EFFECT OF TEMPERATURE AND MEDIUM ADDITIVES DURING LIQUID STORAGE ON DEVELOPMENTAL COMPETENCE IN MATURED BOVINE OOCYTES

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

Suranaree University of Technology

Academic Year 2015

ผลของอุณหภูมิและสารเคมีในการเก็บรักษาไข่พร้อมปฏิสนธิในสารละลายต่ออัตราการเจริญเติบโตของตัวอ่อน

นางสาวทยิตา สุทธิโรจน์พัฒนา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
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ปีการศึกษา 2558

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

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ทยิตา สุทธิโรจน์พัฒนา : ผลของอุณหภูมิและสารเคมีในการเก็บรักษาไข่พร้อมปฏิสนธิในสารละลายต่ออัตราการเจริญเติบโตของตัวอ่อน (EFFECT OF TEMPERATURE AND MEDIUM ADDITIVES DURING LIQUID STORAGE ON DEVELOPMENTAL COMPETENCE IN MATURED BOVINE OOCYTES) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.รังสรรค์ พาลพ่าย, 129 หน้า.

การเก็บรักษาไข่ระยะสั้นโดยปราศจากการแช่แข็ง เช่น การขนส่งระยะไกลหรือการใช้ประโยชน์ในการผลิตตัวอ่อนในห้องปฏิบัติการสามารถนำไปใช้ประโยชน์ในด้านการค้าและปศุสัตว์ได้ การทดลองแรกมีวัตถุประสงค์เพื่อศึกษาผลของอุณหภูมิ (4°C, 15°C, 25°C และ 38.5°C) ในการเก็บรักษาระยะสั้นของไข่โคพร้อมปฏิสนธิต่ออัตราการเจริญเติบโตของตัวอ่อนโค คุณภาพของตัวอ่อนระยะบลาสโตซิส ระดับ ATP และ กลูตาไธโอน และการเกิด apoptosis จากผลการทดลองพบว่า กลุ่มที่เก็บที่อุณหภูมิ 25°C และ 38.5°C ให้ผลการเจริญไปเป็นตัวอ่อนระยะบลาสโตซิสสูงที่สุดเมื่อเปรียบเทียบกับกลุ่มที่เก็บรักษาในที่อุณหภูมิอื่นๆ อย่างไรก็ตามการเก็บรักษาที่ 38.5°C มีจำนวนเซลล์ทั้งหมดในตัวอ่อนระยะ บลาสโตซิสต่ำ การเก็บรักษาที่อุณหภูมิ 4°C ลดระดับเมตาโบลิซึ่มโดยวัดจากระดับของ ATP ที่ลดลงเมื่อเปรียบเทียบกับกลุ่มที่เก็บรักษาที่อุณหภูมิอื่นๆ ระดับกลูตาไธโอนลดลงอย่างมีนัยสำคัญทางสถิติในทุกกลุ่มที่เก็บรักษาที่อุณหภูมิต่างๆ การเก็บรักษาที่ 4°C และ 15°C เพิ่มอัตราการเกิด apoptosis ในไข่อย่างมีนัยสำคัญทางสถิติ แต่ไม่พบความแตกต่างในกลุ่มที่เก็บรักษาที่อุณหภูมิ 25°C และ 38.5°C เมื่อเทียบกับกลุ่มควบคุม จากผลการทดลองนี้สรุปได้ว่าการเก็บรักษาที่อุณหภูมิ 25°C เป็นอุณหภูมิที่เหมาะสมที่สุดในการเก็บรักษาแบบระยะสั้นของไข่โคพร้อมปฏิสนธิเนื่องจากความสามารถในการเจริญไปเป็นตัวอ่อนระยะบลาสโตซิสและคุณภาพของตัวอ่อนระยะบลาสโตซิส

การทดลองที่สองมีวัตถุประสงค์ที่จะเพิ่มอัตราการเจริญของตัวอ่อนโคในการเก็บรักษาระยะสั้น โดยใช้การเก็บไข่โคพร้อมปฏิสนธิในน้ำยา HEPES buffered TCM 199 (base medium) นาน 20 ชั่วโมงที่อุณหภูมิ 25°C จากผลการทดลองแรกเป็นกลุ่มควบคุม พบว่าการเติม 10% (v/v) new born calf serum หรือ 10.27 mM pyruvate ไม่สามารถเพิ่มอัตราการเจริญไปเป็นตัวอ่อนระยะบลาสโตซิส อย่างไรก็ตามการเติมสารทั้งสองตัวพร้อมกันสามารถเพิ่มอัตราการเจริญไปเป็นตัวอ่อนระยะ บลาสโตซิสได้อย่างมีนัยสำคัญทางสถิติ การเติม DL-dithiothreitol (DTT) ใน holding medium ไม่มีผลต่ออัตราการเจริญของตัวอ่อนบลาสโตซิสในกลุ่มที่เก็บรักษา ถึงแม้ว่า cyclosporine (CsA) สามารถลดระดับการเกิด apoptosis ในไข่ได้อย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับกลุ่มที่เติม BAPTA AM และ ruthenium red การเติม CsA ไม่สามารถเพิ่มอัตราการเจริญไปเป็นตัวอ่อนระยะ บลาสโตซิสได้ สรุปได้จากการทดลองว่าการเติม pyruvate และ new

born calf serum มีผลร่วมกันในการเพิ่มอัตราการเจริญไปเป็นตัวอ่อนระยะบลาสโตซิส ในขณะที่ mitochondrial membrane pore inhibitor CsA และ antioxidant DTT ไม่มีผลต่อการเจริญของตัวอ่อนโค

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2558 ลายมือชื่ออาจารย์ที่ปรึกษา _____

TAYITA SUTTIROJPATTANA : EFFECT OF TEMPERATURE AND MEDIUM ADDITIVES DURING LIQUID STORAGE ON DEVELOPMENTAL COMPETENCE IN MATURE BOVINE OOCYTES. THESIS ADVISOR : ASSOC. PROF. RANGSUN PARNPAI, Ph.D., 129 PP.

BOVINE/TEMPERATURE/LIQUID STORAGE/MEDIUM ADDITIVES

Short term preservation without freezing the oocytes, such as during long distance transport or subsequent *in vitro* embryo production would be useful for the flexible timing of their use in assisted reproduction techniques. The first experiment aimed to investigate the effect of temperature (4°C, 15°C, 25°C and 38.5°C) during the temporal preservation of matured bovine oocytes compared with control without storage on embryo development, blastocyst quality, cytoplasmic ATP, glutathione level and apoptosis status. The results suggested that after storage in HEPES TCM 199 (base medium) for 20 h, oocytes stored at 25°C and 38.5°C showed the highest rates of blastocyst formation compared with other storage groups. However, storage at 38.5°C reduced the total cell numbers in resultant blastocysts. Storage at 4°C reduced the metabolic activity characterized by cytoplasmic ATP compared with other groups. The glutathione content was significantly decreased in all storage groups. Storage at 4°C and 15°C significantly increased the proportion of apoptotic oocytes but not in those stored at 25°C and 38.5°C compared with control group. These results suggest that preservation at 25°C is the most suitable temperature for temporal storage of

mature bovine oocytes due to the ability to develop to blastocyst stage and the quality of resultant blastocysts.

The second experiment aimed to improve the developmental competence of matured bovine oocytes during liquid storage. *In vitro* matured oocytes were stored at 25°C according to the best temperature of the first experiment. Supplementation of the medium with 10% (v/v) new born calf serum or 10.27 mM pyruvate alone did not improve the blastocyst development. However, their simultaneous addition significantly improved the blastocyst rate. Addition of DL-dithiothreitol (DTT) in holding medium did not improve the embryonic developmental competence of stored oocytes. Although cyclosporine (CsA) could significantly reduce the percentage of apoptotic oocytes unlike BAPTA-AM and ruthenium red, CsA supplementation could not improve the proportion of blastocyst rate. In conclusion, pyruvate and new born calf serum had a synergistic effect to increase the blastocyst rate whereas the mitochondrial membrane pore inhibitor CsA and antioxidant DTT did not affect the embryo development.

School of Biotechnology Student's Signature _____

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ACKNOWLEDGEMENT

This research was supported by The Royal Golden Jubilee (RGJ) Ph.D. Program and NARO Institute of Livestock and Grassland Science (NILGS), Japan.

First, I wish to express my sincere thanks to my advisor, Assoc. Prof. Dr. Rangsun Parnpai who provided me an opportunity for my Ph.D study at Suranaree University of Technology, for his guidance help and encouragement throughout this research. I appreciated his knowledge and skill in many areas which are very valuable suggestions and ideas for me. I would like to specially thank to my co-advisor, Dr. Tamas Somfai for his enthusiasm, insightful comments and encouragement. He provided me with direction, technical support and motivation during my stay in Japan.

I would like to thank my committee members, Assoc. Prof. Dr. Montarop Yamabhai, Assoc. Prof. Dr. Mariena Ketudat-Cairns, and Dr. Siwat Sangsritawong, for their thoughtful comments of my thesis.

Appreciation also goes to the lab members in NILGS for all of their materials and equipment during my research in Japan. In addition, I was grateful for all members in Embryo Technology and Stem cell Research Center (ESRC) for their help and assistance in the period of this work.

I would like to express my sincerely thanks to Drs. Takashi Nagai, Masaya Geshi and Satoko Matoba for their assistance and thoughtful comments in my research papers. I would like to thank Drs. Junko Noguchi, Hiroyuki Kanko and Kazuhiro Kikuchi at The National Institute of Agrobiological Sciences (NIAS) for providing the opportunity to perform ATP and GSH measurement.

Finally, I expressed my warm thank to my parent, two younger sisters and elder brother. They were always cheering me up and supporting me throughout my Ph.D. study and my life

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LIST OF ABBREVIATIONS

APF	=	aging-promoting factor
ATP	=	adenosine triphosphate
BAPTA AM	=	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
COCs	=	cumulus-oocyte complexes
CsA	=	cyclosporine A
CSF	=	cytosolic factor
DO	=	denuded oocyte
DTT	=	dithiothreitol
GSH	=	glutathione
H_2O_2	=	hydrogen peroxide
ICM	=	inner cell mass
IVC	=	*in vitro* culture
IVF	=	*in vitro* fertilization
IVM	=	*in vitro* maturation
IVP	=	*in vitro* production
MAPK	=	mitogen activated protein kinase
mL	=	milliliter
MPF	=	maturation promoting factor
MII	=	metaphase II

LIST OF ABBREVIATIONS (Continued)

NO	=	nitric oxide
PN	=	pronuclear
PTP	=	permeability transition pore
ROS	=	reactive oxygen species
RR	=	ruthenium red
TE	=	trophectoderm
β-ME	=	2-mercaptoethanol
μg	=	microgram
μl	=	microliter
μM	=	micromolar

CHAPTER I

INTRODUCTION

1.1 Introduction

The *in vitro* production (IVP) of embryo which includes *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) is particularly important for providing an opportunities to produce the embryos in large scale farm animals (Viana et al., 2010) and for research purposes. As we known, the mature mammalian oocytes are arrested at metaphase II (MII) stage following ovulation by elevation of maturation promoting factor (MPF) and mitogen-activated protein kinase (MAPK) until insemination (Masui and Clarke,1979; Smith et al., 1989). If the oocytes are not fertilized in the optimal window period, they undergo time dependent aging process (Yanagimachi and Chang, 1961) called as post ovulatory aging oocytes. Post ovulatory aging oocytes are defined as anomalies in morphology and cellular changes resulting in low fertilization rate and embryo development (Badenas et al., 1989; Tarin et al., 1998; Takahashi et al., 2010). Furthermore, oocyte aging altered many function such as polyspermy, apoptosis, metabolic activity (ATP) and redox status (GSH) (reviewed by Miao et al., 2009). In previous studies, the developmental competence of bovine oocytes was significantly decreased approximately 30 h after IVM (Long et al., 1994; Ward et al., 2002; Agung et al., 2006; Koyama et al., 2014). Recently, they have reported successful preservation of mouse matured oocytes without cryopreservation (Li et al., 2012; Wang et al., 2013). Short term

preservation of matured oocytes would be useful for oocyte transportation and making the planning of IVF more flexible in time and space. Moreover, it could also reduce the production cost of embryos. For example, when bovine oocytes are collected from superstimulated cattle by OPU, about 25% of the oocytes are still immature and these oocytes can be utilized by IVM which requires approximately 20 h (Matoba et al., 2014). Efficient storage matured oocytes during this period would be possible to utilized all oocytes for IVF at once that could reduce the production cost especially sex-sorted sperm which is expensive.

Nowadays, preservation of oocytes can be utilized by cryopreservation and keep in liquid medium. Cryopreservation of oocytes and embryos is crucial step for conservation of genetic resources. However, the oocyte cryopreservation is very sensitive to chilling and cryoprotectant (Parks and Ruffing, 1992; Arav et al., 1996). In some mammalian species such as bovine and porcine, the high chilling sensitivity of oocytes was found due to their high intracellular lipid level and caused poor subsequent embryonic development (Nagashima et al., 1994; Martino et al., 1996). They have reported the low blatocyst rate (5-10%) after cryopreservation of mature bovine oocytes (Otoi et al., 1998; Dinnyes et al., 2000; Chian et al., 2004). Preservation oocytes without freezing may be an alternative way to keep the mature oocytes free from cryoprotectant toxicity. There have been reported the benefit of additives supplementation during oocyte storage and found that the additives supplementation during storage in liquid medium could delayed the aging process for instance, dithitothreitol (DTT), dithiol agent which counteracts with thiol oxidation, could improve blastocyst rate in aging mouse and ovine oocytes (Tarin et al., 1998; Liu et al., 1999; Ye et al., 2010). Li et al. and Wang et al. reported that low

temperature combined with pyruvate supplementation had positive effect on mouse oocytes during storage by maintain the GSH content which is important to prevent oxidative stress (Li et al., 2012; Wang et al., 2013). Zhao et al. delayed the aging mature bovine oocytes by supplemented with chemicals that regulated the calcium concentration (Zhao et al., 2015). The Ca^{2+} concentration is associated with the function of oocytes including egg activation and fertilization (Malcuit et al., 2006). In oocyte aging, the Ca^{2+} oscillation was impaired by dysfunction of Ca^{2+} regulation (Igarashi et al., 1997). Abnormal of Ca^{2+} elevations induced the fragmentation of cells and apoptosis cascade in aged oocytes (Malcuit et al., 2006).The above mentioned reports demonstrate, that the rocess of oocyte aging is greatly affected by the physical and chemical conditions around and therefore can be potentially controlled by medium additives and temperature adjustment.

However, optimization for storage conditions of *in vitro* matured bovine oocytes maintaining their abiity for embryo development has not been resolved to date. Although numerous studies have been performed on oocyte ageing during liquid preservation in mice, only few data has been published on storage of mature bovine oocytes. Oocytes of mice and cattle differ in several aspects such as their size, lipid content (Genicot et al., 2005) which may affect their sensitivity to stresses.

The objective of this study was to optimize conditions for *in vitro* matured bovine oocytes during their storage in a liquid medium for up to 20 h maintaining their ability for embryo development both by optimizing the storage temperature and testing medium additives. The effects of storage conditions on the quality of resultant blastocysts was also investigated.

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CHAPTER II

LITERATURE REVIEW

2.1 *In vitro* production of bovine embryo

In vitro production (IVP) of embryo has been widely used to generate embryos of farm livestock for commercial industry and research purposes. These techniques can improve the production rate of farm animals and reduced the cost per one embryo. Moreover, it can also solve the fertility problems especially in endangered species or individuals with high genetic values (Betteridge et al., 1989; Gordon, 1991). Bovine IVP which consists of three major steps including *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) is now considered as a reasonably efficient procedure. The demand of bovine tends to increase every year so that the IVP technique would be applied in large scale farm animals (Thornton, 2010). However, the production rate of embryos derived from *in vitro* has not been very effective compared with *in vivo* (Rizos et al., 2002). The rate of blastocyst production in bovine species does not exceed 40% when oocytes are matured *in vitro*. These differences are probably caused by several factors such as breed, oocyte quality, follicular environment, fertilization and embryo culture condition (Camargo et al., 2006). The limited developmental competence of bovine oocytes also contributed to infertility problems. Preservation of oocytes or embryos is an important procedure that increased the opportunities to preserve genetic resources and store for future use.

2.2 Oocyte storage

Long term preservation of embryos and gametes is a crucial for the *ex-situ* conservation of genetic resources (Dinnyes et al., 2007), whereas their short term preservation may be useful for the optimization of timing and effective utilization of materials in reproductive technologies such as artificial insemination, embryo transfer, MOET or IVF. Animal cells can be preserved either under subzero temperatures (cryopreservation) or above the freezing point of water in liquid medium. The former enables long term preservation by the complete sustention of biological activities in cells whereas the latter is feasible for short term preservation. Cryopreservation is possible either by slow (equilibrium) freezing or vitrification. Recently, vitrification has become a very popular method for the cryopreservation of the mammalian embryos and oocytes because it is very simple and effective (Rall and Fahy, 1985). Many investigators have achieved excellent outcomes of embryo cryopreservation by vitrification (Vajta et al., 1998; Hamawaki et al., 1999; Lane et al., 1999). In contrast, cryopreservation of oocytes is much more difficult compared with embryos and it is still a problematic field of cryobiology. Due to the low surface to volume ratio and low permeability coefficient of plasma membrane, cryopreservation of oocytes is more challenging than that of embryos (Fabbri et al., 2000; Diez et al., 2012). After freeze-thaw process, the survival rate is relatively high (approximately 80-90%); however, the developmental competence was still low especially in bovine and porcine oocytes which were sensitive to low temperature due to high lipid contents (Hamano et al., 1992; Otoi et al., 1992; Martino et al., 1996; Vajta et al., 1998; Chian et al., 2004; Shi et al., 2006). In cryopreservation process, oocyte suffer considerable morphological and functional changes such as severe

disorganization of meiotic spindle and microtubules after oocyte cryopreservation (Vincent et al., 1989; Aigner et al., 1992; Eroglu et al., 1998; Chen et al., 2003; Stachecki et al., 2004). Moreover, the cryopreservation process induced the premature cortical granule exocytosis which leading to zona pellucida hardening and impair fertilization rate (Matson et al., 1997; Mavrides and Morrol, 2005; Ghetler et al., 2006).

Preservation without freezing is the alternative way to preserve the oocytes without any cryoprotectant which are toxic to oocytes. Kept in a liquid medium above the freezing point of water animal cells do not sustain completely the biological processes therefore such approach allows preservation of cells for a limited time interval. Recently, the successful preservation of mouse oocytes was reported by keeping them in liquid medium at 15-25°C temperature without reducing the competence of embryo development for up to 48 h (Li et al., 2012 and Wang et al., 2014). At the present time, few efforts reported the suitable condition for bovine oocytes and the success rate of embryo development was not satisfied after storage.

2.3 Oocyte aging

After ovulation, the oocytes of most mammalian species are arrested at the metaphase II (MII) stage which is maintained by the elevation of intracellular levels of maturation promoting factor (MPF) and mitogen activated protein kinase (MAPK) until fertilization (Liu et al., 1998). If the oocytes are not fertilized within an optimum window period, the oocytes undergo time dependent aging process called as post ovulatory aging which is associated with the numerous abnormalities in morphology

and cellular functions (Tarin et al., 2002; Miao et al., 2009). These alterations are discussed below.

Impairment of fertilization ability

A decrease of fertilization rate has been related with aged oocytes in several species including human (Wilcox et al., 1998), mice (Tarin et al., 1998a; Liu et al., 2009; Lord et al., 2013), cattle (Koyama et al., 2014; Zhao et al., 2015), ovine (Ye et al., 2010) and porcine (Hao et al., 2009; Lee et al., 2014). Post ovulatory aging oocytes cause many deleterious effects in oocytes such as partial cortical granule exocytosis and zona hardening (Nigues et al., 1988; Ducibella et al., 1990; Fukuda et al., 1992; Diaz and Esponda, 2004). These changes can limit the sperm penetration into oocytes.

Ca^{2+} oscillation

At fertilization, the intracellular Ca^{2+} ($[Ca^{2+}]_i$) dramatically changes by a single long lasting in $[Ca^{2+}]_I$ followed by short repetitive transients of $[Ca^{2+}]_i$ for several hours as called Ca^{2+} oscillation (Cuthberson and Cobbold, 1985; Miyazaki et al., 1986; Kline and Kline, 1994). These oscillations appear to be prerequisite for oocyte activation, pronuclear formation and embryo development (Schultz and Kopf, 1995). It was reported the dysfunction of intracellular Ca^{2+} regulation occurred during oocyte aging (Igarashi et al., 1997; Hao et al., 2009). Compared with fresh oocytes, the frequency of Ca^{2+} oscillation tended to increase but the amplitude of the oscillations was significantly smaller than fresh oocytes (Igarashi et al., 1997). Takahashi et al. found that the poor embryo development in aged oocytes may be related to alteration of Ca^{2+} oscillations (Takahashi et al., 2009). The cytosolic molecules involved in the regulation of Ca^{2+} homeostasis and maintaining the balance between anti- and

proapoptotic proteins (e.g. BCL-s and Bax proteins). In aged oocytes, abnormal Ca^{2+} oscillation triggered the DNA fragmentation and signal apoptosis cascade (Gordo et al., 2002).

MPF and MAPK

Before fertilization process, the oocytes remain in the M phase of cell cycle (metaphase II or MII arrest) by MPF; a dimer formed by the protein cyclin B1 (regulatory subunit) and protein $P34^{cdc2}$ (catalytic subunit) (Homa, 1995; Masui and Markert, 1971). In oocytes, MPF activity reaches its peaks during metaphase stages of the meiotic division. Once the second meiotic metaphase is reached, the oocyte remain at a meiotic arrest by elevated of cytosolic factor (CSF), MPF and mitogen-activated protein kinase (MAPK) until fertilization. During oocyte ageing the activity of MPF gradually decreases until it reaches a threshold level which initiates the exit from MII arrest leading pathway resembling parthenogenetic activation such as the extrusion of the second polar body and the formation of a female pronucleus without participation of a fertilizing spermatozoon (O'Neill and Kufman, 1988; Kikuchi et al., 2000, 2002; Tian et al., 2002; Petrova et al., 2004). This process is associated with increases rates of DNA fragmentation in aged oocytes (Kikuchi et al., 2000, 2002).

Mitochondria dysfunction

Aging oocytes show signs of mitochondrial dysfunction. Ma et al. reported that mitochondrial were relocated during aging process (Ma et al., 2005; Hao et al., 2009) which was related to altered metabolic function of oocytes. It is well known that mitochondrial is the main energy source which generates adenosine triphosphate (ATP) for cellular function comprising maturation, fertilization and embryo

development (Chappel, 2013). The ATP content may be associated with the mitochondrial relocation during oocyte aging. It was reported that ATP content was declined in aged mouse and porcine oocytes (Igarashi et al., 2005; Hao et al., 2009; Duran et al., 2013) but not with bovine oocytes (Koyama et al., 2014). Since mitochondria play important roles not only in ATP production but also in the regulation of the redox status and Ca^{2+} levels in cytosol (Dumollard et al., 2007), their damage is often associated with the altered functions of these cytoplasmic features (Anesti and Scorrano, 2006).

Cytoskeletal

During aging process, these oocytes had greatly disrupted microtubule and microfilament structures and which was associated with increased the incidences of chromosomal misalignment (Kim et al., 1996; Suzuki et al., 2002) either through increased ROS levels and/or reduced ATP (Tarin , 1996).

Redox status

During oocyte ageing the concentrations of reactive oxygen species (ROS) increase (Miao et al., 2009) which – at high concentrations –are very detrimental to the developmental competence by damaging membranes, DNA integrity and cytoskeletal elements in cells (Takahashi et al., 2012). ROS are usually generated by hydrogen peroxide production which is a biological byproduct of glucose metabolism and by superoxide production under high environmental oxygen levels (oxidative stress) leading to the generation of free hydroxyl radicals (Figure 2.1) (Takahashi et al., 2012). It is well known that glutathione (GSH) play a crucial role in biological functions which are involved in preventing oxidative stress such as ROS (Meister and

Tales, 1976) and fertilization process and early embryo development (Gardiner and Reed, 1994). In oocytes, the level of GSH may be an indicator to assess the oocyte cytoplasmic maturation. GSH production in oocytes is an ATP dependent process (Krisher et al., 2007). Moreover, GSH has an important role to maintain the meiotic spindle function (Zuelke et al., 1997). These GSH levels are associated with sperm nuclear decondensation and male pronuclear formation (Yoshida, 1993; Sutovsky and Schatten, 1997). In aging oocytes, GSH concentration was significantly decreased which increased susceptibility to oxidative stress (Hao et al., 2009; Li et al., 2012). As anomalies in aged oocytes, there was not only affected to the fertilization rate and embryonic development but also the production of offspring.

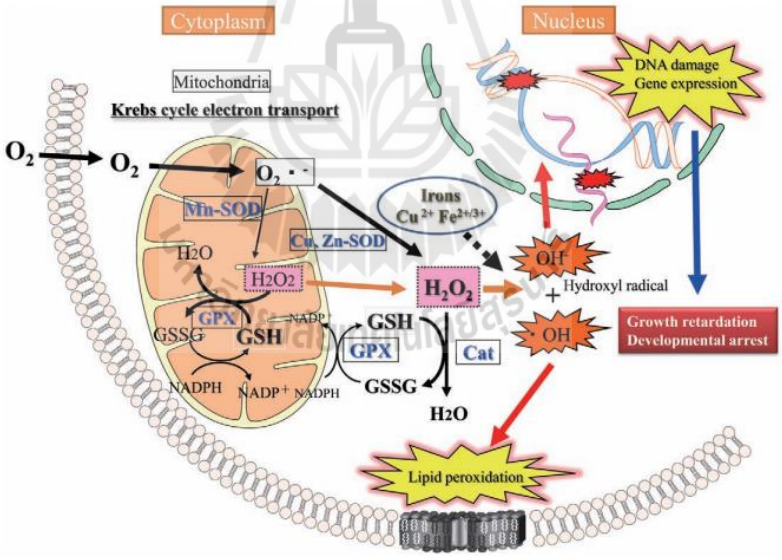

Figure 2.1 A schematic diagram of ROS generation and their effects on cellular functions (Takahashi, 2012).

Apoptosis

Apoptosis - or programmed cell death – is characterized by DNA fragmentation, the activation of pro-apoptotic genes and the activation of proteolytic enzymes such

as Caspases which eventually kill the cells (Elmore, 2007). In oocytes apoptosis can be triggered by high intracellular ROS levels causing DNA fragmentation (Takahashi et al., 2009) and abnormal Ca^{2+} regulation (more specifically extended high Ca^{2+} levels in the cytosol (Dumollard et al., 2007). The concentration of ROS significantly increase in aged oocytes which cause the imbalance of GSH/GSH disulfide ratio resulting in DNA fragmentation (Tarin, 1996; Goud et al., 2008a; Tang et al., 2013; Koyama et al., 2014). Accordingly, increased manifestation of apoptosis characterized by caspase activation, accumulation of apoptotic protein Bax and reduction of anti-apoptotic protein Bcl-2 have been detected in aged oocytes (Gordo et al., 2002; Ma et al., 2005; Tatone et al., 2006)

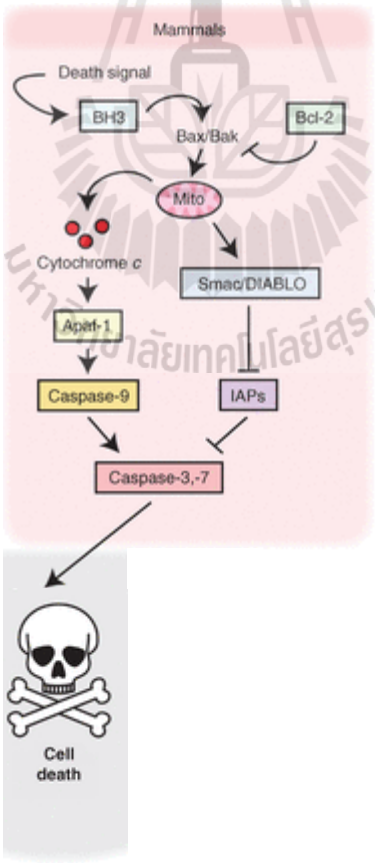

Figure 2.2 Schematic representation of apoptotic signaling (Parrish et al., 2013).

2.4 Factor affecting the aging oocytes

2.4.1 Temperature

As mentioned above, detrimental effects of oocyte aging is greatly displayed by high levels of ROS. ROS are produced as byproducts of mitochondrial metabolism. The activity of mitochondrial metabolism in cells is greatly affected by temperature (Guderley and St-Pierre, 2002). Therefore, temperatures might affect the process of oocytes ageing through the activity of oxidative metabolism and therefore ROS production. In accordance decreasing temperatures in the presence of pyruvate was reported to down-regulate metabolism and thus ROS accumulation which slowed down the aging process in murine oocytes (Li et al., 2012). Abnormally high temperatures are known to cause heat shock (heat stress) which trigger aging-like changes in oocytes such as ROS elevation (Sakatani et al., 2004, 2008). Heat shock can initiate the apoptosis in mammalian oocytes. Exposure COCs to heat stress during maturation reduced the cleavage and blastocyst rate. Moreover, the proportion of TUNEL-positive oocytes was increased (Roth and Hansen, 2004). Zhu *et al.* demonstrated that the aging process could induce apoptosis affected by releasing soluble Fas ligand (sFasL) (Zhu *et al*., 2015) a major pathway in apoptosis cells and tissues (Chu *et al*., 1995; Poulaki *et al*., 2001). The apoptosis cells in aged oocytes may be a factor that reduced the developmental competence of oocytes. In regard of temperature, cumulus cells seem to play a role in ageing regulation. It was reported that the temperature accelerates the aging process of oocytes in cumulus oocyte complexes (also known as cumulus oocyte complexed: COCs) but not in denuded oocytes (DOs). In COCs group, the increasing temperature trigger oocyte activation and decline of MPF activity. In contrast to DOs, the increasing temperature did not

activate oocytes; however, the activated oocytes were significantly higher in DO cultured in conditioned with COCs. Therefore an aging promoting factor (APF) is believed to be produced by the cumulus cells (CCs) as the temperature was rising (Qiao et al., 2008). Nevertheless, hypothermia may also be detrimental to oocytes by triggering a release of Ca^{2+} from intracellular stored leading to apoptosis (Mattioli et al., 2003).

Although temperatures for the liquid storage of matured oocytes has been optimized in mice, the optimum temperature for the storage of matured cattle oocytes has remained unknown.

2.4.2 Cumulus cells

CCs displays important biological functions including ovulation, maturation and fertilization (Tanghe et al., 2002). As mention above, during aging process *in vitro* or *in vivo* oocytes, the spontaneous oocyte activation was significantly increased whereas the MPF activity was declined in the presence of CCs nut not in DOs. Moreover, the aged *in vitro* and *in vivo* COC partial released the cortical granules at higher rates than DOs. These results confirmed that CCs accelerate the aging progression of both *in vivo*-matured and *in vitro*-matured oocytes (Miao et al., 2005) and suggest that they release an APF into the culture medium. (Qiao et al., 2008). A recent study reported that CCs surrounding aged oocytes released sFasL which accelerated the aging process by binding to Fas receptors leading to apoptosis (Zhu et al., 2015). Maalouf et al. reported the aged COCs greatly decreased the blastocyst rate compared with fresh COCs. In contrast to DOs group, the blastocyst rate between fresh and aged DOs groups were not significantly different. These results confirmed that the COCs accelerated the aging progression of oocytes which in turn affects to embryo development. Nevertheless, the blastocyst rate of fresh

COCs was significantly higher than that of fresh DOs group (Maalouf et al., 2009). Complete removal of cumulus cells can negatively affect embryo production since reducing sperm penetration and pronuclear formation in IVF systems (Chian et al., 1995; Zhang et al., 1995; Wongsrikeao et al., 2005). Furthermore, during oocyte maturation, cumulus cells play an important role for energy production and maintaining GSH levels in oocytes by the utilization of glucose through glycolysis and the pentose phosphate pathway (PPP) which prevent the oocyte aging by producing pyruvate (Li and Kazgan, 2011). Without cumulus cells oocytes can utilize pyruvate or lactate but not glucose.

2.5 Medium supplementation with additives

It has been demonstrated that matured bovine oocytes maintained the competence for embryo development for 5-6 h after maturation (Long et al., 1994; Ward et al., 2002; Agung et al., 2006). After this period, the proportion of oocytes which could develop to blastocyst stage embryos was significantly declined because of the cellular and functional changes during aging process (Miao et al., 2009). Delayed aging process of oocytes could promote the developmental competence of oocytes. In order to increase the developmental ability of aged oocytes, the substances involved in the mechanisms of oocyte aging should be neutralized to postpone the aging period and to extend the developmental competence of oocytes in time. To date, several reports have demonstrated that chemical reagents can interact with oocyte aging delaying its detrimental effects on embryonic development.

2.5.1 Antioxidants

Thiols

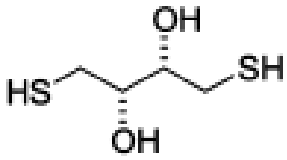

Figure 2.3 Chemical structure of dithiothreitol.

The most widely used thiol reagents are 2-mercaptoethanol (βME) and dithiothreitol (DTT, dithiol reagent, Figure 2.3) (Netto and Stadtman, 1996). In case of 2-mercaptoethanol (mono thiol), the mixed disulfide could be occurred. DTT can resolve this problem by forming the six member ring. The reaction of DTT is intramolecular and involves the formation of two products from one reactant, then converted to a stable cyclic disulfide (Cleland, 1964). DTT was more preferable than βME. Nowadays, DTT was supplemented in the medium to counteract thiol oxidation in aging oocytes (Tarin et al., 1998a, b). DTT supplementation had beneficial effect on developmental competence to blastocyst stage in aging mouse and ovine oocytes (Tarin et al., 1998a; Rausell et al., 2007; Ye et al., 2010). The precise mechanism of DTT in aging oocytes remained to be clarify; however, it may protect the aging oocytes by increasing the GSH levels which decrease the DNA fragmentation (Rausell et al., 2007; Ye et al., 2010).

Cystine/ cysteine/ cysteamine

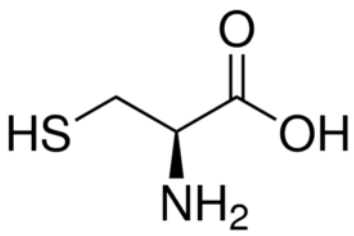

Figure 2.4 Chemical structure of cysteine

Glutathione (gamma-glutamyl-cysteinyl-glycine or GSH) is an endogenous thiol compound containing tripeptide including cysteine, glycine and glutamic acid (Meister and Anderson, 1983) and plays a key role in cellular protection from oxidative stress. Moreover, GSH has many important functions on protein and DNA synthesis by altering the redox status, metabolism and enzyme activity. In reproductive function, GSH is associated with maturation and fertilization process such as sperm decondensation (Perreault et al., 1988; Sutovsky and Schatten, 1997), pronuclear formation rate (Grupen et al., 1995; Yoshida et al., 1993; Yamauchi and Nagai, 1999) and oocyte maturation (Yoshida et al., 1993; Miyamura et al., 1995). GSH display in two forms [(reduced from (GSH) and oxidized form (GSSH)] by the regulation of glutathione peroxidase and glutathione reductase (Luberda, 2005). A decrease of GSH was reported to be early event in apoptosis cascade (Armstrong et al., 2002; Mytilineou et al., 2002). It has been demonstrated that the amino acid cysteine (Figure 2.4) regularly oxidized into cystine form in extracellular cells which is the rate limiting step of GSH synthesis (Bannai and Tateishi, 1986; Ishii et al., 1987). Bannai and Tateishi reported the limited uptake capacity of cystine caused by the transport system which is quite low in cells (Bannai and Tateishi, 1986). Synthesis of GSH under *in vitro* condition may be impaired due to the deficiency of cysteine and easily oxidized to cysteine form (Bannai, 1984). Addition of cysteamine in maturation medium increased the efficiency of *in vitro* blastocyst production by increasing the level of GSH (De Matos et al., 1995, 1996; Anand et al., 2008). During aging process of oocytes, the level of GSH was significantly decreased compared with fresh oocytes (Hao et al., 2009; Liu et al., 2009; Li et al., 2012; Wang et al., 2014). Wang et al. enhanced the oocyte competence by adding pyruvate, α-Tocopherol, cysteamine and cysteine in the medium (Wang et al., 2014). Moreover, the

antioxidant agents prevent the oocytes from reactive oxygen species which can damaged the embryo development (Guerin et al., 2001).

Nitric oxide (NO)

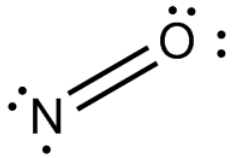

Figure 2.5 Chemical structure of nitric oxide.

Nitric oxide (Figure 2.5) is a free radical that plays significant role in physiological systems. It is not only involved in oocyte maturation process (Sengoku et al., 2001; Tao et al., 2004) but also the embryo development and implantation (Gouge et al., 1998; Sengoku et al., 2001; Tranguch et al., 2003). Moreover, NO increases the calcium rise in fertilization which causes sperm-egg fusion (Kuo et al., 2000). Goud et al. reported the role of NO supplementation in aged oocytes is to maintain the oocyte quality during aging oocytes by supporting the normal fertilization and development to blastocyst stage and also prevent the apoptosis cell in embryos (Goud et al., 2008b). As the intracellular Ca^{2+} was abnormal in aged oocytes and induced the apoptotic cells. NO may prevent this apoptotic process by regulating the Ca^{2+} release and maintaining the ER Ca^{2+} stores (Goud et al., 2008b). Altogether, NO could delay the oocyte aging and improve the integrity of the microtubular spindle (Goud et al., 2005).

Melatonin

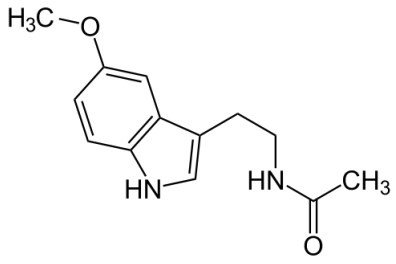

Figure 2.6 Chemical structure of melatonin.

Melatonin (Figure 2.6), a pineal secretary hormone, plays an important role in part of physiological function (Reiter, 1991; Cardinali and Pevet, 1998; Srinivasan et al., 2005). Numerous studies have been indicated that melatonin has a potent effect for scavenger the oxidative stress and antioxidant in mammalian cells (Zang et al., 1998; Reiter et al., 2000; Galano et al., 2011). Melatonin inhibited hydrogen peroxide (H_2O_2) which induced the cell death through apoptotic pathway by regulating the Bax expression and inhibiting the caspase-3 activation (Juknat et al., 2005). Evidence supported that melatonin could improve fertilization and embryo development in mouse and porcine oocytes (Ishizuka et al., 2000; Jang et al., 2005). Recently, melatonin treatment was reported to optimize fertilization in aged oocytes. Furthermore, supplementation with melatonin decreased the early apoptosis cells and ROS levels resulting in high blastocyst formation compared with non-treatment (Lord et al., 2013).

2.5.2 Maintenance MPF and MAPK activities

Caffeine

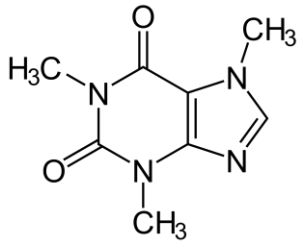

Figure 2.7 Chemical structure of caffeine.

MPF and MAPK are associated with the regulation of cell cycle. When the oocytes are arrested at metaphase of the second meiotic division (MII), the MPF and MAPK activities remained high levels. Nevertheless, these levels decreased with oocyte aging. The abrupt inactivation of MPF trigger the Ca^{2+} release resulting in an escape from the MII arrested (Lee et al., 2008; Kikuchi et al., 2000). It was reported that caffeine (Figure 2.7) treatment could prevent decline of MPF and MAPK activities (Kikuchi et al., 2000; Lee and Campbell, 2008). IP_3R1-mediated Ca^{2+} release are irregular during postovulatory aging oocytes and these abnormal Ca^{2+} release compromised to embryo development. Supplementation with caffeine reduced the changes in aging oocytes through IP_3R1-mediated Ca^{2+} signal, maintained the intracellular Ca^{2+} near normal levels and decrease the embryo fragmentation (Zhang et al., 2011). There has been many reports, suggesting that the free radical oxygen species are overproduced in postovulatory aging oocytes which has detrimental effect on embryo production (Fissore et al., 2002; Goud et al., 2008a; Takahashi et al., 2009; Li et al., 2012). Treatment with caffeine significantly increased the GSH contents (Ye et al., 2010), and reduced the ROS levels and apoptotic embryos (Choi et al., 2013). Moreover, caffeine also exhibited the benefit on rearrangement of cytoskeleton (Ono et al., 2011; Choi et al., 2013) and capacity for PN formation (Choi et al., 2013). They reported the benefit of caffeine on embryo development and quality of resultant

blastocysts in aged ovine and bovine species (Ye et al., 2010; Choi et al., 2013) but in contrast with Lee and Campbell, the developmental competence to blastocyst was not improved in ovine nuclear transfer oocytes (Lee and Campbell, 2008).

2.5.3 Ca^{2+} regulation

Poor embryo development in aged oocytes has been reported to be implicated with Ca^{2+} homeostasis. The Ca^{2+} oscillation regulated many molecular mechanism including maturation, fertilization and embryo development. Impairment of Ca^{2+} regulation leaded to apoptosis of oocytes (Gordo et al., 2002). Moreover, the apoptotic cascade was recognized in aged oocytes due to the Ca^{2+} regulation (Gordo et al., 2002). Changes in Ca^{2+} homeostasis signal apoptosis in aged oocytes. For instance an increase of Ca^{2+} in cytosol can trigger apoptosis in porcine oocytes (Mattioli et al., 2003). The level of apoptotic protein, Bax increased whereas the anti-apoptotic protein, Bcl-2 decreased. Such changes can induced the DNA fragmentation in aged oocytes and limited the embryo development (Gordo et al., 2002; Ma et al., 2005; Tatone et al., 2006). The regulation of ooplasmic free Ca^{2+} to prevent its accumulation may delayed the aging process.

1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA AM)

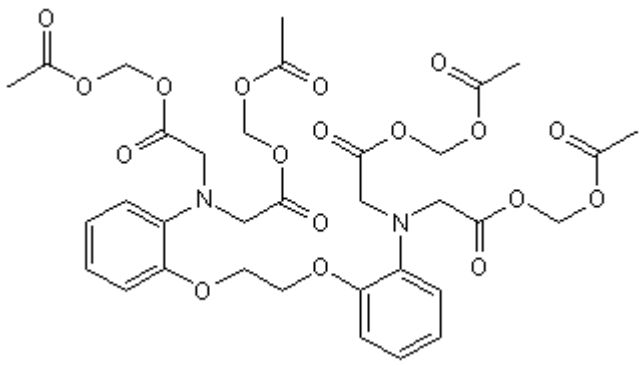

Figure 2.8 Chemical structure of BAPTA AM.

BAPTA (Figure 2.8), an intracellular Ca^{2+} chelator is widely used to study the effect of Ca^{2+} oscillations on oocyte activation and fertilization events (Lawrence

et al., 1998; Petr et al., 2005; Gardner et al., 2007). Recently, Zhao et al. reported that supplementation with BAPTA could reduce the intracellular Ca^{2+} concentration during oocyte aging and produced the high blastocyst compared with aged oocytes without treatment (Zhao et al., 2015). There has been reported that BAPTA inhibited the Ca^{2+} dependent apoptosis pathway (Inanami et al., 1999; Olofsson et al., 2008).

Ruthenium red (RR)

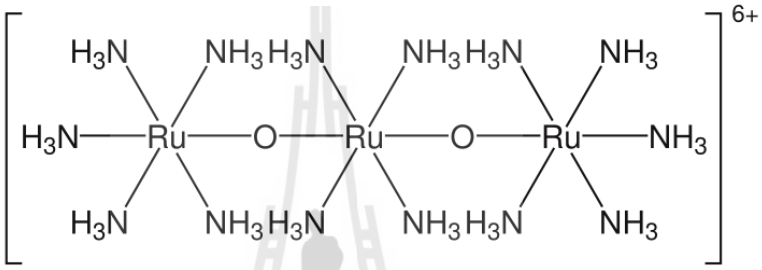

Figure 2.9 Chemical structure of ruthenium red.

Ruthenium red (Figure 2.9), a hexavalent polysaccharide stain is an inhibitor of Ca^{2+} uniporter. Mitochondria play an important role in a regulation of intracellular Ca^{2+} level. The mitochondrial Ca^{2+} uniporter is a gated ion channel which uptake the Ca^{2+} accumulation across the inner mitochondrial membrane (Gunter et al., 2000; Patron et al., 2013). The mitochondria Ca^{2+} implicated with apoptosis and necrosis cascade (Kruman and Mattson, 1999; Anderson et al., 2004). When the mitochondrial Ca^{2+} uptake is overloaded, it affects the permeability transition pore (PTP) formation causing the loss the mitochondrial membrane potential (Kroemer and Reed, 2000). Permeability transition pore formation caused the mitochondrial swelling and triggers the apoptosis factors (Gunter and Pfeiffer, 1990; Giorgi et al., 2002; Halestrap, 2009). Inhibition of mitochondrial Ca^{2+} uniporter prevented this process. Evidence showed that ruthenium red inhibits the mitochondrial Ca^{2+} uniporter and Ca^{2+} influx (Kruman and Mattson, 1999; Gunter et

al., 2000). Moreover, ruthenium red inhibited curcumin induced apoptosis by blocking cytochrome c release and activation of caspase3 (Bae et al., 2003). Mattioli et al. indicated that supplementation with ruthenium red reduced the DNA fragmentation in cold stress porcine oocytes during cold stress (Mattioli et al., 2003). The regulation of Ca^{2+} concentration of ruthenium red may postpone the postovulatory aging oocytes. However, to the best of our knowledge, ruthenium red has not been tested on oocyte aging to date.

Cyclosporin A (CsA)

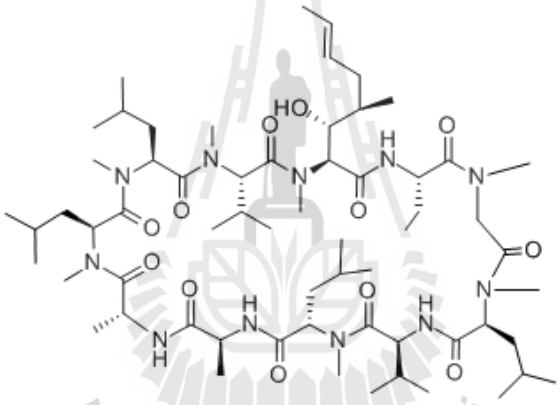

Figure 2.10 Chemical structure of cyclosporine A.

CsA (Figure 2.10) is an immunosuppressive agent for allograft rejection; however, its serious side effects are limited to treat with CsA due to nephrotoxicity, neurotoxicity and hepatotoxicity (Min and Monaco, 1991; Bennett et al., 1996; Erer et al., 1996; Bechstein, 2000). Besides of the immunosuppressive action of CsA, it was reported that CsA affect to mitochondria function, inhibiting Ca^{2+} uptake through Ca^{2+} uniporter (Montero et al., 2004). Inhibition of mitochondrial Ca^{2+} uptake depended on the cytosolic Ca^{2+} concentration which its activation is strongly in low micromolar range (Montero et al., 2004). Many papers investigated the effect of CsA on PTP and found that the inhibition of PTP occurred at lower concentration of CsA (Smaili et al., 2001; Montero et al., 2004). It was reported that CsA inhibited the PTP

by interacting with cyclophilin D which is believed to be a component of the PTP (Halestrap and Davidson, 1990; McGuinness et al., 1990; Tanveer et al., 1996; Elrod et al., 2013). Inactivation of cyclophilin D was insensitive to CsA (Basso et al., 2005) which supported the thought that CsA inhibited PTP via cyclophilin D. As described above, PTP is involved in necrotic and apoptosis caspase including cytochrome C release and triggering the Bax cascade (Giorgi et al., 2002; Morin et al., 2004; Zhang and Armstrong, 2007) resulting in cell death (Bernati and Forte, 2007; Grimm and Brdiczka, 2007). Regarding those mechanisms, we hypothesized that CsA may have positive effect on aging oocytes by maintaining the mitochondria Ca^{2+} concentration through Ca^{2+} uniporter and PTP.

2.5.4 Receptor

Revesratrol

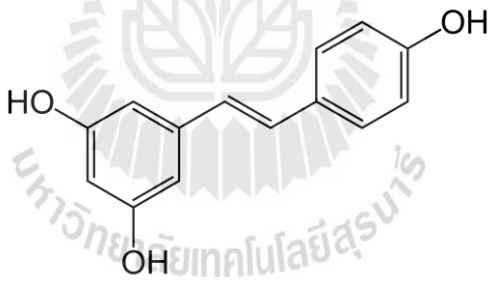

Figure 2.11 Chemical structure of resveratrol.

Resveratrol (3,5,4'-trihydroxystilbene) (Figure 2.11), a phytoalexin synthesized by grapevines, pines, legumes, peanuts and mulberries, is in response to protect the plant from fungal infection or injury i.e. UV light (Langcake and Pryce, 1976). Many studies have reported that resveratrol exerted many biological effects such as anticancer, anti-inflammatory, antioxidant, antiproliferative and cardioprotective (Gusman et al., 2001; Pervaiz and Holme, 2009). Recently, resveratrol has been applied in livestock species to study the beneficial effect on

oocyte maturation and embryo development (Lee et al., 2010; Kwak and Hyun, 2012; Salzano et al., 2014; Itami et al., 2015). Kwak and Hyun reported that treatment of resveratrol during IVM effectively reduced ROS levels, increased GSH concentration and enhanced the developmental competence of oocytes. Moreover, the expression of apoptosis related gene was significantly decreased (Kwak and Hyun., 2012). Supplementation culture medium with resveratrol improved the quality of embryos (Lee et al., 2010; Salzano et al., 2014) and hence the resistance to cryopreservation (Salzano et al., 2014).

It was reported that resveratrol had a beneficial effect in aged oocytes by improving the spindle morphology and chromosome alignment. As we known, the cortical granule was abnormal in aged oocytes, the treatment of resveratrol could decreased the disruption of cortical granule. Additionally, the abnormal mitochondrial were improved by resveratrol treatment (Ma et al., 2015). Resveratrol was found to act as a potent activator of SIRT1 which is one of sirtuin family. Sirtuins, a family of highly conserved protein modifying enzymes found by yeast silent information regulator (Sir2) protein, are (nicotinamide adenine dinucleotide) NAD+-dependent protein deacetylases and ADP-ribosyltransferases (Imai et al., 2000; Landry et al., 2000). In yeast, Sir2 plays an important role to maintain the silent chromatin (Guarente, 2000). Interestingly, sirtuins has been reported to link with regulation of cellular process including energy metabolism and stress response to tumorigenesis and aging in other organisms (Bishop and Guarente, 2007; Haigis and Sinclair, 2010). In mammalian genome encodes, there have seven sirtuin homolog named SIRT1 to SIRT7 (Frye, 2000). SIRT1 appears to be closest mammalian homolog to Sir2. Due to this homology, many studies focused on biology of SIRT1. The data showed that this SIRT1 is associated with metabolic sensor and regulation of gene expression (Li,

2013). Moreover, it plays crucial function in metabolism, development, reproduction and also in part of biological function such as aging and disease (Hargis and Sinclair, 2010; Li and Kazgan, 2011). According to these mechanisms, regulation of Sirt1 may be an important factor to delayed aging process in oocytes. In aged oocytes, the expression of Sirt1 was decreased compared with fresh oocytes. Addition of resveratrol, a potent activator of Sirt1 provided beneficial effect in aging process by improved cellular functions such as mitochondria distribution, spindle and chromosome alignments (Ma et al., 2015).

2.5.5 Metabolism

Pyruvate

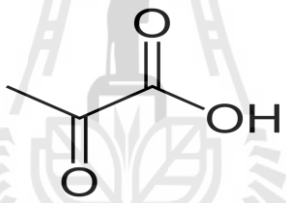

Figure 2.12 Chemical structure of pyruvate.

Cow oocytes prefer to use pyruvate as a main energy source which supports the meiotic maturation process (Rieger and Loskutoff, 1994). Studies have shown that pyruvate (Figure 2.12) supplementation prevent oocyte aging in mouse oocyte (Liu et al., 2009; Li and Kazgan, 2011; Li et al., 2012; Wang et al., 2014). Liu et al. suggested that *in vitro* aging of oocytes may cause by insufficiency of energy (Liu et al., 2009). Addition of pyruvate decreased susceptibility to activation stimuli whereas maintained the MPF activity; it increased the anti-apoptotic Bcl-2 level and prevent premature cortical granule exocytosis (Li et al., 2012). Pyruvate supplementation also improved the redox status GSH, fertilization rate and embryo development in aging oocytes (Li et al., 2012; Wang et al., 2014).

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CHAPTER III

THE EFFECT OF TEMPERATURE DURING LIQUID STORAGE OF *IN VITRO* MATURED BOVINE OOCYTES ON SUBSEQUENT EMBRYO DEVELOPMENT

3.1 Abstract

The aim of the present study was to optimize the temperature for the temporal storage of matured bovine oocytes. *In vitro* matured bovine oocytes were preserved in HEPES-buffered TCM199 medium supplemented with 10% newborn calf serum at different temperatures (4°C, 15°C, 25°C and 38.5°C) for 20 h. Embryo development and blastocyst quality after *in vitro* fertilization, cytoplasmic ATP and glutathione levels in oocytes and the frequency of apoptotic oocytes were compared among storage groups and a control group without storage. Among the storage groups, those at 25°C and 38.5°C showed the highest rates of blastocyst development (19.3% and 24.5%, respectively) compared with those stored at 4°C and 15°C (8.5% and 14.9%, respectively); however, blastocyst formation rates in all storage groups were lower than that in the control group (39.8%) ($P<0.05$). Storage at 38.5°C and 15°C were associated with reduced cell numbers in resultant blastocysts compared with the

control and the 25°C storage groups. Storage at 4°C reduced metabolic activity of oocytes characterized by their lower ATP levels compared with the other groups. Storage for 20 h significantly reduced the glutathione content in oocytes in all groups in a similar manner, irrespective of the temperature. Storage at 4°C or 15°C but not at 25°C and 38.5°C significantly increased the percentage of apoptotic oocytes compared with the control group. In conclusion, 25°C was found to be the most suitable temperature for the temporal storage of matured bovine oocytes regarding both the developmental competence of oocytes and the quality of resultant blastocysts.

3.2 Introduction

In vitro production (IVP) of embryos employing *in vitro* maturation (IVM) of oocytes and subsequent *in vitro* fertilization (IVF) and *in vitro* culture (IVC) is a technology that allows the efficient production of bovine embryos available for embryo transfer in large quantities (Pontes et al., 2011; Viana et al., 2010). Using either IVM oocytes or *in vivo* matured ones (obtained by Ovum Pick-Up (OPU) from pre-ovulatory follicles), IVP technology has been proven to be a cost-effective way to produce embryos using sex-sorted sperm (Matoba et al., 2012, 2014; Pontes et al., 2010). The matured mammalian oocyte is arrested at the metaphase stage of the second meiotic division (also known as the metaphase-II or MII stage) by a high level of maturation promoting factor (MPF) until it is penetrated by a spermatozoon. The sperm entry into the oocyte induces Ca^{2+} oscillations lasting for several hours (Cuthbertson and Cobbold, 1985; Kline and Kline, 1992; Miyazaki et al., 1993) which trigger oocyte activation, pronucleus formation and hence embryo development

(Schultz and Kopf, 1995). However, if matured oocytes are not fertilized for an excessive duration, they undergo a time dependent aging process which greatly reduces their developmental competence (Yanagimachi and Chang, 1961; Wilcox et al., 1998). The aging process is characterised by profound, complex and harmful cytoplasmic changes such as the reduction of MPF activity leading to spontaneous activation causing cortical granule exocytosis and zona hardening, the alterations of mitochondrial activity and ATP levels, altered redox status caused by the accumulation of reactive oxygen species (ROS), the disruption and displacement of microtubules and microfilaments and the activation of the apoptotic cascade (reviewed by Miao et al. (Miao et al., 2009)). Accordingly, the developmental competence of bovine oocytes has been reported to decrease significantly from approximately 5 h after they reached the MII stage (Agung et al., 2006; Koyama et al., 2014a; Long et al., 1994; Ward et al., 2002). Because of the aging of matured oocytes mentioned above, the optimum timing for their use for IVF is predominantly determined by the timing of their retrieval. This fact appears to be a hitch for the planning of IVP programs when MII oocytes are obtained at awkward time points or spatially far from the site of IVF. Therefore, preservation of matured oocytes without reducing their competence even for an extended period would be greatly advantageous by making the planning of IVF programs more flexible in time and space. Efficient storage of matured oocytes could also reduce the production costs of embryos. For instance, when bovine oocytes are collected from superstimulated cows by OPU from follicles larger than 5 mm, approximately 25% of the oocytes are still immature and can be utilized by IVM (Matoba et al., 2014). In our IVM system, nuclear maturation of bovine oocytes is finished after 20 h of culture. Storage of

already matured oocytes during this period would make it possible to utilize all oocytes for IVF at once, using a single straw of frozen sperm which would reduce production costs of each transferable embryos especially when expensive (such as sex-sorted) sperm is to be used.

To date, oocyte storage is possible either by cryopreservation or by keeping them in a medium (liquid storage). Cryopreservation by vitrification is a relatively simple and rapidly developing technique (Rall and Fahy, 1985); however, the current vitrification methods greatly reduce the developmental competence of bovine oocytes (Martino et al., 1996a; Otoi et al., 1998; Rho et al., 2002). Another alternative way to store oocytes for a short term is to keep them in a medium without freezing under conditions that prevent the aging process and thus prolong the developmental competence (usability for IVF) of matured oocytes. Nevertheless, to date, few efforts have been reported for the storage of matured bovine oocytes. Therefore, the optimum environment for the liquid storage of matured bovine oocytes and the cytoplasmic alterations that may compromise competence during the process have remained unknown. In a recent report, matured mouse oocytes were successfully stored in medium without reducing their developmental competence for up to 36 h under low temperatures which seemed to prevent aging of oocytes (Li et al., 2012). The aim of the present study was to define the optimum storage temperature for liquid preservation of MII stage bovine oocytes for the first time and to reveal cytoplasmic alterations during the process. We stored cumulus-enclosed IVM oocytes for 20 h in a simple HEPES -buffered tissue culture medium at different temperatures and compared their developmental competence in terms of 1) fertilization and embryo development after IVF, 2) the quality of resultant blastocysts characterised by their

hatching ability and cell numbers, 3) overall metabolic activity (ATP levels), 4) cytoplasmic redox status (levels of glutathione; a natural antioxidant), and 5) the onset of apoptosis in oocytes.

3.3 Materials and Methods

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

The oocyte collection and IVM were performed as described by Imai et al. (Imai et al., 2006). Briefly, bovine ovaries were obtained from slaughterhouse and kept in plastic bag at 25°C. Then, the ovaries were transported to laboratory. After bovine spongiform encephalopathy (BSE) test according to Abattoir Law of Japan was confirmed to be negative, the ovaries were washed several times in 0.9% (w/v) sodium chloride solution. Cumulus-oocyte complexes (COCs) were aspirated from small follicles (2-6 mm in diameter) with an 18-gauge hypodermic needle connected to a 10-mL syringe. Groups of 20 COCs which had homogeneous cytoplasm and compacted cumulus cells were selected and cultured in 100 μL droplets of 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) under paraffin oil (Paraffin liquid, Nacalai Tesque Inc., Kyoto, Japan) overlay for 20-21 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air.

3.3.2 *In vitro* preservation of oocytes

The medium for oocyte preservation was a TCM-199 medium (Medium 199 powder, 31100-035, GIBCO Invitrogen) buffered with 11 mM of HEPES (Sigma-Aldrich Co., St. Louis, MO, USA), 9 mmol/L of Na-HEPES (Sigma-Aldrich Co.), and supplemented with 5 mmol/L of sodium bicarbonate and 10% (v/v) NCS with a pH adjusted to 7.3 and the osmolarity is approximately 0.290 osm. After maturation, the cumulus-enclosed oocytes were washed twice in the storage medium, and then they were transferred into 1.5-mL Eppendorf microfuge tubes in 1.3 mL of storage medium (Figure 3.1). The tubes were then closed, sealed air tight with parafilm and kept at either 4°C, 15°C, 25°C or 38.5°C for 20 h.

Figure 3.1 Oocyte preservation in liquid medium.

3.3.3 *In vitro* fertilization (IVF)

Frozen semen of a single proven Japanese Black bull was thawed in 37°C for 40s and centrifuged in 3 mL of 90% Percoll (Sigma-Aldrich Co.) solution at 740 x *g* for 10 min. Then, the pellet was re-suspended in 5.5 mL of IVF 100 medium (Research Institute for Functional Peptides Co., Ltd., Yamagata, Japan) and centrifuged at 540 x *g* for 5 min. The pellet was re-suspended with IVF 100 medium and the final concentration was adjusted to 3 x 10^6 sperm/mL. The matured oocytes were washed twice in IVF 100 medium and co-cultured with sperm in 100 µL

droplets of IVF 100 medium under paraffin oil overlay for 5 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air (20 oocytes per droplet).

3.3.4 *In vitro* embryo culture (IVC)

After insemination, presumptive zygotes were completely denuded from cumulus cells by gentle pipetting with fine glass pipette in preincubated embryo culture 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. Then, twenty zygotes were cultured in droplets of 100 μL culture medium under paraffin oil overlay at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90 % N_2 for up to 9 days.

3.3.5 Assessment of fertilization status

The fertilization status of oocytes was assessed 18 h after IVF. Oocytes were mounted on glass slides and fixed with acetic alcohol (1:3 acetic acid: ethanol) for at least 3 days, then stained with 1% (w/v) orcein (Sigma-Aldrich Co.) in acetic acid, rinsed in glycerol: acetic acid : water (1:1:3) and examined under a phase-contrast microscope with ×40 and ×100 objectives. The presence and numbers of female and male pronuclei and/or a sperm head(s), and extrusion of the two polar bodies (PBs), were then investigated. An oocyte was considered to be activated if a female pronucleus was detected in the cytoplasm. Oocytes were considered to have been penetrated when a sperm head(s) or a male pronucleus(ei) with the corresponding sperm tail(s) were detected in the cytoplasm. Oocytes with one penetrating sperm in the cytoplasm were defined as monospermic.

3.3.6 Evaluation of total cell number in blastocysts

Blastocysts on Day 9 (Day 0 = IVF) were fixed in 99.5% ethanol supplemented with 10 µg/mL Hoechst33342 (Calbiochem, San Diego, CA, USA) overnight at 4°C. After washing in ethanol, embryos were mounted on glass slides in glycerol droplets, flattened by cover slips and total nuclei were counted under UV light with excitation at 330–385 nm and emission at 420 nm under an epifluorescenct microscope (Nikon Eclipse E600, Tokyo, Japan) (Figure 3.2).

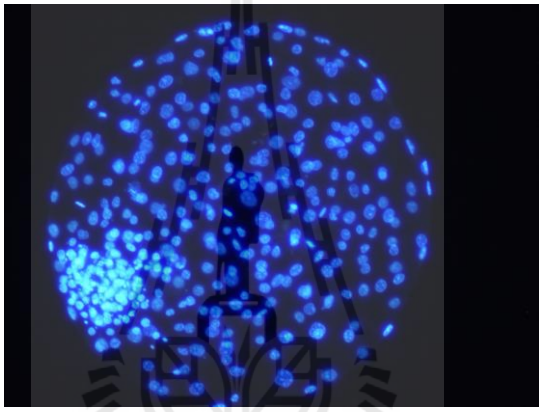

Figure 3.2 Total cell number of blastocyst after Hoechst staining.

3.3.7 Measurement of ATP content in oocytes

The ATP content of matured oocytes was measured using a commercial assay (FL-ASC; Sigma-Aldrich Co.) based on the luminescence reaction of luciferin/ luciferase, according to the method of Stojkovic et al. (Stojkovic et al., 2001) with modifications. Briefly, oocytes were completely denuded from cumulus cells. Pooled samples of 10 oocytes in each treatment group were rinsed three times in a Ca^{2+} and Mg^{2+} free Dulbecco's phosphate buffer saline (PBS-) supplemented with 3 mg/mL polyvinyl pyrrolidone (PVP, P0930; Sigma-Aldrich Co.), then placed in plastic tubes with 50 µL of PBS- on ice/water and the samples were stored at -20°C until analysis. To measure ATP contents sample tubes were thawed and kept on ice. Then, 100 µL

of ice-cold somatic cell reagent (FL-SAR) was added to all tubes that were incubated for 5 min on ice/water. Subsequently, 100 µL of ice cold assay mix solution (dilution 1:25 with ATP assay mix dilution buffer, FL-AAB) was added, and the tubes were kept for 5 min at room temperature in the dark. The ATP content of the samples was measured using a luminometer (TD-20/20, Turner Designs, Sunnyvale, CA, USA) with high sensitivity (0.01 pmol/tube). A seven-point standard curve (0–60 pmol/tube) was routinely included in each assay. The ATP content in samples was determined from the formula for the standard curve (linear regression).

3.3.8 Assay of intracellular glutathione (GSH) content

Total GSH concentrations in matured oocytes were measured by 5,5′-dithio-*bis*-2-nitrobenzoic acid (DTNB) – glutathione disulfide reductase recycling assay, as described by Anderson (Anderson et al., 1985). In brief, denuded oocytes were stored in groups of 10 in 5 µL of 10 mM Na_2-EDTA–containing sodium phosphate buffer (0.2 M, pH 7.2) and 5 µL of 1.25 M phosphoric acid in a 1.5-mL microfuge tube at –80 °C until assay. Assay samples were warmed at room temperature for 15 min after the addition and mixing of 175 µL sodium phosphate buffer containing 0.33 mg β-nicotinamide adenine dinucleotide phosphate (reduced form; NADPH, Sigma-Aldrich Co.), 25 µL of 6 mM DTNB (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and 40 µL water. Subsequently, 5 µL of 125 IU/mL GSH disulfide reductase (Wako Pure Chemical Industries, Ltd.) was added to the microfuge tube to initiate the reaction. Absorbance was recorded 6 times by spectrophotometer at 30s intervals at a wavelength of 412 nm. GSH standards of 250, 100, 50, 25 and 10 pmol/tube and a blank sample were also assayed. The test was replicated three times.

3.3.9 Assay of live/dead status and apoptosis in oocytes

The onset of apoptotic events in oocytes was assayed by the Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit (Molecular Probes, Eugene, OR, USA) according to Anguita et al. (Anguita et al., 2007). This assay is based on the annexin V binding which is applied to determine phosphatidylserine (PS) externalization on the plasma membrane. Annexin V, a member of the phospholipid-binding annexin family, binds most effectively to PS, which is externalized on the outer membrane of cells exposed to apoptotic stimuli. The assay also applies propidium iodide (PI), a membrane impermeable stain, to discriminate between live and dead (membrane damaged) cells. PI has ability to enter the cell when the cytoplasmic membrane has lost its integrity. Oocytes were washed three times with PBS- and then transferred to 100 μL of binding buffer (Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit, Molecular Probes) with 5 μL of Alexa Fluor Annexin V, 1 μL of PI (100 μg/mL) and 0.2 μL of 5 mg/mL Hoechst 33342 for 20 minutes at 38.5°C in the dark. After incubation, the oocytes were washed three times in binding buffer and subsequently mounted on the glass slides. The samples were examined with an epifluorescence microscope (Eclipse E-600; Nikon) at an excitation wavelength of 480 nm and emission at 510 nm. Oocytes were classified in three groups (Anguita et al., 2007) (Figure 3.3). Viable oocytes were characterized by PI negative nuclei (appearing in blue color by Hoechst 33342) and no annexin staining on the membrane (A-/PI-). Early apoptotic membrane intact (live) oocytes had PI negative nuclei (appearing in blue color by Hoechst 33342 at 350 nm excitation) and a homogeneous annexin positive signal on the membrane (A+/PI-). Dead oocytes which showed PI positive

red nuclei, indicating membrane damage with or without annexin staining on the membrane (PI+).

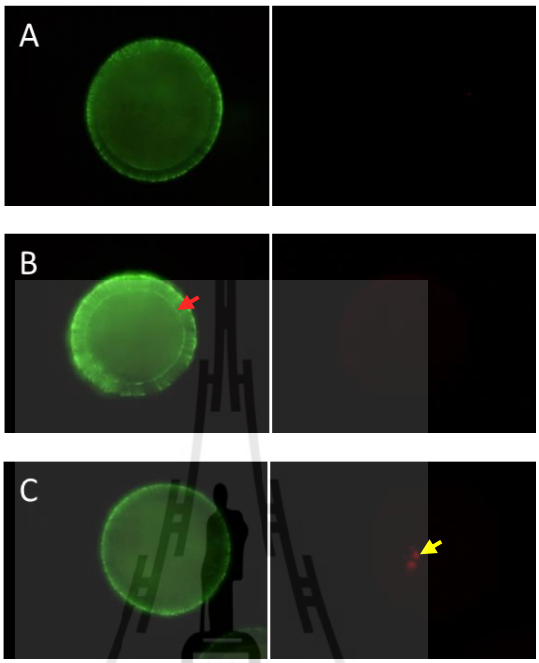

Figure 3.3 Oocyte classification by annexin V staining (A) viable non-apoptotic cell; (B) early apoptotic oocytes with homogeneous annexin positive signal in the membrane (red arrow); (C) dead oocytes which showed PI positive red nuclei (yellow arrow). Left images are of Annexin V and right ones for PI.

3.3.10 Experimental design

Experiment 1 investigated the effect of storage at different temperature on the developmental competence of oocytes. After IVM, cumulus-enclosed oocytes were stored for 20 h either under 4°C, 15°C, 25°C or 38.5°C. Then oocytes were subjected to IVF and IVC as detailed above. Cleavage rates on Day 2, blastocyst formation rates on Days 7-9, hatching rates of blastocysts on Days 8-9 and blastocyst cell numbers on Day 9 were compared among stored groups and the control group (oocytes were fertilized without storage, immediately after IVM). *In vitro* embryo

development was investigated in seven replications whereas, the total cell numbers in embryos were analyzed in five replications.

Experiment 2 investigated the effect of storage at different temperature on fertilization of oocytes. After IVM, cumulus-enclosed oocytes were stored for 20 h either at 4°C, 15°C, 25°C or 38.5°C. Then oocytes were fertilized *in vitro* as described above. Eighteen hours after IVF, oocytes were fixed and stained with orcein. The percentages of oocyte activation, sperm penetration and monospermy were compared among storage groups and control group fertilized without storage. Four replications were performed.

Experiment 3 investigated the effect of storage at different temperatures on ATP and GSH levels of oocytes. After IVM, cumulus-enclosed oocytes were stored for 20 h either at 4°C, 15°C, 25°C or 38.5°C. The ATP and GSH content of oocytes were compared among stored groups and the control group without storage. Three replicates for the ATP content analysis and the GSH content analysis were performed in each assay (10 oocytes/group in each replicate).

Experiment 4 investigated the effect of storage at different temperatures on the onset of apoptosis in oocytes. After IVM, cumulus-enclosed oocytes were stored for 20 h either at 4°C, 15°C, 25°C or 38.5°C. The percentages of apoptotic oocytes were compared among storage groups and the control group without storage. Three replicates were performed.

3.3.11 Statistical analysis

Statistical analysis was performed using software SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA). All data were analyzed by one way ANOVA. The significant differences between mean was compared by a post-hoc Fisher's protected

least significantly difference test. Data were expressed as mean ± SEM, and $P<0.05$ was considered statistically significant. Percentage data were arcsine transformed before analysis.

3.4 Results

3.4.1 Developmental competence of bovine oocytes stored at different temperatures and the quality of resultant embryos

As summarized in Table 3.1, there was no significant difference in cleavage rates among the control group and those stored at 15°C, 25°C and 38.5°C (65.8%, 66.4%, 75.6% and 71.0%, respectively), whereas storage at 4 °C resulted in a significantly lower cleavage rate compared with other groups ($P<0.05$). The percentage of blastocyst development on Day 9 in the control group (39.8%) was significantly higher compared to those in all storage groups ($P<0.05$). Compared among storage groups, the 38.5°C storage group (24.5%) showed significantly higher in blastocyst rate than 4°C and 15°C storage groups (8.5% and 14.9%, respectively) ($P<0.05$) but similar with 25°C storage group (19.3%). In 4°C storage group, only a small proportion of embryos progressed to the blastocyst stage (8.5%) which was significantly lower than those in the groups stored at 25°C and 38.5°C ($P<0.05$). On Days 8 and 9, there was no difference in the percentage of blastocyst stage embryos undergoing hatching among all groups (Table 3.2).

As shown in Table 3.3, the total cell numbers of blastocyst on Day 9 were the highest in the control and the 25°C storage groups (204.9±22.6 and 206.2±21.0, respectively) among all the groups. Compared to these groups, significantly lower cell numbers were detected in blastocysts from the 15°C and 38.5°C storage groups (146.5

± 16.9 and 134.4±19.1, respectively) (P<0.05) whereas the cell numbers in blastocysts of the 4°C storage group (180.1±10.5) did not differ statistically from those in other groups .

3.4.2 Susceptibility of oocytes to normal fertilization after storage at different temperatures

There was no difference among the groups in the rates of sperm penetration and oocyte activation (Table 3.4). A significantly higher rate (76.1%) of control oocytes had 2PBs compared to all storage groups (P<0.05). Furthermore, the 2PBs emission rate in the 38.5°C storage group (16.2%) was significantly lower than those in the 4°C storage group (48.8%) (P<0.05), but not differ from 15°C and 25°C storage groups (44.7% and 39.7%, respectively). The rate of monospermic fertilization was not significantly different among control and storage groups. However, in the 38.5°C storage group the frequency of monospermic fertilization was tendentiously lower compared with the control and the 15°C storage groups (P=0.073 and P=0.066, respectively). The proportion of penetrated oocytes with normal male pronuclear formation in the control group (97.9%) was significantly higher than those in the 4°C, 15°C and 38.5°C storage groups (84.8%, 87.3% and 87.2) (P<0.05), whereas the rate in the 25°C storage group did not differ statistically from the other groups.

3.4.3 ATP and GSH contents in oocytes after storage at different temperatures

The ATP content of oocytes stored at 4°C was significantly lower than those of other groups (P<0.05, Figure3.1). On the other hand, the ATP content in the

group stored at 25°C and 38.5°C was significantly increased compared with control group (P<0.05) whereas oocytes in the group stored at 15°C had ATP levels similar to the control group.

The GSH levels of all storage groups were significantly lower than that of the control group (P<0.05, Figure3.2). However, no significant difference in the GSH levels was found among the storage groups.

3.4.4 The frequency of apoptotic oocytes after storage at different temperatures

The percentage of oocytes classified as viable non-apoptotic ones (A-, PI-) in the control group (91.9%) was statistically higher than that of the storage groups (P<0.05, Table 3.5). In storage groups, a significantly higher percentage of viable non-apoptotic oocytes (A-, PI-) belonged to oocytes stored at 38.5°C (75.0%) compared with the 4°C and 15°C storage groups (38.6% and 36.0%, respectively) (P<0.05); however, this value did not differ significantly from that of the 25°C storage group (61.8%). The proportions of early apoptotic (A+, PI-) oocytes in the 4°C and 15°C storage groups were significantly higher compared with the control group (P<0.05) but did not differ from one another (P>0.05). The proportions of early apoptotic (A+, PI-) oocytes in the 25°C and 38.5°C storage groups did not differ significantly from that in the control; however, the value was tendentiously increased in the 25°C storage group (P=0.09). Furthermore, in groups stored at 4°C, 15°C and 25°C, the percentages of dead oocytes (PI+) were significantly higher than that in control group (P<0.05).

Table 3.1 Effects of storage of IVM oocytes for 20 h at different temperatures on their development after IVF.

Group	Total number (No.) of oocytes examined	No. (%) of oocytes cleaved on Day 2 (Mean ± SEM)	No. (%) of blastocyst obtained on (Mean ± SEM)		
			Day 7	Day 8	Day 9
Control	300	197 (65.8 ± 4.6^{a})	109 (36.2 ± 4.5^{a})	118 (39.2 ± 4.4^{a})	120 (39.8 ± 5.2^{a})
4°C	281	97 (34.5 ± 6.1^{b})	19 (6.7 ± 1.9^{b})	23 (8.1 ± 2.3^{b})	24 (8.5 ± 2.2^{b})
15°C	288	191 (66.4 ± 3.2^{a})	33 (11.5 ± 1.4^{bc})	40 (13.9 ± 3.0^{bc})	43 (14.9 ± 1.5^{bc})
25°C	289	218 (75.6 ± 3.0^{a})	53 (18.3 ± 2.9^{c})	55 (19.0 ± 3.0^{c})	56 (19.3 ± 2.9^{cd})
38.5°C	283	199 (71.0 ± 4.4^{a})	51 (17.8 ± 2.1^{c})	63 (22.1 ± 2.5^{c})	70 (24.5 ± 3.0^{d})

Seven replications were performed. Percentage values with different superscripts in the same column differ significantly ($P<0.05$). Day 0 = the day of IVF.

Table 3.2 Effects of storage of IVM oocytes for 20 h at different temperatures on the hatching ability of resultant blastocysts after IVF/IVC.

Group	Total number of blastocysts examined	% Hatching[$] (Day 8) (Mean ± SEM)	%Hatching[$] (Day 9) (Mean ± SEM)
Control	120	22 19.3 ± 6.3	74 61.8 ± 8.7
4°C	24	7 38.4 ± 12.1	17 65.0 ± 11.7
15°C	43	14 31.6 ±11.2	24 53.9 ± 9.6
25°C	56	21 33.6 ±9.1	37 70.1 ± 6.1
38.5°C	70	21 32.9 ±5.3	36 52.3 ± 7.7

Seven replications were performed. Day 0 = the day of IVF.
$Cumulative percentage of hatching and hatched blastocysts

Table 3.3 Effects of storage of IVM oocytes for 20 h at different temperatures on total cell numbers of resultant blastocysts obtained after IVF on Day 9.

Group	Total number (No.) of oocytes examined	Total cell No. (Mean ± SEM)
Control	88	204.9 ± 22.6^{a}
4°C	19	180.1 ± 10.5^{ab}
15°C	29	146.5 ± 16.9^{b}
25°C	33	206.2 ± 21.0^{a}
38.5°C	44	132.4 ± 19.1^{b}

Five replications were performed. Percentage values with different superscripts in the same column differ significantly ($P<0.05$). Day 0 = the day of IVF.

Table 3.4 Effects of storage of IVM oocytes for 20 h at different temperatures on their fertilization status after IVF.

Group	Total number (No.) of oocytes examined	No. of penetrated oocytes (% total) (Mean ± SEM)	No. of activated oocytes (% total) (Mean ± SEM)	No. of oocytes with 2PBs (% penetrated) (Mean ± SEM)	No. of monospermic oocytes (% penetrated) (Mean ± SEM)	No. oocytes with male PN (% penetrated) (Mean ± SEM)
Control	95	87 (90.2 ± 4.6)	91 (95.1 ± 2.3)	68 (76.1 ± 9.6^{a})	53 (59.3 ± 3.9)	85 (97.9 ± 2.1^{a})
4°C	88	74 (85.8 ± 5.8)	69 (80.5 ± 6.4)	35 (48.8 ± 8.2^{b})	29 (41.6 ± 13.5)	62 (84.8 ± 6.1^{b})
15°C	82	74 (90.8 ± 1.8)	76 (93.2 ± 2.5)	32 (44.7 ± 6.7^{bc})	43 (59.0 ± 10.3)	65 (87.3 ± 2.8^{b})
25°C	89	73 (81.7 ± 1.2)	73 (84.0 ± 5.8)	28 (39.7 ± 6.2^{bc})	40 (54.6 ± 7.3)	67 (90.3 ± 4.5^{ab})
38.5°C	83	76 (91.6 ± 4.1)	73 (88.6 ± 6.6)	12 (16.2 ± 7.0^{c})	24 (32.2 ± 9.4)	66 (87.2 ± 2.7^{b})

Four replications were performed. Percentage values with different superscripts in the same column differ significantly ($P<0.05$).
PB = polar body; PN = pronucleus

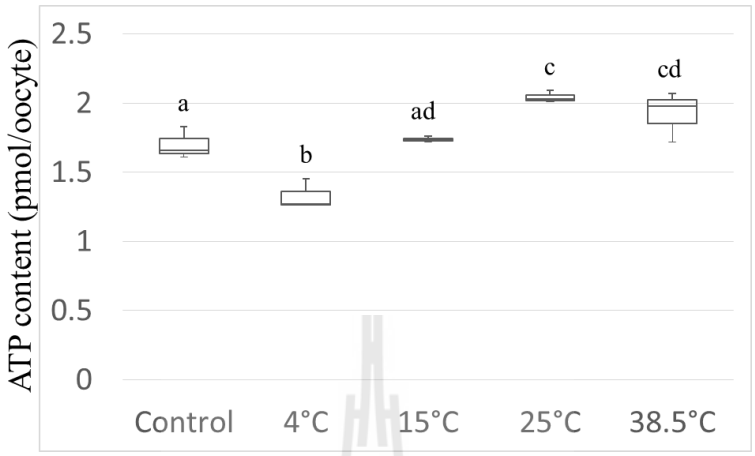

Figure 3.4 Effects of storage of IVM oocytes for 20 h at different temperature on their ATP content.

Bars with different superscript letter differ significantly (P<0.05).

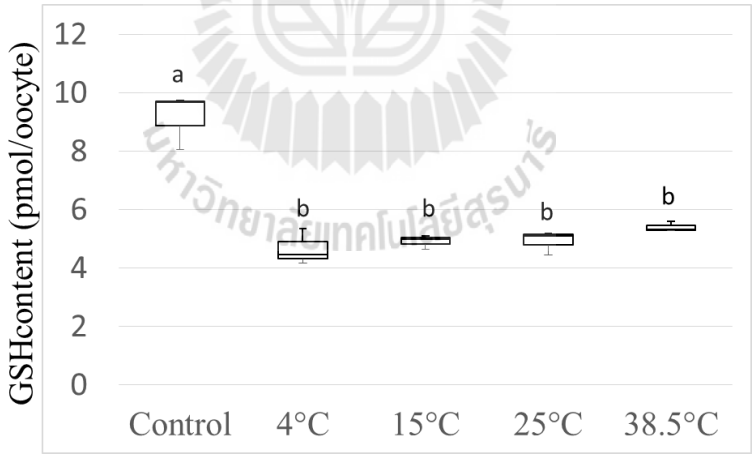

Figure 3.5 Effects of storage of IVM oocytes for 20 h at different temperature on their GSH content.

Bars with different superscript letter differ significantly.

Table 3.5 Effects of storage of IVM oocytes for 20 h at different temperatures on their live/dead status and apoptosis.

Group	Total number of oocytes examined	% of oocytes with (A-/PI-) (Mean ± SEM)	% of oocytes with (A+/PI-) (Mean ± SEM)	% of oocytes with(PI+) (Mean ± SEM)
Control	84	91.9 ± 5.0^{a}	6.6 ± 5.4^{a}	1.6 ± 1.6^{a}
4°C	87	38.6 ± 13.0^{b}	38.5 ± 9.3^{bcd}	22.9 ± 6.3^{b}
15°C	88	36.0 ± 3.9^{b}	48.3 ± 6.0^{c}	15.6 ± 2.3^{bc}
25°C	88	61.8 ± 4.9^{bc}	23.6 ± 3.8^{ad}	14.6 ± 4.9^{bd}
38.5°C	87	75.0 ± 3.4^{c}	19.9 ± 4.8^{ad}	5.1 ± 3.0^{acd}

Three replications were performed. Percentage values with different superscripts in the same column differ significantly ($P<0.05$). A = Annexin-V; PI = Propidium iodide. A-/PI- = non-apoptotic, membrane intact oocyte; A+/PI- = apoptotic, membrane intact oocyte; PI+ = membrane damaged (dead) oocyte.

3.5 Discussion

3.5.1 Effects of temperature during oocyte storage on developmental competence

Our results indicated greatly reduced developmental competence of bovine matured oocytes after 20 h of storage in all groups, in agreement with previous studies showing an oocyte aging associated with limited developmental competence of resultant embryos (Koyama et al., 2014b; Petrova et al., 2005; Wakayama et al., 2004). Although in many reports the cellular and molecular changes during oocyte aging have been studied, only a few investigated the suitable conditions for temporal storage of oocytes. Previous studies in the mouse have demonstrated that the temperature of preservation affects competence of MII stage oocytes (Li et al., 2012; Tsuchiya et al., 2001; Wakayama et al., 2004; Wang et al., 2014), and that reducing temperatures below 30°C prevented the aging process of matured oocytes resulting in blastocyst development even after their storage for up to 24-36 h (Li et al., 2012; Wakayama et al., 2004) in contrast with preservation at 37°C which failed blastocyst production (Tsuchiya et al., 2001; Wakayama et al., 2004). Furthermore, it was suggested that the suitable temperature for the temporal preservation of mouse oocytes was at 25-27°C (Wakayama et al., 2004). Our results demonstrated that when bovine oocytes were stored for 20 h, their developmental competence to the blastocyst stage remained the highest and statistically similar at 38.5°C and 25°C. However, reducing the storage temperature to 15°C reduced embryo development compared with that at 38.5°C and storage at 4°C resulted in very low rates of cleavage and blastocyst formation (8.5%). This result coincided with previous results on mouse

oocytes stored at 4°C (5.0% blastocyst formation rate by Wakayama et al. (Wakayama et al., 2004)), but differed from the results of Tsuchiya et al. who reported that 20% of the mouse oocytes stored at 5°C had the ability to develop to the blastocyst stage (Tsuchiya et al., 2001). The difference between the outcome of the two reports is believe to be related to the composition of the storage medium, especially its serum content which seems to be protective to the oocyte during low temperature storage; Wakayama and colleagues used serum free media (Wakayama et al., 2004) whereas Tsuchiya and colleagues employed as much as 33.3% fetal calf serum (Tsuchiya et al., 2001). Furthermore, it is important to point out species-specific differences in the sensitivity to oocyte storage between mouse and bovine oocytes. The decisive factors may be the differences between the oocytes of the two species in terms of cytoplasmic features of microtubules and lipid. Microtubules are the main structural elements of the meiotic spindle in oocytes and also they play pivotal roles in the movement and distribution of mitochondria in the cytoplasm (Sun et al., 2001a, 2001b). After cooling, the spindles of mouse oocytes have been reported to be reorganized normally during re-warming (Li et al., 2012; Magistrini et al., 1980; Sun et al., 2004) whereas in bovine oocytes, only a low frequency of spindle recovery was observed (Aman and Parks, 1994). Also, bovine oocytes are known to contain higher amounts of lipid compared with their mouse counterpart (Genicot et al., 2005) and high lipid content in oocytes and zygotes had been known to be responsible for their increased sensitivity to low temperatures (Nagashima et al., 1994).

Recent research has revealed that non-frozen preservation protocols for mature mouse oocytes dramatically extend their developmental competence by reducing oxidative stress (Wang et al., 2014). In their paper, Wang et al. reported that

the mouse oocytes could be stored for different durations under different temperatures (such as for 9 h at 37°C, 30 h at 25°C, 48 h at 15°C and 24 h at 5°C) without reducing developmental competence when cytoplasmic GSH was adjusted to a normal level by supplementation of the storage medium with suitable concentrations of pyruvate, α-tocopherol and/or cysteamine/cystine (Wang et al., 2014). In a previous report, the same group has demonstrated that reducing temperatures to 15-25°C contributes to the maintenance of developmental competence by the down regulation of metabolic activity of oocytes preventing the accumulation of its byproducts, the ROS, which are known to be detrimental to cells (Li et al., 2012). Accordingly, in the presence of 10.27 mM pyruvate, mouse oocytes maintained their developmental competence as well as the GSH content for 24 h at 25°C, and for 36 h at 15°C (Li et al., 2012). GSH has been described to play many critical biological roles in cells; its major role is known to protect the cell from oxidative stress (Deneke and Fanburg, 1989). It maintains redox state against oxidative stress by converting reduced form (GSH) to oxidized form (GSSG) in oocytes (Gasparrini et al., 2006). Accordingly, the cytoplasmic GSH concentration has been considered as an indicator of good cytoplasmic maturation (de Matos and Furnus, 2000). Furthermore, GSH is essential for the decondensation of the sperm head and subsequent male pronuclear formation in fertilized oocytes (Sutosky and Schatten, 1997; Yoshida et al., 1992). However, our results on ATP and GSH levels in stored bovine oocytes were only partly accordant with the results of Li et al. (Li et al., 2012) reported in mice. After storage at 4°C, bovine oocytes contained lower ATP levels than those stored at higher temperatures. This confirms that similarly to mouse oocytes, low temperatures during storage down regulated metabolism in bovine oocytes. On the other hand, the GSH content in

oocytes was similar among all storage groups irrespective of the temperature suggesting that it is not the redox status which determines the optimum storage temperature for bovine oocytes. Nevertheless, the importance of maintaining GSH concentrations during oocyte preservation cannot be ruled out since in our study reduced GSH concentrations were detected in all stored groups (in most cases associated with reduced frequencies of male pronuclear formation) compared with the control group, which showed the best embryo developmental rate. Therefore, further research is necessary to normalize the GSH levels in stored bovine oocytes. Although ATP is necessary for GSH production in oocytes (Dumollard et al., 2007), similar GSH levels but different ATP levels among stored groups suggest that reduction of GSH levels during storage was not ATP dependent. Intracellular ATP plays important roles in oocyte maturation, fertilization and embryo development (Cummins, 2002; Chappel, 2013). The ATP content in oocytes has been reported to positively correlate with their quality, which is manifested in the developmental competence of resultant embryos (Stojkovic et al., 2001; Van Blerkom et al., 1995). On the other hand, excessively high ATP content in mature oocytes seems to impair the embryo development (Nagano et al., 2006a, 2006b). Our results indicated that during 20 h of storage at 25°C and 38.5°C, the ATP levels increased in oocytes in a similar manner to approximately 2 pmol, compared to non-preserved control oocytes (1.7 pmol). These results support the previous report of Koyama et al. (Koyama et al., 2014b) who described an increase in ATP content of bovine oocytes during aging, but are in contrast with previous studies in mice, hamsters and pigs which reported decreased ATP levels in aged oocytes (Hao et al., 2009; Igarashi et al., 2005; Simsek-Duran et al., 2013) suggesting differences between species. Nevertheless, storage at 15°C

maintained intracellular ATP at the same level to that of non-preserved oocytes but without improving developmental competence compared with storage at 25°C or 38.5°C. This suggests that beyond 1.7 pmol, the ATP content of the oocyte does not affect embryo developmental competence, which is in agreement with the previous study (Somfai et al., 2012). Our results also revealed that when storage temperature was reduced to 4°C, the ATP levels dropped to 1.3 pmol which was associated with a dramatic decrease in developmental competence already at the level of cleavage. Although a severe reduction in oocyte ATP content may prevent sperm penetration (Somfai et al., 2012), analysis of fertilization events demonstrated normal sperm penetration rates in oocytes stored at 4°C in the present study. On the other hand, their ability to support male pronuclear formation from the penetrating sperm was compromised. Furthermore, reducing storage temperature to or below 15°C induced membrane damage and triggered the apoptotic cascade in surviving oocytes at higher rates compared to those stored at 25°C and 38.5°C. Previous studies have reported aging-induced apoptosis in oocytes (Fujino et al., 1996; Ma et al., 2005; Tatone et al., 2006; Takahashi et al., 2009;Tang et al., 2013). In fact, in the present study, the frequency of membrane intact but apoptotic oocytes was increased either significantly or tendentiously in all groups stored at or below 25°C compared with the control group. However, we did not detect significant difference in terms of the frequency of apoptosis between oocytes stored at 38.5°C and the control oocytes. Furthermore, the frequencies of apoptotic oocytes statistically increased by reducing temperatures during storage. The cold-induced apoptosis is likely to be caused by chilling, to which oocytes with high lipid content (such as those of pigs and cattle) are sensitive (Arav et al., 1996; Martino et al., 1996b; Mattioli et al., 2003). In porcine oocytes, chilling has

been reported to cause the release of Ca^{2+} ions into the cytoplasm which triggers the apoptotic cascade leading to DNA fragmentation and compromised developmental competence (Mattioli et al., 2003; Barboni et al., 2003). Taken together, the combined effect of insufficient metabolism, altered male pronuclear formation and chilling-induced apoptosis of oocytes likely contribute to the extremely low embryo development after storage at 4 °C.

3.5.2 Effects of temperature during oocyte storage on the quality of resultant blastocysts

Our results also revealed that although the blastocyst formation rates were similar between the groups preserved at 25°C and 38.5°C, the quality of resultant blastocysts in terms of total cell numbers was compromised after preservation at 38.5°C whereas blastocyst quality was similar to that of the non-preserved control when oocytes were stored at 25°C and even at lower temperatures. Analysis of fertilization events has revealed that although fertilization rates were not affected, the percentage of fertilized oocytes having the second PB was either significantly or tendentiously reduced after preservation at 38.5°C compared with the group preserved at lower temperatures. This suggests the failure of the second PB emission which may occur as a result of spindle malformation (Maro et al., 1986; Sun et al., 2001a). This result is consistent with the previous studies reporting low incidences of the second PB emission in aged mouse and porcine oocytes (Maro et al., 1986; Suzuki et al., 2002). A plausible reason for this phenomenon may be the degradation of the cytoskeleton as a result of ageing since the second PB emission greatly depends on the cytoskeletal organization (Maro et al., 1986) which has been known to undergo

degradation during oocyte ageing (Miao et al., 2009). Our results suggest that this process is enhanced at higher temperatures. Furthermore, the incidences of monospermic fertilization were tendentiously reduced (i.e. polyspermy was increased) in the 38.5°C storage group compared with the control group, whereas in other storage groups the percentages of monospermy were similar to that of the control group. Previous studies have reported increased frequencies of polyspermy as a result of oocyte ageing (Badenas et al., 1989; Grupen et al., 1997; Wang et al., 2003). Our results suggest that reducing the temperature to 25°C or below might prevents this process. Nevertheless, we cannot rule out the possibility that monospermic/polyspermic fertilization rates were affected via the cumulus compartment since after 20 h of storage at 38.5 C we observed a higher grade of cumulus expansion than after storage at lower temperatures or without storage. Cumulus cells greatly affect sperm penetration in bovine IVF systems (Cox et al., 1993; Chian et al., 1995; Tanghe et al., 2003). Taken together, both the failure of the second PB emission and polyspermic penetration result in the formation of embryos with abnormal (polyploid) chromosome numbers (Fraser et al., 1976; Iwasaki and Nakahara, 1990; Lechniak, 1996; Suzuki et al., 2003; Somfai et al., 2008). Polyploid bovine embryos are known to be able to develop to the blastocyst stage; however, they have reduced cell numbers compared to diploid ones (Kawasky et al., 1996). Therefore, it is possible that after oocyte storage at 38.5°C, the quality of blastocysts is compromised by abnormal chromosome numbers caused by the cumulative effects of tendentiously increased incidences for the failure of the second PB emission and polyspermic penetration.

3.6 Conclusions

In the present study, storage at 25°C was found to be the most suitable temperature for the temporal storage of matured bovine oocytes regarding both their developmental competence and the quality of resultant blastocyst stage embryos. Lower temperatures trigger apoptosis during storage leading to reduced developmental rates whereas at higher temperatures increased frequencies of fertilization anomalies compromise the quality of resultant embryos. Considering the similar quality of blastocysts obtained from non-stored oocytes and those stored at 25°C, this result provides a basis for the development of an efficient method to preserve matured bovine oocytes temporarily in the future. Nevertheless, oocytes stored at 25°C for 20 h were still characterized by reduced GSH contents and increased rates of apoptosis. For the future improvements of the preservation system, these alterations must be addressed.

This part of this work was published in Theriogenology on February 2016.

3.7 References

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CHAPTER IV

EFFECT OF MEDIUM ADDITIVES DURING LIQUID STORAGE ON DEVELOPMENTAL COMPETENCE OF *IN VITRO* MATURED BOVINE OOCYTES

4.1 Abstract

Our aim was to improve the developmental competence of bovine oocytes during their liquid storage by using additives. *In vitro* matured oocytes were stored for 20 h at 25°C in HEPES buffered TCM 199 medium (base medium). After storage, *in vitro* embryo development after IVF was compared to those of non-stored (control) ones. Addition of 10% (v/v) new born calf serum or 10.27 mM pyruvate alone to the base medium did not improve blastocyst formation rates in stored oocytes; however, their simultaneous addition significantly improved the rate compared with those stored in base medium ($P < 0.05$). Supplementation of the holding medium with DL-dithiothreitol (DTT) at any concentrations did not improve embryo development from stored oocytes. Although supplementation with cyclosporine A (CsA) significantly reduced apoptosis and membrane damage rates during storage, it did not improve the developmental competence of oocytes. BAPTA-AM and ruthenium red had no effect on oocyte apoptotic rates. Blastocyst formation rates in all stored groups remained significantly lower than that of the control. In conclusion, pyruvate and serum had a

synergic effect to moderate the reduction of oocyte quality during storage whereas mitochondrial membrane pore inhibitor CsA and the antioxidant DTT did not affect their developmental competence.

4.2 Introduction

In vitro fertilization (IVF) of matured oocytes at the metaphase of the second meiotic division (metaphase-II: MII) has become an important technology for assisted reproduction in farm animals allowing the efficient use of gametes cryopreserved in gene banks, gender-preselected semen with low fertilization ability or the genetic and health diagnosis of the resultant embryos. Nevertheless, IVF technology required a laboratory which limits its availability.

Although MII oocytes remain arrested at this stage until fertilization, excessive delay of fertilization (insemination) causes the reduction of oocyte developmental competence due to a process called post-ovulatory oocyte aging (Fissore et al., 2002). Therefore, in each species, there is a time window for optimal fertilization. In the cattle, signs of oocyte ageing have been detected from approximately 5 h after reaching the MII stage. Prolonging the optimum time window for fertilization would greatly facilitate the use of oocytes for IVF such as allowing the transportation of oocytes from remote areas to laboratories and the flexible planning of experiments in time and space.

Postovulatory aging has been known to cause complex cellular and molecular changes in oocytes (Miao et al., 2009; Takahashi et al., 2013) which are detrimental for the developmental competence of oocytes (Wilcox et al., 1998) and also for the production of offspring (Tarin et al., 1999, 2002). These biochemical changes include

1) the reduction of metaphase-promoting factor (MPF activity) in cytoplasm causing premature cortical granule exocytosis, zona hardening (Xu et al., 1997; Abbott et al., 1998) and parthenogenetic activation (Xu et al., 1997; Abbott et al., 1998; Petrova et al. ,2005), 2) apoptosis caused by the leakage of Ca^{2+} from mitochondria to the cytosol (Smaili et al., 2000) and 3) various cellular damages such as DNA fragmentation and microtubule (spindle) deformation caused by an increase of the intracellular levels of reactive oxygen species (ROS) (Takahashi et al., 2003; Goud et al., 2008; Tang et al., 2013). The magnitude of these processes is affected by temperature and the composition of the medium (Wakayama et al., 2004; Li et al., 2012; Wang et al., 2014). Physiological temperature seems to be harmful during oocyte storage because it upregulates mitochondrial metabolism causing the accumulation of its byproducts, ROS, thus the reduction of intracellular glutathione (GSH) (Li et al., 2012; Venditti et al., 2013) whereas low temperatures may cause the release of Ca^{2+} from mitochondria triggering apoptosis (Giorgi et al. 2012). Recently, we have determined that the optimum temperature range for the temporary storage of IVM bovine oocytes is 15-25°C (Suttirojpattana et al., in press). Nevertheless even at this temperature, we observed significantly reduced levels of intracellular GSH and increased frequencies of apoptosis in stored oocytes associated with their reduced developmental competence. Moreover, the expression of the antiapoptotic protein Bcl-2 was decreased and the rate of TUNEL-positive oocytes was reportedly increased in aged oocytes (Ma et al., 2005; Tatone et al., 2006). Previous studies have demonstrated/suggested that the biochemical processes beyond oocyte aging can be moderated to some extent by specific modifications of medium composition. Increasing pyruvate concentration and neglecting glucose in medium combined with

hypothermic conditions have been reported to prevent aging in mouse oocytes by the downregulation of mitochondrial metabolism (Li et al., 2012; Wang et al., 2014). Under hypothermic conditions serum also seems to act positively on the viability of bovine embryos (Ideta et al., 2013). Antioxidants such as α-tocopherol, cysteamine, cysteine (Wang et al. 2014), melatonin (Lord et al. 2013) or dithiotreitol (DTT) (Tarin et al., 1998; Rausell et al., 2007) have been reported to prevent/moderate the detrimental effects of ROS in aged mouse oocytes. Furthermore, in bovine oocytes, Zhao et al. (Zhao et al., 2015) reported that 1-octanol and BAPTA-AM which reduce cytosolic free Ca^{2+} levels in oocytes could improve the proportion of blastocyst formation rates in stored oocytes after parthenogenetic activation.

The purpose of this study was to test if supplementation of the holding medium with pyruvate, serum, the reducing agent DTT, the Ca^{2+} chelating agent 1,2-bis(2-aminophenoxy) ethane N,N,N',N'-tetraacetic acid tetrakis-acetoxymethyl ester (BAPTA-AM) or mitochondrial membrane pore inhibitors cyclosporine A (CsA) and ruthenium red (RR) can prevent the aging process in bovine oocytes during a 20 h storage and improve their ability to develop to the blastocyst stage after IVF.

4.3 Materials and methods

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

Collection and IVM of bovine oocytes were performed as described by Imai et al. (Imai et al., 2006). In brief, bovine ovaries were collected at slaughterhouse and transported to the laboratory and then washed in 0.9% (w/v) sodium chloride solution. Cumulus-oocyte complexes (COCs) were aspirated from small follicle (2-8mm in diameter) using a 10- mL syringe with an 18 gauge needle. The maturation

medium was 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). Only COCs with compacted cumulus cells were selected, washed twice with maturation medium and then cultured in 100 μL droplets of maturation medium under paraffin oil (Paraffin liquid, Nacalai Tesque Inc., Kyoto, Japan) (20 COCs per droplet) for 20-21 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air.

4.3.2 *In vitro* preservation of oocytes

The medium for oocyte preservation consisted of TCM-199 medium (Medium 199 powder, 31100-035, GIBCO Invitrogen) buffered with 11 mmol/L of HEPES, 9 mmol/L of Na-HEPES, and supplemented with 5 mmol/L of sodium bicarbonate with a pH adjusted to 7.3 and the osmolarity to approximately 0.290 Osm. The storage medium was supplemented with chemicals which were described in experimental designs. After oocyte maturation, the COCs were washed twice in the storage medium and then transferred into 1.5-mL Eppendorf microfuge tubes in 1 mL of storage medium. The tubes were then closed, sealed air tight with parafilm and kept at 25°C for 20 h.

4.3.3 *In vitro* fertilization (IVF)

Frozen semen of Japanese Black bull was thawed in 37°C water for 40 sec and centrifuged in 3 mL of 90% Percoll (Sigma-Aldrich Co., St. Louis, MO, USA) solution at 740 × g for 10 min. Then the pellet was re-suspended in 5.5 mL of IVF 100 medium (Research Institute for Functional Peptides Co., Ltd., Yamagata, Japan) and

centrifuged at 540 × g for 5 min. Then the pellet was re-suspended with IVF 100 medium and the final concentration was adjusted to 3×10^6 sperm/mL. The mature oocytes were washed twice in IVF 100 medium and co-cultured with sperm for 4 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air (20 oocytes/100μL droplet).

4.3.4 *In vitro* embryo culture (IVC)

After IVF, the putative zygotes were gently denuded from cumulus cells with a fine glass pipette. Fifteen to twenty zygotes were cultured in each 100 μL droplet of Charles Rosenkrans 1 (CR1) medium (Rosenkrans et al., 1993) supplemented with amino acids (CR1aa; Imai et al., 2002) and 5% NCS at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90 % N_2 for up to 9 days.

4.3.5 Evaluation of total cell number in blastocysts

Blastocysts obtained on day 9 (day 0 = IVF) were fixed in 99.5% ethanol supplemented with 10 μg/mL Hoechst33342 (Calbiochem, San Diego, CA, USA) overnight at 4°C. The embryos were then washed in ethanol, mounted on glass slides in glycerol droplets and flattened by cover slips. The total numbers of nuclei were counted under UV light with excitation at 330–385 nm and emission at 420 nm under an epifluorescence microscope (Nikon Eclipse E600, Tokyo, Japan).

4.3.6 Assay of live/dead status and apoptosis in oocytes

Staining was performed with an Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit (Molecular Probes, Eugene, OR, USA) according to Anguita et al. (Anguita et al., 2007). This assay is based on the annexin V binding which is applied to determine phosphatidylserine (PS) externalization on the plasma membrane. Annexin V, a member of the phospholipid-binding annexin family, binds most

effectively to PS, which is externalized on the outer membrane of cells exposed to apoptotic stimuli. The assay also applies propidium iodide (PI), a membrane impermeable stain, to discriminate between live and dead (membrane damaged) cells. PI has ability to enter the cell when the cytoplasmic membrane has lost its integrity. Oocytes were washed three times with PBS- and then transferred to 100 μL of binding buffer (Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit, Molecular Probes) with 5 μL of Alexa Fluor Annexin V, 1 μL of PI (100 μg/mL) and 0.2 μL of 5 mg/mL Hoechst 33342 for 20 minutes at 38.5°C in the dark. After incubation, the oocytes were washed three times in binding buffer and subsequently mounted on the glass slides. The samples were examined with an epifluorescence microscope (Eclipse E-600; Nikon) at an excitation wavelength of 480 nm and emission at 510 nm. Oocytes were classified in three groups (Anguita et al., 2007). Viable oocytes were characterized by PI negative nuclei (appearing in blue color by Hoechst 33342) and no annexin staining on the membrane (A-/PI-). Early apoptotic membrane intact (live) oocytes had PI negative nuclei (appearing in blue color by Hoechst 33342 at 350 nm excitation) and a homogeneous annexin positive signal on the membrane (A+/PI-). Dead oocytes which showed PI positive red nuclei, indicating membrane damage with or without annexin staining on the membrane (PI+).

4.3.7 Experimental design

Experiment 1 investigated the effect of serum and/ or pyruvate added to the holding medium (base medium) alone or in combination on the developmental competence of oocytes after storage. After IVM, COCs were stored for 20 h in base medium (HEPES-TCM 199) supplemented either with 10% (v/v) NCS or 10.27mM

pyruvate or both or without any supplementation at 25°C. Then, the oocytes were subjected to IVF and IVC as described above. The cleavage rates, blastocyst formation rates, hatching/hatched rate of blastocysts and blastocyst cell numbers were checked on day 2, days 7-9, days 8-9 and day 9, respectively. In each experimental replication a group of IVM oocytes without storage was also investigated as a control group. Five replicates were performed.

Experiment 2 investigated the effect of the reducing agent DTT on developmental competence in stored oocytes. After IVM, COCs were stored for 20 h in base medium consisting 10.27 mM pyruvate with or without DTT (Sigma, St. Louis, USA) supplementation at different concentrations (0.05 mM, 0.5 mM, 1.0 mM and 5.0 mM). Then, the oocytes were subjected to IVF and IVC as described above. The cleavage rates, blastocyst formation rates, hatching/hatched rate of blastocysts and blastocyst cell numbers were checked on day 2, days 7-9, days 8-9 and day 9, respectively. In each experimental replication a group of IVM oocytes without storage was also investigated as a control group. Three replicates were performed.

Experiment 3 investigated the effects of the Ca^{2+} chelating agent BAPTA-AM (Tang et al., 2013; Zhao et al., 2015), and mitochondrial membrane pore inhibitors RR (Nakagawa et al., 2008) and CsA (Thouas et al., 2004) on apoptosis in stored oocytes. After IVM, COCs were stored for 20 h at 25°C in base medium consisting 10.27 mM pyruvate supplemented with or without either 50 μM BAPTA-AM (Sigma, St. Louis, USA), 1 μM RR (Sigma, St. Louis, USA) or 33 μM CsA (Sigma, St. Louis, USA). The concentration for each reagent was selected according to previous studies given as references. In each experimental replication a group of IVM oocytes without storage was also investigated as a control group. The rate of

apoptotic oocytes were compared among control and storage groups. Three replicates were performed.

Experiment 4 investigated the effect of CsA on developmental competence in stored oocytes. After IVM, cumulus-enclosed oocytes were stored for 20 h in base medium consisting 10.27 mM pyruvate supplemented with different concentration of CsA (1μM and 33μM) (according to previous studies (Nakagawa et al., 2008; Zhao et al., 2011). Then, the oocytes were IVF and IVC as described above. The cleavage rates, blastocyst formation rates, hatching/hatched rate of blastocysts and blastocyst cell numbers were checked on day 2, days 7-9, days 8-9 and day 9, respectively. In each experimental replication a group of IVM oocytes without storage was also investigated as a control group. Five replicates were performed.

4.3.8 Statistical analysis

All data were exposed as mean ± SEM. Statistical analysis was performed with one way ANOVA by using software SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA). The percentage data were subjected to arcsine transformation. The significant differences between mean were compared by a post-hoc Fisher's protected least significantly difference test. Different values were considered statistically significant when P value was less than 0.05.

4.4 Results

4.4.1 Effects of serum and pyruvate supplementation during oocyte storage on developmental competence of stored bovine IVM oocytes and the quality of resultant embryos

As shown in table 4.1, there was no difference in cleavage rates among the control and stored groups. However, the blastocyst formation rate in the control group was significantly higher than those of storage groups ($P < 0.05$) at all time points of IVC (Table 4.1). Compared within storage groups, supplementation of the base medium with NCS + pyruvate resulted in significantly the higher blastocyst formation rate ($P < 0.05$) compared with the group stored in base medium at all time points of IVC. However, when oocytes were stored in base medium supplemented with either NCS or pyruvate, blastocyst formation rates did not differ significantly from those stored without supplementation (Table 4.1). The hatching ability of blastocysts after IVF and IVC of oocytes stored in base medium was significantly lower than that of the control group ($P < 0.05$, Figure 4.1) whereas blastocysts of the other storage groups showed intermediate values of hatching (Figure 4.1). The total cell numbers of blastocysts on day 9 in stored groups were significantly lower than those of the control group ($P < 0.05$) (Figure 4.2).

4.4.2 Effect of DTT supplementation during oocyte storage on the developmental competence of bovine IVM oocytes and the quality of resultant embryos

As shown in table 4.2, there was no significant difference in cleavage rates among the control and storage groups with DTT supplementation of 0.05mM – 1mM.

However, the cleavage rate in 5 mM DTT treated group was 36.5% which was significantly lower than those in the other groups ($P < 0.05$). The incidences of embryos developing to the blastocyst stage in all stored groups were significantly lower than those of the control group at all time points ($P < 0.05$). Treatment of oocytes with 0.05 mM, 0.5 mM, 1.0 mM and 5.0 mM DTT could not improve the blastocyst formation rate compared with the group stored without DTT; moreover, 5 mM DTT significantly reduced the embryo development. The hatching ability of IVF-derived blastocysts was significantly reduced in 1.0 mM and 5.0 mM DTT treated groups compared with control ($P < 0.05$); however, significant difference was not detected among the control and the other treatment groups (Figure 4.3). The total cell numbers of blastocysts on day 9 from stored oocytes were significantly decreased in all treatment groups compared with the control group ($P < 0.05$, Figure 4.4).

4.4.3 Effect of cytosolic Ca^{2+} modulating agents on the onset of apoptosis and membrane damage in oocytes during storage

In this experiment, the effects of 50 μM BAPTA-AM, 1 μM RR and 33 μM CsA) on apoptosis in stored oocytes were compared to those stored in base medium + pyruvate and control without storage (Table 4.3). The percentage of viable non apoptotic oocytes (A-, PI-) in the CsA treated group was not significantly different from that in the non-stored control group whereas in all other treatment groups the rates were significantly lower than that in the control group ($P < 0.05$). A significantly increased rate of membrane intact apoptotic oocytes (A+, PI-) was detected in RR treated group ($P < 0.05$) whereas the percentage of (A+, PI-) oocytes did not differ significantly among the other groups. A significantly increased proportion of membrane damaged oocytes (PI+) were observed in the group stored in base medium

+ pyruvate and the RR treated group compared with those of the other groups whereas BAPTA-AM treated group showed intermediate values.

4.4.4 Effect of CsA on developmental competence of bovine oocytes and the quality of resultant embryos

Since in the previous experiment, status of stored oocytes treated with CsA resembled the most to non-stored control oocytes, we tested the effect of CsA treatment of oocytes at different concentrations during storage on their developmental competence after IVF and IVC. As shown in table 4.4, at all time points of culture the blastocyst formation rate of the control group was significantly higher than those of all the stored groups irrespective of the medium additive ($P < 0.05$). Supplementation of the holding medium with 1 μM or 33 μM CsA did not affect cleavage and blastocyst formation rates. No significant difference ($P > 0.05$) was observed between control and preserved oocytes in either hatching ability or total cell number irrespective of the use of CsA (Figure 4.5 and 4.6).

Table 4.1 Effect of storage medium on embryo development after IVF.

Group	Total	Cleavage (% Mean±SEM)	Blastocyst (% Mean±SEM)		
			day 7	day 8	day 9
Control	210	155 (73.1 ± 4.6)	87 (40.8 ± 3.9^{a})	94 (44.0 ± 4.1^{a})	94 (44.0 ± 4.1^{a})
Stored Base medium	214	149 (68.9 ± 5.3)	14 (6.1 ± 2.2^{b})	16 (7.2 ± 2.3^{b})	16 (7.2 ± 2.3^{b})
Stored Base medium + NCS	208	159 (76.6 ± 4.7)	21 (10.3 ± 1.7^{bc})	23 (11.4 ± 2.0^{b})	23 (11.4 ± 2.0^{b})
Stored Base medium + Pyru	211	152 (71.4 ± 3.9)	28 (13.2 ± 1.2^{bc})	29 (13.8 ± 1.8^{bc})	30 (14.3 ± 1.7^{bc})
Stored Base medium + NCS + Pyru	219	180 (81.8 ± 4.0)	38 (17.1 ± 2.0^{c})	46 (21.0 ± 3.1^{c})	46 (21.0 ± 3.1^{c})

Five Replicates were performed. Values in the same column with no common superscripts differ significantly (P<0.05). Day 0 = the day of IVF.

NCS = 10% (v/v) newborn calf serum

Pyru = 10.27 mM pyruvate

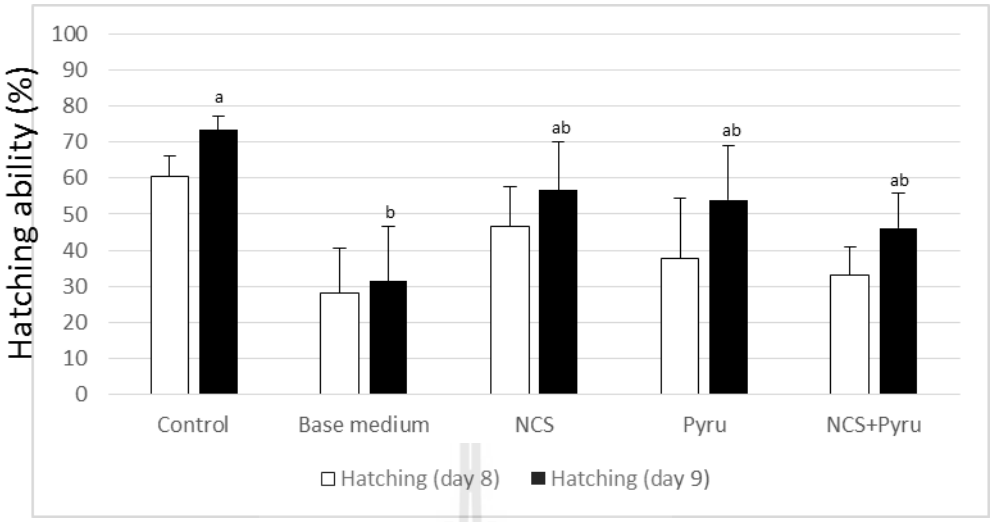

Figure 4.1 Effect of storage medium on ability to hatching after IVF/IVC.

Bars with different superscript letter differ significantly.

NCS = 10% (v/v) newborn calf serum; Pyru = 10.27 mM pyruvate

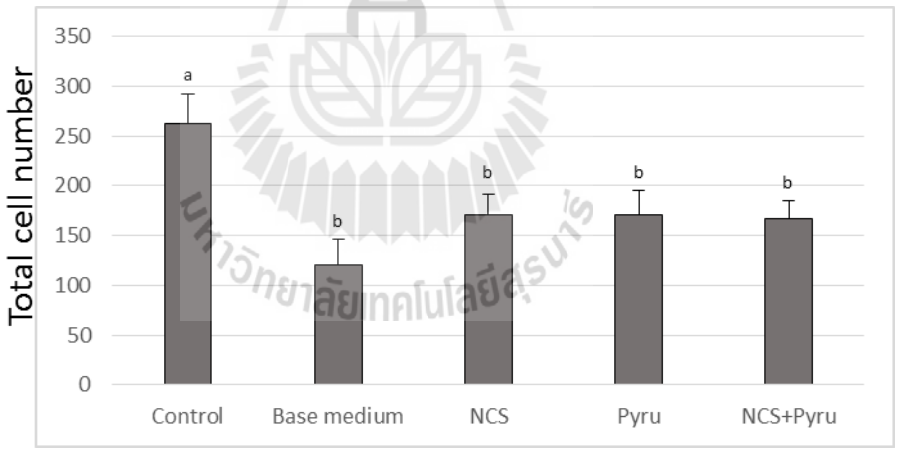

Figure 4.2 Effect of storage medium on total cell number of blastocysts derived from IVF.

Bars with different superscript letter differ significantly.

NCS = 10% (v/v) newborn calf serum; Pyru = 10.27 mM pyruvate

Table 4.2 Effect of DTT supplementation during oocyte storage for 20 h on subsequent embryo development after IVF

Treatment	DTT concentration (mM) during storage	Total	Cleavage (% Mean±SEM)	Blastocyst (% Mean±SEM)		
				day 7	day 8	day 9
Control	NA	149	105 (71.5 ± 5.2^{a})	55 (37.5 ± 5.9^{a})	55 (37.5 ± 5.9^{a})	55 (37.5 ± 5.9^{a})
Stored	0	146	112 (74.9 ±6.0^{a})	24 (16.2 ± 1.5^{bc})	26 (17.7 ± 2.2^{bc})	27 (18.2 ± 2.4^{c})
Stored	0.05	142	111 (78.8 ± 1.7^{a})	28 (20.2 ± 1.9^{c})	30 (21.3 ± 0.8^{c})	30 (21.3 ± 0.8^{c})
Stored	0.5	137	85 (63.8 ± 6.4^{a})	18 (13.3 ± 0.5^{bc})	19 (14.2 ± 1.3^{bc})	19 (14.2 ± 1.3^{bc})
Stored	1	131	86 (66.0 ± 1.6^{a})	23 (18.0 ± 2.2^{c})	23 (18.0 ± 2.2^{c})	23 (18.0 ± 2.2^{c})
Stored	5	104	39 (36.5 ± 8.8^{b})	9 (8.8 ± 1.8^{b})	9 (8.8 ± 1.8^{b})	9 (8.8 ± 1.8^{b})

Three replicates were performed. Values in the same column with no common superscripts differ significantly (P<0.05). Day 0 = the day of IVF. The holding medium for oocyte storage was base medium supplemented with 10.27 mM pyruvate.
DTT = dithiothreitol

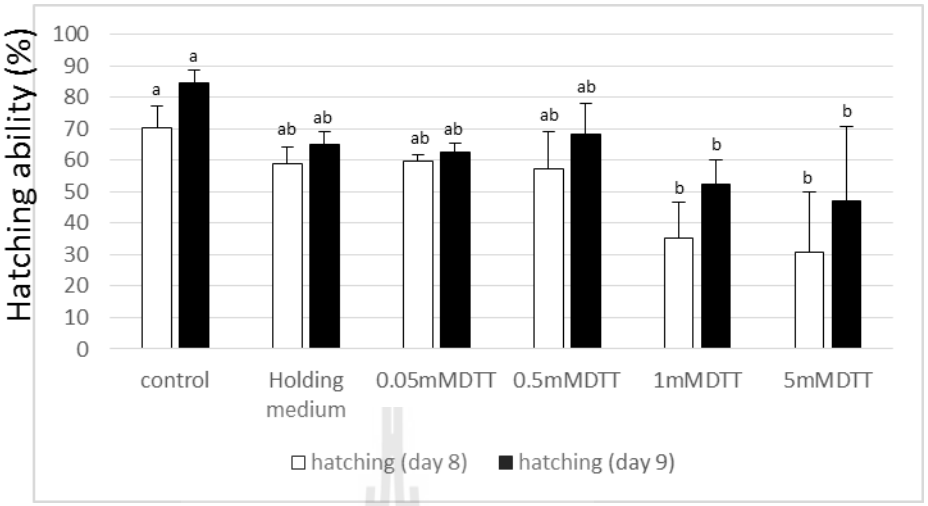

Figure 4.3 Effect of DTT supplementation in holding medium during oocyte storage for 20 h on the ability of resultant blastocysts.

Bars with different superscript letter differ significantly.

The holding medium for oocyte storage was base medium supplemented with 10.27 mM pyruvate. DTT = dithiothreitol

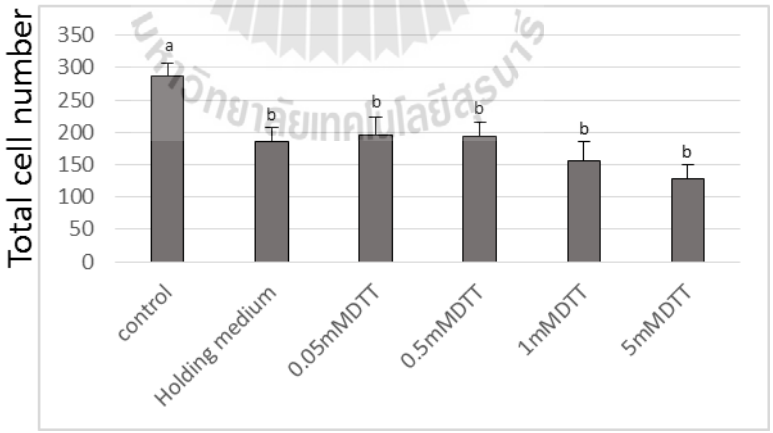

Figure 4.4 Effect of DTT supplementation in holding medium during oocyte storage for 20 h on total cell number of resultant blastocyst.

Bars with different superscript letter differ significantly.

The holding medium for oocyte storage was base medium supplemented with 10.27 mM pyruvate. DTT = dithiothreitol

Table 4.3 Effects of cytosolic Ca2+ modulating agents during storage of IVM oocytes on their live/dead status and apoptosis.

Group	Additive during storage	Number of oocyte examined	A-/PI- (% Mean ± SEM)	A+/PI- (% Mean ± SEM)	PI+ (% Mean ± SEM)
Control	**NA**	140	131 (94.0 ± 3.5^{a})	6 (4.0 ± 3.0^{a})	3 (2.0 ± 1.2^{a})
Stored	**-**	144	108 (74.6 ± 6.0^{bc})	13 (9.2 ± 3.6^{a})	23 (16.1 ± 2.8^{c})
Stored	**BAPTA**	146	118 (80.9 ± 2.0^{bc})	18 (12.3 ± 1.9^{a})	10 (6.8 ± 0.3^{ab})
Stored	**RR**	141	87 (61.7 ± 3.2^{b})	37 (26.2 ± 3.6^{b})	17 (12.0 ± 0.6^{bc})
Stored	**CsA**	143	129 (90.0 ± 5.9^{ac})	9 (6.5 ± 3.4^{a})	6 (4.3 ± 2.2^{a})

Three replicates were performed. Values in the same column with no common superscripts differ significantly (P<0.05). The holding medium for oocyte storage was base medium supplemented with 10.27 mM pyruvate.
RR = Ruthenium red
CsA = Cyclosporin A
A-/PI- = viable non-apoptotic cell; A+/PI- = early apoptotic cell; PI+ = dead oocyte

Table 4.4 Effect of CsA supplementation during oocyte storage for 20 h on subsequent embryo development after IVF

Treatment	CsA concentration (µM) during storage	Total	Cleavage (% Mean±SEM)	Blastocyst (% Mean±SEM) day 7	day 8	day 9
Control	NA	284	196	102	108	113
			68.5 ± 2.6[a]	(35.6 ± 2.2[a])	(37.6 ± 1.9[a])	(39.3 ± 1.7[a])
Stored	0	273	197	46	50	50
			72.9 ± 4.0[a]	(18.1 ± 3.8[b])	(19.8 ± 4.2[b])	(19.8 ± 4.2[b])
Stored	1	267	189	29	32	34
			71.1 ± 2.0[a]	(10.9 ± 2.0[b])	(11.9 ± 1.6[b])	(12.6 ± 1.2[b])
Stored	33	278	230	52	55	55
			83.9 ± 3.1[b]	(18.7 ± 2.5[b])	(19.9 ± 2.1[b])	(19.9 ± 2.1[b])

Five Replicates were performed. Values in the same column with no common superscripts differ significantly ($P<0.05$). Day 0 = the day of IVF. The holding medium for oocyte storage was base medium supplemented with 10.27 mM pyruvate.
CsA = Cyclosporin A

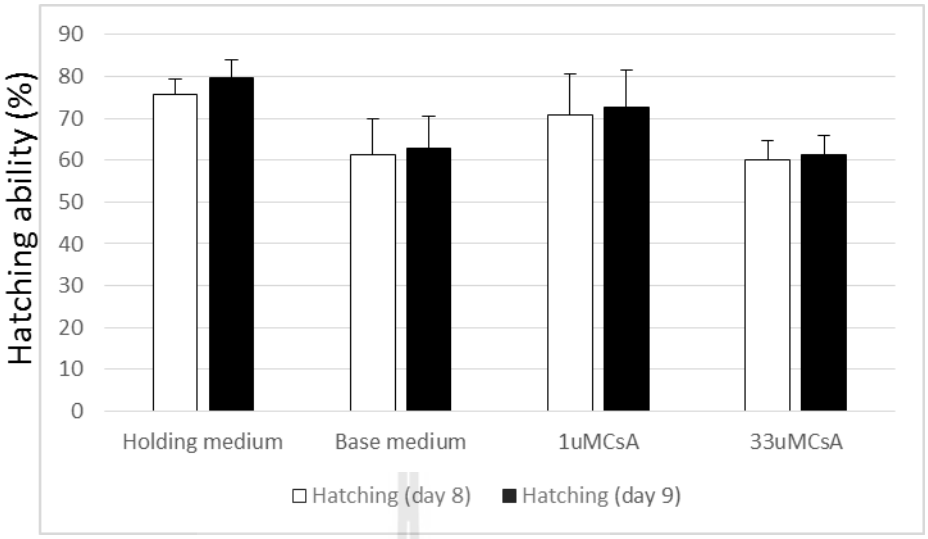

Figure 4.5 Effect of CsA supplementation during oocyte storage for 20 h on the hatching ability of resultant blastocysts.

Bars with different superscript letter differ significantly.

The holding medium for oocyte storage was base medium supplemented with 10.27 mM pyruvate. DTT = dithiothreitol

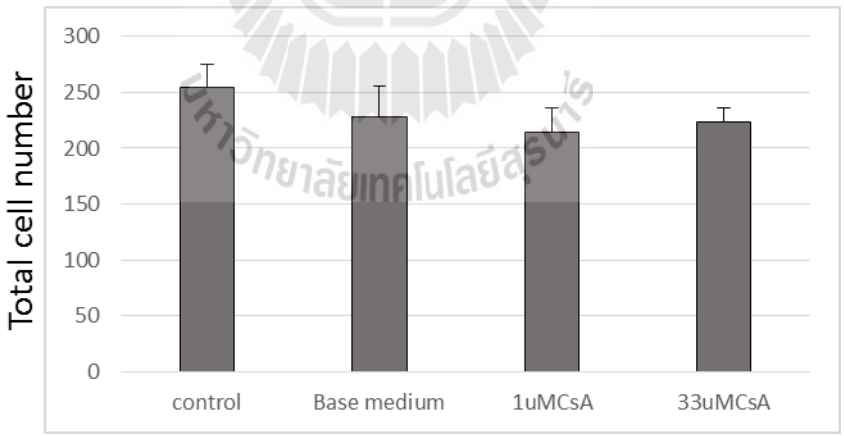

Figure 4.6 Effect of CsA supplementation during oocyte storage for 20 h on total cell number of resultant blastocysts derived from IVF.

Bars with different superscript letter differ significantly.

The holding medium for oocyte storage was base medium supplemented with 10.27 mM pyruvate. DTT = dithiothreitol

4.5 Discussion

The results of Experiment 1 indicated that the supplementation of the base medium with either serum or pyruvate alone did not significantly improve the blastocyst formation rates compared with oocytes stored in base medium. Previous studies reported the benefit of serum supplementation during oocyte storage at 4°C in mouse, but not at 37°C (Tsuchiya et al., 2001; Wakayama et al., 2004). Similarly, serum supplementation during the hypothermic storage of bovine embryos was reported to maintain their viability (Ideta et al., 2012). Furthermore, supplementing a basic holding medium with serum was reported to maintain the viability of immature porcine oocytes preserved under ambient temperatures (Yang et al., 2010). In contrast with these reports, serum supplementation alone did not enhance the developmental competence of bovine oocytes during 20 h storage at 25°C in the present study. Since serum has been reported to exert its preventive effect on bovine embryos concentration-vise, in an additional experiment we have increased the concentration of serum in holding medium during oocyte storage up to 50%; however, there were no significant effects on blastocyst formation rates (data not shown). It is possible that serum exerts its positive effect to support viability of oocytes/embryos only under low temperatures such as 4°C.

In previous studies, an increased concentration (10.27 mM) of pyruvate could maintain the developmental potential of matured mouse oocytes stored under 25 °C for 24 h by regulating intracellular redox status and energy supply (Li et al., 2012; Wang et al., 2014). Furthermore, Liu et al. reported that pyruvate supplementation could delay the aging process by increasing the MPF activity and blocking the premature exocytosis of cortical granules (Liu et al., 2009). In addition, pyruvate also

increased the levels of antiapoptotic Bcl-2 proteins (Liu et al., 2009) and the GSH/GSSH in aged mouse oocytes (Kim and Schuetz, 1991, Liu et al., 2009). According to Li et al. addition of 10.27 mM pyruvate could improve developmental competence in stored mouse oocytes by maintaining the GSH levels (Li et al., 2012) which have an important role to cope with oxidative stress (Deneke and Fanburg, 1989). These reports in mice contradict to our results in cattle oocytes. Recently, we demonstrated the significant decrease of GSH contents in matured bovine oocytes after storage for 20 h, irrespective of the storage temperature compared with oocytes without storage (Suttirojpattana et al., in press). However, in the present study the blastocyst formation rates and embryo quality of stored bovine oocytes were not significantly improved by supplementing the base medium with 10.27 mM pyruvate. This may suggest either a difference in the biology of the ageing process in oocytes between cattle and mice or that the optimum (effective) concentrations of pyruvate may differ for mouse or bovine oocytes. Interestingly, the combination of 10% (v/v) serum and 10.27 mM pyruvate supplementation significantly improved the development of stored bovine oocytes to the blastocyst stage suggesting a synergic effect of these additives on developmental competence of the oocytes. Furthermore, the hatching ability which is an important criteria of embryo quality (Balaban et al., 2000; Yuan et al., 2003) was significantly reduced when oocytes were stored in base medium compared with the non-stored control without storage. However, the supplementation of the base medium with either serum or pyruvate during oocyte storage prevented the significant reduction of hatching ability of resultant blastocysts. Based on these results, in further experiments of the study we used a Hepes-buffered TCM-199 medium supplemented with 10.27 mM pyruvate as a defined storage

medium to avoid possible interactions of the tested reagents with undefined factors of serum.

In animal cells, the mitochondria are the primary intracellular site of oxygen consumption and the major source of ROS (Mari et al., 2009). Previously Tarin reported that a mechanism based on the ROS related to mitochondrial dysfunction during aging (Tarin, 1996). Acceleration of ROS damages mitochondria resulting in decreased redox potential of GSH/GSSH, which leads to the loss of potential to prevent the detrimental effects of excessive ROS (Mari et al., 2009). In accordance, a number of studies have demonstrated increased levels of ROS associated with reduced levels of GSH in aged oocytes (Hao et al., 2009; Wang et al., 2014). Also, in recent experiments we have observed significantly reduced GSH levels in IVM bovine oocytes after 20 h storage in base medium supplemented with 10% serum, irrespective of the temperature (Suttirojpattana et al., in press). DTT, a disulfide reducing agent, is known to be effective to diminish the thiol oxidative damage in mouse zygotes and blastocysts (Tarin et al., 1998; Liu et al., 1999). DTT has been reported to delay the aging process in mouse (Tarin et al., 1998; Liu et al., 1999; Rausell et al., 2007) and ovine oocytes (Ye et al., 2010). To date, there have been no reports on the effect of DTT on developmental competence of aging bovine oocytes. Therefore, we have tested if this compound was an effective additive to maintain blastocyst developmental competence of bovine IVM oocytes after storage. Our results indicated that DTT added at various concentrations did not improve the developmental competence of bovine oocytes which was inconsistent with the results of previous reports in other species (Tarin et al., 1998; Liu et al., 1999; Rausell et al., 2007; Ye et

al., 2010). Moreover, at a high concentration (5mM) DTT exerted a negative effect on blastocyst development for stored oocytes.

In animal cells cytoplasmic Ca^{2+} plays important roles in signal transduction. When mammalian oocytes are fertilized, the penetrating sperm triggers repetitive Ca^{2+} oscillations in the oocyte cytoplasm which have a crucial role for inducing oocyte activation and thus embryonic development (Bos-Mikich et al., 1997; Swann and Lai, 1997; Miyazaki et al., 1998; Gordo et al., 2000). In normal case, each of the fertilization-related Ca^{2+} oscillations are characterized by a step rise to the peak followed by a quick return to the base line since extended increase in cytosolic Ca^{2+} triggers apoptotic events in animal cells (Gordo et al., 2002). However, abnormal Ca^{2+} oscillations were found in aged oocytes (Jones and Whittingham, 1996; Igarashi et al., 1997; Gordo et al., 2000; Hao et al., 2009; Takahashi et al., 2009; Tang et al., 2013) which was associated with abnormal activation and apoptosis (Gordo et al., 2002; Tatone et al., 2006; Koyama et al., 2014) resulting in poor embryo development (Takahashi et al., 2009). In oocytes, the level of cytosolic Ca^{2+} is regulated by the smooth endoplasmic reticulum (SEM) and mitochondria (Bootman et al., 2001). It has been speculated that during ageing, Ca^{2+} might leak from the intracellular stores (i.e the SEM and mitochondria) into the cytosol which may trigger apoptosis (Takahashi et al., 2013). In accordance, in a recent report, Zhao et al. treated bovine oocytes with 1-octanol to diminish the extracellular Ca^{2+} and BAPTA-AM to control the intracellular Ca^{2+} and both of these substances could enhance the embryo development in aged oocytes (Zhao et al., 2015). Regarding the above mentioned, we attempted to test the effects of different substances which affect cytosolic Ca^{2+} levels and regulation on the onset of apoptosis during oocyte storage. The membrane

permeable Ca^{2+} chelator BAPTA-AM was used to block cytosolic free Ca^{2+}. Furthermore, inhibitors of mitochondrial permeability transition (mPT) were tested. CsA is a chemical inhibitor of mPT pore formation (Montero et al., 2004), thus it restrains the release of proapoptotic factors (e.g. cytochrome-c) and Ca^{2+} which are potent apoptotic stimulation factors (Zamzami et al., 1996; Green and Reed, 1998). RR is a non-conpetitive inhibitor of mitochondrial Ca^{2+} uniporter which diminishes Ca^{2+} influx (Kruman and Mattson, 1999; Belous et al., 2003) and specifically prevents the release of cytochrome c and activation of caspase3 (Bae et al., 2003). Our results have revealed significantly lower rates of viable non apoptotic (A-, PI-) oocytes in stored groups compared with the non-stored control group except for the CsA treated and stored group which showed similar rates of A-, PI- oocytes to the control. Moreover, CsA treated group presents the lowest percentages of live apoptotic oocytes (A+, PI-) and dead (membrane damaged, PI+) oocytes. Although a significant increase in the percentage of live apoptotic oocytes was only detected when oocytes were stored in the presence of RR, the percentage of oocytes with damaged membrane were significantly increased when oocytes were stored without additive or with RR. It is possible that oocytes undergoing apoptosis during the early stages of storage might lose the ability to maintain membrane integrity by the end of the storage period.

Based on the results of *Experiment 4*, we tested the effects of CsA addition during oocyte storage on subsequent embryo development after IVF. We hypothesized that CsA would enhance the embryo developmental competence of stored bovine oocytes; however, irrespective of the concentration used it had no effect on the blastocyst formation rate and blastocyst quality. It is possible that under the present oocyte

storage conditions apoptotic events in oocytes might not be the primarily cause of reduced embryo development.

In conclusion, our results demonstrate that supplementation of the oocyte storage medium with serum and pyruvate synergistically improved the developmental competence of stored bovine oocytes whereas treatment with only serum or pyruvate did not enhance the blastocyst rate. Supplementation of the storage medium with the antioxidant agent DTT did not improve the blastocyst formation rate. Among the cytosolic Ca^{2+} modulating agents, only CsA could maintain the percentage of non-apoptotic live oocytes in storage groups at a level similar to that of non-stored oocytes. However, when applied during the storage of matured oocytes, CsA had no effect on subsequent embryo development. Further research will be needed to clarify and address other mechanisms involved in the aging process to improve the developmental ability of stored oocytes.

This part of this work was accepted in Animal Science Journal on January 2016.

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CHAPTER V

OVERALL CONCLUSION

Preservation of oocytes is becoming a very important tool for storage genetic resources and efficient preservation protocols have been developed to maintain ability of embryo development after storage. This study concluded that storage matured bovine oocytes at all temperature significantly reduced the blastocyst formation rate compared with control without storage group. Compared among storage groups the 25°C and 38.5°C produced the highest blastocyst rate. However, the quality of resultant blastocysts in term of total cell number was reduced in 38.5°C. Moreover, the abnormal fertilization (any penetrated oocytes that does not fit this description in normal fertilization including 2PB, 1MPN and 1FPN) was higher in 38.5°C compared with other storage groups. Stored oocytes at low temperature (4°C and 15°C) induced the apoptotic oocytes compared with other storage temperature.

As a result, the 25°C was set in optimal temperature for oocyte preservation in second study and test the ability to maintain embryo development during storage oocytes by supplemented with medium additives compared with control (non-storage group). Aging oocyte in the presence of pyruvate and serum significantly improved the embryo development to blastocyst rate compared with other storage groups but did not different from pyruvate alone. Supplementation with disulfide reducing agent DTT and Ca^{2+} regulating agent CsA could not improve the proportion of blastocyst during storage of matured oocyte.

BIOGRAPHY

Ms. Tayita Suttirojpattana was born on 30 March 1987 in Bangkok, Thailand. She finished high school at Saint Joseph Convent School in 2004. After that, she graduated with a Bachelor's degree in pharmacy from Chiang Mai University. Then, she worked at drugstore for 1 year. In 2011, she decided to study Ph.D. course in School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology. She received the scholarship from the Royal Golden Jubilee Ph.D program. In 2014, she has an opportunity to do research in part of her thesis in Japan with Dr. Tamas Somfai for 13 months. The research topic is "Effect of temperature and medium additives during liquid storage on developmental competence in *in vitro* matured bovine oocytes" First part of this work was published in Theriogenology on February 2016 (DOI: 10.1016/j.theriogenology). Second part of this work was accepted in Animal Science Journal on January 2016.