 0.5 M, 0.25 M and 0 M sucrose). After the oocytes were frozen for a week and then thawed there was no significant difference between this two groups in the oocyte survival rate (155/160, 96.9% and 158/160, 98.6%, respectively; $P \geq 0.05$). There was no significant difference between this 2 groups in embryo development at stage of 2-cell, 4-cell, 8-cell, morula and expanded blastocyst. However, compared with fresh control, both vitrified groups had significantly lower rates of embryos at the 8-cell stage (62/110, 56.7%; 62/110, 56.7%

and 80/114, 70.2%,respectively), morula stage (43/110, 39.1%; 43/110, 39.1% and 73/114, 63.1%, respectively) and expanded blastocyst stage (30/110, 27.3%; 32/110, 29.1% and 63/114, 55.3%, respectively); ($P< 0.05$). All three groups showed no significant differences in the ICM/TE cell counts (0.41 ± 0.11, 0.42 ± 0.10 and 0.45 ± 0.11; respectively; $P\geq0.05$). These results indicated that both the Cryotop and the HS-CS methods have similar oocyte survival and embryo development rates, as well as similar ICM/TE cell counts, but slightly lower embryo development rates when compared with fresh control.

3.2 Introduction

Oocyte cryopreservation has signifcantly contributed to the assisted reproductive technology (ART) programs. It allows patients to cryopreserve their oocytes when they have no partner, want to delay delivery or are about to lose their ovarian function due to surgery, radiotherapy, or chemotherapy (Lee and Yoon, 2009). Also, it avoids the ethical issues and legal restrictions of egg and/or embryo donations. The earlier researches of oocytes cryopreservation were carried out by the slow freezing method using different cryoprotectant agents such as DMSO and 1,2 propandiol or PROH (Tsunoda et al., 1976 and Gook et al., 1993; 1995). More recently vitrification has emerged as an alternative to traditional slow freezing methods as it avoided the chilling injury and ice crystals formation (Rall et al., 1985 and Gupta et al., 2007). Traditionally, vitrification used a combination of cryoprotectant agents such as a mixture of PROH and DMSO (Shaw et al., 1992; Wood et al., 1993 and Liebermann et al., 2002). Several researchers have also suggested that EG is a suitable cryoprotective agent for the vitrification of mammalian

oocytes (Rayos et al., 1994; Hotamisligil et al., 1996 and Chen et al., 2000). Because of its low molecular weight, it is less toxic to mammalian oocytes and embryos, including those of humans (Martino et al., 1996; Zhu et al., 1996; Sommerfeld et al., 1999 and Emiliani et al., 2000).

In vitrification, rapid cooling rates are achieved by placing oocytes in small volumes of freezing media directly into LN_2 or SN_2 (Cha et al., 2011). Several open-carrier systems had been used, including OPS (Kuleshova et al., 1999), EMG (Yoon et al., 2003), cryoloops (Saki and Dezfuly, 2005) and cryotop (Kuwayama et al., 2005). However, with open systems vitrification there is a hypothetical risk of disease transmission if the cells are directly exposed to an accidentally contaminated LN_2 (Bielanski et al., 2000 and 2003). While sterilization of LN_2 by UV radiation (Parmegiani et al., 2010) was an option closed container systems, which had also been used to eliminate direct contact of vitrified oocytes with LN_2 (Bielanski et al., 2000).

Vutyavanich et al. has reported a modified, home-made, closed-system solid surface vitrification by using a hemi-straw (ø 0.25 mm.) loaded with 2-cell embryos, which was inserted into a pre-cooled outer straw (ø 0.50 mm.). The outer straw was then sealed at both ends before directly immersed into the LN_2 (Vutyavanich et al., 2009). This method showed high survival rates, cleavage and blastocyst formation rates of post-thawing oocytes. It is concluded that the potential risk of cross-contamination during cryopreservation could be greatly reduced or eliminated by this method. In addition, this method is less costly, as all materials and instruments are readily made or modified.

After freezing-thawing of oocytes, ICSI is used for assisted fertilization by injecting the sperm directly into the cytoplasm of the oocyte. At present, ICSI is

widely the treatment of human infertility. It is also widely used for training and/or study of mammalian development in a genetically tractable mouse model. For this purpose, piezo micromanipulation is used in the mouse (Kimura and Yanagimachi, 1995) as mouse oocyte plasma membranes can be exquisitely sensitive and survival rates following conventional ICSI rarely exceed 50%, compared to nearly 100% with piezo-ICSI (Yoshida and Perry, 2007). It appeared that piezo micromanipulation enhances the penetration of membranes and matrices, for which the mouse ICSI is a major application.

The objective of this study was to compare the efficience of Cryotop and HS-CS methods on mouse embryo production by using vitrified MII oocytes followed by ICSI with piezo micromanipulator. Oocytes survival, embryo development, and blastocyst formation rates, as well as ICM/TE counts of expanded blastocysts, were determined in Cryotop, HS-CS and fresh control groups.

3.3 Materials and methods

3.3.1 Experiment design

In vivo matured oocytes were randomly separated into three groups (Cryotop, HS-CS and fresh control). Effects of vitrification carriers Cryotop and HS-CS were assessed by comparing the survival rates and ICSI-derived embryo developmental competence of vitrified-warmed oocytes. Embryo development was observed and recorded at 26, 38, 50, 62, 80 and 96 hours post ICSI and at 96-100 h the ICM/TE ratios were determined in the sample of expanded blastocysts.

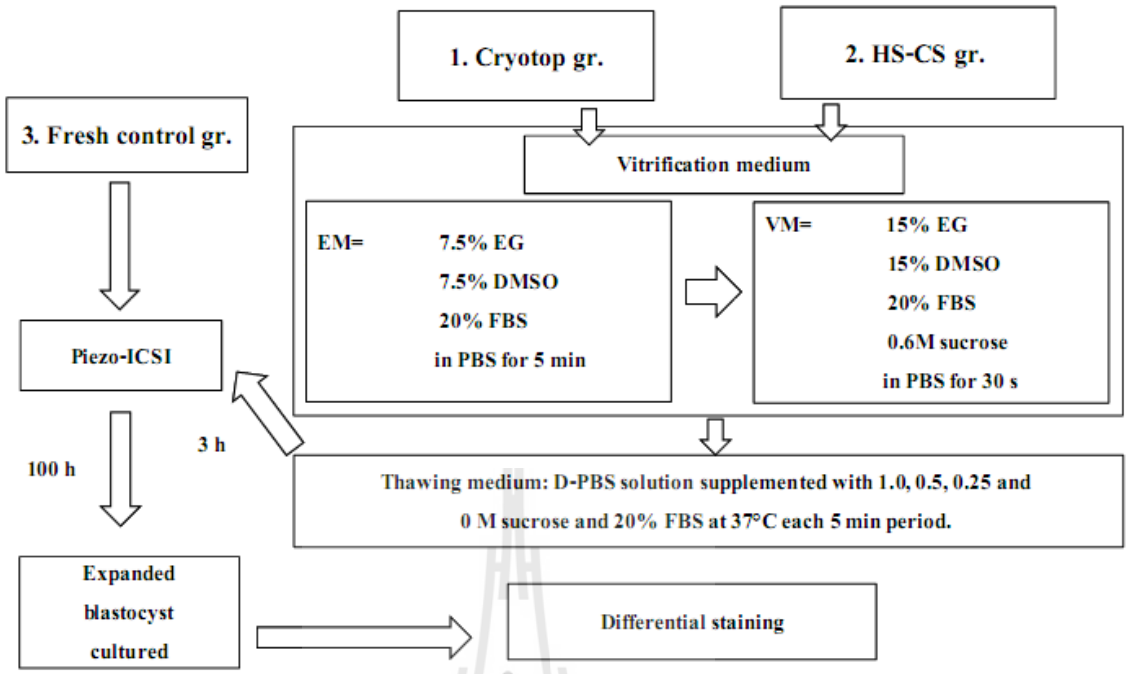

Figure 3.1 Experiment design.

3.3.2 Chemicals and media

All reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated. Chatot Ziomek Bavister-Hepes (CZB-H) was used as holding medium that contains 20 mM HEPES, 81.62 mM NaCl, 4.83 mM KCl, 1.18 mM $MgSO_4.7H_2O$, 1.70 mM $CaCl_2$, 1.18 mM KH_2PO_4, 0.11 mM EDTA.2Na, 31.30 mM Na.lactate, 25.12 mM $NaHCO_3$, 0.27 mM Na.pyruvate, 0.10 mg/mL Bovine Serum Albumin (BSA), 1.00 mM D-glutamine, 10.00 mg/ml phenol red (0.5% (w/v) in Dulbecco's phosphate-buffered saline (D-PBS), pH 7.4 (Chatot et al., 1989). Preparations were made with double-distilled or higher purity water. For embryo culture, CZB-H was sterile-filtered and store at 4ºC for up to a month.

KSOM medium was used as an alternative media for embryo culture. KSOM+ AA contains amino acids, 95.00 mM NaCl, 2.50 mM KCl, 0.20 mM $MgSO_4.7H_2O$, 1.71 mM $CaCl_2$, 0.35 mM KH_2PO_4, 0.01 mM EDTA.2Na, 10.00 mM

Na.lactate, 25.00 mM $NaHCO_3$, 0.20 mM Na.pyruvate, 0.20 mM glucose, 0.10 mg/mL BSA, 1.00 mM D-glutamine, and 10.00 mg/mL phenol red (0.5% (w/v) in D-PBS, pH 7.4 (Lawitts et al., 1993 and Erbach et al., 1994). Preparations were made with double-distilled or higher purity water. KSOM+ AA medium was sterile-filtered and stored at 4ºC for up to a month for embryo culture.

3.3.3 Preparation of MII oocytes and sperm

3.3.3.1 MII oocytes preparation

Outbred ICR (Institute Cancer Research) mice were purchased from the National Animal Institute, Mahidol University, Bangkok, Thailand. They were kept at the Animal Husbandry Building. The Center for Scientific and Technology Equipment, Suranaree University of Technology, in a temperature-and-humidity-controlled room under a 12 h light: 12 h dark cycles. For mature oocyte were collected from 6 to 8 weeks old ICR female mice that superovulated by an intraperitoneal injection (IP) of 7.5 IU pregnant mare serum gonadotropin (PMSG) (Folligon®, Intervet, UK), followed by an IP injection of 7.5 IU human chorionic gonadotropin (hCG) (Chorulon®, Intervet, UK) 48 h later. At 14 to 16 h post hCG injection, the female ICR mice were sacrificed by cervical dislocation for the collection of mature oocytes which were already located in the ampulla of the oviduct. Cumulus-Oocytes-Complexes (COC) were collected by using a 27 G needle for dissection of the ampulla. COC were denuded in CZB-H medium containing 0.2% hyaluronidase, and then the denuded oocytes were washed with fresh medium several times. The washed denuded oocytes were pooled in a drop of 75 µL KSOM medium covered with oil at 37ºC in an atmosphere of 6% CO_2 in air.

3.3.3.2 Sperm preparation

Eight to ten weeks old ICR male mice were sacrificed by cervical dislocation for the collection of sperm. Epididymal sperm was dissected out and suspended in 200 µl of CZB-H medium with 0.50 ml Eppendrof tube. Sperm was swum up to the top of the medium in the incubator at 37ºC, in an atmosphere of 6% CO_2 in air for one hour. Progressive motile sperm were selected for ICSI procedure.

3.3.4 Vitrification of oocytes

3.3.4.1 Vitrification

The MII oocytes were vitrified-warmed by using Cryotop or HS-CS methods.

Cryotop method (group I)

Five good quality oocytes were selected and transferred into the equilibration solution of 7.5% EG and 7.5% DMSO for 5 min and then exposed in the vitrification solution of 15% EG, 15% DMSO and 0.6 M sucrose for 30 s. Oocytes in a small volume of vitrification solution (<1µL) were loaded and placed on the plastic holder tip of the Cryotop strip and immediately immersed into LN_2. The hard plastic cover was caped to the Cryotop strip under LN_2. Finally the Cryotop strip with the vitrified oocytes was moved into the LN_2 tank for storage at least 1 week before thawing.

HS-CS method (group II)

Home-made aluminum cylinders were pre-cooled in LN_2 for approximately 15 to 20 min, as described previously (Vutyavanich et al., 2009). The outer straws with an inner diameter of ø 0.50 mm were sealed at the bottom end and inserted into the holes of the pre-cooled aluminum cylinder for 5 min. Five good quality oocytes were selected and transferred into the equilibration solution of 7.5%

EG and 7.5% DMSO for 5 min and then exposed in the vitrification solution of 15% EG, 15% DMSO and 0.6 M sucrose for 30 s. After that, oocytes were loaded in a small volume of vitrification solution (<1µL) into the tip of a hemi-straw with an inner diameter of ø 0.25 mm. The loaded hemi-straws were inserted into the pre-cooled outer straws and the top end of the outer straws was then sealed to form a completely closed system. The outer straws with the inner hemi-straws were then directly immersed into the LN_2 tank for storage at least 1 week before thawing.

Figure 3.2 HS-CS vitrification method: (A) Two of Aluminum cylinders, (B) Inner cylinder separated from outer cylinder, (C) Pre-cooled outer straw were inserted into equilibrated cylinders, (D) Outer straws were heat-sealed after inserted inside them by hemi-straw with oocytes.

3.3.4.2 Thawing of oocytes

After the vitrified oocytes were stored in the LN_2 tank for 1 week, they were thawed in four serial thawing of D-PBS solutions supplemented with 1.0 M, 0.5 M, 0.25 M and 0 M sucrose and 20% FBS, with an interval of 5 min at 37ºC. The same formula of thawing solution was used in both vitrified-warmed groups.

Cryotop thawing method

The Cryotop strip was taken out of the storage LN_2 tank and then the hard plastic cover was removed. The tip of cryotop strip was immediately immersed into four serial thawing solutions of D-PBS solutions supplemented with 1.0 M, 0.5 M, 0.25 M and 0 M sucrose and 20% FBS, with an interval of 5 min at 37ºC. The survived oocytes were washed several times in KSOM medium and incubated in this medium for 3 h.

HS-CS thawing method

The top end of the outer straw was cut while it was still in the LN_2, the hemi-straw with the vitrified oocytes was taken out and immediately immersed into four serial thawing solutions of D-PBS solutions supplemented with 1.0 M, 0.5 M, 0.25 M and 0 M sucrose and 20% FBS, with an interval of 5 min at 37ºC. The survived oocytes were washed several times in KSOM medium and incubated in this medium for 3 h before the ICSI.

3.3.5 Evaluation of oocytes vitrification

The oocytes viability post-warming was evaluated on the basic of the integrity for the oocyte membrane and the zona pellucid together by morphologically observation with the homogeneity of the cytoplasm.

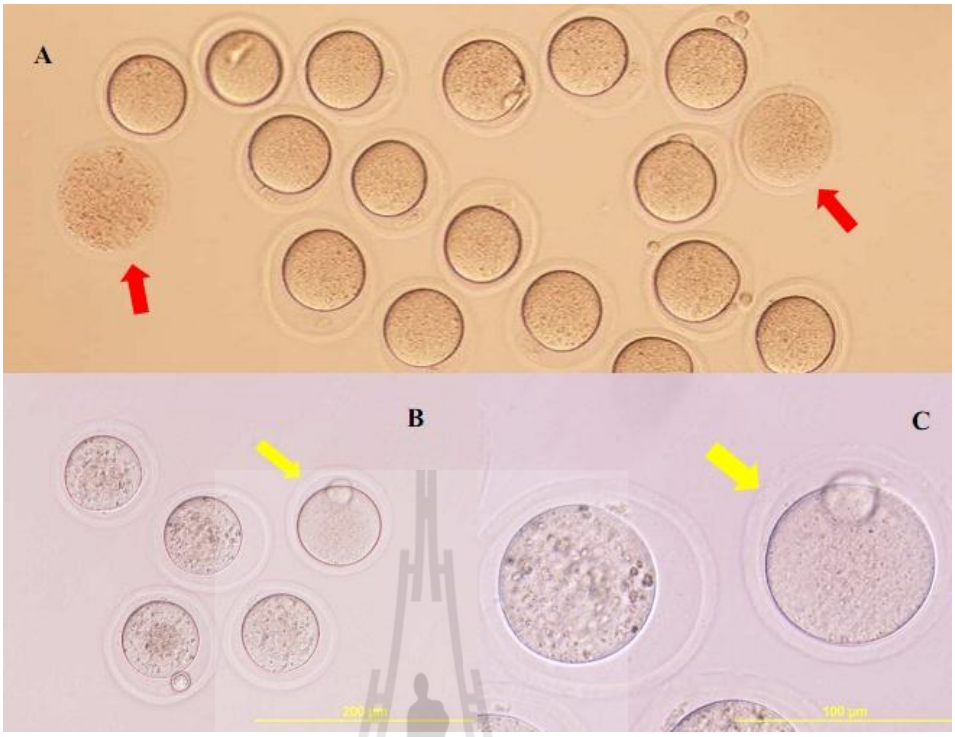

Figure 3.3 The evaluation of oocytes vitrification. (A) Most of vitrified-warmed oocytes still survive, only two oocytes died (red arrow showed the dead oocyte after warming (B) 100X and (C) 200X magnification, showed good quality of vitrified-warmed oocyte (yellow arrow).

3.3.6 ICSI procedure

Microinjection pipette preparation

Borosilicate glass capillary (B 100-75-10, Sutter instrument, Novato, CA, USA), was pulled on a P-97 Flaming/Brown Micropipette Puller (Sutter instrument), equipped with a 4.5 mm box filament with initial setting for ICSI-piezo: heat = 870; pull = 10; velocity = 120 and time = 200. Pulled micropipettes were generated the blunt-ended ICSI-piezo pipette by a MF-900 microforge machine (Narishige, Tokyo, Japan). Pipettes were considered suitable for piezo ICSI when the inner diameter was 7.0 to 8.0 µm. The pipettes were bent by heated glass-ball to ~ 20

degree from the horizontal. Before use, ICSI-piezo pipette was applied 2 to 3 mm mercury through the original end of pipette by using a disposable 1-ml syringe fitted with a 27 G needle.

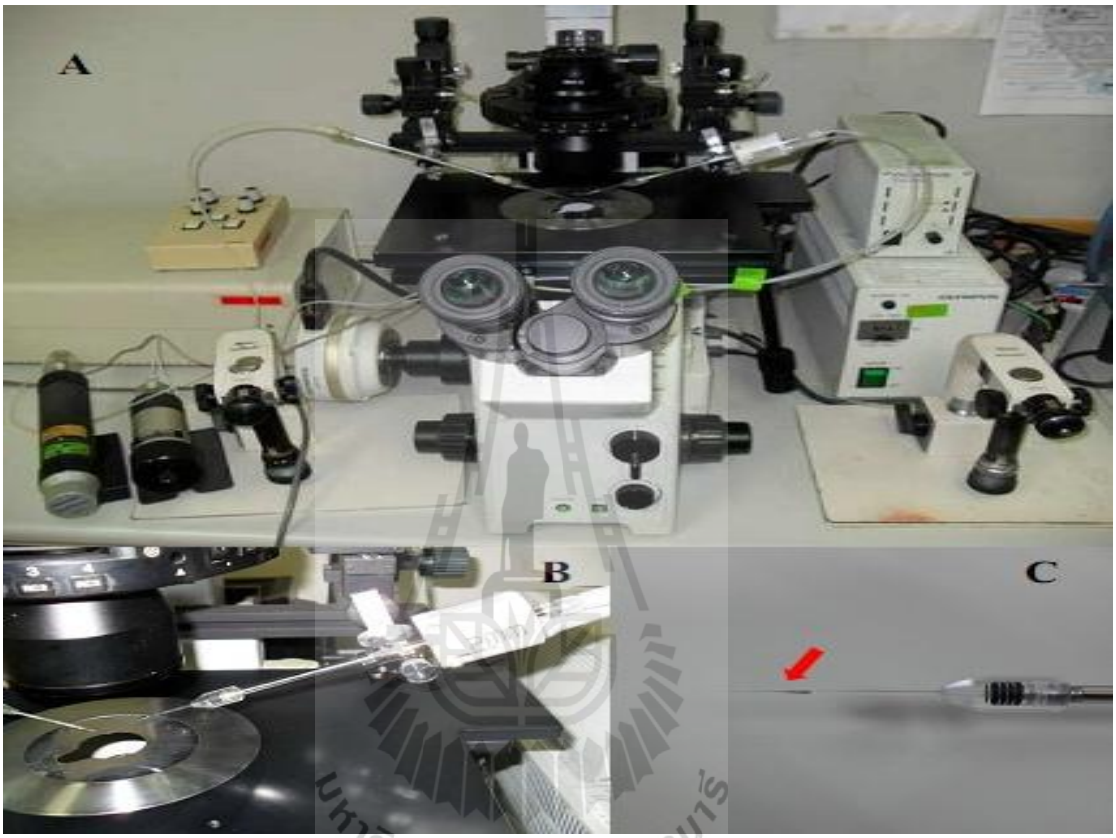

Figure 3.4 Piezo micromanipulator. (A) Piezo machine was connected with inverted microscope. (B) The peizo-injector was connected with the blunt-end tip pipette. (C) The piezo-ICSI pipette, with mercury loaded inside (red arrow).

Piezo- ICSI procedure

Motile sperm were suspended in 50 µL of 7% PVP medium, in a 90 mm culture dish (as ICSI dish) covered with oil. Piezo micromanipulator (PMAS-CT150; Prime Tech, Ibaraki, Japan), with right hand side inverted microscope (IX71;

Olympus, Tokyo, Japan) was used at piezo intensity = 3 and frequency = 3 to sever the sperm head. The blunt tip of the ICSI-piezo pipette with 7 to 8 µm inner diameter was used to draw intact sperm and to separate the sperm head from the tail at the head-midpiece boundary by applying several pulses with a foot switch. About 5 sperm heads were drawn into the ICSI-piezo pipette. Five to ten survived oocytes were transferred to an ICSI dish with CZB-H medium drop approximately 3 hours after thawing. A pulled glass pipette, holding pipette with inner diameter = 20 µm and outer diameter about 90-100 µm, connected to the micromanipulator (M0188NE; Narishige, Tokyo, Japan) was used to hold oocyte at the 9 O'clock position with gentle suction. The metaphase plate of the oocyte was rotated to the 6 or 12 O'clock position using the blunt tip of the ICSI-piezo pipette. Five sperm heads were drawn into the ICSI-piezo pipette and the blunt-end of the ICSI-piezo pipette was used to cut the zona pellucida at the same piezo unit level used to sever the sperm head (intensity = 3 and frequency = 3). The blunt-end of the ICSI-piezo pipette was pushed through the zona pellucida and perivitelline space against the plasma membrane almost half-way into the oocyte cytoplasm, while the plasma membrane is still intact. The plasma membrane was penetrated by switching on the piezo set up and applying one piezo pulse with intensity = 1 and frequency = 1. Only one sperm head was injected into the cytoplasm and then the ICSI-piezo pipette was gently pulled out from the oocyte. This procedure was repeated until all sperm heads in the pipette were injected. The injected oocytes remained in the ICSI dish for 10 min at room temperature and then transferred to KSOM culture medium in the incubator at 37ºC with an atmosphere of 6% CO_2 in air (for 96 to 100 h, until the expanded blastocyst stage).

3.3.7 Embryo development

After the ICSI, injected oocytes were cultured for 96-100 h in KSOM medium at 37ºC with an atmosphere of 6% CO_2 in air. Embryo development was recorded at 26 h (2-cell), 38 h (4-cell), 50 h (8-cell), 62 h (morula), 80 h (early blastocyst) and 100 h (expanded blastocyst).

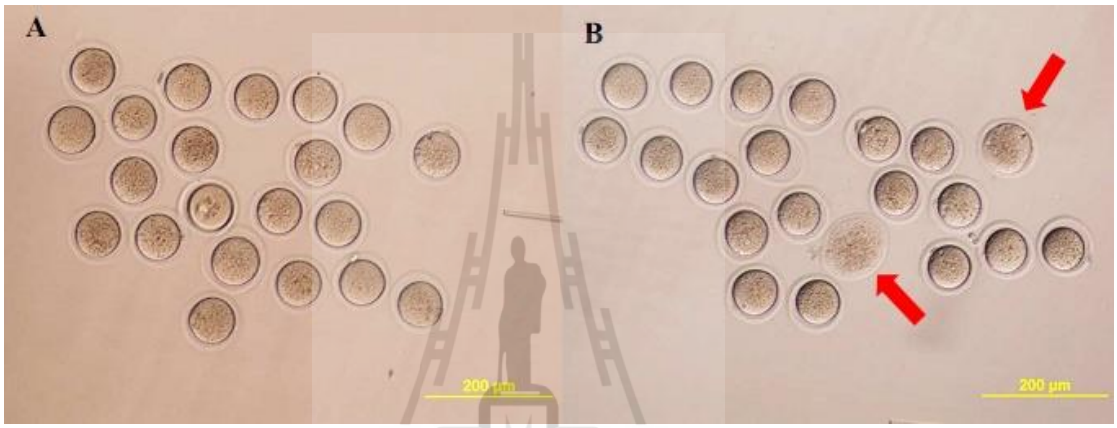

Figure 3.5 Morphology of oocytes before-ICSI (A) and Post-ICSI (B), red arrow showed the dead oocyte after ICSI.

3.3.8 Differential staining of embryos

Differential staining of the expanded blastocysts was performed as previously described (Thouas et al., 2001), with slight modifications. Briefly, expanded blastocysts were treated with 0.1 mg/mL propidium iodide (PI) and 0.2% Triton X-100 dissolved in D-PBS for 35 s. Then, the expanded blastocysts were treated with 25 µg/mL Hoechst 33342 in 99.5% ethanol for 4 min, and mounted on glass slides in a glycerol droplet. Finally, the expanded blastocysts were flattened by glass cover slips and examined under UV light with an excitation wavelength of 330-385 nm with an epifluorescence microscope (IX-71; Olympus, Tokyo, Japan). The nuclei of the TE cells were stained by both PI and Hoechst solutions, which appeared

red or pink. The nuclei of the ICM cells were stained only by the Hoechst solution showing blue. A digital image of each expanded blastocyst was taken, and the counts of both cell types were recorded. Therefore, the numbers of ICM and TE were counted separately in expand blastocysts that had clearly distinguishable populations of blue and red nuclei.

3.3.9 Statistical analysis

The survival rates of frozen/thawed oocytes, fertilization rates, embryo development rates and embryo cell counts were arcsine transformed and analysed by ANOVA, using a statistical analysis software (SAS, Inst. Inc., Carry, NC). Differences between groups were considered to be statistically significant at a probability value of 0.05 or less. Six replications of the experiments were performed.

3.4 Results

3.4.1 Effect of vitrification method on viability and fertilization of oocytes

The viability of vitrified oocytes were evaluated by morphologic observation. As shown in table 3.1, there was no significant difference in the oocytes survival rates between two vitrification groups (155/160, 96.9% and 158/160, 98.8%, for the Cryotop method and HS-CS methods, respectively). Furthermore, there was no significant difference among three groups in fertilization rate (fresh control; 114/160, 71.3%, Cryotop; 110/155, 70.9% and HS-CS; 110/158, 69.6, respectively; $P \geq 0.05$).

3.4.2 Effect of vitrification method on embryo development after piezo-ICSI of surviving oocytes

As shown in table 3.1, embryo development to the 2-cell stage of the Cryotop group, HS-CS group and the fresh control group were not significantly

different (102/110, 92.7%, 104/110, 94.6% and 110/114, 96.45%, respectively; $P \geq 0.05$).

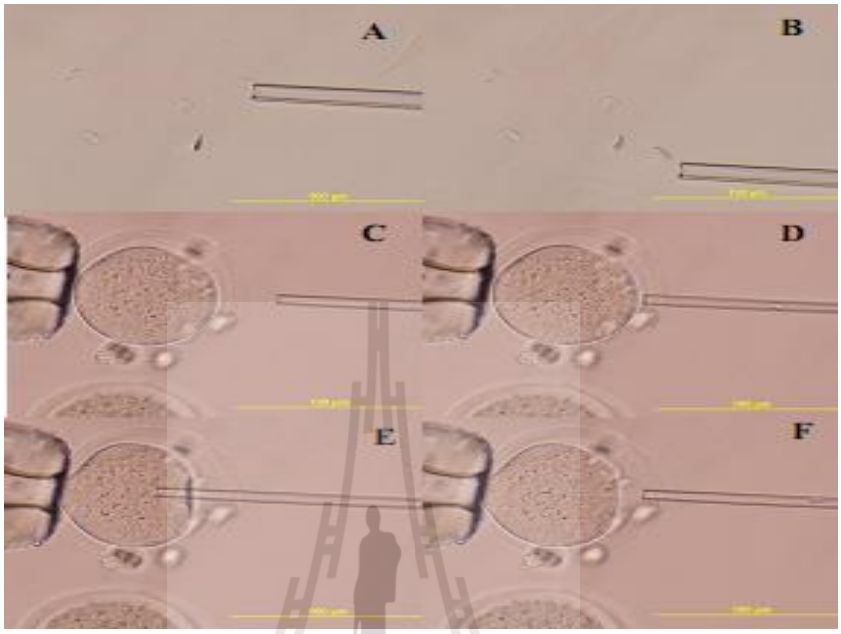

Figure 3.6 Piezo-ICSI process (A) and (B) Blunt end pipette cutting sperm head. (C), (D), (E) and (F) Injection pipette of sperm head penetrated zona pellucida and cytoplasm.

Development to the 4-cell stage was not significantly different between the 2 vitrification groups (90/110, 81.8% and 88/110, 80.0%; $P \geq 0.05$), but there was a significant difference between the HS-CS group and fresh control group (88/110, 80.0% and 100/114, 87.7%, respectively; $P<0.05$). There was no significant difference between the Cryotop group and the fresh control group (90/110, 81.8% and 100/114, 87.7%, respectively; $P \geq 0.05$). Embryo development to the 8-cell stage, morula and expanded blastocyst was similar in both vitrification groups. But when compared with fresh control group, both Cryotop and HS-CS groups showed significantly lower rates of embryo development to the 8-cell stage (80/114, 70.2%, vs. 62/110, 56.4% and

62/110, 56.4%; respectively), to the morula stage (73/114, 63.1% vs. 43/110, 39.1% and 43/110, 39.1% ; respectively) and to the expanded blastocyst stage (63/114, 55.3% vs. 30/110, 27.3% and 32/110, 29.1%; respectively), ($P< 0.05$).

3.4.3 Effect of vitrification method on cell counts of expanded blastocysts after piezo-ICSI of surviving oocytes

As shown in table 3.2, the mean number of TE (45.50±11.60, 45.40±16.79 and 57.80±14.88), ICM (18.00±3.80, 18.00±3.86 and 25.20±5.33) and the ICM/TE ratios (0.41±0.11, 0.42±0.10 and 0.45±0.11) of expanded blastocysts from the Cryotop, HS-CS and fresh control oocytes, respectively were not significantly different ($P\geq0.05$). Six replications were performed in this experiment.

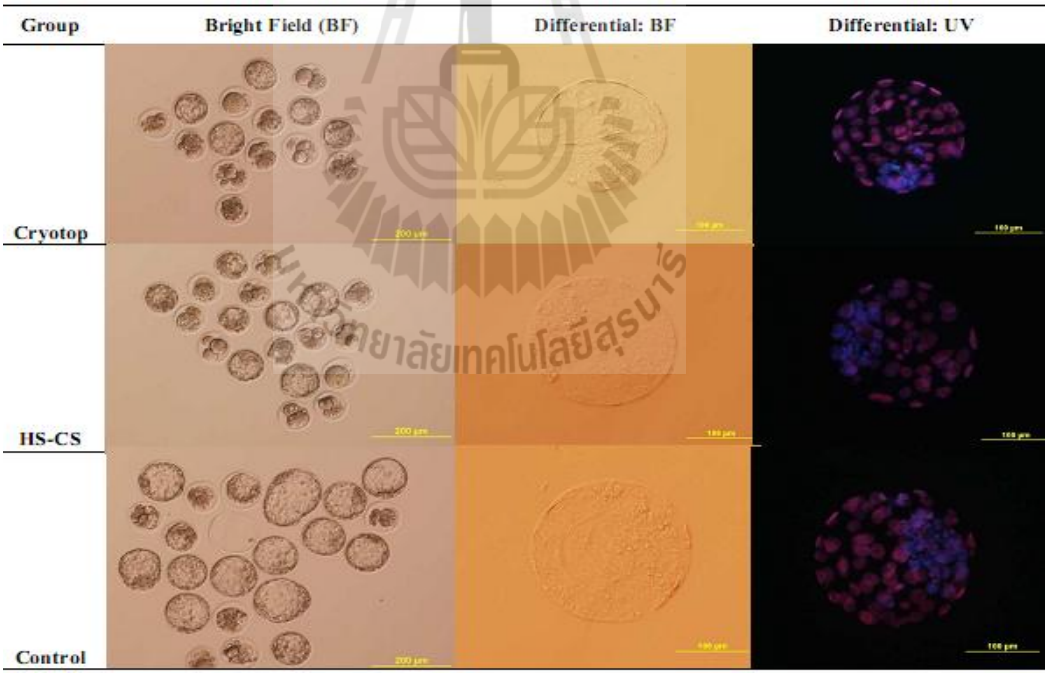

Figure 3.7 Expanded blastocysts of the experimental (vitrification) and fresh controlgroups at bright field (BF), differentially stained at BF, and differential staining at UV light; (Red color represents TE cells and blue represents ICM cells).

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Table 3.1 Post-thawing survival rates of oocyes vitrified by the HS-CS and the Cryotop methods and embryo development after piezo-ICSI of thawed and fresh-control oocyes.

Group	Oocytes	Survived/thawed	Cultured/ICSI	2-C	4-C	8-C	Mo	BL
Cryotop	160	155/160	110/155	102/110	90/110	62/110	43/110	30/110
		96.86%	70.97%	92.73%	81.82%[ab]	56.36%[b]	39.08%[b]	27.27%[b]
HS-CS	160	158/160	110/158	104/110	88/110	62/110	43/110	32/110
		98.75%	69.62%	94.55%	80.00%[b]	56.36%[b]	39.08%[b]	29.09%[b]
Fresh control	160	_	114/160	110/114	100/114	80/114	73/114	63/114
			71.25%	96.49%	87.72%[a]	70.18%[a]	64.05%[a]	55.26%[a]

Six replications were performed.

Values in the same column with different superscripts (a , b) are significantly different, $P<0.05$, (ANOVA)

3.5 Discussions

This study examined the production of mouse blastocysts from closed-system vitrified oocytes after they were thawed and subjected to ICSI by piezo micromanipulator. We used a novel method, the HS-CS method, and compared it with the Cryotop open vitrification method, which is considered a "gold standard", subjected to ICSI. The HS-CS method produced slightly higher rates of expanded blastocysts than the Cryotop method (29.09% *vs* 27.27%, respectively). Both vitrification methods in this study used an identical freezing medium (equilibration medium, 7.5% EG + 7.5% DMSO and vitrification medium, 15% EG + 15% DMSO + 0.6 M sucrose), and thawing mediums (solutions of 1.0 M, 0.5 M, 0.25 M and 0 M sucrose) and similar time durations of the freezing-thawing process. The concentrations of the freezing and thawing media were initially described by Kuwayama et al., for the Cryotop strip, which consists of a transparent polypropylene film attached to a plastic handle and equipped with a cover straw (Kuwayama et al., 2005). However, other concentrations of the vitrification medium may also be suitable and can be slightly modified, depending on the relation of concentration and duration time (cooling rate of method), or the species of the oocytes (Fahy et al., 2004, Vutyavanich et al., 2009 and Cha et al., 2011). The major limitations of cryopreservation are cryoprotectant toxicity, ice crystals formation, chilling injury, solution effect, and osmotic shock; these should be eliminated or minimized for the method to be successful. Generally, EG was used as a highly permeable cryoprotectant with moderate toxicity (Ishimori et al., 1992), and DMSO was reported to increase the permeability of ethylene glycol, resulting in decreased concentrations

Table 3.2 Cell counts of expanded blastocysts in the vitrification groups (HS-CS and Cryotop method) and the fresh control group.

Group	Blastocysts (N)	TE Mean±S.D.	ICM Mean±S.D.	TC Mean±S.D.	ICM/TE ratios Mean±S.D.
Cryotop	10	45.5±11.6	18.0±3.8	63.5±12.3	0.41±0.11
HS-CS	10	45.4±16.7	18.0±3.8	63.4±19.9	0.42±0.10
Fresh control	10	57.8±14.8	25.2±5.3	83.0±18.0	0.45±0.11

Six replications were performed.

There was no statistically significant difference between the groups ($P \geq 0.05$, (ANOVA).

and toxicity of EG (Vicente and Garcia-Ximenez, 1994). Thus, equal proportions of DMSO and EG appear to be the most efficient combination and using a two-step equilibration and supplementation with sucrose in the final concentration. Using a minimum drop size (MDS) of approximately 0.1–0.5 µL to hold the oocytes or embryos (Arav, 1992; Arav et al., 1993), not only offered the obvious benefit of increasing both the cooling and warming rates, but also decreased the chance of ice crystals formation in the small sample. MDS may of benefit to avoid zona pellucida and embryo fracture damage, which occurs often when oocytes or embryos are cryopreserved in conventional standard insemination straws and warmed rapidly afterwards. Fracture damage rarely occurs in open-system vitrification by using small sample volumes and it can be entirely eliminated with appropriate adjustment of the warming parameters (Kuwayama et al., 2007).

The Cryotop method has gained wide acceptance as a novel, standard vitrification method, which uses a small sample volume of the vitrification solution. Once oocytes are loaded onto the Cryotop, almost the entire loading solution is evaporated before direct immersion into the LN_2; thus, the final volume is only 0.1 µL. Therefore, this device offers an extremely fast cooling rate (~23,000ºC/min) and chilling injury is avoided or minimized. This extremely low volume is also useful to achieve high warming rates (~42,000ºC/min), thereby avoiding ice crystals formation during warming. However, very high cooling rates are not always necessary for successful vitrification. Nowshari and Brem showed that increasing the freezing rate of mouse embryos from 1,200°C/min to 10,300°C/min was of no advantage to their vitrification system (Nowshari and Brem, 2001). Kuleshova and Shaw reported that

Table 3.3 Summary of mouse oocytes vitrification methods published in the literature and their success.

Editor-year	Vitrification method	Vitrification solutions	Survival rate (%)	Fertilization method	Cleavaged rate (%)
Chen et al., 2001.	CPS	1.5 M EG 5 min	79	Std.IVF	90 (2-c), 63 (BL)
	Conv. Straw	5.5 M EG 1 min	77		88 (2-c), 59 (BL)
	OPS		63		88 (2-c), 59 (BL)
	EM Grid		39		80 (2-c), 58 (BL)
Endoh et al., 2007.	Cryotop	8% EG + 8% DMSO, 5 min 16% EG+16%DMSO+ 10 mg/ml Ficoll, + 0.65 M sucrose, 1 min	93-100	Piezo-ICSI	> 85 (2-c) ET to surrogated
Turk et al., 2007.	Cryotop	7.5%EG+7.5%DMSO, 5 min 15%EG+15%DMSO, 30 s	94.3	Partial zona dissection ICSI	90.3 (2-c)

Table 3.3 (Continued).

Editor-year	Vitrification method	Vitrification solutions	Survival rate (%)	Fertilization method	Cleavaged rate (%)
Vutyavanich et al., 2009	HS-CS	7.5%EG+7.5%DMSO, 5 min 15%EG+15%DMSO+ 0.6 M suc.+20% FCS, 30 s	95.7	std.IVF	87.1 (2-c), 49.4 (BL)
Cha et al., 2011	EM Grid	7.5%EG+7.5%DMSO, 2.5 min 15%EG+15%DMSO+ 0.5 M suc.+20% FCS, 20 s	60.0 (LN_2) 79.0 (SN_2)	ND	ND
Our study, 2012	HS-CS vs Cryotop*	7.5%EG+7.5%DMSO, 5 min 15%EG+15%DMSO+ 0.6 M suc.+20% FCS, 30 s	98.8 96.7	piezo-ICSI	94.6 (2-c), 29.1 (BL) 92.7 (2-c)*, 27.3 (BL)*

* Means the percentage of cleavage rate derived from cryotop vitrification method.

vitrification in a double straw, with a cooling rate of 400°C/min, is effective for the cryopreservation of mouse embryos (Kuleshova and Shaw, 2000). It was also reported that a moderately low cooling rate of 120°C/min was effective for the vitrification of human embryos at all stages of development (Mukaida et al., 1998). Our study also appeared to suggest that the cooling rate of the HS-CS method (-36°C/min to -964°C/min) (Vutyavanich et al., 2009) is suitable for successful vitrification of mouse oocytes, not significantly different from the Cryotop method. Another advantage of the Cryotop method is the use of concentration reduced permeable CPA (30%), minimizing potentially toxic effects (Kuwayama et al., 2005 and 2007).

It should be noted, that Cryotop vitrification is an open method, in which direct contact exists between the LN_2 and the solution containing the oocytes or embryos. This raises a hypothetical risk of disease transmission during the vitrification procedure, if the cells are directly immersed into a contaminated LN_2 (Bielanski et al., 2000 and 2003). While LN_2 typically produces very low microbial counts, during storage and transfer it can become contaminated with microorganisms such as fungal spores, yeasts, bacteria and viruses (Fountain et al., 1997 and Morris et al., 2005). It has been shown that the hepatitis B virus can be transmitted to recipients of bone marrow tissue stored in contaminated LN_2 (Tedder et al., 1995). This highlighted concerns about the potential contamination hazard from infectious pathogens found in LN_2. On the other hand, the HS-CS cryopreservation method is a closed system (sealed straws), which eliminates the risk of disease transmission through contaminated LN_2 or metal surfaces during freezing and storage.

ICSI was used for fertilization to overcome the zona pellucida hardening in oocytes after cryopreservation (Schalkoff et al., 1989, Pickering et al., 1991, Matson et al., 1997 and Ghetler et al., 2006). After thawing, a 3 h re-warming at 37ºC before

ICSI was attempted to allow restructuring of the meiotic spindles (Ambrosini et al., 2006).

In this study piezo-ICSI was used instead of conventional ICSI, because the plasma membrane of the mouse oocyte is sensitive and ICSI with conventional pipettes generally results in oocyte injury and death (Kimura and Yanagimachi, 1995; Yoshida and Perry, 2007). In humans, although conventional ICSI was generally used successfully, piezo-ICSI was also used. Yanagida et al reported that piezo-ICSI was possible to conduct Ca^{2+} fertilization while minimizing damage to oocytes. Improvements in the viability rate and fertilization rate were considered due to the elimination of failures in the injection of sperm (Yanagida et al., 1988). However, success rate with piezo-ICSI was increased by filling in injection pipette with a small amount of mercury. Yanagida et al confirmed that the mercury in the injection needle does not influence embryo development; in their experiment, only (0.6 µg/L) of mercury was detected in the injection drop. The mercury concentration of blood in healthy humans, using piezo-micromanipulator, was reported to be 0.3–1.05 µg/L (Abraham et al., 1984 and Snapp et al., 1989). The mean concentration of mercury in the cervical mucus of women was 220 µg/L and the concentration in the seminal plasma of men was 52.4 µg/L. Therefore, the value of mercury concentration in the injection drop is thought to be comparatively low and safe (Yanagida et al., 1988). In contrast, it is emphasized that mercury should be avoided in human ICSI because elemental mercury represents a cumulative neurotoxin. Therefore, fluorinert FC-77 was used, instead of mercury, though it is not toxic but the efficiency of piezo-ICSI using mercury is less high (Yoshida and Perry, 2007).

3.6 Conclusions

This is the first report of mouse blastocysts production from HS-CS vitrified oocytes, subjected to ICSI with piezo micromanipulator. The oocyte viability, fertilization and development to the expanded blastocyst stage were not significantly different between HS-CS and Cryotop methods.

The ICSI-derived blastocysts,were similar in ICM and TE ratios among HS-CS, Cryotop and fresh control group. Furthermore, the HS-CS method eliminated the risk of contamination and disease transmission with microorganisms, such as fungal spores, yeasts, bacteria and viruses.

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CHAPTER IV

VITRIFICATION OF MOUSE EMBRYOS AT VARIOUS STAGES PRODUCED BY PIEZO-ICSI: COMPARISON OF CRYOTOP AND HEMI STRAW CLOSED SYSTEM METHODS

4.1 Abstract

The objective of this study was to examine two vitrification methods (Cryotop vs HS-CS) applied on various stages of mice embryos. Piezo-ICSI of fresh mouse oocytes were used to generate 2-cell, 4-cell, 8-cell, morula and early blastocyst embryos, which were then vitrified by 7.5% EG and 7.5% DMSO for 5 min before placed in 15% EG, 15% DMSO and 0.6 M sucrose for 35 s. After thawing in sucrose solutions of decreasing osmolarities), survival rates were determined and the embryos returned to culture conditions until the expanded blastocyst stage. Non-vitrified piezo-ICSI embryos at the various stages of development served as controls. Our results showed no statistically significant difference in embryo survival between the Cryotop and HS-CS methods, at all embryonic stages of vitrification. (2-cell: 95.0%, 93.8%; 4-cell: 96.3%, 97.5%; 8-cell: 97.5%, 100.0%; morula: 100.0%, 100.0% and early blastocyst: 98.8%, 98.8%, respectively; $P \geq 0.05$). Also embryo development rates after thawing were similar in both vitrification groups. However, both vitrified groups showed lower rates of expanded blastocyst formation when compared with fresh

control (2-cell vitrification: 60.0% vs. 42.1% and 44.0%; 4-cell vitrification: 63.8% vs. 51.9% and 53.9%; 8-cell vitrification: 78.8% vs 75.6% and 72.5%; morula vitrification: 92.5% vs. 80.0% and 82.5%; and early blastocyst vitrification: 96.3% vs. 81.0% and 84.8%; respectively, $P< 0.05$). The trophectoderm (TE), inner cell mass (ICM) and total cell counts and the ICM/TE ratios of expanded balstocysts were not differ significantly among 2 vitrification methods and fresh control. It is concluded that the HS-CS method is as effective as the Cryotop for embryo vitrification, and it is also simple to operate, inexpensive to build and offers protection against contamination and disease transmission.

4.2 Introduction

ICSI is being widely used in clinical applications for male-factor infertility. For training and/or study of mammalian embryo development, a mouse model is widely used. However, MII oocytes are quite sensitive and ICSI with conventional pipettes can result in high oocyte damage and death. This problem can be overcome by piezo micromanipulation or the piezo-driven ICSI procedure, in which the injection pipette is driven by a series of piezo-pulses with adjustable frequencies, amplitudes and durations. The use of the piezo-ICSI method results in approximately 80% survival rate of sperm injected mouse oocytes, 70% of which develop to the blastocyst stage. The piezo-drill oocytes applied in ICSI has also been commonly used in other species (i.e., human, bovine and others) (Yanagida et al., 1988; Katayose et al., 1999; Galli et al., 2003; Wei and Fukui, 2002; Oikawa et al., 2005). A small amount of mercury inside the Piezo-ICSI injection pipette can significantly improved its success rate (Huang et al., 1996; Kimura et al., 1998; Katayose et al., 1999). It is suggested

that piezo micromanipulation enhances the penetration of membranes and matrices, and mouse ICSI is a major application (Kimura and Yanagimachi, 1995; Yoshida and Perry, 2007)

Cryopreservation technology applied on mammalian gametes and zygotes has been greatly improved over the past 30 years (Bernard and Fuller, 1996; Walters and Graves, 1997), and is currently used for supporting a wide range of assisted reproductive technologies in reproductive medicine. Successful cryopreservation opens several important opportunities, such as banking of gametes and embryos, which avoid the ethical and moral issues of donations.

Over the last several years cryopreservation by vitrification offered significant advantages over the conventional programmable slow-freezing method in terms of high success rate, simplicity and time requirement (Vutyavanich et al., 2009; Zhang et al., 2009). Vitrification is usually combined with different cryoprotective agents, such as a mixture of PROH and DMSO (Shaw et al., 1992; Wood et al., 1993; Liebermann et al., 2002). Several researchers have also reported that EG may be a suitable cryoprotective agent for vitrification (Rayos et al., 1994; Hotamisligil et al., 1996; Chen et al., 2000) because of its low molecular weight. It is also less toxic to mammalian oocytes or embryos including those of humans (Martino et al., 1996; Zhu et al., 1996; Sommerfeld et al., 1999; Emiliani et al., 2000). The vitrification technique involves very rapid cooling and warming rates and the use of high concentrations of viscous cryoprotectants in a small volume of solution (<1 µl) (Kuwayama et al., 2005). Vitrification brings about the transition of liquid into a glass-like physical state without ice crystallization. The vitrification method is rapid,

quite cheap, and has been used to cryopreserve embryos at various stages of development in several mammalian species (Vajta and Nagy, 2006).

Vitrification has been widely used for the cryopreservation of human oocytes and embryos (Kuwayama et al., 2005; Kuwayama et al.,2007) in the pronuclear stage (Kumasako et al., 2005; Al-Hasani et al., 2007), cleavage stage (Desai et al., 2007; Balaban et al., 2008; Rama Raju et al., 2005; Rama Raju et al., 2009) and blastocyst stage (Hiraoka et al., 2004; Zech et al., 2005; Youssry et al., 2008) of development. A variety of open-container systems have been used for vitrification, including OPS (Kuleshova et al., 1999), Flexipet (Liebermann et al., 2002), hemi-straws (Vanderzwalmen et al., 2003), EMG (Yoon et al., 2003), cryoloops (Saki and Dezfuly, 2005) and cryotop (Kuwayama et al., 2005). However, there is a hot issue concerning the risk of disease transmission through LN_2 during the vitrification procedure if the cells are directly plunged into accidentally contaminated LN_2 (Bielanski et al., 2000 and 2003). To assure absolute sterility of the vitrification procedure, the UV radiation has been used to sterilize LN_2 (Parmegiani et al., 2010). On the other hand, container with closed systems has also been proposed to eliminate or minimize direct contact between the LN_2 and the vitrified oocytes (Bielanski et al., 2000). The Cryotip (Angle, 2007; Valbuena et al., 2012) and the closed-system solid surface vitrification (SSV) (Vutyavanich et al., 2009) was proposed to solve the LN_2 contamination problem. The latter (SSV) is a modified, home-made closed-system solid surface vitrification method, by which hemi-straw (ø 0.25 mm.) is inserted into a pre-cooled outer straw (ø 0.50 mm.), sealed at both ends and plunged directly into liquid nitrogen. This method eliminates or greatly reduces the potential risk of cross-contamination, which is simple

and is less costly, because its materials and instruments are readily available and easily modified (Vutyavanich et al., 2009).

The aim of this study was to examine the efficiency of the HS-CS and Cryotop methods in the cryopreservation of mouse embryos at various developmental stages (2-cell, 4-cell, 8-cell, morula and early blastocyst). We also aimed to gain further insight into the issue of the most appropriate stage for human embryo vitrification.

4.3 Materials and methods

4.3.1 Experimental design

The experiment was divided into three groups (Cryotop, HS-CS and fresh control). The piezo-ICSI method was used for fertilized in all three groups. In the vitrification groups, embryos were vitrified at the stage of 2-cell, 4-cell, 8-cell, morula and early blastocyst. Survival rate of the vitrified-warmed embryos from each stage were determined by morphologically observation. Surviving embryos were continute cultured until expanded blastocyst stage. The embryo development or further cleavage rate were observed in this step. The sampling of expand blastocyst from all three group in each stage of frozen-thawed were investigated in the ICM/TE rate and compared the statistical value among all groups.

4.3.2 Chemicals and media

All reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated. CZB-H was used as holding medium, and KSOM medium was used as an alternative media for embryo culture.

4.3.3 Preparation of mature (MII) oocytes and sperm

4.3.3.1 MII oocytes preparation

Outbred ICR mice were purchased from the National Animal Institute, Mahidol University, Bangkok, Thailand. They were kept at the Animal Husbandry Building. The Center for Scientific and Technology Equipment, Suranaree University of Technology, in a temperature-and-humidity-controlled room under a 12 h light: 12 h dark cycles. For mature oocyte collection, six- to eight-weeks old ICR female mice were superovulated by an IP of 7.5 IU PMSG, followed 48 h later by an

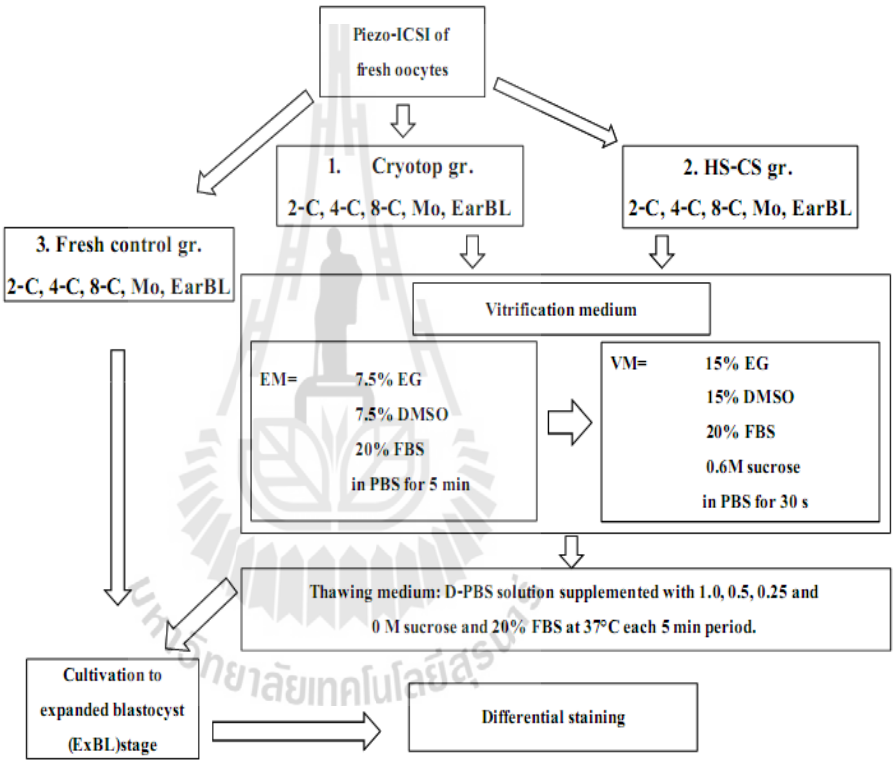

Figure 4.1 Experimental II design**.**

IP injection of 7.5 IU hCG. At 14 to 16 h post hCG injection, the female ICR mice were sacrificed by cervical dislocation for the collection of mature oocytes which were already located in the ampulla of the oviduct. COC were collected by using a 27 G needle for dissection of the ampulla. COC were denuded in CZB-H medium containing 0.2% hyaluronidase, and then the denuded oocytes were washed

with CZB-H medium several times. After that oocytes were kept in KSOM medium covered with oil at 37ºC in an atmosphere of 6% CO_2 in air.

4.3.3.2 Sperm preparation

Eight to ten weeks old ICR male mice were sacrificed by cervical dislocation for the collection of sperm. Epididymal sperm was dissected out and suspended in 200 µl of CZB-H medium in 0.50 ml Eppendrof tube. Sperm was swum up to the top of the medium in the incubator at 37ºC, in an atmosphere of 6% CO_2 in air for one hour. Progressive motile sperm were selected for ICSI procedure.

4.3.4 ICSI procedure

4.3.4.1 Microinjection pipette preparation

Borosilicate glass capillary, (B 100-75-10, Sutter instrument, Novato, CA, USA), was pulled on a P-97 Flaming/Brown Micropipette Puller (Sutter instrument), equipped with a 4.5 mm box filament with initial setting for ICSI-piezo: heat = 870; pull = 10; velocity = 120 and time = 200. Pulled micropipettes were generated the blunt-ended ICSI-piezo pipette by a MF-900 microforge machine (Narishige, Tokyo, Japan). Pipettes were considered suitable for piezo ICSI when the inner diameter was 7.0 to 8.0 µm. The pipettes were bent by heated glass-ball to ~ 20 degree from the horizontal.

4.3.4.2 Piezo-ICSI procedure

Motile sperm were suspended in CZB-H containing 7% PVP medium, in a 90 mm culture dish (as ICSI dish) covered with oil. Piezo-actuated micromanipulator (PMAS-CT150; Prime Tech, Ibaraki, Japan), with right hand side inverted microscope (IX71; Olympus, Tokyo, Japan) was used at piezo intensity = 3 and frequency = 3 to sever the sperm head. The blunt tip of the ICSI-piezo pipette

with 7 to 8 µm inner diameter was used to draw intact sperm and to separate the sperm head from the tail at the head-midpiece boundary by applying several pulses with a foot switch. About 5 sperm heads were drawn into the ICSI-piezo pipette. Five to ten fresh oocytes were transferred to ICSI dish into CZB-H medium drop. Holding pipette, a pulled glass pipette with inner diameter approximately 20 µm and outer diameter about 90 to 100 µm that connected with micromanipulator (M0188NE; Narishige, Tokyo, Japan) from the left, was used to hold oocyte at the position of 9 O'clock with applied gentle suction. Metaphase plate of oocyte was rotated to 6 or 12 O'clock position by the blunt tip ICSI-piezo pipette. Five sperm head were drawn into ICSI-piezo pipette (depend on experience, small number for save time) while, blunt-end ICSI-piezo pipette was used cut zona pellucida of 3 O'clock position of oocyte at the same piezo unit level as sperm head cutting (intensity = 3 and frequency = 3). After zona pellucid was cut, blunt-end ICSI-piezo pipette was pushed through perivitelline space and plasma membrane more than half of oocyte while, plasma membrane still intact. Immediately, plasma membrane was broken by switching the piezo set up unit to braking plasma membrane mode with intensity = 1 and frequency = 1 and applied one time of piezo pulse for membrane braked . Only one sperm head was blown into cytoplasm of oocyte then gentle moved out the blunt-end ICSI-piezo pipette from injected oocyte. Injecting next oocyte until sperm head inside ICSI-piezo pipette was empty. After the last injected oocyte, were not immediately transferred the injected oocytes to culture in the incubator at 37ºC because the rapidly temperature changing caused to cytoplasm lysis. Therefore, after the last injected should rested the injected oocyte at room temperature for 10 min, later transferred to culture in KSOM medium in the incubator at 37ºC in an atmosphere of 6% CO_2 in air approximately 26

h (2-cell formation), 38 h (4-cell formation), 50 h (8-cell formation), 62 h (morula formation) and 80 h (early blastocyst formation), after that cutured to 100 h (expand blastocyst formation).

4.3.5 Vitrification of embryos

4.3.5.1 Vitrification

Vitrification was performed following Cryotop or HS-CS methods.

Cryotop method (group I)

Group of five embryos at each stage (2-cell, 4-cell, 8-cell, morula and early blastocyst) were selected to wash in CZB-H medium before transferred into equilibration solution of 7.5% EG and 7.5% DMSO for 5 min and then exposed in the vitrification solution of 15% EG, 15% DMSO and 0.6 M sucrose for 30 s. The embryos with small volume of vitrification solution (<1µL) were loaded and placed on the plastic holder tip of cryotop strip and immediately immersed into LN_2. The hard plastic cover was caped to cryotop strip under LN_2. The cryotop strip with vitrified embryos then moved into the LN_2 tank for storage at least 1 week before thawing.

HS-CS method (group II)

The aluminum cylinders of home-made vitrification system were pre-cooled in foam box with LN_2 for approximately 15 to 20 min to equilibrate before used, as described in Vutyavanich et al., 2009. Briefly, the aluminum cylinders were immersed into LN_2 for 20 min to equilibrate. Straws size ø 0.50 mm were sealed one end at the bottom and used as the outer straw, then were inserted into a hole inside the pre-cooled aluminum cylinders for 5 min to pre-cooled the outer straw. For vitrification, group of five embryos at each stage (2-cell, 4-cell, 8-cell, morula and

early blastocyst) were selected to wash in CZB-H medium before transferred to equilibration solution of 7.5% EG and 7.5% DMSO for 5 min and then exposed in the vitrification solution of 15% EG, 15% DMSO and 0.6 M sucrose for 30 s and loaded into the tip of hemi-straws (size ø 0.25 mm.) with small volume of vitrification solution (<1µL). Then, hemi-straws was inserted into the pre-cooled outer straw and then the outer straw was sealed the top end for confirm that complete closed system. After that, HS-CS was directly immersed into the LN_2 tank for storage at least 1 week before thawing.

4.3.5.2 Thawing of embryos

After the vitrified embryos were stored in the LN_2 tank for 1 week, vitrified embryos were thawed in four serial thawing medium of D-PBS solution supplemented with 1.0 M, 0.5 M, 0.25 M, 0 M sucrose and 20% FBS with an interval of 5 min at 37ºC. The same formula of thawing solution was used in both experimental groups.

Cryotop thawing method

Cryotop strip from the LN_2 tank was held up in the air. Immediately, The hard plastic cover was taken out and the tip of cryotop strip was directly immersed into four serial thawing solution medium of D-PBS solution supplemented with 1.0 M, 0.5 M, 0.25 M, 0 M sucrose and 20% FBS with an interval of 5 min at 37ºC. Finally, the survived embryos were washed several times and incubated in KSOM medium.

HS-CS thawing method

The top end of the outer straw was cut while it was still inside the LN_2, the hemi-straw with vitrified embryos were taken out and directly immersed the

tip of hemi-straw with vitrified embryos into four serial thawing solution medium of D-PBS solution supplemented with 1.0 M, 0.5 M, 0.25 M, 0 M sucrose and 20% FBS with an interval of 5 min at 37ºC. Finally, the survived embryos were washed several times and incubated in KSOM medium.

4.3.6 Observation of the viability and development of embryos

After warming, the vitrified embryos survival rate was detected by morphologically observation on stereo microscope. And piezo-ICSI procedure of all three group (gr.1; Cryotop method, gr.2; HS-CS method and gr.3; fresh control) were done. After cultivation for 26 h, embryos were observed and recorded the result of 2-cell formation rate. Also, at 4-cell for 38 h, 8-cell for 50 h, morula for 62 h, early blastocyst for 80 h and expand blastocyst for 100 h were observed and recorded together.

4.3.7 Differential staining of inner cell mass (ICM) and trophectoderm (TE)

Differential staining of ICM and TE nuclei at expand blastocyst was performed as previously described (Thouas et al., 2001), with slight modifications. Briefly, expand blastocyst were simultaneously treated with 0.1 mg/mL PI and 0.2% Triton X-100 dissolved in the D-PBS for 35 s. Then, expand blastocyst were treated with 25 µg/mL Hoechst 33342 dissolved in 99.5% ethanol for 4 min, after that mounted on glass slides in glycerol droplet, flattened by glass cover slips and examined under UV light with an excitation wavelength of 330-385 nm by using an epifluorescence microscope (IX-71; Olympus, Tokyo, Japan). A digital image of each expanded blastocyst was taken, and the cell number of both cell types was counted. Therefore, the numbers of ICM and TE were counted separately in expand blastocysts that had clearly distinguishable populations of blue and red nuclei.

4.3.8 Statistical analysis

All data analyzed for the survival rate of frozen-thawed oocytes, embryo development rate and embryo cell number were arcsine transformed and carried out by ANOVA in statistical analysis system (SAS, Inst. Inc., Carry, NC). The differences between groups were considered to be statistically significant at a probability value of 0.05 or less. Four replications were performed in the experiment.

4.4 Results

4.4.1 Effect of the vitrification method on embryo viability anddevelopment after freezing-thawing of 2-cell stage embryos.

The viability of vitrified 2-cell stage embryos was determined by morphologic observation after warming. As shown in table 4.1, there was no significant difference in the survival rates and development rate of vitrified 2-cell stage embryos between the two different vitrification methods: the Cryotop and HS-CS methods (76/80= 95.00% and 75/80= 93.75%, respectively; $P \geq 0.05$). However, compared with fresh controls, the vitrified embryos showed significantly lower rates of morula, early and expanded blastocyst development, while development to the earlier stages (4- and 8-cell) was not significantly different. (4-cell: 75/80, 93.8% vs 72/76, 94.7% and 72/75, 96.0%, 8-cell: 61/80, 76.3% vs 55/76, 72.4% and 56/75,

74.7%, morula: 52/80, 65.0% vs 40/76, 52.6% and 42/75, 56.0%, early blastocyst: 50/80, 62.5% vs 36/76, 47.4% and 38/75, 50.7%, and expanded blastocyst: 48/80, 60.0% vs 32/76, 42.1% and 33/75, 44.0%; respectively, $P<0.05$).

4.4.2 Effect of the vitrification method on embryo viability and Development after freezing-thawing of 4-cell stage embryos.

As shown in table 4.2, there was no significant difference in the survival and development rates of vitrified 4-cell stage embryos between the Cryotop and HS-CS methods (77/80, 96.3% and 78/80, 97.5%, respectively; $p>0.05$), except for a significantly lower rate of early blastocyst embryos in the Cryotop vs the HS-CS (55.8% vs 60.3%, respectively). However, when compared with fresh controls, vitrified embryos showed similar embryo development rates until the morula stage and significantly lower rates of development to the early and expanded blastocyst stages (8-cell: 71/80, 88.8% vs 68/77, 88.3% and 70/78, 89.7%, morula: 55/80, 68.8% vs 50/77, 64.9% and 53/78, 67.9%, early blastocyst: 53/80, 66.3%, vs 43/77, 55.8% and 47/78, 60.3%, and expanded blastocyst: 51/80, 63.8% vs 40/77, 51.9% and 42/78, 53.9%; respectively, $P<0.05$).

4.4.3 Effect of the vitrification method on embryo viability and development after freezing-thawing of 8-cell stage embryos.

As shown in table 4.3, there was no significant difference in the survival and development rates of vitrified 8-cell stage embryos between the Cryotop and the HS-CS methods (78/80, 97.5% and 80/80, 100.0%, respectively; $P\geq0.05$), except for a significantly lower rate of expanded blastocyst embryos in the HS-CS vs the Cryotop (72.5% vs 75.6%, respectively). When compared with fresh controls, vitrified embryos showed similar embryo development rates until the expanded blastocyst

stage, except for a significantly lower rate of development to the expanded blastocyst stages only in the HS-CS group (morula: 72/80, 90.0% vs 71/78, 91.0% and 71/80, 88.8%, early blastocyst: 66/80, 82.5% vs 64/78, 82.1% and 65/80, 81.3%, and expanded blastocyst: 63/80, 78.8% vs 59/78, 75.6% and 58/80, 72.5%; respectively, $P<0.05$).

4.4.4 Effect of the vitrification method on embryo viability and development

after freezing-thawing of morula stage embryos.

As shown in table 4.4, there was no significant difference in the survival and development rates of vitrified morula stage embryos between the Cryotop and the HS-CS methods (80/80, 100% and 80/80, 100%, respectively; $p\geq0.05$). However, compared with fresh controls, the vitrified embryos showed significantly lower rates of early and expanded blastocyst development. (early blastocyst: 77/80, 96.3% vs 68/80, 85.0% and 69/80, 86.3%, and expanded blastocyst: 74/80, 92.5% vs 64/80, 80.0% and 66/80, 82.5%; respectively, $P<0.05$).

4.4.5 Effect of the vitrification method on embryo viability and development

after freezing-thawing of early blastocyst stage embryos.

As shown in table 4.5, there was no significant difference in the survival and development rates of vitrified early blastocyst stage embryos between the Cryotop and the HS-CS methods (79/80, 98.8% and 79/80, 98.8%; respectively, $P\geq0.05$). However, compared with fresh controls, the vitrified embryos showed significantly lower rates of expanded blastocyst development (77/80, 96.25% vs 64/79, 81.0% and 67/79, 84.8%; respectively, $P<0.05$).

4.4.6 Effect of the vitrification method on total cell number, Trophectoderm (TE) and Inner cell mass (ICM) cell counts and their ratios (ICM/TE) in expanded blastocyst embryos.

Ten expanded blastocysts were sampled from each of the vitrified experimental groups and the fresh control group and were subjected to differential staining in order to count the TE and ICM cells of each embryo. There was no significant difference in total cell counts, TE and ICM, and their ratios (ICM/TE) in expanded blastocyst embryos, which were vitrified at different stages of development and fresh control blastocysts. (Table 4.6).

4.5 Discussions

In this study, we examined the effects of two different embryo vitrification methods on embryo survival and further development after thawing by the piezo-ICSI mouse model. We also examined the effect of vitrification at various embryo stages (2-cell, 4-cell, 8-cell, morula and early blastocyst stages) on embryo survival and further development. Embryos were vitrified by the Cryotop and HS-CS methods. Piezo-ICSI was used in this study on fresh oocytes to produce 2-cell, 4-cell, 8-cell, morula and early blastocyst stages embryos. At each embryonic stage embryos were either vitrified by Cryotop method or HS-CS method, using the same freezing and thawing media. Our data showed no significant difference in the survival and embryo development rates after thawing between the Cryotop and the HS-CS methods, at all the embryonic stages of vitrification (2-cell, 4-cell, 8-cell, morula and early blastocyst). However, compared with non-vitrified embryos, vitrification resulted in significantly lower rates of embryo development as early as the morula stage (in 2-cell stage vitrification) and lower rates of blastocyst development when

vitrification was carried out in later stages (4-cell, 8-cell, morula and early blastocyst). It seems as if earlier vitrification (at the 2-cell stage) is associated with more significant decrease in embryo development compared with non-vitrified embryos. These findings suggested that vitrification exerts some negative effects on embryonic development,

Table 4.1 Survival rate and embryo development after freezing-thawing of 2-cell stage embryos.

Group	Stage of embryo freezing	N	Survival rate	Cultured	Embryo developed to				
					4-cell	8-cell	Morula	Early BL	Expanded BL
Cryotop		80	76/80	76	72/76	55/76	40/76	36/76	32/76
			95.0%		94.7%	72.3%	52.6%[b]	47.3%[b]	42.1%[b]
HS-CS	2-cell	80	75/80	75	72/75	56/75	42/75	38/75	33/75
			93.7%		96.0%	74.6%	56.0%[b]	50.6%[b]	44.0%[b]
Fresh	2-cell	80	-	80	75/80	61/80	52/80	50/80	48/80
control					93.7%	76.2%	65.0%[a]	62.5%[a]	60.0% [a]

Four replications were performed. Values in the same column with different superscripts (a, b) are significantly different, $P<0.05$, (ANOVA).

Table 4.2 Survival rate and embryo development after freezing-thawing of 4-cell stage embryos.

Group	Stage of embryo freezing	N	Survival rate	Cultured	Embryo development to			
					8-cell	Morula	Early BL	Expanded BL
Cryotop	4-cell	80	77/80	77	68/77	50/77	43/77	40/77
			96.2%		88.3%	64.9%	55.8%[c]	51.9%[b]
HS-CS	4-cell	80	78/80	78	70/78	53/78	47/78	42/78
			97.5%		89.7%	67.9%	60.2%[b]	53.8%[b]
Fresh control	4-cell	80	-	80	71/80	55/80	53/80	51/80
					88.7%	68.7%	66.2%[a]	63.7%[a]

Four replications were performed. Values in the same column with different superscripts (a, b, c) are significantly different, $P<0.05$, (ANOVA).

Table 4.3 Survival rate and embryo development after freezing-thawing of 8-cell stage embryos.

Group	Stage of embryo freezing	N	Survival rate	Cultured	Embryo development to		
					Morula	Early BL	Expanded BL
Cryotop	8-cell	80	78/80	78	71/78	64/78	59/78
			97.5%		91.0%	82.0%	75.6%[a]
HS-CS	8-cell	80	80/80	80	71/80	65/80	58/80
			100%		88.7%	81.2%	72.5%[b]
Fresh	8-cell	80	-	80	72/80	66/80	63/80
control					90.0%	82.5%	78.7%[a]

Four replications were performed. Values in the same column with different superscripts (a,b) are significantly different, $P<0.05$, (ANOVA).

Table 4.4 Survival rate and embryo development after freezing-thawing of morula stage embryos.

Group	Stage of embryo freezing	N	Survival rate	Cultured	Embryo development to	
					Early BL	Expanded BL
Cryotop	Morula	80	80/80 100%	80	68/80 85.0%[b]	64/80 80.0%[b]
HS-CS	Morula	80	80/80 100%	80	69/80 86.2%[b]	66/80 82.5%[b]
Fresh control	Morula	80	-	80	77/80 96.2%[a]	74/80 92.5%[a]

Four replications were performed. Values in the same column with different superscripts (a, b) are significantly different, $P<0.05$, (ANOVA).

Table 4.5 Survival rate and embryo development after freezing-thawing of early blastocyst stage embryos.

Group	Stage of embryo freezing	N	Survival rate	Cultured	Embryo development to Expanded BL
Cryotop	Early blastocyst	80	79/80 98.7%	79	64/79 81.0%[b]
HS-CS	Early blastocyst	80	79/80 98.7%	79	67/79 84.8%[b]
Fresh control	Early blastocyst	80	-	80	77/80 96.2%[a]

Four replications were performed. Values in the same column with different superscripts (a, b) are significantly different, $P<0.05$, (ANOVA).

Table 4.6 Total cell number, Trophectoderm (TE) and Inner cell mass (ICM) cell counts and their ratios (ICM/TE) in expanded blastocyst embryos after vitrification at various embryo stages and in non-vitrified fresh controls.

Group	Stage of embryo frozen/thawed		TE (Mean±S.D.)	ICM (Mean±S.D.)	TC (Mean±S.D.)	ICM/TE (Mean±S.D.)
Cryotop	2-cell	0	47.1±10.6	21.0±4.7	68.1±12.1	0.46±0.13
HS-CS	2-cell	0	50.1±18.1	21.2±4.2	71.3±19.9	0.47±0.17
Control	2-cell	0	57.3±17.3	28.2±5.3	85.5±20.6	0.52±0.14
Cryotop	4-cel	0	45.4±10.6	20.8±4.9	66.2±14.1	0.46±0.10

HS			46.			
-CS	4-cell	0	4±13.8	21.5±5.4	67.9±19.0	0.47±0.07
Co			57.			
ntrol	4-cell	0	3±17.5	28.1±5.5	85.4±20.6	0.52±0.14
Cr			48.			
yotop	8-cell	0	6±13.2	22.4±4.9	71.0±17.1	0.48±0.12
HS			47.			
-CS	8-cell	0	4±8.8	22.8±6.6	70.2±13.2	0.48±0.12
Co			57.			
ntrol	8-cell	0	8±17.2	28.6±5.5	86.4±20.6	0.52±0.14
Cr			58.			
yotop	Morula	0	2±18.6	25.8±5.8	84.0±22.3	0.47±0.13
HS			56.			
-CS	Morula	0	3±9.3	27.4+6.8	83.7±15.0	0.49±0.09
Co	Morula		58.	29.2±5.3	87.5±20.4	0.53±0.14

ntrol		0	3±17.1			
Cr	Blastocy		59.			
yotop	st	0	3±17.4	28.2±5.3	87.5±21.3	0.50±0.15
HS	Blastocy		60.			
-CS	st	0	4±16.6	29.7±6.7	90.1±22.3	0.51±0.12
Co	Blastocy		58.			
ntrol	st	0	3±17.3	29.1±5.5	87.4±20.8	0.53±0.14

Four replications were performed.

There was no significant difference among these groups, $P \geq 0.05$, (ANOVA).

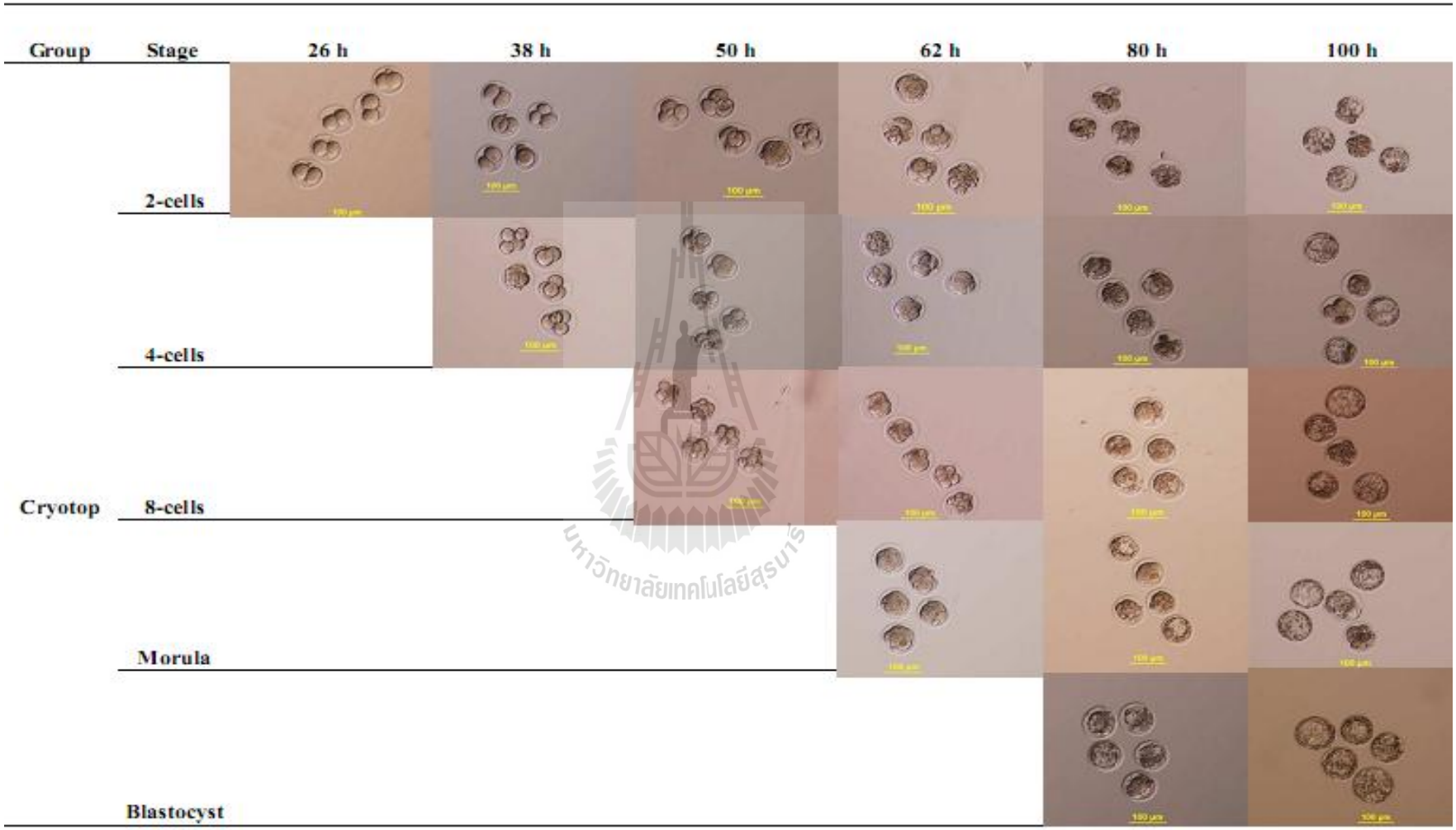

Figure 4.2 Morphology and development of various stages of frozen-thawed embryos compared with fresh control group.

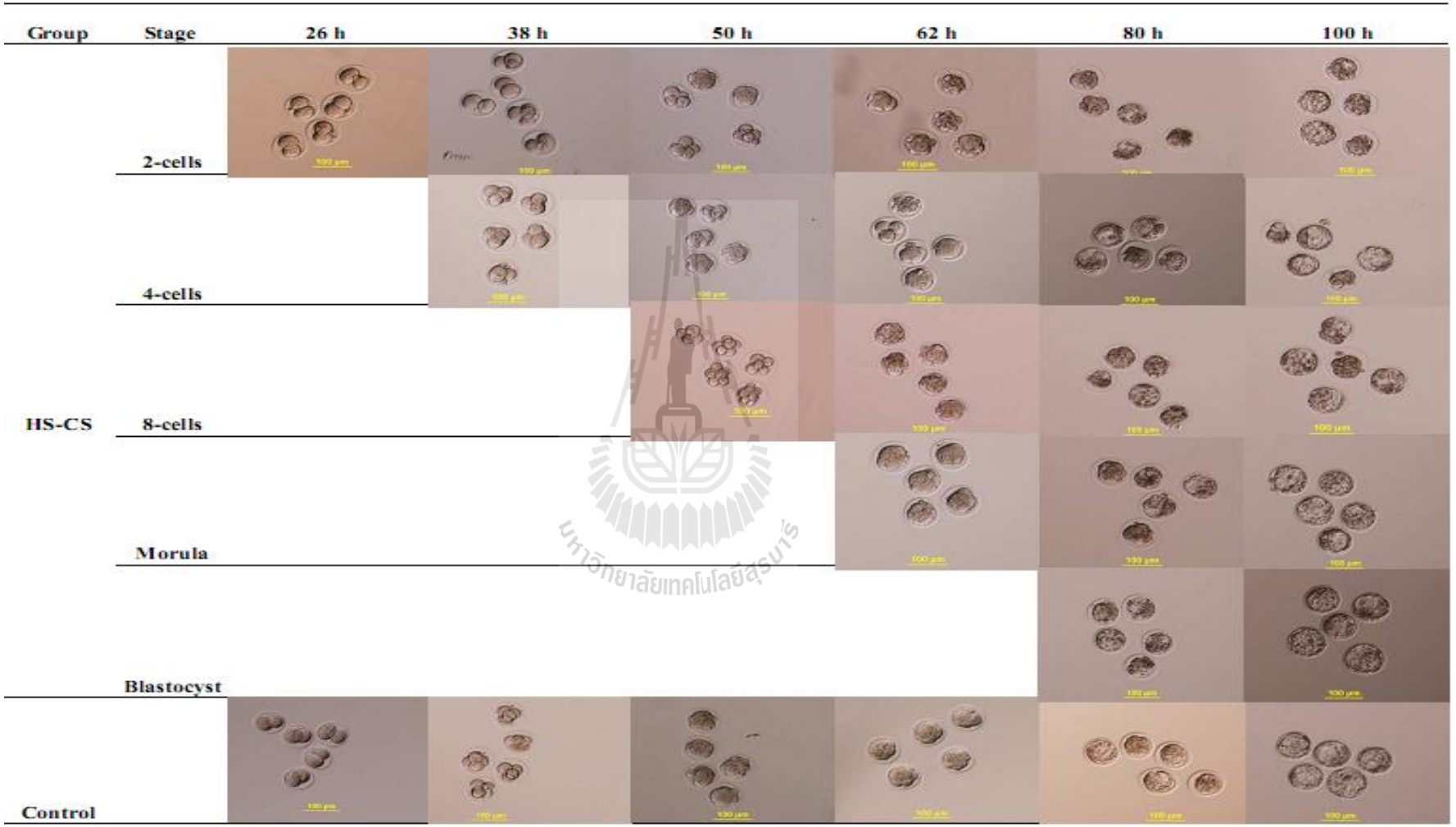

Figure 4.2 (Continued).

especially when carried out at an early stage. Zhang et al. also reported cleavage arrest at the 2-cell stage which occurs in normal mouse embryos and is commonly referred to as the two-cell block (Zhang et al., 2009). Since overcoming the 2-cell block and progress to the next developmental stage requires energy, it may be speculated that it is affected by freezing. Similar to our findings, Lane et al. demonstrated that vitrification of hamster 2-cell embryos inhibited the activities of both the Na^+/H^+ antiporter and HCO^-/Cl^- exchanger, resulting in reduced subsequent embryonic development. These two transport proteins are responsible for regulation of intracellular pH, energy production, and cell division, which seemed to account for the cleavage stage arrest caused by vitrification of 2-cell hamster embryos (Lane et al., 2000). Another potential adverse effect on embryonic development in this study is the use of piezo-ICSI for fertilization. Although this method is more successful than conventional ICSI in the mouse model, showing lower blastocyst formation rate than standard IVF or natural fertilization (*in vivo*). In contrast to our findings, Yan et al. performed vitrification of IVF mouse embryos at the 2-cell stage in OPS and noted similar blastocyst formation (71.8-89.5%) and hatching (61.1-69.6%) rates compared to controls (Yan et al., 2008).

Our results suggested that vitrification at later embryonic stages (4-cell and beyond) are associated with less marked decreases in embryonic development and at the balstocyst stages. Pedro et al. also reported that vitrified 8-cell stage embryos had the highest blastocyst formation and hatching rates, which implied that this stage was the most appropriate for vitrification. This could be due to the higher permeability of cryoprotectants at the 8-cell stage, as compared with earlier developmental stages (Pedro et al., 2005). There are also numerous reports of successful human vitrification

at the cleavage stage embryo (Rama-Raju et al., 2005; Desai et al., 2007; Balaban et al., 2008; Valojerdi et al., 2009; Zhang et al., 2009).

Although our findings showed no significant difference between this 2 methods of embryo vitrification, it should be pointed out that Cryotop vitrification is an open-system method, where there is direct contact between the LN_2 and the solution containing the oocytes or embryos (Kuwayama et al., 2007) and there is the potential risk of disease transmission. Kuwayama et al. also noted that both open- (Cryotop) and close- (Cryotip) system vitrification of human embryos resulted in similar survival and pregnancy rates of frozen-thawed blastocysts (97% vs 93% and 59% vs 51%, respectively). However, only the Cryotip was confirmed to eliminate the risk of cryopreservation contamination (Kuwayama et al., 2005). Vutyavanich et al. also reported higher survival and blastocyst formation rates of mouse 2-cell embryos derived by standard IVF and vitrified by the HS-CS method, compared with the slow freezing method (95.7% vs 77.6% and 79.3% vs 58.6%, respectively) (Vutyavanich et al., 2009).

Nowadays, blastocyst culture is required to produce embryos at blastocyst stage. Because blastocyst culture and transfer have resulted in improved IVF pregnancy rates and reduced multiple pregnancy rates. Therefore, the excesses blastocyst from fresh embryo transfer cycle must be vitrified with the effective method. From our results, frozen-thawed embryo at morula and early blastocyst stage from two vitrified group (Cryotop and HS-CS) methods showed high survival rates (morula; 100% and 100%, early blastocyst; 98.7% and 98.7%, respectively) and high expanded blastocyst formation rates (morula; 80% and 82.5%, early blastocyst; 81.0% and 84.8%, respectively).

Finally, the total cell (TC) and the ICM/TE ratio are well-established parameters for evaluating the blastocyst developmental competence. Our results showed that expanded blastocysts obtained after vitrification at all embryonic stages and thawing were not significantly different. This can't be the reason to explain the blastocyst obtain no significantly different, while this is only a morphological and quantitative finding. We suggest that these embryos are functionally competent after vitrification and thawing.

4.6 Conclusions

This is the first report that compared HS-CS and Cryotop methods for embryo vitrification at the 2-cell, 4-cell, 8-cell, morula and early blastocyst stages in the piezo-ICSI embryo mouse model. Our results indicated that HS-CS method is as effective as Cryotop method in both embryo survival and development after thawing. We have also noted that vitrification, by either method, is associated with some reduction in embryo development compared with fresh controls, which was mostly apparent when vitrification was carried out at the 2-cell stage. However, the vitrified-derived expanded blastocysts were not significantly different from non-vitrified embryos in terms of cell counts and ICM/TE ratios, suggesting their functional competence.

The HS-CS method is an effective method for mice embryo vitrification at various stages and specially for morula and early blastocyst stage of embryos. Which is simple to operate, inexpensive to establish and also eliminates or minimize the risk of contamination. We believe it is time to move toward its application in human embryo cryopreservation.

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CHAPTER V

OVERALL CONCLUSIONS

This is the first report of mouse blastocysts production from HS-CS vitrified oocytes compare with Cryotop vitrified method, following thawing and ICSI with piezo micromanipulator.

Experiment I: HS-CS vitrified oocytes method was no significant difference in the survival rate and expanded blastocyst formation rate when compared with Cryotop method. Therefore, HS-CS method is an effective method for mice oocyte vitrification as well as Cryotop method.

Experiment II: HS-CS method is as effective as Cryotop method in embryo survival and development in various stages of embryos after thawing.

Finally, HS-CS method is an effective method for mice oocyte and embryo vitrification at various stages and specially for morula and early blastocyst stage of embryos, which is simple to operate, inexpensive to establish and also eliminates or minimize the risk of contamination. We believe it is time to move toward its application in human embryo cryopreservation.

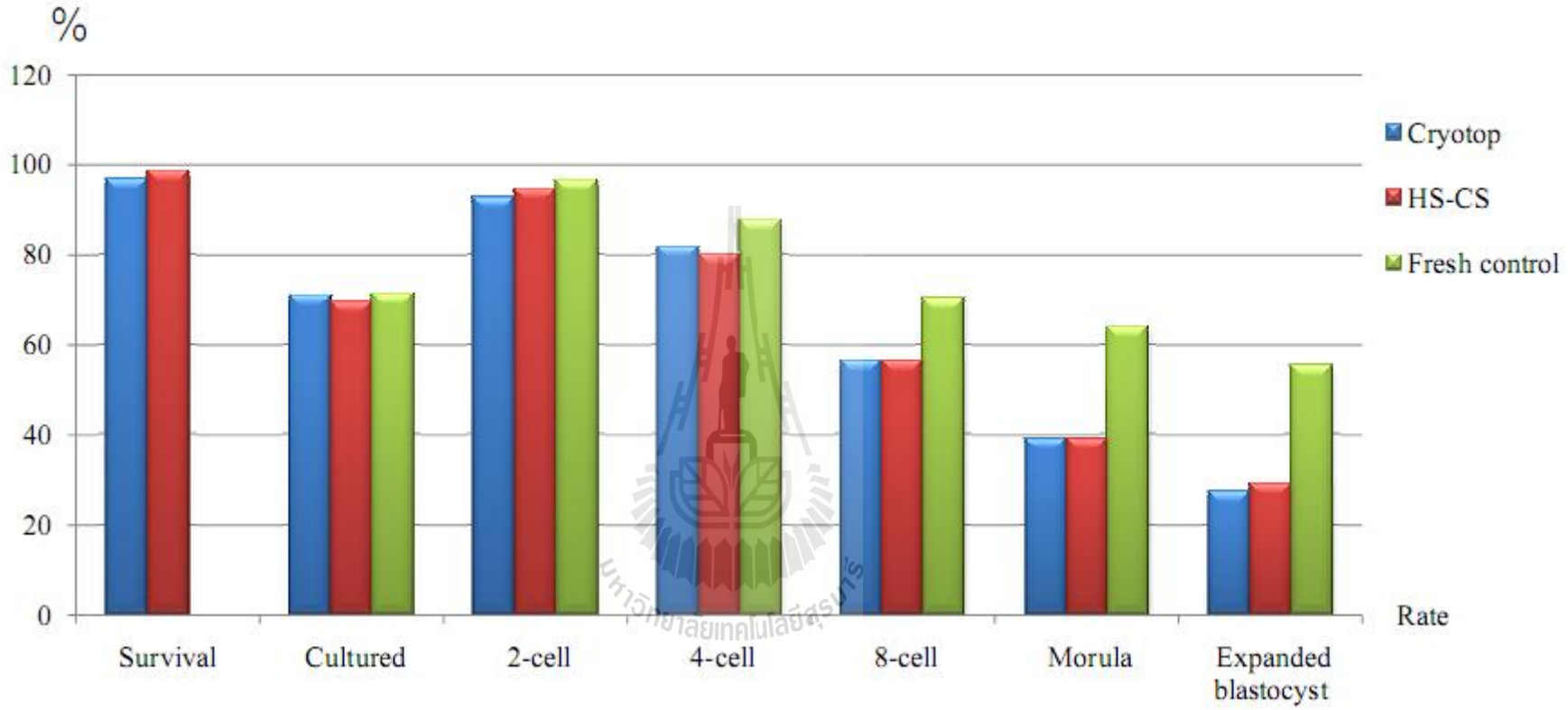

Figure 4.3 Conclusion of experiment I showed survival rate and embryos development from frozen-thawed oocyte by compared Cryotop method, HS-CS method with fresh control.

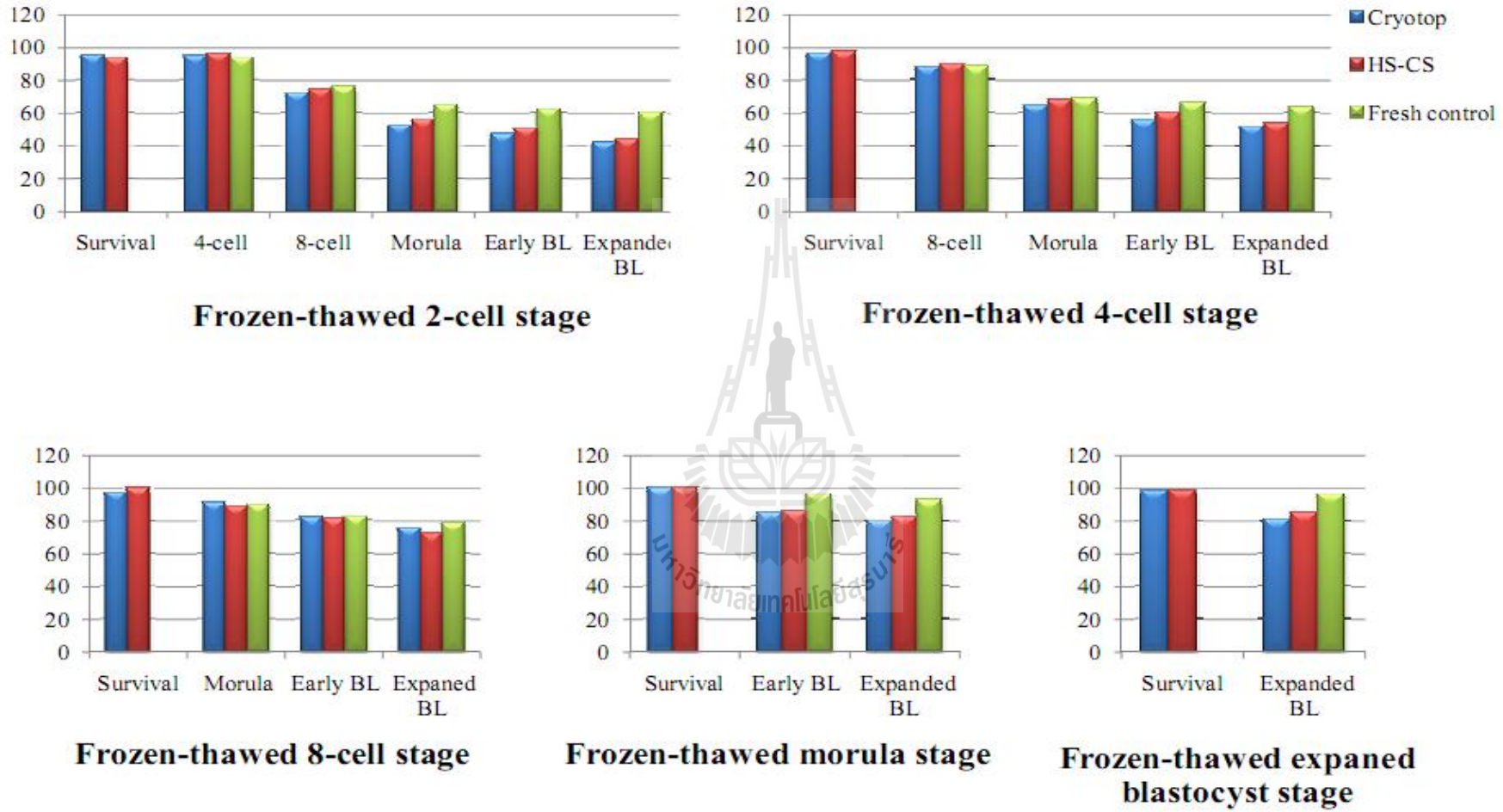

Figure 4.4 Conclusion of experiment II showed survival rate and embryos development from frozen-thawed embryos at various stages by compared Cryotop method, HS-CS method with fresh control.

BIOGRAPHY

Mr. Aniruth Aree-uea was born on April 10, 1979 in Roi-Et, Thailand. In 1998, he finished high school from Yasothornpittayakom School, Yasothorn, Thailand. In 2002, he graduated from Chiang mai University, Thailand with Bachelor's degree (B.Sc., Medical Technology). In the same year, he started work at Kullapat medical polyclinic, the Assisted Reproductive Technology centre in Chaing mai, Thailand. His work concerned about assisted conception in human such as semen analysis, Std. IVF, ICSI, etc. In 2009, he studied Master (M.Sc.) at school of biotechnology, institute of agricultural technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand. His research topic was "Vitrification of mouse matured oocytes and embryos at various stage: comparison of Cryotop and hemi straw closed system methods". Part of this study have been presented as poster presentation in the 3rd SUT graduate conference 2010 on November, 21-23, 2010 at Suranaree University of Technology, Nakhon Ratchasima, Thailand.