THE EFFECT OF BOVINE OOCYTE AND EMBRYO VITRIFICATION ON EPIGENETIC CHARACTERISTICS AND DEVELOPMENTALLY IMPORTANT GENES EXPRESSION

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ผลของการแช่แข็งไข่และตัวอ่อนโคแบบเนื้อแก้วต่อคุณลักษณะทางอีพิจีนิติก และการแสดงออกของยีนที่สำคัญต่อการเจริญเติบโต

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การเก็บรักษาไข่และตัวอ่อนของโคในระยะยาวได้มีการประยุกต์ใช้ในเทคโนโลยีช่วยการเจริญพันธุ์ และการปรับปรุงพันธ์มาอย่างแพร่หลาย ในปัจจุบันการแช่แข็งแบบเนื้อแก้วเป็นวิธีมาตรฐานในการเก็บรักษาไข่และตัวอ่อน อย่างไรก็ตาม วิธีนี้มีอัตราการรอดชีวิตและการเจริญเติบโตต่ำ เมื่อเทียบกับไข่และตัวอ่อนที่ไม่ได้แช่แข็ง ในการทดลองแรกมีวัตถุประสงค์เพื่อตรวจสอบผลของความเข้มข้นของสารป้องกันการแช่แข็งในช่วงการปรับสมดุลต่อประสิทธิภาพการแช่แข็งตัวอ่อนโคแบบเนื้อแก้วในระยะบลาสโตซีสต์และการแสดงออกของยีนที่สำคัญต่อการเจริญเติบโตของตัวอ่อน โดยทำการปรับสมดุลตัวอ่อนโคที่ผลิตจากกระบวนการในหลอดแก้วในน้ำยาที่มีส่วนผสมของ 7.5% เอทิลีนไกลคอล (EG) และ7.5% ไดเมทิลซัลฟอกไซด์ (DMSO) เป็นกลุ่ม Va หรือใช้ 2% EG+2% DMSO เป็นกลุ่ม Vb จากนั้นทำการแช่แข็งแบบเนื้อแก้วโดยใช้น้ำยาที่มีส่วนผสมของ 16.5% EG+16.5% DMSO+0.5M ซูโครส โดยใช้อุปกรณ์ Cryotop หลังจากทำละลายนำตัวอ่อนไปเลี้ยงเป็นเวลา 48 ชั่วโมง ทำการเปรียบเทียบอัตราการรอดชีวิต การฟักตัว และจำนวนเซลล์ที่เสียหายทั้งหมดระหว่างกลุ่มแช่แข็งทั้งสองและกลุ่มตัวอ่อนสดที่เป็นกลุ่มควบคุม ผลการทดลองพบว่าไม่มีความแตกต่างอย่างมีนัยสำคัญระหว่างกลุ่มแช่แข็ง ในส่วนของอัตราการรอดชีวิต จำนวนเซลล์ทั้งหมด และความเสียหายต่อเยื่อหุ้มเซลล์ การแช่แข็งแบบเนื้อแก้วเพิ่มจำนวนเซลล์ที่เสียหายของทั้งสองกลุ่มการแช่แข็ง แต่พบความเสียหายมากกกว่าในกลุ่ม Vb การแช่แข็งแบบเนื้อแก้วเพิ่มการแสดงออกของยีน HSP70 อย่างมีนัยสำคัญ ($P<0.05$) ในกลุ่ม Va เทียบกับกลุ่มควบคุม แต่ไม่แตกต่างจากกลุ่ม Vb ส่วนการแสดงออกของยีน IGF2R SNRPN HDAC1 DNMT3B BAX OCT4 and IFN-t ของทั้งกลุ่มแช่แข็งและกลุ่มควบคุมไม่แตกต่างกัน สรุปได้ว่าความเข้มข้นของสารป้องกันการแช่แข็ง ในช่วงการปรับสมดุลนั้นไม่มีผลกระทบต่อออัตราการรอดชีวิตของตัวอ่อนโคแช่แข็งแบบเนื้อแก้ว อย่างไรก็ตามสารป้องกันการแช่แข็งในช่วงการปรับสมดุลที่ความเข้มข้น 15% สามารถคงไว้ซึ่งจำนวนเซลล์ปกติโดยที่มีความเกี่ยวข้องกับการเพิ่มขึ้นของการแสดงออกของยีน HSP70

ในการทดลองที่สองมีวัตถุประสงค์เพื่อเปรียบเทียบผลของการแช่แข็งไข่โคแบบเนื้อแก้วที่ระยะ GV และ MII ต่อคุณลักษณะทางอีพิจีนิติกและการเจริญเติบโต จากผลการทดลองพบว่าการแช่แข็งไข่โคทั้งสองระยะทำให้การเจริญเติบโตของตัวอ่อนลดลงอย่างมีนัยสำคัญทางสถิติภาย

หลังจากกการทำการปฏิสนธิภายในหลอดแก้ว อย่างไรก็ตาม การแช่แข็งไข่โคที่ระยะ GV ให้ผลการเจริญเติบโตของตัวอ่อนถึงระยะบลาสโตซีสต์สูงกว่าระยะ MII การแช่แข็งไข่โคแบบเนื้อแก้วไม่มีผลกระทบต่อระดับของ 5mC ในสายดีเอ็นเอของไข่ อย่างไรก็ตามตัวอ่อนระยะบลาสโตซีสต์ที่เกิดจากไข่โคแช่แข็งแบบเนื้อแก้วทั้งสองระยะมีระดับ 5mC ลดลง การแช่แข็งไข่โคแบบเนื้อแก้วไม่มีผลต่อระดับของ H3K9me3 และ acH3K9 ในทั้งไข่และตัวอ่อนที่เกิดจากไข่แช่แข็งดังกล่าว จากผลการทดลองแสดงให้เห็นว่าการแช่แข็งไข่โคแบบเนื้อแก้วกระทบต่อระดับการเติมหมู่เมทิลในตัวอ่อนที่ผลิตได้ ถึงแม้จะไม่มีสามรถพบได้ในไข่ก็ตาม การค้นพบดังกล่าวนี้แสดงถึงว่าการแช่แข็งแบบเนื้อแก้วนั้นส่งผลทำให้รบกวนการเปลี่ยนแปลงระดับอีพิจีนิติก โดยการลดระดับของการเติมหมู่เมทิลของดีเอ็นเอ ซึ่งอาจเป็นสาเหตุของความบกพร่องในการเจริญเติบโตของตัวอ่อนโคที่เกิดจากการแช่แข็งไข่โคที่แตกต่างกันทั้งสองระยะ นอกจากนี้การแช่แข็งไข่แบบเนื้อแก้วไม่มีผลต่อระดับการเติมหมู่อะเซทิล และหมู่เมทิลของฮิสโตนในไข่ และตัวอ่อนที่เกิดจากไข่แช่แข็งดังกล่าวอีกด้วย

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2563 ลายมือชื่ออาจารย์ที่ปรึกษา _____

THATAWAT YODRUG : THE EFFECT OF BOVINE OOCYTE AND EMBRYO VITRIFICATION ON EPIGENETIC CHARACTERISTICS AND DEVELOPMENTALLY IMPORTANT GENES EXPRESSION. THESIS ADVISOR : ASSOC. PROF. RANGSUN PARNPAI, Ph.D., 113 PP.

BOVINE/OOCYTE/EMBRYO/VITRIFICATION/EPIGENETIC

Long-term preservation of bovine oocyte and embryo have widespread applications in assisted reproductive technologies and genetic improvement of livestock species. Vitrification is the current gold standard of bovine oocyte and embryo cryopreservation. However, this technique still has inadequate survivability and developmental competency of oocyte and embryo compare with non-cryopreservation. The first experiment aimed to assess the effects of cryoprotectant concentration during equilibration on the efficiency of bovine blastocyst vitrification and the expression of selected developmentally important genes. In vitro produced bovine blastocysts were equilibrated in either 7.5% ethylene glycol (EG)+7.5% DMSO (Va group) or in 2% EG+2% DMSO (Vb group) then vitrified on Cryotop sheets in 16.5% EG+16.5% DMSO+0.5M sucrose. After warming, embryos were cultured for 48 h. Re-expansion, hatching, and the numbers of total and membrane damaged cells were compared among vitrified groups and fresh embryos as a control. There was no significant difference between the vitrified groups in survival, total cell numbers and the extent of membrane damage. Vitrification increased the number of membrane-damaged cells in both groups, however, in a greater extent in the Vb group. Vitrification increased ($P<0.05$) the expression of the *HSP70* gene in Va but

not in Vb embryos. The expression of *IGF2R*, *SNRPN*, *HDAC1*, *DNMT3B*, *BAX*, *OCT4*, and *IFN-t* genes were similar in control and vitrified groups. In conclusion, the concentration of cryoprotectants during equilibration did not affect the survival rate; however, normal cell numbers could be maintained only by equilibration in 15% cryoprotectants which was associated with an increased *HSP70* expression.

The second experiment aimed to compare the effect of bovine oocytes vitrification either at the GV or MII stages on epigenetic characteristics and subsequently developing embryos. The results showed that vitrification of oocytes at both meiotic stages significantly reduced blastocyst development after IVF compared with the fresh control. Oocyte vitrification did not affect 5-methylcytosine (5mC) immunostaining intensity in oocyte DNA. Nevertheless, at both stages of oocyte it caused a similar reduction of 5mC levels in DNA of subsequently developing blastocysts. Vitrification of the oocyte had no effect on the intensity of H3K9me3 and acH3K9 immunostaining in oocytes and subsequent blastocysts. The results showed that oocyte vitrification alters global methylation in resultant embryos although such alteration in the oocytes was not detected. It can be inferred from this finding that vitrification also alters epigenetic modification by reducing DNA methylation level and might be a cause of impaired developmental competency of bovine embryo derived vitrified oocyte in both developmental stages. Furthermore, oocyte vitrification did not affect histone acetylation and methylation in oocytes and resultant embryos.

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Student's Signature _____

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LIST OF ABBREVIATIONS

5mC	=	5-methylcytosine
acH3K9	=	acetylation on H3K9
ARTs	=	assisted reproductive technologies
ATP	=	adenosine triphosphate
BSA	=	bovine serum albumin
BM	=	base medium
BWS	=	Beckwith-Wiedemann syndrome
-CH3	=	methyl group
CpG	=	at cytosine-guanine dinucleotides
COCs	=	cumulus-oocyte complexes
CR1	=	Charles Rosenkrans 1
CPAs	=	cryoprotective agents
DMRs	=	differentially methylated regions
DMSO	=	dimethyl sulfoxide
DNA	=	deoxyribonucleic acid
DNMT	=	DNA methyltransferases
DNMT1o	=	DNA methyltransferase1o
DO	=	denuded oocyte
D-PBS	=	Dulbecco’s phosphate buffered saline
E	=	embryonic day

LIST OF ABBREVIATIONS (Continued)

EG	=	ethylene glycol
EM	=	electron microscope
ES	=	equilibration solution
FSH	=	follicle stimulating hormone
GV	=	germinal vesicle
GVBD	=	germinal vesicle breakdown
h	=	hour
H19	=	H19 imprinted maternally expressed transcript
H3K9me3	=	H3K9 methylation
HATs	=	histone acetyltransferases
HDACs	=	histone deacetylase
HMTases	=	histone methyltransferases
HSP	=	heat shock protein
ICM	=	inner cell mass
ICRs	=	imprint control regions
IGF2	=	insulin-like growth factor 2
IGF2R	=	insulin-like growth factor 2 receptor
IVC	=	*in vitro* culture
IVF	=	*in vitro* fertilization
IVM	=	*in vitro* maturation
IVP	=	*in vitro* production
KCNQ1O1T	=	KCNQ1 overlapping transcript 1

LIST OF ABBREVIATIONS (Continued)

LOS	=	large offspring syndrome
M	=	molar
MEST	=	mesoderm specific transcript
min	=	minute
miRNA	=	micro RNAs
ml	=	milliliter
mm	=	millimeter
MII	=	metaphase II
NCS	=	newborn calf serum
NNAT	=	Neuronatin
PBAT	=	postbisulfite adaptor tagging
PBS	=	phosphate buffered saline
PEG3	=	paternally expressed 3
PEG10	=	paternally expressed 10
PFA	=	paraformaldehyde
PGCs	=	primordial germ cells
PI	=	propidium iodide
PN	=	pronuclear
PVP	=	polyvinylpyrrolidone
RNA	=	ribonucleic acid
ROS	=	reactive oxygen species
RRBS	=	reduced representation bisulfite sequencing

LIST OF ABBREVIATIONS (Continued)

RT	=	room temperature
sec	=	second
siRNA	=	small interfering RNAs
SNRPN	=	small nuclear ribonucleoprotein polypeptide N
SOF	=	synthetic oviduct fluid
TE	=	trophectoderm
TWGBS	=	tagmentation-based WGBS
UV	=	ultraviolet
VS	=	vitrification solution
WGBS	=	whole-genome bisulfited sequencing
XIST	=	X-inactive specific transcript
β-ME	=	2-mercaptoethanol
µg	=	microgram
µl	=	microliter
µM	=	micromolar
ºC	=	Degree Celsius

CHAPTER I

INTRODUCTION

1.1 Introduction

Mammalian oocyte and embryo cryopreservation has been applied in the assisted reproductive technology (ART), applied in medical therapy and agricultural technology, including infertility treatment, animal reproduction, conserving the endangered animal species, and maintaining or improving livestock's genetic diversity. The current gold standard for long-term oocytes and embryos preservation of livestock species, human and laboratory animals is cryopreservation. This method uses for preserving living cells by using cryogenic storage with ultralow very temperature in liquid nitrogen. There are two main strategies that generally use for mammalian oocyte and embryo cryopreservation including conventional slow freezing and rapid freezing or vitrification.

Vitrification is the alternative method which have more advantages than slow freezing in more simple technique, effective and short time proceed, by using a few minutes compared with the conventional method which required more than 1 hour. It can minimize exposure time to sub-physiological conditions (Brambillasca et al., 2013). Particularly, the benefits of vitrification including reducing associated chilling injuries, no need for expensive programmable freezing equipment and higher survival rate post warming compare with slow freezing. Nowadays, vitrification of oocyte successfully introduced in humans (Kuwayama et al. 2005) and many mammalian

species, such as cattle (Hamano et al., 1992), rabbit (Al-Hasani et al., 1989), horses (Maclellan et al., 2002), and porcine (Somfai et al., 2014). In addition to oocyte, successful vitrification of embryo has been achieved for livestock animals including cattle (Massip et al., 1986), goats (Yuswiati et al., 1990), sheep (Széll et al., 1990) and pigs (Dobrinsky, 1996). Especially, cryopreserve of bovine embryos are the most widely used and it has rapidly developed. Following to the International Embryo Transfer Society in 2017, two hundred thousand frozen embryos were applied around the world (Viana, 2017).

Despite vitrification is the most popular method for cryopreserved female gametes of mammalian species. Nevertheless, the embryonic development competence of vitrified oocyte was still low. Several important characteristics for embryo vitrification, such as plasma membrane permeability, variation of different livestock species, each vitrification procedures, and post thawed period (Lieberman, 2002) are involved in the efficiency of oocyte cryopreservation. Different from embryo, oocyte has large size and susceptible to low temperature and cryoprotectant due to the fragility of the cell structure (Fahy et al., 2004). Cryoprotectants can activate amount of intracellular calcium level in oocyte cytoplasm while vitrification due to enhance zona pellucida hardening and influence the sperm penetration during fertilization (Larman et al., 2006). Different developmental stage and species of the oocyte are also the main factor that effect the developmental capacity of vitrified oocytes. For instance, there is a previous research referred that vitrified germinal vesicle breakdown (GVBD) stage oocyte have more developed than germinal vesicle (GV) and metaphase II (MII) after thawing (Khosravi et al., 2010). Another study shown that bovine has more oocyte freezability than porcine (Isachenko et al., 1992)

since porcine oocytes contain more cytosolic lipid than other livestock species due to increasing freezing sensitivity and critical to oocyte maturation and development (Ruffing et al., 1993). The concerns still exist regarding the effects of vitrification on embryo developmental quality, especially considering the stresses from cooling rate and cytotoxicity of cryoprotective agents (CPAs) (Alison et al., 2011). Recently, there are many studies that have shown oocyte vitrification may influence the modification of epigenetic, including DNA methylation (Liang et al., 2012) and histone acetylation (Yan et al., 2010).

Epigenetic is highly dynamic and tissue-specific in most cells of an organism (Li et al., 2006). Although cells in different organs and tissues share the same DNA sequence, they may have differential expression profiles (Suzuki and Bird, 2008). This mechanism occurs gametogenesis and preimplantation embryonic development (Reik et al., 2001). During cell proliferation and differentiation, epigenetic modification regulates gene expression that keeping specificity of the cell type. (Borgel et al., 2010). The other mechanisms involved in epigenetic inheritance include noncoding and coding RNA such as maternal stored mRNAs, long noncoding RNAs, and small RNAs. (Heard and Martienssen et al., 2014).

In vitro embryo production (IVP) which include using vitrification for storage oocytes and embryos, this cryopreservation technique is also an essential part of routine procedure in assisted reproductive technologies (ARTs). Nevertheless, very few studies have been done on the effect of vitrification on epigenetic related gene expression alteration, but there also was an interesting study reported the alteration of epigenetic related gene expression from cryopreserving oocyte influence embryo development competence (Sprícigo et al., 2014). This is involved between

vitrification procedure and epigenetic mechanism related changing in gene expression (Denomme and Mann, 2012). Moreover, ART procedures affect the epigenomic profiles of oocytes and embryos, which may cause imprinting disorders (Petrussa et al., 2014). Imprinting disorders are the main reason for alternating of multiple imprinted growth regulatory genes expression. (Choufani et al., 2013).

Genomic imprinting refers as single allele expression related epigenetic modification with multiple steps and sex specific mechanism (Verona et al., 2003). Previous study found that fetuses derived IVP embryo reduced insulin-like growth factor type 2 receptor (*IGF2R*) gene expression level in muscle and liver (Farin et al., 2010). This imprinted gene was regulated the cattle development of fetal and placental (Constância et al., 2002). In addition, each cryopreservation techniques can alter gene expression pattern (Vergouw et al., 2012). Vitrification also decreased expression of DNA methyltransferase1o (*DNMT1o)* in mouse MII oocytes (Zhao et al., 2013) and significant increased H19 Imprinted Maternally Expressed Transcript (*H19*) methylation level in 2-cell stage embryo (Zhao et al., 2012). Another study showed vitrification significantly increased the imprinted gene such as Paternally Expressed 10 (*PEG10*), X Inactive Specific Transcript (*XIST*), and KCNQ1 overlapping transcript 1 (*KCNQ1O1T*) (Cheng et al., 2016). In murine, vitrification of oocyte can decrease methylation level of *H19*, Paternally Expressed 3 (*PEG3*), and Small Nuclear Ribonucleoprotein Polypeptide N (*SNRPN*) in blastocysts (Cheng et al., 2014). Nevertheless, there is a researcher demonstrated no expression of Mesoderm Specific Transcript 1 (*MEST1*), Neuronatin (*NNAT*), *PEG3* and *SNRPN* imprinted genes in blastocyst (Cruz et al., 2008).

In addition to epigenetic alteration, ARTs trend to cause environmental stress on oocyte and embryo. Oxidative stress and DNA damage causing cell death and apoptosis (Ricci et al. 2003). Stress response in embryo cryopreservation is regulated by heat shock protein (*HSP*) gene. *HSP70* was responded to heat stress in mammalian (Santoro, 2000). The relative abundance of *HSP70* expression upregulated in cryopreserved embryos compare with fresh control embryos, causing damage resulted in increasing apoptosis related gene expression (Park et al., 2006).

This study was aim to determine the effect of vitrification on epigenetic characteristics including, DNA methylation and histone modification, and evaluate the developmentally important genes expression, which compose of putative imprinted gene, apoptosis-related gene, and stress response gene in bovine vitrified embryo and blastocyst derived vitrified oocyte at different developmental stages.

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CHAPTER II

LITERATURE REVIEWS

2.1 *In vitro* production of bovine embryo

In vitro embryo production (IVP) is currently one of the crucial biotechnologies in livestock industries. In cattle, it is established as a commercial enterprise that includes the multiplication of elite genetics cattle, as well as large-scale embryo production from slaughterhouse ovaries (John, 1998). The main purpose of IVP is to accelerate genetic improvement of especially the local cattle populations and the export of embryos to other countries (Gibbons et al., 1994). As the demand of good genetics cattle tends annually increasing application of IVP technique in large scale livestock farm (Thornton, 2010). Moreover, IVP was initially developed to produce many embryos for research purposes and to investigate the basic physiological events occurring during early embryonic development.

Bovine embryos production *in vitro* has three steps process involving *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and in vitro embryo culture (IVC) of subsequent derived zygote. Nevertheless, the efficiency of in vitro embryo production is still low with only 20-30% of blastocyst derived oocyte development, compared with the in vivo embryo development from female reproductive tract (Thompson and Duganzich, 1996). Therefore, the embryo quality is also more unreliable for the in *vitro*-produced blastocyst, but the survival after transfer of in *vivo*-produced embryos is higher (Wright and Ellington, 1995). In addition, there is a lack of understanding

related to the physiological consequences during development following in vitro production, despite the growing evidence that abnormalities exist (Walker et al., 1996). In contrast to in vitro development, in vivo development occurs in a complex dynamic environment reflected by the adaptability of embryos to develop (Massip et al., 1995; Thompson 1996).

Although there are many improving development such as modifying of culture medium, but blastocyst derived in vitro oocyte development rate are also unusually reach to 40% (Lonergan and Fair. 2008; Stroud, 2012). There are also considerable evidences blastocyst quality is determined by post-fertilized cultural development, such as culturing with sheep oviduct resulted in increasing blastocyst quality (Enright et al., 2000; Lazzari et al., 2010). Nevertheless, there are evidence demonstrates that oocyte quality is determine the blastocyst rate. Therefore, post-fertilized environment might not mainly involve the capability of oocyte developing blastocyst (Rizos et al., 2002). Despite the challenges involved in successfully culturing embryos in vitro to blastocyst stage are depend on various factors, for example animal species, culture condition during fertilization and embryo development (Camargo et al., 2006).

2.2 Bovine oocyte and embryo cryopreservation

Preservation of oocyte and embryo is an important part of the ARTs. Cryopreservation of bovine oocyte and embryo has applied in various species including human (Kuwayama et al. 2005), cattle (Hamano et al., 1992), rabbit (Al-Hasani et al., 1989), horse (Maclellan et al., 2002), and porcine (Somfai et al., 2014). Approximately 25 species live offspring resulted from transfer of cryopreserved oocytes or embryos (Gajda and Smorąg, 2009).

There are two major methods using for cryopreserved oocyte and embryo which compose of slow freezing (conventional) and rapid freezing (vitrification). The system of these technique is to control the cooling rate by exchanging cellular water by inducing the ice crystal formation in extracellular region (Karlsson and Toner, 1996). Slow freezing has been applying for direct embryo transfer, it can make more practical using of cryopreservation in the field (Leibo, 1984). Although conventional freezing has reducing the cryoprotectant concentration, but this concentration ability is limited to decrease forming of ice crystal (Pereira and Marques, 2008). During this process there was an increase in the solute concentration outside and inside the cell due to ice crystal formation, this can be detrimental to cell survival (Mavrides and Morroll, 2002).

According to the results from of cryopreserved human oocytes, the survival rate of slow freezing ranged from 74% to 90% (Chen and Yang, 2009), and there was 85% normal morphology of mature oocyte that used slow freezing technique (Sinha et.al, 2014). The previous research reported 80% survival rate after thawing and 60% cleavage rate of the cryopreservation of human oocyte (Chen, 1986, 1988). However, this technique was still not efficiency choice for cryopreservation because of its low success rates, also led to cause cryoinjury and apoptosis (Djuwantono et al., 2011). Moreover, there was reports about susceptibility of the cell to cryoinjury especially in pig embryo. This can be caused by the relatively high lipid component in the pig cytoplasm of oocytes and embryos (Nagashima et al., 1994; McEvoy et al., 2000). Previous report evaluated slow frozen pig oocyte and found the optimal cooling rates by evaluating ice crystal forming characteristics of porcine germinal vesicle cumulus cell complexed oocytes (Yang et. al, 2012).

For the alternative cryopreservation technique, vitrification employed high concentration of cryoprotective agents (CPAs) solution and cooling rates. This technique can solidify the freezing solution turn to glass form without forming ice crystal by plunged directly into liquid nitrogen. (Arav, 2014). Vitrification was first successful reported in 1985, it was demonstrated as murine embryo cryopreservation (Rall and Fahy, 1985). Since that time, literally thousands of articles on vitrification, many in the field of reproductive biology, have appeared in the scientific literature (Fahy and Rall, 2007) and consecutive continuing to applied in livestock farming include cattle (Massip et al., 1986), goats (Yuswiati et al., 1990), sheep (Széll et al., 1990) and pigs (Dobrinsky, 1996).Vitrification continues to be an active area of research repeatable methods for preserving agriculturally important species, having a method to cryopreserve allow improvement in the genetics of domestic and international livestock using ARTs (Mullen and Fahy, 2012). Although there was still has problems related to cryopreservation also remain, but vitrification is still now routine procedure for cryopreserve mammalian oocytes and embryos alternating to slow freezing technique.

Improving the survival rate of IVP cattle embryos after cryopreservation have been attempted by comparing with in *vivo*, embryo derived *in vitro* culture has more susceptibility to cryopreservation by reducing pregnancy rate (Agca et al., 1998). Differ from the embryo, oocyte has lower successful vitrification since the lager size of their cell cause susceptible to injured from low temperature and CPAs by freezing (Fahy et al., 2004). Although many embryos vitrification protocols have been introduced to test in oocyte, but there was still low survival outcome compare with the

embryo due to maintain the post thawing developmental competence which is the most important factor involved in the effective oocyte cryopreservation (Lieberman, 2012).

Moreover, different developmental stage and species of the oocyte are also the main factor that effect the developmental capacity of vitrified oocytes. For example, there is a previous research referred that vitrified germinal vesicle breakdown (GVBD) stage oocyte have more developed than germinal vesicle (GV) and metaphase II (MII) after thawing (Khosravi et al., 2010). There are many reports compared between GV and MII oocytes in term of survivability after warming or developmental competency of vitrified-derived embryo in various species. For equine, mare oocyte either at the GV stage or at the MII stage can equally survived cryopreservation (Tharasanit et al., 2006). In bovine, MII stage oocyte has less susceptibility to cryoinjury that GV one (Lim et al, 1992; Otoi et al, 1995). Nevertheless, some report suggested not significantly different in cleavage among both GV and MII vitrified oocyte (Hochi et al. 1998). In addition, other reports also mentioned that the survival rate of bovine vitrified oocyte and subsequent development in GV stage higher than MII stage, due to the higher membrane permeability of MII more than GV oocyte (Zhou et al., 2010; Wang et al., 2010). For mouse oocyte, most results from overall studies have same tendency by showing a significant decrease survivability of both different stages vitrified oocyte compared with the fresh control group (Cao et al., 2009; Yazdanpanah et al., 2013), but mostly was no significant difference between both vitrified oocyte groups (Cha et al., 2011; Rajaei et al., 2013). In porcine, MII-stage pig oocyte was very susceptible to vitrification by reducing oocyte viability with cytoskeletal changing (Galeati et al., 2011), likewise with another study shown that porcine oocytes bovine has lower freezability than in bovine (Isachenko et al., 1992).

Previously, many reports have been demonstrated that offspring from several species obtained from oocyte vitrification, for instance establishing of live offspring in various species, for example in mouse (Whittingham, 1977), human (Kuleshova et al., 1999), and cattle (Vieira et al., 2002).

Continuing to improve the oocyte vitrification efficiency has become the further investigation for that period, because oocyte vitrification is most optional way for oocyte cryopreserving with successful outcome (Fahy et al. 1984). Vitrification technique was using over the past years for cryopreserving oocyte and embryo that have been developed for providing sufficiently rapid cooling rate developed like solid surface (Dinnyes et al., 2000), cryoloop (Lane et al., 1999), microdrop (Arav et al., 1987), cryotop (Hamawaki et al., 1999), electron microscopy grids (Martino et al., 1996), nylon mesh (Matsumoto et al., 2001), and open-pulled straws (Vajta et al., 1997). Cryotop method was proving to be the most practical used method of vitrification technique, because of its ability to remove maximum volume after loaded the embryo onto the tip of Cryotop stick. Application of Cryotop method was applying for cryopreservation in many animal species, including in cattle (Chian et al., 2004), rabbit (Hochi et al., 2004), buffalo (Muenthaisong et al., 2007), pig (Du et al., 2007) and human (Kuwayama et al. 2005). The factors that affect the efficiency of vitrification which compose of cryoprotectant viscosity, sample volume, and cooling rate. CPAs have two main types, first is membrane permeating which has small molecule for replacing water thus reduce water content in the cell, such as glycerol, ethylene glycol (EG), and dimethylsulfoxide (DMSO). Another CPAs type is non permeating, it can increase osmolarity in extracellular space thus reduce water content in cell and stabilization of membranes. This sub type is mostly found in sugar, for

instance sucrose, trehalose, and raffinose. The second sub type of non-permeating CPAs was acting by inhibition of extracellular ice crystal growth by binding to ice crystals, for example antifreeze proteins and synthetic ice-blockers.

Glycerol is the first CPAs shown to have cryoprotective capabilities successfully preserved the poultry sperm (Polge et al., 1949). Additional cryoprotectant, ethylene glycol (EG) has proved to be nontoxic for murine embryos (Zhu et al., 1996) and may improve the survival rate of frozen-thawed bovine IVP embryos (Saha and Suzuki, 1997). This agent has high penetrating ability and low toxicity; thus, it not need to diluting after thawed (Dochi et al., 1995). EG was successful employed the freezing of in vivo produced bovine embryos (Bracke and Niemann, 1995; McIntosh and Hazeleger et al., 1994). Another popular cryoprotectant is dimethyl sulfoxide (DMSO), it was demonstrated in 1959 usefully cryoprotectant because of highly penetration (Saha et al., 1996). Nevertheless, there was a report that cryopreservation of murine embryo to gain pub porn combine with glycerol and DMSO (Whittingham et al., 1972). Combination of CPAs CPAs ex. glycerol, EG, and DMSO was used in vitrification (Paul et al., 2014; Inaba et al., 2016). Reducing the sample volume and increasing cooling rate permit the moderate decrease in CPAs concentration, cause minimize its toxic and osmotic hazardous effects (Yavin et al., 2009). Combining these three factors can result in the following general equation for the probability of vitrification: Probability of vitrification = Cooling rate x Viscosity/ Volume (Saragusty and Arav, 2011).

2.3 Cryobiology of bovine vitrified oocyte and embryo

Cryopreservation process allow oocyte and embryo exposing to very low temperature to preserve the structure and function for long term (Paynter, 2005). This occurs during before freezing stage of cryopreservation oocyte and embryo with high osmolarity cryoprotectant solution (Pedro et al., 1997). The others negative effect from cryopreservation also includes chilling injuries that the freezing alters intracellular lipid droplets, especially in pig embryos. On the other hand, vitrification significantly decreases cryoinjury opportunity of cryopreserved oocyte and embryo by exposing very shortly of danger temperature region (Rall, 1987). Hence, effective cryopreservation of porcine embryos can be achieved only through vitrification (Berthelot et al., 2001). Cryoinjury cause by cryopreserved embryos depend on several factors, including CPAS type, cryopreserving protocol, and characteristic integrity of the embryos (Vajta and Kuwayama, 2006). Moreover, embryo cryopreservation is related with harmful outcome which induce detrimental effect on the post thawing survival and embryo developmental competency, included osmotic stress and intracellular ice crystal formation, which can harm the cytoskeletal and organelle of the cell.

2.4 Epigenetic modifications

The term “Epigenetic” was explaining the connection between phenotype and genotype during development (Jaenisch and Bird, 2003). Epigenetics is defined as inheritable functional changes that do not involve gene sequence changing (Bird, 2002). Although cells in different organs and tissues share the same DNA sequence, they may have differential expression profiles (Suzuki and Bird, 2008). Epigenetic modification act as a main role in genomic reprogramming and establishment of

embryo development patterns (Bird, 2007; Okano et al., 1999). During cell proliferation and differentiation, epigenetic modification regulates gene expression, keeping specificity of cell type specificity a one-way procedure (Borgel et al., 2010). The epigenetic changes involved in epigenetic inheritance in mammalian cells include different chromosome related mechanisms such as DNA methylation and histone modifications, noncoding/coding RNA such as maternal stored mRNAs, long noncoding RNAs, and small RNAs (Heard and Martienssen, 2014).

DNA methylation is mechanism involved process of methyl group adding at 5^{th} carbon atom of cytosine ring base in DNA sequence at CpG island area. This process is associated silencing of gene expression (Biermann and Steger, 2007). Another mechanism, histone modification is the adding of methyl or acetyl group at the N-terminal of the histone tail, this mechanism occurred after translation (Rajender et al., 2011). For the noncoding/coding RNA related process, small noncoding RNAs including micro RNAs (miRNA) and small interfering RNAs (siRNAs) was regulated post translational gene expression (Bartel, 2009), and it related with modification of transcription involving in the cell phase determination (Brait and Sidransky, 2011).

2.5 DNA methylation

In general, DNA methylation is major related in epigenetic modification, this mechanism is involved in regulating genomic imprinting (Li et al., 1993), X-chromosome inactivation (Panning and Jaenisch, 1996), silencing sequence (Walsh et al., 1998), and modulating chromosome stability (Xu et al., 1999). There are enzymes that regulated in generate and maintain DNA methylation, which are DNA methyltransferases family (DNMTs). DNMTs compose of many types, but not all of

them are mainly involve in the methylation. DNMT1 is response for maintaining of the methylation levels, there are 3 sub-types include DNMT1o, DNMT1s, and DNMT1p which splicing of the first exon (Ko et al., 2005). DNMT1o is expressed in mouse oocytes and preimplantation embryos. Lack of DNMT1 has been reported in the DNMT1^{-} deficient mouse embryos, X-chromosome inactivation was unstable because of hypomethylation due to maintain the X inactivation in embryos (Sado et al., 2000). On the other hand, overexpression of *DNMT1* caused abnormal hypermethylation in the genome, imprinted loss, and embryonic death (Biniszkiewicz et al., 2002). In addition, either DNMT3A and DNMT3B are regulated de novo methylation and maintenance of methylation (Arand et al., 2012).

However, there was a previous study reported both enzymes not maintained methylating (Okano et al., 1999). 5-methylcytosine (5mC) is the methylated form of the fifth carbon of cytosine, which is importance in controlling gene regulation (Lister and Ecker, 2009). It located at CpG islands which are the connection between cytosine and guanine base with phosphodiester bond. In mammals, approximately 60%-80% of CpG sites are methylated. However, about 10% of CpGs in CpG islands are frequently hypomethylated, and these hypomethylated CpGs are associated with gene promoters (Ren et al., 2017). In various mammalian species, reporting by immunofluorescence studies have shown paternal methylation loss in zygote (Beaujean et al., 2004; Fulka et al., 2004). In addition, the labeling of 5mC is weaker in the inner cell mass (ICM) than in trophoblast (TE) (Fulka et al., 2004). DNA methylation was decreased step by step to morula in mouse, it was observed in ICM of preimplantation embryos (Santos et al., 2002). In human, 5mC is very high in the 4-cells embryo and declines thereafter.

Investigation of DNA methylation using immunofluorescence established the first methylation reprogramming map in mouse preimplantation embryos (Santos et al., 2002). Other techniques, such as methylation-specific polymerase chain reaction and bisulfite sequencing after replication, are options for evaluating methylation status (Herman et al, 1996). Reduced representation bisulfite sequencing (RRBS) is used to analyze genomewide methylation profiles on a single nucleotide level with high base‐pair resolution and enrichment for the CpG-dense regions (Smith et al., 2009). There are many using of RRBS to study the DNA methylation pattern in murine oocytes, spermatozoa, and embryos by evaluated zygotes through the post implantation stage (Smith et al., 2012). In 2013, single-cell RRBS was developed to analyze the methylation profile at a single-base pair level within an individual cell (Guo et al., 2013). Currently, whole-genome bisulfited sequencing (WGBS) is the most practical use for detecting DNA methylation profiles with more than 90% reports in human genome (Miura et al., 2012). The post-bisulfite adaptor tagging (PBAT) method allows the assessment of 5mC in a single cell (Smallwood et al., 2014). Tagmentation-based WGBS (TWGBS) is an advanced method that can theoretically cover the complete methylome with a small quantity of cells (Wang et al., 2013).

2.6 Histone modifications

In the nucleosome of eukaryotes, histone proteins are major component of the chromatin in the nucleosome of the chromosome and have an important impact on gene expression. The nucleosome is the basic unit of DNA packaging (Zentner and Henikoff, 2013). In the histone tails, the modification can occur at the N-terminal which can affect their charge and function, including methylation, acetylation, phosphorylation, and

ubiquitination (Cedar and Bergman, 2009). Histone acetyltransferases (HATs) enzyme is involved in histone modification which regulated transcription (Pazin and Kadonaga, 1997; Wolffe, 1997). Histone deacetylase (HDACs) enzyme is regulated in a reversible pathway that related with repression of gene transcription (Pazin and Kadonaga, 1997; Wolffe, 1997). It has been reported that histone modification can establish the DNA methylation characteristic in the early embryo (Hatanaka et al., 2015). In addition, there were histone residues such as H3K4me, H3K9me and H3K27me are detectable (Erhardt et al., 2003). Histone methyltransferases (HMTases) enzyme involved in deacetylation for H3K4me (Wang et al., 2001), H3K9me (Tachibana et al., 2002) and H3K27me (Dodge et al., 2004). Moreover, there is a report that immunofluorescent staining showed differential expression of H3K9me and H3K27me between male and female pronuclei, which may explain the differences in the DNA demethylation processes between paternal and maternal genomes (Santos et al., 2005).

2.7 Epigenetic reprogramming

Epigenetic reprogramming is the deletion of epigenetic markers and readjust them to the different setting in normal mammalian development (Surani, 2001). It exists while gametogenesis and preimplantation embryo development (Reik et al., 2001), since it involved in zygotic genome activation and determination of cell fate (Chen et al., 2016). (Figure 1).

2.7.1 Reprogramming in Germ Cells

During gametogenesis, parental genome of mammal gamete has higher methylation more than somatic genome (Bestor et al., 2000). Reprograming at the germ cell occur in in primordial germ cells (PGCs) that have the removal of parental imprinted

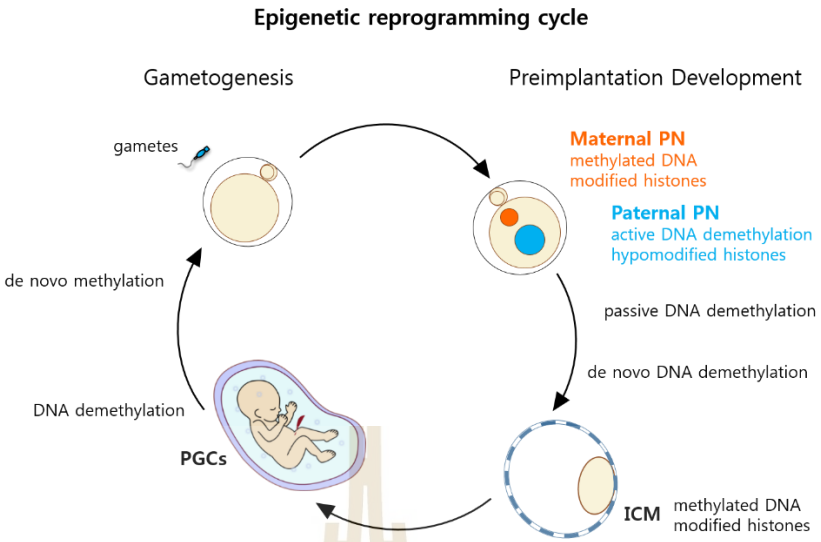

Figure 2.1 Epigenetic reprogramming cycle (Hugh et al., 2005).

genome and totipotency establishment (Figure 2A). Paternal demethylation has occurred in order to reprogrammed the paternal imprinted genome (Reik and Walter, 2001), it requires the activation of embryonic genome transcription which is activating prior maternal genome (Aoki et al., 1997). Remethylation happen after and it occur in paternal genome (Coffigny et al., 2006). Thus, remethylation appears at the same time as the establishing of imprints gene (Ueda et al., 2000). This germ cell reprogramming use for remodeling of imprints and erasure of epigenetic characteristic influencing by genetic and environment (Engler et al., 1991; Reik, 1993). Epigenetic occur to be inherited through the germ cell and incomplete erasure after fertilization (Romer et al., 1997; Morgan et al., 1999). Additionally, demethylation in germ cell can decrease the mutation which cause by DNA methylation (Duncan and Miller, 1980).

2.7.2 Reprogramming in Early Embryos

After fertilization, the preimplantation embryo immediately undergoes DNA methylation reprogramming at the genome level by the processes of

demethylation and methylation (O'Neill et al., 2006). There were both active and passive processes that take place in the reprograming of preimplantation embryo (Figure 2B). After fertilization, demethylation of paternal genomes occurs in hours before DNA replication (Dean et al., 2001).

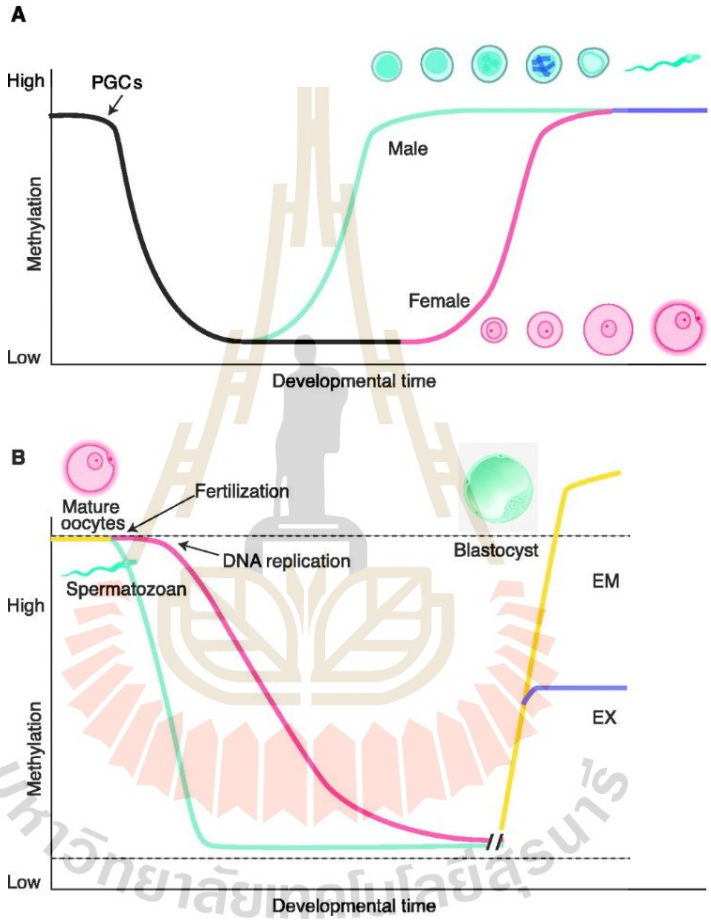

Figure 2.2 Reprogramming in the germ line (A) and preimplantation embryos (B) (Reik et al., 2001).

In this period, maternal genome has passive demethylation by keeping out DNMT1 enzyme from nucleus (Howell et al., 2001), and then follow by de novo methylation (Santos et al., 2002). De novo methylation appears after demethylation for maintain

the methylation level after preimplantation development of embryo which regulated by DNMT3A and DNMT3B (Feil and Khosla, 1999).

2.8 Genomic imprinting

According to above-mentioned, the epigenetic reprograming is significantly associated with on imprinting mechanisms (Figure 3) (Ferguson-Smith and Surani, 2001). Genomic imprinting is related with parental genome asymmetry (Christine and Heiner, 2003), this mechanism is epigenetic regulating to allele specific gene expression in mammalian genome (Bartolomei and Ferguson-Smith, 2011). It regulated with embryo development, placental performance, and neurobehavior (Tilghman, 1999). Following to mammalian genomic DNA is methylated mainly at cytosine-guanine dinucleotides, the imprinted genes are located in the area that contained different marker at CpG rich domain, called imprint control regions (ICRs) or differentially methylated regions (DMRs) (Delaval and Feil, 2004). Methylation of allele specific is typical for DMRs and, the methylation pattern is implicated the expression of imprinted gene regulation (Constância et al., 2000; Murrell et al., 2001). Actually, the epigenetic mechanism of the imprinted genomes empowers the parental mammalian ally gene expression (Preece and Moore et al., 2000). Paternal DMRs obtain methylation in the prespermatogonia (Lucifero et al., 2004). For maternal DMRs, methylation of imprinted gene still unevaluated while early embryonic development (O'Doherty et al., 2015).

In cattle, there were about 16-21 the imprinted gene which was verified and have single allele expression (Khatib, 2007). Previously, the researcher studied the status of the imprinted genes in different developmental stage of bovine

preimplantation embryos, they reported no expression of *PEG3 MEST1* and *SNRPN* in blastocyst. Many imprinted genes are involved in embryo development regulation, cell differentiation and in reproductive outcome, mostly are epigenetically regulated (Wang et al., 2010; Corry et al., 2009), for example; paternally expressed 3 (*PEG3*), X-inactive specific transcript (*XIST*), mesoderm specific transcript (MEST), insulin-like growth factor 2 (*IGF2)*, insulin-like growth factor 2 receptor (*IGF2R*), and small nuclear ribonucleoprotein polypeptide n (*SNRPN*).

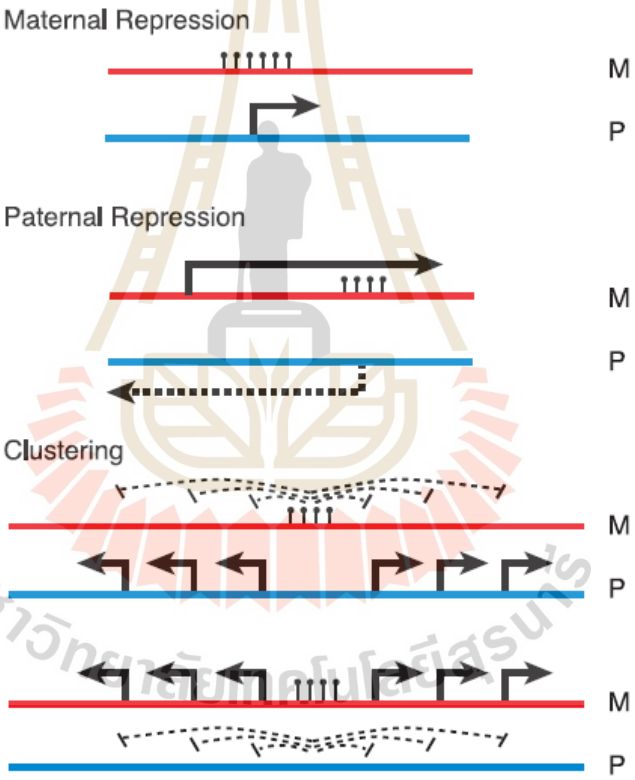

Figure 2.3 Reprogramming on imprinting mechanisms (Reik et al., 2001).

Although imprinting disorders are quite rare, but manipulations used in ARTs may interfere with genomic imprinting (Rossignol et al., 2006), many studies reported the effect of ARTs on imprinting mechanism in animal (Rivera et al., 2008; Market-

Velker 2010; de Waal E, et al. 2012) and and human (Vermeiden and Bernardus, 2013). There were many reports show that offspring derived ARTs inducing imprint disorder, such as Beckwith-Wiedemann syndrome (BWS) in human (Lim, et al. 2009) and large offspring syndrome (LOS) in ruminants (Bertolini et al., 2002; Farin et al., 2006). Another research links ART to increased incidences of low birth weights and caused imprinting disorders, such as Angelman syndrome (Maher, 2005), these findings in domestic animal species indicates that epigenetic mechanisms are likely affected by in vitro production. Furthermore, genes under epigenetic regulation could be candidates for producing the LOS phenotype and the reduced efficiency of IVF (Sadie et al., 2009). In sheep, aberrant expression and methylation of *IGF2R* was found in most lambs that were afflicted with LOS after in vitro culture, it has been characterized associated with methylation abnormalities within the DMR of *IGF2R* (Young et al., 2001). Likewise, for murine preimplantation embryos, the use of serum adversely effected the *H19* methylation expression (Doherty et al., 2000; Khosla et al., 2001).

In additions, it has been supposed that *in vitro* culture of embryos is involved in epigenetic alteration in embryonic genome which associated with TCM and SOF culture medium condition (Wrenzycki et al., 2001). The effects of these two culture systems on *IGF2R* gene expression were also investigated, significant differences were found in the among those two culture systems (Yaseen et al., 2001). On the other hand, few studies refer to the other kind of ART altered imprinted gene include cryopreservation. Especially, vitrification also has effect involve in epigenetic reprograming and imprinted gene expression. There was a study reported that vitrification was an intense stress factor causing heavy impact effect on epigenetic

regulation while preimplantation embryo development which can cause reducing of development competence (Chen et al., 2016).

2.9 Vitrification induce apoptosis and stress in bovine embryo

Dysfunction of mitochondria is bovine embryos development efficiency, because of reducing ATP production from mitochondria tend to relate with embryo developmental failure (Brevini et al.,2005). Cryopreservation can affect mitochondrial dysfunction and swelling (Valojerdi and Salehnia, 2005; Hochi et al., 1996), abnormal shape and mitochondrial membranes rupture (Wu et al., 2006; Turathum et al., 2010). Interestingly, there was reported that cryopreservation can cause detrimental consequence of the apoptotic and oxidative stress gene (Moussa et al., 2014; Lin and Tsai, 2012). The alteration of gene expression is related with physical damage of DNA, there was a report that slow freezing can affect embryo DNA integrity same equally like vitrification (Kader et al., 2008). In addition, vitrification can reduce or increases methylating in bovine embryos gene (Zhao et al., 2012).

Apoptosis is a programmed cell death mechanism which composes of two pathway types, include extrinsic pathways which is death receptors recruit adaptor proteins. For mitochondrial pathway, death stimuli target transduced by proapoptotic BAX and BAK. (Figure 4.)

Stress response in embryo cryopreservation is regulated by heat shock protein (HSP) gene. HSP family (HSPs) have several members, for instance HSP47, HSP60, HSP70, and HSP90 have been identified in animals (Keller et al., 2008). Upregulation of HSPs gene expression is responded to cellular stress such as heat stress and reactive oxygen species (Whitley et al. 1999). Especially, HSP70 was responded to heat stress

in mammalian tissue (Santoro, 2000). Moreover, the relative abundance of *HSP70* expression was upregulated in cryopreserved embryo in comparison with the fresh controls. This can show that cryopreservation caused damaging which resulted in an increase apoptosis-related gene transcription (Park et al., 2006).

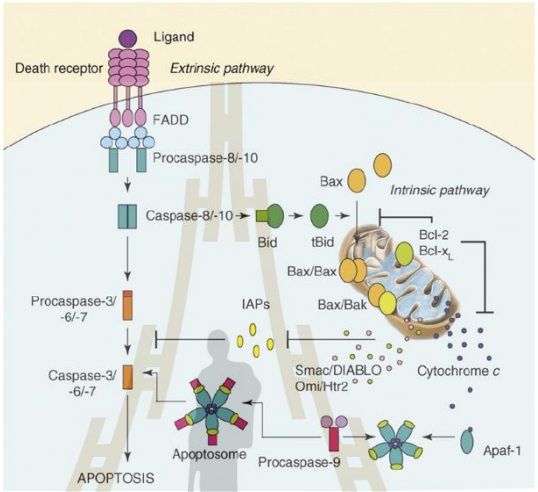

Figure 2.4 Extrinsic and intrinsic apoptotic pathways (Rita et al., 2008).

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CHAPTER III

THE EFFECTS OF VITRIFICATION AFTER EQUILIBRATION IN DIFFERENT CONCENTRATIONS OF CRYOPROTECTANTS ON THE SURVIVAL AND QUALITY OF BOVINE BLASTOCYSTS

3.1 Abstract

This research evaluated CPAs concentration effect on bovine embryo vitrification at blastocyst stage in an equilibrated solution and the developmental related gene expression. Bovine blastocysts derived IVP were equilibrated in either 7.5% ethylene glycol (EG)+7.5% DMSO (Va group) or in 2% EG+2% DMSO (Vb group) then vitrified in 16.5% EG+16.5% DMSO+0.5M sucrose on Cryotop® sheets device. Embryos were cultured for 48 h after warming and compare the percentage of re-expansion, hatching, total cell and membrane damage cells were compared between fresh control blastocyst and vitrified blastocyst groups. The results were founded that survival rate, total cell number and the membrane damage cell number were not significantly different among both vitrified groups. Although both vitrified groups have increased damage cell numbers. Nevertheless, only Vb group had significant higher damage cell numbers than control group ($P<0.05$), while Va group was not. Moreover, vitrification significant increased *HSP70* gene expression in Va group higher than control group, but not differenced from Vb group. On the other hand, the

remained genes expression was similar within those three groups, including *SNRPN*, *IGF2R*, *HDAC1*, *DNMT3B*, *BAX*, *OCT4*, and *IFN-t*. These results can conclude that CPAs concentration in equilibration solution did not influenced the survivability of embryos. However, 15% CPAs concentration of equilibration solution could maintain normal cell number and survivability which was associated with the upregulation of *HSP70* gene expression.

3.2 Introduction

Long term preservation of embryo using cryopreservation is important for reproductive technology in cattle (Viana, 2018). Cryopreservation, which compose of slow freezing and vitrification, has many efficiencies in embryo preservation (Inaba et al., 2016). Nevertheless, there was a report referred causing damage of cell membrane and DNA (Inaba et al., 2016). Blastocyst vitrification in pig did not cause cell membrane damage, but it retarded blastomere proliferation when post warmed culture (Nguyen et al., 2018). In addition, blastocyst vitrification can alter the imprinted genes expression, for example *IGF2R* in pigs (Bartolac et al., 2018) and *SNRPN* in mice (Chen et al., 2014). In mouse and pig, vitrification was reported altering the important developmental gene expression, including heat shock protein gene (*HSP70*) (Castillo-Martín et al., 2015) and octamer-binding transcription factor 4 gene (*OCT4*) (Jahangiri et al, 2018). Furthermore, oocyte vitrification can alter epigenetic related gene expression in murine (Cheng et al., 2014) and bovine (Chen et al., 2016) embryos.

Recently, different vitrification methods had been applied on bovine embryo with various types of cooling device and CPAs solution protocol. (Inaba et al., 2011; Paul et al., 2014; Leme et al., 2020). Vitrification protocol using 7.5% ethylene glycol

(EG) and 7.5% dimethyl sulfoxide (DMSO) for equilibration solution is the most practical used for providing high efficiency cooling (Sansinena et al., 2011). However, there was a previous study reported reducing of equilibration concentration solution in to 4% CPAs using EG and DMSO, it can reduce toxicity of cryoprotectant (Papis et al., 1999; Dinnyés et al., 2000).

3.3 Material and Methods

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

Bovine oocytes were collected as described previously by Imai et al. (Imai et al., 2006). Briefly, the cow ovaries were acquired from slaughterhouse and transfer to laboratory, then washed in 0.9% (w/v) sodium chloride solution for many times and stored in Dulbecco's phosphate buffered saline (D-PBS) supplemented with 50 mg/ml gentamicin at 15℃ for 15 h. Cumulus-oocyte complexes (COCs) were aspirated from 3-6 mm in diameter follicles using a 10-ml syringe with 18-gauge needle only good quality oocytes which had good morphology were chosen After selection, COCs were washed with IVM medium and cultured 50 COCs per droplet in IVM medium 500 µl droplets, which was a HEPES-buffered TCM-199 medium (Medium 199, 12340-030, GIBCO Invitrogen, Life Technologies, Auckland, New Zealand) supplemented with 5% newborn calf serum (NCS, S0750-500; Biowest SAS, Nuaillé, France) and 0.02 Armor Units/ml follicle stimulating hormone (FSH; Antrin R10; Kyoritsu Seiyaku Co., Tokyo, Japan), covered by paraffin oil (Paraffin Liquid; Nacalai Tesque Inc., Kyoto, Japan). COCs were *in vitro* cultured in 4-well dishes (Nunclon Multidishes, Nalge Nunc International, Rochester, NY, USA) for 22 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air.

3.3.2 *In vitro* fertilization (IVF) and *in vitro* culture (IVC)

IVF was done as reported previously by Imai et al. (2006). Briefly, Japanese Black bull frozen semen in 0.5-ml straw was thawed in a 37°C water bath for 30 s, put in 3 ml of 90% Percoll solution in 10-ml conical tubes (As One, Osaka, Japan), and centrifuged at 770×g for 10 min. The pellet was re-suspended in 6 ml of IVF 100 medium (Research Institute for Functional Peptides Co., Ltd., Yamagata, Japan) and centrifuged again at 570×g for 5 min and adjusted the final concentration to 3×10^6 spermatozoa/ml. Post harvested sperm was co-culture with *in vitro* matured in 100 µl droplets of IVF 100 medium (20 oocytes per droplet) in 35-mm plastic dishes (Nunclon Multidishes) overlay with paraffin oil, incubated at 38.5°C in 5% CO_2, 5% O_2 and 90% N_2 humidified atmosphere for 5 h. Day 0 was defined after fertilization start. After *in vitro* fertilized, the cumulus cells of presumptive zygotes were completely denuded until zona pellucida was cleared by gentle pipetting a fine glass pipette. The denuded zygotes were washed and cultured in IVC medium using Charles Rosenkrans 1 (CR1) medium (Rosenkrans et al., 1993) supplemented with amino acids (CR1aa) and 5% CS (Imai et al., 2002). Twenty zygotes were cultured in a droplet contain 100 µl of CR1aa medium in 6-well dishes (Research Institute for Functional Peptides Co., Ltd.), overlay with paraffin oil, incubated at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 for 7 days.

3.3.3 Blastocyst vitrification

Following to the guidelines of the International Embryo Transfer Society (Stringfellow and Givens, 2010), excellent graded of expanded blastocysts were cryopreserved on Cryotop® sheets as previously modification described by Inaba et al. (2016). Briefly, expanded blastocyst stage embryo were incubated for 3 min in an

equilibration solution that composed of HEPES-buffered TCM-199 medium supplemented with 20% (v/v) CS and either 7.5% (v/v) EG (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 7.5% (v/v) DMSO or 2% (v/v) EG (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 2% (v/v) DMSO. Embryos in both groups were immersed into a vitrification solution after equilibration, it composed of a HEPES-buffered TCM-199 medium supplemented with 16.5% (v/v) EG, 16.5% (v/v) DMSO, 0.5M sucrose, and 20% (v/v) CS. One to three embryos were placed on a Cryotop® deviec (Kitazato BioPharma Co. Ltd., Fujinomiya, Japan) in a small vitrification solution volume (<1 µl) within 1 min before plunging into liquid nitrogen. Then, embryo warming by Cryotop® device insertion into 2.5 ml warming solution, which was composed of HEPES-buffered TCM-199 medium supplemented with 0.5M sucrose and 20% CS in 35-mm plastic dishes (Nunclon Multidishes) at 38.5°C for 5 min, transferred into 2.5 ml rinsing solution, which was composed of a HEPES-buffered TCM-199 medium supplemented with 20% (v/v) CS for 5 min at 38.5°C. After warming, embryos were consequently cultured as previously described.

3.3.4 Evaluation of embryo viability

Embryos were washed in CR1aa medium supplemented with 5% (v/v) CS and 0.1 mM β-mercaptoethanol after warming and cultured 5-8 embryos/50 ml in the same culture medium overlayed with mineral oil at 38.5°C in 5% CO_2, 5% O_2 and 90% N_2 for 24 h (Saito et al., 1994). After 24 hours recovery, the embryos were showed blastocoel re-expansion and defined as alive. After 48 hours warming, the re-expansion rate was measured by using hatching and hatched blastocyst rate percentage. (Figure 3.1).

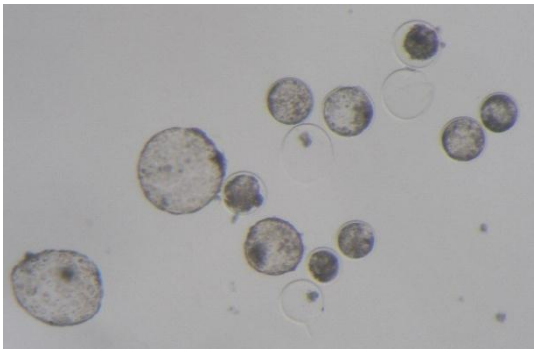

Figure 3.1 hatching and hatched embryos after 48 h warmed.

3.3.5 Total cell and damage cell numbers evaluation in survived embryos

Survived blastocysts were incubated 10 min at 38.5°C in a 5% CO2 incubator with 3 mg/ml of propidium iodide (PI) in the culture medium for labelling the membrane-damaged cells nuclei. Washing embryos in D-PBS supplemented with 3 mg/ml polyvinylpyrrolidone and treated with 0.25 mg/ml Hoechst 33342 (Calbiochem, San Diego, CA, USA) dissolved in 99.5% ethanol at 4°C for overnight to fix the embryos and stain the cell nuclei. The fixed embryos were mounted in glycerol droplets, flattened by cover slips on glass slides, and examined under UV light with excitation at 330-385 nm and emission at 420 nm using an epifluorescence microscope (Nikon Eclipse E600, Tokyo, Japan). Damage cells nuclei were labelled using PI and Hoechst 33342 appearing in red or pink colours (Figure 3.2)., whereas the cells nuclei labelled by Hoechst 33342 appearing in blue (Figure 3.3). Each embryo was capture to the digital images (Nikon Eclipse E600, Tokyo, Japan) and cell numbers were counted using NIH ImageJ (v. 1.52) software.

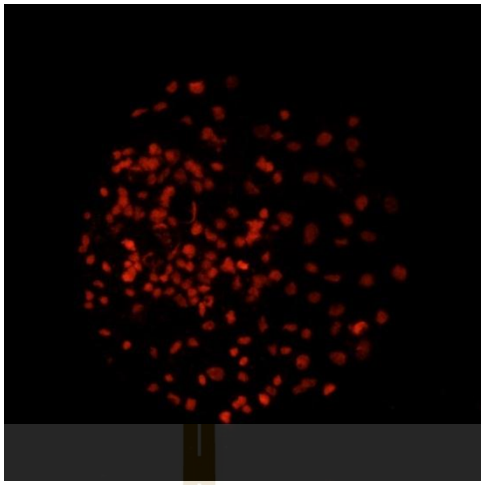

Figure 3.2 Membrane damaged cells of blastocyst after PI staining.

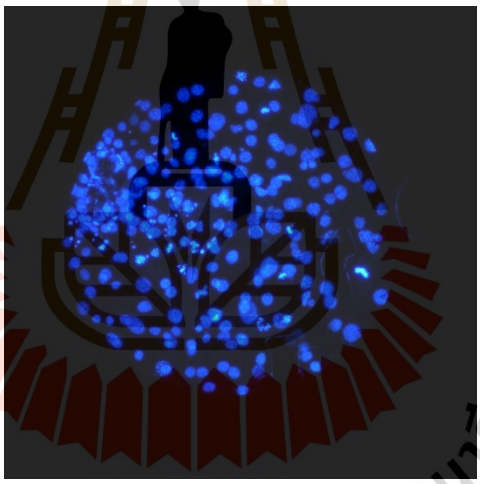

Figure 3.3 Total cells of blastocyst after Hoechst 33342 staining.

3.3.6 Gene expression evaluation in blastocysts

Gene expression of the blastocysts was analysed by reverse transcription quantitative PCR (RT-qPCR) was done as described previously by Takahashi et al. (Takahashi et al., 2013). Briefly, 5-8 blastocysts sample was lysed in RLT buffer (RNeasy Micro Kit; QIAGEN, Hilden, Germany) and stored at -80°C

until analysis. Three biological replications were performed. Total RNA was extracted and purified from pooled samples using the RNeasy Micro Kit (QIAGEN) according to the manufacturer's instructions. cDNA synthesis was performed using the PrimeScript™ II 1st strand cDNA synthesis kit (Takara) by following the manufacturer's protocol. The studied genes and their primers are listed in Table 3.1. Quantitative mRNA expression analysis in samples was performed by RT-qPCR at 1: 20 dilutions using the Light Cycler 480 SYBR Green I Master (Roche Applied Science, Penzberg, Germany) according to standard protocol. PCR conditions were 95°C for 5 min and 45 cycles of 95°C for 10 s, 60°C for 5 s and 72°C for 10 s. The quantification of transcripts in samples was analysed using the Light Cycler 480 Instrument (Roche). In each sample, cDNA levels of each gene were calculated directly by Light Cycler 480 software, comparing measured values to standard curves prepared by the measurement of pooled standards diluted 1 10 and 100 times. For each experimental sample, the amount of mRNA of each transcript was determined from the appropriate standard curve. Subsequently, the quantity of each transcript was divided by that of *GAPDH* to obtain a normalized value for each transcript. Target values were compared with that of the control, which was assigned a value of 1 in each replication.

3.3.7 Experimental design

Experiment 1 investigated the effect of pCPA concentration during equilibration on survival and membrane damage of vitrified blastocysts. Day 7 blastocysts were vitrified either after equilibration in 7.5% EG+7.5% DMSO (Va group) or in 2% EG+2% DMSO (Vb group), warmed immediately and subsequently cultured. Live-dead status of embryos based on re-expansion and hatching were compared to that

of a non-vitrified group (Control) at 24 h and 48 h after warming, respectively. Thereafter, numbers of total cells and membrane damaged cells were compared among groups as described above. The experiment was replicated five times.

Experiment 2 investigated the effect of vitrification after equilibration in low or high concentration of pCPA on the expression of developmentally important genes in surviving blastocysts, On Day 7, blastocyst embryos were vitrified either after equilibration in 7.5% EG+7.5% DMSO (Va group) or in 2% EG+2% DMSO (Vb group), warmed immediately and subsequently cultured. Twenty-four h after warming, live embryos in each group were pooled and subjected for RT-qPCR to assess expression of *DNMT3B*, *HDAC1*, *IGF2R*, *SNRPN*, *BAX*, *HSP70*, *OCT4*, and *INF-t* genes. Gene expression in vitrified groups were compared to that of a non-vitrified control cultured until Day 8. The experiment was replicated three times.

3.3.8 Statistical analysis

Data are expressed as mean±SEM. Percentage data were subjected to arcsine transformation before analysis. Results were analysed using the Kruskal-Wallis test followed by Dunn's multiple comparisons test using GraphPad Prism software (Ver 7.02 for Windows, GraphPad Software, La Jolla, California, USA). $P < 0.05$ was defined as the significance level.

3.4 Results

3.4.1 The effect of pCPA concentration during equilibration on survival and membrane damage of vitrified blastocysts

The re-expansion rates of vitrified blastocysts during 24 h of culture after warming in both vitrified groups are summarized in Table 3.2. There was no

significant difference between the Va and Vb groups in the re-expansion rates at 24 h after warming. The mean re-expansion rate in Vb group was significantly lower than control. Nevertheless, the re-expansion rate in the Va group did not differ significantly from those of the Vb and control groups. Similarly, at 48 h after warming, the rate of hatched blastocysts in the Vb group was significantly lower than the control but did not differ from that in the Va group, (Table 3.2). The percentages of hatching and hatched blastocysts in the Vb group were significantly lower from that of the non-vitrified control group. However, there was no significant difference between the Va and Vb groups and between the control and Va groups in the percentages of hatching and hatched blastocysts, (Table 3.2).

The surviving embryos of the Vb group, but not the Va group, had significantly lower total cell number compared with the control (Table 3.3), and there was no difference between the Va and Vb groups. The numbers of dead cells in surviving embryos in both the Va and Vb groups were significantly higher than that of the control group. However, the percentage of membrane damaged cells was significantly higher only in the Vb group when compared with the control, (Table 3.3).

3.4.2 The effect of vitrification after equilibration in low or high concentration of pCPA on the expression of developmentally important genes in surviving blastocysts

The relative gene expression levels are summarized in Figure 3.4. There was no significant difference between the control, Va and Vb groups in the expression of *SNRPN*, *IGF2R*, *HDAC1*, *DNMT3B*, *BAX*, *OCT4*, and *INF-t* genes. The relative expression level of *HSP70* gene was significantly higher ($P<0.05$) in the Va

group than in the control, whereas the expression level of this gene in the Vb group did not differ significantly from the other groups.

Table 3.1 The list of genes and their primers used in the study.

Gene	Accession no.	Primer sequences	Product size (bp)
*GAPDH**	NM_001034034	F- CTCCCAACGTGTCTGTTGTG	70
		R- TGAGCTTGACAAAGTGGTCG	
SNRPN	NM_174463	F- GGGACCGTTTACACTTGAGAC	153
		R- GGAAATCCACCACAGGTACT	
IGF2R	JO3527	F- CGCCTACAGCGAGAAGGGGTTAGTC	393
		R- AGAAAAGCGTGCACGTGCGCTTGTC	
HDAC1	XM_004005023.1	F- GGAGATGTTCCAGCCCAGTG	153
		R- CCTCCCAGCATCAGCATAGG	
DNMT3B	XM_004014483.1	F- GACGTAGAGGGCAGAGATGC	103
		R- GCCACCAGGAGAAACCCTTG	
HSP70	U09861	F- TGGGGGACAAGTCGGAGAA	127
		R- TTCGTGGGGATGGTGGA	
BAX	NM_173894	F- TCTGACGGCAACTTCAACTG	135
		R- TCGAAGGAAGTCCAATGTCC	
OCT4	NM_174580	F- TGCAGCAAATTAGCCACATC	68
		R- AATCCTCACGTTGGGAGTTG	
INF-tau	X65539	F- TGTCTGAGGACCACATGCTAGGT	121
		R- ACCATCTCCTGAGGAAGACCAA	

*Endogenous reference gene.

Abbreviations: F, forward primer; R, reverse primer.

Table 3.2 Comparison of survival and hatching rate of re-expanded blastocyst between two different vitrification procedures.

Treatment	No. of vitrified embryos	No. (%) of re-expanded blastocyst at 24 h after warming	No. (%) of hatched blastocyst at 48 h after warming	No. (%) of hatching & hatched blastocyst at 48 h after warming
Control	71	71 (100.0±0.0) [a]	45 (74.5±14.4) [a]	62 (93.0±6.9) [a]
Vitrified-A (Va)	74	53 (74.8±9.5) [ab]	13 (23.6±11.8) [ab]	32 (53.6±13.3) [ab]
Vitrified-B (Vb)	74	45 (63.9±4.3) [b]	9 (17.1±9.8) [b]	25 (43.1±12.4) [b]

Five replicates were performed. Percentage values are presented as mean±SEM. Different superscripts (a and b) denote a significant difference in the same column ($P < 0.05$). Vitrified-A = blastocysts vitrified after equilibration in 15% pCPA. Vitrified-B = blastocysts vitrified after equilibration in 4% pCPA.

Table 3.3 Cell numbers and the extent of membrane damage in vitrified re-expanded blastocysts.

Treatment	No. of evaluated embryos	No. of total cells	No. of membrane damaged cells	% of membrane damaged cells
Control	47	140.1±10.7 [a]	0.6±0.1 [a]	0.5±0.1 [a]
Vitrified-A (Va)	36	112.7±11.8 [ab]	1.9±0.4 [b]	1.8±0.5 [ab]
Vitrified-B (Vb)	32	88.3±5.1 [b]	4.2±1.1 [b]	5.5±1.3 [b]

Five replicates were performed. Values are presented as means ± SEM. Different superscripts (a and b) denote a significant difference ($P < 0.05$) in the same column. Vitrified-A = blastocysts vitrified after equilibration in 15% pCPA. Vitrified-B = blastocysts vitrified after equilibration in 4% pCPA.

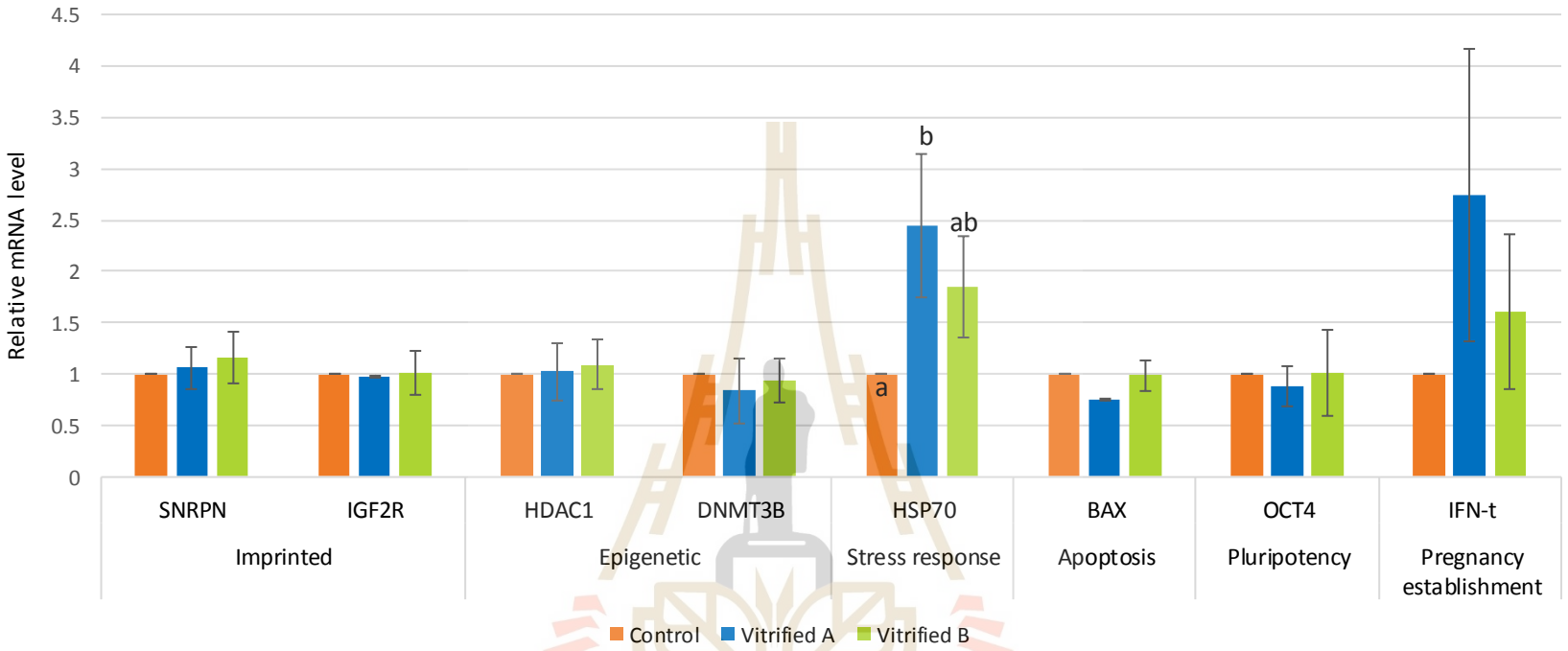

Figure 3.4 Relative expression of genes with different functions in surviving control and vitrified bovine blastocysts. Data are presented as means±SEM. Different superscripts (a and b) denote a significant difference ($P<0.05$). Vitrified-A = blastocysts vitrified after equilibration in 15% pCPA. Vitrified-B = blastocysts vitrified after equilibration in 4% pCPA.

3.5 Discussion

3.5.1 The effect of pCPA concentration during equilibration on survival and membrane damage of vitrified blastocysts

The results of this study showed no significant difference in the re-expansion, hatching and the quality of embryos in terms of total cell numbers, membrane damage and the expression of studied genes between bovine blastocysts vitrified after equilibration in 15% or 4% pCPA. Although vitrification increased the number of membrane damaged cells in both groups, the increase in membrane damage cells was less pronounced after equilibration in 15% CPA since after this treatment total cell number and percentage of blastomeres with membrane damage were not significantly different from those of the control. On the other hand, when embryos were vitrified after equilibration in 4% pCPA, total cell number was reduced, and percentage of membrane damaged cells was significantly increased compared with the control. Interestingly, compared with the control group, vitrification after equilibration in 15% CPA (which from a cytological viewpoint seemed better), was associated with significantly increased expression of *HSP70* gene whereas vitrification after equilibration 4% pCPA was not. The *HSP70* (also known as *HSPA1A*) gene encodes "heat-stress protein" which in mammalian cells can be activated by various stresses such as thermal or oxidative stress (Keller et al., 2008; Bernardini et al., 2004; Sonna et al., 2002). Our results are in accordance with a previous study on porcine embryos reporting the upregulation of this gene in vitrified blastocysts (Castillo-Martín et al., 2015). The role of heat stress protein is the response to a stressful environmental stimulus, such as oxidative stress generated during embryo culture (Bernardini et al., Upregulation of the *HSP70* gene by

vitrification causes an increase in heat stress protein production in cells which acts as a cell stress activated signal in response to cooling and rewarming of mammalian tissues (Sonna et al., 2002). Production of HSP70 might play a role in the recovery from sublethal damages in cryopreserved embryos since it was reported to prevent proteins from damages (Muller et al., 2013) and even inhibit apoptosis (Beere et al., 2000). Therefore, is possible that increased HSP70 production contributed to the better cytological properties in the Va group. However, further investigations will be needed to clarify this point. Previous studies have demonstrated that equilibration in reduced concentration (4%) of pCPA was beneficial for the survival and subsequent developmental competence of vitrified bovine oocytes (Papis et al., 1999) and porcine oocytes (Somfai et al., 2015). Our results revealed that this is not the case in Day 7 bovine blastocysts which suggests a difference in the requirements for pCPA treatment and/or sensitivity to pCPA toxicity between oocytes and blastocysts in cattle.

3.5.2 The effect of vitrification after equilibration in low or high concentration of pCPA on the expression of developmentally important genes in surviving blastocysts

In present study, the most (approximately 70%) of embryos survived vitrification in both groups. However, their ability to induce pregnancy and to develop to healthy offspring remains unknown. To have an insight on their quality, we analysed the expression levels of some developmentally important genes such as *IGF2R*, *SNRPN*, *HDAC1*, *DNMT3B*, *BAX*, *OCT4*, which were reported to be affected by cryopreservation in other cell types or species (for references see Introduction) and/or considered to be reliable markers for the health and competence of bovine

blastocysts to induce pregnancy such as the *IGF2R* and *IFN-t* (Sugimura et al., 2012). However, our results revealed similar expression of these genes between control and vitrified blastocysts, irrespective of the CPA treatment used. We found similar expression of the imprinted *IGF2R* and *SNRPN* genes in vitrified and non-vitrified bovine blastocysts. However, in previous studies vitrification was reported to alter the expression of these genes in porcine and murine blastocysts, respectively (Bartolac et al., 2018; Chen et al., 2014). This suggests possible differences between species in their reaction to vitrification.

Previous researched showed that vitrification of bovine oocytes caused alterations in the expression of epigenesis-regulating genes such as the *HDAC1* later, at the blastocyst stage (Chen et al., 2016). Furthermore, in mice, vitrification at the blastocyst stage altered the expression of other epigenesis-regulating genes including the *DNMT3B* (Cheng et al., 2014). Also, in a previous study, cryopreservation of human embryos by freezing caused delayed expression of *DNMT3B* (Petrussa et al., 2014) suggesting that cryopreservation has an effect on DNA methylation by disturbing gene expression particularly for *DNMT3B* which is the major de novo methylation enzyme at the blastocyst stage. However, we found no difference between the vitrified groups and their fresh counterparts in the expression of the *HDAC1* and *DNMT3B* genes. These results again might suggest differences between species and cell types. According to previous research, cryopreservation can damage DNA and mitochondrial functions in mammalian embryos thus increasing the frequency of apoptotic cells (Inaba et al., 2016; Somfai et al., 2015; Ahn et al., 2002; Men et al., 2006) which is reflected by an increase in the expression of pro-apoptotic genes such as *BAX* (Kuzmany et al., 2011). However, in the present study it might not

be the case since the expression of *BAX* in surviving vitrified embryos in both groups were similar to that in the control.

OCT4 (also known as *POU5F1*) is a pluripotency gene in mammalian embryos which was reportedly altered in blastocyst by vitrification in pigs (Castillo-Martín et al., 2015) and mice (Jahangiri et al., 2018). Furthermore, slow freezing was reported to reduce the expression of this gene in bovine blastocysts (Mori et al., 2015). However, in the present study vitrification of bovine blastocysts did not alter the expression of this gene (irrespective of the equilibration treatment) which might indicate difference between species in terms of their reaction to vitrification and also differences between vitrification and slow freezing in their effect on bovine blastocysts. *IFN-t* encodes the protein interferon tau which in bovine embryo has an important role in signalling molecule during pregnancy in ruminants (Bazer, 2013). Hence, expression level of this gene is considered as an indicator of developmental competence in bovine embryos. In a previous study, cryopreservation by freezing was reported to alter the expression of this gene in bovine embryos (Mori et al., 2015). Our results reveal that vitrification did not cause a significant difference in the expression of this gene in bovine blastocysts which is in accordance with a recent study reporting similar pregnancy rates when vitrified and non-vitrified bovine embryos were transferred to recipients (Do et al., 2018). Also, our results might reflect a difference between vitrification and slow freezing in their effect on blastomeres.

3.6 Conclusions

There was no significant difference between equilibration in low (4%) or high (15%) concentration of pCPA in survival, cell numbers and the expression of the selected developmentally important genes in bovine blastocysts after vitrification; however, vitrified embryos equilibrated in 15% pCPA seemed better since they did not differ significantly from control. This way of vitrification caused only a slight increase in the number of membrane damaged cells which was associated with increased expression of the HSP70 gene in surviving embryos. Irrespective of pCPA treatment, vitrification had no effect on the expression of *IGF2R*, *SNRPN*, *HDAC1*, *DNMT3B*, *BAX*, *OCT4*, and *IFN-t* genes.

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CHAPTER IV

EFFECT OF VITRIFICATION AT DIFFERENT MEIOTIC STAGES ON EPIGENETIC CHARACTERISTICS OF BOVINE OOCYTES AND SUBSEQUENTLY DEVELOPING EMBRYOS

4.1 Abstract

Vitrification by the Cryotop method is frequently used for bovine oocyte cryopreservation. Nevertheless, vitrified oocytes still have reduced developmental competency compared with fresh counterparts. The objective of this study was to compare the effect of vitrification either at the GV stage or at the MII stage on epigenetic characteristics of bovine oocytes and subsequently developing embryos. Our results demonstrated that vitrification of oocytes at each meiotic stage significantly reduced blastocyst development after IVF. However, vitrification at the GV stage resulted in higher blastocyst development than did vitrification at the MII stage. Irrespective of the meiotic stage, oocyte vitrification did not affect 5-methylcytosine (5mC) immunostaining intensity in oocyte DNA. However, at both stages it caused a similar reduction of 5mC levels in DNA of subsequently developing blastocysts. Oocyte vitrification had no effect on the intensity of H3K9me3 and acH3K9 immunostaining in oocytes and subsequent blastocysts. The results suggest that irrespective of meiotic stage, oocyte vitrification alters global methylation in

resultant embryos although such alteration in the oocytes was not detected. Oocyte vitrification might not influence histone acetylation and methylation in oocytes and resultant embryos. Vitrification at the immature stage was more advantageous for blastocyst development than at the mature stage.

4.2 Introduction

The cattle industry has benefited the most from the application of cryopreserved gametes over the past decades. Cryopreservation has evolved substantially for gametes and embryos, resulting in a significant increase in the proportion of fertilizable sperm, viable oocytes, and transferable embryos. (Huang et al., 2018). Oocytes are more difficult to cryopreserve than spermatozoa because of the greater size of cells and - in farm animals the high intracellular lipid content (Prentice & Anzar et al., 2011). For the cryopreservation of oocytes, vitrification offers an alternative to the standard slow freezing with many advantages such as simplicity, cost effectiveness and quickness (Mochida & Ogura 2010). Vitrification has become the routine method for oocyte cryopreservation in human (Arindam et al., 2018). Oocyte vitrification has numerous applications in many animal species, such as cattle (Hamano et al., 1992), rabbit (Al-Hasani et al., 1989), horse (Maclellan et al., 2002), and pigs (Somfai et al., 2014). The Cryotop method has become the gold standard for oocyte vitrification in various species including human (Kuwayama et al., 2005), rabbit (Hochi, 2004), cattle (Sprícigo et al., 2017), horse (Maclellan et al., 2002), and pig (Galeati et al., 2011).

However, oocyte vitrification in cattle still faces a challenge since it reduces embryonic development outcomes (Gutierrez-Castillo, 2018). Among many factors,

the meiotic stage of the oocyte affects its survival and the developmental capacity after vitrification (Moa et al., 2014). Nevertheless, there seems to be some discrepancy among studies regarding the optimum stage for cryopreservation of cattle oocytes. In some studies, cattle oocytes at the metaphase II (MII) stage have been reported to survive cryopreservation procedures than those at the germinal vesicle (GV) stage (Chaves et al., 2017; Otoi et al., 1995). On the other hand, other studies indicated that vitrification of cattle oocytes is more advantageous at the GV stage (Zhou et al., 2010; Hardin, 2016) or during germinal vesicle breakdown (GVBD) (Sprícigo et al., 2014).

Although in the recent decades several studies investigated the effects of different vitrification procedures on survival and subsequent development of bovine oocytes, the exact mechanisms leading to their compromised embryo development remained unclear and only very few studies investigated the effect of vitrification on the quality of resultant blastocysts. Cryopreservation is known to induce various types of epigenetic modifications in many different cell types of mammalian species (Chatterjee et al., 2017). Recent studies reported that oocyte vitrification at the MII stage causes epigenetic modifications such as alterations in histone acetylation and methylation and DNA methylation in preimplantation embryos in mice (Yan et al., 2010; Liang et al., 2012) and cattle as well (Chen et al., 2016). In addition, oocyte vitrification can influence genes expression, including epigenetic related gene and putative imprinted gene (Chen et al., 2016). Furthermore, vitrification of bovine and murine oocytes was reported to cause alterations in the expression of different epigenetic related genes in blastocysts (Chen et al., 2016; Cheng et al., 2014). Nevertheless, it remained unclear if vitrification at the GV stage affects epigenetic

characteristics in the same manner. Based on the above mentioned, the objective of this study was to compare the effect of vitrification either at the GV stage or at the MII stage on epigenetic characteristics of bovine oocytes and subsequently developing embryos in order to clarify which stage is better for cryopreservation. DNA methylation and histone methylation and acetylation were assayed by immunohistochemistry both at the MII stage of oocytes and at the blastocysts stage after in vitro fertilization (IVF) and embryo culture.

4.3 Methods and methods

4.3.1 Chemicals

All chemicals were purchased from the Sigma-Aldrich Corporation (St. Louis, Mo, USA), unless otherwise indicated.

4.3.2 Oocyte collection and in vitro maturation (IVM)

The collection and IVM of bovine oocytes were performed as described previously by Imai et al. (Imai et al., 2006) with modifications. Briefly, bovine ovaries were obtained from slaughterhouse, transported to the laboratory, and then washed several times in 0.9% (w/v) sodium chloride solution. The ovaries were then stored in Dulbecco's phosphate buffered saline (D-PBS) supplemented with 50 µg/ml gentamicin at 15°C for approximately 15 h. Cumulus-oocyte complexes (COCs) were aspirated from small follicles (3-6 mm in diameter) using a 10-ml syringe connected with 18-gauge needle and used for IVM. Collected COCs with homogeneous cytoplasm and more than one layer of compacted cumulus cells were selected, washed twice with IVM medium, and cultured in 500 µl aliquots of IVM medium in 4-well dishes (Nunclon Multidishes, Nalge Nunc International, Rochester, NY, USA) (50

COCs/well) covered with paraffin oil (Paraffin Liquid; Nacalai Tesque Inc., Kyoto, Japan). The IVM medium was composed of a HEPES-buffered TCM-199 medium (Medium 199, 12340-030; GIBCO Invitrogen, Life Technologies, Auckland, New Zealand) supplemented with 5% newborn calf serum (NCS, S0750-500; Biowest SAS, Nuaillé, France) and 0.02 Armor Units/ml follicle stimulating hormone (FSH; Antrin R10; Kyoritsu Seiyaku, Tokyo, Japan). The COCs were cultured for 22 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air.

4.3.3 Oocyte vitrification and warming

COCs oocytes were vitrified either before IVM (at the GV stage) or after IVM (at the MII stage). Before vitrification, the cumulus compartment of MII stage oocytes was trimmed after 22 h of IVM by a short treatment with 1000 IU/ml hyaluronidase to facilitate the easy handling of COCs. This treatment was not employed for immature COCs. COCs were vitrified according to the method described previously by Tashima et al. (2017). Briefly, oocytes were rinsed with a base medium (BM) which was composed of a HEPES-buffered TCM-199 supplemented with 20% (v/v) NCS. Then COCs were incubated for 3 min at an ambient temperature (25°C) in an equilibration solution (ES) which was composed of BM supplemented with 7.5% (v/v) ethylene glycol (EG; Wako Pure Chemical Industries, Ltd., Osaka, Japan), 7.5% (v/v) dimethyl sulfoxide (DMSO). The COCs were then transferred into a vitrification solution (VS) which composed of BM supplemented with 15% (v/v) EG, 15% (v/v) DMSO and 0.5M sucrose (Wako Pure Chemical Industries). Ten COCs were placed on a Cryotop® (Kitazato BioPharma, Fujinomiya, Japan) sheet in a small volume (<1 µl) of VS. Within 1 min, the Cryotop® was plunged into liquid nitrogen. The vitrified oocytes were warmed by

insertion of the Cryotop® sheet into 2.5 ml of a warming solution which was composed of BM supplemented with 1 M sucrose, in 35-mm plastic dishes (Nalge Nunc International) at 38.5°C and incubated for 1 min. Then, the COCs were transferred to reduced concentrations of sucrose in BM in a stepwise manner (0.5, 0.25, and 0 M for 3, 5, and 5 min, respectively). After warming, oocytes vitrified at the GV stage were subjected to 22 h of IVM as described above whereas oocytes vitrified at the MII stage were returned to IVM medium and cultured for additional 1 hour before in vitro fertilization.

4.3.4 *In vitro* fertilization (IVF)

IVF was carried out as reported previously by Imai et al. (2006) with modifications. Briefly, a 0.5-ml straw of frozen semen from a proven Japanese Black bull was thawed in a 37°C water bath for 30 s., then transferred to 3 ml of 90% Percoll solution in a 10-ml test tube (As One, Osaka, Japan), and centrifuged at 770×g for 10 min. Then, the pellet was re-suspended in 6 ml of IVF 100 medium (Research Institute for Functional Peptides, Yamagata, Japan) and centrifuged at 570×g for 5 min. The pellet was re-suspended with IVF 100 medium and the final concentration was adjusted to 3×106 spermatozoa/ml. The matured COCs were washed twice in IVF 100 medium and co-incubated with sperm in 100 μL droplets of IVF 100 medium in 35-mm plastic dishes under paraffin oil for 5 h at 38.5°C in a humidified atmosphere of 5% CO2, 5% O2 and 90% N2 (20 oocytes per droplet). The start of IVF (i.e., insemination) was considered as 0 h of fertilization.

4.3.5 Evaluation of oocyte survival

After insemination, presumptive zygotes were completely denuded from cumulus cells and spermatozoa attached to the zona pellucida by gentle pipetting

through a fine glass pipette in pre-incubated in vitro culture (IVC) medium which was a Charles Rosenkrans 1 (CR1) medium (Rosenkrans et al., 1993) supplemented with amino acids (CR1aa) (Imai et al., 2002) and 5% NCS. Denuded oocytes were comprehensively checked under a stereo microscope for morphology and the extrusion of the first polar body. Oocytes with spherical shape and a clear, smooth surface (intact cell membrane) were classified as “live” ones. Live oocytes with at least one extruded polar body were defined as matured.

4.3.6 *In vitro* culture (IVC) of embryos

Live presumptive zygotes of each group were cultured in 100 µl drops of IVC medium (10-20 zygotes/drop) in repro-plates (Research Institute for Functional Peptides), covered with paraffin oil at 38.5°C in a humidified atmosphere of 5% CO2, 5% O2 and 90 % N2 for up to 9 days without changing the medium. The cleavage rate was recorded on Day 2 (Day 0 = IVF), and the numbers of blastocysts were recorded on Day 8- 9.

4.3.7 Immunofluorescent assay of epigenetic makers

To assay global methylation levels, 5-methylcytosine (5mC) was labeled in MII stage oocytes and blastocysts as described by Yu et al. (2018) with modifications. First, denuded MII stage oocytes and blastocysts from all groups were washed in PBS containing 0.3% (v/w) polyvinylpyrrolidone (PBS-PVP) then fixed with 4% paraformaldehyde (PFA) (163-20145, Wako Pure Chemical Industries) for 40 min at room temperature (RT). Then, the fixed samples were permeabilized using 0.5% (v/v). Triton X-100 in PBS for 40 min at RT which was followed by incubation in 4N HCl for 50 min at 37 °C. Then the samples were washed 4 times in 0.05% (v/v) Tween-20 in PBS-PVP. Then, oocytes were blocked in 2% (v/w) bovine serum

albumin (BSA) in PBS for 1 h and incubated overnight at RT with mouse anti-5mC antibody (NA81; Merck Millipore, Tokyo, Japan) in PBS-PVP (1:150). Then, the samples were washed 3 times in PBS-PVP and incubated with Alexa Fluor 488–labeled goat anti-mouse IgG (A-10680; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) in PBS-PVP (1:150) for 1 h at 37°C in the dark. Then after two consecutive washings in PBS-PVP the samples were incubated in 100 µg/ml propidium iodide (PI) in PBS-PVP for 20 min at 37°C. Then the samples were washed twice again in PBS-PVP and once in antifade buffer (S2828, Invitrogen) and mounted on glass slides in a glycerol-based antifade solution (S2828, Invitrogen). For each oocyte and embryo, images of total chromatin labelled by PI and 5mC labelled by Alexa Fluor 488 were recorded under a fluorescence microscope (Nikon Eclipse E600, Tokyo, Japan) at the same position, same magnification using the same camera settings (i.e., gain, exposition) within 60 sec of exposition to UV light using the G-2A (excitation wavelength 510-560 nm) and B-2A (excitation wavelength 450-490 nm) filters, respectively. In each replication, control samples incubated without the primary antibody were also processed for negative controls, to verify the specificity and accuracy of assay. In each group, 5-10 oocytes and 1-7 blastocysts were examined for each experimental replication. Quantification of fluorescence intensity for 5mC was performed as described below.

Methylation and acetylation of histone in oocytes and embryos were assayed by immunostaining of H3K9me3 and acH3K9 as described by Chen et al. (2016) with modifications. In brief, washing samples in PBS-PVP, fixation in 4% PFA and permeabilization with 0.5% Triton X-100 was performed as described earlier. Then samples were blocked in 2% BSA in PBS for 1 h. The samples were then

washed 3 times in PBS-PVP supplemented with 0.01% Triton X-100 (PBS-T) and incubated with rabbit anti- H3K9me3 (ab8898; Abcam, Tokyo, Japan) or rabbit anti-acH3K9 primary antibody (ab10812; Abcam) in PBS-T (1:500 and 1:1000, respectively) for 2h at 38.5°C. Then the samples were washed 3 times in PBS-T supplemented with 0.1% Tweeen-20 (PBS-TT) and incubated with Alexa Fluor 488–labeled goat anti-rabbit IgG (A-11008; Invitrogen) in PBS-TT (1:800) for 1 h at 38.5°C. Then the samples were washed 3 times in PBS-PVP and once in antifade buffer on glass slides in a glycerol-based antifade solution (S2828, Invitrogen) supplemented with 0.01 mg/ml Hoechst 33342 (Calbiochem, San Diego, CA, USA).

For each oocyte and embryo, images of total chromatin labelled by Hoechst 33342 and H3K9me3 or acH3K9 labelled by Alexa Fluor 488 were taken under a fluorescence microscope at the same magnification and same camera settings (i.e., gain, exposition) within 60 sec of exposition to UV light using the UV-1A (excitation wavelength 365 nm) and B-2A (excitation wavelength 450-490 nm), respectively. In each replication, control samples incubated without the primary antibody were also processed for negative controls, to verify the specificity and accuracy of assay. In each group, 5-10 oocytes and 2-6 blastocysts were examined for each experimental replication. Quantification of fluorescence intensity for H3K9me3 and acH3K9 was performed as described below.

4.3.8 Quantification of fluorescence intensity

Quantification of fluorescence intensity for 5mC, H3K9me3 and acH3K9 labelled by Alexa Fluor 488 was performed using Image J software (v. 1.52, NIH, Bethesda, MD, USA). In brief, each image for 5mC, H3K9me3 and acH3K9 was stacked with its total chromatin counterpart. On the stacked image, the total area

of chromatin in PI or Hoechst-stained images was outlined by “wand tool” on the PI or Hoechst-stained images and from this area the mean signal intensity was measured for 5mC, H3K9me3 and acH3K9 stained image counterparts. To eliminate the influence of background colors, the optical density was calibrated, and the average cytoplasmic intensity was measured for normalization to background. Relative fluorescence was calculated from the mean signal intensity (expressed in arbitrary units) where the control group was 1.

4.3.9 Experimental design

Experiment 1 The effect of vitrification at the GV and MII stage on global methylation levels in oocytes and embryos. Bovine oocytes collected on the same day were vitrified either before IVM at the GV stage (GV-vitrified) or after IVM at the MII stage (MII-vitrified) or processed without vitrification (Control). At 22 h IVM, in each group, representative oocytes at the MII stage were sampled for 5mC assay as described above. The rest of the oocytes were subjected to IVF and IVC. Oocyte survival was recorded after IVF (Day 0). Cleavage was recorded on Day 2. Blastocyst development rates were recorded on Day 8-9. On Day 9, resultant blastocysts were harvested to compare total call numbers and 5mC expression in chromatin as described above. Five experimental replications were performed.

Experiment 2 The effect of vitrification at the GV and MII stage on histone methylation levels in oocytes and embryos. Bovine oocytes collected on the same day were vitrified either before IVM at the GV stage (GV-vitrified) or after IVM at the MII stage (MII-vitrified) or processed without vitrification (Control). At 22 h IVM, in each group, representative oocytes at the MII stage were sampled for H3K9me3 assay as described above. The rest of the oocytes were subjected to IVF

and IVC. Oocyte survival was recorded after IVF (Day 0). Cleavage was recorded on Day 2. Blastocyst development rates were recorded on Day 8-9. On Day 9, resultant blastocysts were harvested to compare total call numbers and H3K9me3 expression in chromatin as described above. Five experimental replications were performed.

Experiment 3 The effect of vitrification at the GV and MII stage on histone acetylation levels in embryos. Bovine oocytes collected on the same day were vitrified either before IVM at the GV stage (GV-vitrified) or after IVM at the MII stage (MII-vitrified) or processed without vitrification (Control). All oocytes were subjected to IVF (Day 0) and IVC. Oocyte survival was recorded on Day 0. Cleavage was recorded on Day 2. Blastocyst development rates were recorded on Day 8-9. On Day 9, resultant blastocysts were harvested to compare total cell numbers and acH3K9 expression on chromatin as described above. Since acH3K9 is not expressed at the MII stage (Liang et a., 2012), this assay was applied only on embryos. Five experimental replications were performed.

4.3.10 Statistical analysis

Data are expressed as mean ± SEM. Percentage data for oocyte survival, maturation and embryo development of Experiments 1-3 were summarized and subjected to arcsine transformation. After verification of normal distribution, the data was analyzed with one-way ANOVA followed by Tukey's multiple comparison test. Values for relative fluorescence after 5mC, H3K9me3 and acH3K9 staining (without normal distribution) were analyzed with Friedman test followed by Dunn's multiple comparison test. The statistical analysis was carried out using GraphPad prism software (Ver. 7.0, La Jolla, California, USA). $P<0.05$ was defined as the significance level.

4.4 Results

4.4.1 Effect of oocyte vitrification at different meiotic stages on post-warming survival and subsequent embryo development

The percentages of survival, maturation and subsequent embryo development of Experiments 1-3 are summarized in Table 4.1. The percentage of live oocytes in both the GV-and MII-vitrified groups were significantly lower than that in the control group. Furthermore, the percentage of live oocytes in the MII-vitrified group was significantly higher than that in the GV-vitrified group. After IVF, the percentages of cleavage and blastocyst formation in both vitrified groups were significantly lower than those in the control group at every timepoint. There was no significant difference in the percentage of cleavage and blastocyst development by Day 8 in cultured oocytes between the MII-vitrified and GV-vitrified groups although the values in the GV-vitrified group were tendentiously higher (P=0.08). Blastocyst development from cultured oocytes in the GV-vitrified group was higher (P<0.05) than in MII-vitrified group on Day 9. However, blastocyst production calculated from total oocytes was similar in GV-vitrified and in MII-vitrified groups and they were significantly lower compared with the control. The number of total cells in Day 9 embryos at or beyond the blastocyst stage in the control group was significantly higher than that in the MII-vitrified group, whereas total cells number of blastocysts in the GV-vitrified group did not differ significantly from those of the other groups (Table 4.1).

4.4.2 Effect of oocyte vitrification at different meiotic stages on global DNA methylation levels in oocytes and embryos

The results of relative fluorescence intensity of 5mC immunostaining are show in Figure 4.1. There was no significant difference in the 5mC level of the

chromatin of MII-stage oocytes between the control, GV-vitrified and MII-vitrified groups (Figure 4.1A, Figure 4.2A). When nuclei of blastocyst stage embryos were compared, 5mC intensity in control group was significantly higher than those in MII-vitrified and GV-vitrified groups (Figure 4.1B, Figure 4.2B). The fluorescence of 5mC was not detected in the negative control group both in the chromatin of MII-stage oocytes and blastocysts (Figure 4.2).

4.4.3 Effect of oocyte vitrification at different meiotic stages on histone methylation levels in oocytes and embryos

Histone methylation levels were evaluated by measuring H3K9me3 immunofluorescence intensity. The results of relative fluorescence intensity of H3K9me3 in oocytes are show in Figure 4.3. There was no significant difference in H3K9me3 level in oocytes among control and vitrified groups ($P>0.05$) (Figure 4.3A, Figure 4.4A). Also, there was no significant difference among control and vitrified groups in H3K9me3 levels in blastocyst stage embryos ($P>0.05$) (Figure 4.3B, Figure 4.4B). The fluorescence of H3K9me3 in the negative control group was not detected in MII-stage oocytes and blastocysts (Figure 4.4).

4.4.4 Effect of oocyte vitrification at different meiotic stages on histone acetylation in subsequently developing embryos

There was no significant difference between the control, GV- vitrified and MII-vitrified groups in the relative acH3K9 intensity levels in blastocysts (Figure 4.5A, Figure 4.5B). The acH3K9 fluorescence was not detected in blastocysts of the negative control group (Figure 4.5).

Table 4.1 Survival and embryo development after IVF of bovine oocytes vitrified at different meiotic stages.

Group	Total	Survived (% total)	Cultured	Cleaved (% cultured)	Blastocyst			Total cell number
					Day 8 (% cultured)	Day 9 (% cultured)	Day 9 (% total)	
Control	689	648 (93.8±1.3) [a]	615	487 (79.4±2.1) [a]	176 (29.3±2.6) [a]	187 (31.1±2.7) [a]	27.8±2.7 [a]	124.7±4.9 [a]
GV-vit	780	427 (53.7±3.7) [c]	391	184 (47.5±2.5) [b]	49 (13.7±2.2) [b]	55 (16.3±2.3) [b]	7.7±1.0 [b]	107.3±9.4 [ab]
MII-vit	797	675 (84.5±2.2) [b]	646	256 (40.1±2.7) [b]	53 (8.7±1.2) [b]	56 (9.5±1.2) [c]	7.8±1.0 [b]	89.3±8.6 [b]

Eighteen replications were performed. Data are presented as mean±SEM values.

[a, b, c] Column with different letters are significantly different ($P<0.05$).

Abbreviations: GV-vit = oocytes vitrified at the GV stage; MII-vit = oocytes vitrified at the MII stage.

4.5 Discussion

In the present study we compared the effect of vitrification of bovine oocytes either at the GV stage or at the MII stage on epigenetic markers in MII stage oocytes and subsequently developing blastocysts with an immunofluorescent approach and investigated its relevance to blastocyst development. Our results demonstrated that vitrification of bovine oocytes both at the GV and MII stages by the Cryotop method reduced viability and subsequent embryo developmental competence. Vitrification at the GV stage resulted in significantly lower oocyte survival than that at the MII stage which confirm the previous reports demonstrating that immature oocytes are more susceptible to membrane damage than matured ones (Chaves et al., 2017; Otoi et al., 1995). On the other hand, oocytes which survived vitrification at the GV stage showed better embryo developmental competence to the blastocyst stage than those vitrified at the MII stage which was represented by a significantly higher blastocyst rate on Day 9. Furthermore, vitrification of oocytes at the MII stage compromised the quality of blastocysts as measured by their total numbers of cells; however, such alterations were not observed when oocytes were vitrified at the GV stage. These results suggest the advantage of vitrification at the GV-stage over vitrification at the MII stage which supports previous studies in cattle (Zhou et al., 2010; Hardin, 2016) and pigs (Li et al., 2016; Egerszegi et al., 2013). Since subsequent embryo development to the blastocyst stage of GV-vitrified oocytes was better, despite of higher survival rate in MII-vitrified group the overall efficacies of the blastocyst formation were similar for both groups. GV stage oocyte has not organized meiotic spindle, therefore, it can bypass the risk of chromosome aberrations during cryopreservation due to the protection from nuclear envelope (Cooper et al., 1998; Isachenko et al., 1999). Moreover, this relaxant of and sheep

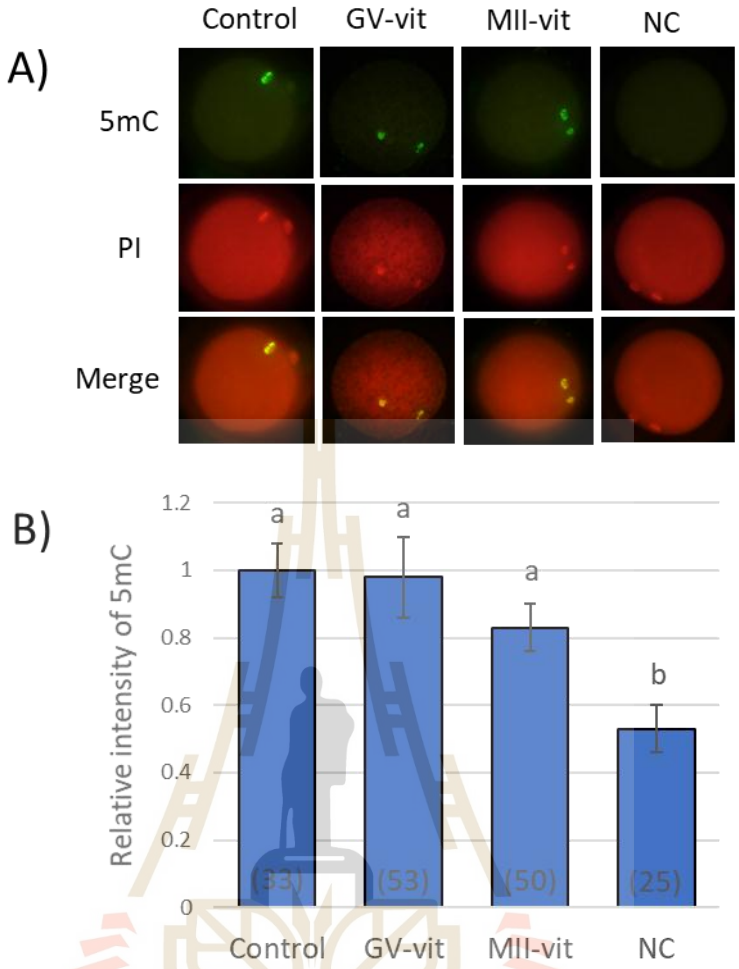

Figure 4.1 Immunostaining of 5mC (A) and its relative intensity (B) in MII-stage bovine oocytes. Five replications were performed. Data are presented as mean±SEM values. [a, b] Column with different letters are significantly different ($P<0.05$) Total numbers of oocytes in each group are shown in parentheses. Abbreviations: GV-vit = oocytes vitrified at the GV stage; MII-vit = oocytes vitrified at the MII stage; NC = negative control; PI = propidium iodide.

microtubule may preserve the functionality of the gap junctions faster and more uniform penetration of the cryoprotants on the vitrification of pig (Fujihira et al., 2004) (Silvestre et al., 2006). On the other hand, mature MII-stage oocyte has complex spindle apparatus with microtubules causing cytoskeletal damage by cryopreservation concerns disruption of the sub-cortical actin network, microtubule depolymerization, abnormal spindle configuration and chromosome scattering (Rho et al., 2002; Mandelbaum et al., 2004).

Reduced ability of surviving oocytes to develop to the blastocyst stage in both vitrified groups and reduced cell numbers in MII- vitrified oocytes suggest sublethal alterations in oocytes and subsequently developing embryos as a result of cryopreservation. In previous studies vitrification of MII stage oocytes reportedly altered epigenetic characteristics of oocytes and subsequently developing embryos in mice (Cheng et al., 2014) and cattle (Chen et al., 2016), respectively. Epigenetics refer to heritable modifications occurring in DNA without changing the DNA sequences, including DNA methylation and histone modifications. DNA methylation is an epigenetic modification mechanism for gene expression mediation (Larrivée et al., 2019). This mechanism is involved in many physiological processes such as genomic imprinting, gene silencing, and X-chromosome inactivation (Smith & Meissner, 2013), by adding a methyl group (-CH3) to cytosine residues in the CpG dinucleotides (Robertson & Wolffe, 2000). Histone modifications are post-translational modifications occurring in the oocyte chromosome during fertilization and early embryonic development including histone methylation and histone acetylation. Histone methylation is the binding of methyl group at the amino-terminal tails of histones for regulating chromatin structure and suppress gene transcription (Qiao et

al., 2010). Methylation of H3K9 is correlated to euchromatic gene silencing (Nielsen et al., 2001; Peters et al., 2002) and has been associated with DNA methylation by acting a downstream role (Tamaru & Selker 2001; Gendrel et al., 2002).

In a previous study, Chen et al. (2016) reported alterations of epigenetic markers such as reduced levels of global DNA methylation (assayed by 5mC immunostaining) and histone methylation (assayed by H3K9me3 immunostaining) and increased levels of histone acetylation (assayed by acH3K9 immunostaining) in bovine oocytes and/or subsequently developing embryos as a result of vitrification at the MII stage. In the present study we compared the same epigenetic markers in both GV and MII-vitrified oocytes and subsequent embryos to those of non-vitrified control oocytes and subsequent embryos. Our results revealed that oocyte vitrification either at the GV or MII stage reduced 5mC immunofluorescence in resultant blastocysts at a similar manner compared with the control. This result is in agreement with the observations of Cheng et al. (2014) on mouse embryos developing from MII-vitrified oocytes and suggest that oocyte vitrification reduces the global DNA methylation levels in resultant embryos irrespective of the meiotic stage. The contribution of reduced DNA methylation levels to developmental competencies to the blastocyst stage and to term is not clarified yet. Chatterjee et al. (2017) mentioned that epigenetic alterations from suboptimal cryopreserved conditions are related with inadequate post-thawing cellular efficiency such as reduced cell survival, differentiation capacity and chromosomal aberrations. Also, cryopreservation reportedly induces epigenetic changes, which occur in cellular response to environmental stress (Chatterjee et al., 2017). In our study, in both vitrified groups, reduced developmental competence to the blastocyst stage was associated with

similarly reduced 5mC levels in blastocysts. On the other hand, the developmental competence of oocytes vitrified at the GV stage was better than those vitrified at the MII stage whereas vitrification of oocytes reduced 5mC staining intensity in embryos in a similar manner. This suggests that DNA methylation may not be the factor that causes difference in developmental competence between GV-vitrified and MII-vitrified oocytes. Other factors such as differences in spindle integrity and fertilization normality could possibly cause better development results of GV-vitrified oocytes compared with MII-vitrified ones which was observed previously in pigs (Egerszegi et al., 2013; Somfai et al., 2012). Further research will be needed to clarify the contribution of reduced DNA methylation to developmental competence of vitrified oocytes. In the present study, we did not detect difference in 5mC immunofluorescence (DNA methylation levels) among the control and the vitrified groups at the MII stage of oocytes. These results are in agreement with those reported earlier in ovine oocytes vitrified at the GV stage (Shirazi et al., 2016). However, unlike our finding, Chen et al. (2016) reported reduced 5mC immunofluorescence in vitrified MII stage bovine oocytes. The reason for this discrepancy remains unclear.

In this study we detected similar H3K9me3 levels in blastocysts obtained from non-vitrified oocytes and those obtained from oocytes vitrified at the GV stage or MII stage. This result corresponds with the results of Chen et al. who demonstrated normal H3K9me3 level in blastocysts obtained from MII-vitrified oocytes (Chen et al., 2016). Our results suggest that irrespective of meiotic stage oocyte vitrification may not alter histone methylation levels in embryos. On the other hand, the results are not in agreement with the reports of Hu et al. (2012) who reported reduced H3K9me3 in oocytes as a result of vitrification.

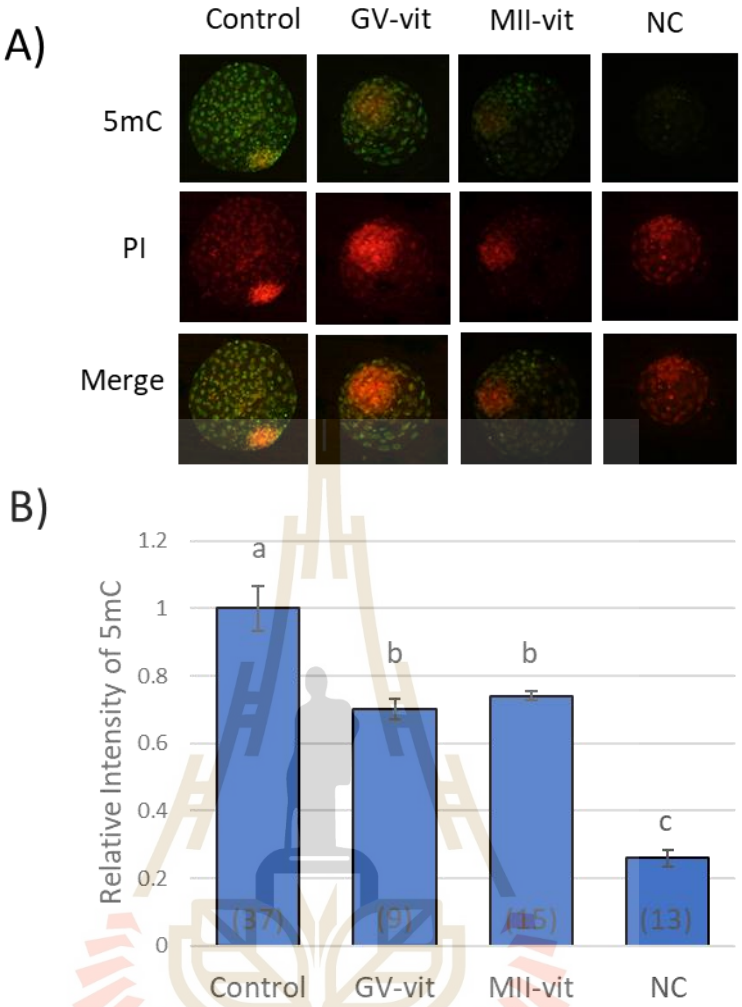

Figure 4.2 Immunostaining of 5mC (A) and its relative intensity (B) in Blastocyst derived vitrified bovine oocytes. Five replications were performed. Data are presented as mean±SEM values. [a, b] Column with different letters are significantly different ($P<0.05$) Total numbers of blastocysts in each group are shown in parentheses. Abbreviations: GV-vit = Blastocyst derived vitrified oocytes at the GV stage; MII-vit = Blastocyst derived vitrified oocytes at the MII stage; NC = negative control; PI = propidium iodide.

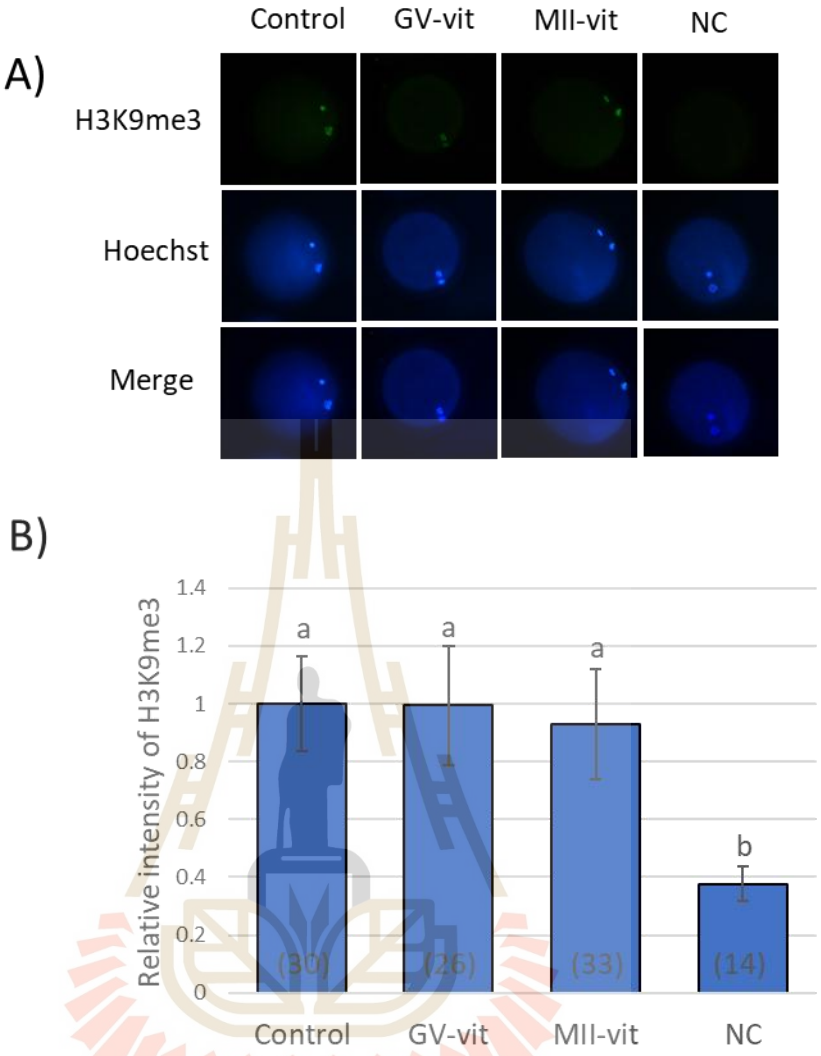

Figure 4.3 Immunostaining of H3K9me3 (A) and its relative intensity (B) in MII-stage bovine oocytes. Five replications were performed. Data are presented as mean±SEM values. [a, b] Column with different letters are significantly different ($P<0.05$) Total numbers of oocytes in each group are shown in parentheses. Abbreviations: GV-vit = oocytes vitrified at the GV stage; MII-vit = oocytes vitrified at the MII stage; NC = negative control; Hoechst = Hoechst 33342.

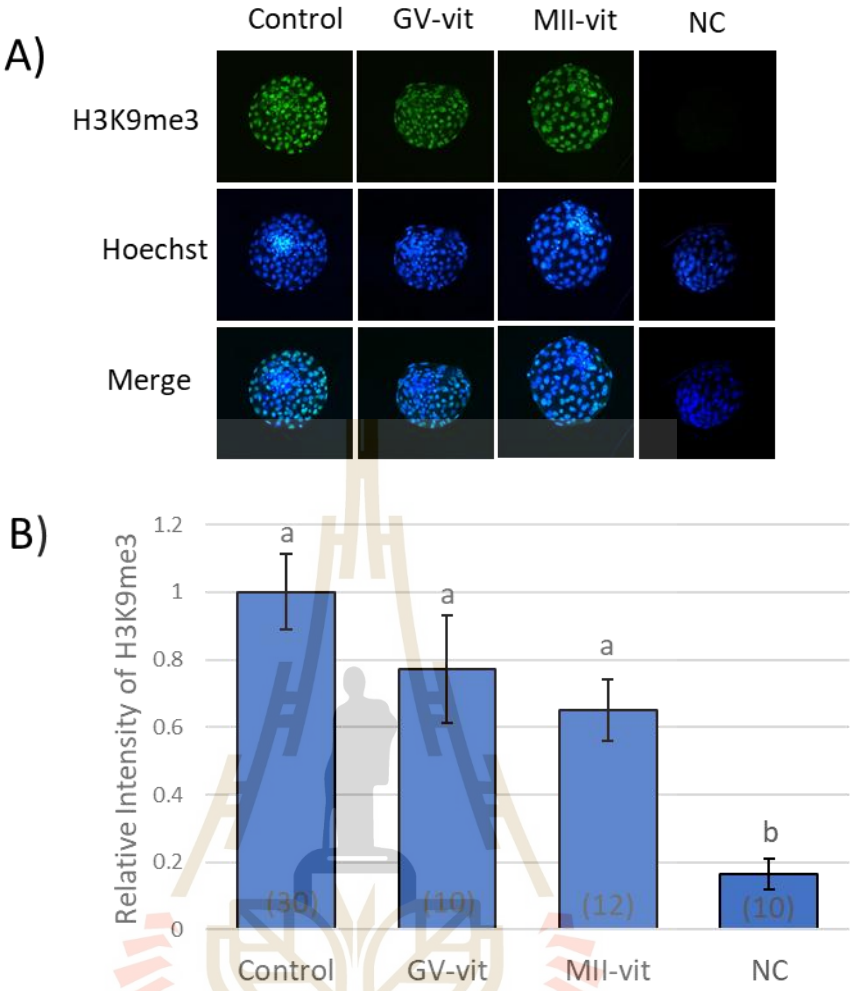

Figure 4.4 Immunostaining of H3K9me3 (A) and its relative intensity (B) in Blastocyst derived vitrified bovine oocytes. Five replications were performed. Data are presented as mean±SEM values. [a, b] Column with different letters are significantly different (P<0.05) Total numbers of blastocysts in each group are shown in parentheses. Abbreviations: GV-vit = Blastocyst derived vitrified oocytes at the GV stage; MII-vit = Blastocyst derived vitrified oocytes at the MII stage; NC = negative control; Hoechst = Hoechst 33342.

Histone acetylation is the addition of acetyl group to specific lysine residues to neutralize the positive charges on N-terminus of the histone protein, which help in relaxing chromatin result in allows the binding of transcription factors to generate transcription (Canovas & Ross, 2016). acH3K9 is an important histone acetylation marker which is involved in epigenetic regulation. Our results revealed similar fluorescence intensity levels for acH3K9 in blastocysts derived from oocytes vitrified either at the GV stage or MII stage and fresh control (P>0.05). This result is in agreement with those of Chacon et al. (2011) who reported that cryopreservation did not alter acetylation levels of H3K9 in bovine fibroblasts. On the other hand, the results are different from those of Chen et al. reporting reduced acH3K9 level in TE cells of blastocyst obtained from vitrified oocytes (Chen et al., 2016). Also, cryopreservation reportedly lowered acH3K9 levels in cat somatic cells (Gómez et a., 2008). On the other hand, Spinaci et al. (2012) reported increased histone acetylation status in porcine oocytes as a result of vitrification.

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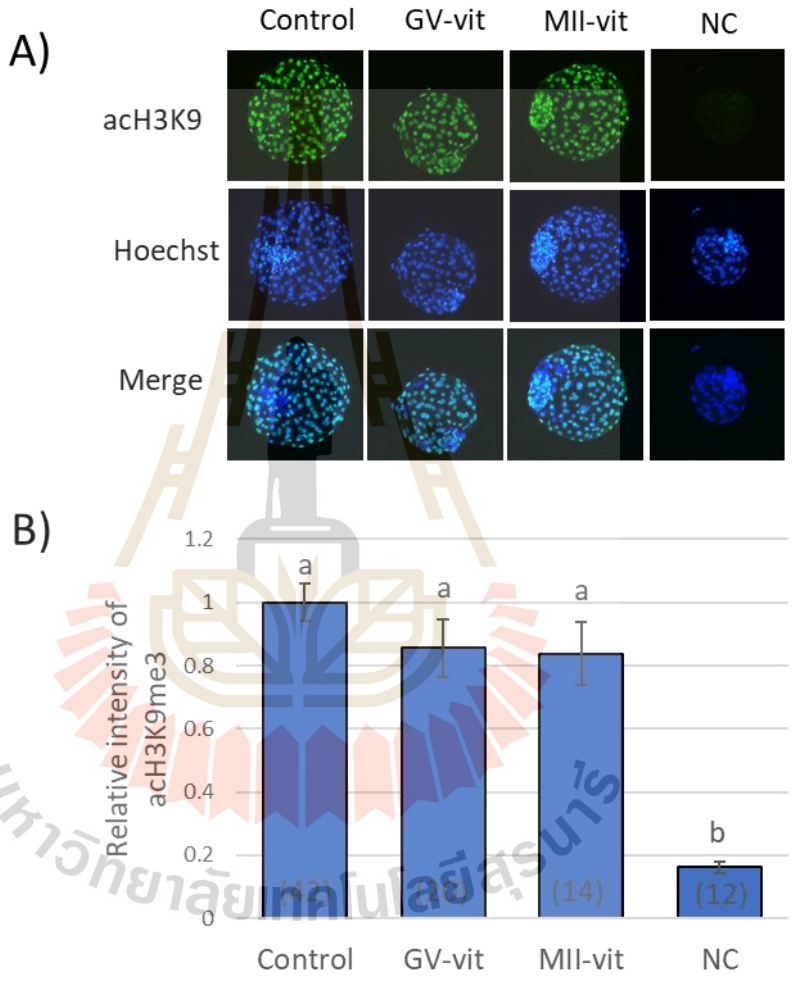

Figure 4.5 Immunostaining of acH3K9 (A) and its relative intensity (B) in Blastocyst derived vitrified bovine oocytes. Five replications were performed. Data are presented as mean±SEM values. [a, b] Column with different letters are significantly different ($P<0.05$) Total numbers of blastocysts in each group are shown in parentheses. Abbreviations: GV-vit = Blastocyst derived

vitrified oocytes at the GV stage; MII-vit = Blastocyst derived vitrified oocytes at the MII stage; NC = negative control; Hoechst = Hoechst 33342.

The use of immunostaining to quantify levels of epigenetic markers may raise the question for reliability and accuracy of the method since non-specific binding or residual fluorochrome in some samples could theoretically affect fluorescence levels. To verify the specificity of the immunostaining and the reliability of fluorescence level analysis used in this study, in each replication of each assay we processed a negative control group (without primary antibody) which always showed significantly lower fluorescent intensity compared with the control group. It is worth to note that in some previous studies such negative control group is lacking. Furthermore, exposure to UV light of stained samples is known to gradually reduce the intensity of fluorochromes via a process called "bleaching" (Diaspro et al., 2010). Therefore, when fluorescence intensity is measured by microscopy, extensive observation of samples under UV light might result in an artifact reading of reduced fluorescence (i.e., caused not by the treatment but by UV). For this reason, the exact timing of recording fluorescent images after the start of exposure to UV light is crucial in order to acquire reliable results. In a preliminary experiment we tested the bleaching of the Alexa 488 stain (used as the marker of 5mC, H3K9me3 and acH3K9) under UV light in our system. The results showed that exposure of stained samples (DNA of MII stage bovine oocytes) to UV light caused a significant reduction of fluorescent intensity after 150 sec intensities (Supplementary Figure S1). Therefore, to minimize the environmental effects on results, images for all samples were recorded within 60 sec after exposure to UV light in the present study.

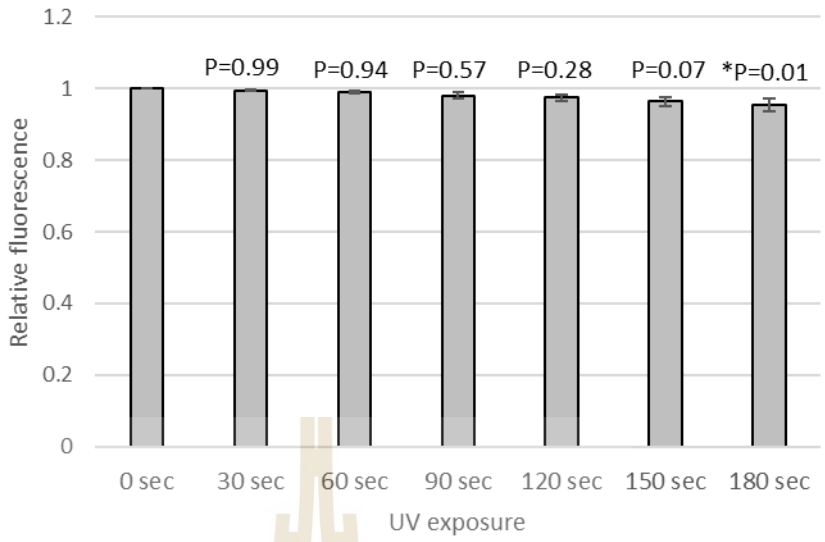

Figure S1 Relative fluorescent intensity of H3K9me3 labelled with Alexa 488 in the chromatin of individual MII-stage oocytes at different time points during continuous exposure to UV light. Five replications were performed. Data are presented as mean±SEM. Aster denotes significant difference compared with initial measurement value.

4.6 Conclusions

In conclusion, our results revealed that bovine oocyte vitrification either at the GV stage or at the MII stage similarly reduced global DNA methylation assayed by 5mC staining in subsequently developing blastocysts; however, in oocytes such difference was not observed. On the other hand, irrespective of meiotic stage, oocytes vitrification had no effect on histone methylation and acetylation measured by H3K9me3 and acH3K9 staining in oocytes and resultant blastocysts. Further research is required to clarify the contribution of reduced DNA methylation to reduced developmental competence in vitrified bovine oocytes.

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CHAPTER V

OVERALL CONCLUSION

Long-term preservation of bovine oocyte and embryo have widespread applications in assisted reproductive technologies and genetic improvement of livestock species, thus vitrification technique is the important tool for bovine oocyte and embryo cryopreservation. This study concluded that there was no significant difference between equilibration in low (4%) or high (15%) concentration of pCPA in survival, cell numbers and the expression of the selected developmentally important genes in bovine blastocysts after vitrification; however, vitrified embryos equilibrated in 15% pCPA seemed better since they did not differ significantly from control. This way of vitrification caused only a slight increase in the number of membrane damaged cells which was associated with increased expression of the HSP70 gene in surviving embryos. Irrespective of pCPA treatment, vitrification had no effect on the expression of IGF2R, SNRPN, HDAC1, DNMT3B, BAX, OCT4, and IFN-t genes.

According to the results found that oocyte vitrification at different developmental stages have different efficient outcome by which MII-stage oocyte has significantly higher survivability than GV-stage oocyte. However, GV-stage oocyte has marginally higher blastocyst development rate more than MII-stage oocyte on Day 9, but blastocyst production calculated from total oocytes was similar in both groups and still lower compared with the control. Although vitrified oocyte has not significantly different in epigenetic immunofluorescence level compare with fresh counterpart, but 5mC intensity in control group was significantly higher than those in MII-vitrified and GV-vitrified groups

in blastocyst stage embryos. This finding can refer that bovine oocyte vitrification either at the GV stage or at the MII stage similarly reduced global DNA methylation assayed by 5mC staining in subsequently developing blastocysts; however, in oocytes such difference was not observed. On the other hand, irrespective of meiotic stage, oocytes vitrification had no effect on histone methylation and acetylation measured by H3K9me3 and acH3K9 staining in oocytes and resultant blastocysts. Further research is required to clarify the contribution of reduced DNA methylation to reduced developmental competence in vitrified bovine oocytes.

BIOGRAPHY

Mr. Thatawat Yodrug was born on 23 December 1991 in Songkhla, Thailand. He finished high school at Mahavajiravudh Songkhla School in 2010. After that, he graduated with a Doctor of Veterinary Medicine (D.V.M.) degree at Faculty of Veterinary Science, Chulalongkorn University in 2016. In the same year, he continued his Ph.D. study course at School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology. He received the scholarship from the Royal Golden Jubilee Ph. D program. In 2019, he has an opportunity to do research in part of his thesis at National Institute of Grassland Science, Tsukuba, Japan with Dr. Tamas Somfai for 12 months. The research topic is "The effect of bovine oocyte and embryo vitrification on epigenetic characteristics and developmentally important genes expression" First part of this work was published in Animal Science Journal on August 2020 (DOI: 10.1111/asj.13451). The second part of this work was submitted to Animal Science Journal in 26 April, 2021 and under review.