การเพิ่มประสิทธิภาพเทคนิคการแช่แข็งแบบ vitrification เพื่อการเจือจางใน ขั้นตอนเดียวหลังการทำละลายของตัวอ่อนโคที่ได้จากการปฏิสนธิ ในหลอดทดลองและผ่านการตัดแบ่งตัวอ่อน

นางสาวกาญจนา ปัญญาไว

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

สาขาวิชาเทคโนโลยีชีวภาพ

มหาวิทยาลัยเทคโนโลยีสุรนารี

ปีการศึกษา 2558

IMPROVEMENT OF VITRIFICATION TECHNIQUE FOR ONE STEP DILUTION OF VITRIFIED BIOPSIED BOVINE IVF-DERIVED EMBRYOS

Kanchana Punyawai

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IMPROVEMENT OF VITRIFICATION TECHNIQUE FOR ONE STEP DILUTION OF VITRIFIED BIOPSIED BOVINE IVF-DERIVED EMBRYOS

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

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กาญจนา ปัญญาไว : การเพิ่มประสิทธิภาพเทคนิคการแช่แข็งแบบ vitrification เพื่อการเจือจางในขั้นตอนเดียวหลังการทำละลายของตัวอ่อนโคที่ได้จากการปฏิสนธิในหลอดทดลองและผ่านการตัดแบ่งตัวอ่อน (IMPROVEMENT OF VITRIFICATION TECHNIQUE FOR ONE STEP DILUTION OF VITRIFIED BIOPSIED BOVINE IVF-DERIVED EMBRYOS) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.รังสรรค์ พาลพ่าย, 97 หน้า.

การแช่แข็งตัวอ่อนโคระยะบลาสโตซีสที่ได้จากการผลิตในหลอดทดลองด้วยวิธี vitrification จำเป็นต้องมีการพัฒนาเพื่อป้องกันการปนเปื้อน และสามารถเจือจางสารแช่แข็งได้ในขั้นตอนเดียวหลังการทำละลาย ซึ่งจะเพิ่มมูลค่าทางเศรษฐกิจของตัวอ่อนโคที่ได้จากการผลิตในหลอดทดลอง การศึกษาแรกเพื่อประเมินประสิทธิภาพของอุปกรณ์แช่แข็ง micro volume air cooling (MVAC) ในการแช่แข็งด้วยวิธี vitrification โดยนำไข่โคที่ผ่านการเลี้ยงให้สุกในหลอดทดลองและตัวอ่อนโคระยะบลาสโตซีสได้จากการผลิตในหลอดทดลอง มาแช่แข็งด้วยอุปกรณ์ MVAC โดยใช้ Cryotop ซึ่งเป็นอุปกรณ์แช่แข็งมาตรฐานที่มีอัตรารอดหลังการทำละลายสูงเป็นกลุ่มเปรียบเทียบ ส่วนการแช่แข็งด้วย MVAC นั้นจะทำการเปรียบเทียบในสองระบบคือ MVAC group ซึ่งไข่หรือตัวอ่อนจะไม่สัมผัสกับไนโตรเจนเหลวโดยตรง ส่วนระบบที่สองคือ MVAC in LN_2 group ระบบนี้ไข่หรือตัวอ่อนจะสัมผัสกับไนโตรเจนเหลวโดยตรง และเป็นระบบเดียวกับการแช่แข็งด้วย Cryotop เมื่อนำไข่หลังการทำละลายจากทั้งสามกลุ่มการทดลอง คือ MVAC, MVAC in LN_2 และ Cryotop มาทำการปฏิสนธิในหลอดทดลอง ผลการทดลองไม่พบความแตกต่างทางสถิติของอัตราการแบ่งตัว และอัตราการเจริญของตัวอ่อนเข้าสู่ระยะบลาสโตซีส (53.1% to 56.6% and 20.0% to 25.5%, ตามลำดับ) ส่วนตัวอ่อนโคระยะบลาสโตซีสได้จากการผลิตในหลอดทดลอง เมื่อนำมาแช่แข็งด้วยวิธีการดังกล่าวข้างต้น ผลการทดลองไม่พบความแตกต่างทางสถิติของอัตราการเจริญสู่ระยะแฮชบลาสโตซิสในตัวอ่อนทุกกลุ่ม

การศึกษาที่สองเพื่อเพิ่มอัตรารอดและการเจริญต่อในหลอดทดลองของตัวอ่อนโคระยะบลาสโตซีสที่ได้จากการผลิตในหลอดทดลอง เมื่อนำมาแช่แข็งโดยใช้อุปกรณ์ 0.25 mL straw ในการทดลองนี้ได้หาปัจจัยร่วมที่เหมาะสมในการแช่แข็งด้วย 0.25 mL straw โดยใช้การแช่แข็งด้วย Cryotop เป็นกลุ่มเปรียบเทียบ ผลการทดลองพบว่าความเข้มข้นของน้ำตาลซูโครสในน้ำยาเจือจางสารแช่แข็ง และวิธีการจุ่มหลอด 0.25 mL straw ลงในไนโตรเจนเหลว ไม่มีผลต่ออัตรารอดและการเจริญต่อในหลอดทดลองของตัวอ่อนโคระยะบลาสโตซีสที่แช่แข็งด้วย 0.25 mL straw นอกจากนี้ไม่พบความแตกต่างทางสถิติของอัตรารอดและการเจริญต่อในหลอดทดลอง เมื่อใช้น้ำยาแช่แข็งที่

ประกอบด้วย EG-DMSO (VS1) และ EG-Gly (VS2) ในการแช่แข็งตัวอ่อนโคระยะบลาสโตซีสที่ได้จากการผลิตในหลอดทดลองด้วย 0.25 mL straw แต่อย่างไรก็ตามพบว่าประสิทธิภาพของการแช่แข็งด้วยวิธี 0.25 mL straw ยังต่ำกว่า Cryotop

การศึกษาที่สามเพื่อศึกษาผลกระทบของการตัดแบ่งตัวอ่อนด้วยไมโครเบลดก่อนการแช่แข็งแบบ vitrification ด้วยอุปกรณ์สองชนิดคือ Cryotop และ 0.25 mL straw ต่ออัตรารอดและการเจริญต่อในหลอดทดลองของตัวอ่อนโคระยะบลาสโตซีสได้จากการผลิตในหลอดทดลอง โดยนำตัวอ่อนโคดังกล่าวนำมาตัดแบ่งเซลล์ออกบางส่วนด้วยไมโครเบลด ส่วนกลุ่มตัวอ่อนที่ไม่ถูกตัดแบ่งเซลล์จะใช้เป็นกลุ่มควบคุม จากนั้นนำตัวอ่อนทั้งสองกลุ่มมาแช่แข็งด้วยวิธี Cryotop และ 0.25 mL straw ผลการทดลองพบว่าอัตรารอดและการเจริญต่อในหลอดทดลองในตัวอ่อนแช่แข็งทั้งสองกลุ่มไม่แตกต่างทางสถิติกับกลุ่มตัวอ่อนสดที่ไม่แช่แข็ง ส่วนในตัวอ่อนที่ผ่านการตัดแบ่งตัวเซลล์ไม่พบความแตกต่างของอัตรารอดและการเจริญต่อในหลอดทดลองในกลุ่มตัวอ่อนแช่แข็งด้วย Cryotop หรือตัวอ่อนสด แต่อย่างไรก็ตามอัตราดังกล่าวในทั้งสองกลุ่มการทดลอง สูงกว่ากลุ่มตัวอ่อนที่ผ่านการตัดแบ่งเซลล์และแช่แข็งด้วย 0.25 mL straw ทางสถิติ นอกจากนี้ผลของค่าเฉลี่ยเซลล์ apoptotic ต่อบลาสโตซีสพบค่าเฉลี่ยสูงในทุกกลุ่มของตัวอ่อนที่ผ่านการแช่แข็งมากกว่าในกลุ่มตัวอ่อนสด ($P<0.05$)

จากการศึกษาดังกล่าวข้างต้นสรุปได้ว่า สามารถพัฒนาการแช่แข็งตัวอ่อนที่ผ่านการตัดแบ่งเซลล์ด้วยอุปกรณ์ 0.25 mL straw ซึ่งช่วยลดการปนเปื้อน และสามารถเจือจางสารแช่แข็งในขั้นตอนเดียวหลังการทำละลายเพื่อการย้ายฝากตรงในภาคสนาม

มหาวิทยาลัยเทคโนโลยีสุรนารี

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา ______________________

ปีการศึกษา 2558 ลายมือชื่ออาจารย์ที่ปรึกษา ____________________

KANCHANA PUNYAWAI : IMPROVEMENT OF VITRIFICATION TECHNIQUE FOR ONE STEP DILUTION OF VITRIFIED BIOPSIED BOVINE IVF-DERIVED EMBRYOS. THESIS ADVISOR : ASSOC. PROF. RANGSUN PARNPAI, Ph.D., 97 PP.

IN VITRO PRODUCED BOVINE BLASTOCYSTS/MICRO VOLUME AIR COOLING/CRYOTOP/0.25 ML STRAW

Vitrification of *in vitro* produced bovine (IVP) blastoycsts is necessary to develop for sanitary and one-step dilution after warming to improve the economic value of IVP bovine embryos. In the first study, to evaluate the efficiency of the micro volume air cooling (MVAC) system, IVM-oocytes and IVP bovine blastocysts were vitrified using the MVAC device without direct contact with liquid nitrogen (LN_2; MVAC group) and directly plunged into LN_2 (MVAC in LN_2 group) as similar to the Cryotop method (without direct contact with LN_2). After warming, vitrified oocytes were fertilized and cultured *in vitro*. Between the three vitrified groups, there were no significant differences in cleavage and blastocyst formation rates, ranging from 53.1% to 56.6% and 20.0% to 25.5%, respectively. In vitrified IVP bovine blastocysts, the rate of development of vitrified-warmed blastocysts changed into the hatched blastocyst stage after 72 h of culturing; there was no significant diffence between the groups.

In the second study, to improve the *in vitro* survival rates of vitrified IVP bovine blastocysts using the 0.25 mL straw method with the optimum combined factors when compared with the standard Cryotop. The result suggested that the sucrose concentrations and the methods of immersion of 0.25 mL straw into the LN_2 did not

affect the *in vitro* survival of vitrified IVP bovine blastocyst with 0.25 mL straw method. Moreover, the vitrification solution which was composed of the mixtures of cryoprotectants (CPAs) between EG-DMSO (VS1) and EG-Gly (VS2) showed equal efficiency for both mixtures of CPAs used for vitrifying IVP bovine blastocysts using 0.25 mL straws. However, the *in vitro* survival rates of the Cryotop were higher than those of vitrification using the 0.25 mL straw method.

In the third study, to evaluate the effects of biopsying with microblade prior to vitrification using the Cryotop or 0.25 mL straw methods on their *in vitro* survival rates after vitrification-warming. IVP bovine blastocysts were subjected to biopsy with microblade and were not subjected to biopsy before vitrified using the Cryotop or 0.25 mL straw methods. *In vitro* survival rates of non biopsied groups were not different when vitrified using the Cryotop, 0.25 mL straw, and fresh control groups. In the biopsy-derived blastocyst groups, the difference in *in vitro* survival rates after vitrification-warming using the Cryotop and fresh control groups were not found. Moreover, they were higher than the rates of vitrification of biopsied-derived blastocysts using 0.25 mL straw. In addition, the numbers of apoptotic cells per blastocyst were higher in all vitrified groups derived from biopsied and non-biopsied blastocysts than those of fresh control groups ($P<0.05$).

In conclusion, this study can develop the 0.25 mL straw device for sanitary vitrification, which enables one step dilution after warming for the direct transfer of biopsied IVP bovine embryos on farm.

School of Biotechnology　　　　Student's Signature_______________

Academic Year 2015　　　　Advisor's Signature_______________

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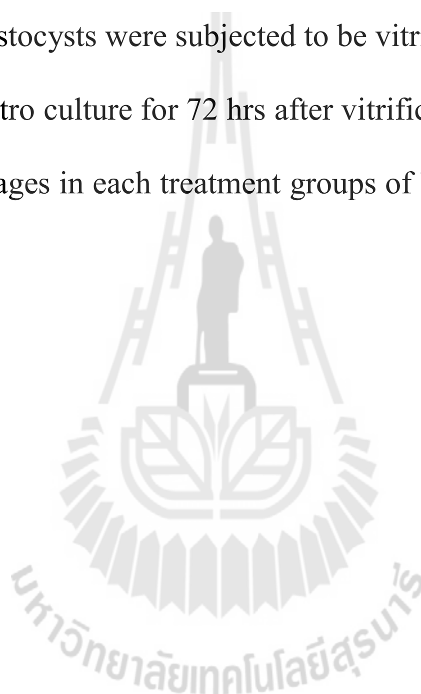

LIST OF ABBREVIATIONS

IVM	=	*in vitro* maturation
IVF	=	in vitro fertilization
IVC	=	*in vitro* culture
IVP	=	*in vitro* production
MII oocytes	=	mature oocytes that are in metaphase of meiosis II
IVM oocytes	=	in vitro maturation of oocytes
COCs	=	cumulus oocyte complexes
AI	=	artificial insemination
ART	=	assisted reproductive techniques
ICM	=	inner cell mass
TE	=	trophectoderm
MVAC	=	micro volume air cooling device
μL	=	microliter
μg	=	microgram
mg	=	milligram
mL	=	milliliter
TALP	=	Tyrode’s Albumin Lactate Pyruvate
DMSO	=	Dimethylsulphooxide
EG	=	Ethylene Glycol
Gly	=	Glycerol

มหาวิทยาลัยเทคโนโลยีสุรนารี

LIST OF ABBREVIATIONS (Continued)

PVP	=	Polyvinylproledone
ET	=	Embryo Transfer
ºC	=	Degree Celsius

CHAPTER I

INTRODUCTION

1.1 Introduction

In vitro embryos production (IVP) has become a useful procedure to enhance genetic improvement in dairy and beef cattle when transvaginal ultrasound-guided follicle aspiration (Ovum Pick-Up: OPU) was established (Hasler, 2003). The OPU technique allowed the collection of oocytes from a living female with high genetic merit. Subsequently, the oocytes were fertilized *in vitro* with superior male sperms which have proved for improving the yield of embryos from the designated donor (Boni, 2012). Because cattle are found to be significant for economy (Hochi, 2003), it is important to cryopreserve those embryos for commercial advantages. Moreover, the cryopreserved embryos can be transferred to a recipient animal at the most convenient time.

Previously, it has been found that IVP bovine embryos have higher thermal sensitivity than *in vivo* produced bovine embryos due to the difference in morphology between each source of embryos (Leibo and Loskutoff, 1993; Massip et al., 1995; Boni et al., 1999; Hochi et al., 2003). IVP bovine embryos have been reported for their swollen blastomeres (van Soom, A. and de Kruif, A., 1992) and poor compaction with darkness of cytoplasm at the morula stage (Hochi et al., 1998; Hochi et al., 2003). Moreover, from a higher ratio of lipid to proteins, it has been hypothesized to explain the darker of cytoplasm in IVP bovine embryos than *in vivo* produced bovine embryos which make IVP bovine embryos weaker to chill (Hochi et al., 2003), and the yield of

pregnancy rates is lower than *in vivo* produced bovine embryos (Farin and Farin, 1995).

A major breakthrough of IVP bovine embryo cryopreservation has been reported in 1985, when Rall and Fahy used the vitrification method for mouse embryo cryopreservation with 0.25 mL straw and glass tube devices. Vitrification method is found to the higher survival rates after vitrification-warming because of the use of minimum volume in combination with rapid cooling achieving the high cooling velocity, transferring heat, and preventing ice crystal formation during vitrification procedure (Arav et al., 2012). Since then, several vitrification devices have been developed: Cryoloop (Lane et al., 1999), nylon loop (Lane et al., 2001), hemi-straw (Vanderzwalmen et al., 2000), electron microscopy grid (Martino et al., 1996), open pulled straw (Vajta et al., 1997), glass capillary (Hochi et al., 1994) and Cryotop (Hamawaki et al., 1999). However, one of the most efficient devices for vitrification of both oocytes and embryos in domestic animals is Cryotop resulting in high survival and developmental rates (Isachenko et al., 2001; Martino et al., 1996; Dhali et al., 2000; Kuwayama and Kato, 2000; Katayama et al., 2003).

Although, the open system devices such as Cryotop are found to be a high probability of successful vitrification, it allows for the possibility of microbial disease transmission and viral contamination when the samples come into direct contact with LN_2 (Abdelhafez et al., 2011). Therefore, the development of a vitrification technique that concerns with sanitary vitrification and high success rates is necessary.

To improve the economic value of IVP bovine embryos, it is extremely important to develop the vitrification technique not only for sanitary, but for embryo transfer under field conditions. A 0.25 mL device is widely used for freezing and

transferring embryos at the industry level (Wright, 1985), because it is commonly used with 0.25 mL transfer gun in the field condition. However, the high cooling rates is not achieved by the close system devices when compared with the open system device (Saragusty and Arav, 2011). Thus, vitrification system of the 0.25 mL straw should improve the survival rates after vitrification-warming.

Embryo biopsy in bovine for sex analysis of preimplantation developmental stage before transferring to recipients is the crucial step on the survival rates after being biopsied. Therefore, cryopreservation of biopsied embryos until the sexing results are obtained, led to the use of biopsied embryos in bovine embryo transfer industry. However, previous reported suggested that bovine sexed embryos after cryopreservation resulted in the reduction of viability and pregnancy rates after embryo transfer (Thibier and Nibart, 1995; Hasler et al., 2002). The question addressed here is the efficiency of 0.25 mL for vitrification of biopsied IVP bovine embryos.

The present study was to investigate the efficiency of the close system device of micro volume air cooling (MVAC) and 0.25 mL straw for vitrification of IVP bovine embryos at the blastocyst stage. Subsequently, the improvement of vitrification with 0.25 mL straw device for sanitary vitrification, which enables one step diluation after warming for the direct transfer of biopsied IVP bovine embryos on farm were investigated on *in vitro* survival rate after vitrification-warming. Moreover, the effect of biopsy and vitrification on the embryos after these procedures was also investigated.

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CHAPTER II

REVIEW OF LITERATURES

2.1 Cryopreservation of mammalian embryos

The first success of cryopreserved mammalian embryos reported by Whittingham and colleagues in 1972. In 1973, the first live calf born was reported from the transfer of a thawed bovine embryo by the studied of Wilmut and Rawson (1973). This technique is accepted to be a safe procedure because of the use of relatively low concentration of cryoprotectants that might not cause serious toxic and osmotic damage. However, as low concentrations of cryoprotectants may be insufficient for avoiding ice crystal formation within the cells, the slow freezing is more time consuming and requires an expensive programmable freezing machine; most of the embryologists are not satisfied with this technique and try to find other cryopreservation protocols.

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The latter technique named vitrification, was first reported by Rall and Fahy in 1985 for cryopreservation of mouse embryos. The physical definition of vitrification is the solidification of solution at low temperature. The water will cool by use ultra rapid cooling rate and it changes into a glassy, vitrify state from the liquid phase. Vitrification technique not created ice crystal by using the extreme elevation in viscosity during cooling, (Fahy et al., 1984). The ultra-rapid cooling rate is based on direct contact between the vitrification solution and LN_2. Pioneering studies on mouse embryos vitrified at freezing rates of -3,000°C per min have resulted in high

percentages of post-thaw viability (Rall and Fahy, 1985). The vitrification technique requires high concentration of cryoprotectants and supplement with macromolecule to dehydrate the cell such as sucrose, Ficoll and Polyvinylpyrolidone (PVP) prior to the initiation of the cooling process. Vitrification solutions share three common properties. First, these vitrification solutions contain a combination of low and high molecular weight cryoprotectants. Low molecular weight cryoprotectants penetrate cell membranes and protect the cytoplasm from damage during freezing. Higher molecular weight cryoprotectants do not pass across the cell membrane. However, they are effective extracellular dehydration agents. Secondly, the final overall concentration of the cryoprotective agents in the mixture is high, to enhance vitrification and thus, avoiding lethal ice crystal formation. Finally, the standard vitrification solution contains an isotonic level of saline (Yavin and Arav, 2007).

During vitrification procedure, viable embryos are equilibrated in a 1.5 M concentration of cryoprotectants. As in the standard slow freezing protocol, embryos will shrink as water leaves the cells in response to the increased concentration of solute. Following a short equilibration period, the cell will return to their initial volume. The embryo will transfer to the final concentration of cryoprotectants in a one-step or two-step procedure prior to plunging into LN_2. This short exposure to high concentrations of cryoprotectant agents causes rapid cellular dehydration, preventing intracellular ice formation in the embryo during the cooling process (Fahy and Rall, 2007).

Vitrification has more advantages than conventional freezing. For example, in case of economic, vitrification technique is cheaper and much simpler than conventional freezing because the programmable freezing machine is not required to

decrease the temperatures. For the operating time, vitrification consumed a few seconds while conventional freezing took as long as 2-3 hours. Moreover, vitrification technique can get away from the formation of intracellular and extracellular ice crystals which are the major cause of cell damage (Jin et al., 2010).

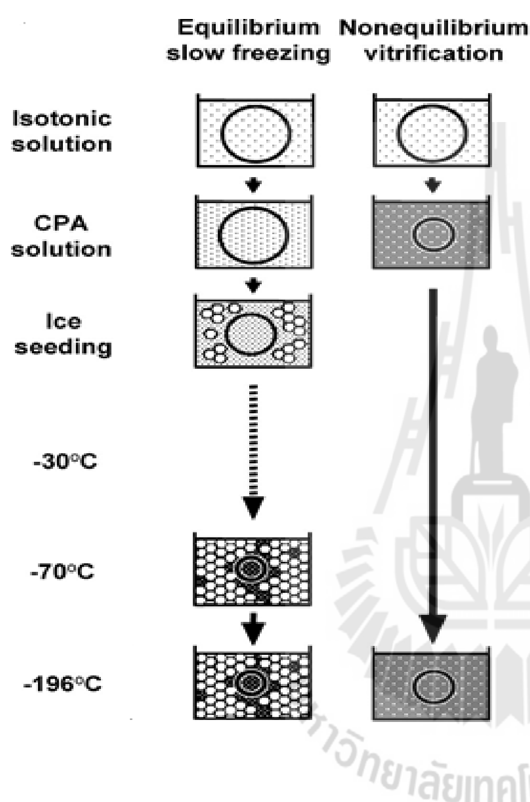

Figure 2.1 Diagram showing volume changes of an embryo (circle) during slow freezing and vitrification. Hexagons represent ice crystals. Concentration of cryoprotectant is shown by darkness of shading. In both processes, final step is immersion in liquid nitrogen (LN_2) (Jin et

2.2 Cryopreservation technology for *in vitro* produced bovine embryos.

Bovine embryos cryopreservation is a fundamental issue in the widespread use of embryo transfer. The progress in cryopreservation of bovine embryos was substantially improved due to factors such as an increase in embryo quality, an adequate selection of the recipient and an appropriate synchronization between donor and recipient (Gordon, 1996). In addition, the improvement of the cryopreservation methods has significantly contributed to the success of bovine embryo freezing. The

development of vitrification method for the cryopreservation of embryos was a major cryobiologic advance (Rall and Fahy, 1985) and the technique has application for IVP of bovine embryos (Agca et al., 1994). Throughout the world, approximately 15% of bovine embryos are produced by *in vitro* technology. However, IVP and cryopreservation have advantages over the *in vivo* methods in terms of cost effectiveness (Mapletoft and Hasler, 2005). In general, IVP embryos are more sensitive to cryopreservation than their *in vivo* embryos (Enright et al., 2000). Therefore, it has been shown that IVP bovine embryos are required the special cryopreservation method to gain the survival rates after cryopreservation.

2.2.1 Vitrification method for one step dilution after warming

For many years, vitrification procedures appear to be more efficient for the cryopreservation of IVP bovine embryos than other freezing methods (Massip et al., 1987; Douchi et al., 1990; Ishimori et al., 1993; Agca et al., 1998; Akiyama et al., 2010; Vieira et al., 2007). The first successful vitrification of bovine embryos was performed using 0.25 mL straw (Massip et al., 1986). Since then several vitrification devices have been developed, each with a specific method of minimizing the volume vitrification solution. Smaller volumes allow better heat transfer, thus achieved the superior cooling rates. Furthermore, the smaller the volume can results in the following general equation for the probability of vitrification:

$$\text{Probability of vitrification} = \frac{\text{Cooling rate} \times \text{Viscosity}}{\text{Volume}}$$

Previously, these devices can generally be separated into two categories: surface techniques and tubing techniques (Saragusty and Arav, 2011). The surface technique include electron microscope grid (Martino et al., 1996), minimum drop size

technique (Arav et al., 1987), Cryotop (Hamawaki et al., 1999), Cryoloop (Lane et al., 1999), solid surface (Dinnyes et al., 2000), nylon loop (Lane et al., 2001), Hemi-straw (Vanderzwalmen et al., 2001), nylon mesh (Matsumoto et al., 2001), Cryoleaf (Chian et al., 2005), direct cover vitrification (Chen et al., 2006), fiber plug (Muthukumar et al., 2008), vitrification spatula (Tsang and Chow, 2009), Cryo-E (Petyim et al., 2009), plastic blade (Sugiyama et al., 2010), and Vitri-Inga (Almodin et al., 2010). For the tubing techniques belongs the plastic straw (Rall and Fahy, 1985), open-pulled straw (Vajta et al., 1997), closed pulled straw (Chen et al., 2001), flexipet-denuding pipette (Liebermann et al., 2002), superfine open-pulled straw (Isachenko et al., 2003), high-security vitrification device (Camus et al., 2006), CryoTip (Kuwayama et al., 2007), pipette tip (Sun et al., 2008), sealed pulled straw (Yavin et al., 2009), Cryopette (Portmann et al., 2010), Rapid-i (Larman et al., 2010) and JY Straw (Wang et al., 2014).

One of the most efficient techniques for vitrification of embryos is Cryotop, resulting in high survival and developmental rates in pigs (Esaki et al., 2004; Ushijima et al., 2004), cattle (Martino et al., 1996; Vajta et al., 1998), buffalo (Duran et al., 2004), and humans (Kuwayama and Kato, 2000). However, because it is an open method in which samples come into direct contact with LN_2, it allows for the possibility of microbial disease transmission and viral contamination (Abdelhafez et al., 2011). Therefore, the development of a vitrification technique that concerns with sanitary vitrification and high success rates is necessary. Moreover, to improve the economic value of IVP bovine embryos, it is extremely important to develop the vitrification technique not only for sanitary, but for embryo transfer under field conditions.

Several methods of vitrification have been developed for IVP bovine embryos (Massip et al., 1987; Douchi et al., 1990; Ishimori et al., 1993; Akiyama et al., 2010), however; they have not been widely adopted for commercial use for bovine embryo transfer (Van Wagtendonk-de Leeuw et al., 1997; Vijta, 2000). Therefore, vitrification requires several steps for cryoprotectant dilution in laboratory setting (Ishimori et al., 1993) because it uses a high concentration of cryoprotectants.

Recently, it has been shown that some of vitrification methods which enable one step dilution after warming before being transferred into the recipients. Considerably, as it would bring embryo transfer on the farm to the same level of artificial insemination (AI) (Kuwayama et al., 1994; Wurth et al., 1994; Vajta et al., 1995; Van Wagtendonk-de Leeuw et al., 1997).

2.3 The preselection of the sex of offspring in bovine

The sex pre-selection of offspring has been a goal of livestock producers for generations. Female is the choice animal for dairy industry whereas the male is the first choice in meat industry. Sex pre-selection is one of the most sought after biotechnology of all times. The hunt has been on for several decades to find the scientific breakthrough that will allow one to use spermatozoa which will produce offspring of the desired sex (Sperm sexing) and sexing concepts before embryo transfer (Embryo sexing) (Prasad et al., 2010).

Both of sperm and embryo sexing have some advantage and disadvantage for each method. Of all the semen sexing techniques available till date, one commonly used method to accomplish this is flow cytometry. With accuracy of 90%, approximately 10 million live sperm of each sex (X and Y) can be sorted per hour

(Siedel, 2003). Sexed semen is currently commercially used in cattle for general AI programs to produced replacement offspring from genetically superior cows (Cran et al., 1995). The disadvantages of sperm sexing is the low percentage of embryo development to blastocyst and pregnancy rates when compared to unsexed semen (Mapletoft and Hasler, 2005). Because during sorting process indicated that the injury of sperm cell occurred from the fluorochrome-stained, the fluorescence emission and the exposure to Hoechst 33342-staining dye. The sperm viability test with SYBR-14 and PI showed that the mechanical stresses of sorting and centrifugation increase the number of dead or damaged sperm (Garner, 2006). Determining the sex of embryos before implantation has been developed to solve the low percentage of blastocyst and pregnancy rates when using sexed semen. Since the development of DNA amplification techniques by Polymerase Chain Reaction and the identification of bovine Y-chromosome specific DNA probes, the idea of embryos sexing has become a reality. Sexing based on detection of specific DNA probes has been used to predict the sex of offspring. Polymerase chain reaction (PCR) is routinely used in the field for sexing (Thibier and Nibart, 1995). This technique enables amplification of a target sequence from a small number of blastomeres. However, these methods require technical skill and are time consuming. Furthermore, PCR has the risk of false positives because of DNA contamination during the handling of the PCR products in duplicate PCR procedures and/or electrophoresis. Loop-mediated isothermal amplification (LAMP) is a novel DNA amplification method reported by Notomi et al (2000). The LAMP method amplifies a target DNA with high specificity, efficiency, and rapidity. The LAMP reaction is carried out under isothermal conditions (range, 60–65°C) using DNA polymerase with strand displacement activity. This reaction requires four specific primers (inner and outer primer sets) recognizing six

independent sequences, and specifically synthesizes a large amount of the amplification products, which are a mixture of stem-loop DNAs with various sizes and cauliflower-like structures with multiple loops. When the target DNA is amplified by LAMP, a white precipitate derived from magnesium pyrophosphate (a byproduct of the LAMP reaction) is observed. It is noteworthy that LAMP does not need special reagents or electrophoresis to detect the amplified DNA (Mori et al., 2001).

2.4 Bovine embryo biopsy for sex preselection of offspring

Biopsy for sample collection of embryonic cells has been applied for sex preselection of offspring. Therefore, a biopsy is a crucial step for the success of embryo sexing which cause of embryos damaged and reduced the quality of embryos after biopsied. In general, biopsy methods for bovine embryo sexing are derived from preimplantation genetic diagnosis (PDG) in human embryos. According to the development stage, two different biopsy procedures are used for bovine embryo sexing: cleavage stage embryo biopsy (early development stage) and biopsy at the blastocyst stage (later development stage).

Previous reports suggested that biopsy at later developmental stage, such as morula or blastocyst is less affected to embryos when compared with biopsy at earlier development stages due to more relatively collected cells (De Vos and Van Steirteghem, 2001). Moreover, it is difficult to biopsy at the morula stage because of the extensive compaction (Van Blerk et al., 1991). Therefore, biopsying at the blastocysts stage by removing off the small portion of trophectoderm is widely used due to its ease and less damage than the other stages of embryos (Evsikov and Verlinsky, 1998).

The biopsy procedure always takes two steps: opening of the zona pellucida and then removal of the cells. Opening of zona pellucida can be done in three ways: mechanically (partial zona dissection or zona slitting), chemically (using acidic Tyrode's solution), and laser technology (De Vos and Van Steirteghem, 2001). For acidic Tyrode's solution has no report in bovine embryo biopsy and its proven to be harmful for oocytes (De Vos and Van Steirteghem, 2001). To our knowledge, laser technology has not yet been reported in bovine biopsy for sexing. Therefore, only the mechanically technique is often used for opening of the zona pellucida of bovine in several reports (Macháty et al., 1993; Thibier and Nibart, 1995; Vajta et al., 1997; Leoni et al., 2000; Cenariu et al, 2012ab)

Various methods have been used for removal of the embryo cells, such as the needle technique (Thibier and Nibart, 1995; Cenariu et al, 2012b), the aspiration technique (Thibier and Nibart, 1995; Vajta et al., 1997), and the microblade technique (Thibier and Nibart, 1995; Leoni et al., 2000). It shows that the microblade technique is widely used for bovine embryo biopsy for sexing as this technique has more rapid and simpler procedure than the other two approaches. It is suitable for sexing a large number of embryos (Cenariu et al, 2012a).

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CHAPTER III

COMPARISON OF CRYOTOP AND MICRO VOLUME AIR COOLING METHODS FOR CRYOPRESERVATION OF BOVINE MATURED OOCYTES AND BLASTOCYSTS

3.1 Abstract

This study was designed to compare the efficiency of the Cryotop method and that of two methods that employ the micro volume air cooling (MVAC) device by analyzing the survival and development of bovine oocytes and blastocysts vitrified using each method. In experiment I, *in vitro*-maturation (IVM)-oocytes were vitrified using MVAC device without direct contact with liquid nitrogen (LN_2; MVAC group) or directly plunged into LN_2 (MVAC in LN_2 group). A third group of IVM oocytes was vitrified using the Cryotop device (Cryotop group). After warming, vitrified oocytes were fertilized *in vitro*. Between the three vitrified groups, there were no significant differences in cleavage and blastocyst formation rates, ranged from 53.1% to 56.6% and 20.0% to 25.5%, respectively; however, these rates were significantly lower ($P<0.05$) than Fresh control group (89.3% and 43.3%, respectively) and in the Solution control group (87.3% and 42.0%, respectively). In experiment II, i*n vitro*-produced (IVP)-expanded blastocysts were vitrified using the MVAC, MVAC in LN_2, and Cryotop methods, then warmed and cultured for survival analysis and comparison with the Solution control group. The rate of developmental of vitrified-warmed

expanded blastocysts into hatched blastocyst stage after 24 h of culturing was lower in the MVAC in LN_2 group than in the Solution control group; however, after 48–72 h of culturing, they did not significantly differ between the groups. These results indicated that the MVAC method without direct LN_2 contact are equally effective to the standard Cryotop method for vitrification of bovine IVM oocytes and IVP expanded blastocysts.

3.2 Introduction

Vitrification was initially used for cryopreservation of mouse embryos (Rall and Fahy, 1985) and has become a viable alternative to traditional freezing protocols, as it prevents chilling injury and ice crystal formation. During the vitrification procedure, cells and tissues are exposed to high concentration of cryoprotectants (CPAs), which effectively dehydrate the cells prior to initiation of the cooling process. Extended exposure to high concentrations of permeating CPAs is detrimental to cells (Hochi et al., 2004). To achieve a high probability of successful vitrification, the volume of the vitrification solution is minimized, which increases cooling velocity and heat transfer and prevents ice crystal formation (Arav et al., 2002). A rapid cooling rate during vitrification is the key to successful vitrification that avoids chilling injury in sensitive cells (Arav et al., 1993).

Oocytes are highly sensitive to chilling because of their low surface-to-volume ratio, which makes it difficult for water and CPAs to move across the cell plasma membranes (Pereire and Marques, 2008). Furthermore, vitrification of mature oocytes that are in metaphase of meiosis II (MII) leads to disorganization or disruption of the meiotic spindle, resulting in chromosome aberration (Arav et al., 1996; Rho et al.,

2002). The first successful vitrification of MII bovine oocytes was performed using electron microscope grids and a 0.25-ml straw as a vitrification device (Martino et al., 1996). Since then, other methods, such as the solid surface vitrification and Cryotop methods, have been found to allow for high-efficiency vitrification of MII bovine oocytes (Dinnyes et al., 2000; Chian et al., 2004). In contrast, embryos are more cryotolerant than oocytes because the properties of the plasma membrane change after fertilization, promoting dehydration and reducing ice formation during cryopreservation (Chen et al., 2003). Massip et al. (1986) were the first to report successful vitrification of bovine embryos using a 0.25-ml straw. Since then, several vitrification devices have been developed, each with a specific method of minimizing the volume of the vitrification solution: Cryoloop (Lane et al., 1999), nylon loop (Lane et al., 2001), hemi-straw (Vanderzwalmen et al., 2000), electron microscopy grid (Martino et al., 1996), open pulled straw (Vajta et al., 1997), glass capillary (Hochi et al., 1994) and Cryotop (Hamawaki et al., 1999).

The Cryotop method is one of the most efficient techniques for vitrification of both oocytes and embryos, resulting in high survival and developmental rates in pigs (Isachenko et al., 2001; Esaki et al., 2004; Ushijima et al., 2004), cattle (Martino et al., 1996; Dinnyes et al., 2000; Vajta et al., 1998), buffalo (Dhali et al., 2000; Duran et al., 2004, Gasparrini et al., 2007), and humans (Kuwayama and Kato, 2000; Katayama et al., 2003). However, because it is an open method in which samples come into direct contact with LN_2, it allows for the possibility of microbial disease transmission and viral contamination (Abdelhafez et al., 2011). Therefore, a new device and the corresponding micro volume air cooling (MVAC) method have been invented to prevent direct contact with LN_2 during vitrification. The MVAC and Cryotop devices

are equally effective for vitrification of *in vivo*-derived porcine expanded blastocysts (Misumi et al., 2013). However, there have been no reports on the application of the MVAC device (both with and without direct exposure to LN_2) to oocytes and embryos at the blastocyst stage in bovine species.

The objective of this study was to compare the efficacy of the MVAC and Cryotop devices for vitrification of both bovine IVM oocytes and bovine IVP embryos at the blastocyst stage by analysis of subsequent *in vitro* development after warming.

3.3 Materials and Methods

3.3.1 Chemicals and media

All reagents were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA), unless otherwise indicated.

3.3.2 Oocyte collection and IVM

Collection and IVM of bovine follicular oocytes were performed as previously described (Imai et al., 2006). Briefly, slaughterhouse ovaries were washed and stored in physiological saline supplemented with 50 µg/ml gentamicin for approximately 20 h at 20 °C. Cumulus-oocyte complexes (COCs) were aspirated from follicles (2–8 mm in diameter) using a 5 ml syringe connected to a 19-gauge needle. The IVM medium consisted of 25 mM Hepes-buffered TCM199 (Life Technologies Inc Gibco-BRL Division, Grand Island, NY, USA) and 5% calf serum (CS; Life Technologies Inc Gibco-BRL Division). COCs were washed twice with IVM medium, then cultured for 20 h in 600-µl droplets of IVM medium (80–100 oocytes/droplet) that were covered with paraffin oil (Nacalai Tesque, Inc., Kyoto, Japan) in 35-mm

plastic dishes (Nalge Nunc International, Roskilde, Denmark) at 38.5 °C under a humidified atmosphere of 5% CO_2 in air.

3.3.3 Vitrification and warming of oocytes

Twenty hours after IVM, cumulus cells were partially removed by repeated pipetting using a fine glass pipette in Dulbecco's phosphate-buffered saline (DPBS; Life Technologies Inc Gibco-BRL Division) supplemented with 0.1% (w/v) hyaluronidase. IVM oocytes with two to three layers of cumulus cells on their surface were subsequently washed five times in holding medium (HM), which consisted of 25 mM Hepes-buffered TCM 199 supplemented with 20% (v/v) CS. Thereafter, they were vitrified using either the MVAC device or the Cryotop device (Kitazato BioPharma Co., Ltd., Shizuoka, Japan) in a vitrification solution, as described previously by Dinnyes et al. (2000). Briefly, three separate groups of 5–10 oocytes were placed in equilibration medium, which was composed of HM supplemented with 4% (v/v) ethylene glycol (EG; Wako Pure Chemical Industries, Ltd., Osaka, Japan), for 12–15 min at 38.5 °C, then transferred into a vitrification solution composed of HM supplemented with 35% EG, 50 mg/ml polyvinyl pyrrolidone, and 0.4 M trehalose. Then, one group of 5–10 oocytes (Cryotop group) was placed on a Cryotop sheet as described previously (Chian et al., 2004), while another group was placed in a small volume of vitrification solution (<1 µl) on the inner surface of a stainless steel sheet in the MVAC device, which was then plunged directly into LN_2 (MVAC in LN_2 group). The third group was treated with the MVAC method (MVAC group), as follows: A 0.25-ml plastic straw was precooled in LN_2 while sealed with a plastic plug to prevent LN_2 from entering the straw. Thereafter, the plug was removed, followed by insertion of the MVAC device containing oocytes entirely into the straw. The straw

containing the MVAC device was then plunged into LN_2 (Misumi et al., 2013). Each cryodevice containing oocytes was kept in LN_2 for at least 24 h.

While submerged in LN_2, cover straws were removed from the vitrification devices used for the Cryotop and MVAC in LN_2 groups, followed by removal of the devices from inner straws. For the MVAC group, the cover straw was removed from the device above the surface of the LN_2, ensuring that the oocytes could be warmed without directly contacting LN_2. Thereafter, the devices were transferred into 3 ml of warming solution, which was composed of HM supplemented with 0.3 M trehalose, in a 35-mm plastic dish at 38.5 °C. Two minutes later, oocytes were consecutively transferred to 500 μl droplets of HM supplemented with 0.15, 0.075, or 0.0375 M trehalose at 38.5 °C, where they were held for 1 min each. They were washed three times with HM at 38.5 °C, and then transferred into IVM medium and incubated for an additional 2 h at 38.5 °C in a humidified atmosphere of 5% CO_2 in air.

3.3.4 *In vitro* fertilization (IVF)

The vitrified-warmed oocytes and non-vitrified oocytes were subjected to IVF as described previously (Imai et al., 2006). Briefly, frozen semen from a Japanese Black bull was thawed in a 37 °C-water bath for 30 sec and then centrifuged in 3 ml of 90% Percoll solution at 740 × *g* for 10 min. The pellet was resuspended and centrifuged in 6 ml of BO medium (Brackett and Oliphant, 1975) supplemented with 10 mM hypotaurine and 4 U/ml heparin (Novo-heparin Injection 1000; Aventis Pharma Ltd., Tokyo, Japan) at 540 × *g* for 5 min. Then, the pellet was resuspended with BO medium supplemented with 20 mg/ml BSA (IVF medium) to reach a final concentration of 3×10^6 spermatozoa/ml. To prepare fertilization droplets, 100 μl-

droplets of the sperm suspension were placed in a 35-mm dish and covered with paraffin oil. A group of 20 oocytes was washed three times in IVF medium. The oocytes were transferred into the fertilization droplets and cultured for 6 h at 38.5 °C under a humidified atmosphere of 5% CO_2 in air.

3.3.5 *In vitro* culture (IVC) of embryos

After IVF, cumulus cells and sperm attached to oocytes were removed by gentle pipetting with a fine glass pipette. IVC was performed in CR1aa medium (Rosenkrans et al., 1993) supplemented with 5% CS and covered with paraffin oil in a 35-mm dish at 38.5 °C under a humidified atmosphere of 5% CO_2 in air. Twenty presumptive zygotes derived from vitrified oocytes and fresh oocytes (Experiment I) were cultured in a 100-µl IVC drop, and 80 presumptive zygotes derived from vitrified oocytes and fresh oocytes (Experiment II) were cultured in a 600-µl IVC drop. After culturing for 48 h, cleavage rates were recorded. The blastocysts were continuously cultured in the same drop without changing of the medium. Blastocyst formation rates were recorded on Days 7, 8, and 9 (Day 0 was defined as the day of IVF).

3.3.6 Vitrification of IVP expanded blastocysts

Grade 1 IVP expanded blastocysts (IETS code 7; n = 345) obtained on Day 7 were vitrified using either the Cryotop or the MVAC device. Briefly, the expanded blastocysts were washed three times in HM consisting of DPBS supplemented with 20% (v/v) CS. The expanded blastocysts were then placed in equilibration medium, which was composed of HM supplemented with 7.5% EG and 7.5% dimethyl sulfoxide (DMSO) for 3 min at 38.5 °C, and then transferred into a vitrification solution composed of HM supplemented with 16.5% EG, 16.5% DMSO, and 0.5 M sucrose (VS33 solution), where they were held for 1 min. Then, a group of

three to five blastocysts was placed either on the inner surface of an MVAC device (Misumi et al., 2013) or on a Cryotop device before vitrification by the MVAC, MVAC in LN_2, or Cryotop method. Thereafter, cryodevices containing IVP expanded blastocysts were immediately plunged into LN_2, where they were stored for at least 24 h.

3.3.7 Culture of vitrified-warmed IVP expanded blastocysts

In each vitrification method, cover straws were removed from the devices in a process similar to the cover straw removal process used during vitrification and warming of oocytes, described above. After being washed in HM three times, the devices were placed in TCM-199 (Life Technologies Inc Gibco-BRL Division) supplemented with 20% CS and 0.1 mM β-mercaptoethanol, and then cultured for 72 h in the same medium (3-4 blastocysts/20 μl) covered with paraffin oil at 38.5 °C in a humidified atmosphere of 5% CO_2 in air. To evaluate blastocyst survival after vitrification-warming, the percentages of vitrified-warmed expanded blastocysts that developed to the hatched blastocyst stage were determined at 24, 48, and 72 h of IVC.

3.3.8 Evaluation of blastocyst cell numbers with differential staining

Differential staining of inner cell mass (ICM) and trophectoderm (TE) nuclei in blastocysts was performed as previously described (Thouas et al., 2001) with slight modifications. Briefly, the blastocysts were simultaneously treated with 0.1 mg/ml propidium iodide (PI) and 0.2% Triton X-100 dissolved in DPBS for 60 sec to permeabilize the membrane and stain the nuclei of TE cells. The blastocysts were then treated for 5 min with 25 μg/ml Hoechst 33342 (Calbiochem, San Diego, CA, USA) dissolved in 99.5% ethanol, and then mounted on glass slides in glycerol droplets that were flattened by cover slips. The blastocysts were examined under UV light with an

excitation wavelength of 330-385 nm using an epifluorescence microscope (IX-71; Olympus Corporation, Tokyo, Japan). The nuclei of TE cells labeled with both PI and Hoechst 33342 appeared pink or red, whereas the nuclei of ICM cells labeled only with Hoechst 33342 appeared blue. A digital image of each blastocyst was captured, and the cell numbers of both cell types were counted using NIH Image J (v. 1.40) software (Abramoff et al., 2004). Numbers of ICM and TE cells were counted separately in blastocysts that had clearly distinguishable populations of red and blue nuclei.

3.4 Experimental design

Experiment I: This experiment was designed to investigate the effect of cryodevices (MVAC and Cryotop) used for vitrification of bovine IVM oocytes on oocyte development rates after IVF. To serve as the Solution control group, some IVM oocytes were exposed to equilibration medium, vitrification medium, and warming solutions without cooling. Untreated oocytes served as the Fresh control group. In vitrified groups, IVM oocytes were randomly vitrified by the MVAC, MVAC in LN_2, or Cryotop method (MVAC, MVAC in LN_2, and Cryotop groups, respectively), and then warmed before IVF. After IVF, all oocytes were cultured *in vitro*. Rates of development of the oocytes to the blastocyst stage and cell numbers of the obtained blastocysts were compared between all five groups.

Experiment II: The objective of this experiment was to compare the efficiency of cryodevices for the vitrification of IVP blastocysts. Grade 1 IVP expanded blastocysts (IETS Quality code 1, Stage code 7; n = 455) were randomly divided into four groups; the expanded blastocysts were 1) exposed to vitrifcation and warming solutions

(Solution control group), 2) vitrified by the MVAC method (MVAC group), 3) vitrified by the MVAC in LN_2 method (MVAC in LN_2 group), or 4) vitrified by the Cryotop method (Cryotop group). Blastocyst survival after vitrification-warming was assessed by their hatching ability during 72 h of additional IVC.

3.5 Statistical analysis

The percentages of IVM-IVF oocytes that developed to the blastocyst stage and survival rates of IVP blastocysts after vitrification-warming were arcsine-transformed. Cell numbers of the embryos were expressed as untransformed means ± standard error of the mean (SEM). Data were analyzed by analysis of variance (ANOVA). Differences were considered to be significant for $P<0.05$.

3.6 Results

3.6.1 Experiment I

The developmental rates of fresh and vitrified-warmed oocytes after IVF are shown in Table 1. Oocytes from all vitrification groups showed lower rates of cleavage and development to the blastocyst stage than those from the Fresh and Solution control groups ($P<0.05$). Cleavage rates of the vitrified oocytes did not differ between the MVAC, MVAC in LN_2, and Cryotop groups. The cleavage rates of oocytes in all vitrified groups were lower than those of oocytes in the Fresh and Solution control groups ($P<0.05$); however, there was no difference in these rates between the two control groups. Rates of oocyte development to the blastocyst stage did not differ between the MVAC, MVAC in LN_2, and Cryotop groups on Days 7, 8,

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or 9; however, they were lower than those of the Fresh and Solution control groups ($P<0.05$).

As shown in Figure 1, there were no significant differences between all five groups in the total number of nuclei in blastocysts obtained on Day 9. Similarly, no significant difference was found in the numbers of ICM and TE cells between all groups.

3.6.2 Experiment II

Rates of development to the hatched blastocyst stage of vitrified-warmed expanded blastocysts after 24 h of IVC differed between the MVAC in LN_2 and Solution control groups (35.1% and 62.4%, respectively; $P<0.05$); however, there were no significant differences in these rates between the Solution control, MVAC, and Cryotop groups. Hatched rates at 48 and 72 h of culture did not differ between the Cryotop, MVAC, and MVAC in LN_2 groups; moreover, no difference was observed in hatched rates between the vitrified and Solution control groups (Table 2).

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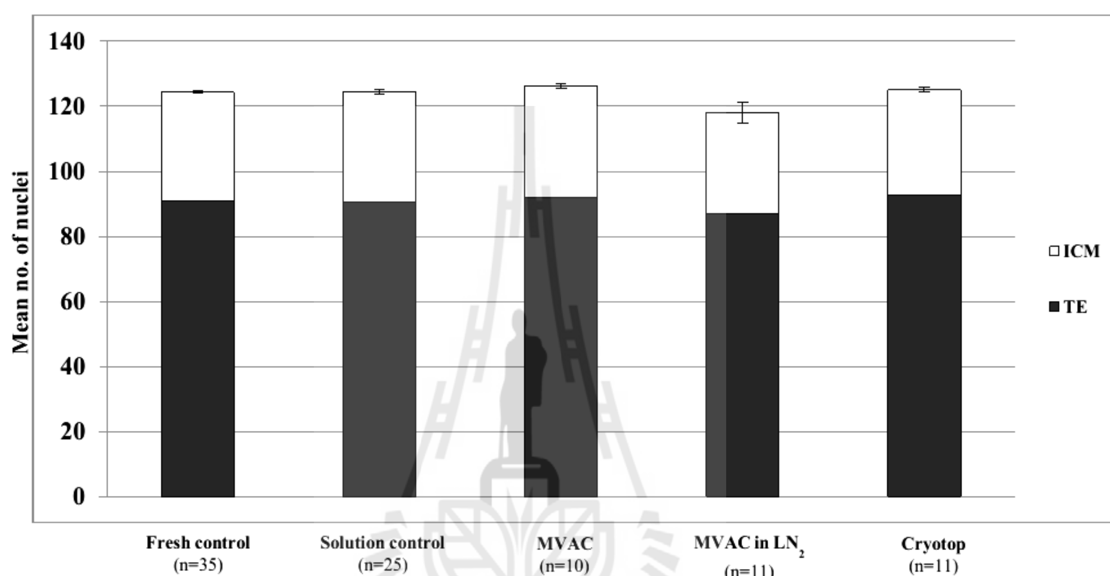
Figure 3.1 Cell numbers in blastocysts derived from fresh IVM bovine oocytes (Fresh control), treated with vitrification solution (Solution control), or vitrified by the MVAC or Cryotop methods after IVF and *in vitro* culture for 9 days.



Data presented as mean no. of nuclei ± SEM.

Fresh control: IVM oocytes without any vitrification treatments. Solution control: IVM oocytes that were exposed to vitrification and warming solutions. MVAC: IVM oocytes that were vitrified by inserting the MVAC device containing them into a precooled 0.25-ml plastic straw. MVAC in LN_2: IVM oocytes that were vitrified by plunging the MVAC device containing them directly into LN_2. Cryotop: IVM oocytes that were vitrified by the Cryotop method. ICM: inner cell mass. TE: trophectoderm. No significant differences in ICM and TE cell numbers were detected between the treatment groups at $P<0.05$ using one-way ANOVA.

Table 3.1 *In vitro* development of fresh IVM bovine oocytes (Fresh control), IVM bovine oocytes treated with vitrification solution (Solution control), and IVM bovine oocytes vitrified using the MVAC or Cryotop device after IVF and *in vitro* culture for 9 days.

Treatment group	No. of IVF oocytes	Oocytes cleaved and developed into blastocysts (%)					
		Cleaved (Day 2*)		Blastocyst (BL)			
		Total	≥5 Cells	Day 7	Day 8	Day 9	Total BL
Fresh control	150	134 (89.3)[a]	90 (60.0)[a]	48 (32.0)[a]	14 (9.3)[a]	3 (2.0)[a]	65 (43.3)[a]
Solution control	150	131 (87.3)[a]	93 (62.0)[a]	43 (28.7)[a]	15 (10.0)[a]	5 (3.3)[a]	63 (42.0)[a]
MVAC	145	77 (53.1)[b]	59 (40.7)[b]	26 (17.9)[b]	6 (4.1)[b]	1 (0.7)[b]	33 (22.9)[b]
MVAC in LN_2	145	81 (55.9)[b]	60 (41.4)[b]	24 (16.6)[b]	4 (2.8)[b]	1 (0.7)[b]	29 (20.0)[b]
Cryotop	145	82 (56.6)[b]	63 (43.5)[b]	30 (20.7)[b]	5 (3.5)[b]	2 (1.4)[b]	37 (25.5)[b]

Five replications were performed. [a,b]Values within a single column that have different superscripts are significantly different at $P<0.05$ using one-way ANOVA. *Day 0 was defined as the day IVF was performed. MVAC: IVM oocytes that were vitrified by inserting the MVAC device containing them into a precooled 0.25-ml plastic straw. MVAC in LN_2: IVM oocytes that were vitrified by plunging the MVAC device containing them directly into LN_2. Cryotop: IVM oocytes that were vitrified by the Cryotop method.

Table 3.2 Development to the hatched blastocyst stage of in vitro-produced expanded blastocysts vitrified using the MVAC or Cryotop device, warmed, and cultured *in vitro* for 72 h

Vitrification	**No. of cryopreserved embryos**	**No. of hatched embryos (%)**		
		24 h	**48 h**	**72 h**
Solution control	117	73[a]	94	109
		(62.4%)	(80.3%)	(93.2%)
MVAC	110	53[ab]	88	102
		(48.2%)	(80.0%)	(92.7%)
MVAC in LN_2	114	40[b]		104
		(35.1%)	(71.9%)	(91.2%)
Cryotop	114	61[ab]	89	102
		(53.5%)	(78.1%)	(89.5%)

Six replications were performed. a,bValues within a single column that have different superscripts are significantly different at P<0.05 using one-way ANOVA. Solution control: IVP expanded blastocysts that were exposed to vitrifcation and warming solutions. MVAC: IVP expanded blastocysts that were vitrified by inserting the MVAC device containing them into a precooled 0.25-ml plastic straw. MVAC in LN2: IVP expanded blastocysts that were vitrified by plunging the MVAC device containing them directly into LN2. Cryotop: IVP expanded blastocysts that were vitrified by the Cryotop method. No significant difference in development to hatched blastocyst stage was detected between the treatment groups at P<0.05.

3.7 Discussion

In this study, it is demonstrated for the first time that both bovine IVM oocytes and IVP expanded blastocysts can be successfully cryopreserved by the MVAC device. The triangular MVAC sheet (1.0 mm wide, 60 mm long, and 0.2 mm thick) is made from stainless steel, which supports high rates of heat exchange. High survival and developmental rates of vitrified-warmed IVM oocytes (Experiment I) and IVP embryos at the expanded blastocyst stage (Experiment II) were also achieved by the MVAC device as a result of rapid heat exchange and high cooling and warming rates.

Although Cryotop vitrification has been reported to be a highly efficient method for cryopreservation of bovine oocytes (Dinnyes et al., 2000; Chian et al., 2004) and embryos (Laowtammathron et al., 2005), the 0.4-mm-wide and 20-mm-long sheet allows for the placement of oocytes and embryos in less than 1 µl of vitrification solution (Kuwayama, 2007). Thus, only 5-10 oocytes can be cryopreserved on each Cryotop sheet (Sripunya et al., 2010). In contrast, the longer sheet used in the MVAC method (60 vs. 20 mm) can hold as many as 25 oocytes or blastocysts, according to our preliminary study (unpublished data).

Many viral and bacterial agents can survive in LN_2 and be transmitted into cryopreserved and banked embryos (Bielanski et al., 2003; Bielanski, 2005). Potential sources of contamination during freezing are the cryopreserved samples and LN_2 themselves (Bielanski, 2012). For example, when an infected embryo is stored in LN_2, cross-contamination between it and LN_2 may occur. Also, as water evaporates, it cools and freezes above the LN_2 tank, forming small ice crystals with a high electrostatic charge. These ice crystals can capture airborne microorganisms, which in turn, fall into the tank (Morris, 2005; Grout and Morris, 2009). Pessoa et al. (2014) reported that up to 84.3% of farms and 100% of companies in Southern Brazil use

LN_2 contaminated with bacteria, fungi, or both. Therefore, an alternative method for cryopreservation that avoids exposing samples directly to LN_2 is required. In this study, the MVAC method without LN_2 contact was shown to be as effective as the MVAC in LN_2 and Cryotop methods, in which samples had direct contact with LN_2. Because the actual heat transfer in the MVAC device was not investigated in Experiment I (because of the closed carriers), the MVAC device was employed in two separate methods to determine which method provided greater heat exchange during cooling. The results suggest that heat exchange and cooling rates associated with direct plunging of the device into LN_2 (MVAC in LN_2) and without direct contact between the device and LN_2 (MVAC) did not differ significantly from each other. A previous report on porcine embryos produced *in vivo* also showed equal efficiency between the MVAC and Cryotop vitrification methods (Misumi et al., 2013). However, because the stainless steel sheet of the MVAC device is non-transparent, loading a small volume of the vitrification solution (<1 μl) requires extensive skills.

In this study, a toxicity test of the vitrification solution showed that treatment with CPAs did not affect the developmental competence of vitrified IVM oocytes. Similar results have been reported previously in bovine IVM oocytes (Martino et al., 1996; Dinnyes et al., 2000; Sripunya et al., 2010).

We achieved relatively high rates of development of vitrified-warmed IVM oocytes to the blastocyst stage, ranging from 20% to 25.5% for all vitrification methods. In general, higher cooling rates are expected to be achieved by open system methods, such as Cryotop and MVAC in LN_2, than by closed system methods, such as MVAC, because conductive heat transfer is very rapid in LN_2 (Liebermann and Tucker, 2002). However, our results showed similar developmental competence of vitrified-warmed IVM oocytes to the blastocyst stage in all vitrification groups

($P<0.05$). These results indicate that all of the cooling systems investigated in this study are equally effective for cryopreservation of bovine IVM oocytes. Moreover, for the MVAC device, recovery rates after warming of both bovine IVM oocytes and IVP expanded blastocysts were nearly 100% and did not significantly differ from those of the standard Cryotop method. Recovery rates for the MVAC device in our study were higher than those measured in a previous study of vitrified mouse oocytes, in which recovery rates were 62% in an open pulled straw (OPS) and 81% in a 0.25-ml plastic straw (Chen et al., 2000). Abdelhafez *et al.* (2011) reported lower recovery rates in mouse embryos vitrified by the Cryotip method than we report here; their recovery rates were 85% for the cleavage stage and 75% for the blastocyst stage. In this study, cleavage rates of vitrified-warmed bovine IVM oocytes after IVF did not differ significantly between the treatment groups, ranging from 53% to 56%; these rates are higher than cleavage rates previously reported by Vajta et al. (1998) (47–50%) using the OPS method. Similarly, Sripunya et al. (2010) reported that the cleavage rate of vitrified-warmed bovine IVM oocytes was 41%. Our findings suggest that bovine IVM oocytes vitrified by the MVAC method, avoiding direct contact between the samples and LN_2, can be fertilized with equal efficiency as oocytes vitrified by other standard methods that allow direct contact with LN_2 (Cryotop and MVAC in LN_2). Furthermore, we found similar blastocyst rates and quality (as measured by the numbers of TE cells and ICM cells, as well as by the total numbers of nuclei in the blastocysts) between the MVAC, MVAC in LN_2, and Cryotop groups.

The results of our comparison study of the effect of cryodevices on the survival of IVP expanded blastocysts after vitrification-warming in experiment II showed that there was no significant difference between the Cryotop, MVAC, MVAC in LN_2, and Solution control groups ($P<0.05$). However, the rate of hatched blastocysts after

warming and 24 h of IVC was lower in the MVAC in LN_2 group than in the Solution control group. The reasons for this low hatched rate in the MVAC in LN_2 group are not clear; however, it may be partially explained by the difficulty of controlling the vitrification solution volume (<1 μl) in the MVAC device. Indeed, a higher volume of vitrification solution, which acts as an insulator surrounding the blastocysts, has been reported to result in decreased cooling viscosity and heat transfer (Arav et al., 2002; Kuwayama, 2007). However, the lower blastocyst viability in our MVAC in LN_2 group was slight; later, at 48 and 72 h after warming, there were no significant differences in the rates of hatched blastocysts between the groups. Although direct comparison between our results and results obtained by previous studies is impossible, our results showed higher survival rates for vitrified-warmed bovine blastocysts treated with the Cryotop method than those previously reported using the same Cryotop method: 75% (De Rosa et al., 2007), 81.9% (Min et al., 2013) and 47.6% (Morató et al., 2014).

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3.8 Conclusion

In conclusion, the results of this study demonstrate that the Cryotop and MVAC cooling systems are equally effective for vitrification of both bovine IVM oocytes and bovine IVP embryos at the expanded blastocyst stage, resulting in high survival and developmental rates. Thus, we suggest that the MVAC system is a new, useful method for vitrification of both bovine IVM oocytes and bovine IVP embryos. Our results also show that the modified MVAC device used in the MVAC method may address biosafety concerns by serving as a closed carrier system that prevents exposure to

LN_2. However, it is necessary for future studies to investigate live calf production from oocytes and embryos vitrified by the MVAC method.

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CHAPTER IV

A COMBINED-FACTORS APPROACH TO SUCCESSFUL VITRIFICATION OF *IN VITRO* PRODUCED BOVINE BLASTOCYSTS BY USING 0.25 ML STRAWS

4.1 Abstract

This study was designed to improve the *in vitro* survival of vitrified IVP bovine blastocysts using the 0.25 mL straw method. The combined factors have been investigated to improve the efficiency of vitrification with the designed straw columns. Experiment 1 was done to evaluate the effect of sucrose concentration in diluting solution of the *in vitro* survival of vitrified IVP bovine blastocysts using the 0.25 mL straw method, IVP bovine blastocysts were exposed to VS1 and then were loaded into 0.25 mL straws each containing three diluting solutions (0 M, 0.2 M and 0.5 M sucrose) before vitrification. Their *in vitro* survival rates of vitrification-warming approach after 24−72 h of culture showing no significant difference among 0 M, 0.2 M and 0.5 M of sucrose solution groups (30−70%, 34.7−66.6% and 35.1−68.9%, respectively); whereas, they were significantly lower when compared with fresh control group ($P<0.05$). Regarding Experiment 2, after vitrification IVP bovine blastocysts using the 0.25 mL straw method, then the straws were sealed and then immersed with the LN_2 or LN_2 vapor method before plunging into LN_2. No significant differences were found in the *in vitro* survival rates between the two methods. For Experiment III, it was designed to compare the efficiency of solutions between VS1 and VS2 that were used to vitrification of IVP bovine

blastocysts by using the 0.25 mL straw method. There was no significant difference in the *in vitro* survival rates between the two groups. In terms of Experiment IV, when IVP bovine blastocysts were vitrified by using the Cryotop or 0.25 mL straw method, there was no significant difference in the *in vitro* survival rates at 24 h of additional IVC between the two methods. However, the *in vitro* survival rates at 48 and 72 h of culture in the Cryotop were higher than those of vitrification using the 0.25 mL straw method. Our results indicated that the use of our straw columns with the optimum combined factors was effective for vitrified IVP bovine blastocysts and it allowed the one step dilution after warming. This could simplify the embryo transfer on the farm.

4.2 Introduction

A 0.25 mL French semen straw or 0.25 mL straw has become the standard use for freezing and transferring embryos at the industry level (Wright, 1985). It has been especially convenient to freeze bovine embryos since the first success of a one step dilution method of bovine embryos has been developed (Leibo, 1984). The one step dilution procedure started with freezing single bovine embryo in one straw. Subsequently, the individual embryo was diluted and warmed in the straw in sucrose solution before being transferred into the recipients. Thereafter, several studies demonstrated the one step dilution method without requiring several steps for cryoprotectants (CPAs) dilution after warming in a laboratory setting (Massip et al., 1987; Voelkel and Hu, 1992; Dochi et al., 1998). This method is commonly used for bovine embryos transfer in the field and it achieved the pregnancy rates slightly less than fresh embryos (Leibo and Mapletoft, 1998).

Although 0.25 mL straws are useful for direct transfer through one step dilution of freezing bovine embryos, they are limited to apply for slow freezing technique (Massip et al., 1987, 1995; Voelkel and Hu, 1992; Dochi et al., 1995, 1998). However, several studies indicated that rapid freezing or vitrification was more proper than slow freezing; because the higher survival rates of *in vitro* produced bovine (IVP) embryos would be the one reason (Leibo and Loskutoff, 1993; Pollard and Leibo, 1994; Agca et al., 1998; Vieira et al., 2007; Rodriguez-Villamil et al., 2012; Caamaño et al., 2015). Moreover, this technique provided rapid, simple and inexpensive procedures (Agca et al., 1994).

Early vitrification experiments have not been widely adopted for commercial use for bovine embryo transfer (Van Wagtendonk-de Leeuw et al., 1997; Vijta, 2000). Therefore, vitrification procedure required several steps for CPAs dilution in laboratory setting due to their toxicity at high concentration of CPAs (Ishimori et al., 1993). Accordingly, the vitrification method of direct transfer to recipients should be developed to simplify this technology for farming applications. With this idea, vitrification of IVP embryos should go through warming and diluting procedures in one step.

Previously, two techniques have been widely used for one step dilution after warming the vitrified IVP bovine embryos: the 0.25 mL straws and the additional devices combined with the 0.25 mL straws. For the first technique, 0.25 mL straws is used for vitrification, followed by warming procedure (Mahmoudzadeh et al., 1995; Saha et al., 1996; Vajta et al., 1996; Ohboshi et al., 1997; Agca et al., 1998; Donnay et al., 1998; Sommerfeld and Niemann, 1999; Pugh et al., 2000; Campos-Chillòn et al., 2006; Akiyama et al., 2010, 2012; Inaba et al., 2011; Na Ha et., 2014). For the second technique, the additional devices is used for vitrification before reloading vitrified

embryos into the plastic straws for warming and diluting CPAs. They include open-pulled straws (OPS) (Vajta et al., 1999), hand-pulled glass micropipettes (GMP) (Vieira et al., 2007), Cryotop (Inaba et a., 2011), Fyberplug with solid surface vitrification (Rodriguez-Villamile et al., 2013; Caamaño et al., 2015) and VitTrans (Morató and Mogas, 2014).

The additional devices are used to gain the highest cooling rate because the higher heat was transferred than that of plastic straws before inserting into plastic straws for diluting and warming (Rodriguez-Villamil et al., 2013). However, using 0.25 mL straws as a vitrification device is still attractive, because this device is commonly used with 0.25 mL transfer gun in the field condition. Therefore, it is not complicated and it simplifies the transfer procedure of vitrification embryos as a basic technique for artificial insemination (Taniguchi et al., 2007; Vajta et al., 1997) because the requirement of involved method in introducing vitrification devices into 0.25 mL straws for warming and diluting is not necessary.

The objective of this study was to improve the efficacy of the 0.25 mL straws for vitrification of IVP bovine blastocysts. Therefore, we investigated the combined factors that affected the survival rates after warming of vitrified IVP bovine embryos in the expanding blastocyst stage using 0.25 mL straws in the sucrose concentration of diluting solution; the immersion methods and the vitrification solutions. Finally, the 0.25 mL straw was compared with the standard Cryotop in terms of the efficacy of IVP bovine blastocyst vitrification.

4.3 Materials and Methods

4.3.1 Chemicals and media

Except where otherwise indicated, all chemicals were purchased from Sigma-Aldrich, Co (St. Louis, MO, USA).

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

The slaughterhouse ovaries of Holstein cows were washed and stored in physiological saline (0.9% NaCl) during transportation to the laboratory at room temperature. Cumulus oocyte complexes (COCs) were collected from follicles (2−8 mm in diameter) using a 10-ml syringe connecting with a 18-gauge needle. The IVM procedure was performed as previously described (Parnpai et al., 1999). Briefly, a group of 20 to 25 COCs was washed five times in modified Dulbecco's phosphate buffer saline (mDPBS) supplemented with 0.1% polyvinyl pyrolidone (PVP). Groups of 20-25 COCs were washed three times with 100-µl droplets of IVM medium, before being cultured for 22 h in 100-µl droplets of IVM that was covered with mineral oil under humidified atmosphere with 5% CO_2 in air at 38.5ºC. The IVM medium consisted of TCM199 supplemented with 10% fetal bovine serum (FBS; Life Technologies Inc Gibco-BRL Division, Grand Island), 50 IU/ml hCG (Intervet UK Ltd., Milton Keynes, UK), 0.02 AU/ml FSH (FSH, Antrin®, Denka Phamaceutical Co., Kanagawa, Japan) and 1 µg/ml 17ß-estradiol.

4.3.3 *In vitro* fertilization (IVF)

The frozen semen was thawed in 37 °C water for 30 sec. Then, the thawed sperm was layered under 2 mL of TALP (Tyrode's albumin lactate pyruvate; Lu et al., 1987) medium in a 5 mL snap tube and incubated at 38.5°C under humidified atmosphere

with 5% CO_2 in air for 40 min to allow live spermatozoa to swim up. The top 1.5 ml from each tube was removed, pooled in a 15 ml conical tube centrifuged at 400 × g for 7 min. The pellets were resuspended in TALP medium to adjust the final concentrations of 2 × 10^6 spermatozoa/ml. One hundred µl drops of sperm suspension were prepared in culturing dish, covered with mineral oil and used as fertilization droplets. The groups of 20–25 oocytes were washed three times in TALP medium. After that, the oocytes were added into 100 µl of fertilization droplets and cultured for 12 h at 38.5 °C under humidified atmosphere with 5% CO_2 in air.

4.3.4 *In vitro* culture (IVC)

IVC was performed in 100 µl droplets of mSOF medium supplemented with 3 mg/ml BSA covered with mineral oil. At the end of insemination, putative zygotes were completely denuded from cumulus cells and spermatozoa by gently pipetting with a fine glass pipette in pre-incubated IVC medium. Twenty to twenty five zygotes were placed in each culture drop and then were cultured at 38.5°C under humidified atmosphere with 5% O_2, 5% CO_2 and 90% N_2 for 2 days. Thereafter, embryos at the 8-cell stage were selected and co-cultured with bovine oviduct epithelium cells (BOEC) in mSOF medium under humidified atmosphere with 5% CO_2 in air at 38.5°C for 5 days (Parnpai et al., 1999). Half volume of mSOFaa was replaced daily and the development of embryos was recorded (Day 0 was defined as the day of IVF).

4.3.5 Blastocyst vitrification and warming using 0.25 mL straws

IVP expanded blastocysts (IETS quality code 1, stage 7) were washed three times in HM consisting of DPBS supplemented with 20% (v/v) FBS. The expanding blastocysts were then placed in equilibration medium, which was composed of HM

supplemented with 7.5% Ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO) for 3 min, and then transferred into a vitrification solution comprising HM supplemented with 16.5% EG, 16.5% DMSO, and 0.5 M sucrose (VS1) or placed in pre-equilibration medium 1, which consisted of mDPBS supplemented with 10% glycerol (Gly), 0.1 M Xylose, 0.1 M Sucrose and 0.1% BSA for 5 min, after that they were transferred into pre-equilibration medium 2, which contained mDPBS supplemented with 10% Gly, 10% EG, 0.2 M Xylose, 0.2 M Sucrose and 0.2% BSA for 2 min. After the pre-equilibration step, blastocysts were exposed to vitrification solution that consisted of mDPBS supplemented with 10% Gly, 30% EG, 0.3 M Xylose, 0.3 M Sucrose and 0.3% BSA (VS2) (depending on the experiments). Subsequently, blastocysts (one blastocyst per straw) were then pipetted into 0.25 mL straws and placed in the inner surface of straws (Fig. 1). The straws contained diluting solution which was HM supplemented with three different sucrose concentrations (0 M, 0.2 M and 0.5 M groups). The loaded straw was immediately heat-sealed and the part of straw containing blastocysts was placed vertically into LN_2 for 1 min (LN_2 group) or placed horizontally above LN_2 about 1-1.5 cm for 1 min to get the cool from LN_2 vapor (LN_2 vapor group) before immersing in LN_2 for storage (depending on the experiment). The process of blastocysts exposure to vitrification solution and their immersion in LN_2 was completed within 30 sec. All vitrification procedure was performed at room temperature (24-26 ºC). After being stored for at least 24 hrs in LN_2 tank, the straws containing embryos were warmed by exposing in the air for 10 sec and then plunged into the 35 ºC water to warm the diluents for 20 sec. Straws were then removed from the water and shaken to mix the columns of the straws. After that, the straws were vertically held in the same water at 35 ºC for 5 min. Thereafter, the

blastocysts were transferred into culturing dishes and cultured *in vitro* for 72 h in TCM-199 supplemented with 20% FBS and 0.1 mM ß-merceptoethanol at 38.5°C in humidified atmosphere with 5% CO_2 to evaluate the survival of embryos after being thawed (Saito et al., 1994).

4.3.6 Blastocyst vitrification and warming using Cryotop

IVP expanded blastocysts (IETS quality code 1, stage 7) were exposed to VS1 as described above. Then, a group of 5 blastocysts was placed on a Cryotop sheet in a small volume of vitrification solution (<1 µl) and then the Cryotop was plunged into LN_2. The process of exposure of the blastocysts to vitrification solution and their immersion in LN_2 was completed within 30 sec. All vitrification procedure was performed at room temperature (24-26 ºC). After being stored for at least 24 h in LN_2 tank, vitrified blastocysts were warmed by inserting the Cryotop sheet into HM at 38.5 ºC for 5 min. After washing them three times in HM, they were transferred into culturing dishes and cultured *in vitro* for 72 h at 38.5 ºC in humidified atmosphere with 5% CO_2 in air with the same medium used in 0.25 mL straw method to evaluate the survival of embryos after being thawed.

4.3.7 Evaluation of blastocyst cell numbers with different staining

Staining inner cell mass (ICM) and trophectoderm (TE) nuclei in blastocysts was performed as previously described (Thouas et al., 2001), with slight modifications. Briefly, the blastocysts were simultaneously treated with 0.1 mg/ml propidium iodide (PI) and 0.2% Triton X-100 dissolved in DPBS for 60 sec to permeate the membrane and stain the nuclei of TE cells. The blastocysts were then treated for 5 min with 25 µg/ml Hoechst 33342 (Calbiochem, San Diego, CA, USA) dissolved in 99.5% ethanol, and then

mounted on glass slides in glycerol droplets that were flattened with cover slips. The blastocysts were examined under UV light with an excitation wavelength of 330-385 nm using an epifluorescence microscope (IX-71; Olympus Corporation, Tokyo, Japan). The nuclei of TE cells labeled with both PI and Hoechst 33342 became pink or red, whereas the nuclei of ICM cells labeled only with Hoechst 33342 became blue. A digital image of each blastocyst was captured, and the cell numbers of both cell types were counted using the NIH ImageJ (v. 1.40) software (Abramoff et al., 2004). With clearly distinguishable population of red and blue nuclei, numbers of ICM and TE cells in blastocysts, were counted separately.

4.4 Experimental design

Experiment 1: Effects of sucrose concentrations of diluting solution on the *in vitro* survival after vitrification-warming approach of IVP bovine blastocysts using 0.25 mL straws

This experiment was to examine the effects of sucrose concentrations for one step dilution on the *in vitro* survival after vitrification-warming IVP bovine blastocysts using 0.25 mL straws. IVP bovine expanded blastocysts (IETS quality code 1, stage 7; n = 288) were exposed to VS1 and then were loaded into 0.25 mL straws which contained one of three groups of diluting solution (0 M, 0.2 M and 0.5 M sucrose). After being sealed, the parts of loaded straws containing blastocysts were placed vertically into LN_2 for 1 min before immersing in LN_2 for storage. Blastocysts that were not vitrified served as a fresh control group. Thereafter, the straws containing blastocysts were warmed and the *in vitro*

survival rates after being vitrification-warming was assessed by their hatching ability to hatch blastocyst stage during 72 h of additional IVC.

Experiment 2: Effects of immersion methods on the *in vitro* survival after vitrification-warming bovine blastocysts using 0.25 mL straws

The effects of immersion methods were examined by exposing IVP bovine expanded blastocysts (IETS quality code 1, stage 7; n = 150) to VS1 and then they were loaded into 0.25 mL straws which contained diluting solution (0 M sucrose). After sealing the straws, they were randomly divided into two groups. For the first group, the parts of straws containing blastocysts were placed vertically into LN_2 for 1 min (LN_2 group). For the second group, they were placed horizontally above LN_2 about 1-1.5 cm for 1 min to get the cool from LN_2 vapor (LN_2 vapor group). Blastocysts that were not vitrified served as a fresh control group. The *in vitro* survival rates after vitrification-warming were assessed. They were similar to Experiment I which was described above.

Experiment 3: Effects of vitrification solutions on the *in vitro* survival after vitrification-warming IVP bovine blastocysts using 0.25 mL straws

The effects of vitrification solutions which were examined by IVP bovine expanded blastocysts (IETS quality code 1, stage 7; n = 314) were exposed to VS1 or VS2 and vitrified using 0.25 mL straws which contained 0 M sucrose of diluting solution. After sealing the straws, the parts of straws containing blastocysts were placed vertically into LN_2 for 1 min before immersing in LN_2 for storage. Blastocysts that were not vitrified served as a fresh control group. The *in vitro* survival rates after vitrification-warming were assessed. They were similar to those of Experiment I and II which were described above.

Experiment 4: Effects of cryodevices on the *in vitro* survival after vitrification-warming IVP bovine blastocysts

This experiment was to compare the efficiencies of the cryodevices for the vitrification of IVP bovine blastocysts. IVP expanded blastocysts (IETS quality code 1, stage 7; n = 315) were exposed to VS1, and then randomly vitrified by 0.25 mL straws which contained 0 M sucrose of diluting solution (0.25 mL straws groups) or vitrified by the Cryotop method (Cryotop group). For the hatching ability during 72 h of additional IVC, the cell numbers and ICM ratio of survival blastocysts at 72 h in each treatment group were compared.

4.5 Statistical analysis

The percentages of embryos hatching rates in each group were subjected to arcsine transformation. Cell numbers of embryos were expressed as untransformed means ± standard error of the mean (SEM). Data were analyzed by the analysis of variance (ANOVA). Differences were considered to be significant when $P<0.05$.

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4.6 Results

4.6.1 Experiment 1

There were no different rates of development in hatching blastocyst stage during 72 h of additional IVC after verifying and warming IVP bovine expanded blastocysts in each concentrated sucrose dilution (0 M, 0.2 M and 0.5 M sucrose) which was loaded into 0.25 mL straws. However, the rates of hatching embryos during 72 h of additional IVC in all vitrified groups were significantly lower than those of fresh control group ($P<0.05$) (Table 1).

4.6.2 Experiment 2

As shown in Table 2, there were no different rates of embryos developing in the hatching blastocyst stage during 72 h of additional IVC after warming and vitrification of IVP bovine blastocysts during the immersion methods (LN_2 vs. LN_2 vapor groups). However, the hatching rates in the vitrified groups were significantly lower than the fresh control group (P<0.05).

4.6.3 Experiment 3

The rates of development in hatching blastocysts stage of vitrification-warming expanded blastocysts during 72 h of additional IVC between the blastocysts that were exposed to VS1 or VS2 groups were not different. However, there were lower rates between groups of vitrified-warmed IVP bovine blastocysts and fresh control groups during 72 h of additional IVC at significant differences (P<0.05) (Table 3).

4.6.4 Experiment 4

As shown in Table 4, there were no differences in the rates of embryos developing to the hatching blastocyst stage at 24 h of additional IVC between the Cryotop or 0.25 mL straw methods. However, the rates of hatching blastocysts at 48 and 72 h of culture in the Cryotop were higher than those of the 0.25 mL straw methods (77.5% and 92.5% vs. 65.3% and 80.8%, P<0.05; respectively).

The total number of nuclei and ICM ratios of the blastocysts survival between the Cryotop and 0.25 mL straw groups after vitrification-warming approach obtaining 72 h of additional IVC was not different. Similarly, no significance was found in total number of nuclei and ICM ratio obtaining 72 h of additional IVC in both devices and *in vitro* culture for 9 days (fresh control group).

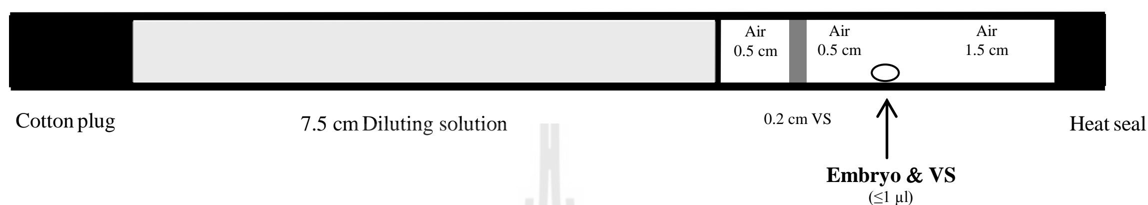

Figure 4.1 The loading columns in 0.25 mL straw. From cotton plug to heat seal: 7.5 cm of diluting solution, 0.5 cm of air, 0.2 cm of vitrification solution was loaded into the straws to prevent the embryo diluted in diluting solution. Then they were removed before petting the embryo with vitrification solution (≤1 µl) into the inner surface of the straws. The rest of the straw is filled with 1.5 cm of air before being sealed by heat *(Petty patent: 9367, Parnpai et al., 2014).*

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Table 4.1 Effects of sucrose concentrations of diluting solution on *in vitro* development of vitrified-warmed IVP bovine blastocysts using the 0.25 mL straw method

Treatment group	No. of embryos	No. of hatching embryos (%)		
		24 h	48 h	72 h
0 M Sucrose	70	21^{b} (30.0%)	37^{b} (52.8%)	49^{b} (70.0%)
0.2 M Sucrose	72	25^{b} (34.7%)	38^{b} (52.7%)	48^{b} (66.6%)
0.5 M Sucrose	74	26^{b} (35.1%)	39^{b} (52.7%)	51^{b} (68.9%)
Fresh Control	72	45^{a} (62.5%)	68^{a} (94.4%)	70^{a} (97.2%)

Seven replications were performed. [a,b]Values within a single column having different superscripts were significantly different at $P<0.05$ using one-way ANOVA. 0 M Sucrose: IVP expanded blastocysts were vitrified by 0.25 mL straw containing sucrose-free in diluting solution. 0.2 M Sucrose: IVP expanded blastocysts were vitrified by 0.25 mL straws containing 0.2 M sucrose in diluting solution. 0.5 M Sucrose: IVP expanded blastocysts were vitrified by 0.25 mL straws containing 0.5 M sucrose in diluting solution.

Table 4.2 Effects of immersion methods on the *in vitro* development of vitrified-warmed IVP bovine blastocysts using the 0.25 mL straw method

Treatment group	No. of embryos	No. of hatching embryos (%)		
		24 h	48 h	72 h
LN_2	50	10^b (20.0%)	26^b (52.0%)	32^b (64.0%)
LN_2 vapor	50	11^b (22.0%)	28^b (56.0%)	34^b (68.0%)
Fresh Control	50	20^a (40.0%)	40^a (80.0%)	49^a (98.0%)

Five replications were performed. [a,b]Values within a single column having different superscripts were significantly different at $P<0.05$ using one-way ANOVA. LN_2 group: IVP expanded blastocysts were vitrified by 0.25 mL straw, after the straws were sealed, the parts of straws containing blastocysts were placed vertically into LN_2 for 1 min before being immersed in LN_2 for storage. LN_2 vapor group: IVP expanded blastocysts were vitrified by 0.25 mL straw, after the straws were sealed, they were placed horizontally above LN_2 about 1-1.5 cm for 1 min to achieve the cool from LN_2 vapor before being immersed in LN_2 for storage.

Table 4.3 Effects of vitrification solutions on *in vitro* survival after vitrification-warming IVP bovine blastocysts using 0.25 mL straws

Treatment group	No. of embryos	No. of hatching embryos (%)		
		24 h	48 h	72 h
VS1	105	35^{6} (33.33%)	69^{b} (60.00%)	87^{b} (82.85%)
VS2	104	41^{b} (39.42%)	68^{b} (65.38%)	84^{b} (80.76%)
Fresh control	105	61^{a} (58.10%)	83^{a} (79.05%)	102^{a} (97.14%)

Twelve replications were performed. ^{a,b}Values within a single column having different superscripts are significantly different at $P<0.05$ using one-way ANOVA. VS1: IVP expanded blastocysts were exposed to VS1 (EG-DMSO) before being vitrified using 0.25 mL straws. VS2: IVP expanded blastocysts were exposed to VS2 (EG-Gly) before being vitrified using 0.25 mL straws.

Table 4.4 Development to the hatching blastocysts stage of IVP vitrified bovine blastocysts using the Cryotop or 0.25 mL device and cultured *in vitro* for 72 h

Treatment method	No. of embryos	No. of hatching			No. of nuclei ± SEM		
		24 h	48 h	72 h	No. of embryos	Total	ICM cells (%)
Cryotop	107	49^{ab} (45.79%)	83^{a} (77.57%)	99^{a} (92.52%)	10	114±1.54	32±1.21
0.25 mL straw	104	41^{b} (39.42%)	68^{b} (65.38%)	84^{b} (80.76%)	11	110±1.91	30±1.37
Fresh control	105	61^{a} (58.10%)	83^{a} (79.05%)	102^{a} (97.14%))	10	116±1.00	33±1.20

Twelve replications were performed. ^{a,b}Values within a single column having different superscripts are significantly different at $P<0.05$ using one-way ANOVA. Cryotop: IVP expanded blastocysts were exposed to VS1 before being vitrified using Cryotop device. 0.25 mL straw: IVP expanded blastocysts were exposed to VS1 before being vitrified using 0.25 mL straws device. No. of nuclei were presented as mean ± SEM. Total: total no. of nuclei in blastocysts. ICM: inner cell mass. No significant differences in total no. of nuclei and ICM ratio in blastocysts were detected between the treatment groups at $P<0.05$ using one-way ANOVA.

4.7 Discussions

In this study, it was demonstrated that our straw columns used in combination with the optimal factors can be successful cryopreservation of IVP bovine blastocysts. We achieved highest survival rates of vitrified-warmed IVP bovine blastocysts with 0.25 mL straw method that were assessed by their development competence in hatching blastocyst stage at 24 to 72 h of additional of IVC, ranging from 33.3% to 82.8%. The 0.25 plastic straw in our study has been designed to reduce the volume of vitrification solution less than 1 µl to achieve the high cooling rates for vitrification, due to minimum volume of vitrification solution which is one of the three factors improving the probability of vitrification by increasing the cooling rates and reducing the probability of ice formation (Arav, 2014).

The efficiency of our straw columns that were used in combination with the optimal factors was investigated. Sucrose solution was used as an osmotic buffer to prevent the rapid hydration leading to over swelling embryos during rehydration and removal of CPAs of the cells which was first introduced by Leibo and Mazur (1978). In this study, 0.2 M and 0.5 M sucrose solutions were used to compare with sucrose-free (0 M sucrose) in diluting solution for one step dilution during warming process. The results suggested that sucrose solution comprising two permeable CPAs (16.5%EG + 16.5%DMSO) as the vitrification solution did not affect the survival ability of embryos (Table 1). In contrast, a previous study reported that the sucrose solution, which was required to improve the survival ability of vitrified-warmed IVP bovine blastocysts, was the two combination of permeable CPAs such as EG and propanediol (PROH) in vitrification solution (Rodriguez-villamil et al., 2013). Besides, they found the survival ability did not improve

when vitrified embryos warmed and diluted in one step with sucrose solution with only one permeable CPA such as EG during vitrification. Similar reports showed the vitrification with EG as only one permeable CPA in equine (Hochi et al., 1994) and bovine embryos (Mahmouzadeh et al., 1995; Saha et al., 1996; Campos-Chillon et al., 2006). However, Inaba et al. (2011) reported the same corresponding results as those of our current study, which did not show the effect on the survival ability of vitrified IVP bovine blastocysts of sucrose in diluting solution comprising 16.5% EG and 16.5% DMSO in vitrification solution. Similarly, Ohboshi et al. (1997) reported that sucrose solution did not help improve survival ability after warning IVP bovine blastocysts vitrified in the solution with the combination of two permeable CPAs like EG and polyethylene glycol (PEG).

It is unclear for the addition of sucrose in diluting solution that affected in different ways on the survival ability of vitrified IVP bovine embryos in various combinations of two permeable CPAs. However, it can be assumed that the components affecting two permeable CPAs were related to the requirement of sucrose solution during warming process. In addition, the characteristic of expanded blastocysts that were used in our study and suggested in a previous study showed greater cryosurvivals due to their more tolerance to an osmotic shock after warming. Therefore, there are low surface-to-volume ratio when compared with embryos at the blastocyst stage, resulting in slower rehydration after warming (Camargo et al., 2011). These indicated that sucrose had no effect on embryo survival after vitrification-warming bovine embryos at the expanded blastocyst stage using the diluting solution comprising 16.5% EG and 16.5% DMSO and following one step dilution in 0.25 mL straw.

The fractures of zona or embryos during the immediate plunge of the straw into LN_2 could occur because of the rapid change of the pressure leading to a fast shrinkage and air bubbles which expanded during the changes of temperature (Vajta et al., 1997a). Therefore, the immersion method which was placing the straws into LN_2 vapor before plunging into LN_2 has been introduced to prevent the fractures of zona or embryos (Rall and Meyer, 1989). However, our results showed an equal efficiency between the two immersion methods for survival ability after warming and vitrifica IVP bovine blastocysts using 0.25 mL straws. This corresponding result was found in Donnay et al. (1998).

Vitrification requires a high concentration of CPAs to avoid the ice-crystals formation and achieve glass transition (Jain and Paulson, 2006). However, the high concentration of CPAs used for vitrification was damaged. It caused cells to have osmotic stress or chemical toxicity (Arav, 2014). These effects can be minimized: (i) stepwise addition of CPAs in vitrification solution (Saha and Suzuki, 1997); (ii) addition of non permeable CPAs such as sugar (Fahy et al., 1984); (iii) decrease the concentration of CPAs and expose the embryos to vitrification solution at low temperatures (Rall et al., 1987); (iv) use the low toxicity of CPAs (Voelkel and Hu, 1992; Kasai et al., 1996); (v) using a mixture of two or more CPAs (Massip et al., 1987). Among most of these methods, the low toxicity of CPAs like EG (Newton and Subramoniam, 1996) is used for embryos as a standard component of most successfully vitrification solutions (Kasai and Mukaida, 2004).

Although EG has high penetrating rates into the cells (Dochi et al., 2006) due to lower molecular possession than the other permeables including glycerol, DMSO and

propylene glycol (62.07 vs. 92.07, 78.13 and 76.10, respectively), it has poor glass forming tendency (Mullen and Critser, 2007). It has been proposed that the mixtures of EG with other CPAs which have greater glass forming characteristic like DMSO or EG (Ali and Shelton, 1993) can achieve the glass-forming tendency, avoid devitrification (MacFarlane et al., 1992), and reduce risks of chemical toxicity of any one permeable CPA (Rall and Fahy, 1985).

To our knowledge, this is the first report to compare the efficacy of vitrification solution which is composed of the mixtures of CPAs between EG-DMSO (VS1) and EG-Gly (VS2) used for vitrification of IVP bovine blastocysts using 0.25 mL straw method. This study suggested the equal efficiency of both mixtures of CPAs used for vitrification of IVP bovine blastocysts using 0.25 mL straws. Our results, however, were slightly lower than those obtained in the previous study, which vitrified IVP bovine blastocysts in the same vitrification solution that was composed of the mixture of EG-Gly in the 0.25 mL straws (Inaba et al., 2011). Nevertheless, the highest *in vitro* survival rates of 82.8% of vitrified IVP bovine blastocysts achieved from vitrification solution comprising the mixture of EG-DMSO in our study are higher than those of previous reports in which the *in vitro* survival rates were 69% at 72 h of additional IVC (Mahmoudzadeh et al., 1995), 63% at 72 h of additional IVC (Vajta et al., 1996), 70% at 72 h of additional IVC (Saha et al., 1996) and 37.1% at 24 h of additional IVC (Na ha et al., 2014).

To investigate the efficiency of our straw columns, IVP bovine blastocysts that were vitrified with the 0.25 mL straw method were compared with those of blastocysts vitrified with the standard Cryotop method (Experiment IV). Our results showed the lower rates of *in vitro* survival after vitrification-warming IVP bovine blastocysts using

our straw columns than those of blastocysts that vitrification-warming by the standard Cryotop method at 48 and 72 h of additional IVC (65.3% and 80.8% vs. 77.5% and 92.5%, $P<0.05$; respectively). These results suggested that the Cryotop method provided a higher efficiency than 0.25 mL straw method for cryopreservation obtained with open system devices, such as The Cryotop of which the cooling rate was reported approximately 20,000 ºC /min (Kuwayama et al., 2005), while the close system devices such as the 0.25 mL straw of which the maximum cooling rate was limited at 2500 ºC/min (Shaw et al., 1991. However, we found the equal qualities when measured by ICM ratios and the total numbers of nuclei in the blastocysts between the IVP bovine blastocysts that were vitrified by using Cryotop and 0.25 mL methods.

4.8 Conclusion

In conclusion, the uses of our straw columns in combination with the optimum factors are effective for vitrified IVP bovine blastocysts using the 0.25 mL straw method. Therefore, we suggest that this method is allowed the one step dilution after warming vitrified IVP bovine embryos of which the transfer can be simplified on the farm to the same level of artificial insemination.

4.9 References

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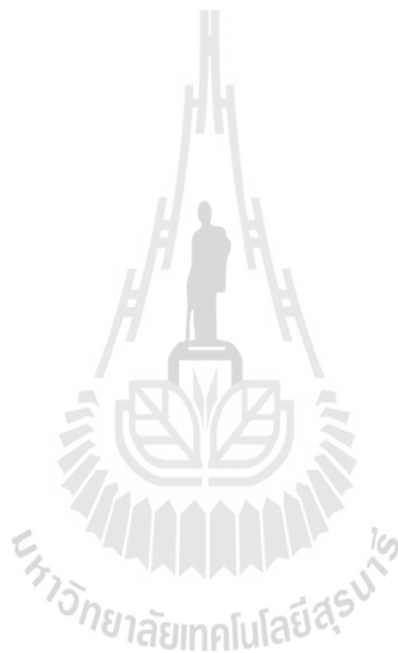

CHAPTER V

THE EFFECTS OF BIOPSY WITH MICROBLADE PRIOR TO VITRIFIED USING THE CRYOTOP OR 0.25 ML STRAW METHODS ON *IN VITRO* SURVIVAL AFTER VITRIFICATION-WARMING

5.1 Abstract

This study was designed to evaluate the effects of biopsying with microblade prior to vitrification using the Cryotop or 0.25 mL straw methods on their *in vitro* survival after vitrification-warming. *In vitro*-produced (IVP) bovine blastocysts were subjected to biopsy with microblade (biopsied-derived blastocyst groups) and were not subjected to biopsy (non-biopsied derived blastocyst groups). Thereafter, both of biopsied and non-biopsied derived blastocyst groups were vitrified using the Cryotop (biopsied + Cryotop and non-biopsied + Cryotop groups) or 0.25 mL straw methods (biopsied + 0.25 mL straw and non-biopsied + 0.25 mL straw groups). After vitrification-warming, the rates of embryos developing to the hatching blastocyst stage at 24 h of additional IVC in biopsied- derived blastocyst were higher than those of non-biopsied derived blastocyst groups. However, the *in vitro* survival rates of biopsying + 0.25 mL straw group had no significant difference than those of vitrified and freshly derived from non biopsied blastocysts. After 48-72 h of culture, the rates of development to hatching blastocyst stage between the vitrification of non-biopsied

derived blastocysts using the Cryotop and 0.25 mL straws devices were not different. Similarly, they did not differ from the rates of fresh control group at 72 h of culture. In biopsied- derived blastocyst groups, the rates of hatching blastocysts after 48-72 h of culture between vitrification of biopsied-derived blastocysts using the Cryotop and fresh control groups were not different; moreover, they were higher than the rates of vitrification of biopsied-derived blastocysts using 0.25 mL straw (83.0-88.0%, 93.7-96.8% vs. 64.4-72.1%, $P<0.05$; respectively). Moreover, the numbers of apoptotic cells per blastocyst were higher in all vitrified groups derived from biopsied and non-biopsied blastocysts than those of fresh control groups ($P<0.05$). These results indicated that biopsy with microblade prior to vitrification using Cryotop method is greater in *in vitro* survival after vitrification-warming as well as as non-biopsied and fresh embryos. However, it is necessary to improve vitrification system for sanitary vitrification and simplify this technique for direct transfer of vitrified sexed bovine embryos.

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5.2 Introduction

Embryo biopsy in bovine has become a useful tool for embryo sexing to produce offspring pertinent to the owner's desire. The advances in state of art has made it possible to sexing embryos by Polymerase Chain Reaction (PCR) and the identification of bovine Y-chromosome specific DNA probes (Kageyama et al., 2004). Nowadays, PCR is routinely used in the field for embryo sexing because this technique enables amplification of a target sequence from a small number of blastomeres (Thibier and Nibart, 1995).

The embryo biopsy is the process to remove the cells from the preimplantation

embryos for diagnosis. Therefore, this process is an invasion that caused embryos damage which reduces the quality of embryos after being biopsied (Polisseni et al., 2010). Various methods have been used for embryo biopsy such as the needle technique (Thibier and Nibart, 1995; Cenariu et al, 2012b), the aspiration technique (Thibier and Nibart, 1995; Vajta et al., 1997), and the microblade technique (Thibier and Nibart, 1995; Leoni et al., 2000). It has been showed that the microblade technique is widely used for bovine embryos biopsy for sexing as this technique has more rapid and simpler procedure than the other two approaches. It is suitable for sexing a large number of embryos (Cenariu et al, 2012a).

Previous reports suggested that biopsy at later developmental stage, such as morula or blastocyst is less affected to embryos when compared with biopsy at earlier development stages due to more relatively collected cells(de Vos and Van Steirteghem, 2001). However, it is difficult to biopsy at the morula stage because of the extensive compaction (Van Blerk et al., 1991). Therefore, biopsying at the blastocysts stage by removing off the small portion of trophectoderm is widely used due to its ease and less damage than the other stages of embryos (Evsikov and Verlinsky, 1998).

The pregnancy rates obtained from the transfer of frozen-warmed biopsied embryos were lower in microblade than those of needle and aspiration techniques (Nibart et al., 1997; Shea, 1999; Cenariu et al.; 2012a). Furthermore, several studies reported that the high rates of pregnancy, ranged from 53 to 62% when transferring biopsied bovine embryos with microblade without frozen (Bredbacka et al., 1996; Herr and Reed, 1991; Thibier and Nibart, 1995; Roschlau et al., 1997).

To our knowledge, there has been little information of *in vitro* survival and

damage of embryos after using frozen-warmed biopsied IVP bovine embryos for vitrification method. Therefore, this study was to evaluate the effects of embryo biopsy with microblade on their subsequent development and their *in vitro* survival when subjected to be vitrified with the Cryotop and 0.25 mL straw methods. Moreover, the damage of embryos after vitrification-warming of biopsied IVP bovine blastocysts was investigated by using the TUNEL assay.

5.3 Materials and Methods

5.3.1 Chemicals and media

Except where otherwise indicated, all chemicals were purchased from Sigma-Aldrich, Co (St. Louis, MO, USA).

5.3.2 Oocytes collection and *in vitro* maturation (IVM)

The slaughterhouse ovaries of Holstein cows were washed and stored in physiological saline (0.9% NaCl) during transportation to the laboratory at room temperature (RT). Cumulus oocyte complexes (COCs) were collected from follicles (2−8 mm in diameter) using a 10-ml syringe connected with 18-gauge needle. The IVM procedure was performed as described previously (Parnpai et al., 1999). Briefly, a group of 20 to 25 COCs was washed five times in modified Dulbecco’s phosphate buffer saline (mDPBS) supplemented with 0.1% polyvinyl pyrolidone (PVP). Groups of 20-25 COCs were washed three times with 100-µl droplet of IVM medium, before being cultured for 22 h in 100-µl droplet of IVM that covered with mineral oil under humidified atmosphere with 5% CO_2 in air at 38.5ºC. The IVM medium consisted of TCM199 supplemented with 10% fetal bovine serum (FBS; Life Technologies Inc Gibco-BRL Division, Grand Island), 50 IU/ml hCG (Intervet UK Ltd., Milton Keynes,

UK), 0.02 AU/ml FSH (FSH, Antrin®, Denka Phamaceutical Co., Kanagawa, Japan) and 1 µg/ml 17ß-estradiol.

5.3.3 *In vitro* fertilization (IVF)

The frozen semen was thawed in a 37°C water bath for 30 sec. Then, the thawed sperm was layered under 2 mL of TALP (Tyrode's albumin lactate pyruvate; Lu et al., 1987) medium in a 5 mL snap tube and incubated at 38.5°C under humidified atmosphere of 5% CO_2 in air for 40 min to allow live spermatozoa to swim up. The top 1.5 ml from each tube was removed, pooled in a 15 ml conical tube centrifuged at 400 × g for 7 min. The pellets were resuspended with TALP medium to adjust the final concentrations of 2×10^6 spermatozoa/ml. One hundred µl drops of sperm suspension were prepared in culture dish, covered with mineral oil and used as fertilization droplets. The groups of 20–25 oocytes were washed three times in TALP medium. After that, the oocytes were added into 100 µl of fertilization droplets and cultured for 12 h at 38.5 °C under a humidified atmosphere with 5% CO_2 in air.

5.3.4 *In vitro* culture (IVC)

IVC was performed in 100 µl droplets of mSOF medium supplemented with 3 mg/ml BSA covered with mineral oil. At the end of insemination, putative zygotes were completely denuded from cumulus cells and spermatozoa by gently pipetting with a fine glass pipette in pre-incubated IVC medium. Twenty to twenty five zygotes were placed in each culture drop and then were cultured at 38.5°C under humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 for 2 days. Thereafter, embryos at the 8-cell stage were selected and co-cultured with bovine oviductal epithelium cells (BOEC) in mSOF medium under humidified atmosphere with 5% CO_2 in air at 38.5°C for 5 days (Parnpai et al., 1999). Half volume of mSOF medium

was replaced daily and the development of embryos was recorded (Day 0 was defined as the day of IVF).

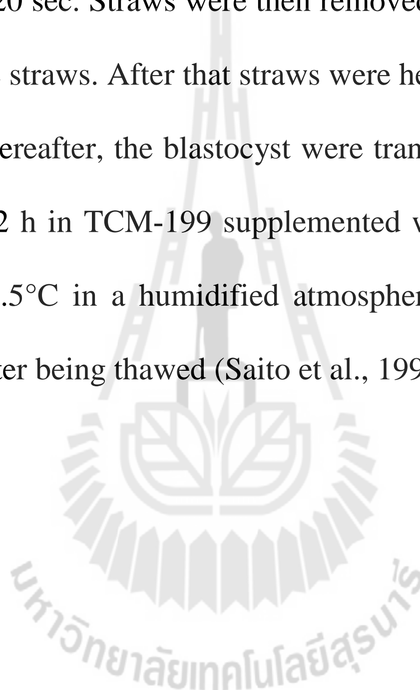
5.3.5 Blastocyst biopsy with microblade technique

IVP blastocysts (IETS quality code 1, stage 6) were placed into a microdrop of DPBS (without $CaCl_2$ and $MgCl_2$) supplemented with 10% (v/v) FBS, in a scratched bottom dish. The scratches were made for stabilizing the embryos as suggested by Bredbacka et al. (1995). All procedure was done under an inverted microscope (IX71, Olympus, Tokyo, Japan) with a micromanipulator (M0188NE, Narishige Co., Ltd., Tokyo, Japan). A small portion of embryo (about 5%) was cut off from the trophoblast of blastocysts by using microblade (Bio-cut blade, Feather Safety Razor Co., Ltd., Osaka, Japan). The biopsied blastocysts were co-cultured with BOEC cells in mSOF medium and under humidified atmosphere with 5% CO_2 in air at 38.5°C for 4-6 h. The survival after biopsy was assessed by their morphological development to hatching blastocyst and subjected to vitrification procedure subsequently.

5.3.6 Blastocyst vitrification and warming using 0.25 mL straws

Biopsied and non-biopsied derived blastocysts were washed three times in HM consisting of DPBS supplemented with 20% (v/v) FBS. The expanded blastocysts were then placed in equilibration medium, which was composed of HM supplemented with 7.5% Ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO) for 3 min, and then transferred into vitrification solution that was composed of HM supplemented with 16.5% EG, 16.5% DMSO, and 0.5 M sucrose. Subsequently, blastocysts (one blastocyst per straw) were then pipetted into 0.25 mL straw and placed on the inner surface of straw. The straw contained diluting solution which was HM. The loaded

straw was immediately heat-sealed and the part of straw containing blastocyst was placed vertically into LN_2 for 1 min before immersing in LN_2 for storage. The process of exposure of the blastocysts to vitrification solution to their immersion in LN_2 was completed within 30 sec. All vitrification procedure was performed at RT (24-26 ºC). After beingstored for at least 24 h in LN_2 tank, the straws containing embryos were warmed by exposing in the air for 10 sec and then plunged into the 35 ºC water to warm the diluents for 20 sec. Straws were then removed from the water and shaken to mix the columns of the straws. After that straws were held vertically in the same water at 35 ºC for 5 min. Thereafter, the blastocyst were transferred into culture dishes and cultured *in vitro* for 72 h in TCM-199 supplemented with 20% FBS and 0.1 mM ß-merceptoethanol at 38.5°C in a humidified atmosphere of 5% CO_2 to evaluate the survival of embryos after being thawed (Saito et al., 1994).



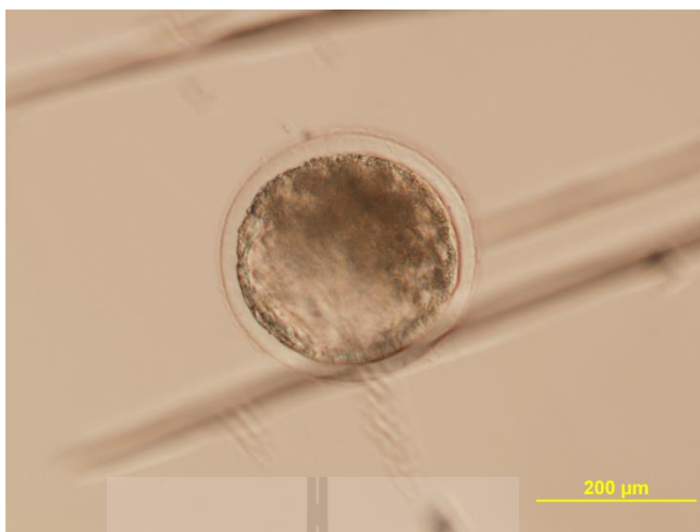


Figure 5.1 IVP bovine blastocysts were subjected to biopsy with microblade in a dish with scratched bottom. All procedure was done under an inverted microscope (IX71, Olympus, Tokyo, Japan) with a micromanipulator (M0188NE, Narishige Co., Ltd., Tokyo, Japan) (magnification 100x).

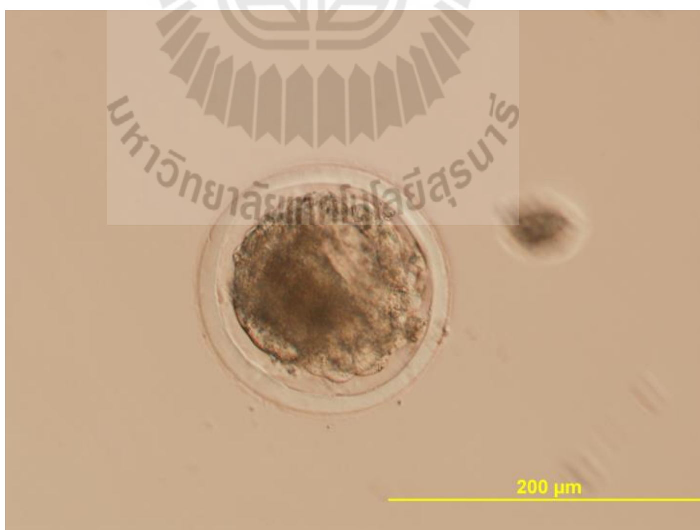


Figure 5.2 A small portion of embryo (about 5%) was cut off from the trophoblast of blastocysts by using microblade (Bio-cut blade, Feather Safety Razor Co., Ltd., Osaka, Japan) (magnification 100x).

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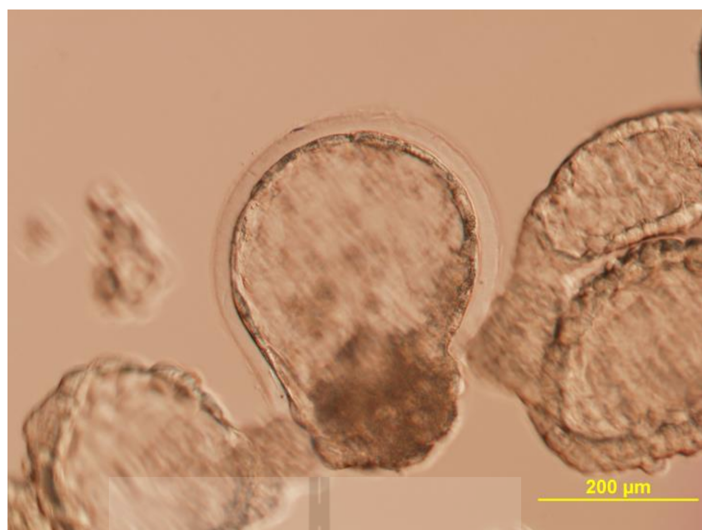

Figure 5.3 The biopsied blastocysts were co-cultured with BOEC cells in mSOF medium and under humidified atmosphere with 5% CO_2 in air at 38.5°C for 4-6 h to check their survival after biopsied (magnification 100x).

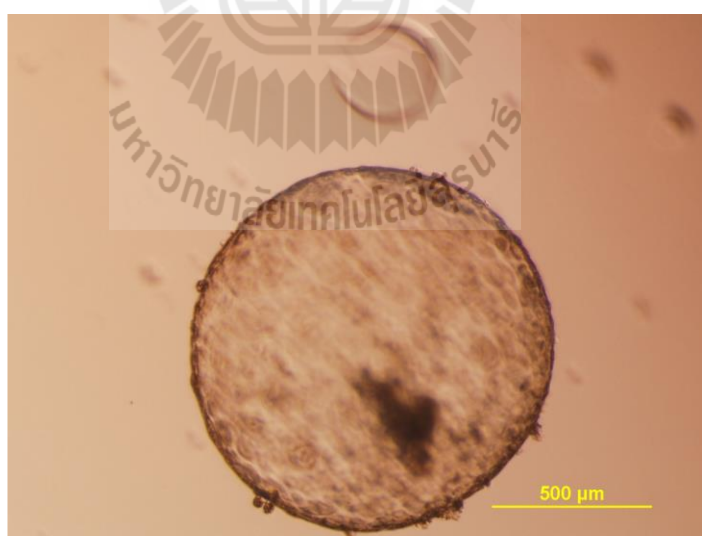

Figure 5.4 The biopsied blastocysts were subjected to be vitrified with the Cryotop method, and their *in vitro* survival after vitrification-warming was assessed by their hatching ability to hatch blastocyst stage during 72 h of additional IVC (magnification 100x)

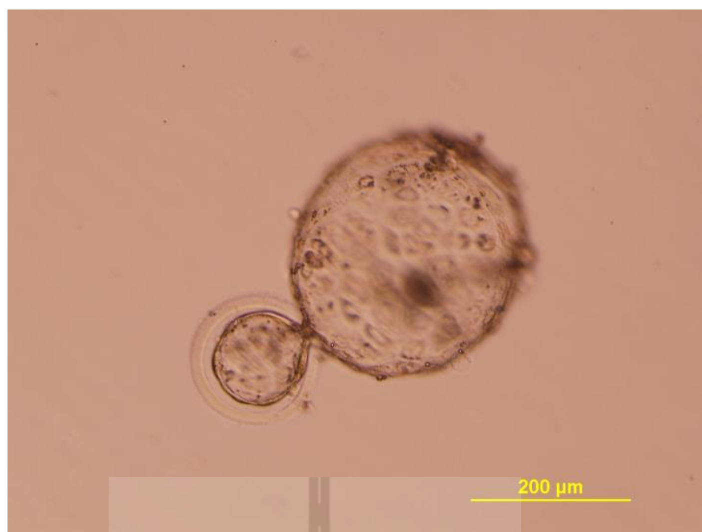

Figure 5.5 The biopsied blastocysts were subjected to be vitrified with the 0.25 mL straw method and in *vitro* culture for 24 h after vitrification-warming (magnification 100x).

Figure 5.6 The biopsied blastocysts were subjected to be vitrified with the 0.25 mL straw method and in *vitro* culture for 72 h after vitrification-warming (magnification 100x).

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5.3.7 Blastocyst vitrification and warming using Cryotop

Biopsied and non-biopsied derived blastocysts were exposed to vitrification solution as used in 0.25 mL straw method. Then, a group of 5 blastocysts was placed on a Cryotop sheet in a small volume of vitrification solution (<1 μl) and then the Cryotop was plunged into LN_2. The process of exposing the blastocysts to vitrification solution to their immersion into LN_2 was completed within 30 sec. All vitrification procedure was performed at room temperature (24-26 ºC). After stored for at least 24 h in LN_2 tank, vitrified blastocysts were warmed by insertion of the Cryotop sheet into HM at 38.5 ºC for 5 min. After washing three times in HM, they were transferred into culture dishes and cultured *in vitro* for 72 h at 38.5 ºC in humidified atmosphere of 5% CO_2 in air with the same medium used in 0.25 mL straws method to evaluate their survival of embryo after being thawed.

5.3.8 TUNEL assay

DNA fragmentation evaluation was performed using TUNEL (*In Situ* Cell Death Detection kit; Roche, USA) as previously described (Park et al., 2007). Blastocysts were washed in phosphate buffer saline (PBS) containing polyvinylpyrrolidone (PVP, 1 mg/ml) (PBS-PVP) before fixation in 3.7% paraformaldehyde in PBS for 1 h at RT. After fixation, blastocysts were permeabilized with 0.5% Triton X-100 in PBS for 1 h at RT. The blastocysts were then washed twice in PBS-PVP and incubated in fluorescein-dUTP and terminal deoxynucleotidyl transferase for 1 h at 37 ºC in the dark. Then, blastocysts were incubated with 50μg/ml RNase A in 40μg/ml propidium iodide (PI) for 1 h at 37 ºC to label all nuclei. Blastocysts were washed in PBS-PVP, mounted on glass slides in glycerol droplets that were flattened by cover slips. The blastocysts were examined under UV light with

an excitation wavelength of 330-385 nm using an epifluorescence microscope (IX-71; Olympus Corporation, Tokyo, Japan). TUNEL-positive nuclei appeared bright yellowish-green and the PI staining allowed counting the total cell number.

5.4 Experimental design

This experiment was designed to evaluate the effects of blastocysts biopsy with microblade prior to vitrification by using Cryotop or 0.25 mL straw methods on their *in vitro* survival after vitrification-warming. IVP bovine blastocysts (IETS quality code 1, stage 6; n = 594) were biopsied with microblade technique, or were not biopsied (control) and then randomly vitrified using Cryotop or 0.25 mL straw methods. Non-vitrified blastocysts from each biopsied or non-biopsied groups served as the fresh control groups. The *in vitro* survival after vitrification-warming was assessed by their hatching ability to hatch blastocyst stage during 72 h of additional IVC. The *in vitro* survival after vitrification-warming, the total number of cells and the number of apoptotic cells per blastocyst were compared between the groups.

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5.5 Statistical analysis

The rates of hatching blastocysts were expressed as percentage and subjected to arcsine transformation. Total numbers of cells and apoptotic cells per blastocyst were expressed as untransformed means ± standard error of the mean (SEM). Data were performed using 2×3 factorial randomized complete block design. Differences were considered to be significant when $P<0.05$.

5.6 Results

As shown in Table 4, the rates of embryos developing to the hatching blastocyst

stage at 24 h of additional IVC after vitrification-warming of biopsied-derived blastocyst groups were higher than those of non-biopsied derived blastocyst groups. However, the hatching rate of biopsied derived-blastocyst and vitrified using 0.25 mL straw (biopsied + 0.25 mL straw) were not significantly different from the hatching rate of non-biopsied derived blastocyst groups. After 48-72 h of culture, the rates of development to hatched blastocyst stage between vitrified non-biopsy derived blastocysts using the Cryotop and 0.25 mL straw devices were not difference. Similarly, they did not differ in the hatching rates with fresh control group at 72 h of culture. In biopsied-derived blastocyst groups, the rates of hatching blastocysts after 48-72 h of culture between vitrified biopsied-derived blastocysts using the Cryotop and fresh control groups were not different; moreover, they were higher in these rates than vitrified biopsied-derived blastocysts using 0.25 mL straw (83.0-88.0%, 93.7-96.8% vs. 64.4-72.1%, $P<0.05$; respectively). Furthermore, we found a negative correlation between the blastocysts biopsy with microblade and vitrify with the 0.25 mL method ($r= -0.72$, $P=0.05$).

The total number of nuclei after vitrification-warmed that obtained at 72 h of additional IVC and *in vitro* culture for 9 days (fresh control) between all groups were not different. However, the numbers of apoptotic cells per blastocyst were higher in all

vitrified groups derived from biopsied and non-biopsied blastocysts than those of fresh control groups ($P<0.05$).

Table 5.1 Development to the hatching blastocysts stage of biopsied and non-biopsied derived blastocyst vitrified using the Cryotop or 0.25 mL device, warmed and cultured *in vitro* for 72 h

Treatment		**No. of cryopreserved embryos**	**No. of hatched embryos**		
			24 h	**48 h**	**72 h**
Biopsied	Cryotop	100	71^{a} (71.0%)	83^{a} (83.0%)	88^{a} (88.0%)
	0.25 mL straw	104	61^{b} (58.6%)	67^{b} (64.4%)	75^{b} (72.1%)
	fresh control	95	81^{a} (85.3%)	89^{a} (93.7%)	92^{a} (96.8%)
Non-biopsy	Cryotop	100	52^{b} (52.0%)	76^{ab} (76.0%)	93^{a} (93.0%)
	0.25 mL straw	100	45^{b} (45.0%)	66^{b} (66.0%)	84^{ab} (84.0%)
	fresh control	95	53^{b} (55.7%)	78^{a} (82.1%)	95^{a} (100.0%)

Ten replications were performed. ^{a,b}Values that have different superscripts within a single column are significantly different at $P<0.05$. **Biopsied + Cryotop**: IVP blastocysts that were biopsied with microblade prior vitrified using Cryotop device. **Biopsied + 0.25 mL straw**: IVP blastocysts that were biopsied with microblade prior to vitrification using 0.25 mL straws device. **Biopsied + fresh control**: IVP blastocysts that were biopsied with microblade but were not subjected to vitrification. **Non-biopsied + Cryotop**: non-biopsied of IVP blastocysts that vitrified using Cryotop device. **Non-biopsied + 0.25 mL straw**: non-biopsied of IVP blastocysts that were vitrified using 0.25 mL straws device. **Non-biopsied + fresh control**: non-biopsied of IVP blastocysts that were not subjected to vitrification.

Table 5.2 Total cell numbers and apoptosis cells per blastocyst derived from biopsied and non-biopsied blastocysts with microblade, and then randomly vitrified using the Cryotop or 0.25 mL straws device, warmed and cultured *in vitro* for 72 h.

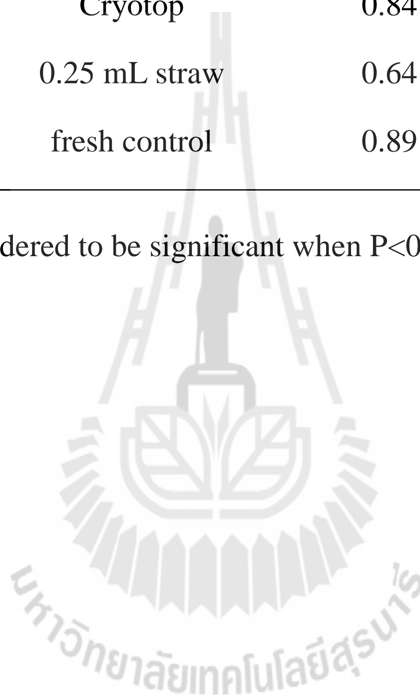
Treatment		No. of blastocyst evaluated	No. of nuclei ± SEM	
			Total cells blastocyst	Apoptosis cells blastocyst
Biopsied	Cryotop	10	103.3±0.3	13.4±9.2[a]
	0.25 mL straw	10	101.7±0.7	15.1±8.5[a]
	fresh control	10	110.3±0.5	0.7±3.0[b]
Non-biopsy	Cryotop	10	123.3±1.5	8.8±10.1[a]
	0.25 mL straw	10	121.5±1.9	9.5± 8.0[a]
	fresh control	11	133.3±1.2	0.5±3.9[b]

Data presented as means ± SEM. [a,b]Values that have different superscripts within a single column are significantly different at $P<0.05$. **Biopsied + Cryotop**: IVP blastocysts that were biopsied with microblade prior to vitrification using Cryotop device. **Biopsied + 0.25 mL straw**: IVP blastocysts that were biopsied with microblade prior to vitrification using 0.25 mL straws device. **Biopsied + fresh control:** IVP blastocysts that were biopsied with microblade but were not subjected to vitrification. **Non-biopsied + Cryotop**: non-biopsied of IVP blastocysts that vitrified using Cryotop device. **Non-biopsied + 0.25 mL straw**: non-biopsied of IVP blastocysts that were vitrified using 0.25 mL straws device. **Non-biopsied + fresh control**: non-biopsied of IVP blastocysts that were not subjected to vitrification.

Table 5.3 Correlation between the blastocysts biopsy with microblade and vitrification methods on their *in vitro* survival after these processes

Treatment		*In vtiro* survival	
		R	***P*-value**
Biopsied	Cryotop	0.70	0.11
	0.25 mL straw	-0.72	0.05
	fresh control	0.86	0.14
Non-biopsy	Cryotop	0.84	0.06
	0.25 mL straw	0.64	0.12
	fresh control	0.89	0.06

Differences were considered to be significant when P<0.05.



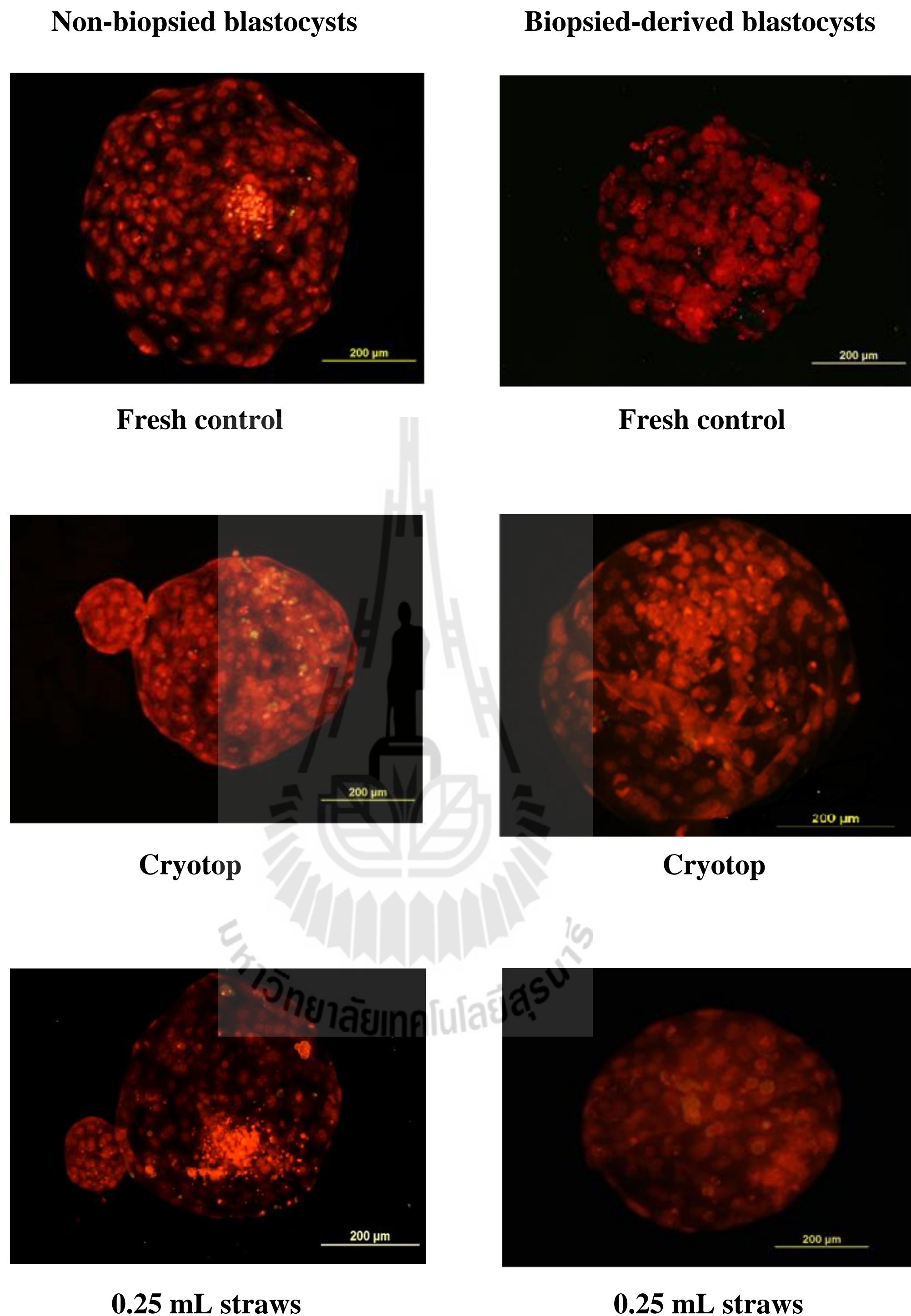


Figure 5.7 Fluorescence images in each treatment groups of bovine blastocysts. Red color of PI stained total nuclei and bright yellowish-green of TUNEL kit stained DNA fragmentation (magnification 100x).

5.7 Discussions

It is known that the biopsy procedure could risk the embryonic damage by reducing the available cells number for differentiation (de Vos and Van Steirteghem, 2001). Moreover, combining embryo biopsy and freezing had more impact on their pregnancy rates or *in vivo* survival (Shea, 1999). All three biopsy techniques that were used for embryo sexing in previous reports, the microblade technique tended to decrease the pregnancy rate in recipients when compared with needle and aspiration technique (12% vs. 22 and 18%, respectively) (Cenariu et al., 2012a). Similarly, Nibart et al. (1997) and Shea (1999) obtained pregnancy rates, ranged from 23 and 28%, respectively, when frozen-warmed with mocroblade biopsies bovine embryos were transferred; whereas, the pregnancy rates ranged from 33 to 66% when the needle or aspiration biopsied were used. Moreover, Vajta et al. (1997) reported that 98% of survival after being biopsied, and 86% of re-expanded rate after vitrification warming with 0.25 mL straw method when a biopsy was performed before vitrification. In addition, they found the less efficient when biopsy after vitrification, which the survival rate in both process was only 69%. In this study, we achieved the highest rate at 88% of *in vitro* survival following microblade biopsied and vitrified using the Cryotop method. The *in vitro* survival after vitrification-warming was assessed by their hatching ability to hatch blastocyst stage during 72 h of additional IVC in our study. Besides, our results supported the *in vitro* survival rate, which obtained from biopsied prior to vitrification with the Cryotop method was not different from fresh and non-biopsied blastocysts (Table 1).

It has already been described that the short-term culture of bovine blastocysts for 2.5 to 5 h after being biopsied and before being subjected to vitrification improved

their *in vitro* survival after warming vitrified IVP bovine blastocysts using 0.25 mL straw method (Ito et al., 1999). In our study, 4 to 6 h of culture after being biopsied and before being subjected to vitrification was used to improve the *in vitro* survival after warming of vitrified IVP bovine blastocysts using the Cryotop method. However, successful rates that were achieved in this study may be occurred by the other factors such as, the high efficiency vitrification system of Cryotop device which achieved the cooling rate approximately 20,000 °C/min (Kuwayama et al., 2005). Moreover, *in vitro* survival after warming of vitrified biopsied blastocysts using 0.25 mL straw was lower than those of the Cryotop and two control groups. In addition, we found a significant negative correlation between the blastocysts biopsy with microblade and vitrify with the 0.25 mL method. These agree with Bredbacka et al. (1995), who showed that microblade biopsy without micromanipulator caused cell lysis approximately 15-30%, after biopsying blastocysts. Therefore, microblade biopsy under micromanipulator is the beneficial effect in this study.

To investigate the quality of IVP bovine embryos derived from biopsy with microblade prior to vitrification using the Cryotop or 0.25 mL straw methods, the total cell number and the number of apoptotic cells per blastocyst was determined. It has been proposed that apoptosis play a potentially important role in early embryonic loss and in cellular responses to stress and suboptimal developmental condition (Betts and King, 2001; Gjørret et al., 2003). Therefore, when the cell number is increasing and smaller number of apoptotic cells may indicate improved embryo health and embryonic viability after being transferred to recipients (Caamaño et al., 2015). Our results showed that no differences between biopsied and non biopsied derived-blastocyst that subjected to vitrification with the Cryotop or 0.25 mL straw method in

total cell number per blastocyst. However, the apoptotic cell per blastocyst was greater in all vitrified groups derived from both of biopsied and non-biopsied blastocysts than those of fresh control groups (P<0.05) (Table 2). These corresponding results were found in Park et al. (2007) and Ha et al. (2014a, b). In addition, we did not findthe significant differences in apoptotic cell per blastocyst between bovine blastocysts derived from biopsied and non biopsied blastocysts. These indicated that microblade biopsy had no effect on increasing apoptotic cells per blastocyst; whereas, the vitrification performed an effect on increasing apoptotic celsl per blastocyst after vitrification-warming.

5.8 Conclusion

In conclusion, the use of microblade for biopsy prior to vitrification using the Cryotop method is the highest *in vitro* survival after vitrification-warming as well as non-biopsied and fresh embryos. When vitrified biopsied IVP bovine blastocysts using 0.25 mL straw, the lower *in vitro* survival after vitrification-warming was found. It may be caused by the combined-effects between microblade biopsy and vitrification with 0.25 mL straw methods which had more impact on their *in vitro* survival. However, it is necessary to improve the vitrification system for sanitary vitrification and simplifies this technique for direct transfer of vitrified sexed embryos on the farm.

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CHAPTER VI

OVERALL CONCLUSIONS AND IMPLICATIONS

Bovine blastocysts produced *in vitro* have been successfully cryopreserved by vitrification method. To improve the economic value of IVP bovine embryos, it is necessary to develop the vitrification technique for sanitary and one step dilution after warming, which supported direct transfer of vitrified bovine embryos under field conditions.

Vitrification of both bovine IVM oocytes and bovine IVP embryos at the expanded blastocyst stage by using Cryotop and MVAC cooling systems are equally effective, resulting in high survival and developmental rates. Thus, this study suggests that the MVAC system is a new, useful method for vitrification of both bovine IVM oocytes and bovine IVP embryos. Our results also show that the modified MVAC device used in the MVAC method may address biosafety concerns by serving as a closed carrier system that prevents exposure to LN_2.

To improve the efficiency of the 0.25 mL straw for vitrification device, our straw columns in combination with the optimum factors have been suggested for vitrified IVP bovine blastocysts. Therefore, this study suggests that this method is allowed the one step dilution after warming of vitrified IVP bovine embryos, which embryo transfer can be simplified on the farm to the same level of artificial insemination.

Using microblade for biopsy prior to vitrification with 0.25 mL straw method, resulting in a decrease after biopsied and vitrified procedures. When vitrified biopsied

IVP bovine blastocysts using 0.25 mL straw, the lower *in vitro* survival rates after vitrification-warming was found. It may be caused by the combined-effects between microblade biopsy and vitrification with 0.25 mL straw methods which had more impact on their *in vitro* survival. However, no differences between biopsied and non biopsied derived-blastocyst that subjected to vitrification with the Cryotop or 0.25 mL straw method in total cell number per blastocyst. Moreover, this study did not find the significant differences in apoptotic cell per blastocyst between bovine blastocysts derived from biopsied and non biopsied blastocysts. These indicated that microblade biopsy had no effect on increasing apoptotic cells per blastocyst.

In this study, we found that vitrification with 0.25 mL straw device can be successful develop for sanitary vitrification and one step dilution after warming for the direct transfer of biopsied IVP bovine embryos on the farm. However, it is necessary for the future studies to investigate *in vivo* survival rates from biopsied and non-biopsied IVP blastocysts that vitrified by 0.25 mL straw method.

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BIOGRAPHY

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